The Expression of Multiple Drug Resistance (MDR) Associated Proteins in Invasive Breast Cancer

A thesis submitted for the degree of Ph.D.

by

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The research work in this thesis was carried out under the supervision of

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I certify that this material, which I now submit for assessment on the programme of study leading to the award of Ph.D. is entirely my own work and has not been taken from the work of others and to the extent that such work has been cited and acknowledged within the text of my work.

Signed: Anne Marie Ken ID No: 97971332

Date: 1/2/02

ABSTRACT

Despite the recent advances in the detection, treatment and management of breast cancer, with an increasing range of hormonal, cytotoxic and more recently MAb targeted drug regimes available in the clinic, between 40% and 50% of patients diagnosed will eventually die from this disease. Identification of biological factors which can function as reliable markers of both prognosis and chemoresponsiveness will allow for more precise targeting of treatment for individual breast cancer patients. During the course of this thesis the expression of a panel of MDR associated proteins was correlated with patient and tumour characteristics and relapse and overall survival in a series of invasive breast carcinomas. In order to address the multifactorial nature of this disease, expression of individual proteins was analysed in relation to other markers. The results presented show that MRP-1 protein at diagnosis is an independent prognostic factor for overall survival in node positive breast cancer patients treated with CMF based chemotherapy, absence of MRP-1 expression at diagnosis was significantly associated with increased overall survival. Results also indicated that this prognostic role of MRP-1 is likely to be stronger in patients with high grade i.e. grade III tumours. It also appeared that expression of cerbB-2 may be associated with reduced relapse and overall survival times in node positive patients treated with a CMF based chemotherapeutic regime. On preliminary analysis it appeared that the co-expression of MRP-1 with cerbB-2 in these patients may be a stronger prognostic tool than either cerbB-2 or MDR-1 alone. MDR-1 and survivin expression levels appeared to correlate in 71% of patients. There was no correlation observed between the other markers studied.

Investigation of MDR-3 Pgp (which has recently been shown to transport several cytotoxic drugs) expression was also investigated in these breast cancer patients using a novel monoclonal antibody (MAb), 6/1G, which was produced during the course of this research; this MAb specifically recognises the MDR-3 encoded gene product. Preliminary results indicate that MDR-3 Pgp was expressed in a high proportion (73.3%) of invasive breast carcinomas studied. The exact significance of this expression and the contribution, if any, of MDR-3 Pgp in the resistance of breast cancer or other solid cancers further investigation. Immunocytochemical studies with

antibody 6/1G on a panel of B cell malignancies suggest that MDR-3 Pgp may possibly be associated with a more malignant phenotype in B-CLL and that its expression may be associated with certain stages of B cell development.

In an attempt to identify new breast cancer drug resistance associated antigens, MAb 5C3 was generated using paraffin wax embedded formalin fixed archival breast tumour tissue from a biopsy showing no P-170 or MRP-1 expression, as an immunogen. Immunohistochemical analysis with this novel MAb of *pre* and *post* treatment tumours however, did not reveal any apparent association with resistance for the antigen recognised by antibody 5C3. Extensive immunocytochemical and Western blot analysis of both cell lines and tissues with antibody 5C3 suggested that the reactive antigen did not appear to be tumour specific and was more likely to be an unidentified breast antigen also expressed in the normal breast. Results of internal sequencing analysis of a 175Kda band which was obtained from immunoprecipitation of the ductal breast carcinoma cell line, ZR-75-1 revealed that MAb 5C3 is recognising a heteropolymer of cytokeratin 6 and cytokeratin 9. The expression of this novel complex in human breast cancer should be further investigated to address any possible prognostic implications.

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This thesis is dedicated to my parents
Brendan and Mary Larkin

Published papers arising from the work presented in this thesis:

A new monoclonal antibody that specifically recognises the MDR-3 encoded gene product
AM. Larkin, E. Moran, D. Alexander, G. Doherty, L. Connolly, SM. Kennedy & M Clynes.
International Journal of Cancer; **80***:* 265-271, 1999.
Preliminary immunocytochemical studies of MDR-1 and MDR-3 Pgp expression in B-cell leukaemiasAM. Larkin, E. Moran, D. Alexander & M. Clynes.
Adv Exp Med Biol; **457:** 65-70, 1999.

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1.Introduction

1.1. Monoclonal antibodies (MAbs)

1.1.1. General introduction

One of the pioneers of Immunology at the beginning of the last century, Paul Ehrlich proposed the concept of "receptors" (which we now know as antibodies) secreted by cells of the immune system in response to foreign antigens. He foresaw their use as "magic bullets" to specifically attack a wide variety of diseases (Ehrlich 1900).

Antibodies are proteins produced by an individual against the presence of a foreign molecule known as "the antigen". The antibody will then bind to the antigen and elicit a range of effector mechanisms to destroy this foreign molecule. Since the discovery of these "magic bullets" pioneering researchers have looked on them as potential therapeutic agents in a wide range of diseases. As early as 1895 cancer cells were injected into animals, and the resultant antiserum administered to patients suffering from various forms of advanced cancer. Although none of these patients were cured, significant improvements in symptoms were observed. During the following decades, these trials were repeated by many researchers, however results proved inconsistent and contradictory. Antisera being used contained mixtures of different antibodies each being directed against a different antigen on cancer cells, in addition many of these antigens were also present on normal cells, thus use of these antibodies could result in harmful side effects.

A major milestone in antibody development was arrived at in 1975 when Kohler and Milstein received the Nobel prize for their work which reported for the first time the production of virtually unlimited quantities of antibody molecules derived from a single antibody producing cell in which all antibodies were identical and of the same precise specificity for a given epitope. During the last 25 years immunology research has exploded with the availability of thousands of MAbs generated to a vast array of antigens which have been exploited in virtually every branch of biomedical science.

1.1.2. Hybridoma Technology

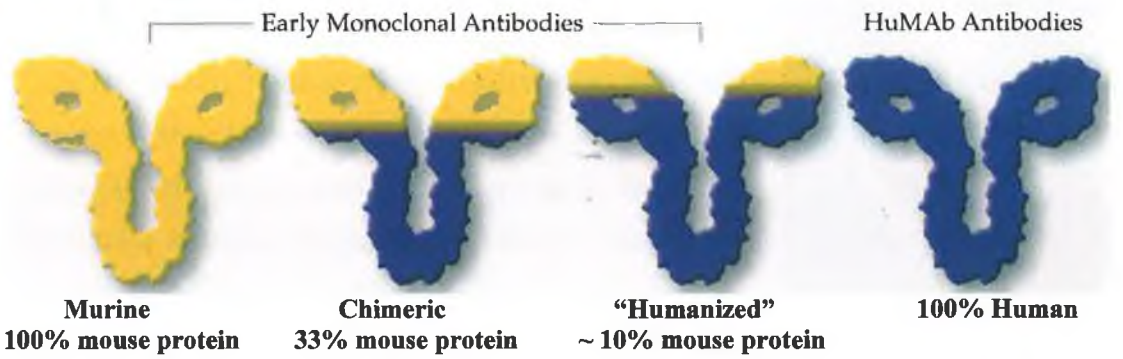


Figure 1.1. : Evolution of antibodies

All hybridoma technology is based on the fundamental Nobel prize winning work of Kohler and Milstein (1975). The evolution of MAbs from 1975 to the present day is represented in Figure 1.1. In 1984 genetic engineering enabled existing murine MAbs to be converted into chimeric MAbs, murine constant regions were replaced with human constant (C) regions (Boulianne *et al.,* 1984, Riechmann *et al.,* 1988, Issacs 1990). These chimeric antibodies however, vary considerably in their immunogenicity when used therapeutically, thus limiting their application in a variety of diseases (Issacs 1990, Kuus-Reichel *et al.,* 1994). Despite this several FDA approved therapeutic MAbs are of this design.

Following on from this, between 1988 and 1991, techniques to successfully humanise murine MAbs were developed where the antigen binding complementarity determining regions (CDRs) are grafted into human V region framework regions (FRs) (reviewed in detail by Clarke, 2000). Framework CDR grafted antibodies are more than 90% human. In addition rodent IgG with human constant regions will have a longer *in vivo* half life. An alternative approach for the generation of fully human MAbs is that of phage display technology. In 1994 Winter *et al.* showed for

the first time this technique could be used to select antigen specific antibodies from libraries made from the spleen cells of immunised mice, thus bypassing the requirement to immortalise the antigen specific B cells as in hybridoma technology (reviewed by Hoogenboom & Chames, 2000).

Another alternative approach to the generation of human MAbs is the genetic manipulation of mice to disable the production of mouse immunoglobulin and to functionally replace them with human antibody producing genes. These *transgenic* mice like the "Xenomouse" developed by Abgenix (CA, US) can then produce MAbs in response to immunisation to a variety of diverse antigens. A lot of success has been achieved with such technology, several high affinity antibodies have been generated (Fishwild *et al.,* 1996, Mendez *et al.,* 1997, Tomizuka *et al.,* 1997, Yang *et al.,* 1999, Tomizuka *et al.,* 2000). Thus using this Xenomouse ™ technology it should be possible to generate high affinity, fully human MAbs.

A human myeloma cell line suitable for the generation of human monoclonal antibodies as a suitable partner for the derivation of human-human hybridomas has recently been described by Karpas *et al.,* (2001). Such a development will ultimately facilitate the isolation of human antibodies which can be used for immunotherapy. The first version of the Human Combinational Antibody library (HuCAL) has also been described, this will allow the rapid and high through-put development of human antibodies (Krebs *et al.,* 2001).

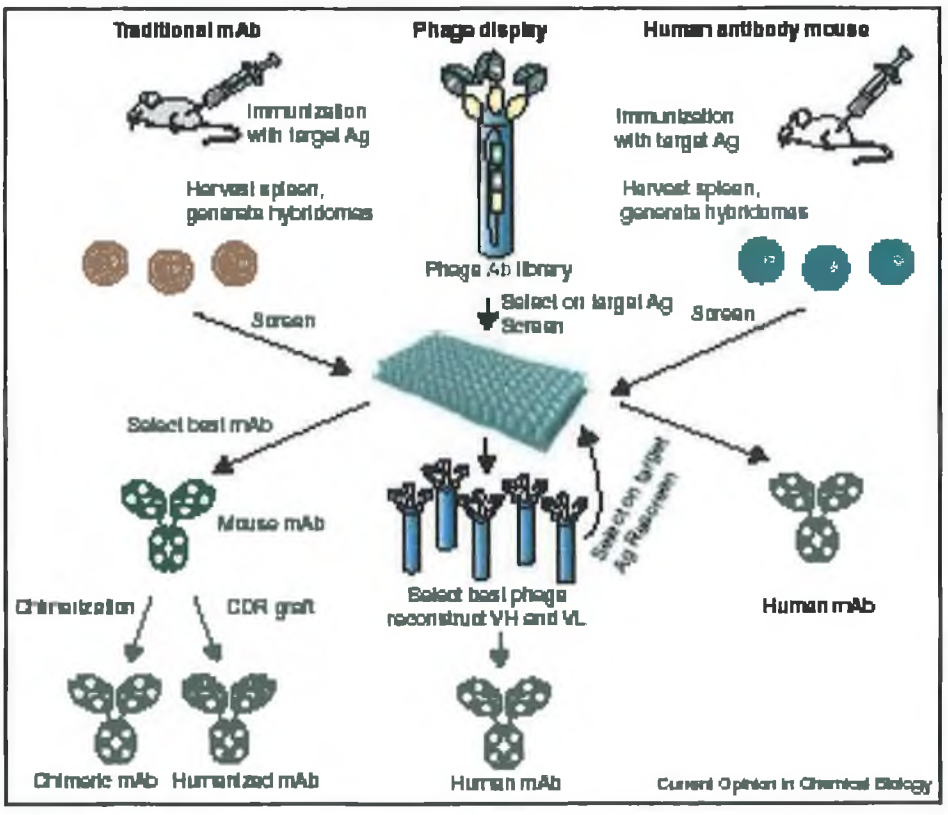


Figure 1.2. Generation of therapeutic human MAbs by obtaining mouse, chimeric and humanised MAbs, via phage display and with the aid of genetically engineered "human antibody" (Ag=antigen, VL=variable light chain)

(Taken from "Next generation therapeutics", Current Opinion in Chemical Biology, 2001; **5**:368-374. Authors, MA van Dijk, JA van de Winkel)

1.1.3. The current state of therapeutic MAbs

As mentioned earlier looking at MAbs as "magic bullets" which potentially can combat tumours and other diseases has challenged scientists for the past 25 years. The first therapeutic MAb used with success was in 1982 when a complete response was observed in a lymphoma patient following administration of a "tailormade"

mouse anti-idiotype (anti-D) MAb (Miller 1982). Despite both academic and commercial interest sparked by this important development, subsequent work has failed to live up to this early success. However the past 5 years have seen a turnaround again thanks to the developments in technologies as has been outlined. MAbs are now emerging as useful adjuncts for cancer therapy.

Antibodies and their derivatives constitute twenty five percent of therapeutics currently in development. Recent developments have helped to reduce or eliminate murine components and fully human MAbs will hopefully become the norm. It is still not fully established exactly how antibodies kill tumour cells; the term antibody dependent cell mediated cytotoxicity (ADCC) is applied widely to describe this process; the recruitment of killer, natural killer and cytotoxic T lymphocytes is also thought to be involved in this process.

The use of MAbs to interfere with cellular processes inside the cell i.e. intracellular signalling is another area of MAb technology which is being exploited for both research and therapeutic purposes. This technology allows antibody genes to be introduced into a cell and expressed to produce intracellular antibody which can then be used to modify cellular processes etc.

Researchers are also looking at a new type of anti-cancer MAb that operates not via a direct effect on the tumour cell but as a result of agonistic interaction with key receptors on cells of the immune system; antigen presenting cells (APCs) or lymphocytes (anti CD-40 and anti CD-137) (Leach *et al.,* 1996, French *et al.,* 1999). Alternatively such antibodies could act in an antagonistic fashion by blocking inhibiting signals delivered by certain receptors (Melero *et al.,* 1997). As a result rapid powerful T cell responses can be stimulated by unknown tumour antigens. The great potential of such reagents lies not only in their ability to augment existing ineffectual responses but in their ability to leave the tumour rechallenged (Glennie & Johnson, 2000). However only data from animal experiments is available yet, it remains to be seen whether this immune stimulation can be achieved in patients.

Another aspect of antibody based therapy for cancer is the use of antibodies to direct cytotoxic agents. Radionucleotides, toxins and pro-drug converting enzymes have all

been conjugated to MAbs and are at various stages of clinical trials. The most promising results have been reported using radioimmunotherapy for lymphoma, where a synergy has been observed between the signalling induced by the anti CD-20 MAb, Ritoxumab and the delivery of β radiation. High response rates were observed in both newly diagnosed patients (Kaminski *et al.,* 1998) and in those patients treated extensively with chemotherapeutic drugs (Press *et al.,* 1995, Kaminski *et al.,* 1996). Preliminary evidence from a trial suggests that this approach is superior to the use of Ritoxumab alone (unconjugated MAb) (Witzag *et al.,* 1999). Enzymes that convert drugs to their active metabolites can be targeted to tumour cells by MAbs; this is known as antibody directed enzyme pro-drug therapy (ADEPT). ADEPT and also immunotoxin therapy are still in the early stages of development so results from patient trials are not available yet.

Earlier therapeutic MAbs were primarily directed to target antigens that were not involved in the regulation of growth of tumour cells. More recently research has revealed that tumour growth may be influenced by the function of discrete oncogene-determined cell surface receptors. The cerbB-2 gene (also known as HER-2/neu or neu) is a proto-oncogene which encodes a transmembrane growth factor receptor (p-185 HER2), which is overexpressed in 25-30% of breast cancer patients. An MAb directed to the oncogene product, Neu, was found to reverse the tumour phenotype *in vitro* and exert antitumour effects *in vivo* (Drebin *et al.,* 1986, 1988). Trastuzumab (Genentech SF, US) is a recombinant DNA derived humanised antibody to HER-2/neu. The exact mechanism of action of this MAb has not been fully elucidated, but this antibody does induce a clear down regulation of the growth factor receptor which may reverse the malignant phenotype. This antibody is known to be capable of activating of a signal transduction pathway that leads to inhibition of tumour cell proliferation and possibly cell death (Goldenberg 1999). Trastuzumab has been shown to be an efficacious adjuvant therapeutic agent for patients with breast cancer who overexpress the Her-2/ *neu* gene (Baselga *et al.,* 1998, Slamon *et al.,* 1998). Recent results have shown that Trastuzumab increases the clinical benefit of first line chemotherapy in metastatic breast cancer in patients whose tumours overexpress HER-2/ *neu* (Slamon *et al.,* 2001).

Along with trastuzumab, Rituximab (anti CD-20) is one of the most successful therapeutic MAbs. Rituximab (or Rituxan) (IDEC Pharmaceuticals Inc, San Diego, CA, US) was discovered in 1990 and was the first MAb approved for the treatment of cancer and the first single agent approved specifically for therapy of lymphoma. Rituximab is a chimeric MAb and is directed against the CD-20 antigen found on the surface of pre-B and mature lymphocytes. This antigen is found on more than 90% of B-cells in non-Hodgkins Lymphoma (NHL), but it is not found on stem cells, pre B cells, normal plasma cells or in other normal tissues. CD-20 is important in cell cycle initiation and differentiation, free CD-20 is not found in the circulation. On binding to its target CD-20, lysis of the B cell takes place, effects on signalling in the cell also take place. Little or no binding has been observed in non-lymphoid tissues. Despite being a chimeric MAb, in a large study of patients treated with a second course of this antibody, none of the 60 patients developed anti-chimeric antibody (HACA) (Davis *et al.,* 1999). Ritoxumab has recently been found to be an active agent for the treatment of follicular cell lymphoma and to be modestly effective in mantle cell lymphoma (Ghielmini *et al.,* 2000). The evidence available to date from clinical trials with these and other FDA approved antibodies suggests these MAbs may have a role in low volume residual disease e.g. when used as adjuvant therapy following surgery to remove primary tumour. It is less likely that they will have a beneficial role in advanced disease.

As well as their role as drugs antibodies can also act as useful tools for molecular characterisation particularly in this "post genomic" era, where more than 40,000 genes have been revealed via the genome map. Antibodies will play a key role in identifying these targets. Estimates from industry suggest that antibody sales will approach $5 billion by 2005. 110 new MAb products are in the developmental stage spanning 50 disease areas from cancer to inflammatory disease. Antibodies represent 16% of drugs in development. The recent clinical success of MAbs is attributable to a number of developments; the identification of specific cell surface markers and molecular targets, the developments in therapeutic MAbs such as CDR grafting to improve therapeutic stability and also in improvements in the methods of production of MAbs. Whether this success will continue with therapeutic and diagnostic MAbs resulting in improvements in the diagnosis and mortality rates of the common diseases remains to be seen.

1.1.4. MAbs as Diagnostics Tools

MAbs directed to a vast array of antigens serve as diagnostic tools in virtually every area of biomedical science. Antibody based immunoassays such as ELISA, radioimmunoassay etc are the most commonly used diagnostic bioassay. Bioassays based on whole cells, receptors and enzymes do not for the most part offer the same unlimited applicability and specificity. Advances in technology have resulted in improved sensitivity, specificity and detection of immunoassays.

Conjugated antibodies may allow recognition of small number of tumour cells in bone marrow or biopsies of tissues that would not be recognised by conventional histological techniques. Specific properties of tumour cells (also other disease conditions) can be identified and quantified using specific MAbs directed to these particular cellular components and thus these MAbs can serve as prognostic/ predictive markers in the diagnosis and management of these diseases. MAbs that recognise antigens that are associated with various cancers or other disease conditions such as autoimmune disorders can be used to detect (and quantify in some cases) the antigen in serum, pleural effusions or tissue biopsies either usually by ELISA or immunocytochemical analysis.

MAbs can therefore play a pivotal role both in routine classification of tumours and also in the diagnosis of tumours of uncertain origin. This area of MAb application is continuously expanding as more and more tumour (and non-tumourogenic) associated antigens are being identified by researchers. MAbs are readily available now for both research and diagnosis that recognise proliferation related antigens, tumour specific markers, apoptosis associated markers, drug resistance pumps (discussed in detail in section 1.6.) and various oncoproteins.

1.1.5. MAbs as immunohistochemical markers

Immunocytochemistry is a well established and widely used technique (in research and routine settings) which enables the localisation of the antigen of interest in cytological as well as histological material at both the light and electron microscope level. Immunocytochemistry plays a vital role in diagnostic tumour pathology when

morphology alone cannot reliably be used to infer the tissue of origin. In addition immunocytochemistry plays a fundamental role in the understanding of the biology of neoplastic growth and has been applied to every aspect of cellular behaviour. This technique involves the use of labelled antibodies as specific reagents for the localisation of tissue components (antigens) *in situ* and can be performed on either frozen sections (fresh tissue that has been cut using a cryostat) or routinely processed formalin fixed paraffin wax embedded archival material.

Formaldehyde is the most widely used fixative in routine histopathology and has become the gold standard for morphological tissue preservation; although other fixatives are sometimes used including Bouins fixative and para-formaldehyde (Merz *et al.,* 1995). Immunostaining of cryostat sections results in a great loss in tissue morphology compared to routinely fixed material where resulting morphology is a lot superior. In addition the availability of fresh frozen tissue can be a problem. However although archival material should be the most useful option; many routinely used antibody markers cannot be detected in routinely processed archival sections. The reason for this is because the determinants recognised by these antibodies may be masked or denatured by the fixation and processing. The molecular mechanisms underlying tissue fixation are not fully understood, it is clear that available immunoreactive sites are progressively lost during the fixation process. Loss of antigen expression when using certain antibodies e.g. in the assessment of P-glycoprotein expression levels using the C219 MAb, has been observed in formalin fixed paraffin embedded tissue sections when compared to frozen tissue sections from the same cell lines and origins (Cordon Cardo *et al.,* 1990).

Researchers have attempted to achieve signal amplification with an acceptable signal to noise ratio in an attempt to address this problem (Merz *et al.,* 1995). More successful results have been achieved using various antigen retrival methods in an attempt to reveal these cross linked proteins. Initially enzyme treatments with trypsin or pronase treatment were investigated, however such methods proved difficult to standardise even within individual laboratories and reproducibility was poor (Huang *et al.,* 1976).

Microwave or pressure cooker based heat treating methods have been applied with some success although several modifications of the basic method may have to be employed to ensure optimum antigen retrival (Cattoretti *et al.,* 1993). In general microwave based heat treatment of MAbs does appear to enhance tissue antigenicity while preserving tissue morphology, although positive staining should be interpreted carefully particularly in the case of new or previously untested MAbs (Cuevas *et al.,* 1994). A new generation of immunohistochemical markers (e.g. Ki-67, Bcl-2, p53, PCNA) are now available for clinical studies of archival tumours thanks to these retrieval methods. However it has been reported that such aggressive techniques may influence immunolocalisation of antigens or in some cases cause detachment of tissue (Cuevas *et al.,* 1994, Szekeres *et al.,* 1994). In addition there is considerable scope for intralaboratory variation.

With these constraints in mind researchers have attempted where possible to develop MAbs that perform well on routinely processed archival tissue i.e. detect "formalin resistant" antigen thus avoiding the use of often aggressive and sometimes inconsistant antigen retrival techniques.

1.2. Tumour specific antigens

Presentation of a certain antigen can be indicative of the histogenesis (i.e. histological origin) of a tumour. Expression of particular antigens can also be helpful in the identification of poorly differentiated tumours of unknown origin and invaluable in the determination of the correct origin of poorly differentiated tumours. A wide range of both polyclonal and monoclonal antibodies are commercially available that react with antigens which are products of embyronic and adult tissue differentiation. Markers of tumour cell differentiation that are commonly used in the diagnosis of tumours include tissue specific proteins such as S-100 (gliomas, melanomas and schwaanomas), prostate specific antigen (PSA), prostatic acid phosphatase (prostate tumours), carcinoembryonic antigen (CEA) (distinquishes adenocarcinomas from mesothelioma), alpha feto protein (hepatocellular carcinoma, ovarian and testicular germ cell tumours), leucocyte common antigen (distinquishes haematopoietic malignancies from non-haematopoietic tumours) and myoglobin

(striated muscle tumours). Intermediate filaments that function as routine markers include cytokeratin (carcinomas, mesotheliomas), vimentin (tumours of mesenchymal origin/ lymphomas/ poorly differentiated carcinomas) and glial fibrillary acidic protein (gliomas, distinguishes glioma from other brain tumours including metastatic ones). Antigenic markers of hormones and related products can also be used, e.g. hormones related to pancreatic islet cells and thyroglobulin are used in the characterisation of islet cell tumours and the diagnosis of neoplasms of thyroid origin respectively, see True (1990) for more detail. (Breast cancer associated antigenic markers will be discussed in section 1.4.2. and 1.4.3)

1.3. Identification of novel antigens/ proteins

Identification of novel/ new tumour specific antigens that may have a role as potential predictive or prognostic markers is a constant challenge for researchers, which has been made easier with the advent of the new MAb technologies outlined above. Various sources of cancer antigens (lymphocytes from metastatic lymph nodes, solublised solid tumour, tumour cell lines etc.) have been used to generate MAbs directed to these components. Many antigens that have been associated with tumours originating from specific organs have been detected with such MAbs, however the isolation of antigens with absolute specificity for a particular type of tumour has proved much more difficult. The first tumour specific antigen was reported by Thierry Boon and his colleagues who described the MAGE-1 antigen in malignant melanoma; this antigen in conjunction with other more recently described melanoma specific antigens has proved useful in the classification of specific melanocytic lesions (van der Bruggen *et al.,* 1991, Xu *et al.,* 2002). Using T cell lines several human tumour antigens have been defined at the molecular level (the majority being malignant melanoma specific antigens) (Boon *et al.,* 1994, Rosenberg, 1997, Xu *et al.,* 2002). Newer strategies such as SEREX (serological identification of antigens by recombinant expression cloning) have been developed that can identify tumour antigens from antibody repertoires of cancer patients. These antibody repertoires are comprised of cDNA libraries constructed from fresh tumour samples, packaged into lambda phage vectors and expressed recombinantly in *e.coli* (Sahin *et al.,* 1997). Tumour antigens identified using this technique include glioma

associated antigens in astrocytoma patients and cancer-testis antigens in gastrointestinal/ breast cancer patients (Mashino *et al.,* 2001, Schmits *et al.,* 2002).

The identification of novel antigens has been facilitated by a number of new technologies availability for the identification and characterisation of proteins in cells and tissues. The term "proteomics" which denotes all of the proteins expressed by a genome was first coined in late 1994 at the Siena 2D Electrophoresis meeting. "Proteomics" is proclaimed as the next step after genomics, the ultimate goal will be to assemble a complete library of proteins. Several new technologies are facilitating this high throughput protein identification and discovery. Tissue cell populations can be isolated using either Affinity Cell Sorting of disaggregated cells from pieces of fresh tissue (Page *et al.,* 1999) or alternatively by Laser Capture Microdissection (LCM) which procures specific tissue cell populations under direct microscopic visualisation of a standard frozen or fixed tissue section (Banks *et al.,* 1999). Isolated cells using both of these methods can then be used for high sensitivity protein analysis using 2-D gels. "Proteomes" from a range of tissues (normal epithelium, benign and dysplastic states, *in situ* cancer, malignant and invasive cancers) can then be used to select antibodies from phage display libraries. These antibodies can then be spotted onto chips and characterised by SELDI mass spectrophotometry. These technologies have enabled for the first time researchers to analyse proteins from every stage of the cancer process (Borrebaeck 2000, Le Naour *et al.,* 2001).

1.4. Breast Cancer

Despite the ever increasing efforts to fully understand the disease process, the incidence of cancer in the population is rising. As a cause of mortality overall in the Western World cancer is second only to cardiovascular disease. Statistics from a recently published joint publication by the Irish Cancer Registry and the Northern Ireland Cancer Registry reveal that breast cancer was after skin cancer, the commonest form of cancer in women and the most common cause of cancer deaths in females. On average each year 2368 new cases of malignant breast cancer are diagnosed in Ireland. (All Ireland Cancer Statistics 1994-1996).

The management of breast carcinoma has changed considerably over the past twenty years. The number of available therapeutic options has widened considerably and there are an increasing range of hormonal, cytotoxic and more recently MAb targeted drug regimes available which can be used in both the adjuvant and *neo*-adjuvant settings. However despite the advances both in the detection and in the treatment of the disease between 40 to 50% of patients diagnosed will eventually die of the disease. It is vital thus to determine the optimal treatment modality for each individual patient and identify sub groups of women for various treatment strategies. Identification of biologic markers that can predict clinical outcome of the disease (*prognostic* markers) and the likelihood of a response to a particular type of adjuvant therapy (*predictive* markers) will facilitate this. There is a constant search for new markers of both prognosis and chemoresponsiveness.

1.4.1. Classification of Breast Cancers

1.4.1.1. non-invasive breast cancers

The majority of breast cancers are adenocarcinomas arising from the epithelium of either the mammary lobules (lobular cancer) or the mammary ducts (ductal cancer). Often invasive breast cancers may be preceded by cancer *in situ*. In some cases the development of invasive disease may be preceded by cancer *in situ* in which the malignant cells proliferate within the ducts or lobules but do not breach the basement membrane (intra ductal cancer or intralobular cancer). Intraductal cancers tend to be more common than intralobular ones.

Ductal carcinoma *in situ* (DCIS) is defined as a proliferation of epithelial cells with cytological features of malignancy within parenchymal structures of the breast and is distinguished from invasive carcinoma by the absence of stromal invasion across the basement membrane. DCIS varies in cell type, growth pattern and extent of disease and may thus represent a group or spectrum of related *in situ* neoplastic processes. Despite the name, most DCIS is thought to arise from the terminal *lobular* units. Previous classifications of these tumours were based on growth patterns, however evidence has emerged that lesions composed of cells of high nuclear grade are more

aggressive (Lagois 1990, Bellamy *et al.,* 1993). Thus, the classification system currently used is based on the nuclear grading of DCIS, tumours are classified as high, intermediate and low grade (Holland *et al.,* 1994). *High nuclear grade* DCIS may exhibit several growth patterns, it may be solid with central comedo type necrosis or may exhibit micropapillary or cribriform patterns again often with central comedo like necrosis (refer to "Pathology Reporting in Breast Cancer Screening", National Coordinating Group for Breast Screening Pathology. NHSBSP Publications, Second edition, 1997).

In *low grade DCIS* the cells are generally arranged in micropapillary and cribriform patterns which are frequently present within the same lesion. Less frequently a solid growth pattern is seen. *Intermediate nuclear grade DCIS* may exhibit a solid, cribriform or micropapillary growth pattern. Comedo type and high nuclear grade in general have a more aggressive clinical course than other DCIS lesions.

Lesions are also classified according to the *Van Nuys Prognostic Index (*VNPI), the histological type, width of the excision margins and tumour size are each assigned a score of 1, 2 or 3. These scores are then added to produce the VNPI which represents a cumulative score ranging from 3-9.

Lobular carcinoma *in situ* (LCIS) lesions arise when the entire cell population of the lobular units consist of characteristic small, rounded cells with granular or hyperchromatic nuclei, inconspicuous nucleoli and high nucleo-cytoplasmic ratio. Unlike DCIS variability of growth pattern is not observed in LCIS

1.4.1.2. invasive breast cancers

'Ductal' no specific type (Ductal – NST)

Invasive tumours originating in the ductal system which exhibit great variation in appearance represent approx. 75% of invasive breast tumours. Pure examples of the following variants constitute only a small percentage of the total number; certain features of each are seen within the main portion of commonly presented invasive/ infiltrating ductal carcinomas.

Infiltrating Lobular Carcinoma

These carcinomas are composed of small regular cells identical to those seen in the *in situ* variant. In its classical form, the cells are dissociated from each other or form single files or targetoid patterns around uninvolved ducts. Other variants include alveolar, solid, tubulo-lobular, pleomorphic and mixed type.

Tubular Carcinoma

These carcinomas are composed of round, ovoid, or angulated single layered tubules in a cellular fibrous or fibro-elastotic stroma.

Invasive Cribriform Carcinoma

These carcinomas are composed of masses of small regular cells similar to those seen in tubular carcinoma. The invasive islands, however exhibit a cribriform rather than a tubular appearance.

Medullary Carcinoma

These rare tumours are composed of syncytical interconnecting masses of large pleomorphic cells with vesicular nuclei and prominent nucleoli. The border of the tumour is well defined and surrounded by a dense chronic inflammatory cell infiltrate, *in situ* components are uncommon.

Mucinous Carcinoma

These tumours are also known as mucoid, gelatinous or colloid carcinoma. They comprise of uniform small cells in lakes of extracellular mucin, *in situ* components are uncommon.

Mixed tumours of the above can also arise.

(Refer to "Pathology Reporting in Breast Cancer Screening", National Coordinating Group for Breast Screening Pathology. NHSBSP Publications, Second edition, 1997).

1.4.1.3. Grading of invasive Breast cancers

The Nottingham method of tumour *grading* is now widely used both in the UK and the US. This system is based on the evaluation of the following histological features: glandular (tubule) formation, nuclear pleomorphism and mitotic count. Following scoring tumours are graded into well differentiated tumours (grade I), moderately differentiated (grade II) and poorly differentiated ones (grade III).

The most commonly used *staging* system for breast cancers is the TNM system recommended by the International Union against Cancer (UICC) which is based on tumour size (T1 – T4), nodal status (N0 –N3) and the presence or absence of distant metastasis (M0-M1).

1.4.2. Established prognostic/ predictive markers in Breast Cancer

Diagnostic histopathology provides for a substantial amount of useful prognostic information from routine analysis of breast tumour samples (Galea *et al.,* 1992). Tumour size is considered to be the most valuable prognostic factor in breast carcinoma and is an important factor in breast cancer screening programmes, lymph node status is also very important (Elston *et al.,* 1999). A recent review of the value of the various prognostic factors in node negative breast cancer concluded that only tumour grade and tumour size remain the only markers which may be clinically useful for these patients; lack of standardisation in measurement techniques for many of the other breast cancer markers limited their current clinical usefulness (Mirza *et al.,* 2002). Generally patients with smaller tumours have a better long term survival than those with larger tumours. Tubular, mucinous, invasive cribriform, medullary and tubular lobular tumour types have all been reported to have a more favourable outcome than invasive carcinoma of no special type (ductal NST). Unusual tumour types such as adenoid cystic carcinoma, adenomyoepithelioma and low grade adenosquamous carcinoma also have been reported to show a more favourable outcome (Elston *et al.,* 1999).

Even though assessment of histological subtype provides a certain amount of prognostic information the effect is shown to be relatively small when evaluated in multivariate analysis (Pereira *et al.,* 1995). Histological grade is considered to be a more powerful prognostic factor. Several studies have confirmed the strong correlation between histological grade and long term survival (reviewed by Henson *et al.,* 1991, Elston *et al.,* 1998). Lymphovascular invasion has shown to be a marker of local recurrence (Elston *et al.,* 1998).

Several studies have shown that patients who have histologically confirmed lymph node involvement have a significantly poorer prognosis than those without nodal metastases (Carter *et al.,* 1989, Galea *et al.,* 1992, Veronesi *et al.,* 1993). The greater the number of nodes involved the worse the prognosis is; in addition metastasis to "higher" level nodes in the axilla or apex is associated with a worse outcome (Veronesi *et al.,* 1993). A newer method for the classification/ staging of axillary lymph nodes in breast cancer is now being employed which is a minimally invasive diagnostic procedure; sentinel node mapping or sentinal lymphonodectomy (the sentinel node is the first lymph node encountered by lymphatics draining from tissues around a tumour). Methods for identifying the sentinel node include radiotracer and blue dye mapping have been advocated by some researchers (Elston *et al.,* 1999, Hill *et al.,* 1999, Linehan *et al.,* 1999).

The significance of the presence of metastatic carcinoma in the adipose tissue surrounding axillary lymph node known as the extra nodal spread or extracapsular metastasis (ECM) is still not clear, studies have shown conflicting results regarding its prognostic significance however it is generally considered a worse prognostic factor in some tumour types (Cascinelli *et al.,* 1987, Donegan *et al.,* 1993, Hetelekidis *et al.,* 2000).

Assessment of vascular invasion (i.e. the presence of tumour emboli in vascular spaces) using immunohistochemical endothelial markers such as CD31 and CD34 has also been used as a potential prognostic marker in breast carcinoma. Vascular invasion has been shown to be a predictor of early recurrence, however its most important prognostic role is thought to be in the prediction of local recurrence (Pinder *et al.,* 1984, Sapino *et al.,* 2001).

Other morphological characteristics of breast carcinoma that may be prognostically significant include the presence of tumour necrosis, stromal fibrosis and stromal elastosis. However although there is some published data regarding a possible prognostic role for these factors, available results are conflicting and larger studies are required to make any conclusion (reviewed by Elston *et al.,* 1999).

It is well established that the presence of estrogen receptor (ER) (ER alpha) can be used to predict response to endocrine therapy in patients with invasive breast cancer. Receptor negative patients do not respond as well to endocrine therapy (tamoxifen) as those patients which are found to be receptor positive. Recent studies have focused on the possible predictive role of ER beta in breast cancer (Jensen *et al.* 2001). Presence of ER is not an independent prognostic factor due to its close relationship with histological grade (Galea *et al.,* 1992). The combination of the assessment of progesterone receptor (PgR) status as well improves the predictiveness (reviewed by Allred *et al.,* 1998).

1.4.3. MAbs raised to breast associated antigens

Over the years several MAbs, both human and murine have been generated using various types of immunisation schedules (use of live cells, membrane preparations, purified molecules) against several breast cancer associated antigens in an attempt to isolate MAbs which would recognise breast tumour specific antigens. Such antibodies could have important clinical diagnostic applications. Although the overall goal has always been to identify antigens that are expressed exclusively by breast tumour cells, in practise this has not tended to be the case; most of these breast associated antigens have been found to be expressed to a varying degree by normal non tumourogenic breast cells and by other tumour cell types. This was the case with most of the earlier work in this area with the generation of several murine MAbs which did not prove to recognise breast cancer specific antigens. However some MAbs were successfully shown to be reactive with specific breast cancer associated antigens, and in addition a small number of these MAbs have been to have potential prognostic significance when screened on patient material. Breast cancer associated

antigens identified in this way include the tumour marker CA15-3 which is recognised by the MAb, DF3 which has shown to have prognostic significance for disease free interval and the nmt55 nuclear protein. Loss of expression of this antigen has been shown in estrogen receptor negative human breast cancer (Traish *et al.,* 1997, Dimas *et al.,* 2000). Many of the MAbs raised to breast cancer associated antigens have been shown to recognise high molecular weight glycoproteins (300-400 to over 1000 KDa). Members of this family include the MAbs B72.3 and CC49 which recognise the tumour associated glycoprotein, TAG-72, which is a 220-400 KDa glycoprotein and MAb 83D4, which was generated against a cell suspension from a paraffin block of human breast carcinoma tissue, and detects a large group of heterogenous of 300- 1000 KDa proteins (Colcher *et al.,* 1981, Pancino *et al.,* 1990, Charpin *et al.,* 1992). A second generation MAb LU-BCRU-G7 which was raised against a 230 KDa novel glycoprotein has been shown to have significant association with early stage breast carcinoma (Rye & Walker 1994). A more recently described novel circulating tumour antigen in breast cancer which was identified using proteomics based approaches is RS/DJ-1, a novel oncogenic protein which regulates RNA protein interaction (Le Naour *et al.,* 2001).

With the advent of human MAb technologies it was hoped that more specific antibodies could be generated and that the reactivity of such antibodies would more closely mimic the *in vivo* response of the malignant cell. However problems associated with the production of human MAbs such as the lack of stable clones and the isolation of IgM isotypes have meant that very few human breast cancer specific antibodies have been produced to date. Hopefully this has started to change with the improvements in the generation of human antibodies as previously discussed. Again there has been some success in the area, antibody JDB1 was generated by fusion of a human lymphoma cell line and peripheral blood lymphocytes from a breast cancer patient (Strelkauskas *et al.,* 1994). This MAb has been shown to identify metastatic infiltrating ductal carcinoma in both axiliary nodes and skin and normal breast. Other malignancies did not show any reactivity with this antibody.

1.4.4. Novel prognostic/ predictive markers in Breast Cancer

1.4.4.1. Miscellaneous cellular markers

Over the years in an attempt to identify possible markers for both the prognosis and chemoresponsiveness of breast cancer; a wide variety of novel variables have been proposed as possible prognostic factors. The majority of these are associated with mechanisms of differentiation, invasion, metastasis or growth rate of the tumour (Elston *et al.,* 1999).

There are several antigenic markers that provide useful diagnostic information in breast cancer but do not appear to be prognostically important. Metallthionein is a protein which is involved in cellular repair, growth and differentiation. Its expression is associated with early phase breast cancer (Iochim *et al.,* 1999). Gelsolin is an actin filament regulatory protein; altered gelsolin expression appears to be involved in progression from normal epithelium to DCIS to invasive breast cancer (Asch *et al.,* 1999). Expression of CD24, a small mucin like glycosylphosphatidylinositol-linked cell surface marker has been suggested as a possible useful marker for breast carcinoma, the intensity of CD24 positivity has been shown to increase with the histological grade of the tumour (Fogel *et al.,* 1999). Recent work suggests that frizzled related protein (Frp) may have a possible prognostic role in invasive breast cancer (Wong *et al.,* 2002).

T1-S, an oncogene inducible, secreted glycoprotein of the immunoglobulin family has been shown to be a novel and independent tumour biological factor possibly associated with reduced progression of lymph node negative breast cancer (Prechtel *et al.,* 2001). Sialyl-Tn (STn), a carbohydrate antigen formed by the premature 2-6 sialylation of N-acetylgalactosamine has been shown to be a marker for short term prediction of breast cancer (Leivonen *et al.,* 2001). Expression of *Fas* protein has been associated with aggressive tumour behaviour in locally advanced breast carcinomas (Pernick *et al.,* 2000).

Complete loss of the breast cancer susceptibility gene, BRCA1 nuclear protein expression (mutation of this gene has been reported in 80% of familial breast cancer)

and its correlation with other poor prognostic markers suggest that its expression may play an important role in the pathogenesis and prognosis of sporadic breast cancer (Lee *et al.,* 1999). Expression of epithelial mucins including MUC1 are thought to be related to the progression of malignant disease (Devine *et al.,* 1992, Croce *et al.,* 1997) and proteases such as Cathepsin D may have prognostic significance in this malignancy (Tetu *et al.,* 2001).

The matrix metalloproteinases (MMPs) are a family of zinc dependant endopeptidases. It is thought that MMPs are involved in breast cancer initiation, invasion and metastasis. MMP-9 overexpression may be an independent prognostic factor in node negative breast cancer (Scorilas *et al.,* 2001). MMP-2 has been shown to offer prognostic value in certain node negative post menopausal patients (Talvensaari-Mattila *et al.,* 2001). Stromelysin-3 has been shown to be a strong independent prognostic factor in node negative patients (Ahmad *et al.,* 1998).

Numerous studies have looked at the expression of another protease, urokinase plasminogen activator (uPA) which can promote tumour invasion, tissue type PA (tPA) and antiproteases including PAI-1 and PAI-2 (Bouchet *et al.,* 1994). Reduced tPA mediated plasmin production has been shown to be an independent adverse prognostic factor in breast cancer (Chappuis *et al.,* 2001). Studies have revealed that evaluation of uPAR the cellular receptor of uPA may serve as predictor of overall survival in breast cancer and as an indicator of recurrence in DCIS (Guyton *et al.,* 2000, Pacheco *et al.,* 2001). In a recent report, uPA and PAI-1 have been shown to have independent prognostic value in lymph node negative breast cancer (Look et al., 2002).

Immunohistochemical expression of cell adhesion molecules such as E-cadherin and α_2 and β_1 integrins (Gonzalez *et al.,* 1999) has also been evaluated. It has been reported that cytokeratin 18 expression indicates a favourable prognosis in breast cancer patients (Schaller *et al.,* 1996). More recently it has been observed that the presence of cytokeratin positive cells in the bone marrow increases the risk of relapse in patients with breast cancer (Braun *et al.,* 2000). The expression of luminal epithelial antigen (LEA-135) has been shown to be an independent and favourable

prognostic marker in invasive primary breast cancer; its expression appears to override the adverse effects of certain oncogenic and cell proliferation associated markers (Liu *et al.,* 2000). CEA immunoreactivity was not shown to be prognostically relevant in a large series of infiltrating breast cancers (Mauri *et al.,* 1998).

1.4.4.2. Proliferation markers

Cell proliferation constitutes a major determinant in the biological behaviour of a tumour. DNA content and proliferative activity of breast tumours have been investigated extensively using various methods including measurement of mitotic index, S phase fraction (SPF) (by flow cytometry) and expression of various proliferation associated markers such as Ki-67 (MIB-1), PCNA and Topo II α, in an attempt to address the possible prognostic and predictive significance of these parametres. It is well recognised that the proliferative activity of a given tumour is an important prognostic factor in primary breast cancer; various markers of proliferation have shown that high proliferative activity is directly related to poor clinical outcome (Clarke *et al.,* 1996).

The most widely investigated proliferation associated marker is the Ki-67 antigen which is expressed during the G1, S, G2 and M phases of the cell cycle and not by non-cycling cells in G0 (Gerdes *et al.,* 1984). Expression of the Ki-67 antigen is considered to be a more accurate marker of proliferation than PCNA which has a longer half life, in addition there are currently Ki-67 MAbs available that recognise formalin resistant epitopes and thus can be used to screen formalin fixed paraffin embedded archival material. Several studies have correlated poor clinical outcome with expression of Ki-67 (Veronese *et al.,* 1996, Ruldoph *et al.,* 1999a,1999b).

Measurement of proliferative activity using Topo II α specific MAbs such as antibody Ki-S4 has shown to be a useful indicator of prognosis in breast carcinoma (Ruldoph *et al.,* 1999b, 1999c). Increased Topo II α mRNA expression has been shown to accompany tumour progression in breast cancer, in the same study there was no difference in expression of this protein in relapsed and non-relapsed patients (Ito *et al.,* 1998). Recent studies have revealed that increased Topo II α protein

expression has been shown to be associated with an aggressive form of breast cancer and cellular dedifferentiation and also predicted disease related death, lymph node metastasis and advanced tumour stage (Depowski *et al.,* 2000, Nakopoulou *et al.,* 2000).

Mitosin is a recently described 350kDa nuclear phosphoprotein that is expressed in the late G1, G2 and M phases of the cell cycle and is not expressed in G0. Preliminary immunohistochemical data suggest that the value of mitosin expression may be superior to that of SPF in its prognostic value (Clarke *et al.,* 1997).

Caspase–3 is a prominent member of the CED-3 family of cysteine proteases which act as intracellular effectors of apoptosis. Increased Caspase–3 positive staining has been observed in both *in situ* and infiltrating breast tumours compared to normal mammary epithelium; the prognostic significance of caspase-3 expression in breast tumours has not been addressed (Krajewski *et al.,* 1999).

Many studies have suggested that investigation of proliferation markers and certain oncogenes such as c-erbB-2 or p53 or other prognostic markers (e.g. receptor status) may provide more prognostic information than proliferative activity alone (Niewiadomska *et al.,* 1998, Wiesener *et al.* 1998, Ruldoph *et al.,* 1999c).

1.4.4.3. Oncogenic markers

Overexpression / activation of genes that promote cellular transformation, tumour growth and/ or dissemination i.e. oncogenes have been detected in a significant proportion of breast tumours (Berns *et al.,* 1992).

Dysregulation of programmed cell death i.e. apoptosis mechanisms plays an important role in the pathogenesis and progression of breast cancer as well as in the responses of tumours to therapeutic intervention and the overall survival of breast cancer patients. Increased apoptotic index has been associated with high tumour grade, large tumour size, DNA aneuploidy, high S phase, high mitotic rate, lack of ER and shorter disease interval and overall survival. It is well established that

increased apoptosis is associated with a worse prognosis in breast carcinoma (Vakkala *et al.,* 1999). Recently apoptosis has also shown to be induced by *neo* adjuvant chemotherapy (also referred to as primary chemotherapy or *pre* operative chemotherapy). *Post* chemotherapy apoptotic index has shown to be correlated with clinical response and increased relapse free and overall survival of patients treated in this fashion (Shao *et al.,* 1999). Interestingly, medullary carcinoma of the breast (which exhibits a high mitotic rate and shows an anaplastic histology) in general shows a better prognosis than ordinary ductal carcinoma, a high frequency of apoptosis in such tumours may be related to a favourable prognosis in these patients (Kajiwara *et al.,* 1999).

Apoptosis is controlled/ regulated positively and negatively by the products of different genes. Overexpression of genes that inhibit apoptosis may play not only in the etiology of various tumours including those of mammary origin, but also in tumour progression. One of the first of these anti-apoptotic/ survival genes to be identified was the *bcl-2* gene. At least 17 *bcl-2* family members have been identified (in mammalian cells and viruses) which show sequence homology with *bcl-2* but possess different functions in the regulation of apoptosis (reviewed in Reed 1998). Interestingly, expression of *bcl-2* is generally associated with favourable prognostic indices and thus with a favourable outcome in breast cancer. However there have also been reports demonstrating that expression of *bcl-2* does not always predict a favourable clinical outcome (Silvestrini *et al.,* 1994). Bcl-2 immunostaining has been correlated with ER and PgR in several studies (Leek *et al.,* 1994, Silvestrini *et al.,* 1994, Jalava *et al.,* 2000). Such Bcl-2 negative patient groups may therefore benefit from additional treatment with anti estrogen therapy. Bcl-2 positive patients (node positive and negative) have shown a better prognosis and an overall better survival rate compared to Bcl-2 negative patients, these studies have included uniformly treated patients with node negative, node positive or metastatic disease (Joensuu *et al.,* 1994, Silvestrini *et al.,* 1996, Zhang *et al.,* 1998). Lack of Bcl-2 expression was independently associated with a poor prognosis in patients with higher grade tumours who were treated with primary chemotherapy, in the same study p53 expression was a independent prognostic marker of relapse free survival (Bottini *et al.,* 2000). Bcl-2 appears to be associated with good prognostic markers both in invasive breast cancer and DCIS tumours (Rehman *et al.,* 2000).

The exact reason of how *Bcl-2* expression is associated with a favourable outcome is not clear but the anti-proliferative effect of *Bcl-2* maybe involved. Alternatively the presence of *Bcl-2* may represent a marker of tumours that have arisen by a less aggressive genetic pathway involving a dependence on steroid hormones (Krajewski *et al.,* 1999). It appears that Bcl-2 immunopositivity may represent a more treatable form of breast cancer, and is therefore associated with longer survival in patients with node negative, node negative and metastatic disease.

There are other genes that promote apoptosis such as wild type *p53* and *c-myc*, Mutant *p53* suppresses apoptosis. It has been reported that the *bcl-2* and *c-myc* oncogenes may act in a synergistic fashion in the promotion of the metastasis process in breast tumours (linked to loss of apoptosis) (Sierra *et al.,* 1999). Expression patterns of the tumour suppressor gene, *p53* have been widely studied in breast cancer. p53 is a transcription factor that participates in cell cycle checkpoint processes and apoptosis. Positive p53 (mutated) immunostaining appears to correlate with larger tumour size, higher proliferation rate, oncogenic amplification and in some studies the absence of hormone receptors (reviewed by Dhingra and Hortobagl 1996). The overall prognostic role of mutated *p53* in breast cancer is still not clear; largely due to discrepancies between p53 immunostaining and single strand confirmation polymorphism (SSCP)/ DNA sequencing methods. A recent study has revealed that increased expression of Bcl-2 as well as p53 is associated with a decreased risk of local recurrence, an increased risk was associated with increased expression of MIB-1 (Jager *et al.,* 2000). p53 immunostaining in primary breast tumours was found to an independent prognostic factor associated with relapse free survival. There appears to be an inverse relationship between Bcl-2 and p53 positive staining in breast cancer (Krajewski *et al.,* 1997). Co-expression of c-erbB-2 and p53 has been shown to be of independent prognostic value (Beenken *et al.,* 2001).

It has been observed that low levels of p27Kip1 and apoptotic index strongly correlated with presence of lymph node metastasis and decreased patient survival; in node negative patients p27Kip1 also had prognostic value for relapse free and overall survival as well as having predictive value for chemotherapy (Wu *et al.,* 1999).

Less is known regarding the role of the other members of the *bcl-2* family in breast cancer. The ratio between the *pro* apoptotic gene *Bax* and *bcl-2* appears to be an important determinant of cellular sensitivity to induction of apoptosis in breast carcinoma. Indeed the exact interaction of all of these regulatory apoptosis remains has not been elucidated to date. However it appears that the ratios of survival versus death related proteins in cells are important as these ratios then function as determinants of either the induction of apoptosis or the promotion of cell survival in response to various insults including that of cytotoxic drugs. Studies of *Bax* expression in breast cancer have revealed that loss of Bax immunostaining represents a prognostic indicator of poor response to therapy as well as reduced survival in women with metastatic disease who are treated with combination chemotherapy (probably due to its role in promoting apoptosis in response to genotoxic injury induced by anti cancer drugs) (Krajewski *et al,* 1995a, Kapranos *et al.,* 1997). The prognostic role of Bax in breast cancer appears to be limited to chemotherapy treated patients although it has been observed that Bax (also Bcl-xl) immunostaining predominates in higher histological grades of ductal carcinomas (grade 111) compared to grade 1/ 11 where Bcl-2 and Bak were predominantly expressed (Sierra *et al.,* 1998). There appears to be no correlation between Bax and p53 immunostaining (Veronese *et al.,* 1996, Yang *et al.,* 1999).

It has been reported that expression of the *pro* apoptotic protein, Bak was associated with high apoptotic rate, high tumour grade, overexpression of p53, expression of ER and low expression of Bcl-2, however no correlation between Bak expression and clinical outcome has been reported (Krajewski *et al.,* 1994, 1995b, Rochaix *et al.,* 1999).

Bcl-xl and *Mcl-1* are anti-apoptotic members of the *Bcl-2* family which have been implicated in cancer chemoresistance (Reed 1997). No correlation of *Mcl-1* expression with either tumour progression or clinical outcome has been reported (Krajewski *et al.,* 1994, Krajewski *et al.,* 1995b). In contrast expression of *Bcl-xl* is commonly reported in breast cancer where intense Bcl-xl positivity has been observed in malignant cells compared to normal epithelia, suggesting a possible prognostic role. Like *Bax* the prognostic role of *Bcl-xl* does not apply to early stage disease which is treated with surgery or radiation, the expression of *Bcl-xl* in

metastatic patients treated with chemotherapy has not been investigated (Krajewski *et al.,* 1994, 1999, Olopade *et al.,* 1997).

The heat shock protein, *Bag-1* (not homologous to *bcl-2*) has an anti-apoptotic function and interacts with several other proteins including Bcl-2 (which it enhances the anti-apoptotic function of), the mechanism whereby it interacts with bcl-2 to suppress apoptosis is not fully understood (Takayama *et al.,* 1995). *Bag-1* appears to be expressed in a majority of breast cancers; this expression varies among patients. Preliminary results indicate that Bag-1 immunostaining, particularly nuclear staining may provide prognostic information in early stage breast cancer; higher levels of Bag-1 appear to be associated with a shorter disease free interval, and shorter overall survival in these patients (Tang *et al.,* 1999). Immunohistochemical expression of Bag-1 was not shown to correlate with either bcl-2 or bcl-xl expression. Breast tumours showed high expression of two isoforms of Bag-1, p46 and p33 in the same study (Yang *et al.,* 1999). As there appears to be no correlation between Bag-1 expression and conventional prognostic indices (except the degree of differentiation), it may constitute an independent predictive factor in breast cancer independent of *bcl-2* and *bcl-xl.* Preliminary results published recently suggest that Bag-1 represents a potential marker of improved survival in early stage breast cancer patients independent of the status of axillary lymph nodes (Turner *et al.,* 2001).

c-myc amplification is detectable in approximately 20% of breast tumours; its expression has been shown to correlate with poor tumour differentiation, inflammatory carcinoma and loss of PR, and its overexpression has in some studies been associated with an adverse prognosis (Berns *et al.,* 1992, Chrzon *et al.,* 2001, Naidu *et al.,* 2002).

Survivin is a recently discovered gene which encodes a structurally unique IAP apoptosis inhibitor (reviewed by Altieri *et al.,* 2001). Survivin is expressed during foetal development and is undetectable in terminally differentiated non-neoplastic adult human tissues, however the expression appears to be switched on again in most common human cancers (and transformed cell lines) (Ambrosini *et al.,* 1997). It has been reported that apoptosis inhibition by survivin alone or in co-operation with *bcl-2* may be a significant prognostic factor of worse outcome in breast carcinoma

(Tanaka *et al.,* 2000). Expression of survivin protein was not correlated with p53 in this study but did correlate with bcl-2 protein levels and reduced apoptotic index.

The AP-1 family of transcription factors (which includes *c-jun, junB, junD, c-fos, fosB, fra1* and *fra2*) show a differential pattern of expression in breast cancers, expression of fosB was found to correlate significantly with positive steroid hormone receptor status and a more differentiated phenotype (Bamberger *et al.,* 1999).

The proto oncogene HER-2/ neu (also known as cerb-B-2) is a member of the epidermal growth factor family and its amplification is one of the most common genetic alterations associated with human breast cancer occuring in approximately 30% of breast carcinomas (Slamon *et al.,* 1987). The HER family plays an important role in the regulation of cell growth, survival and differentiation. HER-2/ neu amplification and protein overexpression have been implicated both in the pathogenesis and clinical behaviour of human breast cancer. Higher expression of HER-2/ neu has been reported in intraductal carcinomas compared to infiltrating tumours; and it may be useful in the determining the invasive potential of DCIS patients (Allred *et al.,* 1992). This oncogene has proved to be one of the most promising markers currently under study in breast cancer both as a marker of poor prognosis and as a predictor of chemoresistance. Preclinical studies suggest that HER-2/ neu overexpression enhances the metastatic potential of breast cancer cells (Sahin 2000). As already mentioned the growth of breast tumours (*in vitro* and *in vivo*) overexpressing HER-2 is inhibited by anti-HER-2 MAbs (Drebin *et al.,* 1986, 1988). With the successful clinical trials of trastuzumab, the establishment of HER-2 tumour status is obviously a prerequisite for therapeutic use of this MAb (Baselga *et al.,* 1998, Slamon *et al.,* 1998, Slamon *et al.,* 2001). Herceptest (Dako Corporation, CA) has been approved by the US Food and Drugs Administration to be used to screen tumours by immunohistochemistry for HER-2 status. However there is still ongoing controversy as regards whether the establishment of HER-2 status of tumours should be done by immunohistochemistry or by fluoresence *in situ* hybridisation (FISH) (measurement of gene amplification). It is generally agreed that the best approach is to combine both immunohistochemistry and FISH (Wang *et al.,* 2000).

The HER-2/ neu oncogene has been extensively studied as a prognostic marker although this role remains controversial. The first report of its prognostic role was in 1987 by Denis Slamon and his colleagues which revealed that HER-2 gene amplification independently predicted overall survival and disease free survival in a multivariate analysis of node positive patients (Slamon *et al.,* 1987). Since this, several studies have confirmed a significant correlation between HER-2/ neu overexpression and poor prognostic markers in node positive disease i.e. a poor degree of differentiation, high nuclear grade, lack of steroid receptors, high proliferative activity and DNA aneuploidy (reviewed by Sahin 2000). Assessment of HER-2/ *neu* status now constitutes one of the most important prognostic factors in node positive breast cancer patients.

The significance of HER-2/ *neu* overexpression in node negative patients is conflicting, some studies as is the case with node positive patients show a poorer prognosis in either certain or all cases while other studies did not find such a correlation. These conflicting results may be explained by the small patient numbers evaluated, the low event rate and the diversity of methods used, presently the assessment of HER-2/ *neu* in node negative cannot be used as a prognostic factor routinely (reviewed by Mirza *et al.,* 2002). It has also been suggested that overexpression of other members of the HER family, c-erbB-3 and c-erbB-4 oncoproteins may play a role in the prognosis of breast carcinoma; increased expression of both of these oncoproteins has been associated with a prognostically favourable ER positive phenotype (Bodey *et al.,* 1997). Higher levels of cerbB-4 expression have been associated with a more differentiated phenotype (Kew *et al.,* 2000).

It has been shown that increased Topo II α expression is associated with an aggressive form of breast cancer featuring erbB-2/ neu amplification and predicts disease related death, lymph node metastasis and advanced tumour stage (Depowski *et al.,* 2000). In DCIS HER-2/ neu and Topo II α expression is associated with poorly differentiated lesions (Shpitz *et al.,* 2000).

1.4.4.4. Markers of Chemosensitivity

Breast cancer is moderately sensitive to multiple chemotherapeutic agents; however the duration of this response is usually short and the majority of these patients will eventually relapse and the tumour will fail to respond to chemotherapy. Chemotherapy resistance constitutes a major problem in the management of breast carcinoma. To date the exact mechanisms of this resistance have not been fully elucidated, it is well recognised that drug resistance in breast cancer is multifactorial and heterogeneous. A better understanding of the underlying mechanisms may result in the expansion of the use of MDR modulators and antibody therapy within the clinic. Identification of parametres that can accurately predict the response of individual patients to chemotherapeutic agents will allow for more precise targeting of these chemotherapeutic drugs in the clinic for breast cancer patients. However predictive markers for chemosensitivity for breast cancer are less well established than prognostic markers in this disease. There appears a lot of conflicting data regarding the contribution of various markers to the prediction of response in the treatment of breast cancer, results from evaluation of HER-2/ neu tumour status and its prediction of response to various chemotherapeutic drugs is probably the most convincing to date.

Expression of the mismatch repair protein (MLH1) has recently been shown to be an independent predictive factor for poor disease free survival in patients treated with *neo*-adjuvant therapy (MacKay *et al.,* 2000).

It is well recognised that high tumour proliferation in addition to being a marker of poor prognosis has a role in the prediction of response to chemotherapy. Retrospective studies have demonstrated that breast cancer tissues with high proliferation indices and low grades of tumour differentiation respond better to chemotherapy (Silvestrini & Daidone 1993). Tumour grade, SPF, Bax expression independently predicted response to treatment (5-FU, epirubicin and cyclophosmide) in metastatic breast cancer patients (Sjostron *et al.,* 1998). Another study reported that Ki-67, DNA ploidy, SPF, and p53 did not predict response to *neo* adjuvant therapy (mitoxantrone, methotrexate +/- mitomycin C and tamoxifen) in primary breast cancer patients (Makris *et al.,* 1997). A later study of SPF and Ki-67

expression has shown that these markers may have a predictive role in the response of patients to *neo* adjuvant chemotherapy (Makris 1999). Breast tumours with a high growth activity by assessment with Ki-67 have been shown to have low chemosensitivity (Itaya *et al.,* 1999). Expression of Ki-67, MDR-1, MRP-1, LRP, DNA Topoisomerases or p53 proteins did predict response to neo adjuvant chemotherapy in a study by Linn *et al.,* (1997). It has been suggested that expression of MIB-1, cerbB-2 and p53 may offer possible predictive value in patients who received primary chemotherapy (Colleoni *et al.,* 1999).

Conclusions from studies looking at evaluation of *p53* as a possible marker of chemosensitivity vary. p53 overexpressing tumours have been shown to predict response to chemotherapy in a number of studies; lower chemosensitivity has been observed in such tumours (Burke *et al.,* 1998, Itaya *et al.,* 1999, Mottolese *et al.,* 2000, Geisler *et al.,* 2001). In patients treated with *neo* adjuvant therapy high response rates were observed in patients whose *pre* treatment tumours were p53 negative/ Pgp negative (Daidone *et al.,* 1999). Other studies however do not support a predictive role for p53 (Rein *et al.,* 2000, Sjostrom *et al.,* 2000, Yang *et al.,* 2000).

Again the possible predictive role of *Bcl-2* in breast cancer remains controversial. It has been suggested that *Bcl-2* may predict response to chemotherapy in a number of studies (Chang *et al.,* 2000, Mottolese *et al.,* 2000, Yang *et al.,* 2000, Geisler *et al.,* 2001). One study has shown that expression of Bcl-2 and Bax proteins and apoptotic index (AI) may be predictive of response to chemotherapy (Wu *et al.,* 2000). Other studies however have shown that Bcl-2 did not predict for response to chemotherapeutic drugs (Takei *et al.,* 1999, Poelman *et al.,* 2000). Investigation of survivin expression levels and response to therapy has not been reported to date.

MIB-1, the protein products of the *mdm-2* (a gene which plays a central role in the regulation of p53) and *p21* (or *WAF1*, the wild type p53 activated fragment) genes were found to have a predictive role in the response to docetaxel and methotrexate/ 5-FU (Sjostrom *et al.,* 2000). Recent work suggests that mRNA levels of the breast cancer susceptibility gene, BRCA2, may predict response to docetaxel (Egawa *et al.,* 2001).

Again there have been several studies directed at the possible predictive role of HER-2/ *neu* in breast cancer. However results to date have proved conflicting. Early studies looking at the possible predictive role of HER-2/ neu in the response of breast cancer patients treated with CMF therapy suggested that this chemotherapeutic regime is of less benefit to patients with HER-2/ neu overexpressing tumours (Allred *et al.,* 1992, Gusterson *et al.,* 1992, Tetu & Brisson, 1994, Stal *et al.,* 1995). It was reported by Slamon *et al.* (1987) that HER-2 gene amplification could independently predict overall survival and disease free survival in node positive breast cancer using multivariate analysis. This observation has been confirmed by several investigators (reviewed by Sahin *et al.,* 1999, Simon *et al.,* 2001).

However opinions on whether HER-2/ neu status should be used to routinely determine which patients might not get benefit from CMF are still mixed, some researchers are waiting for more validation or results before recommending such a predictor in the clinic (Ravidin 1999). Association of HER-2/ neu with response to doxorubicin containing regimes has also been studied; results have suggested that HER-2/ neu overexpression may determine which patients have tumours which may be particularly sensitive to such drug regimes (Paik *et al.,* 1998, Thor *et al.,* 1998). Furthermore preliminary results suggest that it may also play a predictive role in response to pacitaxel (taxol) (Baselga *et al.,* 1998).

Amplification of the cerbB-2 oncogene is thought to be followed by a complex sequence of genetic aberrations which leads to the amplification of the Topo II α gene (Muss *et al.,* 1994, Jarviven *et al.,* 1999). Topo II α amplification and deletion are thus common in cerbB-2 amplified breast cancers, which may explain the altered chemosensitivity to Topo II inhibitors reported in cerbB-2 amplified breast cancers (Jarvinen *et al.,* 2000).

1.5. Classification of Haematological malignancies

1.5.1. Acute Leukaemias

Acute leukaemias are neoplastic disorders marked by uncontrolled proliferation of haematopoietic cells with a predominence of immature lymphoid or myeloid cells (more than 30%) in the bone marrow and peripheral blood. Acute leukaemias may be designated as lymphoblastic (ALL) or nonlymphoblastic (myleoblastic/ monocytic) (ANLL or AML). The incidence of these malignancies increases with age. ALL is the most common childhood malignant neoplasm, 80% of adults with leukaemia have ANLL.

AML is characterised by both the proliferation and the accumulation of leukaemic blast cells of myeloid origin that cannot enter normal granulo-monocytic differentiation. AML consists of hierarchical subpopulations including a minority of proliferarting progenitors. This malignancy is classified according to the French-American-British (FAB) study by morphological and histochemical criteria.

	FAB Classification of AML
MO	myeloblastic, without maturation, mainly differentiated
M1	myeloblastic, without maturation
M2	myeloblastic, with maturation
M3	pro-myelocytic
M4	myelomonocytic
M5A	monoblastic
M5B	pro-monocytic
M6	erthroleukaemia
M7	megakaryoblastic

Table 1.1. : FAB Classification of AML

1.5.2. Chronic Leukaemias

Chronic Lymphocytic Leukaemia (CLL)

CLL is a malignant haematological disorder characterised by a persistant absolute increase in "mature" appearing lymphocytes in the peripheral blood and bone marrow. The vast majority of cases are of B cell (B-CLL) origin, the T cell variant is only seen in about 5-10% of patients diagnosed with CLL. B-CLL is the most common type of haematological malignancy in the Western world, particularly affecting older age groups. Prolymphocytic leukaemia (PLL) is a variant of CLL which usually occurs in elderly patients. The T cell variant of PLL is rarely seen and follows a less predictable prognosis.

LGL Leukaemia

Clonal diseases of large granular lymphocytes can be either T cell (T-LGL) of natural killer cell origin NK-LGL.

Hairy Cell Leukaemia (HCL)

This is a rare malignancy which is also known as leukaemic reticuloendotheliosis. The characteristic cells which are lymphoid appearing with "hairy" cytoplasmic projections are always of B cell origin and the disease is therefore, considered a variant of CLL.

Chronic Myelogenous leukaemia (CML)

CML also referred to as chronic granulocytic leukaemia is a clonal myeloproliferative disorder arising from neoplastic proliferation at the level of the pleuripotent stem cell. This malignancy is rare in childhood and peaks in the mid fifth decade.

1.5.3. Non Hodgkins Lymphoma (NHL)

NHLs are a diverse group of malignancies of the lymphoreticular system that have heterogenous histopathologic, immunologic, cytogenetic and clinical characteristics. The median age for this disease is 50 years, the incidence increases with age. NHL

are graded histologically as low, intermediate, high and miscellaneous grade. Mantle cell lymphoma (MCL) and follicle centre lymphoma (FCL) which belong to the NHL group of malignancies are also classified as sub groups of B-cell chronic lymphoproliferative disorders (B-CLPD).

1.5.4. Multiple Myeloma (MM)

MM and related disorders (plasma cell dyscarias) comprise a spectrum of diseases that are characterised by autonomous proliferation of differentiated lymphoid cells and plasma cells whose physiological function is to secrete immunoglobulins. MM is the most common of these diseases representing 75% of dyscarias, is an incurable cancer that accounts for 1% of all cancers and 10% of haematological malignancies. Waldenstrom's macroglobulinemia represents 20% of dyscarias, the remainder consisting of other types of heavy chain disease.

1.6. Multiple Drug Resistance (MDR)

Human malignancies including breast cancer are routinely treated with chemotheraptic drugs and/ or hormone therapy, usually following surgery. Radiation therapy is given to some patients. However although chemotherapy has the potential to cure certain malignancies, in approximately 84% of patients who receive chemotherapy, drug resistance will develop. Resistance to these chemotherapeutic agents remains the major obstacle in the successful therapy of many human cancers. Certain malignancies do not respond to chemotherapy; these tumours display intrinsic resistance. Alternatively other malignancies can be initially sensitive to a chemotherapeutic regime, leading to a partial or complete response of the tumour (generally resulting in a decrease in tumour size). Unfortunately these tumours can then recur (if a small number of tumour cells has become resistant to the chemotherapeutic agent) and then display resistance not only to the initial chemotherapeutic agent but also to other drugs to which the tumour has not

previously been exposed. This is termed acquired or secondary resistance (Gerlach *et al.,* 1986).

MDR refers to a phenomenon whereby tumour cells acquire resistance to a broad range of structurally and functionally diverse chemotherapeutic drugs including anthracyclines, vinca alkaloids, epipophyllotoxins and taxol following exposure to a single agent. Several mechanisms have been identified that can render tumour cells multidrug resistant. The first mechanism to be discovered and the most widely studied of these is the overexpression of the MDR-1 gene product, P-glycoprotein also referred to as P-170/ Pgp (Gros *et al.,* 1986). Other MDR mechanisms include altered levels of multidrug resistance associated protein (MRP), a 190 kDa membrane protein (Cole *et al.,* 1992), the lung resistance related protein (LRP) (Scheper *et al.,* 1993), topo II (Hoffmann & Mattern, 1993), glutathione S transferase (GST) (Moscow & Dixon, 1993) and possibly the transported associated with antigen processing (TAP) (Izquierdo 1996).

1.6.1. P-glycoproteins/ Pgps

P-glycoproteins (Pgps) are members of the superfamily of ATP binding cassette (ABC) transporter proteins (Schinkel *et al.,* 1991). ABC transporters are membrane proteins that contain multiple membrane spanning regions and at least 1 ATP binding site (Walker *et al.,* 1982). Various Pgp isoforms have been identified; all of these isoforms are encoded by a group of closely related genes. Two Pgp genes have been identified in humans, MDR-1 and MDR-3, these two genes show 80% homology (Roninson *et al.,* 1986, Van Der Bliek *et al.,* 1987).

Pgp class	Human	Mouse	Hamster
1	*mdr 1*	*mdr 3 (mdr 1a)*	*pgp 1*
11		*mdr 1 (mdr 1b)*	*pgp 2*
111	*mdr 3*	*mdr 2*	*pgp 3*

Table 1.2. : Mammalian P-glycoprotein isoforms

1.6.2. MDR-1 P-glycoprotein

The MDR-1 gene product acts as an energy dependent efflux pump that actively transports drugs out of the cell before they can reach cytotoxic concentrations. It is thought that this transport of drugs by MDR-1/ Pgp involves a translocation or flippase mechanism (Grottseman & Higgins 1992). Pgp is typically localised to the apical surface of the cells that express it. P-gp is involved in the normal physiological functions of the cell and is thought to have a protective role i.e. the transport and excretion of naturally occurring xenobiotics (Schinkel *et al.,* 1994). The tissue distribution pattern of P-170 reflects this protective role, however the physiological functions of MDR-1/ Pgp have been poorly defined. High expression of Pgp has been found in human adrenal cortical cells, the brush border of renal proximal tubule epithelium, the lumenal surface of biliary hepatocytes, small and large intestinal mucosal cells and pancreatic ductules. P-glycoprotein is also expressed at lower levels in capillary endothelial cells of the brain and testis, the placenta, lung, prostate, NK cells and in CD34 positive bone marrow stem cells (reviewed by Bellamy 1996).

1.6.3. MDR-1/ P-glycoprotein in Breast Cancer

Over the last 10 years several researchers have attempted to address the question of the precise clinical relevance of MDR in breast cancer. Most of this work has focused on the role of *MDR-1/* Pgp in this disease; despite huge variability and conflicting results it is likely that *MDR*-1/ Pgp is expressed in a significant proportion of breast cancers; higher levels are seen in tumours exposed to MDR substrates. An extensive meta analysis of *MDR-1/* Pgp in breast cancer examined 31 different studies in detail and reports that *MDR-1/* Pgp is expressed in 41.2% of breast tumours; this expression is associated with treatment and with a poor response to chemotherapy. This report has also concluded that *MDR-1/* Pgp is not acting as a surrogate for another prognostic marker (Trock *et al.,* 1997).

A factor which probably contributes to the huge variability of results in assessing MDR-1/ Pgp in breast cancer and other malignancies is that majority of studies have used only one P-170 specific antibody. Trock *et al. (*1997) report that 67% of studies

using immunohistochemistry used only one P-170 antibody. It is generally accepted now that at least two antibodies directed to different epitopes of P-170 should be employed in any study (reviewed by Beck *et al.,* 1996). In addition some of the P-170 antibodies have been shown to cross-react with other cellular components; JSB-1 and C294 cross react with pyruvate carboxylase, the C219 MAb cross reacts with the c-erbB-2 protein. C219 may therefore yield false positive results in breast tumours which overexpress c-erbB-2, careful interpretation and caution must therefore be employed when examining such tumours (Liu *et al.,* 1997, Chan & Ling 1997).

Several studies have showed *MDR-1/* Pgp expression associated with either recurrence free survival or overall survival. In a group of 14 patients with non-metastatic locally advanced breast carcinoma; strong P-170 positive staining was significantly correlated with no initial response to chemotherapy and with a shorter progression free survival (Verrelle *et al.,* 1991). Evaluation of *mdr-1* RNA was not found to predict response to therapy in primary breast cancers; but expression was associated with a metastatic phenotype (Hennequin *et al.,* 1993). Another study of locally advanced breast cancers found high Pgp expression was related to a poor response to chemotherapy and a short disease-free survival (Botti *et al.,* 1993).

However the debate still goes on as to whether there is enough evidence to support a contributory role for *MDR-1/* Pgp in the MDR of some breast tumours and if so what is this exact role. Some researchers feel that *MDR-1/* Pgp may be just a marker of a more aggressive phenotype, high levels of this protein and indeed other MDR markers may be part of the initial phenotype of the malignant cells (Buser *et al.* 1997).

This relationship between P-170/ Pgp positivity and survival in a range of tumour types is discussed in relation to breast cancer in an editorial by Kaye (1997) where it is suggested that P170 expression may be a marker of cell behaviour and metastasis (Pinedo *et al.,* 1995). Giaccone *et al. (*1995) have suggested that Pgp expression in tumour and in desmoplastic stroma cells may identify a subgroup of very aggressive tumours; Pgp was also more often associated with metastatic than primary tumours. This group also showed a correlation between Pgp expression in primary tumour and development of axillary lymph node metastasis. Buser *et al.* (1997) found that Pgp

was expressed in a higher number of metastatic than early breast cancer tumours. Pgp expression in tumour cells and especially when accompanied by Pgp expression in fibroblasts in desmoplastic stroma has been shown to be likely to be a marker of a more malignant phenotype (Linn *et al.*, 1996).

Recent studies suggest that protein kinase C (PKC) may be involved in the expression status of the MDR associated genes, MDR-1, MRP and LRP (Beck *et al.*, 1998). Expression of Ki-67 has been found to correlate with Pgp expression; in the same study estrogen receptor expression was associated with low levels of Pgp (Seymour *et al.*, 1995). Linn *et al.* (1996) showed that co-expression of p53 and Pgp was predictive of shorter survival time in locally advanced disease. Mutant p53 has been shown to regulate *MDR-1* gene expression (Chin *et al.*, 1992). Sequential assessment of Pgp and S-phase fraction has been shown to predict response to neoadjuvant chemotherapy (Chevillard *et al.*, 1997). A high PCNA score was not found to be predictive of poor response to chemotherapy and a short disease free interval (Botti *et al.*, 1993).

Using flow cytometry expression of Pgp was found to be positively correlated with expression of *c-erbB-2, c-myc* and *c-jun* but not ER or EGF-R; GST π was positively associated with *c-erbB-2* and *c-myc* but not *c-jun,* ER or EGF-R (Brotherick *et al.*, 1996). The majority of work to date points to the multifactorial nature of MDR in the breast cancer clinic as indeed in all malignancies; it is imperative therefore that several MDR associated parameters are investigated in breast cancer patients.

1.6.4. Non Pgp mediated MDR in Breast Cancer

Lower levels of topo II α are associated with drug resistance (Hoffmann & Mattern 1993). Topo II enzymes represent the key target for a number of anticancer drugs including those used in the treatment of breast cancer such as doxorubicin, mitoxantrone and VP-16 thus expression patterns may relate to the sensitivity/ resistance of the tumour in particular chemotherapeutic regimes. In general however there have been limited reports of topo II α levels correlating with chemosensitivity patterns. This may be in part due to the heterogeneous expression of the protein and

the high proportion of non-cycling cells; such observations have been reported by Doyle (1994) and Kaufmann *et al.* (1994).

The chromosome location of *topo II α* is similar to that of *c-erbB-2* (Muss *et al.*, 1994). Recently it has been reported that topo II α gene amplification and deletion are common in erbB-2 amplified breast cancers and are associated with increased or decreased sensitivity to topo II inhibitors *in vitro*, respectively (Jarvinen *et al.*, 2000). An earlier study by the same group did not show topo II α to be predictive in the response to topoisomerase II inhibitors in advanced breast cancer (Jarvinen *et al.*, 1998).

High topoisomerase II α levels are usually found in tumours with a high proliferation index and aggressive clinical behaviour, topoisomerase II α expression has been associated with poor prognostic markers namely, high tumour grade/ large tumour size/ nodal status and the distant metastases at the time of diagnosis (Hellemans *et al.*, 1995). Topoisomerase II α expression levels have been correlated with tumour proliferation rates and with c-erb-2 expression (Lynch *et al.*, 1997, Jarvinen *et al.*, 1996). High levels of topoisomerase II α (and cyclin A) gene expression were observed in high grade (G3) tumours which are less well differentiated, show enhanced proliferation activity and exhibit a better response to chemotherapy compared to intermediate grade (G2) tumours (Beck *et al.*, 1998). Thus it appears that elevated topoisomerase II α expression represents a marker of proliferation in breast cancer and furthermore may play a predictive role in breast cancer i.e. high levels of expression appear to identify those tumours which are most chemosensitive to certain chemotherapeutic protocols.

Some researchers suggest a weak correlation between GST π and Pgp expression in untreated breast cancer (Keith *et al.*, 1990). High levels of GSH, GST and glutathione peroxidase (GPx) were associated with favourable clinical characteristics and good prognosis in untreated breast cancer patients; low GSH and GST activity were associated with more aggressive/ advanced disease (Buser *et al.*, 1997). Immunohistochemical staining of GST π has been shown to inversely correlate with disease free interval and overall survival rates in node negative patients (Gilbert *et*

al., 1993, Sochacki *et al.,* 1994). Several other groups have failed to show such correlations (Kim *et al.,* 1991, Wright *et al.,* 1992, Peters *et al.,* 1993).

1.6.5. The MRP family of drug transporters

MRP is also a member of the ABC family of transporters which has been shown to confer resistance. MRP is 1531 amino acid Mr 190,000 glycosylated integral membrane protein that is overexpressed in several MDR cell lines (Cole *et al.,* 1992). Like P-glycoprotein MRP1 transports drugs out of the cell, reducing the concentration at the intracellular target.

MRP-1 has been detected in several normal tissues with high levels of the protein being detected in adrenal gland, lung, heart and skeletal muscle and lower expressions detected in liver, spleen, kidney and erythrocyte membranes (Flens *et al.,* 1996). MRP-1 protein expression has also been detected in several human cancers and has been implicated in the clinical resistance in a number of solid malignancies and in certain haematological malignancies (see section 1.6.7).

Since the initial discovery of MRP-1 and the recognition of the importance of MRP in MDR, seven additional members of the MRP family have been described, MRP2-8 (Kool *et al.,* 1997, 1999, Bera *et al.,* 2001, Hopper *et al.,* 2001,).

High levels of MRP-1 gene expression have been associated with poor prognosis in childhood neuroblastoma (Bordow *et al.,* 1994, Peaston *et al.,* 2001). In adult patients with soft tissue sarcomas, expression of MRP-1 has been associated with high histological grade and poor porgnosis (Oda *et al.,* 1996). It is thought that expression of MRP-1 may play a part in the clinical resistance of malignant melanoma (Ichihashi *et al.,* 2001). One recent study has suggested that expression of this protein along with that of Topo II alpha may have a possible predictive role in the chemosensitivity of ovarian and endrometrial carcinomas (Koshiyama *et al.,* 2001), however another study of ovarian carcinomas did not show that MRP-1 was predictive for chemosensitivity (Goff *et al,* 2001). Again MRP-1 expression is thought to be an important factor for chemotherapy failure in bladder cancer (Chen *et*

al., 2001). It has been suggested that MRP-1 expression may be related to the intrinisic resistance in human gliomas, recent studies have suggested that MRP-3 may modulate drug sensitivity to certain anti cancer agents in human gliomas (Mohri *et al.*, 2000, Haga *et al.,* 2001). In addition MRP-1 expression may be involved in the clinical resistance of oral small cell squamous carcinomas (OSCCs) (Cho & Kim, 2001).

The functions and their role, if any, in clinical drug resistance of the newly discovered MRP family members have not been fully elucidated to date. Several studies investigating such roles for these proteins are currently underway. MRP1, MRP2 and MRP3 have all been shown to cause resistance to neutral organic drugs and MRP4 overexpression has been associated with resistance to nucleoside analogues used in immunodeficiency virus drugs (reviewed by Borst *et al.,* 2000). Recently MRP2 has been implicated in the resistance to cisplatin treatment in colorectal cancer (Hinoshita *et al.,* 2000). A recent report has shown that expression of MRP-2 and MRP-3, but not that of MRP-1 can contribute to the MDR phenotype of human hepatocellular carcinomas (Nies *et al.,* 2001). It has been suggested that MRP5 may play a role in some cases of unexplained resistance to thiopurines in acute lymphoblastic leukemia (Wijinholds *et al.,* 2000). Recent work has also suggested that MRP-3 may contribute to the intrinsic resistance of NSCLC cells (Young *et al.,* 2001). Previous studies have suggested that expression of MRP-1 may be significant in non small cell lung cancer, with moderate to high levels of expression being associated with a poor prognosis (Ota *et al.,* 1995, Chuman *et al.,* 1996, Giaccone *et al.,* 1996).

1.6.6. MRP1 protein expression in breast cancer

It is known that MRP-1 is expressed by most breast carcinomas. It has been reported in one study that *pre* chemo Pgp and *pre* and *post* chemo MRP-1 staining predicted tumour recurrence and patient death (Mechetner *et al.,* 1997). A study by Nooter *et al.,* (1997a) has concluded that MRP-1 expression is an important predictor of poor prognosis in patients with *recurrent* breast cancer who were treated with chemotherapy. MRP-1 expression was more positive in non-responding tumours than

in responding ones; these MRP-1 positive patients also showed shorter time to disease progression than MRP-1 negative tumours. In a large series of *primary* breast cancers, the same group showed that MRP expression might be associated with shorter relapse free survival in patients with small tumours and in node negative tumours; expression was also shown to be predictive of overall survival in patients who received adjuvant systemic chemotherapy with CMF. This work suggests that MRP-1 expression in primary breast cancer might be related to a more aggressive phenotype (Nooter *et al.,* 1997b). In an earlier study of MRP-1 and MDR-1 gene and protein expression in a large series of primary breast cancer patients, no correlation was found between these 2 markers. *MDR-1* RNA was detected in 60% of patients whereas *MRP* RNA was detected in 100%; protein levels showed a similar pattern although the overall level of expression was reduced compared to RNA levels. Strong MRP-1positive staining was observed more frequently in the primary tumours of patients with metastatic disease and in locally advanced tumours (Filipits *et al.,* 1996). An earlier study on MRP-1 in various cancers showed relatively low levels of MRP-1 RNA in a series of 32 breast cancers; protein was also detected in a small percentage of patients (levels similar to Filipits *et al.,* 1996*)* (Nooter *et al.* 1995). No significant difference in MRP protein expression was found between advanced local breast cancers and primary operable cancers in a study carried out by Linn *et al.* 1997; whereas significantly higher protein levels of Pgp, Ki-67 and P53 were observed in locally advanced cancers. MRP-1 negative tumour status was associated with an excellent prognosis in another immunohistochemical study, suggesting again that MRP-1 is of important prognostic value in breast cancer (Filipits *et al.,* 1999a). MRP-1 protein expression was more pronounced in lymph node metastasis than in corresponding primary tumour in a recent report by Zochbauer-Muller *et al.* (2001). Increased MRP-1 expression has previously been reported in primary tumours with distant metastases (Filipits *et al.,* 1996).

A correlation has been observed between protein kinase C (PKC) and MDR-1, LRP and MRP gene expression, furthermore higher levels of MRP and LRP expression were observed in grade I compared to grade II tumours. These results (also associated higher levels of topo II α in G3 tumours) may explain the better response of grade III tumours to chemotherapeutic regimes (Beck *et al.* 1998). Using RT-PCR

a clear relationship has been suggested between GSTπ and MRP (i.e. the glutathione conjugate carrier function of MRP) gene expression in breast tumours (Lacave *et al.,* 1998).

Again as with *MDR-1/* Pgp there appear to be conflicting results regarding MRP expression in breast cancer; however it does appear to be expressed in significant number of tumours and recent work suggests a predictive role for MRP in breast cancers. Suggestions that MRP may be associated with advanced disease and/or may be a marker of biological tumour behaviour need to be investigated further. Interestingly, MRP-8 the newest member of the MRP superfamily has been shown to be highly expressed in breast cancer, thus this gene may also possibly constitute a potential marker in this malignancy (Bera *et al.,* 2001). Low expression of the breast cancer resistance protein (BCRP) compared to other MDR genes was observed in breast cancer patients, suggesting that BCRP does not have a significant role in breast carcinoma (Kanzaki *et al.,* 2001).

1.6.7. MRP family expression in haematological malignancies

In some studies the expression of MRP1 and not Pgp has been found to be higher at relapse than at diagnosis in AML patients. However in general MRP1 expression at diagnosis is not associated with clinical response to induction chemotherapy and survival in most studies. It has been reported that intermediate or high MRP1 expression may be associated with shorter overall survival compared to patients with low MRP-1 expression (Filipits *et al.,* 1999b, Han *et al.,* 2000). AML (subtype M4E0) patients with an inversion of chromosome 16 (inv[16]) leading to the deletion of the MRP1 gene, have a relatively favourable outcome which suggests that MRP1 may play a role in determining clinical outcome in these patients (Kuss *et al.,* 1996). In CLL MRP1 mRNA expression has been detected in both chemotherapy treated and untreated CLL (Burger *et al.,* 1994, Beck *et al.,* 1994). There has been one report that MRP-1 is involved in the drug resistance of chronic B-cell lymphoproliferative disorders (CLL and NHL) in patients treated with Pgp transportable drugs (Webb *et al.,* 1998). A small number of studies investigating the expression of MRP1 in ALL

have reported that MRP1 expression at diagnosis is not associated with response and long term survival (reviewed by Sonneveld 2000). MRP1 expression does not appear to be of prognostic value in mylelodysplastic syndromes (MDS) (Poulain *et al.,* 2000).

The expression/ clinical significance of the other members of the MRP family members in various haematological malignancies has not been reported. Relatively high expression of another recently discovered ABC transporter, breast cancer resistance protein (BCRP) has been observed in 30% of AML cases investigated, suggesting a possible role for this protein in the drug resistance of certain leukaemias (Ross 2000, Sargent *et al.,* 2000).

1.6.8. LRP/mvp

LRP also known as the major vault protein was first described in non Pgp drug resistant cell lines (Scheper *et al.,* 1993). Like Pgp and MRP, LRP is widely expressed in both normal tissues and various tumour types (Izquierdo *et al.,* 1996).

1.6.9. LRP/mvp expression in breast cancer

Although LRP is frequently expressed in breast cancers, it is not thought to play a role in the resistance of these tumours. LRP is neither associated with known prognostic factors nor is it a prognostic factor itself in breast carcinomas, expression of this protein does not appear to predict response to CMF or CAF therapy (Pohl *et al.,* 1999, Schneider *et al.,* 2000, 2001). However it has been reported that over expression of LRP prior to *neo* adjuvant therapy (and associated MDR-1 positivity following chemotherapy) appears to be associated with axillary nodal metastasis after chemotherapy (Schneider *et al.,* 2001).

1.6.10. LRP/mvp expression in haematological malignancies

Expression of LRP has been shown to be of significance in certain haematological malignancies. MM is a clonal plasma cell neoplasm characterised by the proliferation of plasma cells in the bone marrow. This disease has a high initial response however drug resistance usually occurs. Both Pgp and LRP have been implicated in drug resistance in MM, MRP is not thought to play a role in this resistance (Rimza *et al.*, 1999). LRP expression is considered to be an important marker for clinical drug resistance in MM (Filipits *et al.*, 1999c, Rimsza *et al.*, 1999). It is frequently expressed in this disease and has been shown to be an independent poor prognostic marker for response and survival in patients treated with melphalan combined with prednisone (MP) (Raaijmakers *et al.*, 1998). LRP predicts for a poor outcome in AML patients (Filipits *et al.*, 1998, Pirker *et al.*, 1999). In a recent univariate analysis of several drug resistance factors in AML LRP was the only factor with independent predictive and prognostic significance (Filipits *et al.*, 2000a). It is also thought that LRP may contribute to drug resistance in certain childhood leukaemias such as childhood AML (den Boer *et al.*, 1998). In diffuse large B cell lymphomas (a subtype of NHL) LRP expression was associated with a worse outcome in previously untreated patients (Filipits *et al.*, 2000b). In the same study MRP1 expression had no impact on the outcome of chemotherapy or survival of the patients. In a recent report the co expression of LRP *and* Pgp was associated with a shorter overall survival in ALL patients (Tsuji *et al.*, 2000). In patients with adult T cell leukaemia LRP was shown to independently predict for a poorer outcome (Ohno *et al.*, 2001).

1.7. MDR-3 P-glycoprotein

MDR-3 is thought to be the most highly conserved Pgp gene amongst various mammalian species and human MDR-3 is closely related to murine mdr-2 (91% identity, 95% similarity) (Gros *et al.*, 1988). Classical Multiple Drug Resistance (MDR) is associated with overexpression of P-170. Specific information regarding the exact role of MDR-3 in normal or resistant cells and in various tissues is limited.

1.7.1. The function of MDR-3 P-glycoprotein

Like the closely related MDR-1 gene a physiological role as a transport pump has been ascribed to MDR-3. By generating mice with a disruption of the mdr-2 gene, this gene has been shown to encode a Pgp located in the canalicular membrane of hepatocytes where it functions as a transporter of phosphatidylcholine into bile (Smit *et al.,* 1993, Ruetz & Gros, 1994, 1995). On analysis these mice showed a defect in the transport of phospholipids (mainly phosphatidylcholine) into bile. It is thought that the MDR-3 and mdr-2 genes may encode a phosphatidylcholine flippase (Ruetz & Gros, 1994, Smith *et al.,* 1994, Crawford *et al.,* 1997), MDR-3 and mdr-2 Pgps have been identified as specific translocators of phosphatidylcholine (van Helvoort *et al.,* 1996). Given the high conservation of amino acid sequence between these two genes and their respective tissue distribution patterns it is likely that human MDR-3 fulfils a similar function to murine mdr-2. It has been confirmed that human MDR-3 Pgp is functionally homologous to murine mdr-2 Pgp (Smith 1998, PhD Thesis). Kino *et al.*, (1996) reported that a yeast strain transformed with the *MDR-3* gene developed low level resistance to the fungicide aureobasidin A. Preliminary results using polarised cell systems suggested that MDR-3 may act as a transporter of certain cytostatic drugs (Smith 1998, PhD Thesis). Confirmation of this earlier work again using polarized membranes of MDR-3 transfected cells has now been demonstrated indicating that MDR-3 can transport a sub-set of MDR-1 P-glycoprotein substrates including digoxin, paclitaxel and vinblastine (Smith *et al.,* 2000). Direct interaction of MDR-3 with these drugs was shown by interference with nucleotide trapping. The rate of this MDR3 mediated transport was low, this may explain why MDR3 has not been shown to be previously involved in MDR.

1.7.2. Tissue Distribution of MDR-3 P-glycoprotein

Excluding the liver, a physiological role for *MDR-3/ mdr-2* has not been established although gene expression studies using PCR and the RNase protection assay have revealed details regarding the distribution of these genes in various normal tissues. *MDR-3* RNA has been detected at high levels in liver and to a lesser extent in kidney, spleen, bone marrow, tonsil, striated muscle and adrenal gland which showed very

low levels (Chin *et al.,* 1989, Smit *et al.,* 1994). Murine *mdr-2* mRNA and rat *mdr-2* mRNA have both been shown to have a similar distribution pattern to that of human MDR-3; mdr-2 has also been detected in mouse lung and rat mammary tissue (Croop *et al.,* 1989, Teeter *et al.,* 1990, Brown *et al.,* 1993, Smit *et al.,* 1994, Zhang *et al.,* 1996). Using mice transgenic for an *MDR-3* mini gene, Smit *et al.* (1996) found that *MDR-3* overexpression in Schwaan cells resulted in dysmyelination of the peripheral nervous system.

1.7.3. The role of MDR-3 P-glycoprotein in liver disease

Progressive familial intrahepatic cholestasis (PFIC) is a heterogenous group of autosomal recessive liver disorders characterised by early onset of choleostasis that progresses to cirrhosis and liver failure before adulthood (Alonso *et al.,* 1994, Whitington *et al.,* 1994). There are three types of PFIC, type 1 which is caused by defects in bile synthesis, type 2 is caused by defective bile secretion and is known as Byler disease; patients with these conditions have normal levels of gamma-glutamyltransferase (GGT). A third subtype of PFIC presents later in life, carries a greater risk of portal hypertension and gastrointestinal bleeding and ends in liver failure at a later age. This subtype shows elevated levels of GGT activity, with ductular proliferation and portal inflammation observed in the liver of these patients. This condition shares histological, biochemical and genetic features with mice lacking mdr-2 (-/-) gene expression. Absence of MDR-3 mRNA in the liver was observed in patients with PFIC (high GGT serum activity) (Deleuze *et al.,* 1996). It has been reported that mutations in the *MDR-3* gene lead to this subtype of PFIC, negative canalicular staining for MDR-3 P-glycoprotein (using a polyclonal antibody) has been observed in two patients with this subtype of PFIC (de Vree *et al.,* 1998). Larger studies have since showed defects in MDR-3 present in at least one third of patients (neonatal period to early adulthood) with PFIC, and in 1 out of 47 Taiwanese infants with PFIC- high GGT (Chen *et al.,* 2001, Jacquemin *et al.,* 2001).

Intrahepatic cholestasis of pregnancy (ICP) is a liver disease of pregnancy with series consequences for the mother and fetus. Recently MDR-3 gene mutations have been

shown to be responsible for an additional phenotype of ICP in women with raised GGT but no family history of PFIC (Dixon *et al.,* 2000). It now appears that mutations in the MDR-3 gene are involved in a number of liver diseases, recently MDR-3 gene defects have been reported in adults with symptomatic intrahepatic and gallbladder cholesterol cholelithiasis (Rosmorduc *et al.,* 2001).

Intrahepatic Mdr-3 mRNA levels were shown to be unaffected in various liver diseases including primary biliary cirrhosis, chronic viral hepatitis and non-choleostatic liver cirrhosis, MDR-3 Pgp levels were not investigated (Dumoulin *et al.,* 1997). Decreased MDR-3 Pgp staining has been reported in liver specimens from patients with intrahepatic calculi, which is characterised clinically by chronic proliferative cholangitis with frequent stone recurrences (Shoda *et al.,* 2001).

1.7.4. The role of MDR-3 and MDR-1 P-glycoproteins in haematological malignancies

It is well established that MDR-1 P-glycoprotein is expressed in several haematological malignancies and that it may play a diagnostic and/ or predictive role in certain malignancies (reviewed by Sonneveld 2000). Data on MDR-3 in the various haematological malignancies is more limited.

There is a possibility that MDR-3 may encode a functional drug pump in certain B-cell lymphocytic leukaemias. MDR-3 expression appears to be limited to leukaemias of B-cell origin. In addition it has been suggested that *MDR-3* expression may be further restricted to certain B-cell developmental stages; no *MDR-3* expression has been found in MM which presents with end stage B-cells (Herweijer *et al.,* 1990). MM remains uncurable despite the initial chemosensitivity of most bone marrow plasma cells. The basis of this resistance is unclear (Pilarski *et al.,* 1999). On analysis of various B-cell malignancies Nooter and Sonneveld (1993) found that malignancies representing early B-cell development and also very mature B-cell tumours (Waldenstrom and MM) had no *MDR-3* expression, whereas B-PLL, CLL and HCL representing the intermediate and mature B-cells have been shown to express intermediate to high levels of *MDR-3.*

1.7.4.1. CLL

CLL has a highly variable clinical course and presents great difficulty in prognosis and therapy. Previous studies have shown that in general B-CLL cases express MDR-1 and MDR-3. MDR-1/ P-glycoprotein over expression appears to be intrinisic rather than acquired in this disease. PLL (pro-lymphocytic leukaemia) is a variant of CLL which is usually refractory to chemotherapy effective in CLL. Sometimes patients have a T-cell variant of this disease which has a less predictable prognosis than B-PLL. High levels of MDR-3 gene expression have been observed in cases of B-PLL with no detectable MDR-1 expression (Nooter *et al.,* 1990, Herweijer *et al.*,1990). MDR-1 has not been detected in B-PLL. *In vitro* drug uptake studies have shown that in PLL and B-CLL cells expressing MDR-3, drug accumulation can be significantly increased upon addition of cyclosporine and to a lesser extent verapamil (Herweijer *et al.,* 1990). However Ludescher *et al.* (1993) found no evidence that overexpression of MDR-3 was related to rhodamine efflux in B-CLL. Increases in intracellular rhodamine levels upon addition of cyclosporin A were observed in MDR-1 Pgp negative/ MDR-3 Pgp positive acute leukaemia patients (Arai *et al.,* 1997). Prior treatment has been associated with higher MDR-3 levels (no influence on MDR-1 levels) in one study (Herweijer *et al.,* 1990). Later work did not show such a correlation (Sonneveld *et al.,* 1992, Ludescher *et al.,* 1993). In patients with advanced B-CLL, MDR-3 mRNA expression (not MDR-1 expression) was significantly higher than in early stage disease (Sonneveld 1992). Ludscher *et al.* (1993) also found a tendency towards higher MDR-3 mRNA expression in patients with advanced stages of CLL. In another study MDR-3 (or MDR-1) gene expression did not predict for clinical response (el Rouby *et al.,* 1993).

1.7.4.2. AML

There has only been one study reporting MDR-3 expression in AML. MDR-3 mRNA was detected in 11 cases of AML, stage M2, 9 of these cases had the t(8;21) chromosomal abnormality which is one of the most common chromosomal abnormalities in AML particularly in subtype M2 (Mizutani *et al.,* 1997). It is thought that expression of MDR-3 may be restricted to certain B cell lineages, AML

has been classified into subtypes representing various stages of B cell development (French American British, FAB classification) therefore it is likely that MDR-3 may show a differential pattern of expression within these various subtypes. There appears to be a high correlation between MDR-1/ P-glycoprotein overexpression and clinical drug resistance in AML (Marie *et al.,* 1996). The *MDR-1* gene product has been shown to be frequently expressed in AML at diagnosis (i.e. prior to treatment) and at relapse. Reports vary regarding the levels of *MDR-1* expression within the various French American British (FAB) subclasses of AML. One study carried out over 6 years showed that Pgp overexpression was detected more frequently in FAB classes M4, M5A and M5B and less frequently in class M3 (Nussler *et al.,* 1996).This M3 promyelocytic sub-type is particularly sensitive to chemotherapy (efficacy of anthracycline treatment) (lack of Pgp may explain this).

1.7.4.3. NHL

The role, if any, of MDR-3 P-glycoprotein in NHL has not been investigated with the exception of a small study which reported *MDR-3* expression in B-NHL (9/21 patients showed *MDR-3* expression) (Nooter & Sonneveld 1993). In contrast several studies have addressed the role of MDR-1 P-glycoprotein in NHL. Most of this work on *MDR-1* encoded Pgp in NHL has been performed on frozen lymph node sections. In general more *MDR-1*/ Pgp expression is seen in treated than untreated patients. There appears to be no difference in Pgp expression between high and low grade lymphomas and between B and T sub-types (Marie *et al.,* 1996). Adult T-cell lymphoma (ATL) has been shown to frequently express *MDR-1*/ Pgp at presentation (Kuwezuru *et al.,* 1990). As to whether *MDR-1*/ Pgp is an indicator of better clinical response; three studies have shown a correlation (Pileri *et al.,* 1991, Cheng *et al.,* 1993, Rodriguez *et al.,* 1993). A larger study did not show such a correlation (Niehans *et al.,* 1992).

1.7.5. Detection of MDR-3 P-glycoprotein

In the majority of the haematological and tissue studies performed to date, the assessment of MDR-3 status was by MDR-3 gene expression using RT-PCR and the

RNase protection assay; MDR-3 encoded Pgp levels have not been investigated extensively, with the exception of a report by Arai *et al.,* (1997). Some work has been performed using polyclonal antibodies generated to the murine mdr-2 and human MDR-3 gene products where mdr-2 and MDR-3 Pgps have been observed in the canalicular membranes of liver (Devault and Gros 1990, Buschman *et al.,* 1992, Smit *et al.,* 1994, de Vree *et al.,* 1998). Excluding this work however, for the most part detection of the MDR-3 encoded gene product has relied on comparison studies with the C219 monoclonal antibody which recognises both the MDR-1 and the MDR-3/ mdr-2 encoded gene products (Georges *et al.,* 1990) and MDR-1 specific monoclonal antibodies such as JSB-1 and MRK16. At the time of commencement of this thesis no MDR-3 specific MAbs were commercially available, however another MAb which specifically recognises the MDR-3 encoded gene product has since been described by Scheffer *et al.* (2000).

In summary, despite the recent advances in the detection, treatment and management of breast cancer, with an increasing range of hormonal, cytotoxic and more recently MAb targeted drug regimes available in the clinic, between 40% and 50% of patients diagnosed will eventually die from this disease. As has been previously outlined identification of biological factors which can function as reliable markers of both prognosis and chemoresponsiveness will allow for more precise targeting of modalities for individual breast cancer patients. Results presented in this thesis, attempt to address the possible prognostic and predictive significance of a number of MDR proteins namely, MRP-1, P-170, LRP and MDR-3 which has recently been shown to transport cytotoxic drugs, and associated apoptosis and oncogenes in invasive breast cancers. Specific information regarding the exact role of the MDR-3 gene product and its contribution, if any, in breast cancer as indeed in other solid malignancies is limited. Results presented here, attempt to address the role of MDR-3 in breast cancer and also further address the role of MDR-3 in certain B-cell malignancies. In an attempt to identify a possible novel breast cancer associated antigen characterisation of the antigen recognised by a new MAb directed against dewaxed formalin fixed paraffin embedded archival ductal breast tumour tissue is also presented.

1.8. Aims of this thesis

1. Carry out an immunohistochemical study of MDR associated proteins in a panel of formalin fixed paraffin embedded invasive breast tumours. In order to identify if any of these proteins have possible prognostic/ predictive value in invasive breast cancer, correlate the expression of these proteins with patient and tumour characteristics and with Relapse Free Survival and Overall Survival. In addition analyse individual expression of these proteins in relation to the other protein markers being studied in order to address the multifactorial nature of this malignancy.
2. Generate and characterise a MAb which successfully recognises the MDR-3 encoded gene product. Attempt to address further the role, if any, of the MDR-3 encoded Pgp in human cancers with particular emphasis on breast and haematological malignancies.
3. Using novel production methodology developed in this laboratory (Moran *et al.*, 1998) generate and characterise a MAb directed against dewaxed formalin fixed paraffin embedded archival ductal breast tumour tissue in an attempt to identify a possible novel breast cancer associated antigen.

2. Materials & Methods

2.1. Cell Culture

- Addresses of suppliers are provided in **Appendix II.**

2.1.1. Cell culture procedures

All cell culture work was carried out in a class II down-flow re-circulating laminar air-flow cabinet (Nuaire Biological Cabinet). Strict aseptic technique was adhered to at all times. The laminar flow cabinet was swabbed with 70% industrial methylated spirits (IMS) before and after use, as were all items brought into the cabinet. Each cell line was assigned specific media and waste bottles. At any time, only one cell line was used in the laminar air-flow cabinet, and upon completion of work with any given cell line the laminar air-flow cabinet was allowed to clear for at least 15 minutes. This was to eliminate any possibilities of cross contamination between the various cell lines. The cabinet itself was cleaned weekly with industrial disinfectants (Virkon, Antec International or TEGO, TH Goldschmidt) as were all incubators used in the culture of cell lines and hybridomas. These detergents were alternated every month.

2.1.2. Subculturing cell lines

During routine subculturing or harvesting of adherent cell lines, cells were removed from their flasks by enzymatic detachment. Waste medium was removed from the cells which were then rinsed with pre-warmed trypsin-versene/EDTA (TV) solution (0.25% (w/v) trypsin (Gibco 043-05090), 0.01% (w/v) EDTA (Sigma) solution in PBS (Oxoid BR14A). This ensured that any naturally occurring trypsin inhibitor in residual serum was deactivated. Fresh TV was then placed in the flask and incubated until the cells were seen to have detached (2-10 minutes). The TV solution was deactivated by the addition of pre-warmed basal medium containing serum. The entire solution was then transferred to a 30ml sterile universal tube (Sterilin 128a) and centrifuged at 1000 rpm (Allegra^{TM} 6KR Centrifuge, Beckman, USA) for 5 minutes. The resulting pellet was then re-suspended in pre-warmed growth medium, cells counted and tissue culture flasks re-seeded at the required density.

Hybridoma and SP/2/0-Ag cells are loosely adherent. Cells were passaged by tapping the flask lightly and/or gentle pipetting with a 10ml sterile pipette (Elkay, Ireland). Cell suspensions were pooled and centrifuged at 1000 rpm for 5 minutes. The cell pellet was then re-suspended in culture medium and a cell count performed (section 2.1.3.) and the cells re-suspended at the desired density. Cells were grown in 5% CO_2.

2.1.3. Cell counting

Cells were trypsinised, pelleted and re-suspended in media as described in section 2.1.2. Cell counting and viability were carried out by using trypan blue (Gibco, 15250) dye exclusion technique. An aliquot of Trypan blue was added to a single cell suspension at a ratio of 1:2. After 3 minutes incubation at room temperature, a sample of the mixture was applied to the chamber of a Neubauer haemocytometer over which a glass cover slip had been placed. Cells in the 4 outer corner grids of the chamber were counted microscopically, an average per corner grid was calculated with the dilution factor taken into account and the final number multiplied by 10^4 to determine the number of cells per ml. Non-viable cells were those which stained blue while viable cells excluded the trypan blue dye and remained unstained.

2.1.4. Cell freezing

Cells of various passage numbers were frozen and cryo-preserved to serve as master stocks. Vials could then be thawed and cultured for study. Cells to be frozen were harvested in the log phase of growth and counted as described in Section 2.1.3. The pellets were re-suspended in foetal calf serum (pre-cooled to 4^0C) and an equal volume of freezing medium (DMSO (Sigma D-5879)/serum 1:9 (v/v)) was added drop-wise to the cell suspension to give a final concentration of at least $5x10^6$ cells per ml. 1.5ml of the cell suspension was quickly placed in a cryovial (Greiner 122278) which was placed in the vapour phase of liquid nitrogen container for 2.5-3.5 hr. After this, the cryovials were stored in liquid nitrogen until required.

2.1.5. Cell thawing

The cryopreserved cells were removed from the liquid nitrogen and thawed at 37^0C. Immediately prior to removal of a cryovial from the liquid nitrogen, a sterile universal tube containing growth medium was prepared for the rapid transfer and dilution of thawed cells (thus reducing the exposure time to DMSO, which is toxic at room temperature). Following the addition of the thawed cell suspension to the growth medium, the suspension was centrifuged at 1,000 rpm for 3 minutes, after which the pellet was re-suspended in fresh growth medium. A viability count was carried out (Section 2.2.3.) and the thawed cells were placed in tissue culture flasks and allowed to attach over night, The following day the cultures were re-fed with growth medium to remove any residual DMSO.

2.1.6. *Mycoplasma* analysis and sterility checking of cell lines

Mycoplasma analysis and sterility checks were carried out routinely on all hybridomas and cell lines used during the course of this work (Appendix III).

2.2. Cell lines

Details pertaining to the cell lines used for the experiments detailed in this thesis are provided in **Table 2.1.** Cell lines were maintained in 25 cm^2 (Costar 3035), 75 cm^2 (Costar 3075) or 175 cm^2 (Nunclon, NUNC) tissue culture flasks at 37^0C and fed every 2-3 days. Cell lines were cultured through 7-10 passages before they were discarded and new cultures grown from frozen stocks. Confluent hybridoma cell lines were grown for at least 7 days with no change of growth media when antibody was being harvested.

TABLE 2.1. Cell lines used during the course of this thesis

CELL LINE	HISTOLOGY	BASAL MEDIA	SOURCE/ REFERENCE
A2780	human ovarian carcinoma	ATC*	Prof. RJ Scheper, Free University Hospital, Amsterdam, The Netherlands.
BRL-3A	Buffalo rat liver	ATC*	ATCC
BSC-1	African green monkey kidney	ATC*	ATCC
BT-20	human ductal breast carcinoma	MEM	ATCC
COR-L-26-S	human large cell lung cancer	RPMI-1640 (Gibco)	Dr. P. Twentyman, Cambridge UK. Twentyman *et al.*, 1986.
COR-L-26-R	human large cell lung cancer MDR variant	RPMI-1640 (Gibco)	Dr. P. Twentymans, Cambridge UK. Twentyman *et al.*, 1986.
DLKP	non-small cell lung cancer	ATC*	NCTCC Law *et al.*, 1992
DLKP-SQ	non-small cell lung cancer	ATC*	NCTCC Mc Bride *et al.*, 1998
DLKP-Taxotere	non-small cell lung cancer MDR variant	ATC*	NCTCC
DLKPA	non-small cell lung cancer adriamycin resistant	ATC*	NCTCC Clynes *et al.*, 1992
DLKPA-5F	clone of above	ATC*	NCTCC Heenan *et al.*, 1995
DLKPA-2B	clone of above	ATC*	NCTCC Heenan *et al.*, 1995
FVB #C	transgenic mouse fibroblast parental	DMEM	Prof. Piet Borst, Dept of Molecular Biology, Netherlands Cancer Institute, Amsterdam, The Netherlands.
HL60-S	human leukaemia promyelocyte	RPMI-1640 (Gibco)	Marsh *et al.*, 1986.
MDA-MB-231	human ductal breast carcinoma	ATC*	ATCC
MDA-MB-435-S	human ductal breast carcinoma	ATC* #	ECACC

OAW42-S	human ovarian adenocarcinoma	ATC*	ECACC
OAW42-SR	human ovarian adenocarcinoma spontaneously resistant	ATC* #	ECACC
OAW42-A	human ovarian adenocarcinoma adriamycin resistant	ATC* #	ECACC
OAW42-A1	human ovarian adenocarcinoma adriamycin resistant (higher)	ATC * #	ECACC
RPMI	human nasal squamous carcinoma	MEM **	ATCC
RPMI-Taxol	human nasal squamous carcinoma MDR variant	MEM**	NCTCC Liang *et al.*, 2001
RPMI-Melphalan	human nasal squamous carcinoma MDR variant	MEM**	NCTCC Liang *et al.*, 2001
SP-2/O-AG14	myeloma mouse	DMEM (Glutamax 1 Gibco, 61965-026)	Shulman *et al.*, 1978
T24	Human bladder carcinoma	ATC* #	ATTCC
VIM # 1	MDR-3 transgenic mouse fibroblast	DMEM	Prof. Piet Borst, Dept of Molecular Biology, Netherlands Cancer Institute, Amsterdam, The Netherlands.
VO1-VO1	MDR-3 transgenic mouse fibroblast	DMEM	Prof. Piet Borst, Dept of Molecular Biology, Netherlands Cancer Institute, Amsterdam, The Netherlands.
ZR-75-1	human ductal breast carcinoma	RPMI-1640 (Gibco)	ECACC Engel *et al.*, 1978.

* ATC basal media consists of a 1:1 mixture of DMEM and HAMS F12

* # ATC basal media consists of a 1:1 mixture of DMEM and HAMS F12 supplemented with 1% sodium pyruvate

** MEM supplemented with 1% (vol/vol) non-essential amino acids (NEAA) (Gibco, 043-001140)

RPMI 1640 media supplied as a 1X stock (Gibco, 52400-025).

DMEM media supplied as a 1X stock (Gibco, 61965-026).

ECACC, European Collection of Animal Cell Cultures, Salisbury, Wiltshire, SP4 OJG.

ATCC American Type Culture Culture Collection, Rockville, MD, USA.

NCTCC : National Cell and Tissue Culture Centre, Dublin City University, Glasnevin, Dublin 9, Ireland.

2.3. Tissue samples

Formalin fixed paraffin wax embedded tissue was kindly provided by the Histopathology Departments of St. Vincents Hospital, Dublin and The National Maternity Hospital, Holles St., Dublin. 5μm sections of tissue blocks were cut using a microtome, mounted onto poly-l-lysine coated slides (section 2.7.1.) and dried overnight at 37° C. Slides were stored at room temperature until required.

2.4. Haematolgical samples

Cytospin preparations of peripheral blood, bone marrow and pleural fluid were kindly provided by the Department of Haematology, Belfast City Hospital, Belfast. Samples were stored at -20° C until required.

2.5. Monoclonal antibodies

Table 2.2. : MAbs used during the course of this work

MAb	*Specificity*	*Reference*
Anti Cytokeratin 6	Keratin 6	Wetzels *et al.*, 1991
Anti Cytokeratin 9 (multiepitope cocktail)	Keratin 9	Langbein *et al.*, 1994
BRI anti MDR-1 (6/1C) #	P-170/ Pgp	Moran *et al.*, 1997
MRK-16 **	P-170/ Pgp	Sugawara *et al.*, 1988
MRP-R1 *	MRP-1	Flens *et al.*, 1994
LRP-56 *	LRP	Scheper *et al.*, 1993
cerbB-2 oncoprotein, clone CB11 (Novacastra, UK)	CerbB-2	Corbett *et al.*, 1990
Anti human Bcl-2 oncoprotein (Dako)	Bcl-2	Zutter *et al.*, 1991
BRI anti Survivin (1C5) #	Survivin	Personal communication Dr. Irene Cleary

\# obtained from Bioresearch Ireland, Dublin.

* kindly provided by Prof. R.J. Scheper, Free University Hospital, Amsterdam, The Netherlands.

** kindly provided by Prof. T. Tsuruo, Department of Pathology, University of Tokoyo, Japan.

2.6. Monoclonal antibody production

- All experiments on animals were carried out under license and in compliance with the rules of The Cruelty to Animals Act, 1876, E.C. Directive 86/609/EC.

2.6.1. Immunogen for generation of archival breast tumour MAb, 5C3

The procedure for generation of this archival breast tumour MAb was based on methodology developed in this laboratory previously (Moran *et al.,* 1998). A *pre* treatment intraductal breast tumour was chosen as an immunogen. Five lots of 10 x 10μm sections were cut on a microtome, placed in 1ml eppendorf tubes and stored at R.T until required for immunisation. For screening of hybridomas and characterisation studies 2 x 5 μm sections were mounted onto poly-l-lysine slides, dried at 37° C overnight and stored at R.T. until required.

2.6.2. Immunogen for generation of the *MDR-3* specific MAb, 6/1G

The peptide used for the production of antibody 6/1G was selected after alignment searches of the EMBL Swiss-Prot protein sequence database using the Mail-FASTA program. The entire amino acid sequence of the *MDR-3* encoded Pgp (deduced from the known cDNA sequence (Van Der Bliek *et al.* 1988)) was obtained from the data bank above using the NETSERV program. From this deduced sequence a 12 amino acid peptide was chosen corresponding to the intracellularly located amino acids 13-24 (RPTSAEGDFELG) of *MDR-3* encoded Pgp which resides in the N-terminal part of the protein. This peptide was chosen because of its insignificant homology with the *MDR-1* encoded Pgp. The peptide was synthesised by BioSyn Ltd. (10, Malone Rd, Belfast BT9 5BN, Northern Ireland) and was purchased in both the free form and conjugated to bovine serum albumin (BSA) for immunisation and screening purposes.

2.6.3. *In vivo* immunisation for generation of archival breast tumour MAb, 5C3

Prior to immunisation of two Balb/c mice, one 10 x 10 μm section was dewaxed as follows: 0.5ml of xylene (BDH) was added to sections in the eppendorf tube and allowed to stand for 3-4 mins before being centrifuged at 13,000 rpm in a microfuge (Hereaus Instruments, Hanau, Germany) for 4 mins. This procedure was repeated one more time with fresh xylene and then with 100%, 90% and 70% ethanol, each twice. Finally the sections were washed twice in d.H_20 and PBS, before being resuspended in 0.5 ml of PBS and an equal volume of Freunds Complete Adjuvant (Sigma, F-5881) for immunisation. Using the BCA method (section 2.9.1.) the protein content of each 10 x 10 μm section was determined to be 600 μg/ ml which was divided equally between 2 mice. Each mouse received an intraperitoneal injection comprising 0.5 ml of combined dewaxed protein/ adjuvant. Following primary immunisation of two mice with one lot of dewaxed tumour in Freunds complete adjuvant (Sigma, F-5506), two booster injections of the same material in Freunds incomplete adjuvant were each administered at 2 weekly intervals. A similar final booster injection was administered 3 days prior to fusion of the immunised spleen cells with SP/2/0- Ag myeloma cells .

2.6.4. Combination *in vivo* and *in vitro* immunisation regime for generation of MDR-3 specific antibody 6/1G

Two balb/c mice received an intraperitoneal injection of peptide (140 μg reconstituted in 250 μl of PBS plus an equal volume of Freund's Complete Adjuvant (Sigma, F-5881) was added. One booster intraperitoneal injection was administered at 14 days (same immunogen plus an equal volume of Freund's Incomplete Adjuvant (Sigma, F-5506) was added). At 28 days the spleen was removed from one mouse and the spleen cells immunised *in vitro* with 60μg of conjugated peptide following instructions provided with the *in vitro* immunisation kit, Cell-Prime (supplied by Immune Systems Ltd., Paignton, UK). Three days after *in vitro* immunisation, the spleen cells were fused with SP/2/0-Ag myeloma cells.

2.6.5. Fusion protocol

The fusion procedure used for the production of monoclonal antibodies, 6/1G and 5C3 was a modification of the protocol outlined by Kohler and Milstein (1975). Prior to the removal of the spleen from the sacrificed mouse, SP/2/O-Ag14 myeloma cells (Shulman *et al.* 1978) were prepared for cell fusion by harvesting 2 x 75 cm^2 flasks and centrifuging at 1,000 rpm for 5 minutes in HEPES-free serum free medium. This step was repeated twice. A cell count was then performed (section 2.1.3.) and the cells kept at 37^0C.

A Balb/C mouse was then sacrificed by cervical dislocation. The animal was swabbed with 70% IMS and the spleen removed in a laminar flow cabinet with sterile dissection instruments. Single cells were obtained by forcing the spleen through a sterile Falcon cell strainer (Becton Dickinson 2360) using the plunger from a sterile 10ml syringe into serum free DMEM (the DMEM referred in this section is DMEM with Glutamax I (high glucose concentration - 4.5mg Gibco 61965-026) containing pyridoxine and without sodium pyruvate or HEPES. This cell suspension was placed in a universal and the volume adjusted to 10mls. Large clumps of cells were allowed to pellet by standing at room temperature for 2-3 minutes. The supernatant was then transferred to a fresh centrifuge tube and centrifuged at 1,000 rpm for 5 minutes. A cell count was performed as before (section 2.1.3.).

Splenocyte and SP/2/O-Ag myeloma cells were mixed in a universal at a ratio of 10:1 (a minimum of $1x10^7$ SP/2 are required for this procedure), centrifuged at 12,000 rpm for 5 minutes and re-suspended in serum free medium. This step was repeated twice. Following the final washing step, 1ml of PEG (polyethylene glycol, Roche Diagnostics GmbH, Germany, 783641) (pre-warmed to 37^0C) was added to the cell pellet with a Pasteur pipette using a gentle swirling and aspirating action for 30 seconds. After 30 seconds the aspiration was discontinued. 75 seconds after the start, 0.5ml of plating medium (DMEM with Glutamax I, 10% heat inactivated FCS (Myoclone Super Plus Foetal Bovine Serum, 16000-044, Gibco BRL Life Technologies US), 5% Briclone (Archport Ltd, Ireland) and 1% HAT (Hypoxanthine, Aminopterin, Thymidine) (Roche Diagnostics GmbH, 644579) was added slowly down the side of the universal while continuing to swirl gently. 5mls of

plating medium was added over the next 5 minutes (at 1 minute intervals followed by the addition of 5mls). Following this step, the cell suspension was centrifuged at 500 rpm for 5 minutes. The supernatant was removed and the cells were re-suspended in 10ml of plating medium and incubated at room temperature for 15 minutes.

Prior to performing the fusion, 0.5ml of plating medium was dispensed into each well of 8x48 well plates (Costar, 3548) and the plates were equilibrated in the CO_2 incubator @ 37^0C. Following the 15 minute incubation at room temperature one drop of fused cells was added to each well and the plates were incubated for 10-12 days at 37^0C, 5% CO_2.

2.6.6. Screening of MDR-3 specific MAb, 6/1G

Hybridomas were allowed to form large colonies and grow for at least 10 days undisturbed before supernatant was removed to screen for specific antibody production. Initially all clones produced were screened by enzyme linked immunosorbent assay (ELISA) against the peptide/ BSA complex and against BSA alone.

For the peptide ELISA, plates were coated with peptide (1μg per well in 100μl carbonate buffer and incubated at 4^o C overnight (BUFFER 1: 1.68g $NaHCO_3$ dissolved in 100ml distilled H_2O; BUFFER 2: 2.12g Na_2CO_3 dissolved in 100ml distilled H_2O. 74ml of BUFFER 1 was mixed with 26ml of BUFFER 2, pH 9.4 - 9.7). The plates were washed once in PBS and then incubated with blocking buffer (1% (w/v) BSA, 0.1% (w/v) Sodium Azide, PBS) for 1.5 hours at room temperature. The plates were washed once in PBS again. 100μl of test hybridoma supernatant was then added to the peptide coated plates and incubated at 37^0C for 1 hr. This solution was then discarded and the plates washed 3 times with wash buffer (0.1% (v/v) Tween 20 (Merck) in PBS). 100μl of secondary antibody, alkaline -phosphatase-linked rabbit anti-mouse immunoglobulins, I_gG, I_gM (Dako, diluted 1/10,000 in PBS 0.1% (v/v) Tween 20) was added to each well and incubated at 37^0C for 1 hr. The secondary antibody was removed and the plates washed 3 times with wash buffer as before. Plates were then incubated with the substrate solution (1 mg/ml p-nitrophenyl

phosphate (PNPP, Sigma 104-0) in 0.1M glycine, 0.001M $MgCl_2$, 0.001 M $ZnCl_2$, pH 10.4) at 37^0C for 0.5-1 hr or until a yellow colour appeared in the wells. The reaction was stopped by the addition of 1M NaOH which also enhanced colour. Absorbencies were read on a Titerex ELISA plate reader at 405nm. Positive reactivity was determined by comparing supernatant containing wells with those which had been incubated with PBS instead of supernatant. Only clones positive for the peptide/ BSA complex and negative for BSA alone were chosen for further expansion. Clone 6/1G was cloned by limiting dilution (section 2.6.9.) to ensure its monoclonal status. The resultant supernatant was used for characterisation of the antibody by western blotting and immunocytochemical methods.

2.6.7. Screening of archival breast tumour MAb, 5C3

Hybridomas were allowed to form large colonies and grow for at least 10 days undisturbed before supernatant was removed to screen for specific antibody production. All clones were screened by immunohistochemistry (section 2.7.3.) using formalin fixed paraffin wax embedded sections of the ductal breast tumour tissue which served as an immunogen i.e. positive control tissue block.

2.6.8 Subculture of hybridomas

Positive clones were further sub-cultured to 6 well plates (Costar) and gradually transferred (fed 2x in HAT medium followed by gradual change into HT (Hypoxanthine, Thymidine) medium - 50:50 HAT: HT) (Roche Diagnostics GmbH, 623091). Eventually (within two weeks) hybridoma clones were weaned off HT (by decreasing HT gradually in feeds every three days) and fed with DMEM medium supplemented with 5% Briclone (Archport Ltd., Ireland) and 10% heat inactivated FCS (Myoclone,Gibco).

2.6.9. Single cell cloning by limiting dilution

Using a multi-channel pipette (eppendorf) 100µl of DMEM growth medium was pipetted into each well of a sterile 96 well tissue culture treated plate. 100µl of cell

suspension from rapidly growing hybridomas at a concentration of $1x10^4$ cells was added to the top left hand well and mixed by pipetting. 1 in 2 doubling dilutions were performed down the left hand row of the plate (8 wells, 7 dilution steps) and mixed by pipetting, ensuring to change the pipette tip each time. 1 in 2 dilutions were also performed across the plate using a multi-channel pipette. Plates were then incubated for 7-10 days at 37^0C, 5 CO_2. Wells with a single colony were chosen. Hybridomas were cultured in 25 cm^2 flasks and the procedure repeated. The selected clones were screened by ELISA, Western blotting and immunocytochemistry and frozen stocks made of positive clones.

2.6.10. Isotype analysis

Isotyping was carried out using the The Isostrip Mouse Monoclonal Antibody Isotyping Kit (Roche Diagnostics GmbH, 1493027).

2.6.11. Quantitation of IgG/ IgM levels

IgG/ IgM levels were determined using a RID kit (radio immunodiffusion) (The Binding Site, UK).

2.6.12. Propagation of hybridomas by ascitic tumour production

Prior to introducing the hybridoma cells into the peritoneal cavity, Balb/c mice were primed with 0.5 mls of Freunds incomplete adjuvant (Sigma, P5506) which was administered into the peritoneum. 24 hours later, 5 x 10^5 hybridoma cells were asceptically resuspended in PBS and injected intraperitoneally. Tumour growth was evident 7-10 days post - injection. The mice were sacrificed and the ascitic tumour fluid drained under sterile conditions by insertion of a sterile 21-gauge needle into the peritoneal cavity. The ascitic fluid was centrifuged at 3,400 rpm for 10 minutes and the supernatant carefully removed, aliquoted and stored at -20° C. Hybridoma cells harvested from the peritoneal cavity were re-cultured in DMEM growth medium.

2.6.12. Purification of the MDR-3 specific MAb, 6/1G

Monoclonal antibody was purified from ascitic fluid using the ImmunoPure IgM purification kit from Pierce (44897). MAb 6/1G ascites was dialysed against two changes of 20 mM Tris/ 1.25M NaCl using a slide-A-lyser dialysis cassette (Pierce, 66425) overnight at R.T. on a stirring platform. The sample was diluted 1:1 with the ImmunoPure IgM binding buffer provided and was applied to the ImmunoPure immobilised MBP column which had been pre-washed and calibrated with 20 mls of binding buffer. All subsequent steps were carried out at 4^{o} C. 1 ml of sample was allowed to completely enter column to which .5 ml of binding buffer was then added and the column allowed to incubate for 30 minutes. Unbound protein was removed using binding buffer. Elution buffer was then applied to column and the eluted fractions were transferred to quartz cuvettes and absorbencies read at 280 nm on a spectrophotometer (Titertek, Multiscan Plus). Those fractions which showed highest absorbencies were pooled and concentrated 5.5X and exchanged with two changes of 150Mm Tris, pH 7.4. using Ultrafree - 15 centrifugal filter devices (Millipore, UFV2BGC10). Purified antibody was stored at -20^{o} until required.

2.7. Immunocytochemical analysis

2.7.1. Preparation of cytospins

Glass slides were coated with poly-l-lysine prior to all immunocytochemical procedures (Huang *et al.,* 1983). Glass slides were washed in 0.5% Tween 20, rinsed thoroughly, immersed in 70% IMS (industrial grade alcohol) for 10 minutes and dried at 37° C. Approx 10µl of poly-l-lysine was applied to one end of slides and spread into an even film over whole surface of slides. Coated slides were stored at R.T. until required.

Cells from actively growing cultures were trypsinised (Section 2.), washed 3 times in 10 minutes in PBS and diluted to a final concentration of $1x10^6$ cells/ml in PBS. 100µl of the cell suspension was then applied to a modified eppendorf component of a cytofuge and spun onto poly-l-lysine coated glass slides at 400 rpm for 4 minutes Cytospins were allowed to air dry over night. Alternatively 100µl aliquots of cell suspension were spotted directly onto coated slides, air dried for 20 minutes after which excess liquid was tapped off. Again slides were allowed to air dry overnight. All slides were foil wrapped and stored at -20^0 C until required.

2.7.2. Immunofluoresence studies on live cells

Viable MDR-3 transgenic cells VIM # 1 and their parental cells, FVB # c were tested for reactivity with antibody 6/1G by indirect immunofluoresence studies. When immunofluoresence studies are performed on viable cells only cell surface antigens are recognised (Schachner *et al.* 1977). Briefly, test cells were adjusted to a concentration of $1x10^6$ cells/ ml in PBS. 100µl of the cell suspension was pipetted into each of two eppendorf tubes and 100µl of antibody 6/1G added (neat supernatant) to one and 100µl of PBS added to the other. The tubes were mixed and incubated for 30 min at room temperature. The primary antibody was removed by centrifugation of cells at 1000 rpm for 4 min. Cells were washed 3x with PBS by the same procedure. 100µl secondary antibody, fluorescein isothicyanate-linked (FITC) sheep anti-mouse IgG (Roche Diagnostics GmbH, Germany) diluted 1/50 in PBS

was added to the tubes, mixed and incubated for 30 min at room temperature. Secondary antibody was removed and cells washed as above. Each cell pellet was resuspended in a mimimum amount of Vectashield (Vector Laboratories, U.K.) mounting medium and cells viewed using a Nikon phase contrast microscope fitted with an FITC filter.

2.7.3. Immunocytochemistry

All immunocytochemical studies on cell lines, haematological samples and paraffin wax embedded archival material were performed according to the method of Hsu *et al.* (1981) using an avidin-biotin horseradish peroxidase (HRP) conjugated kit (ABC) plus an appropriate secondary antibody.

Briefly, cytospin preparations were fixed for optimal time in ice cold acetone (MDR-1: 1 minute, MDR-3: 2 minutes, antibody 5C3: 4 minutes) and allowed to air dry for at least 15 mins prior to immunostaining. Tissue sections were dewaxed in xylene (2 x 5 mins), rehydrated in graded alcohols and placed in Tris Buffered Saline (TBS) / 0.05% (v/v) Tween 20. Endogenous peroxidase activity was quenched by placing cytospins in 0.6% (v/v) H_2O_2/ methanol and tissue sections in 3% (v/v) H_2O_2/ distilled H_2O for 5 mins. Antigen retrival was carried at this point if required as outlined below. All slides were blocked for non-specific staining with 20% (v/v) normal rabbit serum for 20 mins. Primary antibodies were applied to each sample optimally diluted in TBS/0.1% (v/v) Tween 20 (antibody 6/1G: 1:2 to 1:10 dilution; antibody 6/1C: neat supernatant or ascites diluted 1:40; MRK-16 used at 50μg/ml; MRP-R1: 1 in 40 dilution, 6.25μg/ml; LRP-56: 1 in 20 dilution; BCL-2: 1 in 50 dilution, 6.4μg/ml; Ki-67: 1 in 50 dilution (9μg/ml), survivin: 1 in 5 dilution). Primary antibodies were incubated overnight at 4°C or for 2 hours at R.T. which was followed by a 30 minute incubation with biotinylated rabbit anti-mouse IgG (1/300 dilution in TBS/ 0.1% (v/v) Tween 20) or biotinylated rabbit anti-rat (1/500 dilution in TBS/ 0.1% (v/v) Tween 20). Finally Vectastain Elite ABC reagent (HRP conjugated) (Vector Laboratories, UK PK-7100) was applied for 25 minutes and the peroxidase substrate 3'-3 diaminobenzidine tetrahydrochloride (DAB) peroxidase substrate kit (Vector Laboratories, UK SK-4100) was then applied for 5-7 mins. All

incubations were carried out at room temperature and slides were washed after each incubation in 3 changes of (TBS/0.1% (v/v) Tween 20 over 15 minutes. Tissues sections and cells were lightly stained with haematoxylin, differentiated in 1% (v/v) acid alcohol and 'blued' in Scott's tap water. Following dehydration in graded alcohols slides were cleared in xylene and mounted in DPX (BDH, UK). Negative control slides in which primary antibody was replaced by control mouse immunoglobulins (I-2000, Vector Laboratories, UK) (used within the manufacturers recommended concentration range) were included in all experiments.

2.7.4. Antigen retrival

The epitopes of certain antigens are masked by tissue fixation processes in order to enable the antibodies that recognise these antigens to work more successfully an antigen retrival technique is necessary prior to immunostaining (Cattoretti *et al.*, 1993). This technique was employed when detecting the Ki-67 antigen and the BCL-2 oncoprotein. Following blocking of endogenous peroxidase activity slides were placed in plastic coplin jars immersed in a freshly prepared 10mM citrate buffer, pH 6.0. and covered in microwave plastic film. Coplain jars were placed at fixed positions in microwave sections and treated at med-high power for a pre determind number of 4 minute cycles for each individual antibody (BCL-2 (Dako) 3 x 4minutes, Ki67 (Dako) 4 x 4 minutes). Citrate buffer was topped up following each cycle. Sections were allowed to stand in buffer for 15 - 20 minutes after which they were rinsed briefly in 1 x TBS/ 0.1% (v/v) Tween 20 before proceeding with immunohistochemical staining method.

2.8. Western Blot analysis

2.8.1. Protein Concentration Determination

Protein concentration was determined by the BCA method (Smith *et al.,* 1985) using a kit obtained from Pierce (Pierce, 23225). Protein samples (crude cell lysates or partially purified membranes) were diluted to a final volume of 100µl in PBS. BSA protein standards of known concentration were also prepared from a 2mg/ml stock solution in PBS in borosicilliate test tubes. Negative controls consisted of PBS. 100µl of the BCA reagent (prepared according to the manufacturers instructions) was added to each protein sample, mixed and incubated at 60^0C for 30 minutes. Following a brief cooling period, samples were transferred to plastic cuvettes (Elkay) and the absorbencies read on a spectrophotometer (Titertek, Multiscan Plus) at 562 nm. A standard curve of absorbance vs. protein concentration of the BSA standards was constructed. The protein concentration of the test samples was then calculated.

2.8.2. Preparation of murine tissues and cell lysates

Whole cell lysates of cell lines were prepared by trypsinisation of cells from culture flasks (1 X 75cm^2 flask), washing 3 x in PBS, sonicating cells with a Braun Labsonic U sonicator (Braun, US) in 250-500µl PBS containing a cocktail of protease inhibitors (Complete ™, 1697498, Roche Diagnostics, GmbH) until all cells were disrupted (when viewed microscopically). Samples were finally resuspended 1:1 in 2X reducing Laemmli sample buffer (Sigma, S-3401) (1.25M Tris-HCL, ph 6.8, 4% SDS, 10% 2-Mercaptoethanol, 20% glycerol and 0.02% bromophenol blue) prior to performing sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Alternatively after washing with PBS cell pellets were resuspended in 350 µl of M-Perm mammalian protein extraction reagent (Pierce, 78501), left to stand at R.T. for 15 minutes and then spun at 7,500 rpm for 15 minutes (Biofuge, Heraseus) to remove debris. Before resuspension in 2X Laemmli sample buffer samples of cell lysates and murine tissues in PBS were taken for determination of protein concentration as outlined above. Equal concentrations (15-30µg), where possible of

all cell membranes or partially purified tissues were applied to appropriate SDS-PAGE gels.

Kidney, liver, heart, spleen and lung were removed from a normal healthy Balb/c mouse and snap frozen in liquid N_2 until required. The tissues were prepared using a modification of the method of Ronchi *et al.* (1989). Briefly, the tissue samples were allowed to thaw whilst being maintained on crushed ice before being homogenised in ice cold PBS containing a cocktail of protease inhibitors (Roche Diagnostics GmbH, UK). The homogenate was centrifuged at 7500rpm for 10 min and the resultant supernatant centrifuged at 38000rpm for 1hr in an ultracentrifuge (Beckman Optima, model XL-80, Beckman U.K. Ltd). The resultant pellet was resuspended in 0.5 ml PBS plus 0.5 ml 2X reducing loading buffer (Sigma, S-3401). Aliquoted samples were stored at –20°C until required.

For detection of the antigen recognised by archival breast tumour MAb, 5C3 all test samples (in 2X sample buffer) were boiled at 100° C for 3 minutes, detection of the MDR-3 or MDR-1 specific antigen did not require heat treatment. All test samples (in 2X sample buffer) were stored at -20° C for short time periods until required for gel electrophoresis.

2.8.3. Gel electrophoresis

Proteins for Western blotting were separated by SDS-PAGE gel electrophoresis (Laemmli *et al*, 1970). Gels were prepared as outlined in table 2.4.3. and poured into clean 10cm x 8cm gel casting cassettes (Mighty Small™ SE 245, Hoefer, US) which consisted of one glass plate and one aluminium plate separated by 0.75cm^2 plastic spacers. The resolving gel was poured first and allowed to set. A layer of saturated isobutanol (50 mls isobutanol:5 mls dH_2O) was gently layered over the resolving gel to prevent drying out. When the resolving gel was set the layer of isobutanol was washed off with several changes of $d.H_2O$. The stacking gel was then poured and a comb was fitted allowing the formation of wells for sample loading. Once the gels had set (at room temperature) they were wrapped in tinfoil and stored at 4^0C if not used immediately. Approx. 15 - 30 μg of protein was applied to each well of the

polyacrylamide gel. Pre-stained molecular weight markers (New England Biolabs, UK) were also loaded onto the gel for the determination of the molecular weight of unknown protein samples. Gels were run at 250 volts and 45 milliamps. (Atto power pack, Atto Corp., Japan) for 1-1.5 hr. (values were halved if only one gel was being run), with 1X Tris/ Glycine/ SDS running buffer (Biorad, 161-0732). When the bromophenol dye front had reached the end of the gel, electrophoresis was stopped, the gel removed and equilibrated in transfer buffer (0.25 M Tris - 1.92M Glycine pH 8.4, 10X, Bio-Rad, US, 161-0734) for 15 minutes.

Table 2.3. Preparation of electrophoresis gels

Stock solutions	*6% Resolving Gel*	*7.5% Resolving Gel*	*Stacking Gel*
30% Acrylamide/ bis-Acrylamide (Sigma, A-3574)	3.04 ml	3.8 ml	0.8 ml
d.H_20	9.87 ml	9.1 ml	3.89 ml
8X Tris-HCl pH 8.8 (BDH, 444102T)	1.88 ml	1.88 ml	_
16X Tris-HCl pH 6.8 (BDH, 444092L)	_	_	0.31 ml
10% SDS (Biorad, 1610416)	150µ	150 µl	50 µl
10% Ammonium persulphate	60 µl	60 µl	17 µl
TEMED (Sigma, T-9281)	10 µl	10 µl	5 µl

2.8.4. Western blotting

Between 8 to 10 sheets of filter paper (Whatman, UK, 1001824) were soaked in transfer buffer (0.25 M Tris - 1.92M Glycine, 10X (Sigma T-4904), diluted 1 in 10 in d.H_2O) and placed on the anode of a semi-dry blotting apparatus (Trans-Blot SD Semi-Dry Transfer Cell, Bio-Rad, US) taking care to remove all bubbles by rolling a glass pasteur pipette across the sheets. PVDF membrane (Roche Diagnostics GmbH, 972206) or Sequi-blot ™ PVDF (Bio-Rad, US 162-0180) (used for transfer of immunoprecipitated proteins) both pre-soaked according to the manufacturers instructions were placed over the filter paper and the gel layered on top of this. A

further 8-10 sheets of pre-soaked filter paper were paced over the gel and any air bubbles removed. The cathode was carefully laid on top of the stack and the proteins transferred from gel to PVDF membrane at 15 volts and 34 milliamps for 25 minutes.

2.8.5. Development of Western blots by enhanced chemiluminescence (ECL)

ECL Western blotting detection is a highly sensitive, rapid, quantifiable, non-isotopic method for detecting proteins immobilised on membranes. The primary antibody is located with the Horseradish-Peroxidase (HRP)-labelled species specific secondary antibody. The HRP-labelled molecule catalyses the oxidation of luminol, resulting in the emission of light - chemiluminescence. Enhanced chemiluminescence (ECL) is the sustained emission of light provided by the inclusion of chemical enhancers in the HRP catalysed oxidation of luminol, and gives approximately 1000 fold more light that the oxidation of luminol alone.

Following Western blotting, the filter paper was removed and the membrane blocked for non-specific binding by incubating for 1- 4 hours on a rocking platform with non-fat milk (Marvel, Cadbury). After the blocking step was complete, the blot was rinsed once in TBS, and incubated with the primary antibody optimally diluted in TBS/0.1% (v/v) Tween 20 overnight at 4^0C or 2 hours at R.T. on a rocking platform (6/1G supernatant diluted 1/2 or ascites diluted 1:50; 6/1C ascites diluted 1 in 200 to 1 in 300 and 5C3 supernatant diluted 1 in 10, cytokeratin 6 specific MAb, Ab-1 (MS-766-P, Neomarkers, Lab Vision UK) used at a concentration of 2µg/ ml, cytokeratin 9 specific MAb cocktail supernatant diluted 1 in 100 (651104, Research Diagnostics, US). Mouse IgG diluted 1/5,000 (Vector labs, UK) in TBS/0.1% (v/v) Tween 20 or irrelevant supernatant served as a negative control. The following day blots were washed 3 times within 45 minutes with TBS/ 0.5% (v/v) Tween 20. Blots were then incubated with a rabbit anti-mouse HRP-conjugated secondary antibody (Dako, P0447) diluted 1/2,000 in TBS/0.1% (v/v) Tween 20 for 1 hour at room temperature on a rocking platform. The blots were washed as before, laid out on a glass plate

covered in parafilm and incubated with ECL reagents (Amersham RPN 2109) for 1 minute at room temperature. Following this the solution was tapped off, the blot laid between two sheets of cling film and exposed to LUMI film, chemiluminescence film (Roche Diagnostics GmbH, 166657) for various time periods and processed using standard x-ray developing procedures. Dried film was aligned with pre-stained molecular weight markers for molecular weight determination.

2.9. Immunoprecipitation studies

2.9.1. Immunoprecipitation

Protein isolated from the ductal carcinoma cell line, ZR-75-1 was directly labelled with the antibody 5C3, immunoprecipitated and then detected by Western blot analysis using the same antibody to re-probe the blot. Briefly at least 4 x 175 cm^2 flasks of ZR-75-1 cells were trypsinised and washed 3x with PBS. Cell pellets were resuspended in 650 µl of M-Perm mammalian protein extraction reagent (Pierce, 78501), left to stand at R.T. for 15 minutes and then spun at 7,500 rpm for 15 minutes (Biofuge, Heraseus). (At this point for one experiment with denatured cell lysates, the samples were boiled for 3 minutes prior to commencement of immunoprecipitation procedure).

0.5 and 0.25 ml test aliquots of supernatants were pre-cleared by incubating a 50:50 mixture of protein-L (P3351, Sigma, UK) and protein-G agarose (Roche Diagnostics GmbH, Germany) (i.e. 50µl of protein-L agarose and 50µl of protein - G agarose was added to all test samples, 25µl of each was added to 0.25 ml aliquots for negative control IgG samples). All samples were incubated with two changes (2 x 2.5 hours) of protein - L/ protein - G agarose beads at 4° C on a rocking platform. Beads were removed by spinning at 2,500 rpm for 10 minutes at 4° C. Supernatants were removed to clean eppendorf tubes and 100 µl of antibody 5C3 (concentrated 3X supernatant, using Millipore Ultrafree-15 centrifuge filter units, Z-36436-0, Sigma, US) was added to 0.5 ml lysate samples; 50 µl of antibody was added to 0.25 ml samples. 50 µl of control mouse IgG (Sigma, 31204) (used at a concentration of 22 µg/ ml) was added to 0.25 ml negative control lysates. Antibody/ lysate mixtures were incubated at 4° C overnight on a rocking platform. The following day in order to precipitate the antibody-antigen complex, protein - L / protein - G agarose was added to the antibody - antigen complex samples as before and incubated at 4° C for 4 hours on a rocking platform. Beads were removed by spinning at 2,500 rpm for 10 minutes at 4° C, *and the supernatant discarded.* To check how much protein was left in cell lysate test samples following immunoprecipitation procedure, some of the supernatants were retained and added to 2X laemmili sample buffer (Sigma, 3401)

and/ or non reducing 3X SDS sample buffer (New England Biolabs, UK), boiled for 1-3 minutes and stored at –20°C. The beads were then washed for 3 x 15 minute periods with buffers as described in Appendix III and pelleted at 2,500 rpm for 10 minutes. Following final wash and spin as much liquid as possible was removed from all samples. 50 µl of 2X laemmili sample buffer (Sigma, 3401) was added to original 0.25 ml lysates and control IgG samples, 100 µl was added to original 0.5 ml lysates. In further experiments, non-reducing 3X SDS sample buffer (New England Biolabs, UK) was added to test lysates. All samples were boiled for 1 minute and stored at -20 ° C until required for SDS-PAGE/ Western blot analysis.

Later experiments employed an optimised 8 hour immunoprecipitation protocol. A lysis buffer was prepared (Appendix II) from materials provided in the cellular labelling and immunoprecipitation kit supplied by Roche Diagnostics, GmbH. ZR-75-1 cell pellets prepared as in earlier experiments were thawed on ice and resuspended in lysis buffer (600µl lysis buffer added to each 175 cm^2 flask pellet), left for 2-3 minutes at R.T. and sonicated briefly (complete cell lysis was confirmed by viewing under an inverted microscope). Test and IgG control lysates were precleared with two changes (2 x 1 hour) of protein-L/ protein-G agarose (i.e. 100µl of protein-L and protein-G agarose was added to 200 µl test lysates/ control Ig lysate). Beads were removed as previously described. 150µl of concentrated MAb 5C3 was added to test lysates and incubated for 1 hour at 4°C , 50µl of mouse IgG was added to negative control lysates (22 µg). Following this 100µl of protein-L and 100µl protein-G were added to all lysates and incubated for a further three hours at 4°C on a rocking platform in order to precipitate the antibody-antigen complex. (Some supernatant at this point was placed in 2X loading buffer, boiled for 3 minutes and stored at –20°C). Pelleted beads were this time washed for 15 minutes intervals with two changes of wash buffer 1 and 2 and one change of wash buffer 3. Following a final wash and spin, as much liquid as possible was removed from all samples. 75 µl of 2X laemmili sample buffer (Sigma, 3401) was added to test lysates and control IgG samples. All samples were boiled for 1 minute and stored at -20 ° C until required for SDS-PAGE/ Western blot analysis. Further immunoprecipitated samples (in 2X loading buffer) were boiled for 10 mins and stored at -20° C.

2.9.2. Gel electrophoresis of immunoprecipitated proteins

Immunoprecipitated proteins were separated on 7.5% and 10% SDS-PAGE electrophoresis as described in section 2.8.3. Retained supernatants (in reducing and non reducing loading buffer) were also separated on 7.5% gels, in addition for some experiments combinations of immunoprecipitated proteins and supernatants were also separated on SDS-PAGE. Following SDS-PAGE western blot analysis was carried as outlined in section 2.8.4 - 2.8.5.. Immunoprecipitates and whole cell lysates of ZR-75-1 cells were probed with antibody 5C3, anti cytokeratin Ab-1 (MS-766-P1, Labvision, UK) or anti cytpkeratin 9 (651104, Research Diagnostics Inc., US). Control IgG ZR-75-1 immunoprecipitates were probed with antibody 5C3.

In order to visualise proteins SDS-PAGE gels were stained for 15-30 mins with a 0.25% (w/v) Coomassie Brilliant Blue R-250 solution (containing 45% methanol (v/v) and 45% d.H_2O (v/v)) and destained with 45% methanol (v/v)/ 10% acetic acid (v/v)/ 45% d. H_2O.

2.9.3. Preparation of isolated proteins for N-terminal sequencing

ZR-75-1 and control mouse immunoprecipitates were separated on 5 well 7.5% SDS PAGE and blotted onto PVDF membrane (Sequiblot ™, Biorad). Following transfer the membrane was washed briefly in d.H_20. The blot was then stained for 5 minutes with Comassie R-250 (0.25% Comassie Blue R-250 in 40% methanol) and destained for 1 hour with 50% (v/v) methanol. The membrane was air-dried for at least 3 hours and stored at -20° C. Protein bands were aligned with molecular weight markers and ECL films of immunoprecipitates which had been probed with antibody. The bands of interest were excised and processed for N-terminal sequencing.

2.9.4. Preparation of isolated proteins for internal sequencing

Following immunoprecipitation and separation on 7.5% SDS-PAGE using a 5 well spacer ensuring that the maximum amount of protein was loaded, gels were stained with Coomassie Brilliant Blue R250 (0.1% (v/v) in methanol, 45% dH_2O(v/v), 10%

acetic acid (v/v) and destained with 45% methanol (v/v) / 10% acetic acid (v/v) / 45% dH_2O (v/v). Staining and destaining protocols were carried out according to recommendations from Eurosequence. The band of interest was excised from gels, placed in eppendorf tubes and sent to Eurosequence BV (Gronigen, The Netherlands).

2. Materials & Methods

2.1. Cell Culture

- Addresses of suppliers are provided in **Appendix II.**

2.1.1. Cell culture procedures

All cell culture work was carried out in a class II down-flow re-circulating laminar air-flow cabinet (Nuaire Biological Cabinet). Strict aseptic technique was adhered to at all times. The laminar flow cabinet was swabbed with 70% industrial methylated spirits (IMS) before and after use, as were all items brought into the cabinet. Each cell line was assigned specific media and waste bottles. At any time, only one cell line was used in the laminar air-flow cabinet, and upon completion of work with any given cell line the laminar air-flow cabinet was allowed to clear for at least 15 minutes. This was to eliminate any possibilities of cross contamination between the various cell lines. The cabinet itself was cleaned weekly with industrial disinfectants (Virkon, Antec International or TEGO, TH Goldschmidt) as were all incubators used in the culture of cell lines and hybridomas. These detergents were alternated every month.

2.1.2. Subculturing cell lines

During routine subculturing or harvesting of adherent cell lines, cells were removed from their flasks by enzymatic detachment. Waste medium was removed from the cells which were then rinsed with pre-warmed trypsin-versene/EDTA (TV) solution (0.25% (w/v) trypsin (Gibco 043-05090), 0.01% (w/v) EDTA (Sigma) solution in PBS (Oxoid BR14A). This ensured that any naturally occurring trypsin inhibitor in residual serum was deactivated. Fresh TV was then placed in the flask and incubated until the cells were seen to have detached (2-10 minutes). The TV solution was deactivated by the addition of pre-warmed basal medium containing serum. The entire solution was then transferred to a 30ml sterile universal tube (Sterilin 128a) and centrifuged at 1000 rpm (Allegra^{TM} 6KR Centrifuge, Beckman, USA) for 5 minutes. The resulting pellet was then re-suspended in pre-warmed growth medium, cells counted and tissue culture flasks re-seeded at the required density.

Hybridoma and SP/2/0-Ag cells are loosely adherent. Cells were passaged by tapping the flask lightly and/or gentle pipetting with a 10ml sterile pipette (Elkay, Ireland). Cell suspensions were pooled and centrifuged at 1000 rpm for 5 minutes. The cell pellet was then re-suspended in culture medium and a cell count performed (section 2.1.3.) and the cells re-suspended at the desired density. Cells were grown in 5% CO_2.

2.1.3. Cell counting

Cells were trypsinised, pelleted and re-suspended in media as described in section 2.1.2. Cell counting and viability were carried out by using trypan blue (Gibco, 15250) dye exclusion technique. An aliquot of Trypan blue was added to a single cell suspension at a ratio of 1:2. After 3 minutes incubation at room temperature, a sample of the mixture was applied to the chamber of a Neubauer haemocytometer over which a glass cover slip had been placed. Cells in the 4 outer corner grids of the chamber were counted microscopically, an average per corner grid was calculated with the dilution factor taken into account and the final number multiplied by 10^4 to determine the number of cells per ml. Non-viable cells were those which stained blue while viable cells excluded the trypan blue dye and remained unstained.

2.1.4. Cell freezing

Cells of various passage numbers were frozen and cryo-preserved to serve as master stocks. Vials could then be thawed and cultured for study. Cells to be frozen were harvested in the log phase of growth and counted as described in Section 2.1.3. The pellets were re-suspended in foetal calf serum (pre-cooled to 4^0C) and an equal volume of freezing medium (DMSO (Sigma D-5879)/serum 1:9 (v/v)) was added drop-wise to the cell suspension to give a final concentration of at least $5x10^6$ cells per ml. 1.5ml of the cell suspension was quickly placed in a cryovial (Greiner 122278) which was placed in the vapour phase of liquid nitrogen container for 2.5-3.5 hr. After this, the cryovials were stored in liquid nitrogen until required.

2.1.5. Cell thawing

The cryopreserved cells were removed from the liquid nitrogen and thawed at 37^0C. Immediately prior to removal of a cryovial from the liquid nitrogen, a sterile universal tube containing growth medium was prepared for the rapid transfer and dilution of thawed cells (thus reducing the exposure time to DMSO, which is toxic at room temperature). Following the addition of the thawed cell suspension to the growth medium, the suspension was centrifuged at 1,000 rpm for 3 minutes, after which the pellet was re-suspended in fresh growth medium. A viability count was carried out (Section 2.2.3.) and the thawed cells were placed in tissue culture flasks and allowed to attach over night, The following day the cultures were re-fed with growth medium to remove any residual DMSO.

2.1.6. *Mycoplasma* analysis and sterility checking of cell lines

Mycoplasma analysis and sterility checks were carried out routinely on all hybridomas and cell lines used during the course of this work (Appendix III).

2.2. Cell lines

Details pertaining to the cell lines used for the experiments detailed in this thesis are provided in **Table 2.1.** Cell lines were maintained in 25 cm^2 (Costar 3035), 75 cm^2 (Costar 3075) or 175 cm^2 (Nunclon, NUNC) tissue culture flasks at 37^0C and fed every 2-3 days. Cell lines were cultured through 7-10 passages before they were discarded and new cultures grown from frozen stocks. Confluent hybridoma cell lines were grown for at least 7 days with no change of growth media when antibody was being harvested.

TABLE 2.1. Cell lines used during the course of this thesis

CELL LINE	HISTOLOGY	BASAL MEDIA	SOURCE/ REFERENCE
A2780	human ovarian carcinoma	ATC*	Prof. RJ Scheper, Free University Hospital, Amsterdam, The Netherlands.
BRL-3A	Buffalo rat liver	ATC*	ATCC
BSC-1	African green monkey kidney	ATC*	ATCC
BT-20	human ductal breast carcinoma	MEM	ATCC
COR-L-26-S	human large cell lung cancer	RPMI-1640 (Gibco)	Dr. P. Twentyman, Cambridge UK. Twentyman *et al.*, 1986.
COR-L-26-R	human large cell lung cancer MDR variant	RPMI-1640 (Gibco)	Dr. P. Twentymans, Cambridge UK. Twentyman *et al.*, 1986.
DLKP	non-small cell lung cancer	ATC*	NCTCC Law *et al.*, 1992
DLKP-SQ	non-small cell lung cancer	ATC*	NCTCC Mc Bride *et al.*, 1998
DLKP-Taxotere	non-small cell lung cancer MDR variant	ATC*	NCTCC
DLKPA	non-small cell lung cancer adriamycin resistant	ATC*	NCTCC Clynes *et al.*, 1992
DLKPA-5F	clone of above	ATC*	NCTCC Heenan *et al.*, 1995
DLKPA-2B	clone of above	ATC*	NCTCC Heenan *et al.*, 1995
FVB #C	transgenic mouse fibroblast parental	DMEM	Prof. Piet Borst, Dept of Molecular Biology, Netherlands Cancer Institute, Amsterdam, The Netherlands.
HL60-S	human leukaemia promyelocyte	RPMI-1640 (Gibco)	Marsh *et al.*, 1986.
MDA-MB-231	human ductal breast carcinoma	ATC*	ATCC
MDA-MB-435-S	human ductal breast carcinoma	ATC* #	ECACC

OAW42-S	human ovarian adenocarcinoma	ATC*	ECACC
OAW42-SR	human ovarian adenocarcinoma spontaneously resistant	ATC* #	ECACC
OAW42-A	human ovarian adenocarcinoma adriamycin resistant	ATC* #	ECACC
OAW42-A1	human ovarian adenocarcinoma adriamycin resistant (higher)	ATC * #	ECACC
RPMI	human nasal squamous carcinoma	MEM **	ATCC
RPMI-Taxol	human nasal squamous carcinoma MDR variant	MEM**	NCTCC Liang *et al.*, 2001
RPMI-Melphalan	human nasal squamous carcinoma MDR variant	MEM**	NCTCC Liang *et al.*, 2001
SP-2/O-AG14	myeloma mouse	DMEM (Glutamax 1 Gibco, 61965-026)	Shulman *et al.*, 1978
T24	Human bladder carcinoma	ATC* #	ATTCC
VIM # 1	MDR-3 transgenic mouse fibroblast	DMEM	Prof. Piet Borst, Dept of Molecular Biology, Netherlands Cancer Institute, Amsterdam, The Netherlands.
VO1-VO1	MDR-3 transgenic mouse fibroblast	DMEM	Prof. Piet Borst, Dept of Molecular Biology, Netherlands Cancer Institute, Amsterdam, The Netherlands.
ZR-75-1	human ductal breast carcinoma	RPMI-1640 (Gibco)	ECACC Engel *et al.*, 1978.

* ATC basal media consists of a 1:1 mixture of DMEM and HAMS F12

* # ATC basal media consists of a 1:1 mixture of DMEM and HAMS F12 supplemented with 1% sodium pyruvate

** MEM supplemented with 1% (vol/vol) non-essential amino acids (NEAA) (Gibco, 043-001140)

RPMI 1640 media supplied as a 1X stock (Gibco, 52400-025).

DMEM media supplied as a 1X stock (Gibco, 61965-026).

ECACC, European Collection of Animal Cell Cultures, Salisbury, Wiltshire, SP4 OJG.

ATCC American Type Culture Culture Collection, Rockville, MD, USA.

NCTCC : National Cell and Tissue Culture Centre, Dublin City University, Glasnevin, Dublin 9, Ireland.

2.3. Tissue samples

Formalin fixed paraffin wax embedded tissue was kindly provided by the Histopathology Departments of St. Vincents Hospital, Dublin and The National Maternity Hospital, Holles St., Dublin. 5μm sections of tissue blocks were cut using a microtome, mounted onto poly-l-lysine coated slides (section 2.7.1.) and dried overnight at 37° C. Slides were stored at room temperature until required.

2.4. Haematolgical samples

Cytospin preparations of peripheral blood, bone marrow and pleural fluid were kindly provided by the Department of Haematology, Belfast City Hospital, Belfast. Samples were stored at -20° C until required.

2.5. Monoclonal antibodies

Table 2.2. : MAbs used during the course of this work

MAb	*Specificity*	*Reference*
Anti Cytokeratin 6	Keratin 6	Wetzels *et al.*, 1991
Anti Cytokeratin 9 (multiepitope cocktail)	Keratin 9	Langbein *et al.*, 1994
BRI anti MDR-1 (6/1C) #	P-170/ Pgp	Moran *et al.*, 1997
MRK-16 **	P-170/ Pgp	Sugawara *et al.*, 1988
MRP-R1 *	MRP-1	Flens *et al.*, 1994
LRP-56 *	LRP	Scheper *et al.*, 1993
cerbB-2 oncoprotein, clone CB11 (Novacastra, UK)	CerbB-2	Corbett *et al.*, 1990
Anti human Bcl-2 oncoprotein (Dako)	Bcl-2	Zutter *et al.*, 1991
BRI anti Survivin (1C5) #	Survivin	Personal communication Dr. Irene Cleary

\# obtained from Bioresearch Ireland, Dublin.
* kindly provided by Prof. R.J. Scheper, Free University Hospital, Amsterdam, The Netherlands.
** kindly provided by Prof. T. Tsuruo, Department of Pathology, University of Tokoyo, Japan.

2.6. Monoclonal antibody production

- All experiments on animals were carried out under license and in compliance with the rules of The Cruelty to Animals Act, 1876, E.C. Directive 86/609/EC.

2.6.1. Immunogen for generation of archival breast tumour MAb, 5C3

The procedure for generation of this archival breast tumour MAb was based on methodology developed in this laboratory previously (Moran *et al.,* 1998). A *pre* treatment intraductal breast tumour was chosen as an immunogen. Five lots of 10 x 10μm sections were cut on a microtome, placed in 1ml eppendorf tubes and stored at R.T until required for immunisation. For screening of hybridomas and characterisation studies 2 x 5 μm sections were mounted onto poly-l-lysine slides, dried at 37° C overnight and stored at R.T. until required.

2.6.2. Immunogen for generation of the *MDR-3* specific MAb, 6/1G

The peptide used for the production of antibody 6/1G was selected after alignment searches of the EMBL Swiss-Prot protein sequence database using the Mail-FASTA program. The entire amino acid sequence of the *MDR-3* encoded Pgp (deduced from the known cDNA sequence (Van Der Bliek *et al.* 1988)) was obtained from the data bank above using the NETSERV program. From this deduced sequence a 12 amino acid peptide was chosen corresponding to the intracellularly located amino acids 13-24 (RPTSAEGDFELG) of *MDR-3* encoded Pgp which resides in the N-terminal part of the protein. This peptide was chosen because of its insignificant homology with the *MDR-1* encoded Pgp. The peptide was synthesised by BioSyn Ltd. (10, Malone Rd, Belfast BT9 5BN, Northern Ireland) and was purchased in both the free form and conjugated to bovine serum albumin (BSA) for immunisation and screening purposes.

2.6.3. *In vivo* immunisation for generation of archival breast tumour MAb, 5C3

Prior to immunisation of two Balb/c mice, one 10 x 10 μm section was dewaxed as follows: 0.5ml of xylene (BDH) was added to sections in the eppendorf tube and allowed to stand for 3-4 mins before being centrifuged at 13,000 rpm in a microfuge (Hereaus Instruments, Hanau, Germany) for 4 mins. This procedure was repeated one more time with fresh xylene and then with 100%, 90% and 70% ethanol, each twice. Finally the sections were washed twice in d.H_20 and PBS, before being resuspended in 0.5 ml of PBS and an equal volume of Freunds Complete Adjuvant (Sigma, F-5881) for immunisation. Using the BCA method (section 2.9.1.) the protein content of each 10 x 10 μm section was determined to be 600 μg/ ml which was divided equally between 2 mice. Each mouse received an intraperitoneal injection comprising 0.5 ml of combined dewaxed protein/ adjuvant. Following primary immunisation of two mice with one lot of dewaxed tumour in Freunds complete adjuvant (Sigma, F-5506), two booster injections of the same material in Freunds incomplete adjuvant were each administered at 2 weekly intervals. A similar final booster injection was administered 3 days prior to fusion of the immunised spleen cells with SP/2/0- Ag myeloma cells .

2.6.4. Combination *in vivo* and *in vitro* immunisation regime for generation of MDR-3 specific antibody 6/1G

Two balb/c mice received an intraperitoneal injection of peptide (140 μg reconstituted in 250 μl of PBS plus an equal volume of Freund's Complete Adjuvant (Sigma, F-5881) was added. One booster intraperitoneal injection was administered at 14 days (same immunogen plus an equal volume of Freund's Incomplete Adjuvant (Sigma, F-5506) was added). At 28 days the spleen was removed from one mouse and the spleen cells immunised *in vitro* with 60μg of conjugated peptide following instructions provided with the *in vitro* immunisation kit, Cell-Prime (supplied by Immune Systems Ltd., Paignton, UK). Three days after *in vitro* immunisation, the spleen cells were fused with SP/2/0-Ag myeloma cells.

2.6.5. Fusion protocol

The fusion procedure used for the production of monoclonal antibodies, 6/1G and 5C3 was a modification of the protocol outlined by Kohler and Milstein (1975). Prior to the removal of the spleen from the sacrificed mouse, SP/2/O-Ag14 myeloma cells (Shulman *et al.* 1978) were prepared for cell fusion by harvesting 2 x 75 cm^2 flasks and centrifuging at 1,000 rpm for 5 minutes in HEPES-free serum free medium. This step was repeated twice. A cell count was then performed (section 2.1.3.) and the cells kept at 37^0C.

A Balb/C mouse was then sacrificed by cervical dislocation. The animal was swabbed with 70% IMS and the spleen removed in a laminar flow cabinet with sterile dissection instruments. Single cells were obtained by forcing the spleen through a sterile Falcon cell strainer (Becton Dickinson 2360) using the plunger from a sterile 10ml syringe into serum free DMEM (the DMEM referred in this section is DMEM with Glutamax I (high glucose concentration - 4.5mg Gibco 61965-026) containing pyridoxine and without sodium pyruvate or HEPES. This cell suspension was placed in a universal and the volume adjusted to 10mls. Large clumps of cells were allowed to pellet by standing at room temperature for 2-3 minutes. The supernatant was then transferred to a fresh centrifuge tube and centrifuged at 1,000 rpm for 5 minutes. A cell count was performed as before (section 2.1.3.).

Splenocyte and SP/2/O-Ag myeloma cells were mixed in a universal at a ratio of 10:1 (a minimum of $1x10^7$ SP/2 are required for this procedure), centrifuged at 12,000 rpm for 5 minutes and re-suspended in serum free medium. This step was repeated twice. Following the final washing step, 1ml of PEG (polyethylene glycol, Roche Diagnostics GmbH, Germany, 783641) (pre-warmed to 37^0C) was added to the cell pellet with a Pasteur pipette using a gentle swirling and aspirating action for 30 seconds. After 30 seconds the aspiration was discontinued. 75 seconds after the start, 0.5ml of plating medium (DMEM with Glutamax I, 10% heat inactivated FCS (Myoclone Super Plus Foetal Bovine Serum, 16000-044, Gibco BRL Life Technologies US), 5% Briclone (Archport Ltd, Ireland) and 1% HAT (Hypoxanthine, Aminopterin, Thymidine) (Roche Diagnostics GmbH, 644579) was added slowly down the side of the universal while continuing to swirl gently. 5mls of

plating medium was added over the next 5 minutes (at 1 minute intervals followed by the addition of 5mls). Following this step, the cell suspension was centrifuged at 500 rpm for 5 minutes. The supernatant was removed and the cells were re-suspended in 10ml of plating medium and incubated at room temperature for 15 minutes.

Prior to performing the fusion, 0.5ml of plating medium was dispensed into each well of 8x48 well plates (Costar, 3548) and the plates were equilibrated in the CO_2 incubator @ 37^0C. Following the 15 minute incubation at room temperature one drop of fused cells was added to each well and the plates were incubated for 10-12 days at 37^0C, 5% CO_2.

2.6.6. Screening of MDR-3 specific MAb, 6/1G

Hybridomas were allowed to form large colonies and grow for at least 10 days undisturbed before supernatant was removed to screen for specific antibody production. Initially all clones produced were screened by enzyme linked immunosorbent assay (ELISA) against the peptide/ BSA complex and against BSA alone.

For the peptide ELISA, plates were coated with peptide (1μg per well in 100μl carbonate buffer and incubated at 4^o C overnight (BUFFER 1: 1.68g $NaHCO_3$ dissolved in 100ml distilled H_2O; BUFFER 2: 2.12g Na_2CO_3 dissolved in 100ml distilled H_2O. 74ml of BUFFER 1 was mixed with 26ml of BUFFER 2, pH 9.4 - 9.7). The plates were washed once in PBS and then incubated with blocking buffer (1% (w/v) BSA, 0.1% (w/v) Sodium Azide, PBS) for 1.5 hours at room temperature. The plates were washed once in PBS again. 100μl of test hybridoma supernatant was then added to the peptide coated plates and incubated at 37^0C for 1 hr. This solution was then discarded and the plates washed 3 times with wash buffer (0.1% (v/v) Tween 20 (Merck) in PBS). 100μl of secondary antibody, alkaline -phosphatase-linked rabbit anti-mouse immunoglobulins, I_gG, I_gM (Dako, diluted 1/10,000 in PBS 0.1% (v/v) Tween 20) was added to each well and incubated at 37^0C for 1 hr. The secondary antibody was removed and the plates washed 3 times with wash buffer as before. Plates were then incubated with the substrate solution (1 mg/ml p-nitrophenyl

phosphate (PNPP, Sigma 104-0) in 0.1M glycine, 0.001M $MgCl_2$, 0.001 M $ZnCl_2$, pH 10.4) at 37^0C for 0.5-1 hr or until a yellow colour appeared in the wells. The reaction was stopped by the addition of 1M NaOH which also enhanced colour. Absorbencies were read on a Titerex ELISA plate reader at 405nm. Positive reactivity was determined by comparing supernatant containing wells with those which had been incubated with PBS instead of supernatant. Only clones positive for the peptide/ BSA complex and negative for BSA alone were chosen for further expansion. Clone 6/1G was cloned by limiting dilution (section 2.6.9.) to ensure its monoclonal status. The resultant supernatant was used for characterisation of the antibody by western blotting and immunocytochemical methods.

2.6.7. Screening of archival breast tumour MAb, 5C3

Hybridomas were allowed to form large colonies and grow for at least 10 days undisturbed before supernatant was removed to screen for specific antibody production. All clones were screened by immunohistochemistry (section 2.7.3.) using formalin fixed paraffin wax embedded sections of the ductal breast tumour tissue which served as an immunogen i.e. positive control tissue block.

2.6.8 Subculture of hybridomas

Positive clones were further sub-cultured to 6 well plates (Costar) and gradually transferred (fed 2x in HAT medium followed by gradual change into HT (Hypoxanthine, Thymidine) medium - 50:50 HAT: HT) (Roche Diagnostics GmbH, 623091). Eventually (within two weeks) hybridoma clones were weaned off HT (by decreasing HT gradually in feeds every three days) and fed with DMEM medium supplemented with 5% Briclone (Archport Ltd., Ireland) and 10% heat inactivated FCS (Myoclone,Gibco).

2.6.9. Single cell cloning by limiting dilution

Using a multi-channel pipette (eppendorf) 100µl of DMEM growth medium was pipetted into each well of a sterile 96 well tissue culture treated plate. 100µl of cell

suspension from rapidly growing hybridomas at a concentration of $1x10^4$ cells was added to the top left hand well and mixed by pipetting. 1 in 2 doubling dilutions were performed down the left hand row of the plate (8 wells, 7 dilution steps) and mixed by pipetting, ensuring to change the pipette tip each time. 1 in 2 dilutions were also performed across the plate using a multi-channel pipette. Plates were then incubated for 7-10 days at 37^0C, 5 CO_2. Wells with a single colony were chosen. Hybridomas were cultured in 25 cm^2 flasks and the procedure repeated. The selected clones were screened by ELISA, Western blotting and immunocytochemistry and frozen stocks made of positive clones.

2.6.10. Isotype analysis

Isotyping was carried out using the The Isostrip Mouse Monoclonal Antibody Isotyping Kit (Roche Diagnostics GmbH, 1493027).

2.6.11. Quantitation of IgG/ IgM levels

IgG/ IgM levels were determined using a RID kit (radio immunodiffusion) (The Binding Site, UK).

2.6.12. Propagation of hybridomas by ascitic tumour production

Prior to introducing the hybridoma cells into the peritoneal cavity, Balb/c mice were primed with 0.5 mls of Freunds incomplete adjuvant (Sigma, P5506) which was administered into the peritoneum. 24 hours later, 5 x 10^5 hybridoma cells were asceptically resuspended in PBS and injected intraperitoneally. Tumour growth was evident 7-10 days post - injection. The mice were sacrificed and the ascitic tumour fluid drained under sterile conditions by insertion of a sterile 21-gauge needle into the peritoneal cavity. The ascitic fluid was centrifuged at 3,400 rpm for 10 minutes and the supernatant carefully removed, aliquoted and stored at -20^o C. Hybridoma cells harvested from the peritoneal cavity were re-cultured in DMEM growth medium.

2.6.12. Purification of the MDR-3 specific MAb, 6/1G

Monoclonal antibody was purified from ascitic fluid using the ImmunoPure IgM purification kit from Pierce (44897). MAb 6/1G ascites was dialysed against two changes of 20 mM Tris/ 1.25M NaCl using a slide-A-lyser dialysis cassette (Pierce, 66425) overnight at R.T. on a stirring platform. The sample was diluted 1:1 with the ImmunoPure IgM binding buffer provided and was applied to the ImmunoPure immobilised MBP column which had been pre-washed and calibrated with 20 mls of binding buffer. All subsequent steps were carried out at 4^{o} C. 1 ml of sample was allowed to completely enter column to which .5 ml of binding buffer was then added and the column allowed to incubate for 30 minutes. Unbound protein was removed using binding buffer. Elution buffer was then applied to column and the eluted fractions were transferred to quartz cuvettes and absorbencies read at 280 nm on a spectrophotometer (Titertek, Multiscan Plus). Those fractions which showed highest absorbencies were pooled and concentrated 5.5X and exchanged with two changes of 150Mm Tris, pH 7.4. using Ultrafree - 15 centrifugal filter devices (Millipore, UFV2BGC10). Purified antibody was stored at -20^{o} until required.

2.7. Immunocytochemical analysis

2.7.1. Preparation of cytospins

Glass slides were coated with poly-l-lysine prior to all immunocytochemical procedures (Huang *et al.,* 1983). Glass slides were washed in 0.5% Tween 20, rinsed thoroughly, immersed in 70% IMS (industrial grade alcohol) for 10 minutes and dried at 37° C. Approx 10µl of poly-l-lysine was applied to one end of slides and spread into an even film over whole surface of slides. Coated slides were stored at R.T. until required.

Cells from actively growing cultures were trypsinised (Section 2.), washed 3 times in 10 minutes in PBS and diluted to a final concentration of $1x10^6$ cells/ml in PBS. 100µl of the cell suspension was then applied to a modified eppendorf component of a cytofuge and spun onto poly-l-lysine coated glass slides at 400 rpm for 4 minutes Cytospins were allowed to air dry over night. Alternatively 100µl aliquots of cell suspension were spotted directly onto coated slides, air dried for 20 minutes after which excess liquid was tapped off. Again slides were allowed to air dry overnight. All slides were foil wrapped and stored at -20^0 C until required.

2.7.2. Immunofluoresence studies on live cells

Viable MDR-3 transgenic cells VIM # 1 and their parental cells, FVB # c were tested for reactivity with antibody 6/1G by indirect immunofluoresence studies. When immunofluoresence studies are performed on viable cells only cell surface antigens are recognised (Schachner *et al.* 1977). Briefly, test cells were adjusted to a concentration of $1x10^6$ cells/ ml in PBS. 100µl of the cell suspension was pipetted into each of two eppendorf tubes and 100µl of antibody 6/1G added (neat supernatant) to one and 100µl of PBS added to the other. The tubes were mixed and incubated for 30 min at room temperature. The primary antibody was removed by centrifugation of cells at 1000 rpm for 4 min. Cells were washed 3x with PBS by the same procedure. 100µl secondary antibody, fluorescein isothicyanate-linked (FITC) sheep anti-mouse IgG (Roche Diagnostics GmbH, Germany) diluted 1/50 in PBS

was added to the tubes, mixed and incubated for 30 min at room temperature. Secondary antibody was removed and cells washed as above. Each cell pellet was resuspended in a mimimum amount of Vectashield (Vector Laboratories, U.K.) mounting medium and cells viewed using a Nikon phase contrast microscope fitted with an FITC filter.

2.7.3. Immunocytochemistry

All immunocytochemical studies on cell lines, haematological samples and paraffin wax embedded archival material were performed according to the method of Hsu *et al.* (1981) using an avidin-biotin horseradish peroxidase (HRP) conjugated kit (ABC) plus an appropriate secondary antibody.

Briefly, cytospin preparations were fixed for optimal time in ice cold acetone (MDR-1: 1 minute, MDR-3: 2 minutes, antibody 5C3: 4 minutes) and allowed to air dry for at least 15 mins prior to immunostaining. Tissue sections were dewaxed in xylene (2 x 5 mins), rehydrated in graded alcohols and placed in Tris Buffered Saline (TBS) / 0.05% (v/v) Tween 20. Endogenous peroxidase activity was quenched by placing cytospins in 0.6% (v/v) H_2O_2/ methanol and tissue sections in 3% (v/v) H_2O_2/ distilled H_2O for 5 mins. Antigen retrival was carried at this point if required as outlined below. All slides were blocked for non-specific staining with 20% (v/v) normal rabbit serum for 20 mins. Primary antibodies were applied to each sample optimally diluted in TBS/0.1% (v/v) Tween 20 (antibody 6/1G: 1:2 to 1:10 dilution; antibody 6/1C: neat supernatant or ascites diluted 1:40; MRK-16 used at 50μg/ml; MRP-R1: 1 in 40 dilution, 6.25μg/ml; LRP-56: 1 in 20 dilution; BCL-2: 1 in 50 dilution, 6.4μg/ml; Ki-67: 1 in 50 dilution (9μg/ml), survivin: 1 in 5 dilution). Primary antibodies were incubated overnight at 4°C or for 2 hours at R.T. which was followed by a 30 minute incubation with biotinylated rabbit anti-mouse IgG (1/300 dilution in TBS/ 0.1% (v/v) Tween 20) or biotinylated rabbit anti-rat (1/500 dilution in TBS/ 0.1% (v/v) Tween 20). Finally Vectastain Elite ABC reagent (HRP conjugated) (Vector Laboratories, UK PK-7100) was applied for 25 minutes and the peroxidase substrate 3'-3 diaminobenzidine tetrahydrochloride (DAB) peroxidase substrate kit (Vector Laboratories, UK SK-4100) was then applied for 5-7 mins. All

incubations were carried out at room temperature and slides were washed after each incubation in 3 changes of (TBS/0.1% (v/v) Tween 20 over 15 minutes. Tissues sections and cells were lightly stained with haematoxylin, differentiated in 1% (v/v) acid alcohol and 'blued' in Scott's tap water. Following dehydration in graded alcohols slides were cleared in xylene and mounted in DPX (BDH, UK). Negative control slides in which primary antibody was replaced by control mouse immunoglobulins (I-2000, Vector Laboratories, UK) (used within the manufacturers recommended concentration range) were included in all experiments.

2.7.4. Antigen retrival

The epitopes of certain antigens are masked by tissue fixation processes in order to enable the antibodies that recognise these antigens to work more successfully an antigen retrival technique is necessary prior to immunostaining (Cattoretti *et al.,* 1993). This technique was employed when detecting the Ki-67 antigen and the BCL-2 oncoprotein. Following blocking of endogenous peroxidase activity slides were placed in plastic coplin jars immersed in a freshly prepared 10mM citrate buffer, pH 6.0. and covered in microwave plastic film. Coplain jars were placed at fixed positions in microwave sections and treated at med-high power for a pre determind number of 4 minute cycles for each individual antibody (BCL-2 (Dako) 3 x 4minutes, Ki67 (Dako) 4 x 4 minutes). Citrate buffer was topped up following each cycle. Sections were allowed to stand in buffer for 15 - 20 minutes after which they were rinsed briefly in 1 x TBS/ 0.1% (v/v) Tween 20 before proceeding with immunohistochemical staining method.

2.8. Western Blot analysis

2.8.1. Protein Concentration Determination

Protein concentration was determined by the BCA method (Smith *et al.,* 1985) using a kit obtained from Pierce (Pierce, 23225). Protein samples (crude cell lysates or partially purified membranes) were diluted to a final volume of 100µl in PBS. BSA protein standards of known concentration were also prepared from a 2mg/ml stock solution in PBS in borosicilliate test tubes. Negative controls consisted of PBS. 100µl of the BCA reagent (prepared according to the manufacturers instructions) was added to each protein sample, mixed and incubated at 60^0C for 30 minutes. Following a brief cooling period, samples were transferred to plastic cuvettes (Elkay) and the absorbencies read on a spectrophotometer (Titertek, Multiscan Plus) at 562 nm. A standard curve of absorbance vs. protein concentration of the BSA standards was constructed. The protein concentration of the test samples was then calculated.

2.8.2. Preparation of murine tissues and cell lysates

Whole cell lysates of cell lines were prepared by trypsinisation of cells from culture flasks (1 X 75cm^2 flask), washing 3 x in PBS, sonicating cells with a Braun Labsonic U sonicator (Braun, US) in 250-500µl PBS containing a cocktail of protease inhibitors (Complete ™, 1697498, Roche Diagnostics, GmbH) until all cells were disrupted (when viewed microscopically). Samples were finally resuspended 1:1 in 2X reducing Laemmli sample buffer (Sigma, S-3401) (1.25M Tris-HCL, ph 6.8, 4% SDS, 10% 2-Mercaptoethanol, 20% glycerol and 0.02% bromophenol blue) prior to performing sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Alternatively after washing with PBS cell pellets were resuspended in 350 µl of M-Perm mammalian protein extraction reagent (Pierce, 78501), left to stand at R.T. for 15 minutes and then spun at 7,500 rpm for 15 minutes (Biofuge, Heraseus) to remove debris. Before resuspension in 2X Laemmli sample buffer samples of cell lysates and murine tissues in PBS were taken for determination of protein concentration as outlined above. Equal concentrations (15-30µg), where possible of

all cell membranes or partially purified tissues were applied to appropriate SDS-PAGE gels.

Kidney, liver, heart, spleen and lung were removed from a normal healthy Balb/c mouse and snap frozen in liquid N_2 until required. The tissues were prepared using a modification of the method of Ronchi *et al.* (1989). Briefly, the tissue samples were allowed to thaw whilst being maintained on crushed ice before being homogenised in ice cold PBS containing a cocktail of protease inhibitors (Roche Diagnostics GmbH, UK). The homogenate was centrifuged at 7500rpm for 10 min and the resultant supernatant centrifuged at 38000rpm for 1hr in an ultracentrifuge (Beckman Optima, model XL-80, Beckman U.K. Ltd). The resultant pellet was resuspended in 0.5 ml PBS plus 0.5 ml 2X reducing loading buffer (Sigma, S-3401). Aliquoted samples were stored at –20°C until required.

For detection of the antigen recognised by archival breast tumour MAb, 5C3 all test samples (in 2X sample buffer) were boiled at 100° C for 3 minutes, detection of the MDR-3 or MDR-1 specific antigen did not require heat treatment. All test samples (in 2X sample buffer) were stored at -20° C for short time periods until required for gel electrophoresis.

2.8.3. Gel electrophoresis

Proteins for Western blotting were separated by SDS-PAGE gel electrophoresis (Laemmli *et al*, 1970). Gels were prepared as outlined in table 2.4.3. and poured into clean 10cm x 8cm gel casting cassettes (Mighty Small™ SE 245, Hoefer, US) which consisted of one glass plate and one aluminium plate separated by 0.75cm^2 plastic spacers. The resolving gel was poured first and allowed to set. A layer of saturated isobutanol (50 mls isobutanol:5 mls dH_2O) was gently layered over the resolving gel to prevent drying out. When the resolving gel was set the layer of isobutanol was washed off with several changes of $d.H_2O$. The stacking gel was then poured and a comb was fitted allowing the formation of wells for sample loading. Once the gels had set (at room temperature) they were wrapped in tinfoil and stored at 4^{0}C if not used immediately. Approx. 15 - 30 μg of protein was applied to each well of the

polyacrylamide gel. Pre-stained molecular weight markers (New England Biolabs, UK) were also loaded onto the gel for the determination of the molecular weight of unknown protein samples. Gels were run at 250 volts and 45 milliamps. (Atto power pack, Atto Corp., Japan) for 1-1.5 hr. (values were halved if only one gel was being run), with 1X Tris/ Glycine/ SDS running buffer (Biorad, 161-0732). When the bromophenol dye front had reached the end of the gel, electrophoresis was stopped, the gel removed and equilibrated in transfer buffer (0.25 M Tris - 1.92M Glycine pH 8.4, 10X, Bio-Rad, US, 161-0734) for 15 minutes.

Table 2.3. Preparation of electrophoresis gels

Stock solutions	*6% Resolving Gel*	*7.5% Resolving Gel*	*Stacking Gel*
30% Acrylamide/ bis-Acrylamide (Sigma, A-3574)	3.04 ml	3.8 ml	0.8 ml
d.H_20	9.87 ml	9.1 ml	3.89 ml
8X Tris-HCl pH 8.8 (BDH, 444102T)	1.88 ml	1.88 ml	_
16X Tris-HCl pH 6.8 (BDH, 444092L)	_	_	0.31 ml
10% SDS (Biorad, 1610416)	150µ	150 µl	50 µl
10% Ammonium persulphate	60 µl	60 µl	17 µl
TEMED (Sigma, T-9281)	10 µl	10 µl	5 µl

2.8.4. Western blotting

Between 8 to 10 sheets of filter paper (Whatman, UK, 1001824) were soaked in transfer buffer (0.25 M Tris - 1.92M Glycine, 10X (Sigma T-4904), diluted 1 in 10 in d.H_2O) and placed on the anode of a semi-dry blotting apparatus (Trans-Blot SD Semi-Dry Transfer Cell, Bio-Rad, US) taking care to remove all bubbles by rolling a glass pasteur pipette across the sheets. PVDF membrane (Roche Diagnostics GmbH, 972206) or Sequi-blot ™ PVDF (Bio-Rad, US 162-0180) (used for transfer of immunoprecipitated proteins) both pre-soaked according to the manufacturers instructions were placed over the filter paper and the gel layered on top of this. A

further 8-10 sheets of pre-soaked filter paper were paced over the gel and any air bubbles removed. The cathode was carefully laid on top of the stack and the proteins transferred from gel to PVDF membrane at 15 volts and 34 milliamps for 25 minutes.

2.8.5. Development of Western blots by enhanced chemiluminescence (ECL)

ECL Western blotting detection is a highly sensitive, rapid, quantifiable, non-isotopic method for detecting proteins immobilised on membranes. The primary antibody is located with the Horseradish-Peroxidase (HRP)-labelled species specific secondary antibody. The HRP-labelled molecule catalyses the oxidation of luminol, resulting in the emission of light - chemiluminescence. Enhanced chemiluminescence (ECL) is the sustained emission of light provided by the inclusion of chemical enhancers in the HRP catalysed oxidation of luminol, and gives approximately 1000 fold more light that the oxidation of luminol alone.

Following Western blotting, the filter paper was removed and the membrane blocked for non-specific binding by incubating for 1- 4 hours on a rocking platform with non-fat milk (Marvel, Cadbury). After the blocking step was complete, the blot was rinsed once in TBS, and incubated with the primary antibody optimally diluted in TBS/0.1% (v/v) Tween 20 overnight at 4^0C or 2 hours at R.T. on a rocking platform (6/1G supernatant diluted 1/2 or ascites diluted 1:50; 6/1C ascites diluted 1 in 200 to 1 in 300 and 5C3 supernatant diluted 1 in 10, cytokeratin 6 specific MAb, Ab-1 (MS-766-P, Neomarkers, Lab Vision UK) used at a concentration of 2µg/ ml, cytokeratin 9 specific MAb cocktail supernatant diluted 1 in 100 (651104, Research Diagnostics, US). Mouse IgG diluted 1/5,000 (Vector labs, UK) in TBS/0.1% (v/v) Tween 20 or irrelevant supernatant served as a negative control. The following day blots were washed 3 times within 45 minutes with TBS/ 0.5% (v/v) Tween 20. Blots were then incubated with a rabbit anti-mouse HRP-conjugated secondary antibody (Dako, P0447) diluted 1/2,000 in TBS/0.1% (v/v) Tween 20 for 1 hour at room temperature on a rocking platform. The blots were washed as before, laid out on a glass plate

covered in parafilm and incubated with ECL reagents (Amersham RPN 2109) for 1 minute at room temperature. Following this the solution was tapped off, the blot laid between two sheets of cling film and exposed to LUMI film, chemiluminescence film (Roche Diagnostics GmbH, 166657) for various time periods and processed using standard x-ray developing procedures. Dried film was aligned with pre-stained molecular weight markers for molecular weight determination.

2.9. Immunoprecipitation studies

2.9.1. Immunoprecipitation

Protein isolated from the ductal carcinoma cell line, ZR-75-1 was directly labelled with the antibody 5C3, immunoprecipitated and then detected by Western blot analysis using the same antibody to re-probe the blot. Briefly at least 4 x 175 cm^2 flasks of ZR-75-1 cells were trypsinised and washed 3x with PBS. Cell pellets were resuspended in 650 µl of M-Perm mammalian protein extraction reagent (Pierce, 78501), left to stand at R.T. for 15 minutes and then spun at 7,500 rpm for 15 minutes (Biofuge, Heraseus). (At this point for one experiment with denatured cell lysates, the samples were boiled for 3 minutes prior to commencement of immunoprecipitation procedure).

0.5 and 0.25 ml test aliquots of supernatants were pre-cleared by incubating a 50:50 mixture of protein-L (P3351, Sigma, UK) and protein-G agarose (Roche Diagnostics GmbH, Germany) (i.e. 50µl of protein-L agarose and 50µl of protein - G agarose was added to all test samples, 25µl of each was added to 0.25 ml aliquots for negative control IgG samples). All samples were incubated with two changes (2 x 2.5 hours) of protein - L/ protein - G agarose beads at 4° C on a rocking platform. Beads were removed by spinning at 2,500 rpm for 10 minutes at 4° C. Supernatants were removed to clean eppendorf tubes and 100 µl of antibody 5C3 (concentrated 3X supernatant, using Millipore Ultrafree-15 centrifuge filter units, Z-36436-0, Sigma, US) was added to 0.5 ml lysate samples; 50 µl of antibody was added to 0.25 ml samples. 50 µl of control mouse IgG (Sigma, 31204) (used at a concentration of 22 µg/ ml) was added to 0.25 ml negative control lysates. Antibody/ lysate mixtures were incubated at 4° C overnight on a rocking platform. The following day in order to precipitate the antibody-antigen complex, protein - L / protein - G agarose was added to the antibody - antigen complex samples as before and incubated at 4° C for 4 hours on a rocking platform. Beads were removed by spinning at 2,500 rpm for 10 minutes at 4° C, *and the supernatant discarded.* To check how much protein was left in cell lysate test samples following immunoprecipitation procedure, some of the supernatants were retained and added to 2X laemmili sample buffer (Sigma, 3401)

and/ or non reducing 3X SDS sample buffer (New England Biolabs, UK), boiled for 1-3 minutes and stored at –20°C. The beads were then washed for 3 x 15 minute periods with buffers as described in Appendix III and pelleted at 2,500 rpm for 10 minutes. Following final wash and spin as much liquid as possible was removed from all samples. 50 µl of 2X laemmili sample buffer (Sigma, 3401) was added to original 0.25 ml lysates and control IgG samples, 100 µl was added to original 0.5 ml lysates. In further experiments, non-reducing 3X SDS sample buffer (New England Biolabs, UK) was added to test lysates. All samples were boiled for 1 minute and stored at -20 ° C until required for SDS-PAGE/ Western blot analysis.

Later experiments employed an optimised 8 hour immunoprecipitation protocol. A lysis buffer was prepared (Appendix II) from materials provided in the cellular labelling and immunoprecipitation kit supplied by Roche Diagnostics, GmbH. ZR-75-1 cell pellets prepared as in earlier experiments were thawed on ice and resuspended in lysis buffer (600µl lysis buffer added to each 175 cm^2 flask pellet), left for 2-3 minutes at R.T. and sonicated briefly (complete cell lysis was confirmed by viewing under an inverted microscope). Test and IgG control lysates were precleared with two changes (2 x 1 hour) of protein-L/ protein-G agarose (i.e. 100µl of protein-L and protein-G agarose was added to 200 µl test lysates/ control Ig lysate). Beads were removed as previously described. 150µl of concentrated MAb 5C3 was added to test lysates and incubated for 1 hour at 4°C , 50µl of mouse IgG was added to negative control lysates (22 µg). Following this 100µl of protein-L and 100µl protein-G were added to all lysates and incubated for a further three hours at 4°C on a rocking platform in order to precipitate the antibody-antigen complex. (Some supernatant at this point was placed in 2X loading buffer, boiled for 3 minutes and stored at –20°C). Pelleted beads were this time washed for 15 minutes intervals with two changes of wash buffer 1 and 2 and one change of wash buffer 3. Following a final wash and spin, as much liquid as possible was removed from all samples. 75 µl of 2X laemmili sample buffer (Sigma, 3401) was added to test lysates and control IgG samples. All samples were boiled for 1 minute and stored at -20 ° C until required for SDS-PAGE/ Western blot analysis. Further immunoprecipitated samples (in 2X loading buffer) were boiled for 10 mins and stored at -20° C.

2.9.2. Gel electrophoresis of immunoprecipitated proteins

Immunoprecipitated proteins were separated on 7.5% and 10% SDS-PAGE electrophoresis as described in section 2.8.3. Retained supernatants (in reducing and non reducing loading buffer) were also separated on 7.5% gels, in addition for some experiments combinations of immunoprecipitated proteins and supernatants were also separated on SDS-PAGE. Following SDS-PAGE western blot analysis was carried as outlined in section 2.8.4 - 2.8.5.. Immunoprecipitates and whole cell lysates of ZR-75-1 cells were probed with antibody 5C3, anti cytokeratin Ab-1 (MS-766-P1, Labvision, UK) or anti cytpkeratin 9 (651104, Research Diagnostics Inc., US). Control IgG ZR-75-1 immunoprecipitates were probed with antibody 5C3.

In order to visualise proteins SDS-PAGE gels were stained for 15-30 mins with a 0.25% (w/v) Coomassie Brilliant Blue R-250 solution (containing 45% methanol (v/v) and 45% d.H_2O (v/v)) and destained with 45% methanol (v/v)/ 10% acetic acid (v/v)/ 45% d. H_2O.

2.9.3. Preparation of isolated proteins for N-terminal sequencing

ZR-75-1 and control mouse immunoprecipitates were separated on 5 well 7.5% SDS PAGE and blotted onto PVDF membrane (Sequiblot ™, Biorad). Following transfer the membrane was washed briefly in d.H_20. The blot was then stained for 5 minutes with Comassie R-250 (0.25% Comassie Blue R-250 in 40% methanol) and destained for 1 hour with 50% (v/v) methanol. The membrane was air-dried for at least 3 hours and stored at -20^o C. Protein bands were aligned with molecular weight markers and ECL films of immunoprecipitates which had been probed with antibody. The bands of interest were excised and processed for N-terminal sequencing.

2.9.4. Preparation of isolated proteins for internal sequencing

Following immunoprecipitation and separation on 7.5% SDS-PAGE using a 5 well spacer ensuring that the maximum amount of protein was loaded, gels were stained with Coomassie Brilliant Blue R250 (0.1% (v/v) in methanol, 45% dH_2O(v/v), 10%

acetic acid (v/v) and destained with 45% methanol (v/v) / 10% acetic acid (v/v) / 45% dH_2O (v/v). Staining and destaining protocols were carried out according to recommendations from Eurosequence. The band of interest was excised from gels, placed in eppendorf tubes and sent to Eurosequence BV (Gronigen, The Netherlands).

3. Results

3. RESULTS

3.1. MDR associated protein expression in invasive breast cancer patients.

The emergence of drug resistance is one of the main obstacles to the successful chemotherapy of breast cancer. As previously discussed in section 1.4.2 and 1.4.4., identification of biological factors which can function as reliable *prognostic* markers and more importantly *predictive* markers will allow for more precise targeting of modalities for breast cancer patients. Results presented here attempt to address the possible prognostic and predictive significance of MDR associated proteins in breast cancer. Immunohistochemical expression of these proteins have been correlated with patient and tumour characteristics, with relapse-free survival and overall survival in patients with invasive breast cancer. In addition immunohistochemical expression of individual MDR markers studied is analysed in relation to other MDR associated proteins. This study was undertaken in collaboration with Dr. Susan Kennedy of the Department of Pathology and colleagues in the Department of Surgery at St. Vincents Hospital, Dublin 2.

Primary breast specimens from a total of 204 patients were included in this study. *Pre* and *post* formalin fixed paraffin embedded archival tumour samples from 46 of these patients were analysed by means of immunohistochemistry for expression of MRP-1, cerbB-2, MDR-1, LRP, Bcl-2 and survivin proteins. MRP-1 protein expression was also studied in an additional 158 *pre* treatment archival tumours (technical work carried out at Royal Victoria Eye and Ear Hospital, Dublin). All of the patients studied were treated with surgery at St. Vincents Hospital between 1982 and 1995.

Following surgery all of these patients received adjuvant chemotherapy or hormonal therapy or combination therapy. 103 (50.4%) of the patients studied received chemotherapy/ combination therapy. Of this group, 56 patients received standard CMF therapy, 26 patients received ACMF, 1 patient received taxol, 1 patient received PMF, 1 patient received VMF/ chloroambucil; the exact details of the chemotherapy administered to 17 patients were not known at the time of compiling

these results. Chemotherapy treated patients referred to, in the results presented in this thesis, represent this group. Of this group of 103 patients; 45 relapsed during follow up and died of disease (status referred to in Tables 3.1.4. and 3.1.5. as dead (d)), 46 are currently disease free (status referred in Tables 3.1.4. and 3.1.5 to as alive (a)) and 8 have relapsed but are currently alive (status referred to in Tables 3.1.4. and 3.1.5 as recurrence (r)). There was a minimum of 5 years follow up for all patients included in this study.

Immunohistochemical analysis of tumours was carried out using a panel of MAbs for various MDR markers (Table 3.1.1.). Tumours stained with appropriate MAbs were *scored* and presented throughout these results, by the *percentage of positive tumour* cells stained with appropriate MAbs.

MDR-1, Mrk-16, LRP-56, MRP-R1, cerbB-2 and Bcl-2 MAbs:
1 = 1-25% of tumour cell stained
2 = 26-50% of tumour cells stained
3 = 51:100% of tumour cells stained

Survivin MAb, 1C5: (note : the difference in survivin scoring system will be taken into account in any comparative analysis with other histochemical markers)
1 = 1-10% of tumour cell stained
2 = 11-49% of tumour cells stained
3 = 50-100% of tumour cells stained

For all MAbs the *percentage of tumour cells stained* is followed by the *intensity* of the staining observed:

Level **1** = weak staining
Level **2** =intermediate level of staining
Level **3** =intense staining.

Unless otherwise stated any positive immunohistochemical score (percentage of tumour cells stained and intensity of staining) was considered positive for analysis of

results presented here. Immunohistochemical results together with clinico pathological details of patients / tumours studied are presented in Tables, 3.1.4. and 3.1.5.

Table 3.1.1.

MAbs used in investigation of MDR associated protein expression in breast cancer patients.

MAb	*Specificity*	*Reference*
BRI anti MDR-1 (6/1C) #	P-170/ Pgp	Moran *et al.*, 1997
MRK-16	P-170/ Pgp	Sugawara *et al.*, 1988
MRP-R1 *	MRP-1	Flens *et al.*, 1994
LRP-56 *	LRP	Scheper *et al.*, 1993
cerbB-2 oncoprotein, clone CB11 (Novacastra, UK)	CerbB-2	Corbett *et al.*, 1990
Anti human Bcl-2 oncoprotein (Dako)	Bcl-2	Zutter *et al.*, 1991
BRI anti Survivin (1C5) #	Survivin	Personal communication Dr. Irene Cleary

\# obtained from Bioresearch Ireland, Dublin.
* kindly provided by Prof. R.J. Scheper, Free University Hospital, Amsterdam, The Netherlands.
** kindly provided by Prof. T. Tsuruo, Department of Pathology, University of Tokoyo, Japan.

Table 3.1.2. Patient and tumour characteristics of invasive breast cancers (n=204)

	preliminary study **n=46**	follow up study **n=158**	total **n=204**
Age			
< 50 years	12 26%	45 28.5 %	57 28%
> 50 years	34 74%	109 69%	143 70%
unknown		4 2.5%	4 2%
ER status			
ER+	15 33%	87 55%	103 50%
ER –	18 39%	26 16.5%	44 22%
unknown	13 28%	45 28.5%	58 28%
Node status			
node positive	40 87%	75 47%	115 56%
node negative	6 13%	80 51%	86 42%
unknown		3 2%	3 2%
Tumour size			
< 2cm	13 28%	55 34.8%	68 33%
2-5cm	18 40%%	84 53.7%	102 50%
>5cm	9 19.5%	9 5.7%	18 9%
unknown	6 13%	8 5%	14 7%
Histological grade			
Grade I	4 9%	18 11%	22 10.8%
Grade II	5 11%	65 41%	72 35.3%
Grade III	25 54%	72 46%	107 49%
unknown	11 24%	3 3%	14 6.8%
Histological classification	28 60.8%	98 62%	126 62%
Infiltrating ductal +/-DCIS	3 6.5%	22 14%	25 12%
Infiltrating lobular +/-LCIS			
lobular tubular/alveolar			
ductal/lobular	1 2.2%	6 4%	7 3%
Infiltrating mucinous	15 32.6%	33 20%	43 23.5%
Nos/ infiltrating			

Table 3.1.3. Patient and tumour characteristics of chemotherapy treated invasive breast cancers (n=103)

	n	%
Age		
< 50 years	18	17.4%
> 50 years	69	67%
ER status		
ER+	48	46.6%
ER -	35	33.98%
unknown	20	19.4%
Node status		
node positive	72	69.6%
node negative	29	28.2%
unknown	3	2.9%
Tumour size		
< 2cm	22	21.4%
2-5cm	55	53.5%
>5cm	18	17.5%
unknown	8	7.8%
Histological grade		
Grade I (low)	9	8.7%
Grade II (intermediate)	29	28.2%
Grade III (high)	52	50.4%
unknown	12	11.6%
Histological subtype		
Infiltrating ductal +/-DCIS	72	69.9%
Infiltrating lobular +/-LCIS lobular tubular/alveolar ductal/lobular	8	7.8%
Infiltrating mucinous	2	1.9%
Nos/ infiltrating	32	31%

Table 3.1.4.

Clinical features of tumours, treatment details of patients and immunohistochemical analysis of MDR associated proteins in pre and post treatment archival tumour material from invasive breast cancer patients (n=46).

Key: **d** =status of these patients: dead of disease, **a** = status of these patients: alive at date last seen, **r** = status of these patients: recurrence of disease, alive at date last seen, **os** = overall survival, **rfs** = relapse free survival, **In du** = infiltrating ductal, **In lob** = infiltrating lobular, **DCIS** = ductal carcinoma *in situ* (comedo/ cribriform/ solid), **In tubular** = infiltrating tubular, **In municous** = infiltrating mucinous, **LCIS** = lobular carcinoma *in situ*, **tum size** = tumour size (cm), **grade** = histological grade, **ER** = estrogen receptor status (pos/neg), **Tam** = tamoxifen therapy, **RT** = radiation therapy (Y/N), **Node** = lymph node status (0/1), **chemo** = adjuvant chemotherapy administered, **CMF** = cyclophosphamide/ methotrexate/5-fluorauacil, **ACMF** = adriamycin/ cyclophosphamide/ methotrexate/ 5-fluorouracil, **meg**= megace, **VMF** = vinblastine, methotrexate, 5-fluouracil, **chlorb** = chlorambucil,. MDR protein marker expression (immunhistochemical analysis of MDR-1, Mrk-16, LRP, MRP-1, cerbB2, Bcl-2, survivin, all performed *pre* treatment, immunohistochemical analysis of *post* treatment samples where available indicated in columns directly following each marker).

no	status	os	rfs	size	grade	ER	Tam	RT	Node	Diagnosis	Chemo	Mdr-1	post	Mrk-1	LRP	post	MRP-1	post	cerbB	post	Bcl-2	post	survivin	post	Mdr-3
1	d	3	3	5.5	3	neg	N	Y	1	R In Du, DCIS-com	**ACMFx12**						3+3		3+3						3+3
2	d	5	2			neg		Y	1	L In Du, DCIS	**CMFx9**	3+3			DCI	1+2	DCIS	neg	neg	neg	3+2	3+(2-3)	2+3	2+3	
3	d	7	6	1.5	3	neg	Y	Y	1	R In Du DCIS-com							neg	1+2	neg	neg					
4	d	9	4	7	3	neg	Y	N	1	In Ca	**ACMFx12**						1(1-2)		neg						
5	d	0	0	9			Y		1	L In Du DCIS	**PMF**	neg	3+1		neg	2+3	neg	neg	3+3	3+3	neg	neg	2+1	3+(1-2)	
6	d	3	1	10				Y	1	L In Ca	**CMF**	2+2	neg		neg	neg	neg	neg	2+1	neg	3+(2-3)	3+(1-2)	2+(1-2)	2+(1-2)	
7	d	11	5	2.2		pos	Y	Y	1	L In Du, DCIS	**CMFx9**				neg	1+1	2+2	neg	neg	1+3	3+3	3+(2-3)			
8	d	1	0	5	3			Y	1	R In Du DCIS	**CMFx9**	neg	2+1		neg	neg	1+1	2+1	neg	neg	3+(1-2)	3+(2-3)	neg	3+(2-3)	
9	d	7	1	4		neg		Y	1	R In Ca	**VMFChlorb**	neg	1+2		neg	1+1	1+3	3+3	(1-2)+	2+2	neg	3+1	1+(1-2)	2+2	
10	d	4	2	6	3	pos	N	N	1	L In Du, DCIS	**CMFx9**	1+2	1+1		neg	neg	1+2	neg	neg	neg	3+3	3+3	3+2	neg	
11	d	6	4		2	pos	Y	Y	1	L In Du	**ACMFx12**						(2-3)+1	neg	3+3	2+3					
12	d	3	3	3	2			Y	1	R In Du	**CMFx9**	neg	2+1		neg	neg	1+1	2+1	neg	neg	neg	neg	2+1	3+(1-2)	
13	d	3	2	3	3	pos	Y		1	L In Du	**CMFx9**						3+3	2+3	neg	neg	3+1	1+1	1+1	neg	
14	d	6	1	3.5	3	pos	Y	Y	1	L In lobular LCIS	**ACMFx12**						neg		neg						2+2
15	d	2	2	4	3				1	L In ca							3+3		2+3						
16	d	3	3	2	3	neg		Y	1	L In Du	**CMFx9**	1+1	1+1		neg	1+1	3+3	3+3	neg	neg	neg	neg	3+2	3+2	
17	d	7	5	6		neg	N		1	R In Ca	**CMFx9**	2+2	2+1		neg	neg	3+2	3+3	neg	1+1	3+3	3+3	3+2		
18	d	2	2	5	3	neg	y	Y	1	L In Du, DCIS	**A4CMFx8**						1+2		2+2						
19	d	2	2	2					0	L In Du, DCIS	**CMFx9**	2+1	1+2		1+2	neg	3+3	3+3	3+3	3+3	2+2	neg	3+3	2+3	
20	d	2	1	2.5	3	neg	Y		0	In Du	**ACMF**						neg		neg						
21	d	6	6			neg		Y	0	L In Du	**CMFx9**				neg	neg	1+2	2+1	3+3	3+3	neg	neg	3+2	2+2	
22	r	2	2	3	3	pos			1	R In Ca	**CMFd1d8x6**						1+1	1+1	3+3	2+2					2+2
23	r	4	4	2.8/4	1	pos		N	1	R In mucinous	**CMFx9**						neg	neg	neg	neg					
24	r	2	2	1	1	pos	Y	Y	0	R In Du	**ACMF**						neg	1+2	neg	neg					
25	a	7	7	0.6	3	neg	N	N	0	L In Du DCIS-com	**CMF X6**						neg		3+3						
26	a	7	7	2.7	3	neg	N	Y	0	R In Du	**CMFx6**						1+1		neg						
27	a	12	12	3	3	neg	N	N	1	L In Ca, NOS	**CMFx9**	1+2		1+2			neg		neg				2+1		neg
28	a	13	13	1.7		neg	N	Y	1	R In Du	**CMFx9**	3+2		neg			1+2		neg				3+(2-3)		3+3
29	a	8	8	1	3	neg	N	N	1	R In lobular	**CMFx9**						neg		neg						
30	a	15	15	2	3	neg			1	L In Ca, NOS	**CMF**	1+2		2+1			neg		neg				2+2		2+2
31	a	13	13	1.5		pos	Y	y	1	L In Du	**CMFx9**			3+2			neg		neg				3+3		
32	a	6	6	2			Y	Y	1	R In Du, DCIS	**CMF**						2+2		neg						
33	a	10	10	2.5		pos			1	R In Du	**CMFx9**	DCIS		neg			neg		neg				1+1		1+1
34	a	8	7	2	2	pos	Y	Y	1	R In Ca	**ACMF**						neg		neg						
35	a	9	9		3		Y		1	R In Ca	**ACMF**	3+3		2+2			1+2		neg				3+1		3+3
36	a	7	7	1.2	1			Y	1	R In Du	**CMFx9 meg**						neg		neg						
37	a	13	13	8	3	pos			1	R In Du	**CMFx9**	1+1		2+3			neg		neg				1+(1-2)		3+3
38	a	10	10	3	3	pos	Y	Y	1	L In Ca, NOS	**CMFx9**	neg		neg			neg		neg				neg		neg
39	a	14	14		3			Y	1	L In Ca	**CMFx9**	1+2		2+1			1+1		neg				2+1		neg

Table 3.1.2. MDR associated protein expression in invasive breast carcinomas (n=46).

no	status	os	rfs	size	grade	ER	Tam	RT	Node	Diagnosis	Chemo	Mdr-1	*post*	Mrk-1	LRP	*post*	MRP-1	*post*	cerbB	*post*	Bcl-2	*post*	survivin	*post*	Mdr-3
40	a	9	9	1	3		N	Y	1	L In Du, DCIS	CMFx9	1+3		neg			neg		neg				neg		2+2
41	d	7	4	3	2	neg		N	1	L In Ca, NOS	Zoldalex						DCIS	neg	1+1	neg					
42	d	0	0	3	3		Y		0	R In Du, DCIS	Tamoxifen				neg	neg	3+(1-2)		3+3	3+3	1+1	3+1	1+1	neg	
43	d	0	0		2	neg	Y	N	1	R In Du, DCIS	Tamoxifen	3+3	3+3		neg	neg	2+2	neg	3+3	3+3	neg	1+1	3+(2-3)	3+(1-2)	
44	d	3	1	3.5	3	pos	Y	Y	1	R In Du	Tamoxifen						1+2		1+1						
45	a	8	8	2	3	pos		Y	1	L In Ca, NOS	Zoldalex						2+2								
46	r	8	3	3	1				1	L in crib tub com	Zoldalex						neg	3+3	neg	neg					3+3

Table 3.1.2. MDR associated protein expression in invasive breast carcinomas (n=46).

Table 3.1.5.

Clinical features of tumours, treatment details of patients and immunohistochemical analysis of MRP-1 protein in pre treatment archival tumour material from invasive breast cancer patients (n=158).

Key: **d** =status of these patients: dead of disease, **a** = status of these patients: alive at date last seen, **r** = status of these patients: recurrence of disease, alive at date last seen, **os** = overall survival, **rfs** = relapse free survival, **In du** = infiltrating ductal, **In lob** = infiltrating lobular, **DCIS** = ductal carcinoma *in situ* (comedo/ cribriform/ solid), **In tubular** = infiltrating tubular, **In municous** = infiltrating mucinous, **LCIS** = lobular carcinoma *in situ*, **tum size** = tumour size (cm), **grade** = histological grade, **ER** = estrogen receptor status (pos/neg), **Tam** = tamoxifen therapy, **RT** = radiation therapy (Y/N), **Node** = lymph node status (0/1), **chemo** = adjuvant chemotherapy administered, **CMF** = cyclophosphamide/ methotrexate/5-fluorauacil, **ACMF** = adriamycin/ cyclophosphamide/ methotrexate/ 5-fluorouracil, **meg**= megace, **VMF** = vinblastine, methotrexate, 5-fluouracil, **chlorb** = chlorambucil, All of these archival tumour samples were *pre* treatment.

no	status	os	rfs	size	grade	ER	RT	Node	Diagnosis	chemo	MRP-1
1	a	8	8	2.4	2	1	y	0	In Du	none	3+3
2	a	8	8	2.5	3	1	n/y ?	0	In Du	none	3+3
3	a	7	7	0.6	2	1	y	1	In Lo		neg
4	a	6	6	2	2	1		0	In Du	None	2+2
5	a	7	7	2.5	3	0	y	1	In Du, DCIS-com		2+1
6	a	7	7	2.5	2	1		0	In Du, DCIS		neg
7	a	7	7	3.9	1	1	y	0	In Ca, NOS		3+2
8	a	7	7	1.5	1	1	y	0	In Lo		2+2
9	a	7	7	3.5	2	1	y	0	In Du		2+1
10	a	6	6	1	2	1	y	0	In Lo, LCIS	none	neg
11	a	6	6	2	1	0	y	0	In Du, LCIS	none	3+2
12	a	0	0	2.5	2	0	n	0	In Du	None +....	3+1
13	a	7	7	3	3	1	y	1	In Du, DCIS-crib	none	neg
14	a	6	6	3	3	1	y	1	In Du + DCIS	none	neg
15	a	7	7	2.5	2	1	Y	1	In Du	None	3+2
16	a	7	7	2.5	3	1	y	1	In Du, DCIS-com	none	3+2
17	a	5	5	4.2	2	1	y	1	In Du, DCIS-sol+com	None	3+3
18	a	7	7	1	2	1	n	0	In Du, DCIS-com	None	2+3
19	a	7	7	2	3	1	Y	1	In Du	None	3+3
20	a	6	6	2.7	3	1	y	1	In Ca, NOS	none	2+3
21	a	0	0	3	2	1	n	0	In Ca	None + ...	2+1
22	a	6	6	1.5	2	0	y	0	In Lo-pleomorphic	None	2-1+1
23	a	7	7	1	2	0	N	0	In Du, DCIS-com+crib	None	neg
24	a			9	1	0	Y	0	Mucinous Ca	megace	1+2
25	a	7	7	2	2			0	In Du, DCIS sol		1-2+1
26	a	7	7	2	2		y	0	In Ca	none	3+1
27	a	7	7	8	3	0		1	In Ca-com		2+2
28	a	6	6	2	3	1	y	0	In Du, NOS	None	neg
29	a	6	6	2.3	3	0	Y	1	In Du	None	3+3
30	a	3	3	2	3	1	y	1	In Du	None + ...	3+3
31	a	5	5	2	3	0	y	1	In Du, DCIS		neg
32	a			3	3	1		0	In Du		3+3
33	a	7	7	1.9	1	1		0	Intraductal Ca-com + crib	None	neg
34	a	7	7	2	2	1	y	1	In Lo	none	neg
35	a	8	8	3	1	1	Y	1	In Lo,DCIS-crib, com	none	neg
36	a	6	6	2.5	2	0	y	0	In com Ca		2+2
37	a	2	2	2	3		y	0	In Ca		neg
38	a	6	6	6	3	1	y	1	In Du	none	2+2

Table 3.1.5. MRP-1 protein expression in invasive breast carcinomas (n=158)

no	status	os	rfs	size	grade	ER	RT	Node	Diagnosis	chemo	MRP-1
39	a	4	4	3	1	1		0	Mucinous Ca		neg
40	a			1.5	2	1		1	In Lo		2+1
41	a	8	8	2	2	1	n	1	In Du	Zoladex	2+2
42	a	7	7	3	2	1	Y	0	In Ca, NOS	none	neg
43	a	8	8	2	2	1	Y	0	In Lo	none	neg
44	a			4.3	2	1	y	0	In Ca, NOS		2+2
45	a	8	8	1.9	1	1	y	0	Mucinous Ca	none	3+1
46	a	5	5	2	3	0	n	0	In Lo, LCIS	None +...	2+2
47	a	7	7	2.5	2	0	y	0	In Du, DCIS	none	2+1
48	a			1.8	2	1	n	0	In Du	None	1-2+3
49	a	8	8			1	y				neg
50	a	6	6	4.2	3	0	Y	0	In Ca, NOS	none	neg
51	d	2	2	2.5	3			0	In Du, NOS		neg
52	d	1	1	2.3	2	1	y	0	In Du	none	2+1
53	d	6	6	1.5	2	1	Y	0	In Du	none	neg
54	d	6	6	3.5	3	0	y	0	In Du, DCIS-com	none	3+3
55	d	3	1	2.3	3	1	n	1	In Du	megace	2+1
56	d	7	7	2	2	0	y	0	In Lo	None	neg
57	d	5	5	1.5	3	1	y	1	In Ca, NOS	Arimidex	2+1
58	d	3	3	3.5	3		y	0	In Du	None	2-3+1
59	d	0	0	3	3	1	y	1	In Du	none	neg
60	d	2	1	2.4	3	1	y	1	In Lo	None	2+1
61	d	2	1	4	3	1	y	0	In Ca, NOS	none	2+3
62	d	2	2	4.5	3	1	n	0	In Du	none	2+3
63	d	6	6	1.3	2	1	y	0	In Du		1+1
64	d	1	0	3.2	3	0	y/n ?	0	In Du, DCIS	none	3+2
65	d	3	3	2.5	2	1	y	0	In Du NOS, DCIS-crib, com	none	3+2
66	d	3	2	4.5	2	1	y	1	In Lo	none	1+1-2
67	d	6	6	2	2	1	y	0	In Du	none	neg
68	d			2	3			1	In Du		neg
69	d	3	3	3.5	3			1	In Du		3+3
70	d	7	7	1.5	2	0	n	1	In Lo, LCIS com	none	1+1-2
71	d	1	2	4.5	3		n	1	In Du	None+ palliative	3+3
72	d	0	0	2	3			0	In Du, DCIS sol, crib		neg
73	r	0	1	0.7	3	1		1	In Ca, NOS		3+3
74	r			1.5	3	0		1	In Du		neg
75	r	7	8	3.5	3	1	y	1	In Du	aredia	neg
76	r	1	6	1.5	2	1	y	0	In tubulo-lobular, LCIS	none	1+2

Table 3.1.5. MRP-1 protein expression in invasive breast carcinomas (n=158)

no	status	os	rfs	size	grade	ER	RT	Node	Diagnosis	chemo	MRP-1
77	r	2	4	3	2	1		1	In Lo		2+3
78	r			2.5	3	1	y	0	In Ca	None	neg
79	r	2		1.8	3	1	y	0	In Du	none	2+3
80	r	5	6	5	2	1	n	0	In Du, DCIS crib, com, pap	None+ palliative	1+2
81	r	2	6	3	1	1	y	0	Mucinous Ca	none	2+2
82	r			1	3	1	n	1	In Du	none	3+3
83	r	7	7	3	2	1	y	0	In Du		neg
84	r	5	5	2.5	2	1	y	0	In Du		1+1-2
85	?			4	2			0	In Du, DCIS com, crib		neg
86	?			3	3	1	Y	1	In Du, DCIS-com		neg
87	?				3		Y		In Ca		neg
88	?			1.5	2	1	Y	0	In Du, DCIS-crib	None	1+2
89	?			4	1	1		1	In Lo		neg
90	?			2	2			0	In Du, DCIS		2+2
91	?			1.5	1			0	In Du		neg
92	?			3.5	1	1		0	In Mucinous, DCIS		neg
93	?			2.2	3			0	NOS		neg
94	?			2.2	3	1		1	In Du, DCIS com, crib, pap		neg
95	?			2.5	3		n	0	In Du	None	1-2+2

Table 3.1.5. MRP-1 protein expression in invasive breast carcinomas (n=158)

no	status	os	rfs	size	grade	ER	RT	Node	Diagnosis	chemo	MRP-1
96	a	7	7	3	3	1	y	0	In Ca, NOS	CMFx9	neg
97	a	6	6	2.5	3	0	n	0	In Du, DCIS	CMFx9	3+3
98	a	7	7	2.6	1	1	n	1	In Du	ACMF	3+
99	a	7	7	4	2	0	n	0	In Ca, NOS	CMF X9	2+2
100	a	6	6	1.2	2	1	n	1	In Du	A4CMF x 8	3+3
101	a	6	6	2	3	1	y	0	In Du	Yes	neg
102	a	7	7	1.5	2	1	y	1	In Lo, DCIS	ACMF	2+2
103	a	5	5	3.5	5	1	Y	1	In Ca	ACMF	1+1
104	a	6	6	2.5	2	1	Y	1	In Du	Yes	3+3
105	a			3	2				In Du, DCIS	ACMF	neg
106	a	5	5	2.5	3	0		0	In Du	CMF x 8	3+3
107	a	7	7	4.3	3	1	n	1	In Lo, In Tubular, crib	Yes	3+3
108	a	2	2	1.5	1	1	y	0	In Ca	A4CMF x 8	3+3
109	a	5	5	5	2	1	y	0	In Du	Yes	3+2
110	a	5	5	4.5	3			0	In Du. DCIS	CMFx9	neg
111	a	6	6	3.2	2	1	y	0	In Du, DCIS-crib, com	Yes	neg
112	a	6	6			1	Y	1		CMFx12	neg
113	a	5	5	3	3	1	y	0	In Lo, DCIS	CMF	neg
114	a	6	6	2.5	2	1	y	1	In Du	CMF x 9	3+3
115	a	1	1	3.8	3	1	y	0	In Du	CMF x 9	1+1
116	a	5	5	3	2	1	n	1	In Du	Yes	2-3+2
117	a	6	6	2	2	1	y	1	In Du	A4CMF x 8	neg
118	a	7	7	3	3	1	y	1	In Du	ACMF	1+1
119	a	4	4	2.5	2		n	0	In Du	Yes	3+2
120	a	7	7	1.8	1	1	n	0	In Ca, NOS	CMF x 7	neg
121	a	6	6	3	2	1	Y	0	In Lo, LCIS	CMF X 6	1+2
122	a	7	7	4	2	0	n	0	In Du	CMF	1-2+3
123	d	2	2	2.5	3	0	n	1	In Du, DCIS	CMF x 9	neg
124	d	3	6	3.5	3	1	Y	1	In Ca, DCIS	ACMF	3+2
125	d	2	2	5	2	1	y	0	In Lo	ACMF,	1+2
126	d	2	3	2.3	2	0	y	1	In Du	CMF,bleomycin,doxirubicin	2+2
127	d	2	2	3	3	0	y	1	In Du	CMF x 9	2+3
128	d	1	2	DCIS			n		In Du, DCIS	CMF	neg
129	d	1	6	3	2	0	y	0	In Du	Yes	3+2
130	d	2	3	3	2	0	Y	1	In Du, DCIS	ACMF	neg
131	d	6	6	2	1	1	n	1	In Du, DCIS	A4CMF x 8	2+1
132	d	3	3	2	2	1	Y	0	In Du	Yes	neg
133	d	6	7	1.8	3	0	n	0	In Du	CMF	1+1-2

Table 3.1.5. MRP-1 protein expression in invasive breast carcinomas (n=158)

no	status	os	rfs	size	grade	ER	RT	Node	Diagnosis	chemo	MRP-1
134	d	3	6	3	3	1	Y	1	In Du	CMF	2+2
135	d	0	0	3.5	3	1		1	In Du	CMFx9	2+3
136	d	1	1	5	3	0	n	1	In Du	ACMF, doxorubicin	3+3
137	d	2	2	2	3	0		1	In Ca- undiff, DCIS-solid	CAF	3+3
138	d	1	2	5	1	0	y	0	In Lo-alveolar	Yes	neg
139	d	3	3	4	2	0	n	1	In Du, DCIS	ACMF	2+1
140	d	4	4	4.5	3	0		1	In Du, DCIS	A4CMF x 8	1+2
141	d	2	2	1.5	3	1	y	1	In Du	ACMF, VNB, Mito C	3+3
142	d	1	2	1.3	2		Y	0	In Du	CMF, Taxotere	2+1
143	d	3	4	4	3		n	1	In Du, DCIS	yes	3+3
144	d	2	2	2.5	3	1	Y	1	In Du	CMFx8	3+2
145	d	4	5	3	3	1	Y	1	In Du, NOS	CMFx9	neg
146	d	0	3	3	2	1		1	In Du	ACMF, zoldalex	2+2
147	d	3	7	2	3	1	y	0	In Lo	CMF	2+2
148	d	3	7	5.5	3	1	y	1	In Du	A4CMF x 8	3+3
149	d	2	4	2.2	2	1	Y	1	Ca	CMFx9, VAT, Taxotere	neg
150	r	4	8	5	2	0		1	In mucinous Ca	Taxol	3+3
151	r	2	3	2.8	3	0	y	1	In Ca, NOS	CMF	1-1+2
152	r	19	21	1.8	2	1	y	0	In Du	CMF x 20	neg
153	r	3	5	2	2	1	y	1	In Du	CMF	neg
154	r	6	7	4	3	1	y	1	In Du	Yes	3+1
155	r	1	7	3.5	2	0		1	In Du, In Lo, DCIS-crib	CMFx6	neg
156	r	2	2	6	3	0		1	In Du	CMF	neg
157	r	1	7	1.5	1		n	1	In Du, DCIS com	yes	2+1
158	?			1.5	3			1		yes	neg

Table 3.1.5. MRP-1 protein expression in invasive breast carcinomas (n=158)

3.1.1. MRP-1 protein expression

MRP-1 protein expression was studied in *pre* treatment tumours from a total of 204 patients with invasive breast cancer. 103 patients who received chemotherapy following surgery were investigated for MRP-1 protein expression *prior* to treatment with chemotherapy, MRP-1 expression was studied in 20 *post* chemotherapy treatment tumours from this group of patients. Immunohistochemical analysis results are presented in Tables 3.1.4. and 3.1.5. Representative tumours stained with the MRP-1 specific MAb, MRP-R1 are presented in Figures 3.2 and 3.3.

MRP-1 protein expression in relation to known clinico-pathological factors i.e. treatment status, node status, age (<50 years/ >50 years), ER status, tumour size, and histological grade is presented in Tables 3.1.6. For analysis purposes, patients were divided into two groups; those who had MRP-1 positive tumours and those who had MRP-1 negative tumours. The association of MRP-1 + tumours and MRP-1 – tumours with clinico-pathological factors is presented in Table 3.1.6. and 3.1.8. In addition, MRP-1 positive tumours which showed an immunohistochemical score of 3+3 are correlated with histological grade in Figure 3.3. (Table 3.1.7.).

Correlation of MRP-1 expression with *relapse free survival* and *overall survival* times in patients who *did not receive chemotherapy* and chemotherapy treated patients is presented in Tables 3.1.9 – 3.1.12. MRP-1 expression is also correlated with relapse free survival and overall survival times in chemotherapy treated patients who received CMF alone (Tables 3.1.15.- 3.1.6) and in combination with adriamycin (ACMF) (Tables 3.1.13.- 3.1.14).

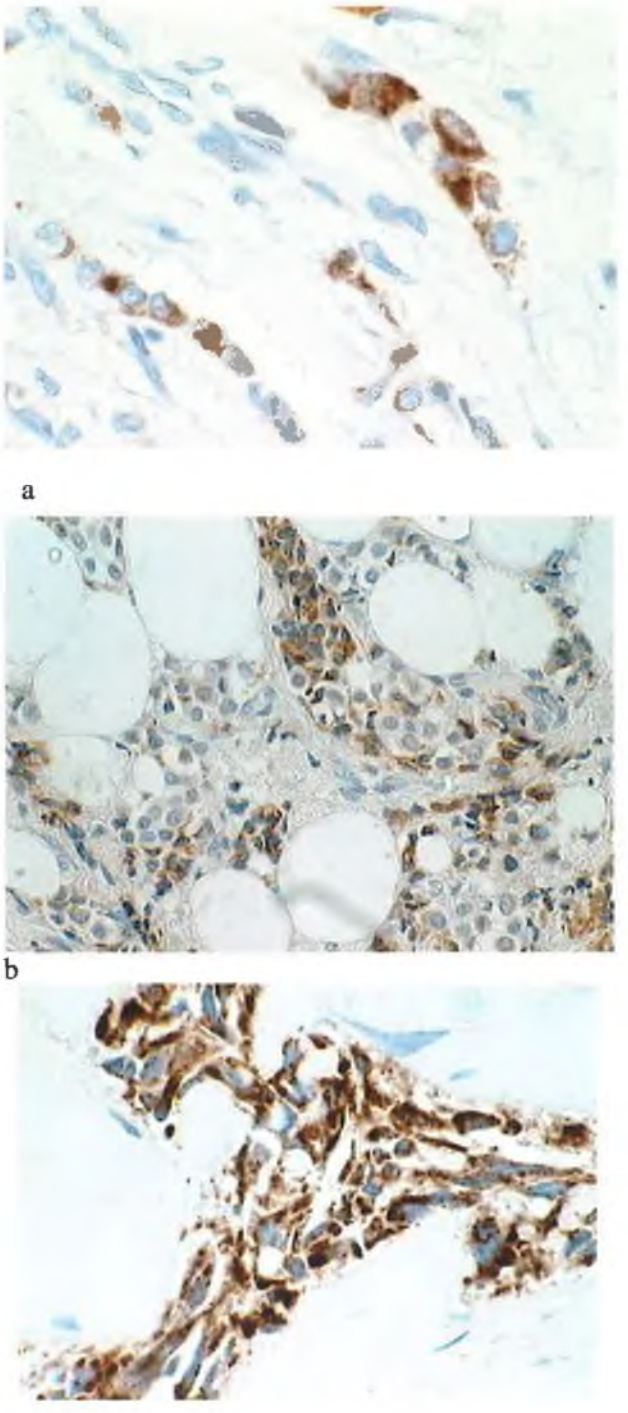
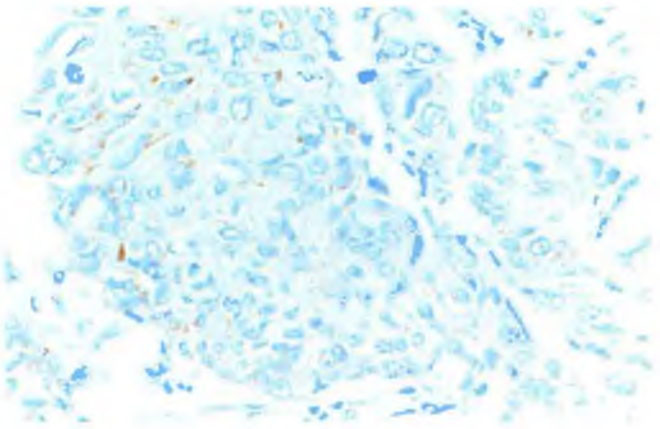
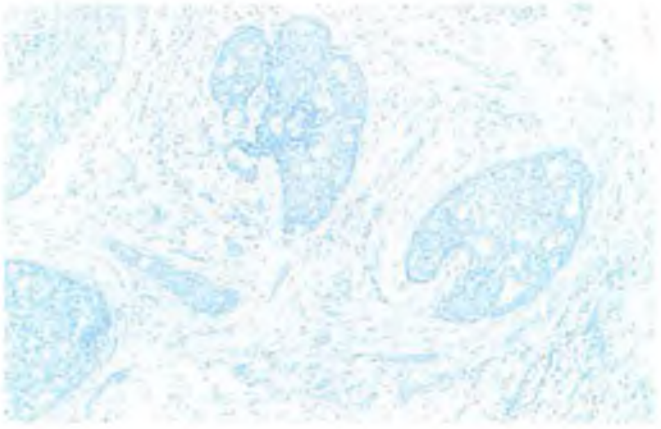


Figure 3.1.1. Immunohistochemical analysis of MRP-1 protein expression (I)

(a) MRP-1 positive tumour (*pre* treatment) from a patient with infiltrating ductal carcinoma (refer to patient no. 17, Table 3.1.4.) stained with MRP-R1 MAb, positive staining (typical granular membranous type staining) can be observed in infiltrating tumour cells (original magnification 60x). **(b)** Following treatment there was a slight increase in the level of MRP-1 positivity observed, intense MRP-1 positive staining can be observed in infiltrating tumour cells at interface of stroma (original magnification 40x). **(c)** Infiltrating carcinoma cells (*pre* treatment) from another patient with infiltrating ductal carcinoma (refer to patient no. 16, Table 3.1.4.) showing intense MRP positive staining, this tumour showed weak Pgp expression (original magnification 60x). Both of these patients relapsed and are now deceased. (Strep/ HRP method using DAB as chromogen, sections were counterstained with haematoxylin).



a



b

Figure 3.1.2. Immunohistochemical analysis of MRP-1 protein expression (II)

Tumour cells from two patients with infiltrating ductal carcinoma (*pre* treatment) stained with MRP-R1 MAb. Both of these patients are currently alive and have not relapsed (refer to patients no. 26 (a) and 38 (b), Table 3.1.4.). Weak MRP-1 expression can be observed in tumour **(a)** (original magnification 40x) while second tumour is negative for MRP-1 expression **(b)** (original magnification 10x) and (StrepABC/ HRP method using DAB as chromogen, sections were counterstained with haematoxylin).

Table 3.1.6.

MRP-1 expression in invasive breast tumours and association with chemotherapy treatment, node status, ER status, age, tumour size and histological grade and histological subytpe of tumour.

	Immunohistochemical analysis of MRP-1 expression (n/%)	
	Total n=204	%
Total group n =204	126/201	62.6%
Chemotherapy treated group n=103	63/103	61.2%
Node Status		
Node positive patients n=115	72/115	62.6%
Node negative patients n=86	54/86	62.7%
unknown n = 3		
Age		
< 50 years	50/79	63.3%
> 50 years	72/119	60.5%
unknown n = 6		
ER status		
ER+	69/102	67.6%
ER –	38/55	69.1%
Tumour size		
< 2cm	35/68	51.47%
2-5cm	64/102	62.7%
>5cm	13/18	77.7%
Histological grade		
Grade I (low)	12/22	52.6%
Grade II (intermediate)	45/67	67.1%
Grade III (high)	66/102	64.5%

Table 3.1.7.

MRP-1 positive tumours with an immunohistochemical score of 3+3 and association with histological grade

Histological grade	
Grade III	19/26 73%
Grade II	6/26 23%
Grade I	1/26 3.8%

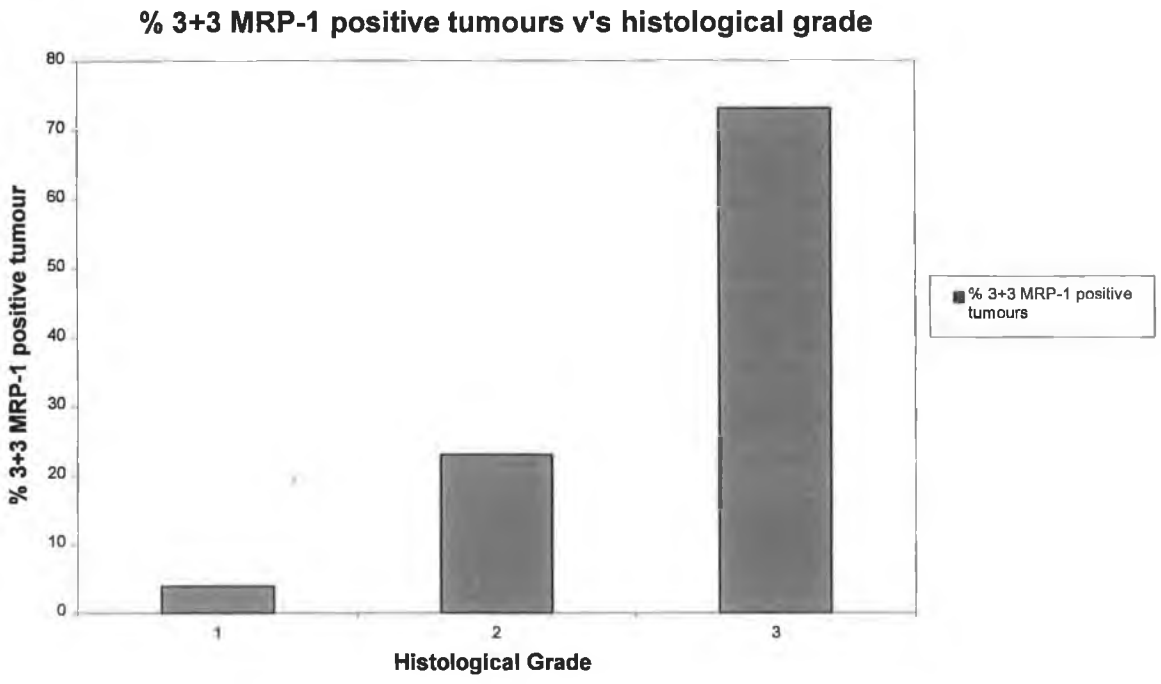


Figure 3.1.3.

Association of MRP-1 positive tumours with an immunohistochemical score of 3+3 and histological grade of tumour.

High histological grade appeared to be strongly associated with MRP-1 positive tumours which had an immunohistochemical score of 3+3. 73% of 3+3 MRP-1 positive tumours were grade III (high grade), this compares to 23% of grade II (intermediate grade) and only 3.8% of grade I (low grade).

Table 3.1.8.

Association of MRP-1 +/ MRP-1 – tumours with node status, ER status, tumour size and histological grade

	MRP-1 +		MRP-1 -	
Node status (n=204)				
Node positive	72/126	57%	43/75	57.3%
Node negative	54/126	42.8%	32/75	42.6%
unknown n = 3				
Node status chemotherapy treated patients (n=103)	46/63	73%	26/38	68.4%
Node positive	17/63	26.9%	12/38	31.6%
Node negative				
ER status (n=204)				
ER +	69/107	64.5%	3/50	66%
ER -	38/107	35.5%	17/50	34%
Tumour size (n=204)				
< 2cm	35/112	31.2%	33/76	43.4%
2-5 cm	64/112	57.1%	38/76	50%
>5 cm	13/112	11.6%	5/76	6.8%
Histological grade (n=204)				
Grade I	10/116	8.6%	12/67	17.9%
Grade II	47/116	40.5%	23/67	34.3%
Grade III	60/116	51.7%	33/27	49.2%

Table 3.1.9.
MRP-1 protein expression (+/-) at diagnosis and correlation with *Relapse Free Survival* in *non chemotherapy* treated patients

MRP-1 +

relapse free survival	*% MRP-1 + tumours node negative*	*% MRP-1 + tumours node positive*	*total*
0-3 years	12/30 40%	9/22 40.9%	21/52 40.3%
4-7 years	16/30 53.3%	9/22 40.9%	27/52 51.9%
8+ years	2/30 6.6%	4/22 18.2%	6/52 11.5%

MRP-1 -

	% MRP-1 - tumours node negative	*% MRP-1 - tumours node positive*	*total*
0-3 years	1/14 7%	2/10 20%	3/24 12.5%
4-7 years	11/14 78.9%	8/10 80%	19/24 79.2%
8+ years	2/14 14.2%		2/24 8.3%

Table 3.1.10.
MRP-1 protein expression (+/-) at diagnosis and correlation with *Overall Survival* in *non chemotherapy* treated patients

MRP-1 +

overall survival	*% MRP-1 + tumours node negative*	*% MRP-1 + tumours node positive*	*total*
0-3 years	9/27 33.3%	7/22 31.8%	16/49 32.6%
4-7 years	16/27 59.2%	11/22 50%	27/49 55.1%
8+ years	2/27 7.4%	4/22 18.2%	6/49 12.2%

MRP-1 -

overall survival	*% MRP-1 - tumours node negative*	*% MRP-1 - tumours node positive*	*total*
0-3 years	1/14 7.1%	1/9 11.1%	2/23 8.6%
4-7 years	11/14 78.69%	7/9 77.7%	18/23 78.3%
8+ years	2/14 14.2%	1/9 11.1%	3/23 13%

Table 3.1.11.
MRP-1 protein expression (+/-) at diagnosis and correlation with *Relapse Free Survival* in *chemotherapy treated* patients

MRP-1 +

relapse free survival	*% MRP-1 + tumours node negative*	*% MRP-1 + tumours node positive*	*total*
0-3 years	5/14 35.5%	23/45 51.1%	28/59 47.5%
4-7 years	9/14 64.3%	17/45 37.8%	27/59 42.4%
8+ years	0/14 0%	7/45 15.6%	7/59 11.8%

MRP-1 -

relapse free survival	*% MRP-1 - tumours node negative*	*% MRP-1 - tumours node positive*	*total*
0-3 years	3/11 27.3%	7/34 20.5%	10/45 22.2%
4-7 years	7/11 63.7%	10/34 29.4%	17/45 37.8%
8+ years	1/11 9%	17/34 50%	18/45 40%

Table 3.1.12.
MRP-1 protein expression (+/-) at diagnosis and correlation with *Overall Survival* in *chemotherapy treated* patients

MRP-1 +

overall survival	*% MRP-1 + tumours node negative*	*% MRP-1 + tumours node positive*	*total*
0-3 years	3/14 21.4%	18/45 40%	21/59 35.6%
4-7 years	11/14 78.6%	22/45 48.9%	33/59 56%
8+ years	10/14 0%	5/45 11.1%	5/59 8.5%

MRP-1 -

	% MRP-1 - tumours node negative	*% MRP-1 - tumours node positive*	*total*
0-3 years	2/11 18.2%	7/34 20.6%	9/45 20%
4-7 years	8/11 72.8%	10/34 29.4%	18/45 40%
8+ years	1/11 9%	17/34 56.6%	18/45 40%

Table 3.1.13.

MRP-1 protein expression (+/-) **at diagnosis in patients who received** ***adriamycin/taxol in combination with CMF therapy*** **and correlation with** ***Relapse Free Survivial*** (figures in brackets = MRP-1 positive tumours with a score of 1+1 were considered negative).

MRP-1 +

relapse free survival	*% MRP-1 + tumours node negative*	*% MRP-1 + tumours node positive*	*total*
0-3 years	1/1 100% (1/1 100%)	8/19 42% (7/14 50%)	9/20 45% (8/15 53.3%)
4-7 years		10/19 52.6% (6/14 42.8%)	10/20 50% (6/15 40%)
8+ years		1/19 5.3% (1/14 7.1%)	1/20 5% (1/15 6.6%)

MRP-1 -

	% MRP-1 - tumours node negative	*% MRP-1 - tumours node positive*	*total*
0-3 years	2/2 100% (2/2 100%)	1/4 25% (2/9 22%)	3/6 50% (4/11 36.3%)
4-7 years		3/4 75% (7/9 77.7%)	3/6 50% (7/11 63.6)
8+ years			

Table 3.1.14.

MRP-1 protein expression (+/-) at diagnosis in patients who received ***adriamycin/taxol in combination with CMF therapy*** **and correlation with** ***Overall Survival*** **in chemotherapy treated patients**

MRP-1 +

overall survival	*% MRP-1 + tumours node negative*	*% MRP-1 + tumours node positive*	*total*
0-3 years	1/1 100% (1/1 100%)	7/19 36.8% (6/14 42.5%)	8/20 40% (7/15 46.6%)
4-7 years		10/19 52.6% (6/14 42.8%)	10/20 50% 6/15 40%
8+ years		2/19 10.5% (2/14 14.3 %)	2/20 10% 2/15 13.3%

MRP-1-

	% MRP-1 - tumours node negative	*% MRP-1 - tumours node positive*	*total*
0-3 years	1/2 50% (1/2 50%)	1/4 25% (2/9 22.2%)	2/6 33.3% (3/11 27.3%)
4-7 years	1/2 50% (1/2 50%)	3/4 75% (7/9 77.7%)	4/6 66.6% (8/11 72.8%)
8+ years			

Table 3.1.15.

MRP-1 protein expression (+/-) at diagnosis in patients who received *CMF therapy* and correlation with *Relapse Free Survivial.*

MRP-1 +

relapse free survival	*% MRP-1 + tumours node negative*	*% MRP-1 + tumours node positive*	*total*
0-3 years	3/12 25%	13/23 56.5%	16/35 45.7%
4-7 years	9/12 75%	7/23 30.4%	16/35 45.7%
8+ years		3/23 13 %	3/35 8.6%

MRP-1 -

	% MRP-1 - tumours node negative	*% MRP-1 - tumours node positive*	*total*
0-3 years	1/9 11.1%	7/20 35%	8/29 27.5%
4-7 years	7/9 77.7%	7/20 35%	14/29 48.2%
8+ years	1/9 11.1%	6/20 30%	7/29 24.1%

Table 3.1.16.

MRP-1 protein expression (+/-) at diagnosis in patients who received *CMF therapy* and correlation with *Overall Survival*

MRP-1 +

overall survival	*% MRP-1 + tumours node negative*	*% MRP-1 + tumours node positive*	*total*
0-3 years	1/12 8.3%	9/22 40.9 %	10/34 29.4%
4-7 years	11/12 91.6%	8/22 3.3%	19/34 55.9%
8+ years		5/22 22.7%	5/34 14.7%

MRP-1 –

	% MRP-1 - tumours node negative	*% MRP-1 - tumours node positive*	*total*
0-3 years	1/9 11.1%	4/20 20%	5/29 17.2%
4-7 years	7/9 77.7%	9/20 45%	16/29 55.1%
8+ years	1/9 11.1%	7/20 35%	8/29 27.5%

In summary, results presented indicate that MRP-1 protein was expressed at diagnosis in 62.6% of invasive breast tumours studied. Analysis of these results suggest that MRP-1 protein expression in this group of 204 invasive breast cancer patients does not appear to be associated with node status, ER status or age of patients at diagnosis (< 50 years/ >50 years) (Table 3.1.6.).

There did however, appear to be a weak association between MRP-1 expression and larger tumour size; higher MRP-1 expression levels (77.7%) were observed in larger tumours (>5 cm) compared to smaller tumours (< 2 cm), of which only 51.5% were MRP-1 positive. Again, it appeared that MRP-1 expression may be associated (weakly) with higher histological grade (Tables 3.1.6. and 3.1.7). Only 8.6% of MRP-1 positive tumours were grade I (low grade). There was a strong association (statistically significant) between MRP-1 positive tumours with an immunohistochemical score of 3+3 and histological grade III (high grade), this is illustrated in Figure 3.1.3. 73% of these MRP-1 positive tumours were grade III, this compares to 23% of grade II (intermediate grade) and only 3.8% grade I.

In patients who did not receive chemotherapy, it appeared that MRP-1 expression may possibly be associated with a shortened relapse free period in *both* node positive and node negative patients; a higher percentage of MRP-1 positive patients relapsed within 1-3 years (40% of node negative patients, 40.9% of node positive patients) compared to MRP-1 negative patients (7% of node negative patients, 20% of node positive patients) (Table 3.1.9.). A similar trend was observed for overall survival again in both node positive and node negative patients, a higher proportion of MRP-1 positive patients survived for less than 3 years than MRP-1 negative patients (Table 3.1.10).

The MRP-1 expression observed in tumours of patients *who received chemotherapy* (61.2%) was similar to that observed in tumours from patients who *did not receive chemotherapy* (62.6%) (Table 3.1.6.). In node positive chemotherapy treated patients, 61% of patients with MRP-1 positive tumours relapsed and are now deceased, compared to only 37.5% of patients with MRP-1 negative tumours. A higher percentage of MRP-1 negative patients compared to MRP-1 positive patients are currently disease free.

On analysis of actual Relapse Free Survival time (years) more MRP-1 positivity at diagnosis appeared to be associated with a shortened relapse free period (1-3 years) (51.1% of MRP-1 positive patients relapsed within the first 3 years, only 20.5% of MRP-1 negative patients relapsed within this period) (Table 3.1.11) in chemotherapy treated node positive patients (not in node negative patients). Again a markedly lower percentage of MRP-1 positive patients (15.6%) showed more than 8 years relapse free survival compared to 50% of MRP-1 negative patients showing 8 or more years relapse free survival. A similar trend was observed on analysis of overall survival of node positive patients (Table 3.1.12). 56.6% of patients with MRP-1 negative tumours at diagnosis survived for at least 8 years, only 11.1% of MRP-1 positive patients survived for this length of time.

When specific chemotherapy treatments were analysed, it appeared that this association appeared to stronger in patients who received standard CMF chemotherapy compared to those patients who received adriamycin in combination with CMF or who received Taxol. Immunohistochemical analysis of *post* treatment tumours from 20 chemotherapy treated patients revealed that expression of MRP-1 was not associated with exposure to chemotherapeutic drugs; MRP-1 expression did not appear to increase following treatment.

MRP-1 protein expression values appeared to correlate with MDR-1 protein expression in 55% of chemotherapy treated patients studied (8/20 positive for both markers, 3/20 negative for both markers). As this figure is very close to 50% this can be viewed as no correlation. MRP-1 protein expression appeared, however, to correlate weakly with survivin protein expression in 63.2% of patients studied (10/19 tumours were positive for both markers, 2/19 tumours were negative for both markers). There did not appear to be any correlation of MRP-1 protein with LRP or Bcl-2 protein expression. MRP-1 expression values in relation to cerbB-2 expression are presented in the following section.

3.1.1.1. Statistical Analysis of MRP-1 expression

The association of MRP-1 expression with patient and tumour characteristics (tumour size, histological grade of tumour, ER status, node status and age of patients at diagnosis) was evaluated using the Chi-squared test. Survival analysis (Overall survival and Relapse free survival probabilities) was performed using the Kaplan and Meier method; differences between survival curves were analysed by means of the log rank test. These parameters were analysed in both the *chemotherapy treated* patient group and in those *patients who did not receive chemotherapy.* Multivariate analysis using Cox's proportional hazards models was applied to all patients, patients who received chemotherapy, patients who did not receive chemotherapy and node positive/ node negative patient groups to evaluate the predictive power of each variable independently of the others. For all tests a P value of *below 0.05* was considered to be statistically significant.

Prognostic significance of MRP-1 expression and clinical-pathological parameters in all invasive breast cancer patients (regardless of nodal status or chemotherapy treatment status)

Multivariate analysis using Cox's proportional hazards models revealed that tumour size (*P*=**.029**) and nodal status (*P*=**.026**) were independent prognostic factors for relapse free survival and that tumour grade (*P*=**.002**) was the only clinical-pathological parameter which had independent prognostic value as regards overall survival (Table 3.1.17.).

MRP-1 expression at diagnosis (*P*=.094) failed marginally to be a significant prognostic factor for overall survival in invasive breast carcinoma patients. It was not shown to be an independent prognostic factor for relapse free survival in these patients.

Table 3.1.17.

Multivariate Cox regression analysis of Relapse free survival and Overall Survival as a function of MRP-1 expression and patient and tumour characteristics in all patients with invasive breast carcinomas (n=163).

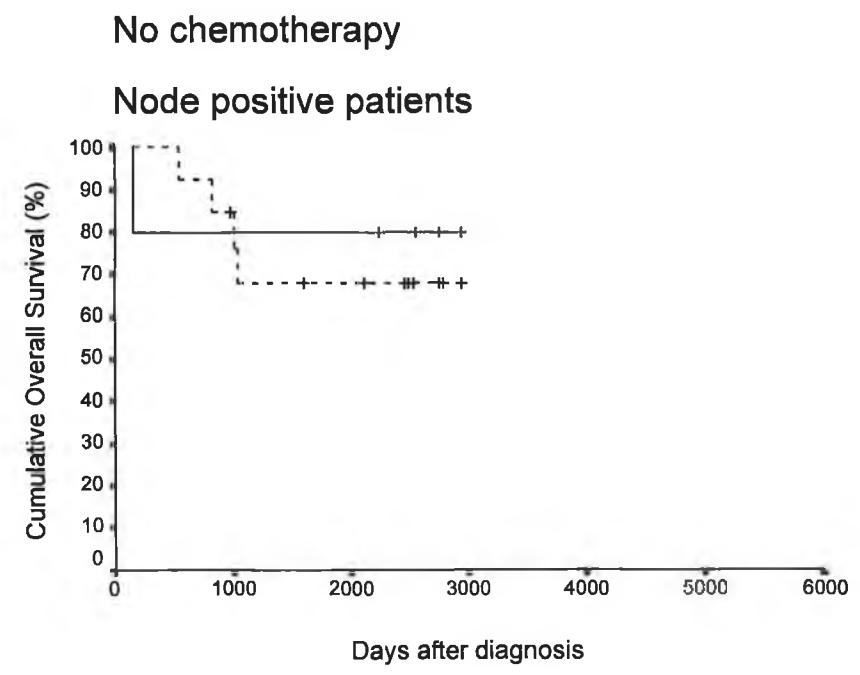
Variable	Relapse free survival *P*-value	Overall survival *P*-value
Tumour size	**.029**	.289
Tumour grade	.328	**.002**
Nodal status	**.026**	.312
ER status	.131	.135
MRP-1	.340	**.094**

Expression of MRP-1 expression and its correlation with clinical-pathological parameters in patients who did not receive chemotherapy

Chi-squared analysis did not reveal any association between MRP-1 expression at diagnosis in patients who *did not receive* chemotherapy and size of tumour, node status, ER status, age of patients at diagnosis or histological grade.

Prognostic significance of MRP-1 expression and clinicopathological parameters in patients who did not receive chemotherapy

Kaplan-Meier analysis did not reveal any association between absence of MRP-1 at diagnosis and either Relapse free Survival time (P=0.7485) or Overall Survival times (P=0.7195) in node positive patients who did not receive chemotherapy (n=18) (Figure 3.1.4.). Similarly in node negative patients; no associations with regards to either Relapse free survival (P=0.1595) or Overall Survival times (P=0.5606) (n=35) (Figure 3.1.5.) were shown. Analysis of node negative patients by histological grade also failed to show any associations between MRP-1 expression and either Relapse free Survival or Overall Survival times in patients with grade II tumours (n=20) (P=0.8602 and P=0.4997, respectively). However in patients with higher grade tumours i.e. grade III (n=11) MRP-1 expression just failed to reach significance, (P=0.1046 and P= 0.1046, respectively, *note* : these P values were identical). There were not enough cases of grade II and grade III tumours node positive tumours from patients who had not received chemotherapy to carry out Kaplan Meier analysis.



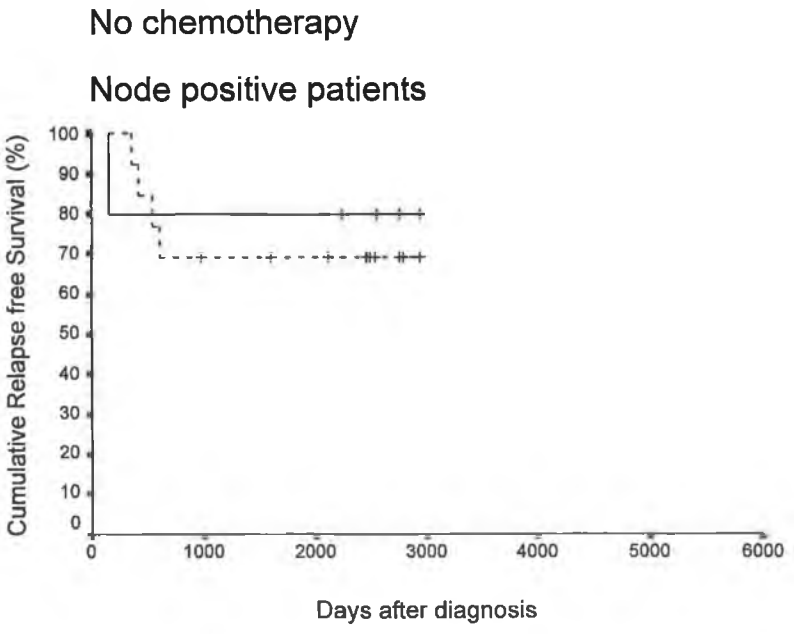


Figure 3.1.4.
Relapse free survival and Overall survival as a function of MRP-1 status i node positive patients who did not receive chemotherapy (n=18)(------MRP-positive patients (13/18), ____ MRP-1 negative patients (5/18)).

No association is observed in these patients between MRP-1 expression a diagnosis and either Relapse free survival (P=0.7485) or Overall free surviva (P=0.7195).

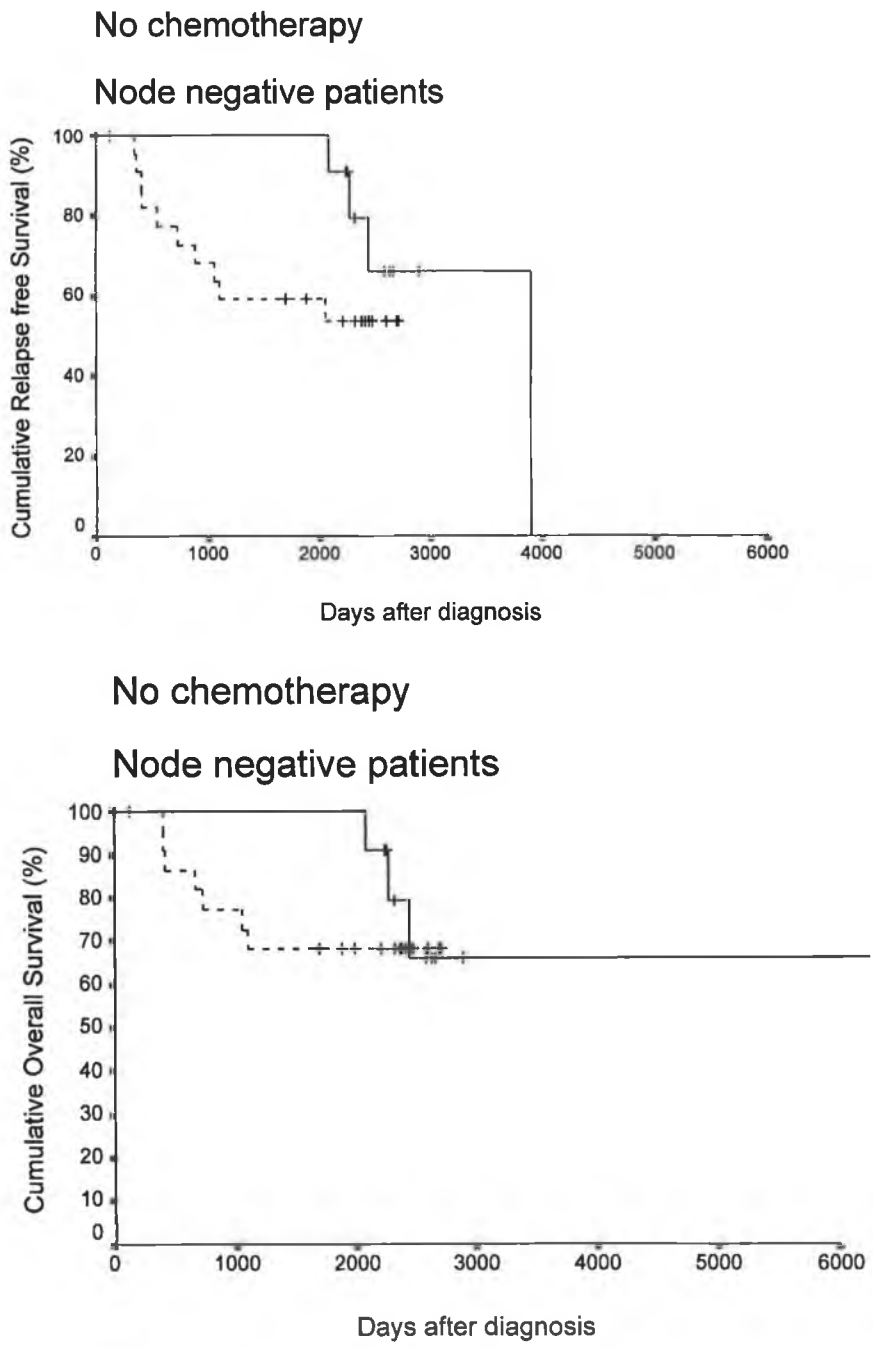


Figure 3.1.5.
Relapse free survival and Overall survival as a function of MRP-1 status i node negative patients who did not receive chemotherapy (n=35) (---- MRP-1 positive patients (24/35), ___ MRP-1 negative patients (11/35)).

No association is observed in these patients between MRP-1 expression a diagnosis and either Relapse free survival (P=0.1595) or Overall survival (P=0.5606).

Multivariate analysis using Cox's proportional hazards models showed that in patients that were *not treated* with chemotherapy (Table 3.1.18. (a)) tumour size (*P*=.049) was the only clinicopathological parameter that was an independent prognostic factor for relapse free survival, although nodal status (*P*=.072) just failed marginally to show significance an independent prognostic factor. Tumour grade (*P*=.019) and ER status (*P*=.007) were identified as prognostic factors for overall survival. Again nodal status just failed marginally (*P*=.079) to have prognostic value. MRP-1 expression was not recognised as an independent prognostic for either relapse free survival (*P*=.589) or overall survival (*P*=.664) in patients who *did not receive* chemotherapy.

Table 3.1.18.

Multivariate Cox regression analysis of Relapse free survival and Overall Survival as a function of MRP-1 expression and patient and tumour characteristics in patients not treated with chemotherapy (n=77).

Variable	Relapse free survival *P*-value	Overall survival *P*-value
Tumour size	**.049**	.436
Tumour grade	.591	**.019**
Nodal status	.072	.079
ER status	.484	**.007**
MRP-1	.589	.664

Patients stratified according to nodal status were also subjected to Cox regression analysis (Table 3.1.19.). In node negative patients neither MRP-1 expression or any other clinical-pathological feature analysed was identified to have independent prognostic power as regards either relapse free survival or overall survival. Likewise in node positive patients MRP-1, tumour grade, tumour size and ER status were not identified as independent prognostic factors for either relapse free survival or overall survival.

Table 3.1.19.

Multivariate Cox regression analysis of Relapse free survival and Overall Survival as a function of MRP-1 expression and patient and tumour characteristics (a) node positive patients not treated with chemotherapy and (b) node negative patients who did not receive chemotherapy.

(a) **node positive patients *not treated* with chemotherapy** (n=17)

Variable	Relapse free survival *P*-value	Overall survival *P*-value
Tumour size	.981	.994
Tumour grade	.981	.994
ER status	.983	.983
MRP-1	.885	.984

(b) node negative patients not *treated* with chemotherapy (n=33)

Variable	Relapse free survival *P*-value	Overall survival *P*-value
Tumour size	.117	.747
Tumour grade	.895	.607
ER status	.800	.584
MRP-1	.178	.612

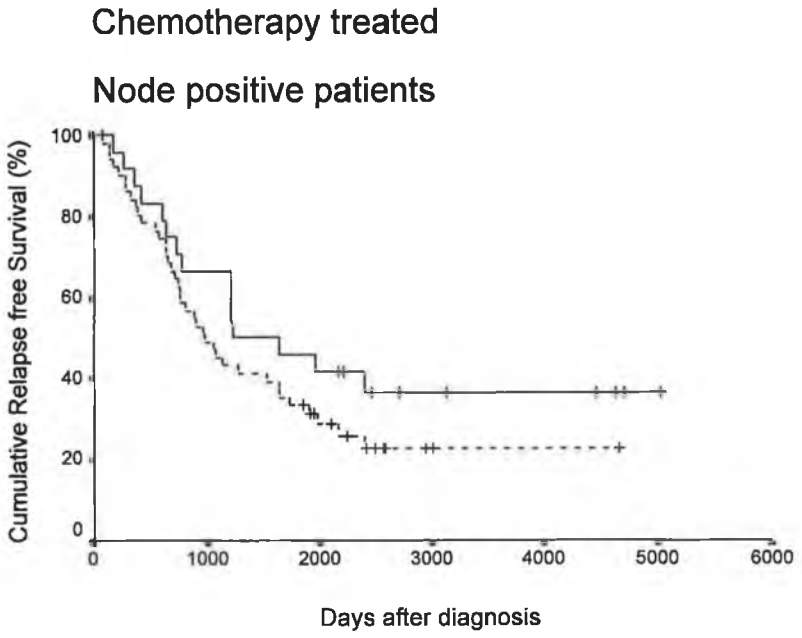
Expression of MRP-1 protein and its correlation with clinical-pathological parameters in patients treated with chemotherapy

Using the Chi-squared method a significant association was observed between MRP-1 expression and high histological grade in chemotherapy treated patients (*P*=**.035**), MRP-1 expression was not significantly associated with any of the other clinicopathological parameters studied.

Prognostic significance of MRP-1 expression and clinical-pathological parameters in patients treated with chemotherapy

Kaplan-Meier analysis demonstrated a significant association between MRP-1 expression at diagnosis Overall Survival in *node positive chemotherapy treated patients* (n=76). Patients with MRP-1 negative tumours at diagnosis showed significantly increased Overall Survival times (*P*=0.0625) compared to those patients with MRP-1 positive tumours (Figure 3.1.4.). No association between MRP-1 expression and Relapse free Survival (*P*=0.2050) was observed in these patients (Figure 3.1.4.). However in node positive patients with grade III tumours (n=41), a significant association was found between MRP-1 expression and both Relapse free survival (*P*=**0.0066**) and Overall survival (*P*=0.**0111**) (Figure 3.1.8.). No association with either Relapse free survival (*P*=0.3214) or Overall survival (*P*=0.8501) were found in patients with grade II tumours (n=19) (Figure 3.1.7.).

In *node negative chemotherapy treated patients* Kaplan-Meier analysis did not show any association between absence of MRP-1 expression at diagnosis and increased Relapse free survival times (*P*=0.8126) or increased Overall survival times (*P*=0.5307) in these patients (n=31). There were not sufficient number of cases of grade II/ grade II node negative chemotherapy tumours to carry out statistical analysis for each histological grade.



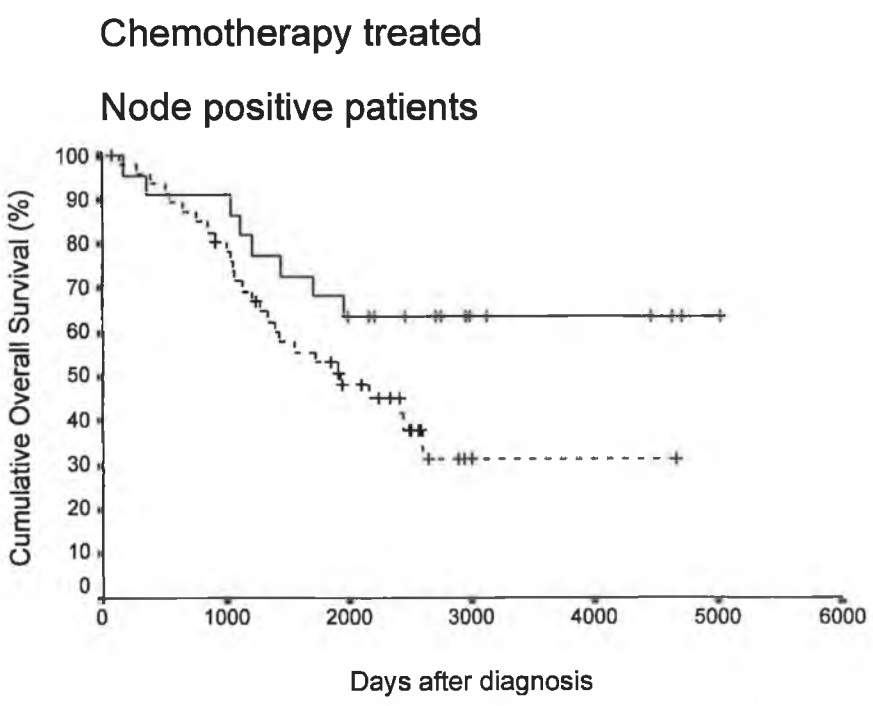
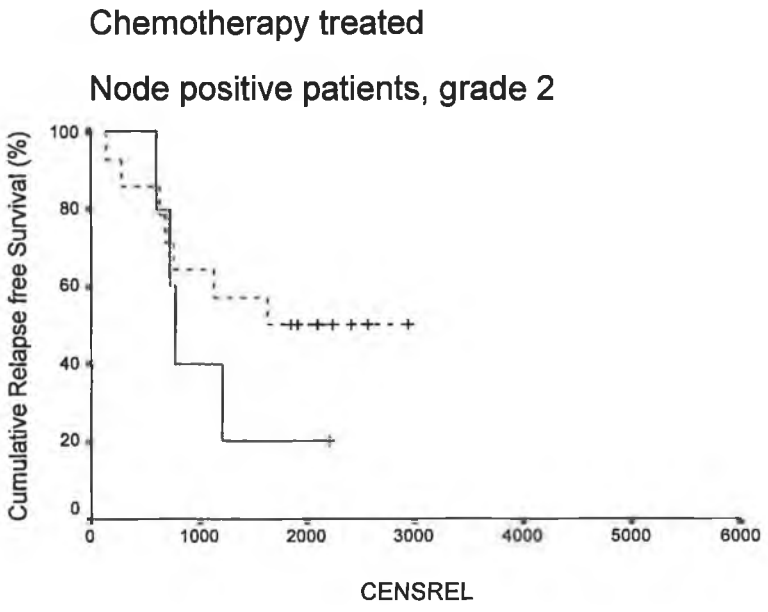


Figure 3.1.6.
Relapse free survival and Overall survival as a function of MRP-1 status i node positive chemotherapy treated patients (n=76) (---- MRP-1 positive patients (51/76), ___ MRP-1 negative patients (25/76).

No association is observed between MRP-1 expression at diagnosis in these patients and Relapse free survival (P=0.2050). An association is observed; which marginally fails to reach statistical significance, with Overall survival (P=**0.0625**). Patients with MRP-1 negative tumours at diagnosis show increased Overall survival compared to those patients with MRP-1 positive tumours.



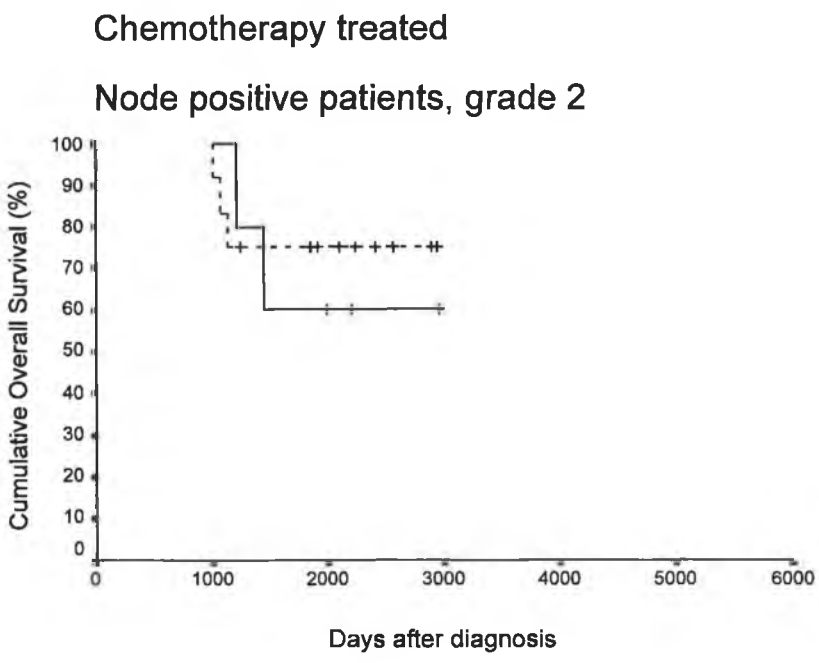
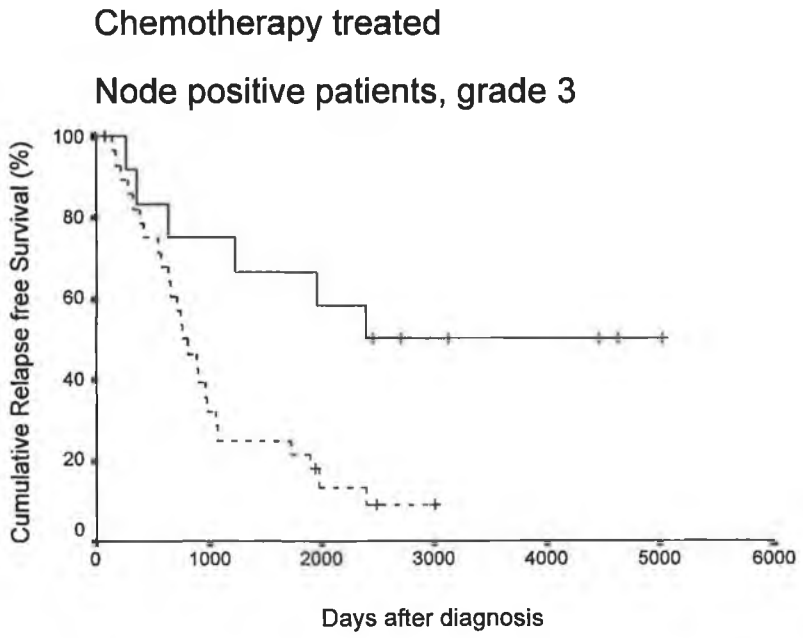


Figure 3.1.7.

Relapse free survival and Overall free survival as a function of MRP-1 status in chemotherapy treated node positive patients with grade II tumours (intermediate histological grade) (n=22) (----- MRP-1 positive patients (n=17/22) , ___ MRP-1 negative patients (5/22).

No association is observed between MRP-1 expression at diagnosis in these patients with grade II tumours and either Relapse free survival (*P*=0.3214) or Overall Survival (*P*=0.8501).



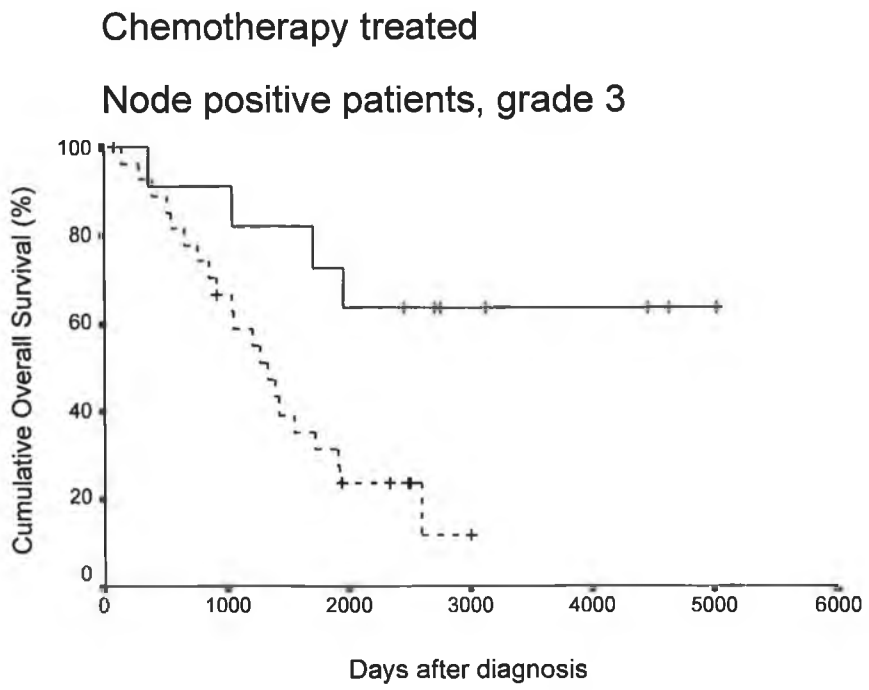
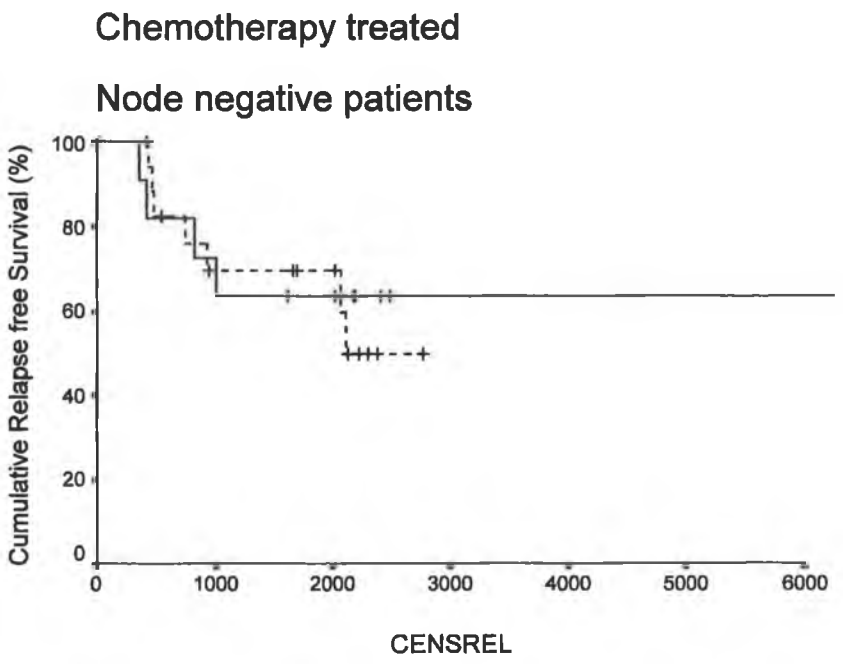


Figure 3.1.8.
Relapse free survival and Overall survival as a function of MRP-1 status i chemotherapy treated node positive patients with grade III tumours (high histological grade) (n=41) (---- MRP-1 positive patients (28/41), ___ MRP-1 negative patients (13/41).

A highly significant association is observed in these patients between MRP- expression at diagnosis and both Relapse free survival (P=0.0066) and Overall survival (P=0.0111). Patients with grade III tumours that are MRP-1 negative at diagnosis show increased Relapse free survival and Overall survival time compared to those patients with grade III tumours that are MRP-1 positive at diagnosis.



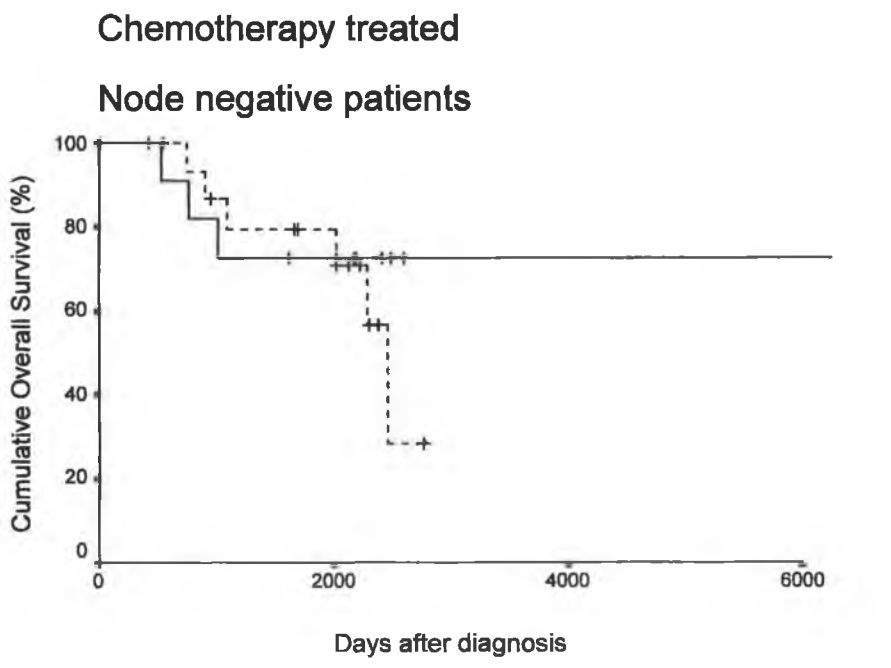


Figure 3.1.9.

Relapse free survival and Overall survival as a function of MRP-1 status in node negative patients who received chemotherapy (n=31) ---- MRP-1 positive patients (19/31), ___ MRP-1 negative patients (12/31).

No association is observed in these patients between MRP-1 expression at diagnosis and either Relapse free survival (P=0.8126) or Overall survival (P=0.5307).

Multivariate analysis using Cox's proportional hazards models showed that in chemotherapy treated patients (Table 3.1.20), MRP-1 expression at diagnosis was an independent prognostic factor for overall survival (*P*=.048) but not for relapse free survival (*P*=.862). Nodal status (*P*=.016) ER status (*P*=.010) were independent prognostic factors for relapse free survival. ER status (*P*=.013) was an independent prognostic factor for overall survival; nodal status (*P*=.088) and histological grade (*P*=.083) failed marginally to show significance as independent prognostic factors.

Table 3.1.20.

Multivariate Cox regression analysis of Relapse free survival and Overall Survival as a function of MRP-1 expression and patient and tumour characteristics in patients treated with chemotherapy.

Variable	Relapse free survival *P*-value	Overall survival *P*-value
Tumour size	.336	.447
Tumour grade	.451	.083
Nodal status	.016	.088
ER status	.010	.013
MRP-1	.862	.048

Patients stratified according to nodal status were also subjected to Cox regression analysis (Table 3.1.21.) In node negative chemotherapy patients MRP-1 was not identified as an independent prognostic factor for either relapse free survival (*P*=.670) or overall survival (*P*=.160). ER status was the only clinicopathological parameter that showed independent prognostic significance in these patients, it was identified as an independent prognostic factor for overall survival (*P*=.037). None of the other clinicopathological factors showed independent prognostic value regarding either relapse free survival or overall survival.

In node positive chemotherapy treated patients, MRP-1 expression at diagnosis was identified as a highly significant independent prognostic factor for overall survival

(*P*=.002), it failed to show independent prognostic significance as regards relapse free survival (*P*=.213). ER status was an independent prognostic factor for relapse free survival (*P*=.008) and overall survival (*P*=.006). Histological grade was also identified as a highly significant independent prognostic factor for overall survival (*P*=.002), but it marginally failed to achieve significance for relapse free survival (*P*=.071).

Table 3.1.21.

Multivariate Cox regression analysis of Relapse free survival and Overall Survival as a function of MRP-1 expression and patient and tumour characteristics in (a) node positive patients treated with chemotherapy and (b) node negative patients treated with chemotherapy

(a) node positive patients *treated* with chemotherapy

Variable	Relapse free survival *P*-value	Overall survival *P*-value
Tumour size	.135	.559
Tumour grade	.071	.002
ER status	.008	.006
MRP-1	.213	.002

(b) node negative patients *treated* with chemotherapy

Variable	Relapse free survival *P*-value	Overall survival *P*-value
Tumour size	.382	.134
Tumour grade	.623	.688
ER status	.240	.037
MRP-1	.670	.160

3.1.2. cerbB-2 protein expression

cerbB-2 protein expression was studied in *pre* treatment tumours from 45 patients with invasive breast cancer. 40 of these patients received chemotherapy following surgery . cerbB-2 expression was studied in 17 *post* treatment tumours from this group of patients. Immunohistochemical analysis results are presented in Table 3.1.4. Representative tumours stained with the cerbB-2 specific MAb, CB 11, are presented in Figures 3.1.10. and 3.1.11.

cerbB-2 protein expression in relation to known clinico-pathological factors i.e. node status, ER status, tumour size, and histological grade is presented in Table 3.1.22. Correlation of cerbB-2 expression with relapse free survival and overall survival in chemotherapy treated patients is presented in Tables 3.1.23 – 3.1.24. Correlation between the co-expression of cerbB-2 and MRP-1 and disease status of patients, relapse free survival and overall survival times is presented in Tables 3.1.25. – 3.1.27.

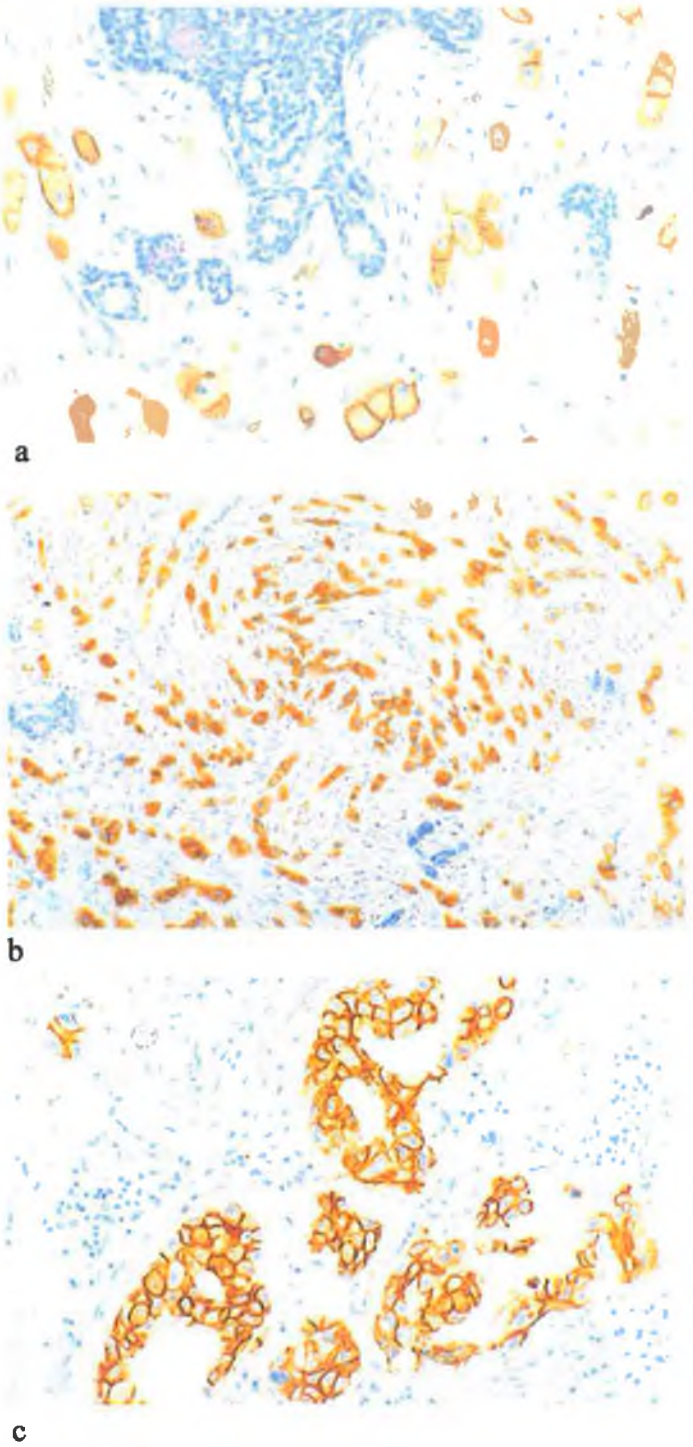
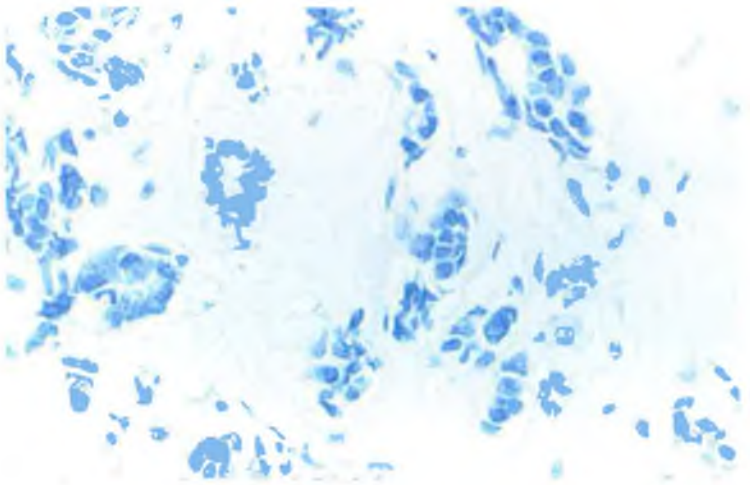
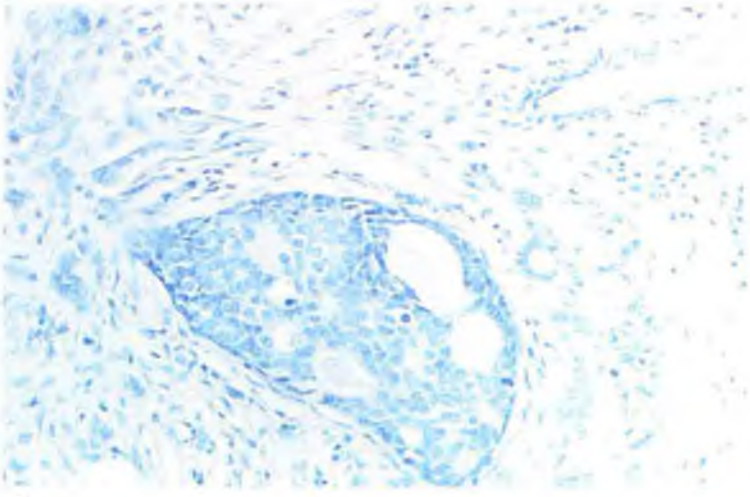


Figure 3.1.10. Immunohistochemical analysis of cerbB-2 protein levels (I)

(a) Infiltrating ductal tumour cells from a patient who relapsed and subsequently died (refer to patient no. 19, Table 3.1.4.), stained with cerbB-2, clone CB11 MAb (Novacastra), intense membranous positivity can be observed in tumour cells at *pre* treatment stage (original magnification 20x). **(b)** Following treatment there was no change in cerbB-2 status, positive staining can be observed in infiltrating tumour cells (original magnification 10x). **(c)** DCIS tumour cells (*pre* treatment) (refer to patient no. 1, Table 3.1.4.) showing intense cerbB-2 membranous positive staining. (original magnification 20x), again this patient relapsed and is now deceased. (StrepABC/ HRP method using DAB as chromogen, sections were counterstained with haematoxylin).



a



b

Figure 3.1.11. Immunohistochemical analysis of cerbB-2 protein levels (II)

(a) High grade infiltrating carcinoma (refer to patient no. 30, Table 3.1.4.) (*pre* treatment) stained with cerbB-2 MAb, clone CB11, no cerbB-2 positivity can be observed in infiltrating tumour cells (original magnification 40x). This patient is currently alive and has not relapsed. **(b)** Ductal carcinoma cells (*pre* treatment) from another patient who is currently alive and well (refer to patient no. 34, Table 3.1.4), stained with cerbB-2 MAb, clone CB11, again no positivity can be observed in tumour cells (original magnification 40x). (StrepABC/ HRP method using DAB as chromogen all sections were counterstained with haematoxylin).

Table 3.1.22.
cerbB-2 protein expression in invasive breast tumours and association with node status, ER status, tumour size and histological grade

	Immunohistochemical analysis of cerbB-2 expression (n/%)	
chemotherapy treated patients n=40	11/40	27.5%
non chemotherapy treated patients n=5	4/5	80%
Node status		
node positive	8/34	23.5%
node negative	1/6	16.6%
ER status		
ER +	2/12	16.6%
ER -	4/15	26.6%
Tumour size		
<2 cm	1/11	9.1%
2-5 cm	3/4	25%
>5cm	3/9	30%
Histological grade		
Grade I	3/3	100%
Grade II	1/1	100%
Grade III	6/22	27.3%

Table 3.1.23.
cerbB-2 protein expression (+/-) at diagnosis and correlation with *Relapse Free Survival* in *chemotherapy treated patients*

cerbB-2 +

relapse free survival	*% cerbB-2 + tumours node negative*	*% cerbB-2 + tumours node positive*	*total*
0-3 years	1/3 33.3%	7/11 63.4%	8/14 57.1%
4-7 years	2/3 66.6%	4/11 36.3%	6/14 42.9%
8+ years		0/11 0%	0/14 0%

cerbB-2 -

relapse free survival	*% cerbB-2 - tumours node negative*	*% cerbB-2 - tumours node positive*	*total*
0-3 years	2/3 66.6%	6/23 26%	8/26 30.8%
4-7 years	1/3 33.3%	7/23 30.4%	8/26 30.8%
8+ years		10/23 43.4%	10/26 38.5%

Table 3.1.24.
cerbB-2 protein expression (+/-) at diagnosis and correlation with *Overall Survival* in *chemotherapy treated patients*

cerbB-2 +

overall survival	*% cerbB-2 + tumours node negative*	*% cerbB-2 + tumours node positive*	*total*
0-3 years	1/3 33.3%	6/10 60%	7/13 53.8%
4-7 years	2/3 66.6%	4/10 40%	6/13 46.2%
8+ years	0/3 0%	0/10 0%	0/13 0%

cerbB-2 -

	% cerbB-2 - tumours node negative	*% cerbB-2 - tumours node positive*	*total*
0-3 years	1/3 33.3%	4/26 15.4%	5/29 17.2%
4-7 years	2/3 66.6%	10/26 38.5%	12/29 41.4%
8+ years		12/26 46.2%	12/29 41.4%

Table 3.1.25.
cerbB-2 / MRP-1 protein expression in *chemotherapy treated* tumours and correlation with *disease status* (n=40).

	MRP-1 +/cerbB-2 -	MRP-1 - /cerbB-2 +	MRP-1 +/ cerbB-2 +	MRP-1 -/ cerbB-2 -
dod	8/21 (38%)	2/21 (9.5%)	7/21 (33.3%)	4/21 (19.4%)
a&w	5/17 (29.4%)	1/17 (5.9%)	0/17 (0%)	11/17 (64.7%)
rec	-		1/3 (33.3%)	2/3 (66.6%)

Table 3.1.26.
cerbB-2 and MRP-1 protein expression at diagnosis and correlation with *Relapse Free Survival* in *chemotherapy treated patients*

	MRP-1 +/cerbB-2 -	MRP-1 - /cerbB-2 +	MRP-1 +/ cerbB-2 +	MRP-1 -/ cerbB-2 –
0-3 years	5/14 35.7%	2/3 66.6%	6/8 75%	4/15 26.7%
4-7 years	5/14 35.7%	1/3 33.3%	2/8 25%	4/15 26.7%
8+ years	4/14 28.6%			7/15 46.7%

Table 3.1.27.
cerbB-2 and MRP-1 protein expression and correlation with *Overall Survival* in *chemotherapy treated* patients

	MRP-1 +/cerbB-2 -	MRP-1 - /cerbB-2 +	MRP-1 +/ cerbB-2 +	MRP-1 -/ cerbB-2 –
0-3 years	5/14 35.7%	2/3 66.6%	5/8 62.5%	2/15 13.3%
4-7 years	3/14 21.4%	1/3 33.3%	3/8 37.5%	6/15 40%
8+ years	5/14 35.7%			7/15 46.7%

In summary, results presented indicate that cerbB-2 protein was expressed in 27% of invasive breast tumours at diagnosis from chemotherapy treated patients. cerbB-2 was expressed 80% (4/5) of tumours from patients who did not receive chemotherapy (Table 3.1.22.). Most of this observed cerb-B-2 positive staining was membranous in a nature, although cytoplasmic positivity was observed in some tumours. Membranous cerbB-2 positivity is generally considered to be specific for HER-2/ neu overexpression and has been shown to correlate with gene amplification. In contrast the significance of cytoplasmic positive staining is still controversial. Only tumours which showed membranous positivity (a small number of these did have some cytoplasmic reactivity) were considered positive. cerbB-2 expression did not appear to be associated with node status, ER status or tumour size (Table 3.1.22). It appears from these results that grade III tumours studied exhibited lower expression levels of cerbB-2 protein.

On analysis of Relapse Free Survival times, results suggest that cerbB-2 expression at diagnosis is strongly associated with a shortened relapse free period in node positive patients (Table 3.1.23). A higher percentage of cerbB-2 + tumours relapsed within 3 years compared to cerbB-2 – tumours, 43.4% of cerB-2 – tumours had a relapse free period of at least 8 years; all of the cerbB-2 + tumours studied relapsed within 8 year. A similar trend was observed on analysis of Overall Survival in node positive patients. It appeared that cerbB-2 expression was strongly associated with a shortened overall survival time in these patients. 60% of cerbB-2 + patients survived less than 3 years following diagnosis compared to 15.4% of cerbB-2 – patients (Table 3.1.24).

When cerbB-2 expression was correlated with expression of MRP-1, results indicated that 75% of patients with MRP-1 + / cerbB-2 + tumours relapsed within 3 years, compared to only 26.7% of patients with MRP-1 -/ cerbB-2 – tumours (Table 3.1.26.). On analysis of overall survival, again co-expression of these two markers was associated with a shortened overall survival (Table 3.1.27.), however in this case MRP-1 -/ cerbB-2 + tumours exhibited similar overall survival times. Tumours which were negative for both markers again showed an increased survival time. These results suggest that co-expression of these 2 markers may be more strongly

associated with a shortened relapse free survival and possibly with a shortened overall survival in node positive patients.

Expression of cerbB2 did not appear to correlate with any of the other markers studied. *Post* treatment tumours from 17 chemotherapy treated patients were also investigated. cerbB-2 expression did not appear to increase following treatment with chemotherapeutic drugs, a slight increase in expression was observed in 2 patients.

3.1.3 MDR-1 Pgp expression

Pgp expression was studied in *pre* treatment tumours from 20 chemotherapy treated patients with invasive breast cancer. Pgp expression was studied in 17 *post* treatment tumours from this group of patients. Immunohistochemical analysis results are presented in Table 3.1.4. Representative tumours stained with the MDR-1 specific MAb, 6/1C, are presented in Figures 3.1.12.

MDR-1 expression in relation to known clinico-pathological factors i.e. node status, ER status, tumour size, and histological grade is presented in Tables 3.1.26. Correlation of MDR-1 Pgp expression (i.e. MDR-1 positive/ MDR-1 negative tumours) with relapse free survival and overall survival in chemotherapy treated patients is presented in Tables 3.1.27 – 3.1.28.

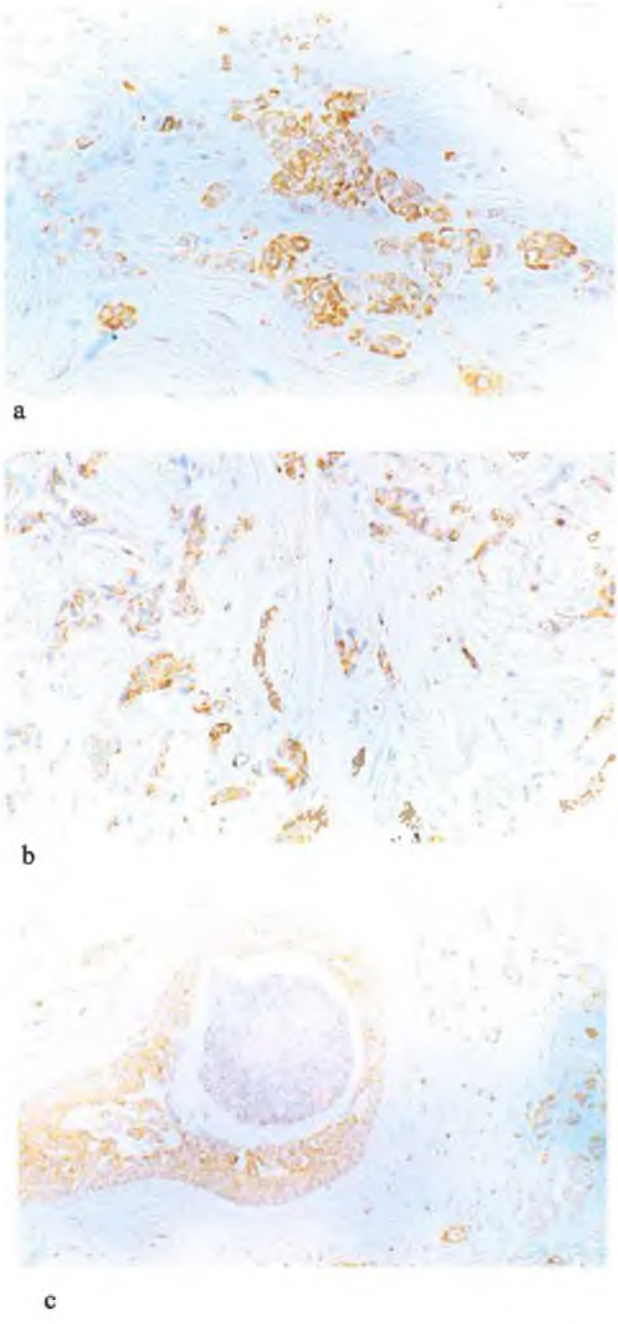


Figure 3.1.12. Immunohistochemical analysis of Pgp expression

(a) *Pre* treatment (original magnification 20x) and **(b)** *post* treatment infiltrating tumour cells (original magnification 20x) from a ductal carcinoma patient (refer to patient no. 17, Table 3.1.4.) stained with BRI anti MDR-1, 6/1C. Intense MDR-1 positivity can be observed in both the *pre* and *post* tumour samples, the level of Pgp positivity was not altered following treatment. **(c)** DCIS tumour, comedo type (centre of tumour has undergone necrosis and does not show any MDR-1 reactivity) (refer to patient no. 2, Table 3.1.4.) (*pre* treatment) showing intense MDR-1 positive staining, positivity can also be observed in surrounding infiltrating tumour cells. Both of these patients relapsed and are now deceased (original magnification 20x). (StrepABC/ HRP method using DAB as chromogen, sections were counterstained with haematoxylin).

Table 3.1.26.

MDR-1 Pgp expression in invasive breast tumours and association with node status, ER status, tumour size and histological grade.

Immunohistochemical analysis of MDR-1 (n/%)

	n = 20	
chemotherapy treated patients n=20	14/20	70%
Node status		
node positive	13/19	68.4%
node negative	1/1	100%
ER status		
ER +	6/7	85.7%
ER -	2/4	50%
Tumour size		
<2 cm	7/7	100%
2-5 cm	2/6	33.3%
>5cm	4/6	66.6%
Histological grade		
Grade I	-	
Grade II	-	
Grade III	7/10	70%

Table 3.1.27.

MDR-1 Pgp expression (+/-) at diagnosis and correlation with *relapse free survival* in *chemotherapy treated patients*

MDR-1 +

relapse free survival	*% MDR-1 + tumours node negative*	*% MDR-1 + tumours node positive*	*total*
0-3 years		8/13 61.5%	8/16 50%
4-7 years		1/13 7.6%	1/16 6.2%
8+ years	3/3 100%	4/13 30.5%	7/16 43.8%

MDR-1 -

	% MDR-1 - tumours node negative	*% MDR-1 - tumours node positive*	*total*
0-3 years		1/3 33.3%	1/6 16.66%
4-7 years			
8+ years	3/3 100%	2/3 66.6%	5/6 83.3%

Table 3.1.28.

MDR-1 Pgp expression (+/-) at diagnosis and correlation with *overall survival* in *chemotherapy treated patients*

MDR-1 +

relapse free survival	*% MDR-1 + tumours node negative*	*% MDR-1 + tumours node positive*	*total*
0-3 years		5/13 38.5%	5/16 31.3%
4-7 years		4/13 30.7%	4/16 25%
8+ years	3/3 100%	4/13 30.7%	7/16 43.8%

MDR-1 -

	% MDR-1 - tumours node negative	*% MDR-1 - tumours node positive*	*total*
0-3 years		1/5 20%	1/5 20%
4-7 years		2/5 40%	2/5 40%
8+ years		2/5 40%	2/5 40%

In summary, MDR-1 was expressed in 70% of tumours at diagnosis in chemotherapy treated patients. Results presented indicate that expression of MDR-1 Pgp does not appear to be associated with node status or ER status (very weak association with ER +). No conclusion can be drawn as regards association with histological grade as MDR-1 Pgp expression was only studied in grade III tumours of which 70% were MDR-1 positive. Again as regards association with tumour size a definite conclusion cannot be reached due to small sample size, the results however do suggest that MDR-1 appears to be associated more strongly with tumours that are less than 2 cm.

77.7% of *pre* chemotherapy treatment tumours from patients currently alive (a) who have not relapsed were MDR-1 positive, 55.5% of those patients who relapsed and are now deceased (d) were MDR-1 positive. Looking at the actual correlation of MDR-1 expression in node positive patients with relapse free survival suggests that MDR-1 + node positive patients are more likely to relapse within 3 years compared to MDR-1 – patients (Table 3.1.27.). As regards overall survival a similar trend was observed, but the association was weaker (Table 3.1.28.). However the numbers of patients studied were very small, thus no statistical analysis could be carried out in order to verify these results. No conclusion as regards node negative patients can be made as there were only 3 patients studied for MDR-1 Pgp expression.

Differences between the 2 Pgp MAbs used in this study were noted both in the intensity of Pgp positive staining observed and in the number of tumour cells stained within individual sections.

4 *pre* treatment tumours which were MDR-1 negative showed MDR-1 Pgp positivity following chemotherapy (all of these 4 patients relapsed within 3 years). There appeared to be no increase in staining in those *post* treatment that were MDR-1 positive at diagnosis. It is not possible to conclude that MDR-1 Pgp expression is associated with chemotherapy treatment in these patients.

As previously outlined, MDR-1 protein expression appeared to correlate with MRP-1 protein expression in 55% of chemotherapy treated patients, however this can be taken as no correlation. Survivin protein levels appear to correlate with MDR-1 values in 15/21 (71.4%) of chemotherapy treated patients (2/21 negative for both

markers, 19/21 positive for both markers). MDR-1 expression did not appear to correlate with cerbB-2, Bcl-2 or LRP protein.

3.1.4. LRP protein expression

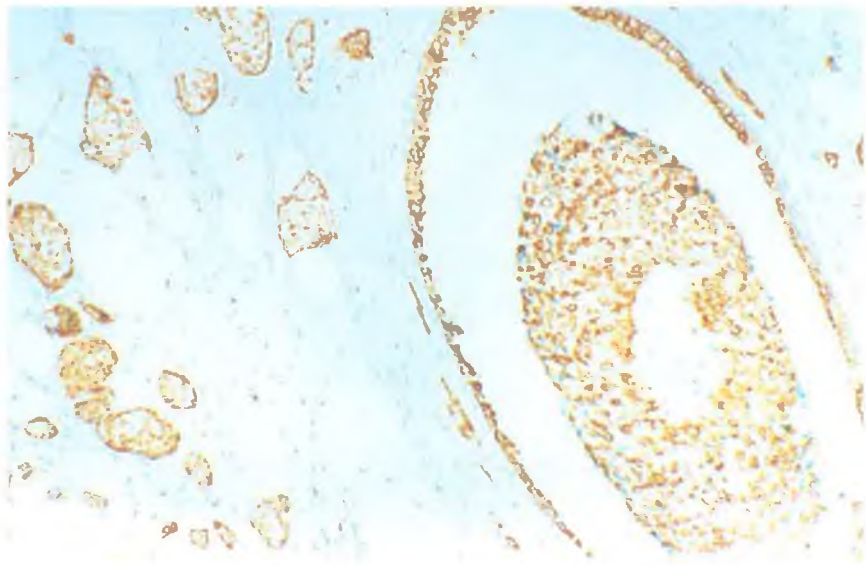
LRP protein expression was studied in *pre* treatment tumours from 14 patients with invasive breast cancer (12 chemotherapy treated). Immunohistochemical results are presented in Table 3.1.4.

4 LRP negative *pre* treatment tumours showed some degree of LRP positivity following chemotherapy (1 patient alive, 3 patients deceased). Again as sample numbers are small it is not possible to conclude that LRP expression is associated with exposure to chemotherapeutic drugs. LRP expression values did not appear to correlate with expression of any other of the markers studied.

3.1.5. BCL-2 protein expression

BCL-2 expression was studied in *pre* treatment tumours from 15 chemotherapy treated patients with invasive breast cancer. Immunohistochemical analysis results are presented in Table 3.1.4. Representative tumours stained with a BCL-2 specific MAb are presented in Figures 3.1.13.

Due to small sample size it was not possible to analyse in detail BCL-2 protein expression in relation to known clinico-pathological factors. The association of BCL-2 + tumours and BCL-2 - tumours with relapse free survival and overall survival in chemotherapy treated patients is presented in Table 3.1.29.



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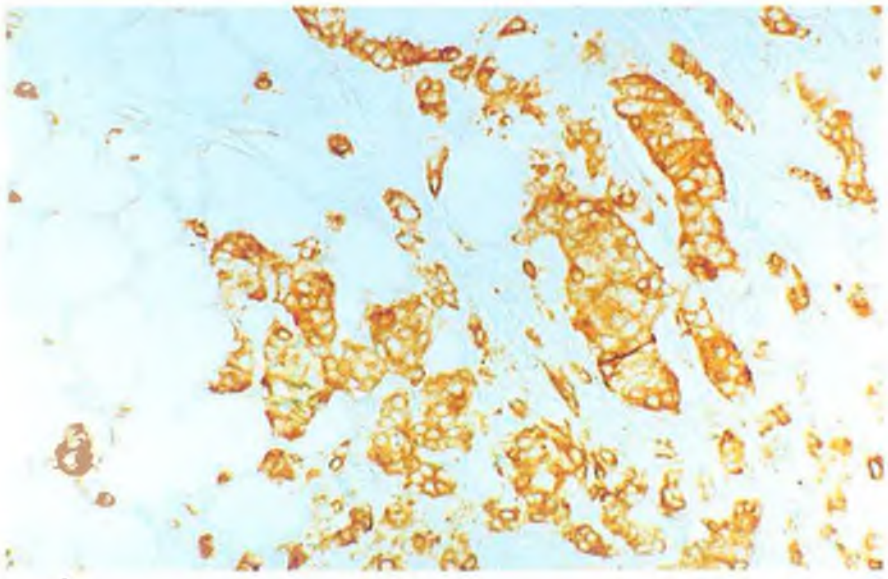


b

Figure 3.1.13. Immunohistochemical analysis of Bcl-2 protein levels

(a) Bcl-2 positive tumour (*pre* treatment) stained with Bcl-2 MAb, clone 124 (Dako) showing intense Bcl-2 positive staining in DCIS component and in surrounding infiltrating tumour cells (original magnification 10x). Bcl-2 status was not altered following treatment. **(b)** *Post* treatment tumour from same patient showing Bcl-2 positivity in infiltrating tumour cells (original magnification 10x). This patient showed a relatively long relapse free period (5 years) (refer to patient no. 17, Table 3.1.4.). (StrepABC/ HRP method using DAB as substrate, sections were counterstained with haematoxylin).

Table 3.1.29.

Association of BCL-2 +/ BCL-2 – tumours with *relapse free survival* and *overall survival* in *chemotherapy treated patients*

relapse free survival (years)	BCL-2 +	BCL-2 -
0-3	7/9 77.7%	3/3 100%
4-7	2/9 22.2%	
8+		

overall survival (years)	BCL-2 +	BCL-2 -
0-3	7/9 77.7%	3/3 100%
4-7	2/9 22.2%	
8+		

Bcl-2 protein was expressed in 61.5% of chemotherapy treated tumours at diagnosis. There did not appear to be any increase in BCL-2 expression following treatment, suggesting that BCL-2 expression is not associated with exposure to chemotherapeutic drugs.

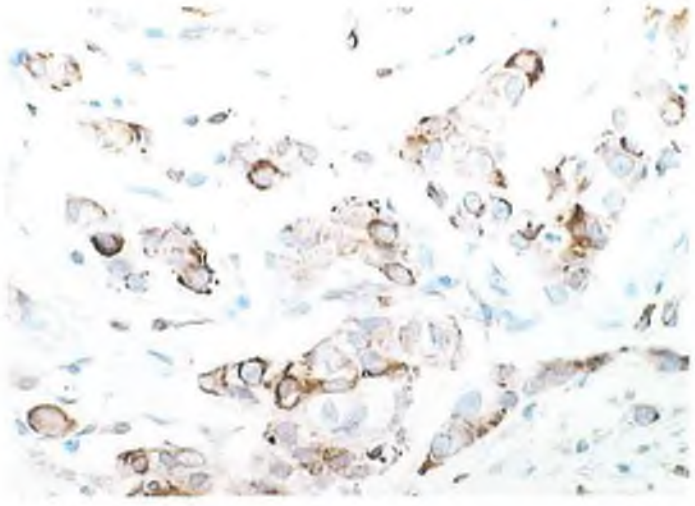
Results suggest that there appeared to be a weak association between BCL-2 negative tumours and a shortened relapse free period and overall survival times (Table 3.1.29.) compared to BCL-1 positive tumours; a higher percentage of BCL-2 – patients relapsed (and died) within 3 years compared to BCL-2 + patients.

Bcl-2 and survivin protein expression values appeared to correlate in 50% of chemotherapy treated patients. There did not appear to be a correlation between BCL-2 and any of the other markers studied.

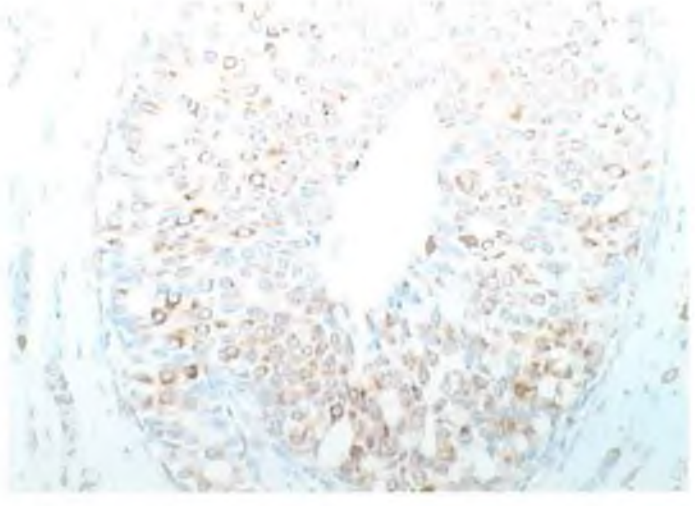
3.1.1.6. Survivin protein expression

Survivin protein expression was studied in *pre* treatment tumours from 24 patients with invasive breast cancer, 22 of these patients received chemotherapy. Immunohistochemical results are presented in Table 3.1.4. Representative tumours stained with survivin specific MAb are presented in Figures 3.1.14.

Due to small sample size it was not possible to analyse in detail survivin protein expression in relation to known clinico-pathological factors. The association of survivin + tumours and survivin - tumours with relapse free survival and overall survival in chemotherapy treated patients is presented in Table 3.1.30.



a



b

Figure 3.1.14. Immunohistochemical analysis of survivin protein levels

(a) Infiltrating tumour cells from a ductal carcinoma (*pre* treatment) (refer to patient no. 16, Table 3.1.4.) showing intense survivin positive cytoplasmic staining with supernatant 1C5 (original magnification 40x). This patient relapsed and is now deceased. **(b)** DCIS tumour cells (*pre* treatment) from a patient who did not relapse and is currently alive and well (refer to patient no. 31, Table 3.1.4.), showing survivin positivity also (original magnification 20x) (StrepABC/ HRP method using DAB as substrate, sections were counterstained with haematoxylin).

Table 3.1.30.

Association of survivin +/ survivin – tumours with *relapse free survival* and *overall survival* in *chemotherapy treated patients*

node negative patients/ node positive patients

relapse free survival (years)	survivin +	survivin -
0-3	9/19 47.4%	1/3 33.3%
4-7	1/19 5.2%	
8+	9/19 47.4%	2/3 66.6%

node negative patients/ node positive patients

overall survival (years)	survivin +	survivin-
0-3	7/21 33.3%	1/7 14.3%
4-7	4/21 19%	4/7 57.1%
8+	8/21 38%	2/7 28.5%

In summary, Survivin protein was observed in 86.3% of chemotherapy treated patients at diagnosis. An increase in survivin positive staining was only observed in 1 patient following chemotherapy treatment indicating that survivin expression is not associated with exposure to chemotherapeutic drugs.

As survivin protein was observed in the majority of tumours studied it was not possible to correlate survivin expression with node status, ER status, tumour size or histological grade.

On initial analysis, results suggested that expression of survivin protein at diagnosis (combined node positive and node negative patients) may be possibly associated with shortened relapse free survival (i.e. 1-3 years); 47.3% of patients with survivin positive tumours relapsed within 3 years compared to 33.3% of patients with survivin negative tumours). However on analysis of overall survival times, it appeared 38% of patients with survivin positive tumours survived for 8 or more years, compared to only 28.5% of survivin negative tumours (Table 3.1.30.) This observation suggested that survivin expression at diagnosis is actually associated with a good prognosis i.e. longer overall survival in chemotherapy treated patients. The numbers studied were small, therefore it was not possible to carry out statistical analysis. Survivin positivity was also observed at diagnosis, in 2 Tamoxifen treated patients; both of these patients relapsed within 1 year following treatment.

As previously outlined Survivin protein expression appeared to correlate with MRP-1 and MDR-1 protein expression chemotherapy treated patients. Bcl-2 and survivin protein expression values appeared to correlate in 50% of chemotherapy treated patients. cerbB-2 and survivin protein expression values appeared to correlate in 36.6% of chemotherapy treated patients studied. There did not appear to be any correlation with LRP protein expression observed.

There appeared to be considerable heterogeneity between the intensity of the staining observed and in the number of tumour cells stained within individual tumours. Nuclear survivin positivity was observed in a number of tumour samples.

3.1.7. Conclusion

Results from an investigation of the possible prognostic/predictive role of a panel of MDR associated markers in invasive breast cancers have been presented in sections 3.1.1.-3.1.6.

In a large series of invasive breast carcinomas (n=204), larger tumour size and positive lymph node status were identified as independent prognostic factors for decreased relapse free survival. Higher histological grade was identified as a highly significant independent prognostic factor for decreased overall survival in these patients. MRP-1 expression at diagnosis just failed to show independent prognostic significance for overall survival.

A highly significant association was shown between MRP-1 expression and both Overall survival and Relapse free survival in *node positive chemotherapy treated patients* with grade III tumours in patients with grade III tumours. Multivariate analysis revealed that MRP-1 expression was an independent prognostic factor for overall survival but not relapse free survival in node positive patients treated with chemotherapy.

No association was shown between MRP-1 expression and either relapse free survival or overall survival in *node negative chemotherapy treated* patients.

ER status was shown to be an independent prognostic factor for both relapse free survival *and* overall survival *in node positive chemotherapy treated* patients, and for overall survival only in *node negative* patients. Histological grade (higher grade associated with a worse outcome) and nodal status (lymph node positive status associated with a worse out come just failed to show significance as independent prognostic factors for overall survival in *node positive* patients chemotherapy treated patients. Small tumour size was not identified as an independent prognostic factor for relapse free survival or overall survival.

MRP-1 expression at diagnosis was significantly associated with higher histological grade in chemotherapy treated patients, no association with other clinicopathological

parameters was found. In patients who did not receive chemotherapy, MRP-1 was not associated with any of the clinicopathological parameters studied.

In patients who *did not receive chemotherapy*; no association was shown between MRP-1 expression at diagnosis and either Relapse free survival or Overall survival, regardless of the node status of the patients. However in *node negative patients with grade III tumours,* it appeared that there may be a trend towards absence of MRP-1 expression at diagnosis being associated with decreased relapse free survival and overall in these patients. None of the clinical-pathological features studied were shown to have any independent prognostic value for either overall survival or relapse free survival in these patients.

Preliminary analysis of results of a smaller group of patients studied for cerbB-2 expression (n=46), indicated that cerbB-2 positivity at diagnosis appeared to be associated with decreased relapse free survival and overall survival in *node positive* patients treated with *CMF based chemotherapy*. Results also suggest that perhaps the co-expression of MRP-1 and cerbB-2 protein may possibly be more strongly associated with shortened Relapse free survival and possibly shortened Overall Survival in *node positive* patients.

MDR-1 Pgp expression did not appear to be associated with any clinicopathological features in 46 patients studied. Preliminary analysis did suggest, however, that there is an association between MRP-1 Pgp expression at diagnosis and shortened relapse free survival and possibly overall survival in chemotherapy treated patients.

Bcl-2 expression at diagnosis appeared to be associated with increased relapse free survival time in chemotherapy treated patients. Survivin protein expression at diagnosis also appeared to be associated with a better outcome, tumours that were survivin positive at diagnosis appeared to show longer overall survival compared to survivin negative tumours. Nuclear survivin positivity, the relevance of which at this point could not be explained, was observed in a number of tumours samples.

MDR-3 expression did not appear to correlate with known patient and tumour characteristics; results suggested that expression of this protein did not appear to have any prognostic value as regards clinical outcome of patients.

Expression of any of the MDR associated proteins studied did not appear to be associated exposure to chemotherapeutic drugs. In addition there not appear to be any correlation between expression of any of these individual proteins and any other of the proteins studied.

3.2. Generation and characterisation of an MDR-3 specific MAb using a combined *in vitro/ in vivo* immunisation system

3.2.1. Background

At the time of commencement of this thesis no MDR-3 specific MAbs were commercially available. The majority of haematological and tissue studies that had been performed previously had focused on MDR-3 gene expression studies, MDR-3 encoded Pgp levels have not been investigated extensively. Excluding a small number of studies (discussed in section 1.7.5.), for the most part most part detection of the MDR-3 encoded gene product had relied on comparison studies with the C219 monoclonal antibody which recognises both the MDR-1 and the MDR-3/ mdr-2 encoded gene products (Georges *et al.,* 1990) and MDR-1 specific monoclonal antibodies such as JSB-1 and MRK16.

In order to generate a MAb that would specifically recognise the MDR-3 encoded gene product, a suitable immunisation peptide was chosen (section 2.6.2. and 2.6.3.). The entire amino acid sequence of the *MDR-3* encoded Pgp (deduced from the known cDNA sequence (Van Der Bliek *et al.* 1988)) was obtained from the EMBL Swiss-Prot protein sequence data bank above using the NETSERV program. From this deduced sequence, using the Mail FASTA programme, a 12 amino acid peptide was chosen which showed insignificant homology with the *MDR-1* encoded Pgp. This peptide corresponded to the intracellularly located amino acids 13-24 (RPTSAEGDFELG) of *MDR-3* encoded Pgp, which resides in the N-terminal part of the protein. FASTA similarity searches indicated that this peptide did not show homology with any other significant proteins e.g. other MDR proteins.

3.2.2. Fusion results

Following fusion using a combined in *vitro/ in vivo* immunisation regime employing an *in vitro* immunisation kit from Immune Systems, Bristol, UK (section 2.6.4.) a total of 384 hybridoma supernatants (100% fusion) were screened by ELISA . Supernatants from 48 clones which were positive for the peptide/ BSA complex alone were chosen for

further expansion. 11 of these hybridomas were studied in detail, based on reactivity patterns with control cell lines. Supernatant 6/1G was chosen for further study and was cloned by limiting dilution prior to further characterisation (section 2.6.9.).

3.2.3. Isotype Analysis of antibody 6/1G

Isotype was determined to be subclass IgM (section 2.6.10.) (Isostrip, Roche Diagnostics GmbH, 1493027).

3.2.4. Western Blotting studies of control cell lines

The MDR-3 transgenic murine ear fibroblast lines , VO1-VO1 and VIM #1 which were used as positive control cell lines, were derived from primary MDR-3 transgenic FVB mice expressing an MDR-3 minigene (Smith *et al.,* 1994). The control (parental) cell line, FVB#C was similarly made from wild type mice (Table 2.2) (Smith *et al.,* 1994). (These cell lines were obtained courtesy of Prof. P. Borst, The Netherlands). On Western blotting of cell lysates of Vim # 1 cells, antibody 6/1G reacted with a band at 170kDa. This band was only very faintly detected on preparations of parental FVB # c cells. A 170 kDa band was also detected on *MDR-3* transgenic V01-V01 cells (data not shown). A faint band was detected on cell lysates of the sensitive DLKP cell line and its adriamycin resistant variant, DLKPA. In contrast a distinct band on cell lysate preparations from DLKP-A cells which overexpress MDR-1 encoded Pgp can be seen with MAb 6/1C, which is specific for the MDR-1 gene product (Figure 3.2.1).

Previous results show lack of positivity of the *MDR-3* transgenic cell lines, VO1-VO1 and Vim # 1 with MAb, 6/1C (specific for the *MDR-1* encoded gene product) (Moran *et al.,* 1997). Taken together these results indicate that 6/1G detects the *MDR-3*, but not the *MDR-1* encoded protein.

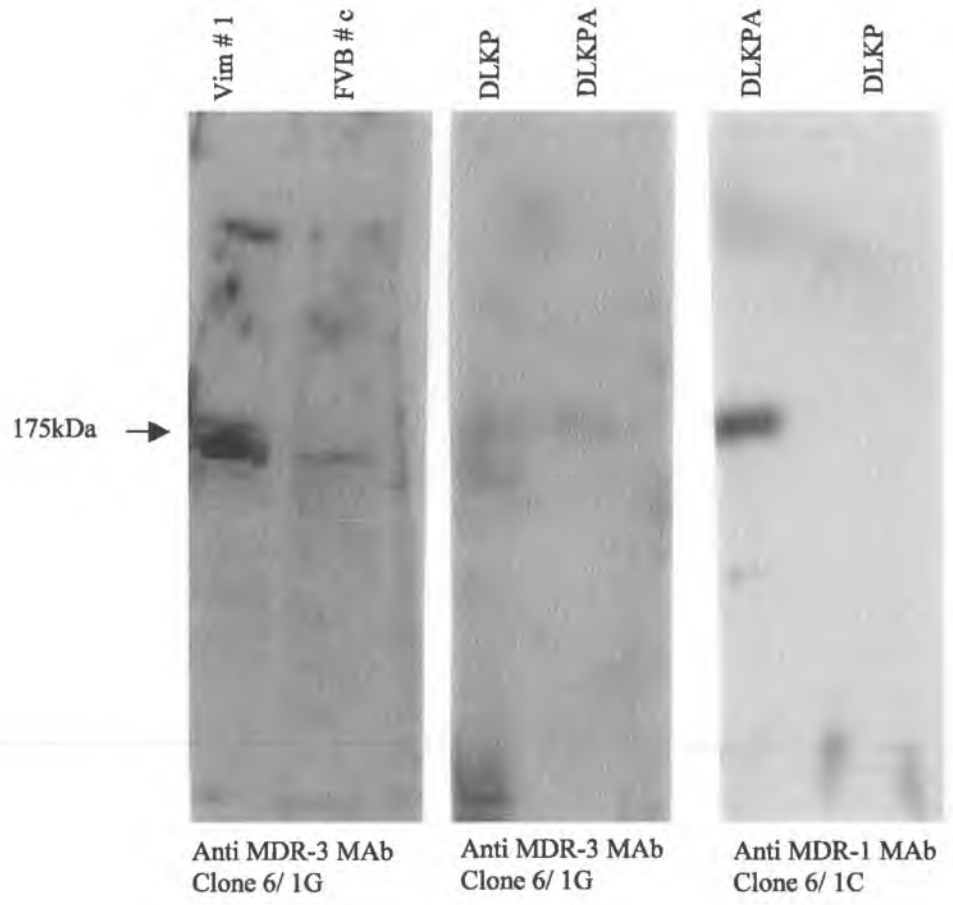


Figure 3.2.1. Western blot analysis of antibody 6/1G and the MDR-1 specific MAb 6/ 1C on the MDR-3 transgenic cell line Vim # 1, its parental cell line FVB # c, the multidrug resistant MDR-1 over expressing DLKPA cell line and its sensitive counterpart DLKP.

Immunoblot of crude cell lysates of the above cell lines which were separated on 7.5% SDS PAGE and probed with antibody 6/ 1G. Antibody 6/1G reacts with a band at 170kDa on Vim # 1 cells, a very faint band is detected on FVB # c cells. A faint band can also be observed on both the sensitive DLKP cell line and its adriamycin variant DLKPA. In contrast DLKPA cells which overexpress MDR-1 encoded Pgp react with a distinct band at 170 kDa when probed with the MDR-1 specific MAb 6/1C, no band is detected on the sensitive DLKP cell line.

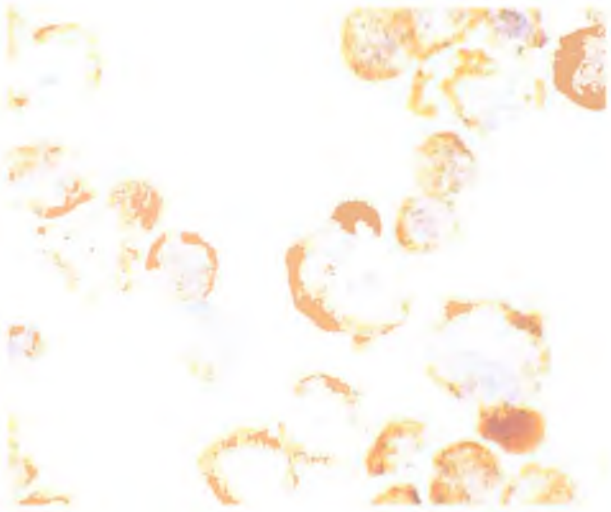
3.2.5. Immunofluoresence studies on live cells

Immunofluoresence studies on live cells (section 2.7.2.) revealed no detectable staining on the *MDR-3* transgenic cell line, Vim # 1 or its parental FVB # c cell line indicating that the epitope recognised by antibody 6/1G was not expressed on the cell surface of these cells .

3.2.6. Immunocytochemical studies of control cell lines

Immunocytochemical studies on cytospins of both Vim # 1 and VO1-VO1 *MDR-3* transgenic cell lines showed consistent cytoplasmic and plasma membranous staining with antibody 6/1G ; negligible staining was observed on parental FVB # c cells (Figure 3.2.2). The C219 MAb which detects both the *MDR-1* and the *MDR-3* gene products, gave a similar pattern of reactivity on VIM # 1 and VO1-VO1 cells (Figure 3.2.3).

Immunocytochemical analysis of resistant DLKP-A and sensitive DLKP cells showed equivalent low level staining with antibody 6/1G (Figure 3.2.3). In contrast previous results showed strong staining on DLKPA cells with negligible staining observed on DLKP cells with both the MDR-1 specific MAb, 6/1C and the widely used anti MDR-1/ MDR-3 gene product MAb, C219 (Heenan *et al.,* 1997, Moran *et al.,* 1997). Analysis of the ovarian carcinoma cell line, A2780 showed negligible reactivity with antibody 6/1G.



a

b

Figure 3.2.2. : Immunocytochemical analysis of antibody 6/1G on the MDR-3 transgenic cell line Vim # 1 and its parental cell line FVB # C.

(a) Vim # 1 cells showing intense cytoplasmic and plasma membrane positivity with antibody 6/1G (original magnification 60x). **(b)** FVB # C cells showing negligable reactivity with antibody 6/1G (original magnification 60x). (Strep ABC/ HRP method using DAB as chromogen, cells were counterstained with haematoxylin)

3.2.7. Immunohistochemical analysis of formalin fixed paraffin embedded tissues

Formalin fixed paraffin embedded normal liver tissue showed 6/1G positive staining in hepatocytes throughout the entire liver lobule with specific staining observed on canalicular membranes. This MDR-3 positive staining can be observed in Figure 3.2.3.

3.2.8. Western Blot analysis of a small panel of human, simian and rat cell lines of varying histological origin with antibody 6/1G.

A distinct band at 170kDa was observed with cell lysate preparations of the Buffalo rat liver cell line, BRL-3A and the human transitional carcinoma cell line, T24. A very faint band was visible at this molecular weight on the African green monkey kidney cell line, BSC-1 (Figure 3.2.4.).

3.2.9. Western blot analysis of normal murine tissues with antibody 6/1G.

Murine kidney and liver tissues preparations showed a single band at 170kDa on Western Blot analysis, when probed with antibody 6/1G (Personal communication, Dr. E. Moran).

3.2.10. Propagation of antibody 6/ 1G by ascitic tumour production

Following introduction of hybridoma cells into the peritoneal cavity of Balb/C mice which were previously primed with pristane (T 7640, Sigma), ascitic fluid was removed and centrifuged and the supernatant tested. Recovered ascites showed consistent reactivity on control cell line both by immunocytochemical and Western blot analysis. Ascitic fluid produced using hybridoma 6/1G grown in protein free medium showed weak immunoreactivity on positive control cell lines.

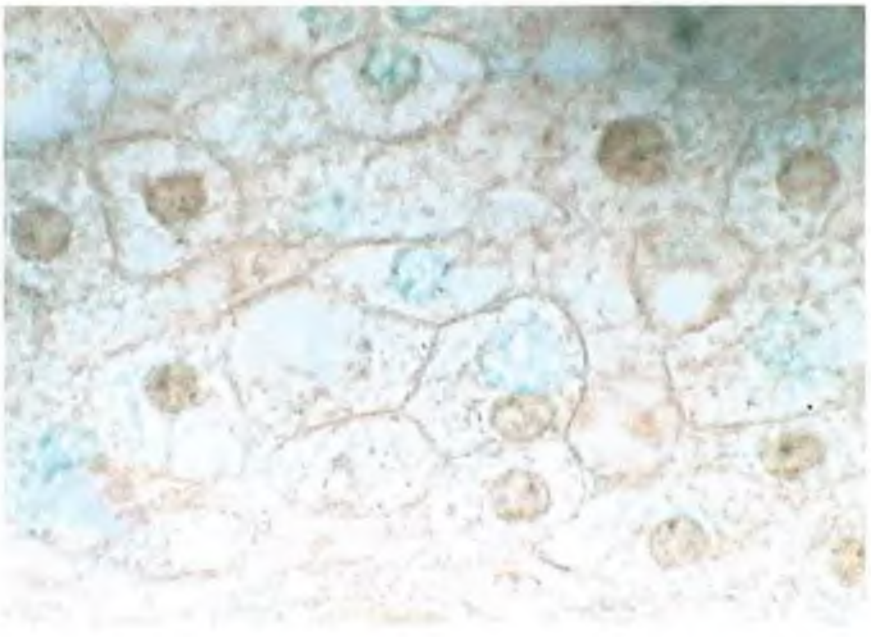


Figure 3.2.3. Immunohistochemical analysis of formalin fixed paraffin embedded human liver

Normal (post necrospy tissue) stained with antibody 6/1G. Diffuse MDR-3 specific staining can be observed throughout the whole liver lobule with intense positive staining localised on canalicular membranes (original magnification 100x) (Strep ABC method using DAB as chromogen, tissue was counterstained with methyl green).

Figure 3.2.4. Western Blot analysis of a small panel of cell lines of varying histological origin with antibody 6/1G.

Immunoblot of crude cell lysates of the MDR-3 transgenic Vim # 1 cell line, the Buffalo rat liver cell line, BRL-3A, the human bladder carcinoma cell line, T24, the human lung carcinoma cell line, DLKP and the African green monkey cell line, BSC-1 seperated on 7.5% SDS PAGE and probed with antibody 6/1G. A distinct band at 170 kDa can be seen in VIM #1, BRL-2A and T24 cells , a very faint band can be observed in DLKP and BSC-1 cells.

3.2.11. Purification of 6/ 1G ascites

Following purification of antibody 6/1G ascites using the Immune Pure IgM purification kit from Pierce, US (section 2.6.12.) recovered fractions of antibody when concentrated failed to show any immunoreactivity on MDR-3 transgenic Vim # 1 cells. Negligible staining was observed of formalin fixed paraffin embedded liver sections. The IgM concentration in purified samples was determined by Radial immunodiffusion (The Binding Site, UK) (section 2.6.11.) to be approx. 1.66 μg/ml .

3.2.12. Production and characteristion of antibody 6/1G in protein free medium (Gibco)

Harvested concentrated supernatants from antibody 6/1G grown in protein free hybridoma medium supplemented with glutamax 1 (Gibco) reacted with a distinct band at 170kDa on Vim # 1 cells; on parental FVB # C cells a very faint band was detected at 170 kDa (Figure 3.2.5.). Similarly consistent cytoplasmic and plasma membranous staining was observed on Vim # 1 cells when stained with protein free supernatants. Negligible reactivity was observed on parental FVB # C cells (Figure 3.2.6). The IgM concentration in these samples as determined by Radial immunodiffusion (The Binding Site , UK) (section 2.6.11.) was in the range of 10 - 15 μg/ ml.

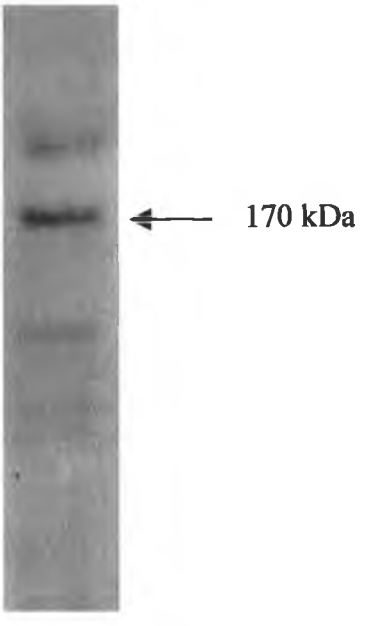


Figure 3.2.5.: Western blot analysis of antibody 6/1G grown in protein free medium (PFHM) (Gibco) on the MDR-3 transgenic cell line Vim # 1

Imunoblot of a crude cell lysate preparation of Vim # 1 cells which were separated on 7.5% SDS PAGE and probed with antibody 6/ 1G (concentrated PFHM supernatant diluted 1:20 in TBS/ 0.1% (v/v Tween 20). Antibody 6/1G reacts with a band at 170kDa on Vim # 1 cells.

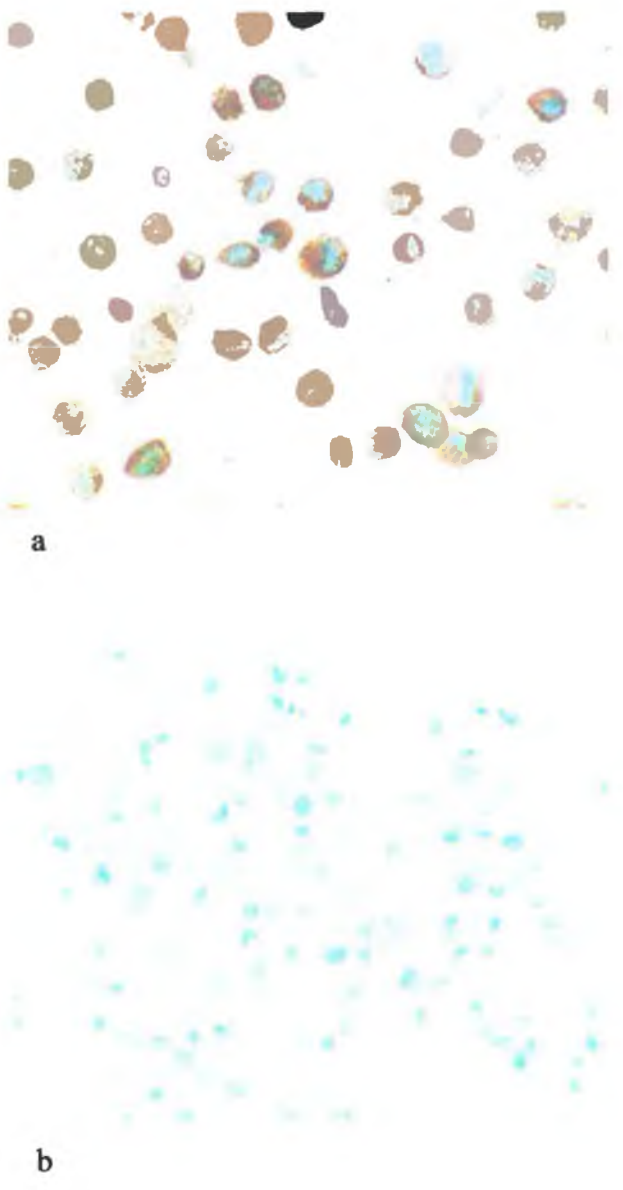


Figure 3.2.6. : Immunocytochemical analysis of antibody 6/1G grown in protein free medium (PFHM) (Gibco) on the MDR-3 transgenic cell line Vim # 1 and its parental cell line FVB # C.

(a) Vim # 1 cells showing intense cytoplasmic and membranous positivity with antibody 6/1G (PFHM supernatant) (original magnification 40x). **(b)** FVB # C cells showing negligible reactivity with antibody 6/1G (PFHM supernatant) (original magnification 40x). (Strep ABC/ HRP method using DAB as chromogen, cells were counterstained with methyl green).

3.2.13. Investigation of MDR-3 encoded Pgp expression in a panel of human tumour cell lines

A panel of human tumour cell lines of varying histological origin together with resistant variants of a number of these were probed with antibody 6/1C . Representative results from this survey can be observed in Figures 3.2.7. and 3.2.8.

CELL LINE	6/1G REACTIVITY
DLKP	+
DLKP SQ	+
DLKP Taxotere selection	+
DLKPA	+
DLKPA 5F	-ve *
DLKPA 2B	+
COR-L26-S	+
COR-L26-R	+
OAW42-S	+
OAW42-SR	+
A2780	+
BT-20	+
MDA-MB-435-S	+
RPMI parental	+/-
RPMI taxol	+/-
RPMI melphalan	+

Key: + detectable MDR-3 Pgp expression
+/- very low/ basal level of MDR-3 Pgp expression
* preliminary result

Table 3.2.1. ***Western Blot Analysis of a panel of human tumour cell lines with the MDR-3 specific MAb, 6/1G.***

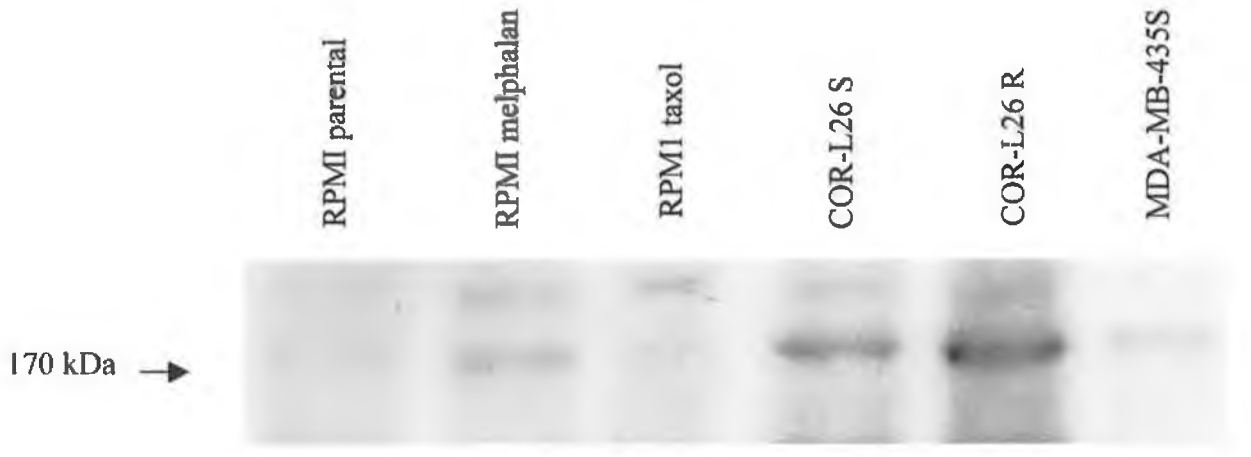


Figure 3.2.7. Western blot analysis of a selection of human tumour cell lines and their drug selected resistant variants with MDR-3 specific antibody 6/1 G (1)

Immunoblot of crude cell lysates of the human nasal carcinoma cell line RPMI 2650 and its melphalan and taxol selected variants (lanes 1-3), the large cell lung carcinoma cell line COR-L26S and its adriamycin selected variant COR-L26R(lanes 4-5) and the ductal breast carcinoma cell line, MDA-MB-435-S (lane 6) separated on 7.5% SDS PAGE and probed with antibody 6/ 1G (PFHM supernatant).

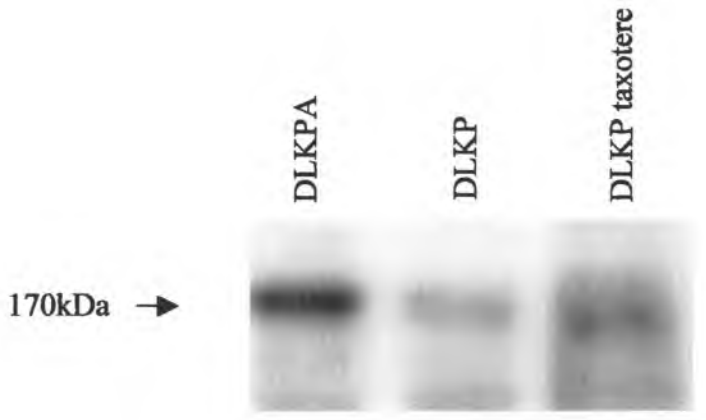


Figure 3.2.8. Western blot analysis of a selection of human tumour cell lines and their drug selected resistant variants with MDR-3 specific antibody 6/1G (2).

Immunoblot of crude cell lysates of the small cell carcinoma cell line DLKP (lane 2) and its adriamycin (lane 1) and taxotere (lane 3) selected variants separated on 7.5% SDS PAGE and probed with antibody 6/ 1G (PFHM supernatant).

3.2.14. Investigation of MDR-3 encoded Pgp expression in archival breast tumour samples

A number of invasive breast tumours investigated (11/15) for MDR-3 Pgp expression using antibody 6/1G appeared to have detectable MDR-3 Pgp expression (Table 3.2.2.). 14 of the patients received adjuvant chemotherapy, 1 patient received primary chemotherapy. A representative MDR-3 positive tumour can be observed in Figure 3.2.7.

Table 3.2.2.

Clinical features of tumours, treatment details of patients and immunohistochemical analysis of MDR-3 Pgp using MAb 6/1G in pre chemotherapy treatment archival tumour material from invasive breast cancer patients (n=14).

Key: **dod** = death of disease, **a&w** = alive and well, **rec** = recurrence of disease, **In du** = infiltrating ductal, **In lob** = infiltrating lobular, **DCIS** = ductal carcinoma *in situ* (comedo/ cribriform/ solid), **In tubular** = infiltrating tubular, **In municous** = infiltrating mucinous, **LCIS** = lobular carcinoma *in situ*, **tum size** = tumour size (cm), **grade** = histological grade, **ER** = estrogen receptor status (pos/neg), **Tam** = tamoxifen therapy, **RT** = radiation therapy (Y/N), **Node** = lymph node status (pos/neg), **chemo** = adjuvant chemotherapy, **CMF** = cyclophosphamide/ methotrexate/5-fluorauacil, **ACMF** = adriamycin/ cyclophosphamide/ methotrexate/ 5-fluorouracil, **meg**= megace, **VMF** = vinblastine, methotrexate, 5-fluouracil, **chlorb** = chlorambucil,.

Patient		Diagnosis	tum	grade	ER	Tam	RT	Node	Chemo	MDR-3
1	dod	R In Du, DCIS-com	5.5	3	neg	N	Y	pos	ACMFx12	3+3
2	a&w	L In Ca, NOS	2	3	neg			pos	CMF	2+2
3	a&w	L In Du	1.5		pos	Y	y	pos	CMFx9	3+3
4	a&w	R In Du	2.5		pos			pos	CMFx9	1+1
5	a&w	R In Ca		3		Y		pos	ACMF	3+3
6	rec	R In Ca	3	3	pos			pos	CMFd1d8x6	2+2
7	a&w	L In Ca, NOS	3	3	neg	N	N	pos	CMFx9	neg
8	rec	L in crib tub com	3	1				pos	Zoldalex	3+3
9	a&w	R In Du	8	3	pos			pos	CMFx9	3+3
0	a&w	L In Ca, NOS	3	3	pos	Y	Y	pos	CMFx9	neg
11	a&w	L Inf Ca		3			Y	pos	CMFx9	neg
12	a&w	L In Du, DCIS	1	3	?	N	Y	pos	CMFx9	2+2
13	dod	L In lobular LCIS	3.5	3	pos	Y	Y	pos	ACMFx12	2+2
14	a&w	R In Du	1.7		neg	N	Y	pos	CMFx9	3+3
15	a&w	L infil ca	1						Adr CMFpost*	neg
									*primary chem	

Table 3.2.2. Immunohistochemical analysis of MDR-3 Pgp in a panel of archival invasive breast tumours

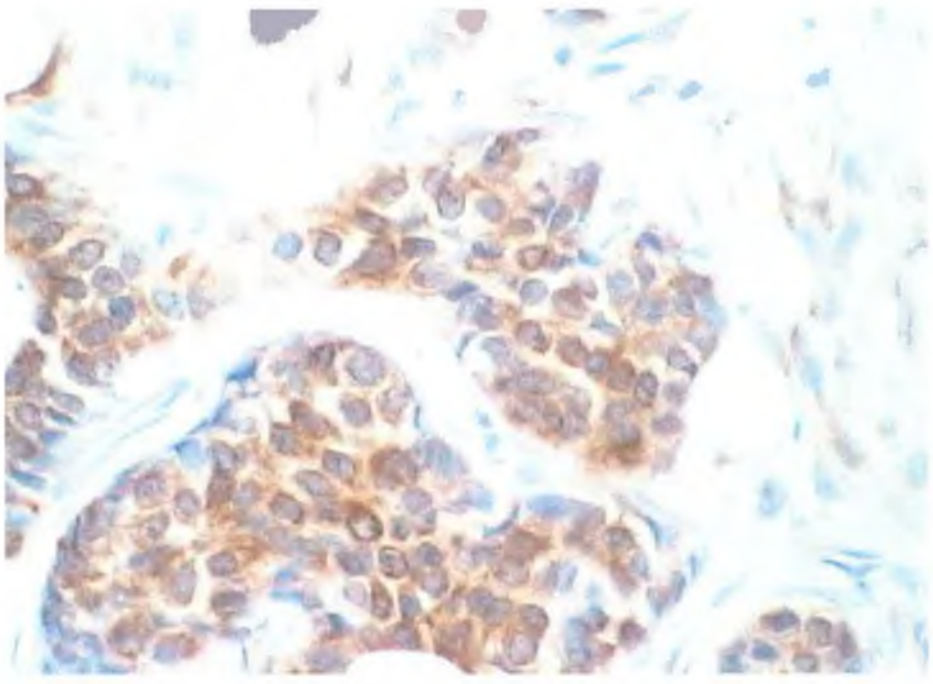


Figure 3.2.9. Immunohistochemical analysis of MDR-3 Pgp in archival breast tumours

Invasive tumour cells from an infiltrating breast carcinoma showing MDR-3 positive staining (*pre* treatment) (original magnification 40x) (Strep ABC method using DAB as chromogen, tissue was counterstained with haematoxylin).

3.2.15. Immunocytochemical analysis of MDR-1 and MDR-3 encoded Pgp expression in B-cell leukaemias

A small series of B-cell haematological malignancies were investigated for MDR-3 Pgp expression using this antibody; it has been indicated that *MDR-3* may be involved in drug resistance in certain B-cell lymphocytic leukaemias (Nooter *et al.*, 1990, Herweijer *et al.*, 1990). For comparison, we also examined MDR-1 Pgp expression levels using BRI MDR-1 MAb, 6/1C.

3.2.15.1 B-cell chronic lymphocytic leukemia (B-CLL)

Two cases of B-CLL (section 1.5.) studied showed MDR-1 positivity using BRI MAb, MDR-1(6/1C) in normal and malignant cell types. Anti MDR-3 MAb, 6/1G showed a differential pattern of staining in both cases of B-CLL with the larger malignant cells (more pleiomorphic, possibly associated with higher grade/ more malignant phenotype) showing more intense MDR-3 positivity than the smaller malignant cell, this staining pattern can be seen in Figure 3.2.10. Both these samples were from patients who had received prior treatment.

3.2.15.2 Acute myleoid leukaemia (AML)

Of three AML (section 1.5) cases included in this study a specimen from a patient presenting with AML, French American British (FAB) classification (see Table 1.1), M0 was negative for MDR-1 and MDR-3 protein expression. An AML, FAB M1 sample showed MDR-1 positivity but did not show any MDR-3 positivity. The third sample, FAB M5a exhibited weak MDR-1 positivity in monoblasts with very intense MDR-3 positivity (which was very granular in nature) also observed in these monoblasts (Figure 3.2.11.). None of these patients had received treatment.

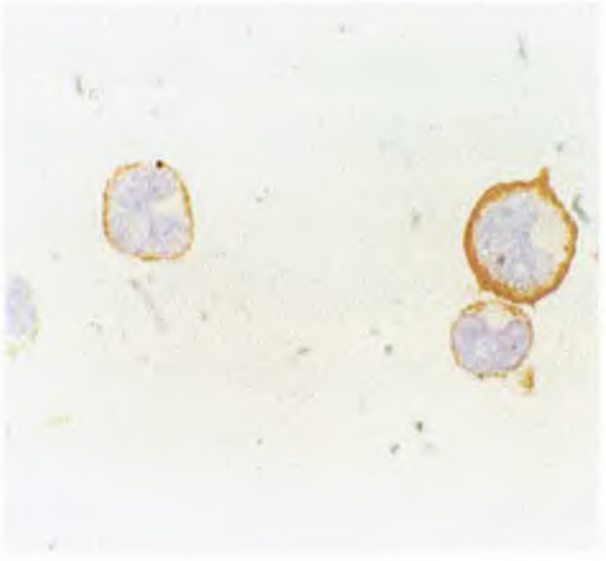


Figure 3.2.10. : Immunocytochemical analysis of MDR-3 and MDR-1 Pgp levels in B-CLL.

Peripheral blood from a B-CLL patient stained with antibody 6/1G, intense MDR-3 positive staining can be observed in the larger possibly more malignant cell type, smaller malignant cells show less positivity (original magnification 100x). (Strep ABC/HRP method using DAB as chromogen, cells were counterstained with haematoxylin).

Figure 3.2.11. : Immunocytochemical analysis of MDR-3 and MDR-1 Pgp levels in AML.

Peripheral blood from an AML stage M5a patient stained with antibody 6/1G showing MDR-3 positive staining in monoblasts (original magnification 40x). (Strep ABC/ HRP method using DAB as chromogen, cells were counterstained with haematoxylin)

3.2.15.3. B-cell non-Hodgkins Lymphoma (B-NHL)

A series of B-NHL (section 1.5.) cases investigated showed varying levels of MDR-3 and MDR-1 Pgp expression; no obvious trend in expression pattern was observed in this preliminary study. However within the particular sub groups of this disease (mantle cell lymphoma, follicle centre lymphoma) some differences in MDR-3/ MDR-1 Pgp expression were noted.

3.2.15.4. Follicle centre lymphoma:

In 3 out 4 cases of Follicle centre lymphoma investigated there appears to be co-expression of MDR-3 and MDR-1 Pgp. In samples from 2 of these patients the level of MDR-3 Pgp expression in malignant cells appeared to be higher than that of MDR-1 Pgp, this can be observed in Figure 3.2.13. where malignant cells show intense MDR-3 positive staining, only weak MDR-1 positivity can be observed. One case of follicle centre lymphoma (atypical CD5 +ve type) showed weak positivity for MDR-1 and did not show any MDR-3 positivity prior to treatment . At 16 days post treatment MDR-3 expression was increased significantly this can be observed in Figure 3.2.12. where intense MDR-3 positivity can be observed in malignant cells; MDR-1 positive staining also increased significantly but not to the same degree as MDR-3 Pgp. Further *post* treatment samples from this patient will need to be investigated.

3.2.15.5. Mantle cell lymphoma:

One case of mantle cell lymphoma showed MDR-3 positivity and was negative for MDR-1; a second case however showed a reversal of these results. Both of these patients had not received treatment. Two further cases of mantle cell lymphoma investigated showed co-expression of both MDR-1 and MDR-3 Pgp, although more intense positive staining was observed with both antibodies in case 1, which also showed very weak MRP-1 positivity. Negative MRP-3 and LRP protein expression was observed in case 2.

3.2.15.6. B-NHL (not further classified):

Peripheral blood samples from 2 B-NHL patients were negative for both MDR-3 and MDR-1 Pgp expression. Neither of these patients had received treatment.

3.2.15.7. T cell clonal disease of large granular lymphocytes (T-LGL):

One case of this very aggressive disease of clonal proliferation included in this study showed weak MDR-3 and MDR-1 expression. This patient had not been treated.

3.2.15.8. Chronic granulamatous leukaemia (CGL):

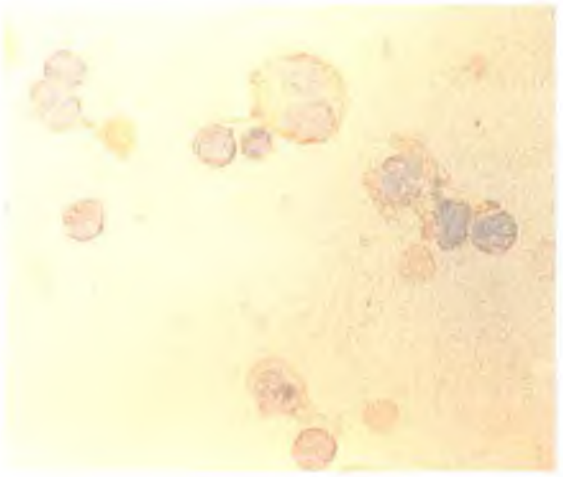
A bone marrow effusion from 1 CGL patient who had not been treated showed intense MDR-3 positive staining. MDR-1 Pgp results were inconclusive.

a

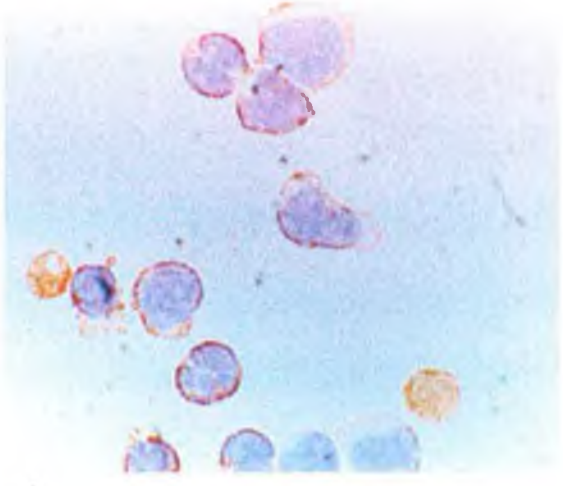
b

Figure 3.2.12. : Immunocytochemical analysis of MDR-3 and MDR-1 Pgp levels in B-NHL (1)

(a) Peripheral blood from a follicle centre lymphoma (atypical type, CD5 +ve) patient stained with antibody 6/1G showing negligible MDR-3 positivity at *pre* treatment stage (original magnification 40x). **(b)** Peripheral blood from same patient at post treatment stage showing intense MDR-3 positive staining (original magnification 60x). (Strep ABC/ HRP method using DAB as chromogen, cells were counterstained with haematoxylin)



a



b

Figure 3.2.13. : Immunocytochemical analysis of MDR-1 and MDR-1 Pgp levels in B-NHL (2).

(a) Pleural fluid from a follicle centre lymphoma patient (CD5 -ve) stained with the MDR-1 specific antibody, 6/1C, showing very weak MDR-1 positivity in some malignant cells (original magnification 60x). **(b)** Pleural fluid from the same patient stained with antibody 6/1G, MDR-3 positive staining can be observed in malignant cells (original magnification 60x). (Strep ABC/ HRP method using DAB as chromogen, cells were counterstained with haematoxolyin)

PATIENT	DIAGNOSIS	MDR-1 EXPRESSION	MDR-3 EXPRESSION
1	B-CLL * PB	+	+ intense MDR-3 positivity associated with larger, possibly more malignant cells
2	B-CLL * PB	+	+ intense MDR-3 positivity associated with larger, possibly more malignant cells
3	? mantle cell PB	+	–
4	? mantle cell PB	–	+
5	Follicle centre PB	+	+ > positive than MDR-1
6	Follicle centre * BM	–	–
7	Follicle centre PF	- / + (v.weak)	+ (weak - moderate)
8	Follicle centre * PB PB PF	- / + + weak -	- + -
9	B-NHL PB	-	- /+
10	B-NHL PB	-	-
11	PL/ CGL BM	?	-
12	T-LGL * PB	+ weak	+ weak
13	AML M0 PB	-	-
14	AML M1 PB	+	- / +
15	AML M5 PB	- / +	+

Table 3.2.3. : Immunocytochemical analysis of MDR-3 and MDR-1 protein expression levels in B-cell leukaemias using antibody 6/1G and the MDR-1 specific MAB, 6/1C.

Key:

*	patients who received treatment
B-CLL	B cell chronic lymphocytic leukaemia
B-NHL	B cell non Hodgkins lymphoma
T-LGL	clonal disease of large granular lymphocytes (T cell origin)
CGL	Chronic granulamatous leukaemia
PB	peripheral blood sample
BM	bone marrow sample
PF	pleural fluid sample

3.2.16. Conclusion

Antibody 6/1C which specifically recognises the MDR-3 encoded gene product was successfully generated by means of a combination of *in vitro* and *in vivo* immunisation regimes and characterised by immunocytochemical and western blotting. Expression of MDR-3 using this antibody was investigated in a series of 13 invasive breast cancer patients, revealing that MDR-3 Pgp was expressed in 73.3% of tumours studied. There did not appear to be any association between this MDR-3 expression at diagnosis and patient out come, exposure to chemotherapeutic drugs or any clinical-pathological features. Investigation of MDR-3 Pgp expression in a very small number of haematological malignancies suggested that this Pgp may possibly be associated with a more malignant phenotype in B-CLL and also may possibly be associated with certain B cell developmental stages.

3.3. Generation and characterisation of a novel MAb raised to formalin fixed paraffin embedded infiltrating breast ductal tumour

3.3.1. Background to this work

A novel production system to generate monoclonal antibodies has been developed in this laboratory using dewaxed formalin fixed paraffin embedded archival material as a source of immunogen. Novel monoclonal antibodies raised to dewaxed formalin fixed paraffin embedded normal kidney tissue have been successfully generated and one of these antibodies, 1/11C has been partially characterised (Moran *et al.,* 1998). The long term aim in developing such a system was to facilitate the isolation of novel antigens which may be overexpressed or indeed underexpressed in breast cancer; this malignancy is of particular interest to this research group. We have access to a large archival tumour tissue base at St. Vincents Hospital Dublin which enables analysis of serial samples from individual patients, thus providing us with an extensive range of breast tumours at different stages of treatment to use as possible sources of immunogens. It was decided therefore to consider a series of *pre* and *post* treatment samples from relapsed breast cancer patients which were being investigated for MDR protein levels (discussed in detail in section 3.1.) as possible immunogens for generation of novel monoclonal antibodies.

3.3.2. Choice of immunogen

Pgp expression levels and MRP protein expression levels in a *small* number of patients (due to time constraints of immunisation schedule) were taken into consideration. Other parameters were also taken into account in the final choice of immunogen such as availability/ number of tissue blocks. A *pre* treatment infiltrating ductal tumour tissue block was chosen as the immunogen. This patient, like the majority in the archival study, was diagnosed with a high grade infiltrating ductal carcinoma of the breast extensive DCIS, comedo type. Following treatment this patient relapsed. The tumour chosen showed low level Pgp positivity at diagnosis, following treatment this expression level was not significantly altered. Small groups of tumour cells in *pre* treatment block showed weak MRP-R1 positivity, no MRP positive staining was observed at *post* treatment stage. It was likely therefore that

neither MDR-1/ Pgp or MRP was responsible for the resistance exhibited by this particular patient, and that overexpression of some other protein maybe be involved.

By using this tumour as an immunogen and screening against *pre* and *post* archival tumour material, it was anticipated that MAbs recognising a novel antigen associated with resistance in breast cancer might be generated. Also, by investigating a larger tissue base which would include normal breast, benign and other breast tumour types the possibility of the antigens recognised by these monoclonal antibodies being associated with a particular cell type in breast cancer could be elucidated.

3.3.3. Fusion results

The protein concentration of the immunogen (i.e. 10 x 10 μm dewaxed sections as described in section 2.6.3.) was determined to be 600 μg/ ml, which was divided equally between two Balb/c mice. A primary immunisation in Freund's complete adjuvant, FCA and 2 boosters in Freund's incomplete adjuvant, FIA at 2 weekly intervals were carried out using freshly dewaxed tissue in each occasion; a final booster in Freunds incomplete adjuvant, FIA was administered 3 days prior to fusion. As was observed with generation of kidney monoclonal antibody (Moran *et al.,* 1998) following excision from mouse, the spleen did not have a healthy appearance as is the case following immunisations with various protein immunogens used routinely in this laboratory The spleen had a darkened haemorragic appearance and looked atrophied. 8 x 24 well plates were used to plate out fused cells.

Following fusion 375 clones, (97% efficiency) were screened by immunohistochemistry on tissue sections from *pre* treatment control tumour block. 36 of these supernatants (10% of total) were considered positive (i.e. they produced specific staining of tumour cells with minimal background) and were rescreened, 16 representative clones (the majority of clones appeared to stain tumour cells in an identical fashion) were screened for a third time. Finally based on reactivity patterns which can be seen in Figure 3.3.1. (i.e. intense cytoplasmic staining) of tumour cells 7 positive clones were chosen for further expansion. Following repeated screening on 3 different *pre* treatment blocks from this patient antibodies 5C3 and 2D5 were

further characterised. When primary antibody was omitted and replaced with either TBS or control mouse IgG, no staining was observed at any stage.

3.3.4. Antibody 5C3 and 2D5 reactivity patterns on control *pre* treatment tumour

Intense cytoplasmic staining of tumour cells was observed on all 3 *pre* treatment tumour blocks with both supernatants. When viewed at 100x magnification this positive staining appeared to be radiating towards the plasma membrane in a cytokeratin like fashion. Within tumour ducts some cells were more intensely stained than others; in some cases tumour cells at the edge of the tumour duct appeared to have more intense positivity than those in centre of ducts. In one *pre* treatment tumour section there was very intense staining of individual tumour cells which appear to be almost in rows (this may represent tumour cells invading lymphatic channels i.e. desmoplasmic stromal reaction which is present according to the histopathology report); this can be observed in Figure 3.3.2. Some low level staining of normal breast ducts was also observed; endothelial cells and muscle layers of some vessels also appear to have some positivity. Preliminary results indicate that antibody 5C3 appears to react weakly on *post* treatment material from this patient.

3.3.5. Isotyping of 5C3 antibody

Isotype was determined to be IgG_1 subclass, (Isostrip, Isostrip, Roche Diagnostics GmbH, 1493027) (section 2.6.10.).

3.3.6. Immunohistochemical analysis of antibody 5C3 on formalin fixed paraffin embedded *pre* and *post* treatment breast cancer tumours

Antibody 5C3 reacted with a number of *pre* and *post* treatment tumours screened. There was considerable heterogeneity in the staining pattern observed. In a number of *pre* and *post* treatment tumours staining with antibody 5C3 showed very intense positivity that was associated with certain infiltrating tumour cells (similar in staining intensity to that observed in invading cells in control *pre* treatment tumour Figure

3.3.2); while weaker reactivity was associated in some cases with non invasive tumour cells i.e. DCIS tumour cells which are often seen along with invasive ductal tumours. An example of this staining pattern can be seen in Figure 3.3.3. A number of *pre* and *post* tumours showed negative reactivity. In all positive tumours screened staining of some normal breast tissue was observed. This antibody also appeared to cross-react with some endothelial cells, muscle and some stromal tissue which may be due to the unpurified nature of the supernatant.

3.3.7. Immunohistochemical analysis of formalin fixed paraffin embedded non-cancer breast tissue

A number of non-cancer breast tissues were investigated for antibody 5C3 reactivity. There appeared to be some degree of reactivity with antibody 5C3 on all of the tissues investigated. The staining intensity however, was less than that observed in tumour cells from patients with infiltrating breast carcinomas.

3.3.8. Immunohistochemical analysis of formalin fixed paraffin embedded normal adult tissues.

A small panel of *available* formalin fixed paraffin embedded tissues were investigated for antibody 5C3 reactivity.

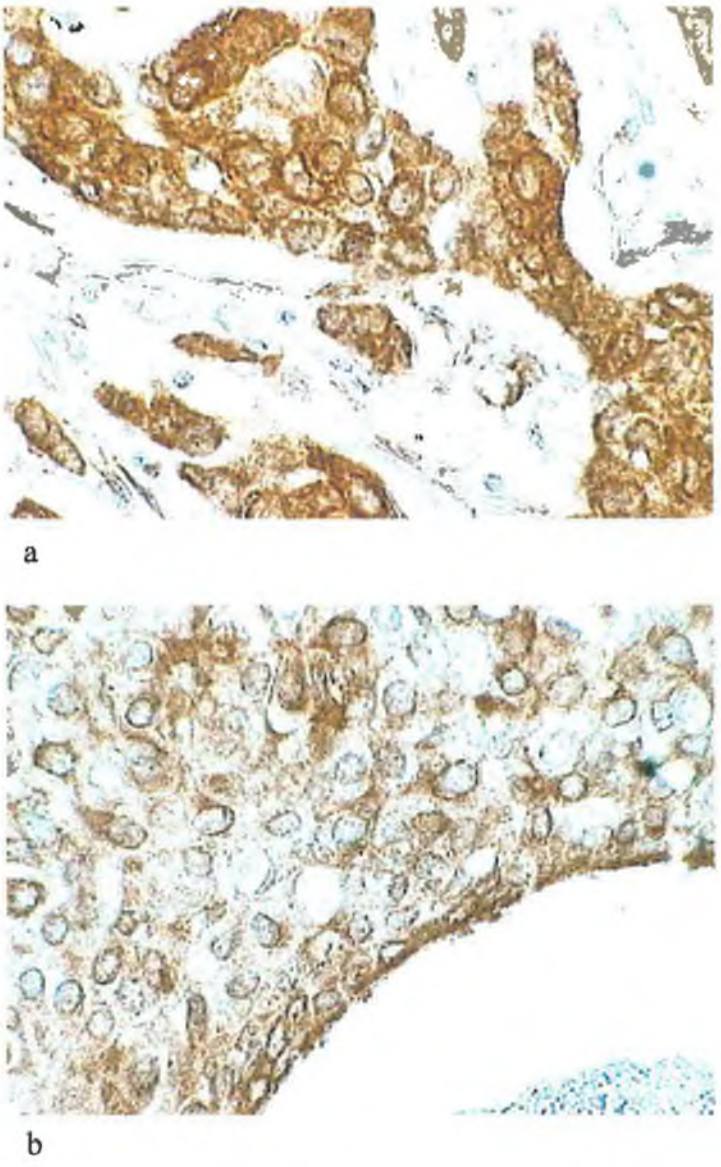
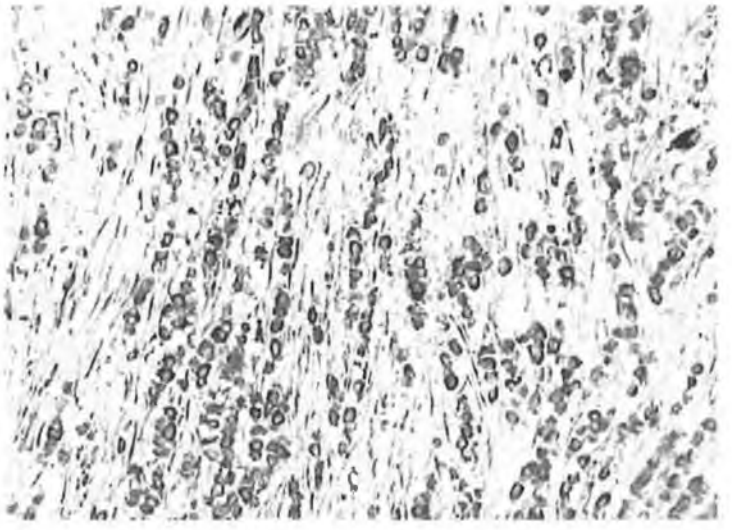


Figure 3.3.1. *: Pre* treatment ductal breast tumour which was used as immunogen stained with supernatant 5C3. This staining pattern was representative of all 7 positive clones which were expanded following a third screening.

(a) Intense cytoplasmic positivity which is granular in nature can be observed in infiltrating tumour cells (original magnification 100x). (b) The DCIS component (comedo type) also shows cytoplasmic positivity in tumour cells however this staining is not as intense as that observed in infiltrating tumour cells; the centre of tumour which has undergone necrosis is negative (original magnification 60x) (StrepABC/ HRP method using DAB as chromogen, sections were counterstained with hematoxylin).

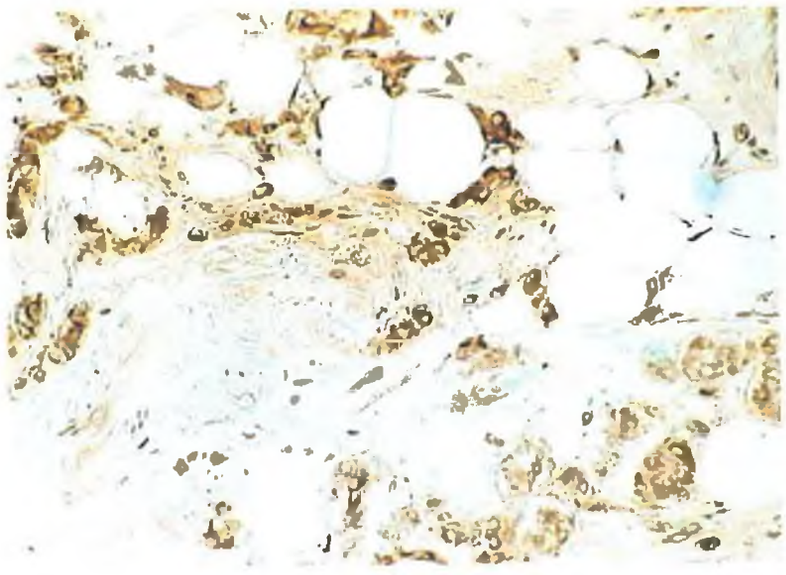


a

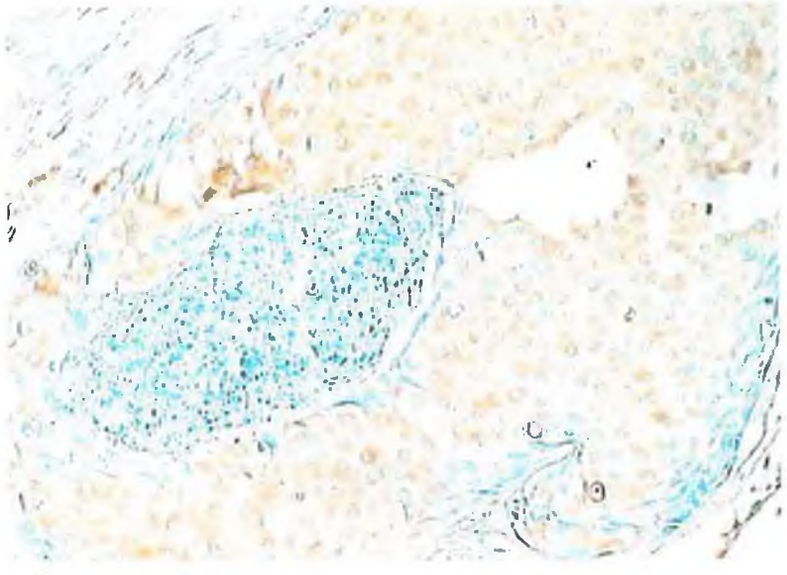
b

Figure 3.3.2. : *Pre* treatment ductal breast tumour block which was used as immunogen stained with antibody 5C3 and negative control section stained with TBS.

(a) Intense cytoplasmic staining of individual infiltrating tumour cells which appear to be organised in rows can be observed in this control tumour section stained with supernatant 5C3, this may represent tumour cells invading lymphatic channels (i.e. desmoplasmic stromal reaction) (original magnification 20x). (b) No staining can be observed in negative control section (original magnification 20x).
(strepABC/ HRP method using DAB as chromogen, sections were counterstained with haematoxylin).



a



b

Figure 3.3.3.: Immunohistochemical analysis of *pre* and *post* treatment breast tumours with antibody 5C3 (1).

(a) *Post* treatment intraductal tumour showing intense 5C3 positive staining in infiltrating tumour cells. This tumour showed a differential pattern of staining with antibody 5C3 (original magnification 20x). The 5C3 positivity observed ranged from very intense staining of infiltrating tumour cells to low level 5C3 positive staining in some DCIS cells, this can be observed in the DCIS (comedo type) tumour in photomicrograph (b) (original magnification 20x). StrepABC/ HRP method using DAB as chromogen, sections were counterstained with methyl green.

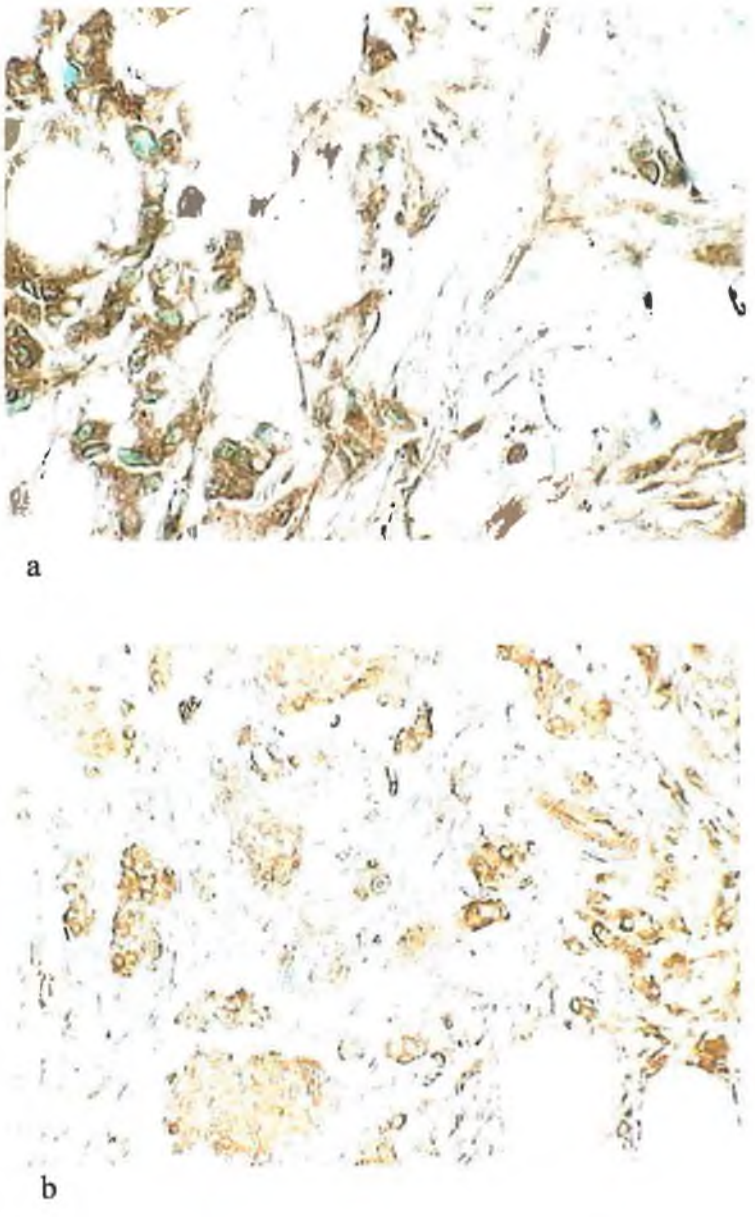


Figure 3.3.4. : Immunohistochemical analysis *pre* and *post* treatment breast tumours with antibody 5C3 (2).

(a) *Pre* treatment infiltrating tumour cells showing intense staining with antibody 5C3, particularly strong reactivity was observed around lipid. Weaker 5C3 positivity was observed in DCIS tumour cells (original magnification 40x). (b) *Pre* treatment tumour from another ductal carcinoma patient again showing strong reactivity in infiltrating tumour cells with antibody 5C3 (original magnification 20x). Strep ABC/ HRP method using DAB as chromogen, sections were counterstained with methyl green).

3.3.9. Immunocytochemical analysis of a panel of cell lines for antibody 5C3 reactivity

A panel of cell lines as outlined in Table 3.3.1. was investigated using immunocytochemistry for 5C3 expression. Representative cells stained with antibody 5C3 can be observed in Figures 3.3.5. and 3.3.6. In all cases cytoplasmic positivity was observed which was similar to the staining observed in individual tumour cells in formalin fixed paraffin embedded tissue sections. All of the ductal breast tumour cell lines, ZR-75-1, MDA-MB-231, MDA-MB-435-S and BT-20 exhibited intense 5C3 positivity, a reduced level of staining was observed in the non-tumourogenic cell line, MCF-10A. Ovarian, lung and leukaemia tumour cell lines also showed varying degrees of 5C3 reactivity, the sensitive non-small cell lung tumour cell line, DLKP and the ovarian adenocarcinoma sensitive and spontaneously resistant variants OAW42-S and OAW42-SR appeared to show weaker 5C3 reactivity than their MDR variants. The MDR nasal carcinoma cell line, RPMI (melphalan selected) did not show any 5C3 positivity. Some very weak staining was observed on negative control slides (stained with control mouse IgG) of some cell lines.

CELL LINE	HISTOPATHOLOGY	5C3 REACTIVITY
ZR-75-1	Breast ductal carcinoma	++
BT-20	Breast ductal carcinoma	++
MDA-MB-231	Breast ductal carcinoma	++
MDA-MB-435-S	Breast ductal carcinoma	++
MCF-10A	Breast non-tumourogenic	-/+
A2780	Ovarian MDR variant	++
OAW42-A1	Ovarian MDR variant	++
OAW42-A	Ovarian MDR variant	++
OAW42-SR	Ovarian spontaneously resistant variant	+
OAW42-S	Ovarian sensitive variant	+
DLKPA	Lung non-small cell MDR variant	++
DLKP	Lung non-small cell sensitive	+
HL-60	Leukaemia	++
RPMI (melphalan)	Nasal carcinoma MDR variant	-

TABLE 3.3.1. : Immunocytochemical analysis of a selection of ductal breast carcinoma cell lines, a non-tumourogenic breast cell line and tumour cell lines of various histological types for antibody 5C3 reactivity.

++ intense 5C3 reactivity
+ low level 5C3 reactivity
-/+ very low level 5C3 reactivity
- negative 5C3 reactivity

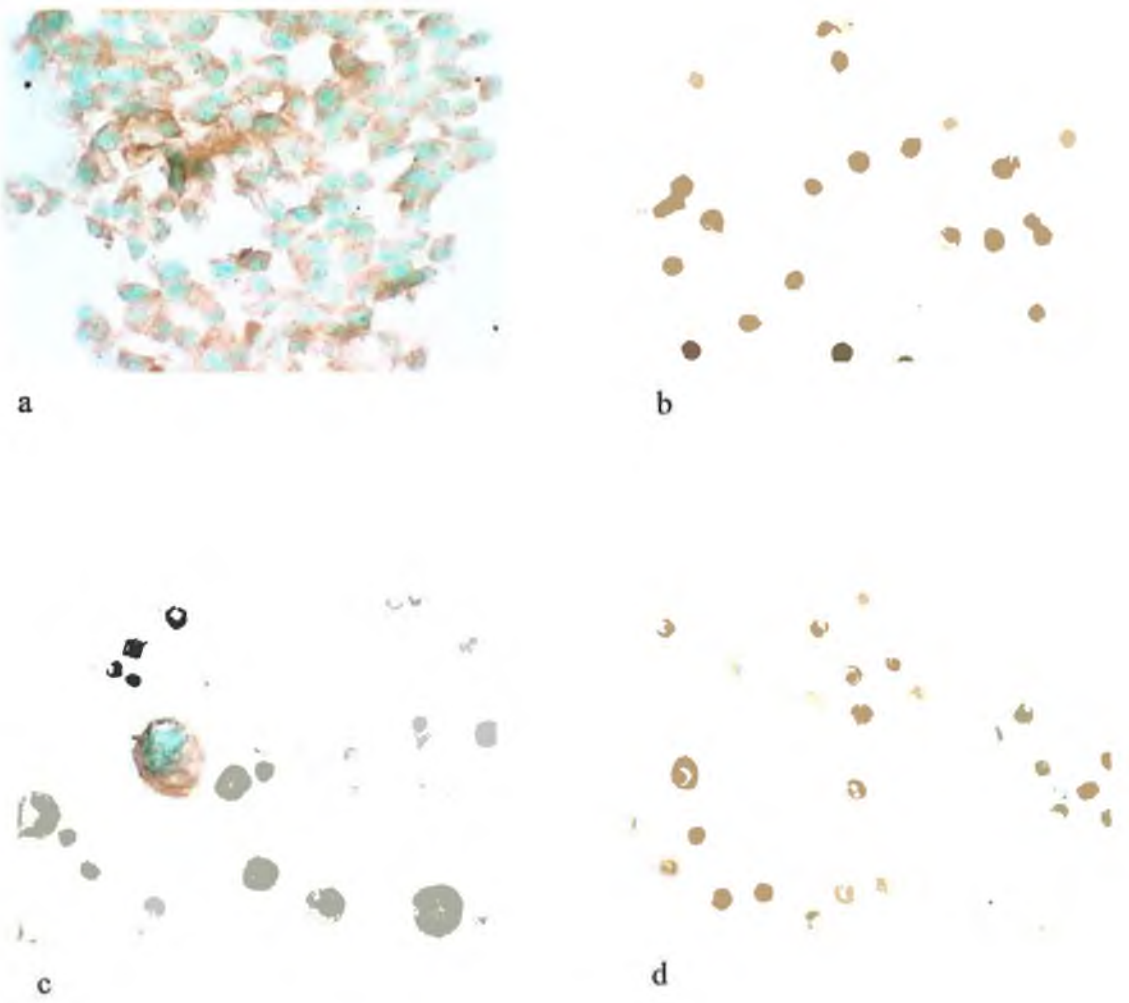


Figure 3.3.5. :Immunocytochemical analysis of ZR-75-1 ductal carcinoma, BT-20 adenocarcinoma, MDA-MB-231 ductal carcinoma and MDA-MB-435-S ductal carcinoma breast cell lines with antibody 5C3.

(a) ZR-75-1 (b) BT-20 (c) MDA-MB-231 and (d) MDA-MB-435-S breast cell lines all showing cytoplasmic reactivity with antibody 5C3. (original magnification of all photomicrographs 40x). (Strep ABC/HRP method using DAB as chromogen, cells were counterstained with methyl green)

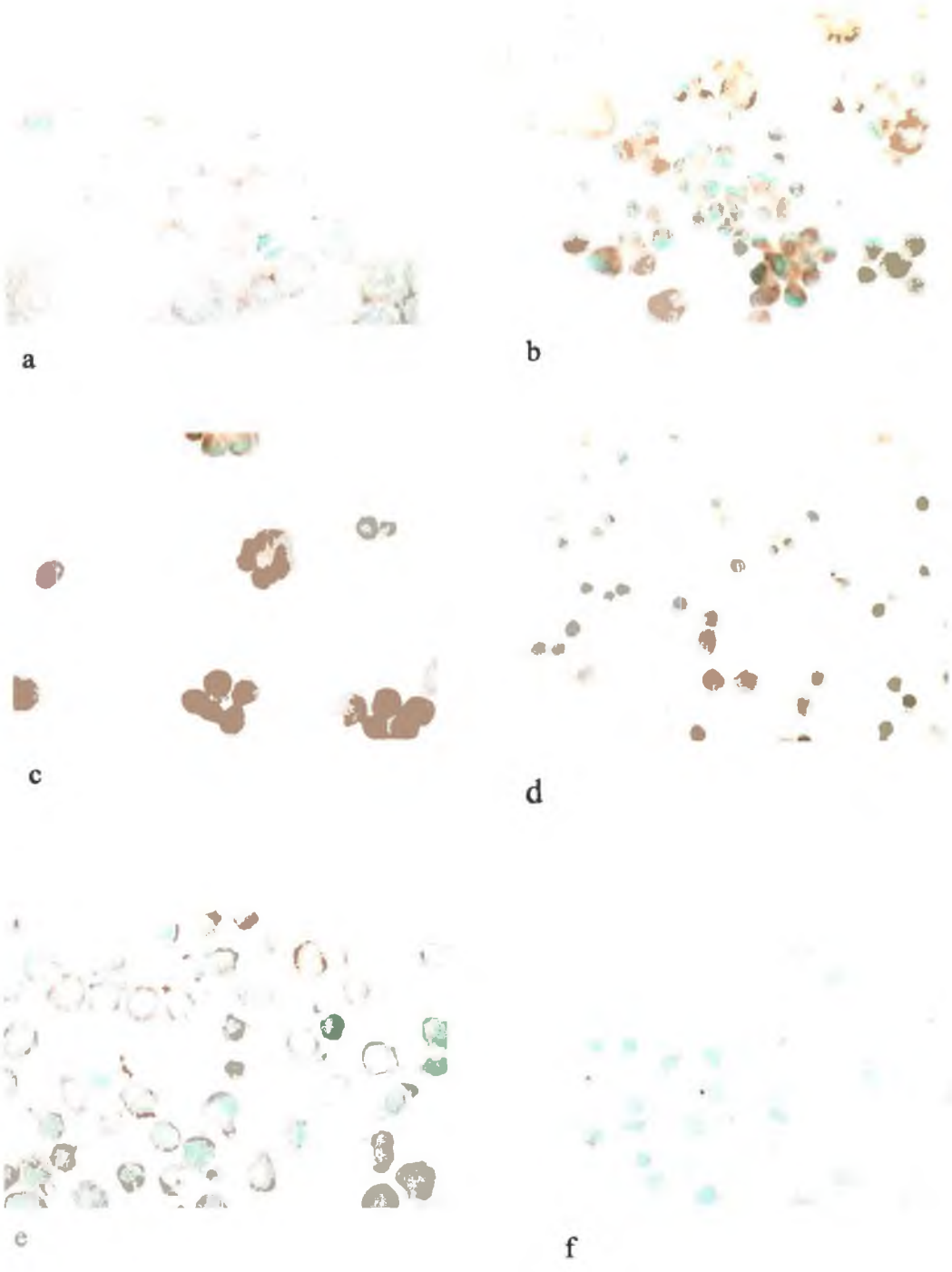


Figure 3.3.6. : Immunocytochemical analysis of OAW42SR spontaneously resistant ovarian carcinoma and resistant variants, OAW42A and OAW42A, A2780 ovarian carcinoma, DLKPA non small cell carcinoma resistant variant and RPMI melphalan resistant nasal carcinoma cell lines with antibody 5C3.

(a) OAW42-SR showing 5C3 positivity.(b) OAW42-A and (c) OAW42-A1 cells showing intense 5C3 positivity. (d) A2780 and (e) DLKPA cells showing intense cytoplasmic 5C3 positivity. (f) RPMI (melphalan) cells showing negligible 5C3 positivity (original magnification of all photomicrographs 40x). (Strep ABC/ HRP method using DAB as chromogen, cells were counterstained with methyl green).

3.3.10. Western blot analysis of a panel of cell lines for antibody 5C3 reactivity

Western blotting analysis was carried out on a selection of ductal breast carcinoma cell lines in order to determine the molecular weight of the antigen reactive with antibody 5C3. In addition, a range of tumour cell lines of various histological origins and MDR status as well as a non-tumourogenic breast cell line were probed with antibody 5C3. Immunoblotting with antibody 5C3 of the ductal breast carcinoma cell lines, ZR-75-1, BT-20, MDA-MB-231 and MDA-MB435-S and the breast carcinoma cell line, MCF-7 identified a series of bands at approx. 210kDa, 150kDa, 95kDa, and 50kDa on crude cell lysates of all of these tumour cell lines. However the relative expression of each these 5C3 reactive bands appeared to vary among some cell lines and individual western blot analysis a representative immunoblot can be seen in Figures 3.3.7. A very distinct band at higher than 210kDa was identified on the ductal carcinoma cell line ZR-75-1. This band was at a slightly lower molecular weight in all other cell lines probed with antibody. In addition this 210kDa band was consistantly detected on all immunoblots regardless of the relative expression of the lower bands.

Immunoblotting of ovarian and colon tumour cell lines revealed similar bands being identified, each with varying relative expression. The 210kDa band was not detected as strongly in these cell lines as was observed in some of the breast tumour cell lines.

3.3.11. Western blot analysis of normal murine tissues for antibody 5C3 reactivity

A range of purified normal murine tissues were investigated for antibody 5C3 reactivity. In contrast to the panel of cell lines investigated probing of these tissues with antibody 5C3 did not reveal bands of similar molecular weights to those seen in the cells lines. The major band identified in these tissues was at approx. 70kDa (specifically spleen and lung), a 50kDa band was identified in all tissues (Figure 3.3.7.). The 70kDa band was not identified in *any* of the cell lines investigated to date.

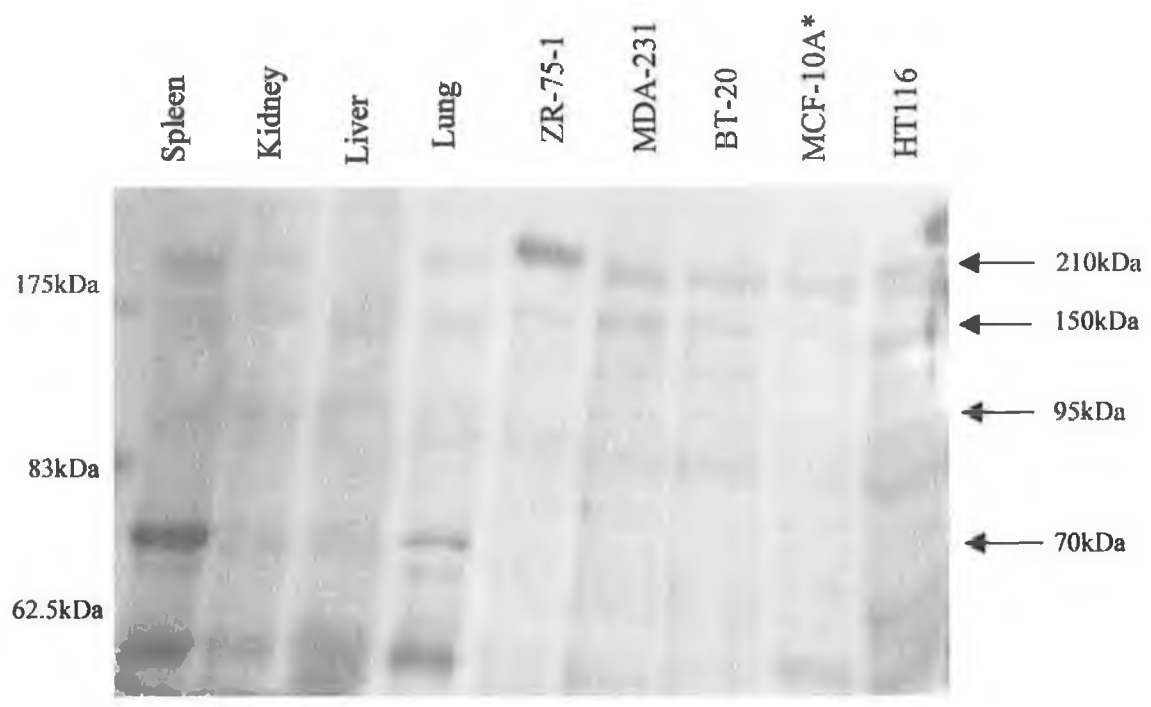


Figure 3.3.7. Western blot analysis of murine spleen, kidney, liver and lung tissues, ductal breast carcinoma cell lines, ZR-75-1, MDA-MB-231 and BT-20, the non-tumourogenic breast cell line, MCF-10A and the colon carcinoma cell line HT116 with antibody 5C3.

Immunoblot of partially purified normal murine tissues and crude cell lysates of the above cell lines which were separated on 6% SDS PAGE and probed with antibody 5C3. Antibody 5C3 appears to recognise a series of bands at 210kDa, 150kDa, 95kDa in all cell lines. These bands are only weakly detected in murine tissues. In contrast a distinct band at approx 70kDa can be seen in spleen and to a lesser degree in lung; a very faint band is detected in kidney and liver. A 50kDa band is detected in all tissue preparations. (20μg protein per lane loaded * MCF-10 only 10 μg protein loaded)

3.3.12. Immunoprecipitation studies

Due to its consistent reactivity with antibody 5C3, the ductal breast carcinoma cell line, ZR-75-1 was thus chosen for immunoprecipitation studies with antibody 5C3. Immunoprecipitation was carried out using a cocktail of protein L and protein G to pull out the 5C3 reactive antigen from crude cell lysates of ZR-75-1 cells. Following separation on 7.5% SDS Page, western blot analysis of the ZR-75-1 immunoprecipitates revealed a band at approx. 175 kDa (Figure 3.3.8) when probed with antibody 5C3 (the predominant band detected by western blotting of ZR-75-1 lysates was at approx. 210 kDa). No band was detected in immunoprecipitates of mouse IgG. Remaining supernatant following incubation of antibody/ lysate with protein L/ protein G was also added to reducing/ non reducing loading buffer and separated on SDS-PAGE. Western blot analysis of reduced supernatants revealed again multiple bands that were observed on ZR-75-1 cell lysate probed with antibody 5C3; the 175kDa band was also detected (Figure 3.3.9). Western Blot analysis of a 1:1 mixture of remaining supernatant and ZR-75-1 immunoprecipitates revealed again a series of bands including the 175kDa (Figure 3.3.9). When non reduced samples of ZR-75-1 immunoprecipitates, remaining supernatant and 1: 1 mixtures of these were separated on SDS-PAGE and probed with antibody 5C3, no distinct bands could be observed (Figure 3.3.9.).

3.3.13. N-Terminal sequence analysis

Further ZR-75-1 immunoprecipitates were transferred to PVDF (Sequiblot, Biorad) and stained with Coomassie Blue R-250, the major band at approx. 175 kDa corresponding to that observed on Western blots of immunoprecipitated cells, was excised from PVDF membrane and processed for N-terminal sequencing (Dr. Kevin Howland, University of Kent, UK) (section 2.9.2.). However results from sequencing revealed that the protein was N-terminally blocked. It was attempted to overcome this by the addition of 0.1% thioglycolic acid to gels, however gels did not resolve correctly. Internal sequencing involves sequencing of amino acid sequences of a protein other that its N or C terminal sequences. This is carried out by N-terminal

sequence analysis of a peptide after digestion and purification from a protein digest. It was decided to proceed with internal sequencing analysis.

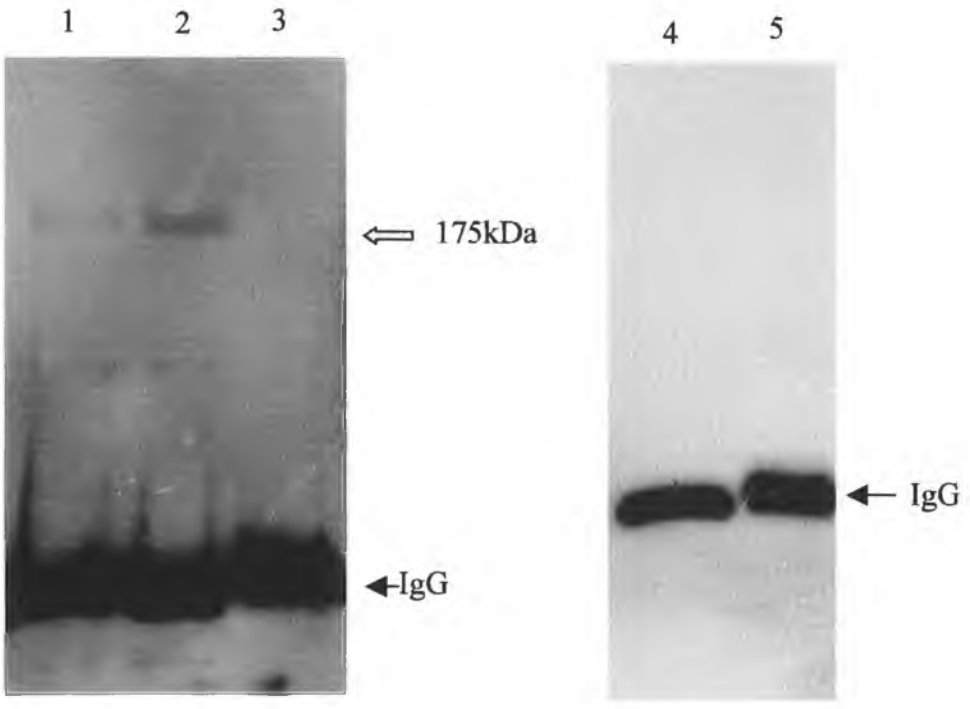


Figure 3.3.8. : Western blot analysis of immunoprecipitates of ZR-75-1 cells probed with antibody 5C3.

Immunoprecipitates of ZR-75-1 ductal breast carcinoma cells (lane 1: 0.5ml crude cell lysate/ 50 µl protein G and protein L, lane 2 : 0.25ml crude cell lysate/ 50 µl protein G and protein L) and control mouse IgG (lane 3) separated on 7.5% SDS PAGE and probed with MAb, 5C3. A band at approx. 175 kDa is detected in ZR-75-1 precipitates, no band is detected in control mouse IgG immunoprecipitate. IgG bands only are detected when primary antibody is omitted (lanes 4: ZR-75-1 immunoprecipitate, lane 5: IgG immunoprecipitate).

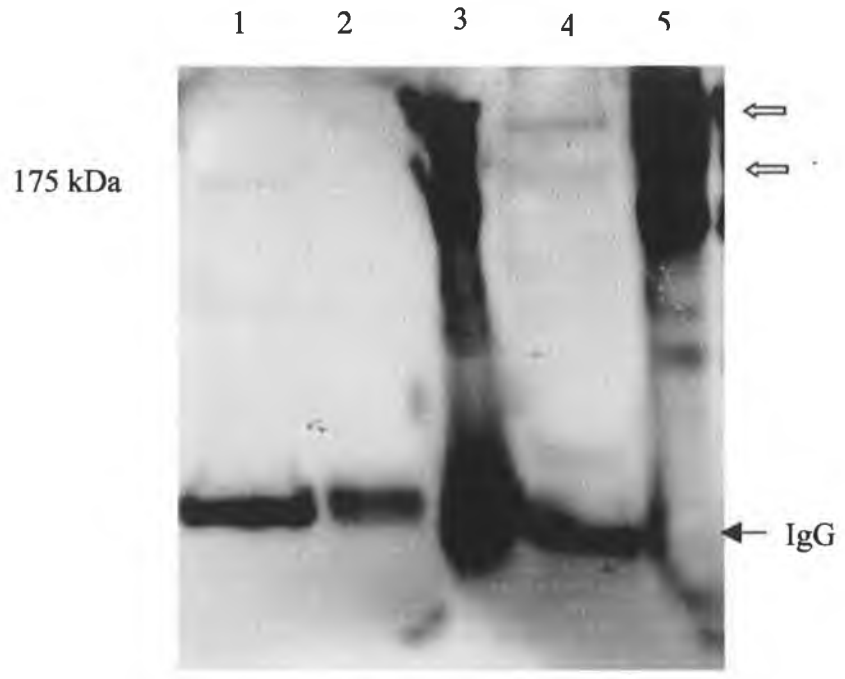


Figure 3.3.9. : Western blot analysis of immunoprecipitates and supernatants of ZR-75-1 cells probed with antibody 5C3.

Reduced (lane 1) and *non* reduced (lane 3) immunoprecipitates of ZR-75-1 cells, control mouse IgG (lane 2), reduced supernatant retained following immunoprecipitation (lane 5) and ZR-75-1 immunoprecipitate/ supernatant (50:50) (lane 4) separated on 7.5% SDS-PAGE and probed with antibody 5C3. A band at approx. 175 kDa is detected in ZR-75-1 immunoprecipitates, this band is also clearly observed in immunoprecipitate/ supernatant sample where the 220 kDa reactive band can also be observed. All 5C3 reactive bands are identified on supernatant sample, no 5C3 reactive bands are detected in non reduced immunoprecipitates.

3.3.14. Internal sequence analysis

Following separation on 7.5% SDS-PAGE, the gel was stained with 0.25% Coomassie Blue and destained accordingly (section 2.9.3., protocols as recommended by Eurosequence). The 175kDa band was excised from the gel and processed for internal sequencing via an in gel digest (Eurosequence BV, The Netherlands). A representative 7.5% SDS-PAGE gel showing the band of interest can be observed in Figure 3.3.10.

An *in situ* trypsin digest of protein (i.e.gel) was carried out by Eurosequence following which resulting peptides were extracted from gel. These were then checked for successful digestion, which in the case of the first sample sent was not proved to be the case. However a second sample of protein was successfully digested, revealing that a mixture of peptides were present. These peptides were then subjected to HPLC for purification. This revealed that a mixture of two peptides were present, their sequence levels were determined to be approx. 1:2. (Appendix IV).

Data base searching carried out by Eurosequence indicated that these peptides corresponded to human cytokeratin 6B (data base analysis subsequently checked at NCTCC suggested a better match with cytokeratin 6D) and human cytokeratin 9. The sequence results showed that the levels were approx. 1:2 (cytokeratin 6: cytokeratin 9). These results suggested that the antigen recognised by antibody 5C3 was a complex consisting of two cytokeratin 9 (type I cytokeratin, 62 kDa) proteins and one cytokeratin 6 protein (type II cytokeratin, 56 kDa). It was likely that the 175 Kda band represented this complex i.e. the sum of these individual cytokeratin m.w.s.

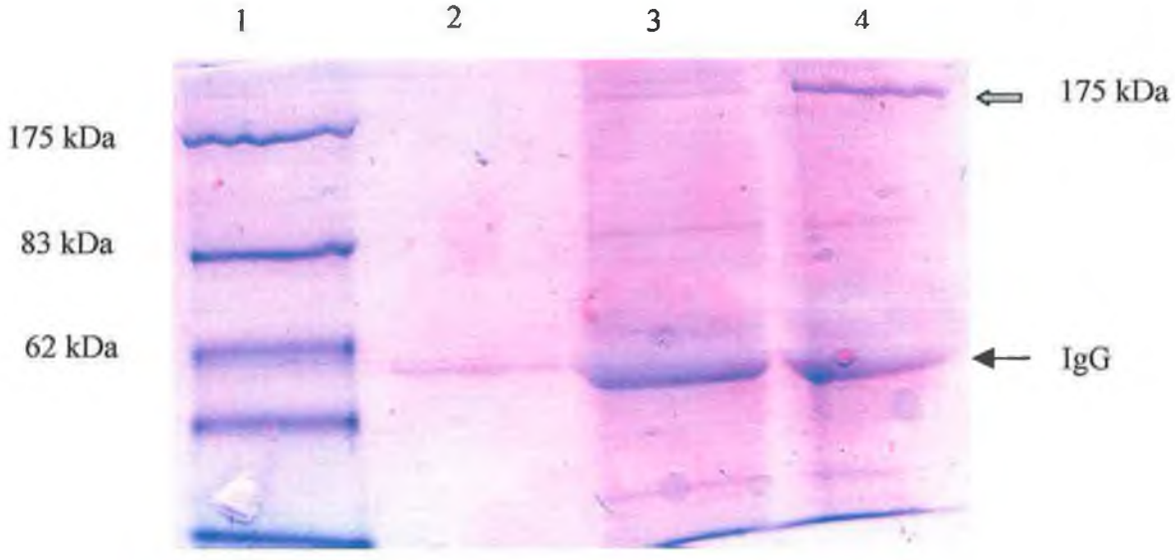


Figure 3.3.10. : Immunoprecipitates of ZR-75-1 cells separated on 7.5% SDS-PAGE and stained Coomassie Blue R-250.

Immunoprecipitates of ZR-75-1 cells (lane 3 and 4) separated on 7.5% SDS-PAGE and stained 0.25% Coomassie Blue R-250 (lane 1: molecular weight markers). (lane 2: no sample loaded, spillover only). A band at approx. 175 kDa is detected on immunoprecipitates, IgG bands are also detected. The 175 kDa band was excised and sent to Eurosequence BV, The Netherlands for internal sequencing.

3.3.15. Western blot analysis of ZR-75-1 immunoprecipitates and cell lysates for Cytokeratin 6 and Cytokeratin 9.

In an attempt to disassociate this proposed complex, further ZR-75-1 immunoprecipitates were boiled for an increased time i.e. 10 mins following immunoprecipitation. These immunoprecipitates were separated on 12.5% SDS-PAGE and stained with 0.25% (w/vol) Coomassie Blue. A band at approx. 60 kDa as well as the 175 kDa was observed (cytokeratin 9 reacts with a 62 kDa band, cytokeratin 6 reacts with a 56 kDa). When probed with antibody 5C3 only IgG bands were detected, no band specific for either cytokeratin 6 or cytokeratin 9 were detected. It appeared therefore that antibody 5C3 appears to recognise hetropolymer, cytokeratin 6/9 when "complexed", but it does not appear to recognise the disassociated complex.

When ZR-75-1 immunoprecipitates were probed with a MAb which specifically recognises cytokeratin 9, a cytokeratin 9 reactive band (62 kDa) was identified on immunoprecipitates which were boiled for 10 mins, a very faint cytokeratin 9 reactive band could be observed on immunoprecipitates which were boiled for 1 min (Figure 3.3.11.). IgG bands were also detected by the secondary antibody. Following probing of ZR-75-1 immunoprecipitates with a MAb that specifically recognises cytokeratin 6, only IgG bands were observed. No cytokeratin 6 reactive bands were detected in immunoprecipitated samples boiled for either 1 min or 10 mins (Figure 3.3.12.).

A cytokeratin 6 (56 kDa) reactive band was identified on crude cell lysates of ZR-75-1 cells when probed with the cytokeratin 6 specific MAb (Figure 3.3.13.). A cytokeratin 9 (62 kDa) reactive band was not observed on *crude cell lysates* probed with the cytokeratin 9 specific MAb (Figure 3.3.14).

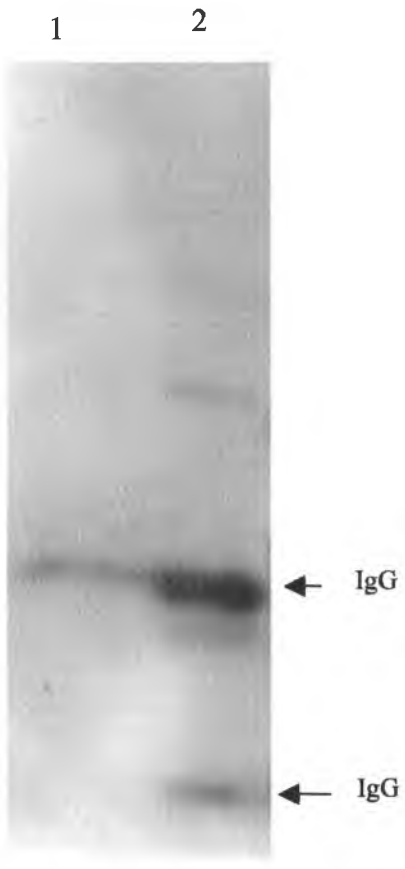


Figure 3.3.11.: Western Blot analysis of ZR-75-1 immunoprecipitates probed with cytokeratin 6 specific MAb.

ZR-75-1 immunoprecipitates boiled for 1 min (lane 2) and in an attempt to disassociate the proposed complex, further samples which were boiled for 10 mins (lane 1) following immunoprecipitation which were separated on 7.5% SDS-PAGE and probed with cytokeratin 6. No cytokeratin reactive band is identified in either immunoprecipitate, IgG (heavy and light chain) bands are only detected .

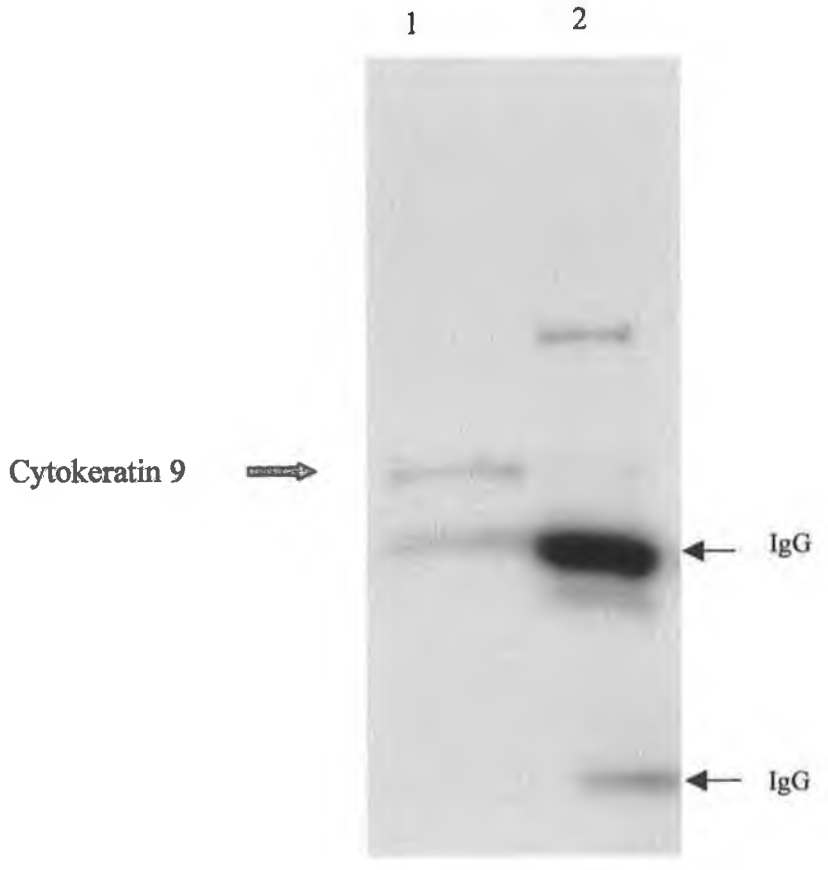


Figure 3.3.12.: Western Blot analysis of ZR-75-1 immunoprecipitates probed with cytokeratin 9 specific MAb.

ZR-75-1 immunoprecipitates boiled for 1 min (lane 2) and in an attempt to disassociate the proposed complex further samples which were boiled for 10 mins (lane 1) following immunoprecipitation which were separated on 7.5% SDS-PAGE and probed with cytokeratin 9. A cytokeratin 9 reactive band is detected at approx. 62kDa in ZR-75-1 immunoprecipitates which were boiled for 10 mins, a very faint band can be observed in immunoprecipitates which were boiled for 1 min. IgG bands (heavy and light chain) are also detected.

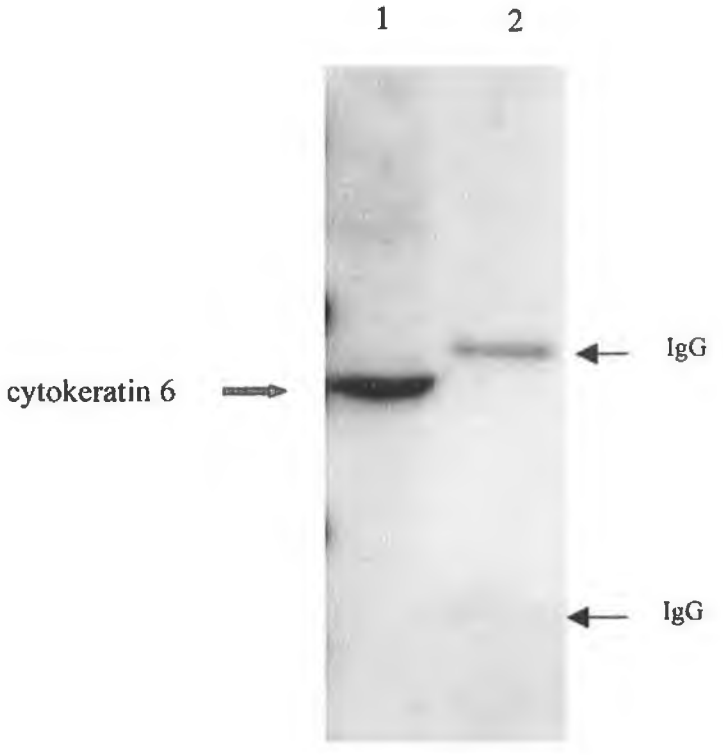


Figure 3.3.13. : Western Blot analysis of ZR-75 crude cell lysates and ZR-75-1 immunoprecipitates probed with anti-cytokeratin 6 specific MAb.

ZR-75-1 crude cell lysates (lane 1) and ZR-75-1 immunoprecipitates (boiled for 10 mins following immunoprecipitation) (lane 2) which were separated on 10% SDS-PAGE and probed with cytokeratin 6 MAb . Cytokeratin 6 reacts with a band at approx. 56 kDa in crude cell lysates of ZR-75-1 cells. No cytokeratin 6 reactive band is detected in immunoprecipitated samples.

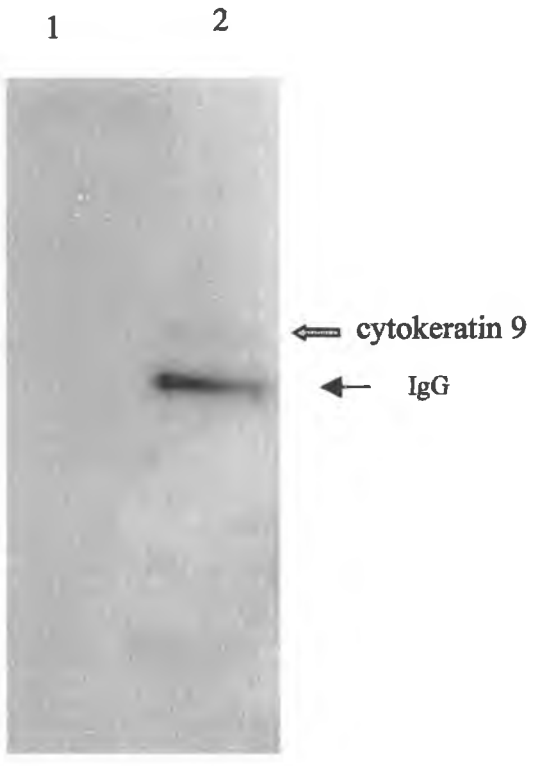


Figure 3.3.14. : Western Blot analysis of ZR-75 crude cell lysates and ZR-75-1 immunoprecipitates probed with anti-cytokeratin 9 specific MAb.

ZR-75-1 crude cell lysates (lanes 1) immunoprecipitates (lane 2) (boiled for 10 mins following immunoprecipitation) which were separated on 10% SDS-PAGE and probed with cytokeratin 6 MAb . A cytokeratin band is identified at approx. 62 kDa in immunoprecipitated ZR-75-1 cells, this band is not observed in crude cell lysates probed with cytokeratin 9 MAb.

3.3.16. Conclusion

Antibody 5C3 was generated using paraffin wax embedded formalin fixed archival breast tumour tissue from a biopsy as an immunogen. There did not appear to be any apparent association with resistance for the antigen recognised by antibody 5C3. Furthermore this antigen did not appear to be tumour specific, it was shown to be expressed in both normal and malignant breast tissue and was expressed in a number of other tumour types. Results of internal sequencing of a 175 Kda band which was obtained from immunoprecipitation of a ductal carcinoma cell line revealed the reactive antigen of antibody 5C3 to be a heteropolymer of cytokeratin 6 and cytokeratin 9.

4. Discussion

4.1. Discussion

In breast cancer, the most frequently used prognostic factors remain those determined by clinical or standard pathological approaches namely lymph node status, tumour size, histological grade, nuclear grade and tumour histology. To date the only valuable predictive factors in this disease are estrogen receptor and progesterone receptor which can predict response to hormonal treatments. Despite all of the studies directed at identifying new molecular markers of both prognosis and chemosensitivity, only cerbB-2 appears to hold any promise as a prognostic and possibly predictive marker. In an attempt to identify additional molecular markers, a panel of MDR and associated apoptosis/ oncogene markers were investigated during the course of this work in a series of invasive breast carcinomas. Immunohistochemical expression of drug transporters, MRP-1, MDR-1 and LRP, the HER-2 neu oncogene and the anti apoptotic proteins, BCL-2 and survivin were correlated with patient and tumour characteristics in patients with invasive breast cancer. Furthermore, expression of these proteins was correlated with Relapse Free Survival and Overall Survival. In order to address further the multifactorial nature of the disease, expression of individual protein markers is also analysed in relation to other markers.

In patients with invasive carcinoma studied here multivariate analysis revealed that in these patients nodal status and tumour size were independent prognostic factors for relapse free survival; tumour grade was an independent prognostic factor for overall survival. It is well recognised that smaller tumour size, lower histological grade and a negative lymph node status (i.e. no spread to the lymph nodes) are indicitive of a better prognosis in invasive breast cancer.

Invasive carcinomas studied were classified into two groups for further analysis; namely patients who received chemotherapy following surgery (endocrine therapy was also given in some cases), and patients who did not receive chemotherapy following surgery (endocrine therapy was given only in the majority of cases). Furthermore patients were also stratified according to node status; namely node positive patients and node negative patients.

Multivariate analysis of patients who *did not receive chemotherapy* revealed that small tumour size was an independent prognostic factor for relapse free survival; nodal status failed marginally to show significance. Histological grade was an independent prognostic factor for overall survival, again nodal status failed marginally to show significance. In these patients ER status was shown to be an independent prognostic factor for overall survival. When these patients were further stratified into node positive and node negative patients; none of the clinicopathological parameters analysed were identified as independent prognostic factors in *node negative* patients. Tumour size and tumour grade are the only features considered to offer any prognostic value in lymph node negative breast cancer (Mirza *et al.,* 2002). Tumours from the node negative patients studied here tended to be lower grade tumours (histological grade II or grade III), there were less grade III tumours which may explain why tumour grade did not have any prognostic significance. Again as tumours not treated with chemotherapy would tend to be smaller tumours anyway, tumour size thus would not be able to offer prognostic value.

In patients treated with chemotherapy multivariate analysis revealed that nodal status and ER status were independent prognostic factors for relapse free survival. ER status was also identified as an independent prognostic factor for overall survival; tumour grade and nodal status failed marginally to show independent prognostic value for overall survival.

Again patients were further stratified according to nodal status, revealing ER status to be a prognostic factor for both relapse free survival and overall survival in node positive patients and for overall survival only in node negative patients. No other clinicopathological parameter was identified as an independent prognostic factor in node negative patients treated with chemotherapy. Only tumours from 23 node negative patients were studied, perhaps this number was too small to reveal any independent prognostic factors, in this patient group.

Histological grade was shown to be a highly significant independent prognostic factor for Overall survival in node positive chemotherapy treated patients, it marginally failed to reach significance for relapse free survival. Tumour size was not

identified as an independent prognostic factor in node positive chemotherapy treated patients.

MRP-1 has been shown to be expressed in several human cancers (section 1.6.5.), in addition expression of this ATP drug transporter has been associated with poor patient outcome in a number of these malignancies including breast cancer (reviewed by Borst, 2000). During the course of this thesis, MRP-1 protein expression was studied by means of immunohistochemical analysis in a total of 204 untreated archival tumours from patients with invasive breast cancer. MRP-1 protein was expressed to varying degrees at diagnosis in 62.6% of these invasive tumours; this observation is in general agreement with previous studies of MRP-1 in breast cancers. The majority of MRP-1 staining observed was membranous with some granular cytoplasmic positivity observed in some tumours; this has been reported previously by Flens *et al.* (1996). Our results also indicated that for the most part when the intensity of the MRP-1 positive staining increased the percentage of tumour cells stained also increased (Table 3.1.2. and 3.1.3.); this has previously been observed by Flens *et al.,* (1996). Previous immunohistochemical studies of MRP-1 protein expression in primary breast carcinomas have ranged from 30% to 100% MRP-1 positivity (Filipits *et al.,* 1996, Nooter *et al.,* 1997, Filipits *et al.,* 1999a). Likewise RNA expression patterns in primary breast carcinomas have also shown variation; mRNA expression levels in untreated tumours of 70% to 100% have been reported (Filipits *et al.,* 1996, Dexter *et al.,* 1998, Ito *et al.,* 1997, Ferrero *et al.,* 2000). The results presented here suggest that in agreement with these previous studies, MRP-1 is detectable in a large proportion of human breast carcinomas.

Previous studies have indicated that MRP-1 mRNA expression and MRP-1 protein expression values when assessed in the same tumours do not always correlate (Filipits *et al.,* 1996, Nooter *et al.,* 1995, Ito *et al.,* 1997). One main contributory factor to such differences is the presence of normal cells as well as tumour cells in bulk detection methods such as RT-PCR and RNase protection assay and in addition tumour cell heterogeneity may be a factor. Breast cancers are known to be heavily infiltrated by normal lymphocytes (Nooter *et al.,* 1995). In parallel with the work presented in this thesis, a study on the expression of MRP-1 mRNA in the same series of breast tumours was also carried out in this laboratory (personnel

communication, Linehan *et al.*,). Preliminary analysis suggested that MRP-1 protein expression and RNA values did not appear to correlate in these invasive breast carcinomas.

Expression of MRP-1 protein, assessed by immunohistochemistry, was not significantly associated with age of patients (<50 years/ >50 years), node status, ER status, tumour size or histological subtype in either chemotherapy-treated patients or non-chemotherapy-treated patients. MRP-1 expression has been associated with estrogen receptor status in only one study; expression was more frequent in estrogen and progesterone receptor negative tumours (Filipits *et al.,* 1999). Other studies have shown that MRP-1 expression at diagnosis is independent of the age of patients at diagnosis, menopausal status, node status and histological subtype, again in agreement with the findings presented here (Filipits *et al.,* 1996, Nooter *et al.,* 1997b).

Preliminary analysis revealed that there was an association between MRP-1 positive tumours and an immunohistochemical score of 3+3 and high histological grade (grade III) 73% of 3+3 MRP-1 positive tumours had a high histological grade whereas only 23% and 3.8% were grade II and grade I, respectively. One previous study has reported more frequent expression of MRP-1 protein (and LRP protein) associated with tumours of an intermediate histological (moderately differentiated) grade (i.e. grade II), compared to those with a high histological grade III (Beck *et al.,* 1998). Grade III tumours are the most aggressive and show the worse prognosis (section 1.4.1.2.). Our results did show that MRP-1 expression was significantly associated with higher histological grade in chemotherapy treated patients. Taken together these results suggested that MRP-1 is possibly associated with a more aggressive tumour phenotype in these patients. This effect was not seen with patients who did not receive chemotherapy, reflecting perhaps, the less aggressive nature of tumours which are not recommended for chemotherapy.

Our initial observation of a possible weak association between MRP-1 expression and larger tumour size in patients studied was not found to be statistically significant. Only one previous study has reported MRP-1 expression being associated with larger tumour size (Filipits *et al.*, 1996). In general, previous studies indicate that

expression of MRP-1 is independent of tumour size and histological grade (Filipits *et al.,* 1996, Nooter *et al.,* 1997a). Increased MRP-1 expression has however, been shown to be associated with primary tumours which have distant metastases and in lymph node metastases compared to primary tumours (Filipits *et al.,* 1996, Zochbauer-Muller *et al.,* 2001).

On preliminary analysis of the data it appeared that a higher proportion of untreated tumours from those chemotherapy treated patients who relapsed following treatment and subsequently died were MRP-1 positive at diagnosis, compared to those patients who did not relapse following treatment and who are currently alive and well. Previous studies have observed both higher MRP-1 gene expression levels and MRP-1 protein expression levels in relapsed primary breast cancer patients compared to non-relapsed patients (Nooter *et al.,* 1997, Ito *et al.,* 1998). Our results indicate that a higher percentage of node positive chemotherapy treated patients with MRP-1 positive tumours at diagnosis (61.7%) relapsed and died compared to those patients with MRP-1 negative tumours (37.5%). However as all of the patients studied were diagnosed at different times, and the follow up period was not similar for individual patients, no firm conclusions could be drawn from these observations as regards MRP-1 expression and its association, if any, with clinical outcome in these patients.

MRP-1 expression was then correlated with relapse free survival and overall survival times in node positive and in node negative patients. In patients *who did not receive chemotherapy*, it appeared that MRP-1 expression at diagnosis may possibly be associated with a shortened relapse free period both in node positive and node negative patients (Table 3.1.9.). This trend was observed more strongly in node negative patients; 40% of these patients with MRP-1 positive tumours relapsed within 3 years following diagnosis compared to only 7% of patients with MRP-1 negative tumours. In node positive patients with MRP-1 positive tumours a similar number of patients relapsed within 3 years (40.9%), however only 20% of these patients with MRP-1 negative tumours relapsed within this time.

On preliminary analysis of overall survival times in these patients it appeared that there may be a similar trend of MRP-1 expression being associated with reduced overall survival times in these patients; a shortened overall survival appeared to be

associated with MRP-1 positive tumours compared to MRP-1 negative tumours. This trend was observed in both node negative and node positive patients (Table 3.1.10). MRP-1 expression has been associated with increased risk for failure in node negative breast cancer patients in two previous immunohistochemical studies (Nooter *et al.,* 1997b, Filipits *et al.,* 1999).

However when these results were analysed statistically using Kaplan Meier analyses, no association was shown between MRP-1 expression and either Overall survival or Relapse free survival, regardless of nodal status, although values for grade III tumours tended to be closer to significance than grade II tumours. This observation suggests that perhaps if more tumours representative of each histological were analysed, MRP-1 expression may hold possible prognostic value in higher grade, III tumours. Multivariate analysis did not however reveal MRP-1 expression to be an independent prognostic factor for outcome in patients *who did not receive* chemotherapy. In conclusion, in patients who *did not receive* chemotherapy MRP-1, expression at diagnosis did not offer any prognostic value in these patients.

Similar levels of MRP-1 protein expression were observed in *pre treatment* biopsies from chemotherapy treated patients (61.6%) and in patients who *did not receive* chemotherapy (62.6%). Immunohistochemical analysis of 20 *post* chemotherapy treated tumour specimens revealed that MRP-1 expression was not markedly increased following treatment, indicating that expression of MRP-1 is not associated with exposure to chemotherapeutic drugs. It has previously been reported that MRP-1 expression was not associated with prior chemotherapy (Nooter *et al.,* 1995).

The majority of these patients received standard CMF treatment. The drugs used in this regime are not in general, considered within the drug profile for MRP-1, although methotrexate has been shown to be a substrate for MRP-1 (Hooijberg *et al.,* 1999). Only one *post* treatment tumour studied was from a patient who received adriamycin in combination with CMF. This patient was negative for MRP-1 at diagnosis but did show MRP-1 positivity following chemotherapeutic treatment. Adriamycin is a substrate for MRP-1 and also MDR-1 Pgp. As only one patient was studied it cannot be concluded that MRP-1 expression is associated with ACMF treatment

On preliminary analysis of *chemotherapy treated* patients, it appeared that in node positive patients, 61% of patients with MRP-1 positive tumours who relapsed are now deceased (d), compared to only 37.5% of patients with MRP-1 negative tumours. This trend was more strongly observed in node negative patients. A higher percentage of MRP-1 negative patients (node negative and node positive) compared to MRP-1 positive patients currently alive and showing no recurrence). Again, as all of the patients studied were diagnosed at different times, follow-up times were not equal so no firm conclusions could be drawn regarding MRP-1 expression and association with clinical outcome by simply comparing "d" with "a" patients (although the results did suggest a possible interesting association).

MRP-1 expression was correlated with actual relapse free survival and overall survival times in these chemotherapy treated patients. On preliminary analysis of the results presented here, more MRP-1 positivity at diagnosis appeared to be associated with a shortened relapse free period (1-3 years) (Table 3.1.11.) in chemotherapy treated node positive patients. This analysis did not reveal such an association in node negative chemotherapy treated patients. A higher percentage of node positive MRP-1 positive patients (51.1%) compared to MRP-1 negative patients (20.5%) relapsed within 3 years of diagnosis. Again a markedly lower percentage of MRP-1 positive patients (15.6%) showed more than 8 years relapse free survival compared to 50% of MRP-1 negative patients showing 8 or more years relapse free survival. A similar trend was observed on analysis of overall survival in node positive patients. 56.6% of patients with MRP-1 negative tumours at diagnosis survived for at least 8 years whereas, only 11.1% of MRP-1 positive patients survived for this length of time (Table 3.1.12). Again from this analysis, MRP-1 did not appear to be associated with a shortened overall survival period in node negative chemotherapy treated patients.

The possibility cannot be ruled out that expression of MRP-1 at diagnosis may be associated with a poor prognosis regardless of the treatment that follows. However, 56% of node positive chemotherapy treated patients with *MRP-1 negative* tumours at diagnosis survived for 8 or more years, compared to only 11% of MRP-1 positive tumours (Table 3.1.12.). Such an association was not observed in non-chemotherapy

treated patients (Table 3.1.0). This preliminary analysis suggested that MRP-1 expression may have predictive value in patients treated with a CMF based regime, in relation to overall survival and possibly a weaker association with relapse.

Kaplan Meier survival analysis of node positive patients, revealed a significant association between MRP-1 expression and Overall survival time in these patients, but not Relapse free survival. A highly significant association was however, shown between absence of MRP-1 expression at diagnosis and increased overall survival in both node negative and node positive chemotherapy treated patients with higher grade tumours (grade III). This effect was not seen in patients with grade II tumours.

Multivariate analysis identified MRP-1 as an independent prognostic factor for overall survival but not relapse free survival in chemotherapy treated patients. When stratified according to nodal status; MRP-1 expression was revealed to be a highly significant independent prognostic factor for overall survival in node positive chemotherapy treated patients, but not in node negative patients.

When specific chemotherapy treatments were analysed, it appeared that this association between the absence of MRP-1 expression at diagnosis and increased Overall survival and Relapse free survival, may possibly be stronger in patients who received standard CMF chemotherapy compared to those patients who received adriamycin in combination with CMF (ACMF) or who received Taxol (Table 3.1.13. – 3.1.1.16). However as the numbers of patients studied is small when the chemotherapy treated group is sub divided by the type of treatment (only 26 patients received ACMF) no definitive conclusions can be drawn regarding the difference between MRP-1 expression and overall and relapse free survival in these two groups. Rather, it can be concluded that absence of MRP-1 protein expression at diagnosis in both node positive and node negative primary breast cancer treated with either standard CMF or CMF in combination with adriamycin is significantly associated with increased survival times in these patients. Nooter *et al.,* (1997b) have reported that in adjuvant chemotherapy treated node positive patients who received CMF, MRP-1 was also associated with shortened relapse-free survival *and* overall survival times. Their study did not however show an association in node negative patients.

It can be concluded that absence of MRP-1 protein expression at diagnosis offers independent prognostic value with regard to overall survival in invasive breast cancer patients treated with CMF based chemotherapy, its prognostic value is significantly stronger in node positive patients. Results presented suggest that the prognostic value of MRP-1 expression is likely to yield the most significant prognostic power in node positive patients with a high histological grade (i.e. grade III) tumours. Its significant association with histological grade suggests that its expression is possibly associated with a more aggressive tumour phenotype in invasive breast carcinoma. associated with a worse outcome in invasive breast carcinomas

The question as to whether MRP-1 has predictive value in those patients who received CMF based chemotherapy is however more difficult to establish. Multivariate analysis of all patients with invasive breast carcinomas showed that MRP-1 expression marginally failed to show significance as a prognostic factor for overall survival regardless of chemotherapy status or nodal status. When patients were stratified according to chemotherapy status and node status, the prognostic value of MRP-1 was shown to be significant in chemotherapy treated patients only, suggesting that perhaps MRP-1 expression may have possible predictive value in patients treated with CMF based chemotherapy. However as numbers of representative tumours in certain subgroups in this study were relatively small, it would be necessary to carry out similar analyses on a larger tumour base to establish if MRP-1 expression at diagnosis has possible predictive power.

MRP-1 protein expression values did not appear to correlate with MDR-1 protein expression. Previous studies have also failed to find a correlation between these two transport pumps (Filipits *et al.,* 1996, Ferrero *et al.,* 2000), although one previous study has reported that *pre* chemo Pgp and *pre* and *post* chemotherapy MRP-1 expression predicted tumour recurrence and patient death (Mechetner *et al.,* 1997). Our results did not, however, suggest any such association between the expression of these two proteins. There did appear to be a slight correlation between MRP-1 and survivin protein expression in 63.2% of patients studied, but the small number of patients analysed for both markers do not allow us to assess if this association is strong enough to be statistically significant. The relationship between these two proteins has not previously been investigated in breast cancer. No apparent

correlation was observed between MRP-1 protein and either LRP or Bcl-2 protein expression.

The HER-2 proto-oncogene also referred to as cerbB-2, encodes a transmembrane tyrosine growth factor that is overexpressed in 25-30% of breast carcinomas. As discussed in the introduction, expression of HER-2 in relation to both prognosis and the response to cytotoxic and hormonal therapies has been investigated by several investigators. Overexpression of this proto-oncogene has been associated with poor clinical outcome in node positive breast carcinomas; the correlation of HER-2 and prognosis in node negative patients has produced more conflicting results (reviewed by Sahin 2000, Simon *et al.,* 2001). HER-2 is currently the most promising marker under study in breast cancer, both as a marker of poor prognosis and as an indicator of cerbB-2 specific therapy. 1998 saw the approval of the use of trastuzamab (Herceptin), a recombinant MAb which targets HER-2 for use in the treatment of breast cancer. It has recently been demonstrated that Trastuzumab increases the clinical benefit of first line chemotherapy in metastatic breast tumours that overexpress HER-2 (Slamon *et al.,* 2001).

During the course of this thesis HER-2 protein expression was studied in a total of 46 untreated tumours from patients with invasive breast cancer. cerbB-2 protein was expressed in 27% (11/40) of invasive breast tumours at diagnosis from chemotherapy treated patients and was expressed in 80% (4/5) of tumours from patients who did not receive chemotherapy. Membranous cerbB-2 positivity is generally considered to be specific for HER-2/ neu overexpression and has been shown to correlate with gene amplification although for borderline cases, FISH is considered more definitive in predicting responsiveness to Herceptin. In contrast the significance of cytoplasmic positive staining is still controversial. Tumours studied which showed only cytoplasmic positivity in this study were not considered positive. There has been a lot of controversy regarding cerbB-2 specific MAbs. The MAb CB11 which was used for this study was the MAb employed in a study which showed that overexpression of cerbB-2 predicted poor clinical outcome (Keshgegian & Cnaan, 1997).

In our study cerbB-2 expression did not appear to be associated with node status, ER status, tumour size or histological sub-type (Table 3.1.7). Overexpression has been associated with the prognostically favourable ER positive phenotype (Bodey *et al.*, 1997).

On analysis of results it appeared that a weak association could be observed between lower expression levels of cerbB-2 protein and higher grade (grade III) tumours. This is an unexpected observation, overexpression of HER-2 is generally associated with poor markers of prognosis which would include high grade carcinomas (i.e. poorly differentiated). As the numbers of grade I (3) and grade II (1) tumours in this group were small i.e. the majority of the tumours (22) were grade III, this observation can probably be discounted.

On preliminary analysis it appeared that a much lower percentage of *pre* treatment tumours from patients who are currently disease free showed cerbB-2 positivity (1/15 node positive patients, 0/2 node negative patients) than those patients who relapsed and died of disease. Tumours from 11/ 17 patients currently disease free were negative for both MRP-1 and cerbB-2. Again as was the case with MRP-1, where all of the patients were diagnosed at different times, no conclusions can be drawn from these observations. On analysis of actual Relapse Free Survival times, results suggest that cerbB-2 expression at diagnosis was strongly associated with a shortened relapse free period in node positive patients (Table 3.1.18). A higher percentage of node positive patients with cerbB-2 positive tumours at diagnosis relapsed within 3 years compared to node positive patients with cerbB-2 negative tumours. 43% of cerB-2 negative tumours had a relapse free period of at least 8 years; all of the cerbB-2 positive tumours studied relapsed within 8 years. This association was not observed in node negative patients.

A similar association was observed on analysis of Overall Survival times in node positive patients. It appeared that cerbB-2 expression was strongly associated with a shortened overall survival time in these patients. 60% of cerbB-2 positive patients survived less than 3 years following diagnosis compared to 15.4% of cerbB-2 negative patients (Table 3.1.19). Again this was not observed in node negative patients.

The first study revealing that HER-2 gene amplification could independently predict overall survival and disease free survival in node positive breast cancer using multivariate analysis was published by Slamon *et al.,* (1987). Since then several studies have confirmed this association in patients treated with CMF, although a small number of these did not find such an association (reviewed by Sahin 2000, Simon *et al.,* 2001). However opinions on whether HER-2/ neu status should be used to routinely determine which patients might not get benefit from CMF are still mixed, some researchers are waiting for more validation or results before recommending such a predictor in the clinic (Ravidin 1999, Hamilton 2000).

cerbB-2 overexpression may also determine which patients have tumours which may be particularily sensitive to doxorubicin containing drug regimes (Paik *et al.,* 1998, Thor *et al.,* 1998, Yamauchi *et al.,* 2001). Furthermore preliminary results suggest that it may also play a predictive role in response to paclitaxel (taxol) (Baselga *et al.,* 1998). Recently it has been reported that topoisomerase II α deletions and amplifications can predict response to epirubicin in cerbB-2 positive tumours (Jarvinen *et al.,* 2000).

Thus results of our study are in agreement with overexpression of cerb-B2 in primary node positive breast cancer possibly being associated with a poor outcome i.e. shortened relapse free survival and overall survival in patients treated with an adjuvant CMF based regime.

To our knowledge, although it has been suggested that there may be a possible link between expression of MRP-1 and expression of certain oncogenes such as cerbB-2, there does not appear to be any reported analysis of these two markers in relation to any possible role for their co-expression (Ito *et al.,* 1998). When the co-expression of both cerbB-2 and MRP-1 proteins were analysed, results indicated that 75% of patients with MRP-1 positive / cerbB-2 positive tumours relapsed within 3 years. Only 26.7% of patients with MRP-1 negative / cerbB-2 negative tumours relapsed within this time (Table 3.1.21.). On analysis of overall survival, again co-expression of these two markers was associated with a shortened overall survival only 13.3% of

MRP-1 negative / cerbB-2 negative tumours survived for less than 3 years (Table 3.1.22.). However in this case MRP-1 negative / cerbB-2 positive tumours exhibited similar overall survival times than those tumours that expressed both proteins.

These results suggest that co-expression of these two markers may be more strongly associated with a shortened relapse free survival and possibly with a shortened overall survival in breast cancer patients, than expression of either of these individual proteins. As the number of node negative tumours studied was very small, it cannot be concluded that this observation of an association between the co-expression of MRP-1 and cerbB-2 patient outcome only applies to node positive patients. In addition again as the numbers studied were small, it was not possible to carry out statistical analysis on the co-expression of these two markers and their association with relapse free survival and overall survival times. This interesting observation should be addressed in a larger tumour base to see if it is indeed statistically significant. In addition this observation should be investigated in node negative patients also.

Apart from MRP-1, expression of cerbB2 did not appear to correlate with any of the other protein markers studied. *Post* treatment tumours from 17 chemotherapy treated patients were also investigated. cerbB-2 expression did not generally appear to increase following treatment with chemotherapeutic drugs although a slight increase in expression was observed in 2 patients. It can be concluded that cerb2B-2 expression is not in general associated with exposure to a CMF based chemotherapeutic regime.

MDR-1/ Pgp expression in breast cancer has been extensively investigated. A meta analysis carried out of MDR-1/ Pgp in breast cancer by Trock *et al.,* (1997) reviewed in detail all of the 31 studies which had been published at that point. This review concluded that MDR-1/ Pgp is expressed in 27.1% of breast tumours using RNAase protection assay methods and 48.5% of tumours using immunohistochemistry methods; the latter have proved more sensitive than gene expression analyses in published studies to date. Trock *et al.,* (1997) also concluded that MDR-1/ Pgp expression is associated with treatment and with a poor response to chemotherapy in breast cancer. Pgp positivity has been associated with poor treatment outcome in

both primary and advanced cancers; there is limited data regarding any possible association with outcome in metastatic disease (Lehart *et al.,* 1996).

In this thesis MDR-1 Pgp expression was studied in 20 untreated tumours from patients who subsequently received chemotherapy; 70% of these chemotherapy treated patients showed MDR-1 positivity at diagnosis (Table 3.1.23). This percentage is higher than the average MDR-1 expression level observed with immunohistochemical studies as reviewed by Trock *et al.,* 1997. However this is the first study of MDR-1 in breast carcinomas using MAb 6/1C (Moran *et al.,* 1997). Differences were observed between MAb 6/1C and MRK-16 expression in individual tumours both in the intensity of the staining and in the percentage of tumour cells stained; this is in agreement with previous reports outlining varying specificity and staining patterns of Pgp specific antibodies (Bittl *et al.,* 1993). Ideally two antibodies directed to different epitopes of MDR-1 Pgp should be employed in such studies (Beck *et al.,* 1996, Trock *et al.,* 1997).

The results presented in this thesis indicate that expression of MDR-1 Pgp does not appear to be associated with ER status, age of patients at diagnosis or node status in these invasive breast carcinomas. There was a very slight association of MDR-1 positivity with ER positive tumours, however as the numbers were small this was discounted. No conclusion can be drawn as regards association with histological grade as all of the tumours studied for MDR-1 Pgp expression were grade III; 70% of these tumours were MDR-1 positive. Again as regards association with tumour size a definite conclusion cannot be reached due to small sample size, the results however do suggest that MDR-1 appears to be associated more strongly with tumours that are less than 2 cm. Previous studies indicate that none of the known prognostic attributes i.e. node status, tumour size, tumour histology, histological grade or ER status are significantly associated with expression of MDR-1 Pgp (reviewed by Trock *et al.,* 1997).

MDR-1 Pgp expression was studied in post treatment tumours from 17 of these patients. 4 *pre* treatment tumours which were MDR-1 negative showed MDR-1 Pgp positivity following chemotherapy (all of these 4 patients relapsed within 3 years). There appeared to be no increase in staining in those *post* treatment tumours that

were MDR-1 positive at diagnosis. As the number of patients examined was small it is not possible to conclude with statistical significance that MDR-1 Pgp expression is associated with chemotherapy treatment, but these preliminary results indicate that the question warrants further investigation.

In previous studies higher expression of MDR-1/ Pgp has been observed in patients who received MDR-1/ Pgp related drugs than that observed in tumours from patients treated with non-MDR-1/ Pgp related drugs (reviewed by Trock *et al.,* 1997). All of the patients studied for Pgp expression with the exception of one who received adriamycin, a Pgp substrate, in combination with CMF, received CMF therapy. The drugs used in the CMF combination (i.e. cytclophosphamide, methotrexate, 5-fluorouracil) are not Pgp substrates.

On preliminary analysis, 77.7% of *pre* treatment tumours from patients currently alive who have not relapsed were MDR-1 positive, compared to 55.5% of those patients who relapsed and are now deceased. This observation suggests that MDR-1/Pgp expression is associated with a good prognosis. Again as with analysis of other markers, as patients were diagnosed at different times no conclusion could be drawn. Trock *et al.,* (1997) reported that tumours with detectable MDR-1 Pgp were three times more likely to exhibit an incomplete response to cytotoxic therapy. To further explain our unexpected results, MDR-1 expression was correlated with relapse free and overall survival times. This analysis suggests that MDR-1 positive node positive patients are more likely to relapse within 3 years compared to MDR-1 negative patients, as regards overall survival a similar trend was observed, but the association was weaker (Tables 3.1.24.- 3.1.25.). Our initial unexpected observation of an association of MDR-1 with a good outcome proved not to be the case, indicating as we have discussed that using the current status of patients does not allow for correct interpretation of markers with clinical outcome. No conclusion as regards node negative patients can be made as there were only 3 patients studied for MDR-1 Pgp expression. Previous studies addressing the expression of MDR-1 Pgp and any possible association with relapse free survival and overall survival times have proved conflicting. A number of studies have associated MDR-1/Pgp expression reduced relapse free survival and overall survival times while other studies have failed to observe this association (reviewed by Trock *et al.,* 1997). These preliminary results

suggest that expression of MDR-1 Pgp may possibly be associated with a shortened relapse free period in invasive breast cancer, however as the numbers are small it is not possible to carry out statistical analysis on these results.

LRP has been detected previously prior to and following treatment in breast cancer but results indicate that it does not play a prognostic/ predictive role in breast cancer, (Izquierdo *et al.,* 1996, Linn *et al.,* 1997, Pohl *et al.,* 1999, Schneider *et al.,* 2001). However a recent report has suggested that the combination of LRP expression after chemotherapy treatment *and* MDR-1 positivity before treatment has prognostic value in the determination whether axillary nodal invasion will occur (Schneider *et al.,* 2001).

LRP protein expression was studied in *pre* treatment tumours from 14 patients with invasive breast cancer (12 chemotherapy treated). 4 LRP negative *pre* treatment tumours showed some degree of LRP positivity following chemotherapy. Again as sample numbers are small it is not possible to conclude that LRP expression is associated with exposure to chemotherapeutic drugs. As the level of expression of LRP expression observed in these tumours was low, it was unlikely that LRP was playing any significant role in these breast tumours. LRP expression values did not appear to correlate with expression of any other of the markers studied.

Bcl-2 is another well established prognostic marker in breast cancer being associated with a favourable outcome. A number of studies have shown that Bcl-2 positivity correlates with positive hormone receptors and is associated with a well differentiated phenotype (Takei *et al.,* 1999, Nakopoulou *et al.,* 1999, Rochaix *et al.,* 1999).

Bcl-2 protein was studied in tumour material from chemotherapy treated patients obtained prior to treatment. Bcl-2 protein was expressed in 61.5% of these tumours at diagnosis. The proportion of patients showing Bcl-2 positive staining is in agreement with previous immunohistochemical studies of Bcl-2 protein expression in breast cancer (Hellemans *et al.,* 1995, Tanaka *et al.,* 2000). There did not appear to be any increase in BCL-2 expression following treatment, suggesting that BCL-2 expression is not associated with exposure to chemotherapeutic drugs. Due to the small numbers

studied it could not be concluded if the expression of Bcl-2 was associated with age of patients, node status, ER status, tumour size or histological grade

The results suggest that there may be a weak association between BCL-2 negative tumours and a shortened relapse free period and overall survival times compared to BCL-1 positive tumours; a higher percentage of BCL-2 negative patients relapsed and died within 3 years compared to BCL-2 positive patients. As the numbers are small statistical analysis cannot be carried out, however it is possible that BCL-2 may be a good prognostic marker in these patients which is not an unexpected result. A collaborative study looking at BCL-2 mRNA expression by RT-PCR in the same large series of invasive tumours as MRP-1, showed that there was a statistically significant association between BCL-2 negativity and reduced relapse rate and reduced survival rate in these patients (personal communication Cronin *et al.,*).

Several studies have reported that Bcl-2 expression is associated with a longer disease free/ relapse free period (Binder *et al.,* 1995, Hellemans *et al.,* 1995, Kobayashi *et al.,* 1997, Jansen *et al.,* 1998, Itaya *et al.,* 1999). Reports regarding BCL-2 as a predictor of response to adjuvant chemotherapy are conflicting with several studies finding that BCL-2 did not predict response to various chemotherapeutic regimes including CMF, epidoxorubicin or doxorubicin alone while other studies have failed to do so (reviewed by Daidone *et al.,* 1999). It is generally felt that there is not enough evidence to date to support such a predictive role for Bcl-2 in breast cancer (Hamilton 2000).

Bcl-2 and survivin protein expression values appeared to correlate in 50% of chemotherapy treated patients, i.e. there was no correlation between these two proteins. There did not appear to be a correlation between BCL-2 and any of the other markers studied. Again as mentioned previously it is not possible to draw any final conclusions regarding comparative studies with survivin immunohistochemistry as the scoring system employed was slightly different to that involving other MAb markers.

At the time of this investigation a new MAb to the recently discovered anti apoptotic survivin protein was generated in this laboratory (Cleary *et al.,* personnal

communication). As the expression of survivin protein had not, then been investigated in breast cancer it was decided to look at the expression of antibody 1C5 in these breast tumours to establish the role, if any of survivin protein expression in this malignancy. Investigation of survivin as a possible marker of chemoresponsiveness has not been reported to date. Presently survivin protein has been associated with a poor prognosis in several human cancers including breast cancer, colorectal cancer and ovarian cancers (Tanaka *et al.,* 2000, Sarela *et al.,* 2000, Yoshida *et al.,* 2001).

Survivin protein expression was studied in 24 untreated breast tumours; 22 of these patients had received chemotherapy. Survivin protein expression was observed in 86.3% of chemotherapy treated patients at diagnosis. This proportion of tumours expressing detectable survivin protein is in agreement with a recent study by Tanaka *et al.,* (1999) where survivin protein was detected in 70% of breast cancer patients investigated. There appeared to be considerable heterogeneity between the intensity of the staining observed and in the number of tumour cells stained within individual tumours. The number of positive tumour cells stained within individual tumours varied between 20 and 100% in the study by Taneka *et al.,* (2000). However the intensity of the staining was usually homogenous in their study; this difference may be accounted by the fact that we were using a MAb recognising a different epitope of survivin protein. Different staining features when using different MAbs have been observed frequently (Bittl *et al.,* 1993)

Nuclear survivin positivity was observed in a number of tumour samples. Survivin positive staining had previously been observed in the cytoplasm of tumour cells, however recent studies have reported detection of this protein primarily in the nucleus of hepatocellular carcinoma cells, in the nuclei of glandular epithelial cells in the normal human endometrium and in gastric tumour cells (Ito *et al.,* 2000, Konno *et al.,* 2000, Okada *et al.,*2001). It has also been shown that nuclear survivin positivity is associated with a shortened disease free survival in ovarian carcinomas (Yoshida *et al.*, 2001). The significance of this nuclear positivity should be addressed in any further study in breast carcinomas.

As previously outlined Survivin protein expression appeared to correlate with MRP-1 (62.3%) and MDR-1 protein (71.4%) expression chemotherapy treated patients. Bcl-2 and survivin protein expression values appeared to correlate in 50% of chemotherapy treated patients, thus it can be concluded that there is no correlation between these two proteins. In addition, as the scoring system for immunohistochemical analysis of survivin (section 3.1) differed from that of the other proteins studied, no direct comparative conclusions can be drawn. In contrast Tanaka *et al.,* (2000) reported a close association between these two proteins; a positive correlation has been shown between these two in other malignancies (Thompson *et al.,* 1995, Yang *et al.,* 1996, Kawasaki *et al.,* 1998, Adida *et al.,* 1998). Perhaps if a larger number of tumours were analysed for these two proteins such an association would also be shown in these invasive breast cancer patients. There did not appear to be any correlation of survivin protein with either cerbB-2 protein or LRP protein expression observed.

As survivin protein was observed in the majority of tumours studied it was not possible to correlate survivin expression with node status, ER status, tumour size or histological grade.

On initial analysis, results suggested that expression of survivin protein at diagnosis may be possibly associated with shortened relapse free survival (i.e. 1-3 years). However on analysis of overall survival times, it appeared 38% of patients with survivin positive tumours survived for 8 or more years, compared to only 28% of survivin negative tumours (Table 3.1.26.). This observation suggested that survivin expression at diagnosis might be actually associated with a good prognosis i.e. longer overall survival in chemotherapy treated patients, but the numbers studied were small, therefore it was not possible to carry out statistical analysis. Tanaka *et al,* (1999) found that survivin expression in breast carcinomas, in association with a reduced apoptotic index was indicative of a poor prognosis. In general survivin expression has been associated with unfavourable prognostic markers, increased risk of recurrence and increased resistance to chemotherapy (reviewed by Altieri *et al.,* 2001). However nuclear survivin expression has been associated with a favourable prognosis in gastric cancer patients (Okada *et al.,* 2001). A larger series of patients

should be investigated in order to address these observations further and see if they are significant.

MDR-3 Pgp has now been shown to transport several cytotoxic drugs including digoxin, pacitaxel, daunorubicin, vinblastine and invermectin (Smith *et al.,* 2000). Previously MDR-3 has been found to be associated with the drug resistance in certain B-cell leukaemias (Herweijer *et al.,* 1990, Nooter *et al.,* 1990). This Pgp has also been demonstrated to be responsible for the drug resistance in an MDR-3 transformed yeast strain (Kino *et al.,* 1996). However the role of MDR-3, if any, in breast cancer and other solid cancers, and a clearer explanation of its possible role in the drug resistance of B-cell malignancies remains to be solved. At the time of commencement of this thesis, there were no MDR-3 specific MAbs available commercially, so it was decided to attempt to generate a MAb which was specific for the MDR-3 encoded gene product. Such an MAb would be useful in establishing further the role, of MDR-3 outside the liver.

This laboratory had successfully produced specific MAbs to the MDR-1 encoded gene product and Topoisomerase IIα using short synthetic peptides as immunogens (Moran *et al.,* 1997). In an attempt to produce a MAb which would specifically recognise the MDR-3 encoded gene product, a peptide corresponding to the intracellularly located amino acids, 13-24 (RPTSAEGDFELG) of MDR-3 encoded Pgp which resides in the N-terminal part of the protein was choosen following alignment searches of the EMBL Swiss-Prot protein sequence database using the Mail-FASTA program. The chosen peptide showed insignificant homology with MDR-1 encoded Pgp. The fusion protein used by Smit *et al.* (1994) to generate the REG-1 polyclonal antibody consisted of amino acids 2-58 from the N-terminal region of MDR-3 Pgp. The AVLCL2 polyclonal also produced by Smit *et al.* (1994), using a 13 amino acid synthetic peptide shared 7 amino acids with the synthetic peptide used for the production of antibody 6/1G. However this antibody appeared to work well on Western blotting only and did not appear suitable for immunohistochemical studies.

Due to time constraints it was decided to carry out a combination of *in vitro* and *in vivo* immunisation with the choosen peptide coupled with the carrier protein BSA in an attempt to generate an MDR-3 specific MAb. Following screening by ELISA supernatant 6/1G was chosen for further study. This hybridoma was cloned by

limiting dilution prior to further characterisation. Isotype analysis of antibody 6/1G revealed that it to be subclass IgM. This was not an unexpected result considering that an *in vitro* immunisation had been used and the number of boosts administered to mouse was small, an effective secondary response may not have been elicited in the animal. Thus there was an increased likelihood of isolating an IgM antibody (section 1.2.).

Preliminary characterisation of antibody 6/ 1G was carried out using the MDR-3 transgenic cell lines, VO1-VO1 and VIM # 1 and FVB # C, their parental cell line. Immunofluoresence studies with antibody 6/ 1G on live cell preparations of these control cell lines revealed no detectable staining indicating that the epitope recognised by antibody 6/ 1G was not expressed on the cell surface. So it appeared that antibody 6/ 1G was recognising an internal epitope which would have been expected given that the peptide chosen corresponded to amino acids which are believed to be intracellularly located in MDR-3 Pgp. Western blotting studies with antibody 6/1G identified a single band at 170kDa on cell lysate preparations of the MDR-3 transgenic cell lines, VO1-VO1 and Vim # 1. In their successful attempt to produce MDR-3 specific polyclonal antibodies Smit *et al.* (1994) observed that the apparent molecular weight of MDR-3 Pgp in VO1-VO1 cells was approximately the same as for MDR-1 encoded Pgp. By comparison with MAb C219 this group showed that VO1-VO1 cells do not express MDR-1 Pgp. Previous work in this laboratory has shown by both immunocytochemistry and Western blotting methods using BRI MAb MDR-1, 6/1C that both the VO1- VO1 and the Vim # 1 cell lines do not express MDR-1 Pgp (Moran *et al.*, 1997). It appeared, therefore, that antibody 6/1G was specifically recognising the MDR-3 gene product. These Western blotting results were confirmed by immunocytochemical studies on fixed cells which revealed consistent cytoplasmic/ plasma membranous staining on both VO1-VO1 and Vim # 1 cells (section 3.3.5). Negligible staining was observed on preparations of FVB # c cells. The C219 MAb, which recognises the MDR-1 and MDR-3 encoded gene products, gave a similar pattern of reactivity on the MDR-3 cell lines. Two of the three polyclonal antibodies; anti-REG 1 and anti-REG 2 produced by Smit *et al.* (1994) showed a similar pattern of staining to that described here with

antibody 6/1G i.e. specific staining of the plasma membrane and cytoplasm in MDR-3 transgenic cells.

For additional characterisation a small number of cell lines of known MDR-3 status were probed with antibody 6/ 1G. As expected these cell lines showed MDR-3 Pgp reactivity patterns which reflected mRNA levels previously studied, indicating again the specificity of antibody 6/ 1G for MDR-3 encoded Pgp. Negligible staining was observed on the ovarian carcinoma cell line, A2780; this cell line has previously been shown not to express the MDR-3 gene (Herweijer *et al.,* 1990). The multi drug resistant non-small cell carcinoma cell line, DLKP-A and the sensitive DLKP cell line showed weak staining with antibody 6/1G on both immunocytochemical and Western blot analysis; DLKP-A and DLKP have previously been shown to have low level *MDR-3* gene expression using RT-PCR (O'Driscoll, PhD thesis 1994).

As the liver is known to express high levels of MDR-3 RNA and protein (section 1.18.2) archival formalin fixed paraffin wax embedded tissue was examined for reactivity with antibody 6/ 1G. An overall high level of reactivity was observed with antibody 6/1G in this tissue. No *pre* treatment of tissue with antigen retrival methods was required, indicating that this antibody recognised a formalin resistant epitope and therefore would be useful for assessment of MDR-3 Pgp status of archival material. Positive staining with antibody 6/1G was observed in hepatocytes, with specific staining observed on canalicular membranes. We have previously shown low level MDR-1 reactivity in liver (Moran *et al.,* 1997). Low level reactivity with antibody JSB-1 (MDR-1 specific) and strong reactivity with MAb C219 (which recognises both the MDR-1 and MDR-3 encoded gene products) has also been observed in liver (Bittl *et al.,* 1993). These results reflect the documented distribution patterns of MDR-1/ MDR-3 (Chin *et al.,* 1989, Smit *et al.,* 1994).

In order to further define a role for MDR-3, it was decided to probe a panel of human tumour cell lines with this MAb. MDR-3 Pgp reactivity was observed in a wide range of tumour cell lines of differing histological origin including ovarian, lung, breast and nasal carcinoma cell lines. Expression was also detected in both sensitive and MDR variants in some of these cell lines. These results suggest that MDR-3 is more widely expressed than thought originally, although its specific role in these

tumours requires further investigation. The fact that MDR-3 has now been proven to transport a range of cytotoxic drugs albeit at a lower rate than MDR-1 (Smith *et al.*, 2000), suggests again that the role of MDR-3 in malignancies warrants further study.

It was decided to purify antibody 6/1G before further studies were carried out on archival tissue. It was thought that purification might aid the interpretation of staining patterns as immunohistochemical analysis of liver showed apparent "sticky" non – specific staining. Since it is well documented that IgM antibodies are unstable and difficult to purify, conventional affinity chromatography methods cannot be utilised for purification of IgM antibodies. A purification system was used which employed immobilized mannan binding Protein (MBP); such a system had previously been successfully used for the purification of IgM from ascites fluid (Nevens *et al.,* 1992). However this system did not result in detectable recovery of IgM. It was decided to use an alternative approach, adaptation of the 6/ 1G hybridoma to grow in protein free medium. Resulting supernatants were tested by Western blotting and immunocytochemistry on control cell lines as before. This measure proved very satisfactory and results indicated that antibody was being successfully produced by this means. As may have been expected RID analysis of the quantity of antibody present in supernatants indicated that the amount of IgM was less that that produced in static flasks with the standard DMEM medium used routinely in this laboratory for growing of hybridomas. However it did appear, particularly on immunocytochemical analysis, that the protein free antibody supernatant resulted in more defined staining with antibody 6/ 1G.

A panel of invasive breast carcinomas was investigated with antibody 6/1G, in order to further elucidate the role of MDR-3, if any, outside the liver. MDR-3 Pgp was expressed to varying extents, in the majority of tumours studied. To our knowledge this is the first report of MDR-3 Pgp expression in invasive breast carcinomas. A small number of *post* chemotherapy treated tumours were studied, MDR-3 levels did not appear to increase following chemotherapeutic treatment indicating that MDR-3 expression was not associated with exposure to chemotherapeutic drugs. MDR-3 expression did not appear to correlate with known patient or tumour characteristics. In addition MDR-3 expression did not appear to correlate with any of the other MDR associated proteins studied in these patients. Taken together with results from cell

lines, it would appear that MDR-3 Pgp is probably expressed in a wider range of tumour types than that was thought originally. However although it may have widespread expression, its exact function in breast cancer and other solid malignancies needs to be addressed.

As the human MDR-3 gene is closely related to the murine mdr-2 gene, normal mouse kidney and liver together with a small number of cell lines from different species available to us were examined with antibody 6/1G. Antibody 6/1G identified a 170kD band on preparations of the Buffalo rat liver cell line BRL-3A but showed very low level reactivity on the African green monkey kidney cell line, BSC-1. A 170kD band was also identified on mouse liver and kidney preparations. These results reflected previously determined mdr-2 mRNA expression patterns for these particular tissues (Croop *et al.,* 1989, Teeter *et al.,* 1990), and indicated that antibody 6/1G cross-reacted with the mdr-2 gene and may therefore be useful for studies of cross-species MDR-3 encoded Pgp expression.

As previous reports of MDR-3 gene expression had been shown to be limited to leukaemias of B cell origin it was attempted to obtain a small panel of both B cell and T cell leukaemias in order to characterise antibody 6/1G further. Pro lymphocytic leukaemia is a variant of chronic lymphocytic leukaemia (CLL) which is usually refactory to chemotherapy effective in CLL. High expression of MDR-3 has been observed in cases of B cell pro lymphocytic leukaemia (PLL) with no MDR-1 expression whereas t cell PLL cases have been shown to have no MDR-3 expression but exhibit high levels of MDR-1 (Nooter *et al.,* 1990, Herweijer *et al.,* 1990).

As it had been indicated that MDR-3 may be involved in drug resistance in certain B-CLL leukaemias, it was decided to look at expression of antibody 6/ 1G in a range of B-cell leukaemias to address further any possible prognostic role for MDR-3 and whether it may be involved in chemotherapy resistance in these haematological malignancies (Nooter *et al.,* 1990, Herweijer *et al.,* 1990). For comparison MDR-1 Pgp levels were also examined using BRI MDR-1 MAb, 6/1C. MDR-1/ P170 is thought to be implicated as a cause of clinical resistance in AML and Multiple Myeloma (MM) and possibly in late stages of ALL and NHL. The significance of

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MDR-1 /P-170 expression in other haematological malignancies remains to be clarified.

Previous studies have shown that in general B-CLL leukaemias exhibit both MDR-1 and MDR-3 gene expression (Herweijer *et al.,* 1990, Sonneveld *et al.,* 1992, Nooter and Sonneveld 1993, Ludescher *et al.,* 1993). MDR-1 and MDR-3 Pgp expression were detected in two post-treatment samples of B-CLL. MDR-1 positivity was observed in both malignant and normal cells whereas MDR-3 positivity was confined to malignant cells. It is not thought that MDR-1 plays a major role in CLL which is in agreement with our results. A differential pattern of MDR-3 positive staining was observed in both cases with intense MDR-3 positivity, possibly indicating a more malignant phenotype being associated with the larger more malignant cells. This was not observed with MDR-1 positive staining.

Prior treatment has been associated with higher MDR-3 gene expression levels (no influence on MDR-1 levels) in one study (Herweijer *et al.,* 1990). Later work did not show such a correlation (Sonneveld *et al.,* 1992, Ludescher *et al.,* 1993). In patients with advanced B-CLL, MDR-3 mRNA expression (not MDR-1 expression) was significantly higher than in early stage disease (Sonneveld 1992). Ludscher *et al.,* (1993) also found a tendency towards higher MDR-3 mRNA expression in patients with advanced stages of CLL. These observations together with these studies suggest that MDR-3 overexpression (in our case as shown by intense MDR-3 positivity using antibody 6/1G) may represent a marker of a more malignant phenotype/ more aggressive disease in B-CLL.

As we also had access to a small panel of acute myleoid leukaemia (AML) cases we also looked at expression of MDR-1 and MDR-3 Pgp in these malignancies. In our study two untreated cases of AML (FAB (see section 1.5.1.), M0 and M1) showed MDR-1 positivity and were both negative for MDR-3. A stage M5a patient showed weak MDR-1 positivity but showed intense MDR-3 positivity. The MDR-1 expression observed is in agreement with previous studies where the MDR-1 gene has shown to be frequently expressed at diagnosis (i.e. prior to treatment) and at relapse. A review on AML by Marie *et al.* (1996) has reported a high correlation

between P-170 overexpression and clinical drug resistance. It is generally accepted that Pgp/ MDR-1 expression constitutes an unfavourable prognostic factor in AML.

Reports vary regarding the levels of MDR-1 expression within the various FAB classes of AML. One comprehensive study has reported that Pgp overexpression was detected more frequently in classes M4, M5A and M5B and less frequently in class M3 (Nussler *et al.,* 1996). This M3 promyelocytic subtype is particularly sensitive to chemotherapy. As the number of cases in our study was small Pgp expression cannot be associated with particular subclasses.

To date MDR-3 expression has not been reported in AML. In our study one AML M5A patient showed intense MDR-3 positivity in monoblasts. Initially it was thought that this AML case could be a conversion from ALL; such a transformation is commonly observed in childhood AML and is associated with treatment with Topoisomerase II α inhibitors. This patient had not, however, received any prior treatment.

Another explanation considered, was that since AML stage M5a has an undifferentiated monoblastic lineage; the possibility existed that MDR-3 is not expressed in certain sub types: M0, M1, M2 and M3, all of which have myeloblastic/ myelocytic morphology. Obviously MDR-3 reactivity in a wider range of AML subtypes would need to be examined to investigate these observations further. Perhaps it is the case that MDR-3 expression is restricted to the M5a and M5b subtypes which have a monocytic morphology. It has already been suggested that MDR-3 expression may be restricted to certain B-cell developmental stages; no MDR- 3 expression has been found in MM which presents with end stage B-cells (Herweijer *et al.,* 1990). MDR-3 expression appears to be restricted to those malignancies which represent intermediate and mature B-cell developmental stages; no MDR-3 was found in malignancies representing early B-cell development or in very mature B-cell tumours such as Waldenstrom and multiple myeloma (MM), in a study carried out by Nooter and Sonneveld (1993).

Interestingly in one B-NHL we examined the malignant cells comprised of lymphoplasmacytoid cells which are late stage B-cells; this sample was negative for both MDR-1 and MDR-3. Our preliminary results suggest that investigation of MDR-3 Pgp in representative cases from AML patients with various sub classes of the disease should be carried out in order to further define the association between MDR-3 and the B cell developmental stage.

Most previous work on MDR-1 encoded Pgp in NHL has been performed on frozen lymph node sections. In general more MDR-1/ Pgp expression is seen in treated than untreated patients. There appears to be no difference in Pgp expression between high and low grade lymphomas and between B and T sub types (Marie *et al.,* 1996). Adult T-cell lymphoma (ATL) has been shown to frequently express MDR-1/ Pgp at presentation (Kuwazuru *et al.,* 1990). As to whether MDR-1/ Pgp is an indicator of better clinical response; three studies have shown a correlation (Pileri *et al.,* 1991, Cheng *et al.,* 1993, Rodriguez *et al.,* 1993). A larger study did not show such a correlation (Niehans *et al.,* 1992). To date only one study has looked at MDR-3 mRNA expression in B-NHL, where expression was observed in 9 out of 21 B-NHL patients (Nooter and Sonneveld 1993). As we have already outlined in the results section, the B-NHL cases in our study (mantle cell, follicle centre and unclassified B-NHL) showed varying levels of MDR-1 and MDR-3 Pgp expression. A high level of MDR-3 positivity appeared to develop following treatment in a case of follicle centre lymphoma (atypical, CD5+ve). More work is obviously required to investigate whether MDR-3 expression is significantly related to treatment in B-NHL, and also to assess the significance of the MDR-1/ MDR-3 expression levels observed in other subtypes of this malignancy.

A new method for the generation of novel MAbs using dewaxed formalin fixed paraffin embedded archival tissue as an immunogen was successfully developed in this laboratory; antibodies directed against archival normal kidney have been generated and one of these, antibody 1/11C has been partially characterised (Moran *et al.,* 1998). As discussed in section 1.9. antibody epitopes are often masked or denatured during routine formalin fixation and processing of tissue; many antibodies therefore cannot be used for immunohistochemical analysis of archival material. Immunising with formalin fixed routinely processed tissue, facilitates the generation of MAbs against cellular antigens preserved during routine fixation and processing and which will therefore be useful in retrospective studies.

In an attempt to isolate possible novel breast cancer antigens, formalin fixed paraffin embedded breast tumour tissue was employed as an immunogen. An archival tumour was chosen from the group of invasive breast cancer tumours being investigated for expression of MDR associated protein expression (section 3.1). This tumour specimen, which was from a patient who relapsed with disease and died, was obtained at surgery, prior to the patient receiving CMF treatment. This *pre* treatment tumour, on immunohistochemical analysis did not appear to show Pgp or MRP-1 immunoreactivity to any great degree, suggesting that neither of these transport pumps was responsible for the observed clinical resistance. Thus, it was hoped that MAbs recognising possible novel antigens associated with resistance in breast cancer would be generated.

Following the method of Moran *et al.,* (1998), with minor modifications, antibodies to dewaxed formalin fixed paraffin embedded breast invasive ductal tumour tissue were successfully generated. The initial screening method i.e. immunohistochemical analysis of 375 positive clones on control breast tumour tissue was very time consuming but proved very discriminating allowing for the isolation of supernatants with very discrete staining patterns.

A possible problem with this system could of been the introduction of potentially toxic/ dangerous substances into the host mouse via the dewaxing of the immunogen and earlier formalin fixation/ tissue processing, but this was proved not to be the case. The excised spleen, on observation did not appear to have a healthy appearance

as was normally observed following immunisation with various protein immunogens routinely employed in this laboratory. The spleen in this case was darker than normal suggesting it was haemorraghic, in addition it also had an atrophic appearance. This was also observed with the generation of the MAb directed to normal kidney; however again, despite this observation, it proved that high numbers of viable spleen cells were indeed available for fusion (Moran *et al.,* 1998).

Following repeated immunohistochemical screening on control tissue sections 7 hybridoma clones were expanded. As all of these supernatants resulted in staining patterns which were very similar i.e. intense cytoplasmic reactivity (and membranous reactivity in some cells) of tumour cells it appeared, that these 7 hybridomas were secreting antibodies which were probably recognising the same antigen or a very closely related antigen. Following further screening, supernatant 5C3 was chosen for further study.

Antibody 5C3 showed intense cytoplasmic reactivity in tumour cells in all 3 control tumour blocks, however some reactivity was also observed in endothelial cells and muscle/ stromal cells. When viewed at high power, antibody 5C3 appeared to show positivity which appeared to be radiating towards the plasma membrane in a manner somewhat similar to that of cytokeratins/ intermediate filaments, suggesting that the antigen being recognised may be an intracellular filament like component. This was only an observation, at this stage it was not possible to assume from antibody staining patterns that the antigen reactive with antibody 5C3 was associated with any specific cell type. Preliminary studies on live cells did not indicate that the antigen was expressed on the cell surface, which was the case with the MAb generated using similar methodology by Pancino *et al.,* (1990). Obviously detailed ultrastructural analysis (i.e. electron microscopy) would need to be carried out to ascertain the target localisation of this antigen.

In one particular tumour control block however very intense staining was observed in individual tumour cells invading lymphatic channels (Figure 3.1.2.). A common feature of malignant tumour cells is their ability to promote the growth of surrounding fibrous tissue; this process is known as desmoplasmia. The desmoplasmic stromal reaction observed in this tumour was confirmed by the

histopathology report. Also, on a number of invasive tumours there appeared to be more intense 5C3 staining associated with infiltrating tumour cells compared to the non-invasive DCIS (comedo type) component, which often accompanies invasive ductal tumours (Figure 3.1.1.).These observations of increased staining with antibody 5C3 on infiltrating tumour cells suggested that the antigen recognised by 5C3 may possibly be associated with the invasion/ metastasis processes in breast cancer. However, this difference was not considered significant enough to confirm such as association with an infiltrating phenotype. Considerable variability in staining patterns was observed in this panel of tumours, ranging from all of tumour cells staining to individual tumour cells exhibiting intense immunoreactivity. Such heterogeneity of antigen expression has been reported previously in breast carcinomas, in different renal cell carcinomas and among tumour cell populations in any particular renal cell carcinoma with MAbs raised to breast carcinoma cells and renal carcinoma tissue (Stacker *et al.,* 1985, Yoshida & Iman 1989). It has been reported that such heterogeneity in the expression of cell surface antigens is cell cycle dependent (Schlom *et al.,* 1985, Grenier *et al.,* 1987). Perhaps the expression reactive 5C3 antigen was also cell cycle dependent, thus contributing to the heterogenous expression observed.

There did not appear to be any increase in 5C3 positivity associated with *post* treatment tumours, suggesting that chemotherapy treatment did not appear to have any effect in the expression of the 5C3 reactive antigen. In addition positive staining was not exclusively associated with tumour cells, normal breast ducts also showed reactivity with antibody 5C3. The expression of antibody 5C3 was also evaluated on a small panel of non malignant breast tissues. Heterogenous expression of antibody 5C3 was observed in these benign breast tumours known as fibroadenomas.

A panel of cell lines derived from various human breast cancers including a number of ductal breast tumours were also studied. As expected strong reactivity was associated with all breast tumour cell lines examined, although there were some differences in the intensity of the staining observed. As antibody 5C3 was apparently showing similar reactivity in ductal breast cells lines to that seen in tumour sections from the ductal tumour block which was used as immunogen, this suggested that this antibody was recognising an antigen directed against a conserved native epitope *in*

vivo rather than to an artificial neo epitope generated during the fixation/ processing. The non tumourogenic breast cell line MCF-10A appeared to have a reduced level of staining compared to breast tumour cell lines. However as this was the only non cancer breast cell line available, no definitive conclusion could be drawn from this observation. In agreement with the heterogenous staining patterns observed on archival breast tumours, considerable variations both in the intensity and in the numbers of tumours cells staining were also observed with cell lines. Thus the 5C3 reactive antigen appeared to be heterogenously expressed by all of the breast tumour cell lines investigated irrespective of their histological subtype and in addition this expression did not appear to be confined to malignant breast phenotypes.

Obviously the best outcome in the generation of MAb against tumour tissue would be the recognition of cellular antigen totally confined to malignant tissue with no expression associated with normal tissue. Although several MAbs have been generated against breast cancer associated antigens, very few of these recognise antigenic determinants which are exclusively expressed in breast cancer and not in normal tissue (section 1.4.3.) These preliminary results suggested that antibody 5C3 was not recognising a specific breast tumour associated antigen, it was more likely that this antigen was an as yet unidentified breast antigen expressed in the normal breast which may become over expressed (or possibly underexpressed) during the malignant process.

A number of archival ovarian tumours together with a panel of tumour cell lines of various histological origins including ovarian, lung and leukaemia showed varying degrees of 5C3 reactivity, indicating that expression of the 5C3 reactive antigen was not confined to breast tumours, it appeared to have a wide spectrum of reactivity among different tumour types. These characterisation studies did reveal that the sensitive variant of the non small cell lung tumour cell line, DLKP and the ovarian adenocarcinoma cell line, OAW42 appeared to show weaker reactivity with antibody 5C3 than their MDR variants, again as this was only observed in 2 cell lines no conclusion could be made. Another observation was a melphalan drug selected variant of the human nasal carcinoma cell line which failed to show any reactivity with antibody 5C3, however until 5C3 expression is studied in the parental RPMI nasal carcinoma cell line, the significance of this observation cannot be addressed.

In agreement with immunocytochemical analysis, results from Western blot analysis with antibody 5C3 indicated that the reactive antigen is heterogenously expressed in number of breast carcinoma cells lines including a number of ductal tumour cell lines and in a non tumourogenic cell line (section 3.3.). Again results indicated that this antigen was not exclusively expressed by breast carcinoma cell lines; expression was also observed in lung, ovarian, and colon tumour cells lines. These Western blotting studies revealed a series of bands reactive with antibody 5C3 at approx. 210kDa, 160kD,a 100kDa and 50kDa on crude cell lysates of the ductal breast tumour cell lines, ZR-75-1, BT-20, MDA-MB-231 and MDA-MB-435S. However the relative expression of these bands appeared to vary among some cell lines and individual Western blot.. Interestingly a lot of background was observed when membranes were blocked for longer than one hour with 5% dried milk powder (Blotto), the standard blocking system used in this laboratory. This observation suggested that the 5C3 reactive antigen was possibly reacting with a component in human milk.

A very distinct band at higher than 210kDa was consistently identified on the ductal carcinoma cell line, ZR-75-1; this band appeared to be at a slightly lower molecular weight in all other cell lines probed with antibody 5C3. This band was consistently identified on all immunoblots regardless of the relative expression of the lower bands, suggesting that this may be the major band. The ovarian and colon tumour cell lines which were probed with antibody 5C3 again revealed similar bands to those identified in breast cell lines, however the 210kD was not detected as strongly in these cell lines as was observed in some of the breast cell lines. One possible explanation for the observed heterogeneity of the 5C3 reactive bands could be that of different glycoslyated forms of this protein being expressed by different cell lines. Glycosylated proteins when analysed on SDS-PAGE generally show higher than expected molecular weights. It is possible that such altered glycosylation could result in different proteins with different antigenic profiles amongst various cell lines studied. Aberrant glycosylation is one of the many molecular changes that accompany malignant transformation. Such altered glycosylation of mucins is thought to probably be responsible for many antigenic differences in mucin expression between carcinomas and adjacent normal epithelial tissues (reviewed by Devine & Mc Kenzie, 1992).

As a supply of normal mouse tissue was readily available, it was decided to probe a panel of partially purified normal murine tissues with antibody 5C3. The major band in these tissues was approx. 70kDa; a distinct band was observed in spleen and to a lesser degree in lung tissue. These results indicated that the antigen being recognised by antibody 5C3 was indeed expressed in murine tissues, suggesting that this antigen was probably conserved amongst species. A possible explanation for the differences in molecular weights of observed bands with these tissues and those observed when crude cell lysates of cell lines were separated, could possibly be attributed to the fact that they were differentially centrifuged and the resultant membrane fractions *only* (not the supernatants) were separated on SDS-PAGE. Thus the observed 5C3 reactive bands from tissues represent the expression of the antigen in the cell membrane fraction and not the cytosol/ cytoplasmic fraction. Crude cell lysates from cells would have included cytoplasmic as well as membrane material. In agreement with immunocytochemical results, this result suggests that the 5C3 reactive antigen is localised to the cell membrane as well as the cytoplasm of the cell. It was also possible that perhaps the distribution of the antigen in question varies among malignant and normal cells types, reflected by the heterogenous expression observed on Western Blot analysis.

It was attempted to probe commercially supplied human tissue lysates with antibody 5C3; however due to problems in the solublisation of the preparations no results were obtained therefore a direct comparison of the expression of the antigen in question between murine and human tissue was not possible.

It was decided to carry out immunoprecipitation studies; immunoprecipitated protein could then be separated on SDS-PAGE and then sequenced. In addition this method might also reveal the precise molecular weight of the 5C3 reactive antigen. As the ZR-75-1 cell line showed consistent reactivity with antibody 5C3 both on immunocytochemical analysis and Western blot analysis it was decided to use this cell line in an attempt to immunoprecipitate the reactive antigen. This cell line was derived from an infiltrating ductal carcinoma of the breast (Engel *et al.*, 1978).

Immunoprecipation was carried out by using a cocktail of protein G and protein L, (the latter being a novel Ig binding bacterial cell wall protein (Bjorck 1988)) to pull

out the 5C3 reactive antigen from crude cell lysates of ZR-75-1 cells which were labelled directly with antibody 5C3. As results were obtained using this direct labelling system, a biotinylation step was not required. Following separation on 7.5% SDS PAGE, Western blot analysis of the ZR-75-1 immunoprecipitates revealed one band at approx. 175kDa when probed with antibody 5C3 (the predominant band detected by Western blotting of ZR-75-1 lysates was at approx. 210 kDa). No other bands were detected, however on some Western blots a very faint band could be observed at approx. 110 kDa. No band except that of IgG was detected in immunoprecipitates of mouse IgG.

The fact that the reactive band had an apparent molecular weight of 175 kDa was an unexpected result, this band was not identified on Western blots. Immunoprecipitation was carried out by pulling out the antigen in its native state from ZR-75-1 cells (although immunoprecipitates were placed in reducing buffer prior to loading on SDS Page), which may explain the difference. Differences in molecular weights of proteins in reducing/ non reducing conditions are well documented. Non-reduced immunoprecipitates were also separated on SDS-PAGE, however these samples did not resolve correctly and therefore no reactive bands could be identified. Another possibility to consider was that the immunoprecipitation procedure was pulling out the antigen in a different glycosylated form.

Supernatants which were retained following antibody incubation with antigen and prior to washing were also separated on SDS-PAGE. All of the 5C3 reactive bands including the 175 kDa band could be observed on Western Blots, when supernatants (and 50:50 mixtures of supernatant/ immunoprecipitate) were probed with antibody 5C3. Although this immunoprecipitation method used here proved sensitive and consistent, it did appear that some of the antigen of interest i.e. the 175 kDa band was not being pulled out. Use of smaller volumes of ZR-75-1 lysate/ antibody /protein L/G and shortened incubation times did result in a stronger reactive band being detected on Western Blots. The final volume is considered crucial to successful immunoprecipitation; volumes used were within the range recommended by Harlow and Lane (1999). As the objective of these studies was to obtain enough protein for sequencing, on assessment of Coomassie R-250 stained gels it was considered that there was enough of the 175kDa band present to be excised and sequenced.

In preparation for N-terminal sequencing further ZR-75-1 immunoprecipitates were transferred to Sequiblot ™ PVDF membrane and stained with Coomassie Blue R-250. The major band at approx. 175 kD when lined up, corresponded to that observed on Western blots of immunoprecipitated cells. This band was excised from PVDF membranes blotted from 5 well SDS PAGE gels and processed for N terminal sequencing. However no sequencing data could be obtained which suggested that the protein was N terminally blocked. Further attempts to avoid N-terminal blockage by the addition of 0.1% thioglycollic acid to the running buffer did not prove successful, lysates appeared to diffuse throughout gel which did not resolve properly. As it was unlikely that N-terminal blockage could be avoided it was decided that the best option would be to attempt to carry out internal sequencing.

Again ZR-75-1 immunoprecipitates were separated on SDS PAGE, and the gel was then stained with Comassie Blue R-250. The band of interest i.e. the 175 kDa band was excised from gel and sent to Eurosequence (The Netherlands) for internal sequencing analysis. Sequencing of HPLC fractions obtained from the 175kDa band revealed that the protein sample (i.e the 175 kDa band) contained a mixture of two peptides, their sequence levels were approx. 1:2. Actual sequence analysis results are presented in Appendix IV.

Data base searching carried out by Eurosequence indicated that that these peptides corresponded to human cytokeratin 6B and human cytokeratin 9. Twice as much of the peptide corressponding to cytokeratin 9 was present compared to the peptide corressponding to cytokeratin 6. Searching of protein databases was also carried out here to confirm results from Eurosequence. The first polypeptide sequence showed 100% homology with a 14 a.a.sequence of human cytokeratin 9. However these searches indicated that this second polypeptide showed 100% homology with a 12 a.a. sequence that was present in cytokeratin 6D (highest score), 6B, 6C, 6E, 6F, 6A, cytokeratin 5 and cytokeratin 2 (lowest score). Our observation of a better match for for this second peptide to cytokeratin 6D rather than 6B, may perhaps be explained by the fact that Cytokeratin 6D has a shorter a.a. sequence than the other isomers of the cytokeratin 6 family and therefore achieved the highest score. It also appeared that all cytokeratin 6 isomers achieved a higher score than cytokeratins 5 or 2. Taken

together these results suggested that this protein represented a member of the cytokeratin 6 family, however it is not possible to fully conclude which cytokeratin 6 isoform this peptide represents. It is thought that there are at least six isoforms of human cytokeratin 6, the 6A being the most common isoform (Takahashi *et al.,* 1995).

Cytokeratins constitute a family of closely related, phylogenetically conserved distinct proteins that are biochemically and immunologically related (Osborn *et al.,* 1982). There are two types of cytokeratins; the smaller (m.w. 40-56.5 kDa) type 1 keratins (cytokeratins 9-20) which are relatively acidic and the larger (m.w. 53-67 kDa) type II cytokeratins which are largely basic-neutral (cytokeratins 1-8) (Moll *et al.,* 1982). Cytokeratins possess a pronounced sequence diversity that is not found in other intermediate filament family proteins. The alpha helical regions of type I cytokeratins share 50-99% sequence identity, whereas type II cytokeratins display 30% homology in these regions (Steinert *et al.,* 1993). During filament formation, cytokeratin filaments form obligate heteropolymers made of type I and type II, usually in a 1:1 molar ratio. Sequence results suggested then the 5C3 reactive 175 kDa band may represent a cytokeratin heteroploymer, composed of the type I cytokeratin 9 and the type II cytokeratin 6. The respective molecular weights of the individual proteins, cytokeratin 9 (63 kDa), cytokeratin 6D (42kDa) and cytokeratin 6B (59 kDa), suggest the most likely partner for cytokeratin 9 would be cytokeratin 6B. However as all of the molecular weights were only estimated it was not possible to conclude fully which cytokeratin 6 isoform was most likely to be the partner involved.

As a follow up to these results it was decided to conduct a small number of experiments to address these findings further. Following immunoprecipitation of ZR-75-1 cells, samples were reduced, this normally would result in any complex if present, being disassociated. However in this case the proposed "complex" appeared to be intact even after reduction and boiling for 1 minute. It is known that during the differentiation process cytokeratin complexes form disulphide bonds to stabilise the complex, perhaps this was the case with the proposed complex detected in ZR-75-1 immunoprecipitates. Therefore it was attempted to disassociate this "complex" by employing a prolonged boiling time of 10 minutes.

Following attempted disassociation these immunoprecipitates were separated on SDS-PAGE. A band at approx. 60kDa in addition to the 175kDa band was observed, indicating that the complex had disassociated to some degree. As the molecular weights of both cytokeratin 9 and cytokeratin 6 are similar it could not be concluded which cytokeratin this approx. 60kDa band represented. When separated proteins were probed with antibody 5C3 however, only IgG bands were detected, no specific 5C3 bands were detected. This result indicated that antibody 5C3 only recognised the hetropolymer, cytokeratin 6/9 when complexed. It still was not clear whether antibody 5C3 recognised a similar epitope on both cytokeratins or whether it only recognised an epitope on 1 cytokeratin but appeared to recognise both as they were complexed.

All intermediate filament proteins contain a central rod domain composed of 4 α - helical segments of conserved size that possess a repeating heptad sequence motif, interspersed with flexible linker sequences, and flanked on the amino and carboxy termini by head and tail domains. The helical rod domain, 2B contains the most highly conserved a.a. sequences amongst the various cytokeratins, the 12 a.a. sequence present in all cytokeratin 6 isomers and in cytokeratin 5 which showed 100% homology with one of the peptides sequenced here was located near this region. A MAb, TSI, directed against cytokeratin 8 has been shown to have a high degree of recognition of all other type II cytokeratins (Johansson *et al.,* 1999). The epitope recognised by MAb TSI has been shown to be contained within a.a. 343-357 within the rod domain of cytokeratin 8 (Johansson *et al.,* 1999). To ascertain the exact epitope of antibody 5C3, it would be necessary to screen peptides covering the entire molecules cytokeratin 6 and 9 and test for antibody binding. This would conclusively reveal if this MAb was recognising just I cytokeratin or similar epitope on both cytokeratins. Such work was beyond the scope of this thesis.

When the immunoprecipitates which were boiled for 10 minutes were probed with a a cytokeratin 9 specific MAb (Research Diagnostics, US), a cytokeratin 9 reactive band at approx. 62 kDa was identified. Immunoprecipitates which were boiled previously for 1 minutes were also probed with the cytokeratin 9 specific MAb, only a very faint band could be identified. Immunoprecipitates were also probed with a

cytokeratin 6 specific MAb (Neomarkers, UK). No cytokeratin 6 reactive bands could be detected on immunoprecipitates which were boiled for either 10 minutes or 1 minute. These results suggested that antibody 5C3 was recognising an epitope on cytokeratin 9 and not on cytokeratin 6, however as these two cytokeratins were complexed it appeared that antibody 5C3 was recognising both cytokeratins.

However when *crude cell lysates* of ZR-75-1 cells were also probed with cytokeratin 6 and 9 specific MAbs, results were unexpected. Cytokeratin 6 reactive bands were identified on separated *crude cell lysates* when probed with cytokeratin 6 MAb, on probing with cytokeratin 9 MAb no specific bands were detected. These results suggested that during the immunoprecipitation process the conformation of the cytokeratin 9 protein is altered somewhat and antigenic sites may have been masked in the crude cell lysates. As there is twice as much of this cytokeratin in complex perhaps this cytokeratin may be masking antigenic sites on cytokeratin 6, and thus it is not detected in *immunoprecipitated samples*. Taken together it would appear that antibody 5C3 recognises this hetropolymer of "complexed" cytokeratin 9 and 6.

It is well recognised that complex cytokeratin polypeptide patterns exist in human cancers (Moll *et al.,* 1982). Expression of the cytokeratin network has been shown to be altered during malignant transformation, distinctive cytokeratin distributions have been observed between breast cancers (Jarasch *et al.,* 1988). The proposed complex presented in these results between this particular type I and type II cytokeratin has not been reported to date.

Cytokeratin 6 has been shown to be induced under hyperproliferative conditions such as benign or malignant tumours, psoriasis and wound healing. This cytokeratin in a complex with cytokeratin 5 has previously been detected both in invasive breast cancers and DCIS breast cancers (Otterbach *et al.,* 2000). This cytokeratin has been shown to be expressed in a distinctive manner in the developing mammary gland (Smith *et al.,* 1990). It is also thought that cytokeratin 6 may be involved in the tumour progression of skin tumours (Bowden 1995).

Cytokeratin 9 expression has not been reported to our knowledge in human cancer, it has only been observed in plantar epidermis and has been shown to be a component

in the perinuclear ring of the manchette of rat spermatids (Mochida *et al.,* 2000). The manchette is a transient tubulin containing structure which is developed during mammalian spermiogenesis, it is thought to have a role in nuclear shaping, however its precise function is not known. Mochida *et al.,* (2000) in an attempt to see if cytokeratin 9 exists with a partner, found expression of another rat keratin which shows homolgy with human keratin 5 associated with the microtubules of the manchette (Mochida *et al.,* manuscript in preparation). The fact that cytokeratin 5 shows high homology with cytokeratin 6 suggests therefore that cytokeratin 9 does indeed form a partner with cytokeratin 6. It cannot be ruled out, that one sequenced peptide here is actually cytokeratin 5, as discussed previously. Interestingly also, epidermolytic palmoplantar keratosis (EPPK) co segregates with a cytokeratin 9 mutation in breast and ovarian cancer (Torchard *et al.* 1994). The finding of cytokeratin 9 complexed with its type II partner cytokeratin 6 (or perhaps cytokeratin 5) in human breast cancer is novel and warrants further investigation to see if expression of this complex has any prognostic significance in this disease.

5. Conclusions and Future Work

Conclusions

The expression of MRP-1 protein was studied in a series of 204 invasive breast cancers; 46 of these tumours were also studied for expression of a range of MDR1 associated proteins. As expected multivariate analysis revealed that nodal status and tumour size were independent prognostic factors for relapse free survival in patients with invasive breast cancer; tumour (histological) grade was identified an an independent prognostic factor for overall survival.

Expression of MRP-1 protein at diagnosis did not offer any prognostic value in those patients who did not receive chemotherapy. In patients who did receive chemotherapy however, a prognostic role for MRP-1 was shown. MRP-1 protein expression at diagnosis was shown to be an independent prognostic factor for overall survival in node positive breast cancer patients treated with CMF based chemotherapy, absence of MRP-1 expression at diagnosis was significantly associated with increased overall survival. It can also be concluded that this prognostic role of MRP-1 was greater in patients with high grade i.e. grade III tumours. A larger study would be required however to establish any possible predictive role for MRP-1 protein.

MRP-1 protein was significantly associated with higher histological grade in chemotherapy treated patients, suggesting that MRP-1 expression is possibly associated with a more aggressive tumour phenotype in these patients.

The expression of cerbB-2 protein was associated with a worse prognosis (shortened relapse free survival times and shortened overall survival times) in *chemotherapy treated* node positive patients. No conclusion could be drawn regarding any possible predictive role as cerbB-2 protein levels were not studied in any tumours from *non chemotherapy* treated patients.

Preliminary analysis did suggest that the *co-expression* of MRP-1 and cerbB-2 may possibly have stronger prognostic value in *node positive* chemotherapy treated patients than expression of either individual marker. However as the numbers of

patients analysed for both markers were small no statistical analysis could be carried out to address the significance of this observation.

cerbB-2, MDR-1, Bcl-2 and survivin protein expression were not significantly associated with the age of patients at diagnosis, node status, tumour size or histological subtype or grade. Conclusions regarding a possible association between MDR-1, Bcl-2 and survivin expression and clinical outcome, cannot be drawn as the numbers studied were too small. Expression of none of the proteins studied was associated with chemotherapy treatment.

A new monoclonal antibody which specifically recognises the MDR-3 encoded gene product was successfully generated and characterised during the course of this thesis. As discussed, it has now been established that MDR-3 can in fact transport certain cytotoxic drugs. MDR-3 Pgp was present in a number of human breast tumours and appears to be present in a range of human tumour cell lines of various histogenic origins including breast and other solid tumour types. Taken together these results suggest that MDR-3 Pgp is present to some degree in a number of human tumour types, but the significance of its presence has yet to be eluciadated.

Studies on a small panel of B cell malignancies suggested that MDR-3 Pgp may possibly be associated with a more malignant phenotype in B-CLL. In addition preliminary results indicate that MDR-3 may be associated with certain developmental stages of B cell development. Results from investigation of B-NHL cases were inconclusive, although MDR-3 Pgp showed a significant increase in expression following treatment in one specific patient.

In an attempt to identify a possible novel breast cancer resistance-associated antigen, MAb 5C3 was generated using a novel source of immunogen i.e. paraffin wax embedded formalin fixed archival breast tumour tissue. The novel source of antigen used to generate MAb 5C3, (although time consuming) proved successful and can therefore be applied to a large number of prospective tumours which are clinically interesting to use as possible immunogens.

It appeared from characterisation studies that antibody 5C3 did not recognise any of the well established breast tumour associated antigens, but was recognising a breast antigen expressed in the normal breast which may become over expressed (or possibly underexpressed) during the malignant process.

Subsequent internal protein sequencing analysis revealed that MAb 5C3 recognises a complex of cytokeratin 9 and cytokeratin 6. A comprehensive literature has shown that such a complex between this particular type I and type II cytokeratin has not been reported in breast cancer, or indeed any other malignancy to date.

Future Work Suggested

1. Further work should include a study of MDR associated proteins in a larger series of invasive breast carcinoma patients. It is essential for any future studies that patient numbers are large enough to allow for statistical analysis.

2. A larger study would establish if perhaps MRP-1 may hold predictive value in certain sub-groups of patients treated with CMF based chemotherapy (i.e. stratified by tumour size, tumour histology, tumour grade etc.). The numbers of patients studied here were too small to investigate the possible predictive role of MRP-1 (and indeed any further prognostic role) in such sub-types of invasive breast carcinomas.

3. Currently for node negative breast cancer patients there are few reliable prognostic markers, so identification of new prognostic markers (and indeed possible predictive markers) for these patients is important. A larger study may address this.

4. The associations between MDR-1 and survivin protein expression and clinical outcome (and the significance of the nuclear staining of survivin) observed in this study needs to be addressed in a larger series of patients. A larger study would also reveal if survivin is actually associated with a better outcome in invasive breast cancer, which in itself, this would be an important observation. The observed correlation between survivin and MDR-1 expression patterns observed should also be addressed in a further study.

5. Also, the observation of the co-expression of MRP-1 and cerbB-2 offering stronger prognostic power than either of the individual proteins as regards clinical outcome should also be addressed further, to see if this observation is significant.

6. It may also be useful to look at the expression of the other members of the cerbB-2 family in tumours from the patients investigated, such a study will further address the role of these proteins in breast cancer.

7. In addition as recently *neo* adjuvant chemotherapy is being used successfully in the breast cancer clinic, it is important to investigate the expression of MDR associated proteins in *pre* and post chemotherapy treated tumours specimens from patients treated in this fashion.

8. When MAbs to other members of the MRP family (including BRCP) which work on archival material become available it may be interesting to look at expression of these MRPs in breast cancer and address any possible prognostic/ predictive significance and investigate their contribution, if any, to chemotherapy resistance in breast cancer.

9. As it has recently been shown that MDR-3 can transport certain cytostatic drugs, antibody 6/ 1G may be useful in further assessment of MDR-3 Pgp status in malignant cells and tissues and establish if indeed MDR-3 is playing a role in drug resistance. As results presented here suggest that MDR-3 Pgp is present in a number of invasive breast tumours, these preliminary observations should be confirmed by immunohistochemical analysis of further breast tumours including other histological subtype of tumours and non-invasive DCIS tumours.

10. Also immunohistochemical studies of other tumour types are required to see if the expression of MDR-3 Pgp observed in the cell lines studied here is reflected *in vivo*; and to establish if this observed MDR-3 Pgp expression in various malignancies is significant i.e. if there any association with clinical resistance in individual patients. Therefore tumours treated with various chemotherapy regimes might be included in a follow up study in order to establish if there is a role for MDR-3 Pgp in malignancies other than haematological ones.

11. As antibody 6/ 1G is the first MAb that specifically recognises the MDR-3 encoded gene product, this antibody should prove useful in further defining the physiological role, if any, of the MDR-3 encoded gene product outside the liver. This antibody will also be useful in the diagnosis of type III PFIC as well as other liver disorders such as Dubin-Johnson syndrome and ICP where mutations in the MDR-3 gene are thought to be involved (discussed in section 1.7.3).

12. To confirm results presented here, regarding MDR-3 expression in B-CLL, AML and B-NHL, further studies with increased patient numbers need to be carried out to define the role of MDR-3 in these malignancies and other haematological malignancies such as MM which is incurable and also subgroups of B-cell chronic lymphocytic disorders (B-CLPD). Such studies may reveal MDR-3 as having possible prognostic value in these patients. In addition the correlation of MDR-3 with the molecular abnormalities associated with these malignancies should be addressed in any future work.

13. Recent studies have suggested that B-CLL can be subdivided into two major subgroups on the basis of whether or not the IgV_H genes are mutated, those patients with unmutated genes have a poor prognosis whereas those with mutated IgV_H have a good prognosis. The possible prognostic contribution of MDR-3 (i.e. a possible reliable surrogate immunophenotypic marker) in the identification of B-CLL patients with unmutated or mutated IgV_H genes might also be addressed.

14. Any further studies should also include investigation of the expression of MDR-1 Pgp and LRP protein, which as discussed previously may be implicated in the chemoresistance of some haematological malignancies.

15. Further work with specific antibodies should confirm which member of the cytokeratin 6 family is actually involved in the proposed complex being recognised by MAb 5C3. By employing a MAb specific for cytokeratin 5 also, it will be possible to deduce if this cytokeratin is involved in the proposed complex instead of cytokeratin 6. The possibility of this being the case cannot be ruled out at present based on analysis of database searching.

16. The expression of this proposed cytokeratin complex needs to be investigated in additional archival samples of normal breast tissues, invasive breast tumours, (representing all histological subtypes of breast cancer), DCIS and other tumours of varying histological origin to address any prognostic or predictive role for co-expression of these two cytokeratins.

17. Expression of this antigen complex should also be investigated in metastatic breast carcinomas including lymphatic nodes completely replaced by metastatic disease of known mammary origin, to see if the antigen is more highly expressed in these tissues compared to primary carcinoma tissue.

18. In order to identify an antigen which may be a possible marker of chemoresistance, it may also be interesting to use *post* chemotherapy treatment archival tumour from the same patient (whose pre treatment archival tumour was used), as a possible immunogen. Thus it may be possible to identify further novel proteins, including tumour specific antigens which may have ultimate prognostic implications.

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7. Appendices

Appendix I

ABBREVIATIONS

ABC/HRP	Streptavidin/biotin-horseradish peroxidase conjugate
ADCC	antibody dependent cell mediated cytotoxicity
ACMF	Adriamycin cyclophosamide methotrexate 5-fluorouracil
ADEPT	antibody directed enzyme pro drug therapy
Adr	Adriamycin
AI	apoptotic index
ALL	acute lymphocytic leukaemia
AML	acute myeloid leukaemia
ANLL	acute non lymphocytic leukaemia
APC	antigen presenting cells
ATCC	American Tissue Culture Collection
BCA	Bicinchoninic acid
B-CLPD	B-cell lymphocytic proliferative diseases
BRCA 1	breast cancer susceptibility gene
BRCP	breast cancer resistance protein
BSA	Bovine serum albumin
CDR's	antigen binding complementarity determining regions
CEA	carcinoembryonic antigen
CLL	chronic lymphocytic leukaemia
CMF	cyclophosamide methotrexate 5-fluorouracil
DAB	Diaminobenzidine
DCIS	ductal carcinoma *in situ*
dH_2O	Deionised water/glass distilled water
DMEM	Dulbeccos Modified Eagles Medium

DMSO	Dimethyl sulfoximide
DNA	Deoxyribonucleic acid
DOX	Doxorubicin (Adriamycin)
EBV	Epstein Barr virus
ECACC	European Collection of Animal Cell Culture
ECL	Enhanced chemiluminescence
ECM	extracapsular metastasis
EDTA	Ethylene diamino tetra-acetic acid
ELISA	Enzyme-linked immunoabsorbent assay
EPPK	epidermolytic keratosis
ER	estrogen receptor
FAB	French American British
FCS	Foetal calf serum
FISH	fluoresence *in situ* hybridisation
FITC	Fluoroscein-isocyanate
GPx	glutathione peroxidase
GST	glutathione –s-transferase
HACA	human anti chimeric antibody
HAMA	human anti mouse antibody
HAT	Hypoxanthine, aminopterin, thymidine
HCL	hairy cell leukaemia
HCL	Hydrochloric acid
HEPES	4-(2-hydroxethyl-)-piperazine ethane sulphonic acid
HGPRT	hypoxanthine guanine phosphoribosyl transferase
HT	Hypoxanthine, thymidine
ICP	intrahepatic choleostasis of pregnancy
IMS	Industrial methylated spirits
kDa	KiloDalton

LCIS	lobular carcinoma *in situ*
LCM	laser capture microscopy
LGL	large cell granulomatous lymphoma
LRP	Lung resistance related protein
MAb/s	Monoclonal antibody/monoclonal antibodies
MBV	mannon binding protein
MCL	mantle cell leukaemia
MDR	Multidrug resistance
MDR1	Multidrug resistance protein 1 gene (P-gp encoding gene)
MEM	Minimum Essential Medium
$MgCl_2$	Magnesium chloride
MM	multiple myeloma
MMP	matrix metalloproteinase
mRNA	Messenger RNA
MRP1	Multidrug resistance-associated protein 1 (protein or gene)
Mw	Molecular weight
NaCl	Sodium chloride
$NaHCO_3$	Sodium bicarbonate
NaOH	Sodium hydroxide
NCTCC	The National Cell and Tissue Culture Centre
NEAA	Non-essential amino acids
NHL	non-Hodgkins lymphoma
NSCLC	Non-small cell lung carcinoma
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PEG	Polyethylene glycol
P-gp	P-glycoprotein or P-170 (MDR1 gene protein product)

Pgr/ PR	progesterone receptor
PKC	protein kinase C
PMSF	Phenylmethyl sulfonyl fluoride
PNAG	Phenyl-N-acetyl-α-D-galactosaminide
PNPP	P-nitrophenyl phosphate
Pristane	2,6,10,14 tetramethylpentadecane
PSA	prostate specific antigen
PVDF	Polyvinyldifluoride (blotting membrane)
RID	radioimmunodiffusion
RNA	Ribonucleic acid
RT	room temperature
SCID	severely combined immune deficiency
SDS	Sodium dodecyl sulphate
SEREX	serological identification of antigens by recombinant expression cloning
SPF	S - phase fraction
SSCP	single strand confirmation polymorphism
TBS	Tris buffered saline
TEMED	N,N,N,N,-tetramethyl-ethylenediamine
tPA	tissue type plasminogen activator
Tris	Tris (hydroxymethyl) aminomethane
TV	Trypsin/versene
UICC	international union against cancer
uPA	urokinase plasminogen activator
UV	Ultra violet
VPNI	Van Nuys prognostic index
vol/vol	Volume to volume ratio
w/vol	Weight to volume ratio

Appendix II

LIST OF SUPPLIERS

- Amersham International plc. Bucks HP7 9NA, UK.
- Antec International Ltd. Sudbury, Suffolk CO16 6XD, UK.
- BDH Laboratory Supplies, Poole, BDH15 1TD, England.
- Beckman Instruments (UK) Ltd. High Wycombe, Bucks HP12 4YH, UK.
- Becton Dickinson UK Ltd, Between Towns Road, Crowley, Oxford OX4 3LY, UK.
- Bio Rad laboratories Ltd. Herts HP2 7TD, UK.
- Bioresearch Ireland, Forbairt, Glasnevin, Dublin 9, Ireland.
- Biosyn Ltd., 10 Malone Road, Belfast, BT9 5BN, Northern Ireland.
- Roche Diagnostics GMBH, Bell Lane, Lewes, East Sussex BN7 1LG, UK.
- Clintech, Clacton-on-Sea, Essex, UK.
- Costar Cambridge MA, USA.
- Dako, 16 Manor Courtyard, Hughenden Avenue, High Wycombe, Bucks HP13 5RE, UK.
- Gelman, United Kingdom Pall Gelman Sciences, Brackmills Business Park, Caswell Road, Northampton NN4 7EZ, UK.
- Gibco, Life Technologies Ltd, 3 Fountain Drive, Inchinnan Buisness Park, Paisley PA4 9RF, UK.
- Greiner Labortechnik Gmbh, Maybachstabe 2, D-72636 Frickenhaussen.
- Hoefer. Hoefer Scientific Instruments, San Fransisco, USA.
- Immune Systems Ltd., PO Box 120, Paignton, TQ4 7XD, UK.
- Lab Vision Corporation, 47790 Westinghouse Dr, Fremont CA, 94539 USA.
- Merck, Magna Park, Little-worth, Leicestershire, UK.
- Novacastra Labs Ltd, Balliol, Business Park West, Benton Lane, Newcastle Upon Tyne, NE12 8EW, UK.
- NUNC A/S. Roskilde, Denmark.
- Chemical Products, R. Borghgraef s.a. Belgium.
- Nuaire, 2100 Fernbrook Lane, Plymouth, Minnesota 55447, USA.
- Oxoid Ltd, Basingstoke, Hampshire, UK.

- Pharmingen, BD Diagnostic Systems, Pottery Road, Kill O'The Grange, Dun Laoghaire, Co. Dublin, Ireland.
- Pierce, Post Office Box 117, Rockford, Illinois 61105 USA.
- Research Diagnostics Ltd. Pleasant Hill Rd, Flanders, NJ 07836, US.
- Sigma Diagnostics, St. Louis, MO 63178 USA.
- Sigma, Sigma-Aldrich Company LTD. Fancy Road, Poole, Dorset, BH12 4QH, UK.
- Sterlin Ltd, Middlesex TW148QS, UK.
- The Binding Site Ltd., Birmingham, UK.
- Vector Laboratories Ltd., 16 Wulfric Square, Bretton, Peterborough PE3 8RF, UK.
- Vector Laboratories, Inc. Burlingame, CA 94010 USA.

Appendix III

1. Water for cell culture

Ultrapure water was used in the preparation of all media and solutions. This water was purified to a standard of 12-18 Mm/cm resistance by a reverse osmosis system (Millipore Milli-RO 10 Plus and Maxima UF water system, ELGA). A digital display provided a visual indication of water quality.

2. Media Preparation

The basal media used during routine cell culture consisted of 10X media (DMEM (Sigma, D5648) and Hams-F12 (Gibco, H6760)) which were added to sterile ultra pure water, buffered with HEPES and $NaHCO_3$ and adjusted to a pH of 7.45-7.55 using sterile 1.5M HCL. The medium was then filtered through a sterile 0.22 μm bell filter (Gelman, G1423S) and stored at 4°C until required. Prior to use aliquots of media were supplemented with 2 mM L-glutamine (Gibco, 043-0503) and 5-15% FCS. Hybridoma and SP2/O-Ag14 myeloma cells were grown in a basic basal medium of commercially available DMEM, DMEM with Glutamax I (Glutamax is L-Amyl-LGlutamine, high glucose concentration – 4.5 mg (Gibco, 61965-026) supplemented with 10 % heat inactivated FCS (Myoclone, Gibco, 10082-147). This was further supplemented for hybridoma growth and hybridoma cloning with 1% Penicillin streptomycin and 5% Briclone (Archport Ltd.). Briclone is a conditioned medium collected from a human cell line, for use in post fusion stages of hybridoma production and cloning, replacing the function of feeder cells. Sterility checks (Tryptone soya broth, Tryptone soya agar and Thioglycollate) were routinely carried out on all media, media supplements and reagents used in cell culture.

3. *Mycoplasma* analysis (indirect staining procedure)

Mycoplasma negative NRK cells (Normal Rat Kidney fibroblasts) were used as indicator cells for this analysis. The cells were cultured with supernatant (harvested from test cell lines after 2-3 days confluency) from test cell lines for 4-5 days, fixed and stained with Hoescht fluorescent stain which binds specifically to DNA. Thus the nucleus of the cell and any *Mycoplasma* infection will be stained, this positive staining is seen as small fluorescent bodies in the cytoplasm of the NRK cells. Positive controls consisted of NRK cells infected with Mycoplasma enriched supernatants. In the case of hybridomas and SP2 cells, cells were passaged 3-4 times in antibiotic free medium prior to *Mycoplasma* testing.

TABLE 1 **Lysis buffer for immunoprecipitation studies**

All materials in for this buffer were supplied in the cellular labelling and immunoprecipitation kit supplied by Roche Diagnostics GmbH (Mannheim, Germany, 1647652).

* Complete ™, 1697498, Roche Diagnostics GmbH, Mannheim, Germany.

STOCK SOLUTIONS	**VOLUMES REQUIRED**
Core buffer	4ml
NaCl	1.8ml
25 X stock protease cocktail *	0.5ml
Nonidet P-40	1.2ml
Sodium deoxtcholate	0.6ml
dH_2O	4.3ml

TABLE 2 **Wash buffers for immunoprecipitation studies**

	WASH BUFFER 1	***WASH BUFFER 2***	***WASH BUFFER 3***
Final volume	24 ml (conc)	24 ml (conc)	24 ml (conc)
1 M Tris buffer	1.2 ml (50mM)	1.2 ml (50mM)	0.24 ml (10mM)
1 M NaCl	3.6 ml (159mM)	12 ml (500mM)	-
Nonidet P-40	0.24 ml	0.24 ml	-
$d.H_20$	19 ml	10.54 ml	23.8 ml

Appendix IV

Internal Sequencing Results from Eurosequence BV.

EUROSEQUENCE bv
Analysis and Synthesis of Proteins and DNA

INTERPRETATION N-TERMINAL PROTEIN SEQUENCE ANALYSIS RESULTS

See also 'Product Information N-terminal Protein Sequence Analysis' for explanation of 'Initial Yield' and amino acids in parentheses.

Our order number >> **010716**
Product code >> **01C318** Sample: **010716-B-T**

Sample amount	: **10%**
Date of analysis	: **August 24, 2001**
Performed	: **6 cycles**
Date of interpretation report	: **September 28, 2001**
Investigator	: **Dr. Wicher J. Weijer**

MAIN SEQUENCE

Position #	1	5
Amino Acid	**???-(Arg/Leu)-(Asp/Tyr)-(Gly/Gln//Gly)-(Met/Leu)-**	
	(Ile/Ser/Arg/Phe)	
Initial Yield:	appr. 1-2 pmol	

REMARKS
1. Your sample "06-07-01/175 kDa" was digested with trypsin and the resulting peptides were extracted from the gel [our sample name after digestion/ extraction: 010716-B-T].
2. A portion of 10% of the extract was analyzed.
3. This analysis was performed to check digestion/extraction.
4. All assignments are tentative because of the presence of a mixture of peptides.
5. Signals are present at a sequence level of appr. 1-2 pmol; this means that peptides in the digest may be present at a level of 2 x 10 x (1-2) = 20-40 pmol, assuming an Initial Yield of sequence analysis of 50%.
6. After this analysis, 90% of the digest was subjected to preparative HPLC (our product code: 01E177).

Signature : ('investigator') . . . . . .

Signature : ('verifier') . . . . . .

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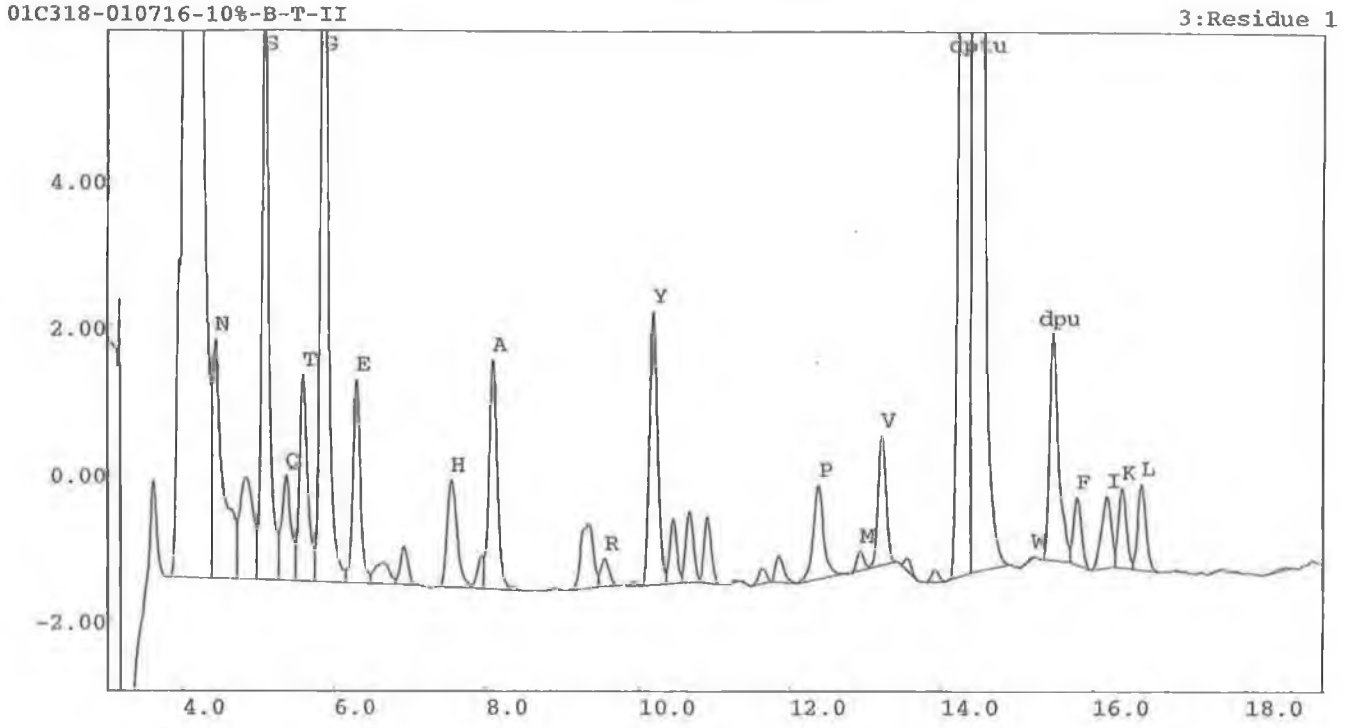
E-mail: eurosequence@chem.rug.nl Internet: www.fwn.rug.nl/eurosequence
Bank: ING Groningen nr. 670447447 Chamber of Commerce Groningen nr. 02043673 VAT nr. NL 0090.18.487.B 01



3:Residue 1, Interpolated baseline

Peak No	RT	Peak ID	Type	Height	Pmol Ht
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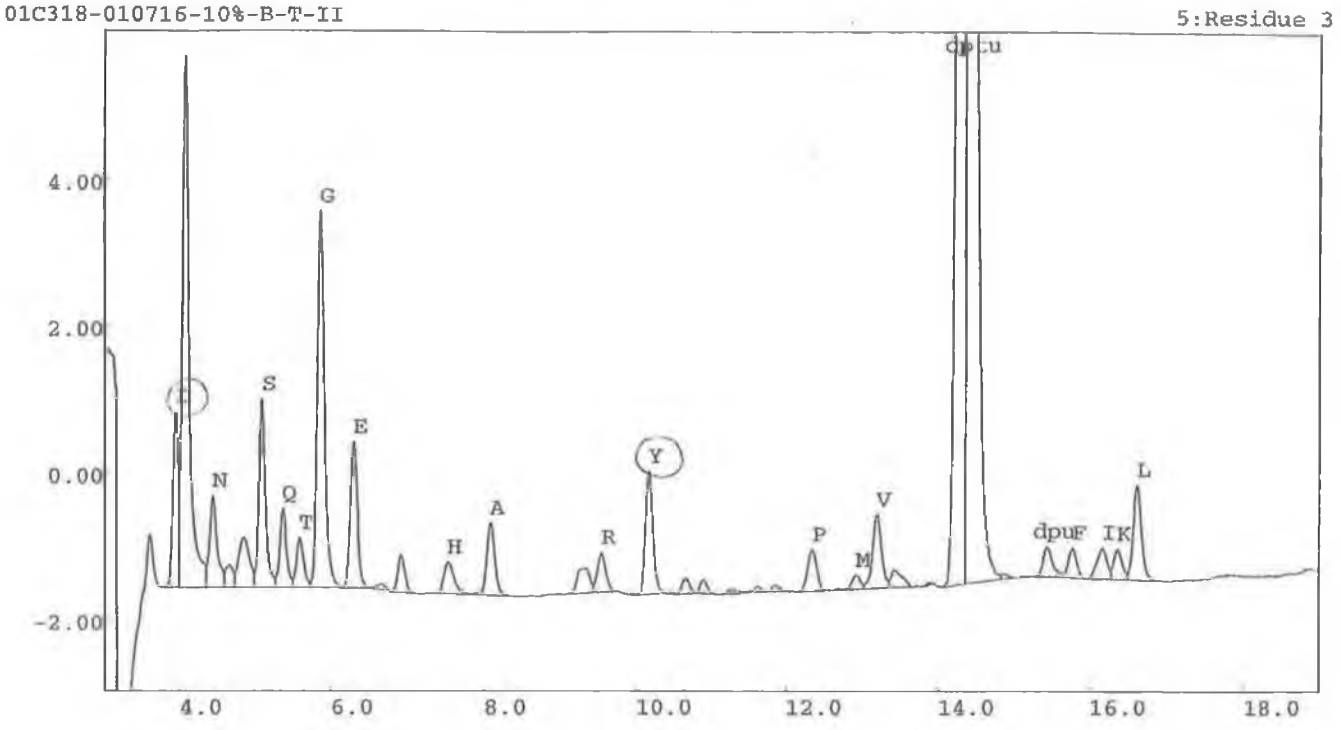
5	5.35	Q	c	11869	5.94
6	5.58	T	c	23143	13.57
7	5.85	G	c	83821	51.59
8	6.29	E	c	22813	10.48
11	7.54	H	c	12103	6.29
13	8.08	A	c	25794	16.40
17	9.54	R	c	3203	2.57
19	10.18	Y	c	30759	19.59
27	12.37	P	c	10472	6.70
28	12.92	M	c	2235	1.81
29	13.20	V	c	14550	10.12
32	14.27	dptu	r	217198	166.65
34	15.18	W	c	628	0.35
35	15.45	dpu		25448	30.63
36	15.78	F	c	7726	5.01
37	16.18	I	c	8174	7.05
38	16.38	K	c	8996	4.60
39	16.63	L	c	9673	6.20



5:Residue 3, Interpolated baseline

Peak No	RT	Peak ID	Type	Height	Pmol Ht
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3	4.43	N	c	10220	4.26
6	5.08	S	c	21032	13.53
7	5.36	Q	c	8807	4.41
8	5.58	T	c	5540	3.25
9	5.86	G	c	42222	25.99
10	6.29	E	c	16383	7.53
13	7.53	H	c	3572	1.86
14	8.09	A	c	8085	5.14
16	9.54	R	c	4407	3.54
18	10.17	Y	c	13800	8.79
25	12.34	P	c	4628	2.96
27	12.92	M	c	1608	1.30
28	13.20	V	c	8307	5.78
31	14.27	dptu	r	136205	104.51
34	15.44	dpu		3412	4.11
35	15.77	F	c	3358	2.18
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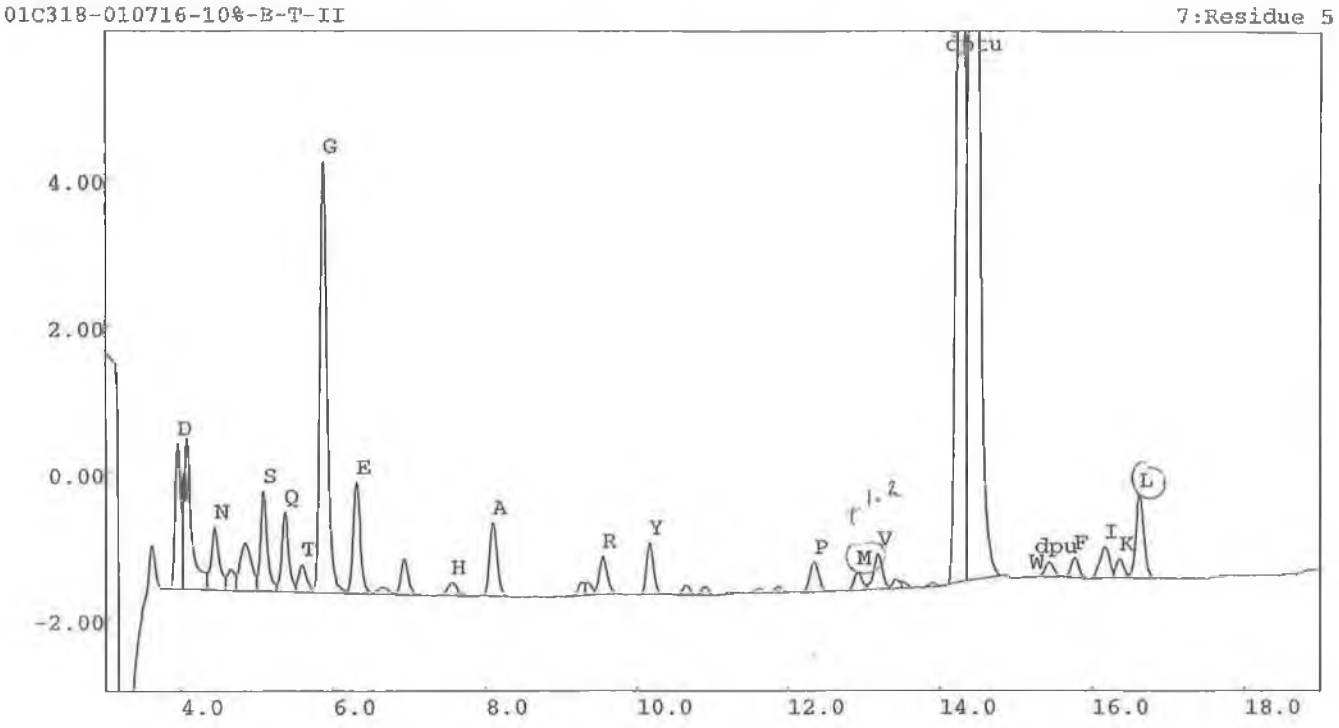
37	16.36	K	c	3380	1.73
38	16.62	L	c	10728	6.87



7:Residue 5, Interpolated baseline

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3	4.44	N	c	7107	2.96
6	5.08	S	c	11217	7.21
7	5.36	Q	c	8952	4.48
8	5.59	T	c	3156	1.85
9	5.86	G	c	48194	29.66
10	6.30	E	c	12565	5.77
13	7.55	H	c	1499	0.78
14	8.09	A	c	8250	5.25
18	9.54	R	c	4260	3.42
19	10.17	Y	c	5792	3.69
27	12.34	P	c	3343	2.14
28	12.91	M	c	2003	1.62
29	13.19	V	c	3983	2.77
33	14.26	dptu	r	105616	81.04
35	15.18	W	c	84	0.05
36	15.43	dpu		1672	2.01
37	15.76	F	c	2273	1.47
38	16.16	I	c	3532	3.04
39	16.36	K	c	2109	1.08
40	16.62	L	c	9326	5.97

EUROSEQUENCE bv
Analysis and Synthesis of Proteins and DNA

INTERPRETATION N-TERMINAL PROTEIN SEQUENCE ANALYSIS RESULTS

See also 'Product Information N-terminal Protein Sequence Analysis' for explanation of 'Initial Yield' and amino acids in parentheses.

Our order number >> **010716**
Product code >> **01C335** Sample: **010716-B-T-fr.33**

Sample amount	: **100%**
Date of analysis	: **September 12, 2001**
Performed	: **14 cycles**
Date of interpretation report	: **September 27, 2001**
Investigator	: **Dr. Wicher J. Weijer**

MAIN SEQUENCE

Position #	1 5
Amino Acid	**(Gly?/Asn/Ser?)-(Leu/Phe)-(Asp/Gly)-(Leu/Gly)-(Asp/Ala)-**
	10
	(Ser)-(Ile/Gly)-(Ile/Gly)-(Ala/Gly)-(Glu/Tyr)-(Val/Ser)-
	14
	(Lys/Ser)-(Ser)-(Gly)
Initial Yield:	**appr. 2-4 pmol**

REMARKS

1. Fraction 33 of HPLC 01E177 was analyzed.
2. No unambiguous sequence can be determined because of the presence of a mixture of two peptides; slight differences in the height of PTH-signals of amino acids allowed the discrimination of signals from each of the two peptides as well as the protein database searches as indicated under remarks 3 and 4.
3. One of the signals at positions 1-14, viz.: (Gly?)-(Phe)-(Gly)-(Gly)-(Ala)-(Ser)-(Gly)-(Gly)-(Gly)-(Tyr)-(Ser)-(Ser)-(Ser)-(Gly), is 100% identical with a sequence from Cytokeratin 9 (e.g. from human; see Appendix III).
4. One of the signals at positions 1-12, viz.: (Asn)-(Leu)-(Asp)-(Leu)-(Asp)-(Ser)-(Ile)-(Ile)-(Ala)-(Glu)-(Val)-(Lys), is 100% identical with a sequence from Cytokeratin 6B (e.g. from human; see Appendix IV).

Signature : ('investigator') . . . . . .

Signature : ('verifier') . . . . . .

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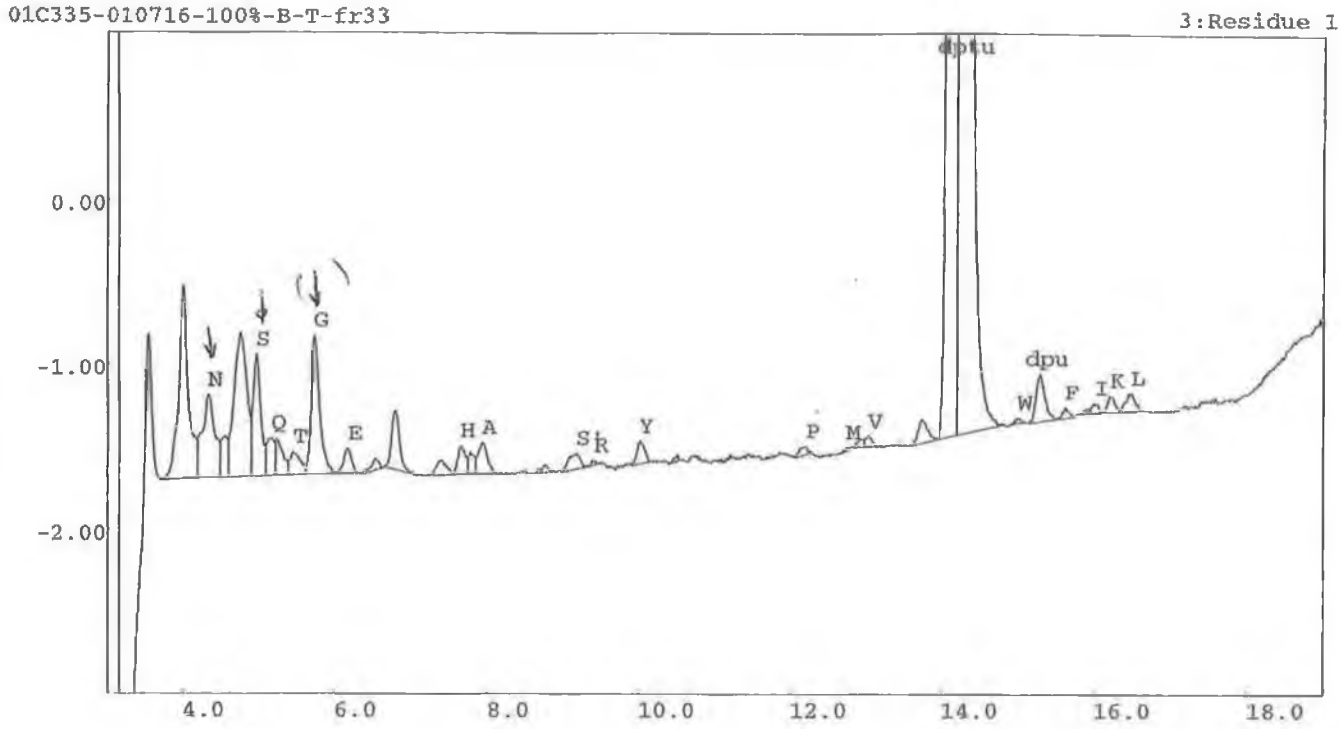
Bank: ING Groningen nr. 670447447 Chamber of Commerce Groningen nr 02043673 VAT nr. NL 0090 18 487.B.01



3:Residue 1, Interpolated baseline

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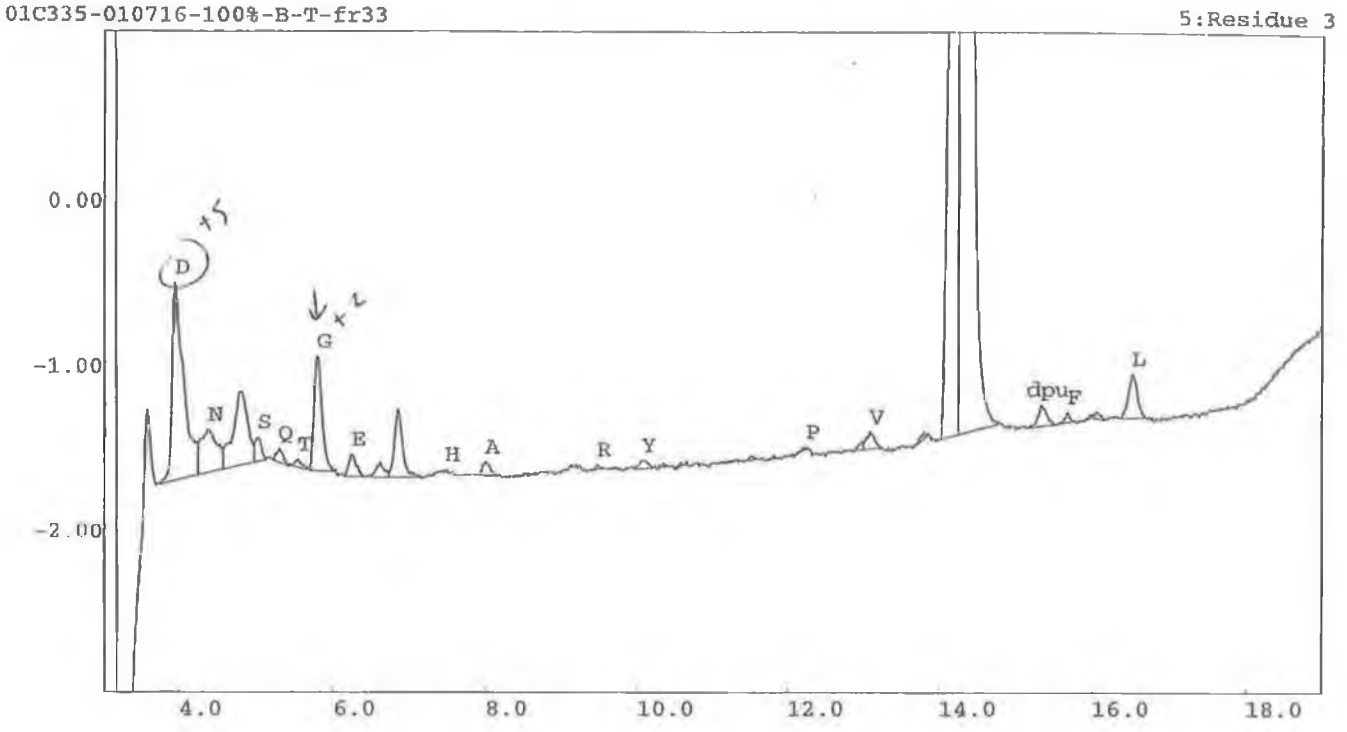
6	5.18	Q	c	1899	0.99
8	5.46	T	c	1155	0.87
9	5.74	G	c	6945	4.39
10	6.18	E	c	1228	0.62
14	7.66	H	c	1438	0.75
16	7.94	A	c	1584	1.05
19	9.18	S'		794	5.72
20	9.42	R	c	200	0.29
21	10.02	Y	c	1064	0.72
25	12.20	P	c	406	0.28
26	12.74	M	c	146	0.11
28	13.03	V	c	528	0.38
30	14.12	dptu	r	79882	62.67
32	15.01	W	c	257	0.15
33	15.29	dpu		2372	2.95
34	15.64	F	c	463	0.32
36	16.02	I	c	502	0.44
37	16.22	K	c	824	0.39
38	16.49	L	c	902	0.60



5:Residue 3, Interpolated baseline

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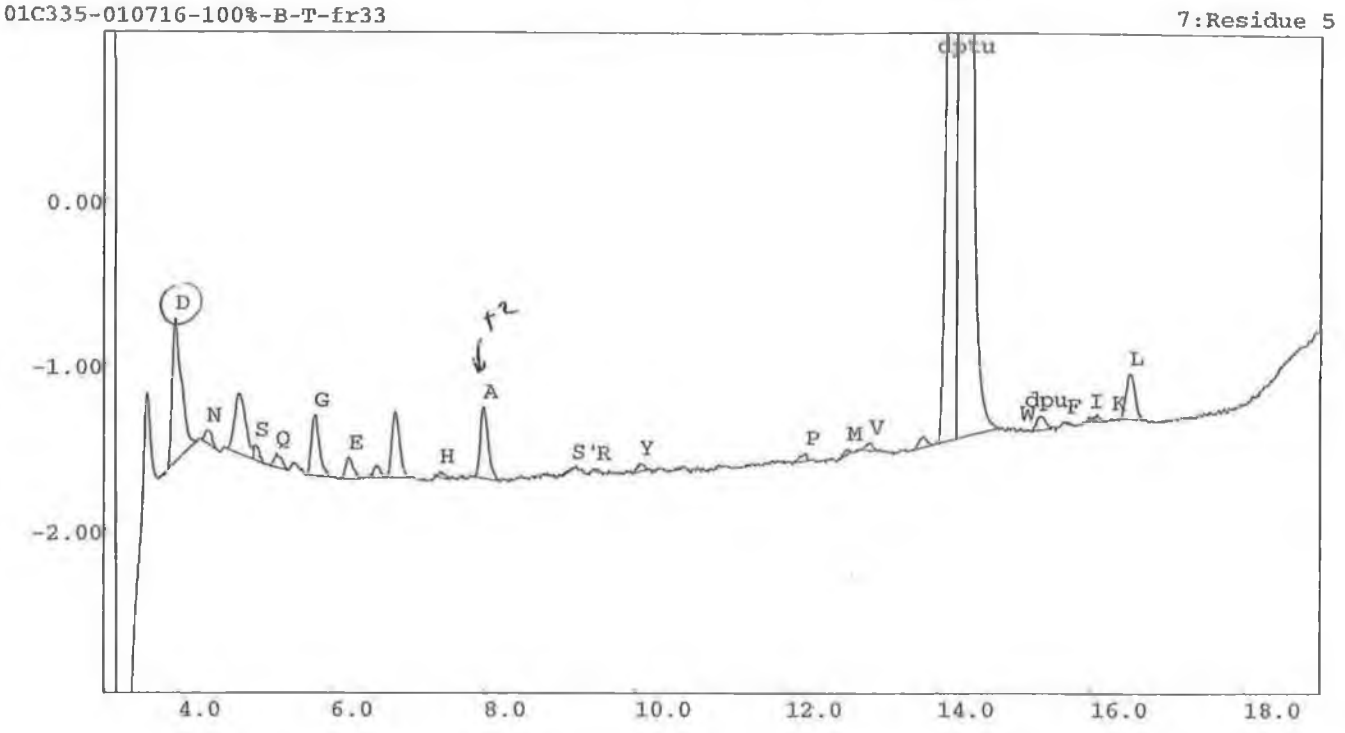
2	4.38	N	c	2148	0.92
4	5.01	S	c	1183	0.79
5	5.29	Q	c	622	0.32
6	5.53	T	c	394	0.30
7	5.79	G	c	5730	3.62
8	6.24	E	c	1081	0.55
11	7.47	H	c	123	0.06
12	8.00	A	c	639	0.42
15	9.47	R	c	215	0.31
16	10.06	Y	c	368	0.25
21	12.23	P	c	221	0.15
23	13.09	V	c	849	0.61
27	15.33	dpu		1035	1.29
28	15.68	F	c	424	0.29
30	16.51	L	c	2174	1.44



7:Residue 5, Interpolated baseline

Peak No	RT	Peak ID	Type	Height	Pmol Ht
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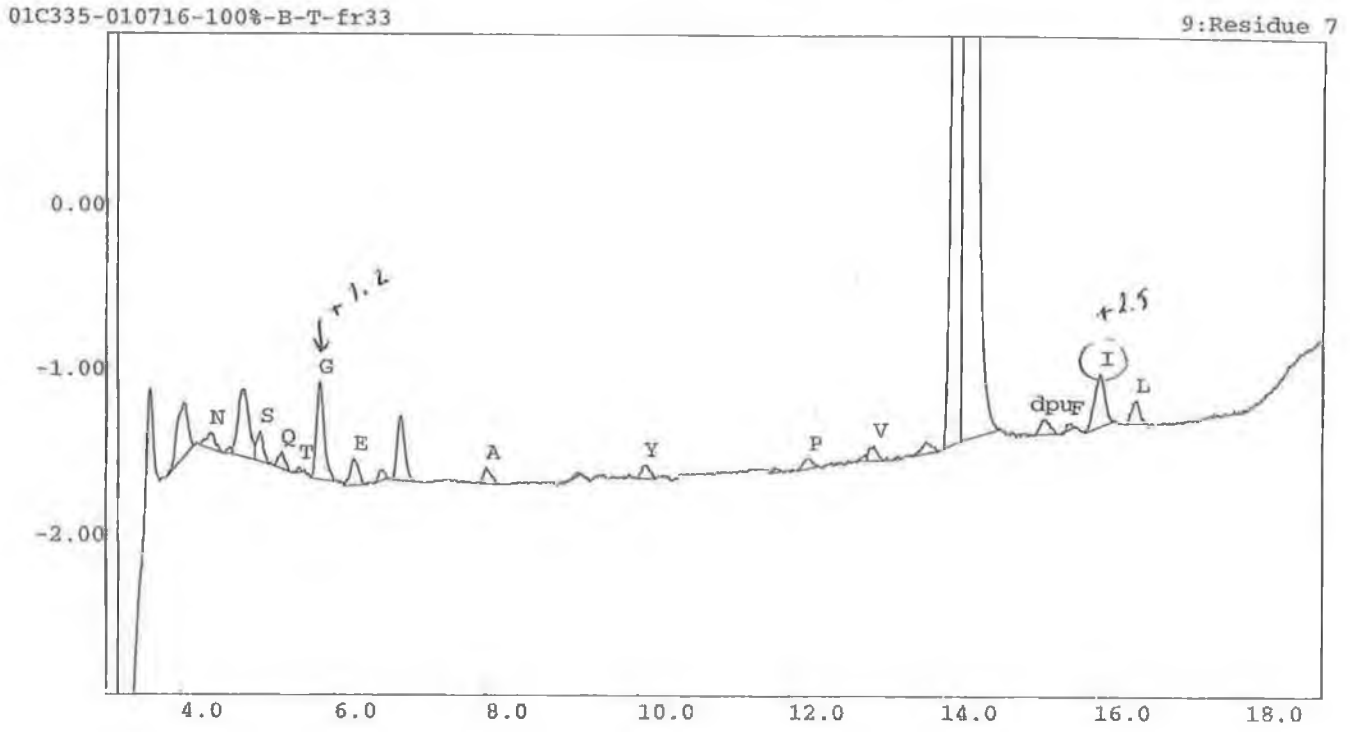
4	5.01	S	c	812	0.54
5	5.28	Q	c	641	0.33
6	5.79	G	c	3033	1.92
7	6.24	E	c	1013	0.51
10	7.43	H	c	221	0.11
11	8.00	A	c	3577	2.36
13	9.15	S'		202	1.46
14	9.48	R	c	144	0.21
15	10.07	Y	c	384	0.26
17	12.25	P	c	356	0.25
18	12.80	M	c	339	0.25
19	13.10	V	c	363	0.26
21	14.17	dptu	r	57369	45.01
23	15.07	W	c	86	0.05
24	15.33	dpu		680	0.84
25	15.68	F	c	116	0.08
26	15.97	I	c	302	0.27
28	16.27	K	c	87	0.04
29	16.51	L	c	2311	1.53



9:Residue 7, Interpolated baseline

Peak No	RT	Peak ID	Type	Height	Pmol Ht
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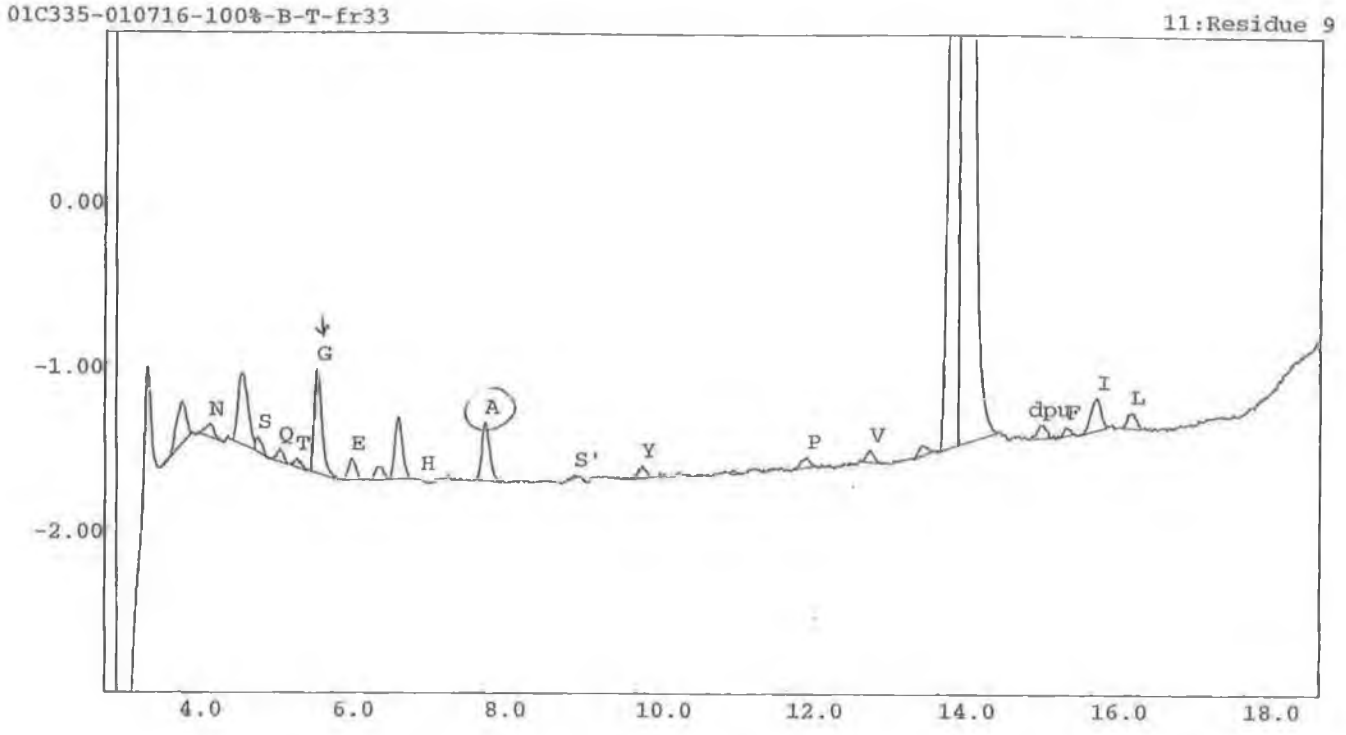
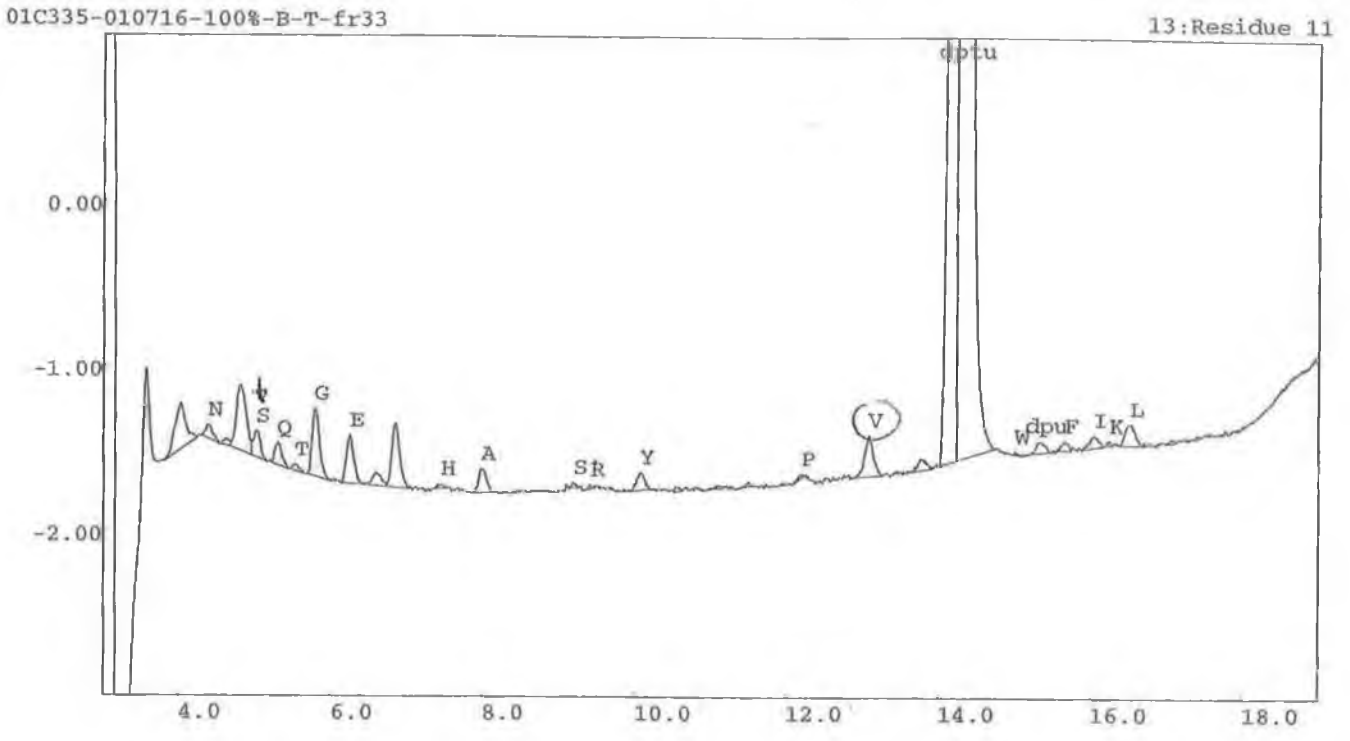
5	5.03	S	c	1492	1.00
6	5.30	Q	c	780	0.41
7	5.53	T	c	192	0.14
8	5.80	G	c	4748	3.00
9	6.24	E	c	1225	0.62
13	8.00	A	c	758	0.50
15	10.08	Y	c	646	0.44
18	12.25	P	c	495	0.34
20	13.12	V	c	713	0.51
25	15.35	dpu		742	0.92
26	15.68	F	c	367	0.25
27	16.07	I	c	2619	2.32
28	16.54	L	c	1152	0.76



11:Residue 9, Interpolated baseline

Peak No	RT	Peak ID	Type	Height	Pmol Ht
2	4.38	N	c	666	0.28
4	5.00	S	c	841	0.56
5	5.29	Q	c	558	0.29
6	5.51	T	c	411	0.31
7	5.79	G	c	5235	3.31
8	6.24	E	c	1028	0.52
11	7.15	H	c	103	0.05
12	8.00	A	c	2923	1.93
14	9.18	S'		239	1.72
16	10.07	Y	c	560	0.38
20	12.25	P	c	505	0.35
21	13.10	V	c	553	0.40
25	15.35	dpu		669	0.83
26	15.67	F	c	411	0.28
27	16.06	I	c	1683	1.49
28	16.51	L	c	790	0.52



13:Residue 11, Interpolated baseline

Peak No	RT	Peak ID	Type	Height	Pmol Ht
2	4.38	N	c	731	0.31
5	5.01	S	c	1378	0.92
6	5.29	Q	c	1126	0.59
7	5.52	T	c	344	0.26
8	5.79	G	c	3451	2.18
9	6.24	E	c	2368	1.20
12	7.45	H	c	210	0.11
14	7.99	A	c	1150	0.76
16	9.20	S'		203	1.46
17	9.43	R	c	137	0.20
18	10.07	Y	c	888	0.60
22	12.20	P	c	283	0.20
23	13.09	V	c	2038	1.47
25	14.16	dptu	r	60476	47.44
27	15.00	W	c	144	0.08
28	15.32	dpu		570	0.71
29	15.66	F	c	410	0.28
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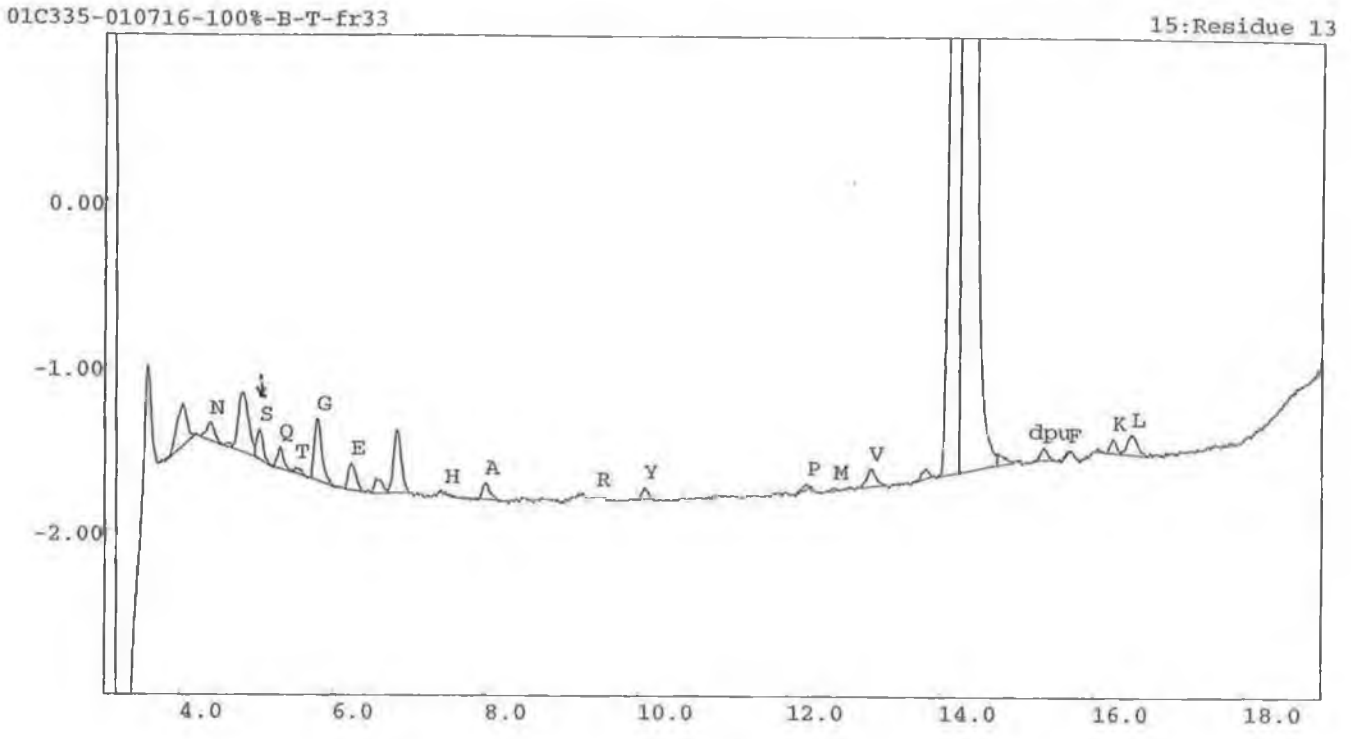
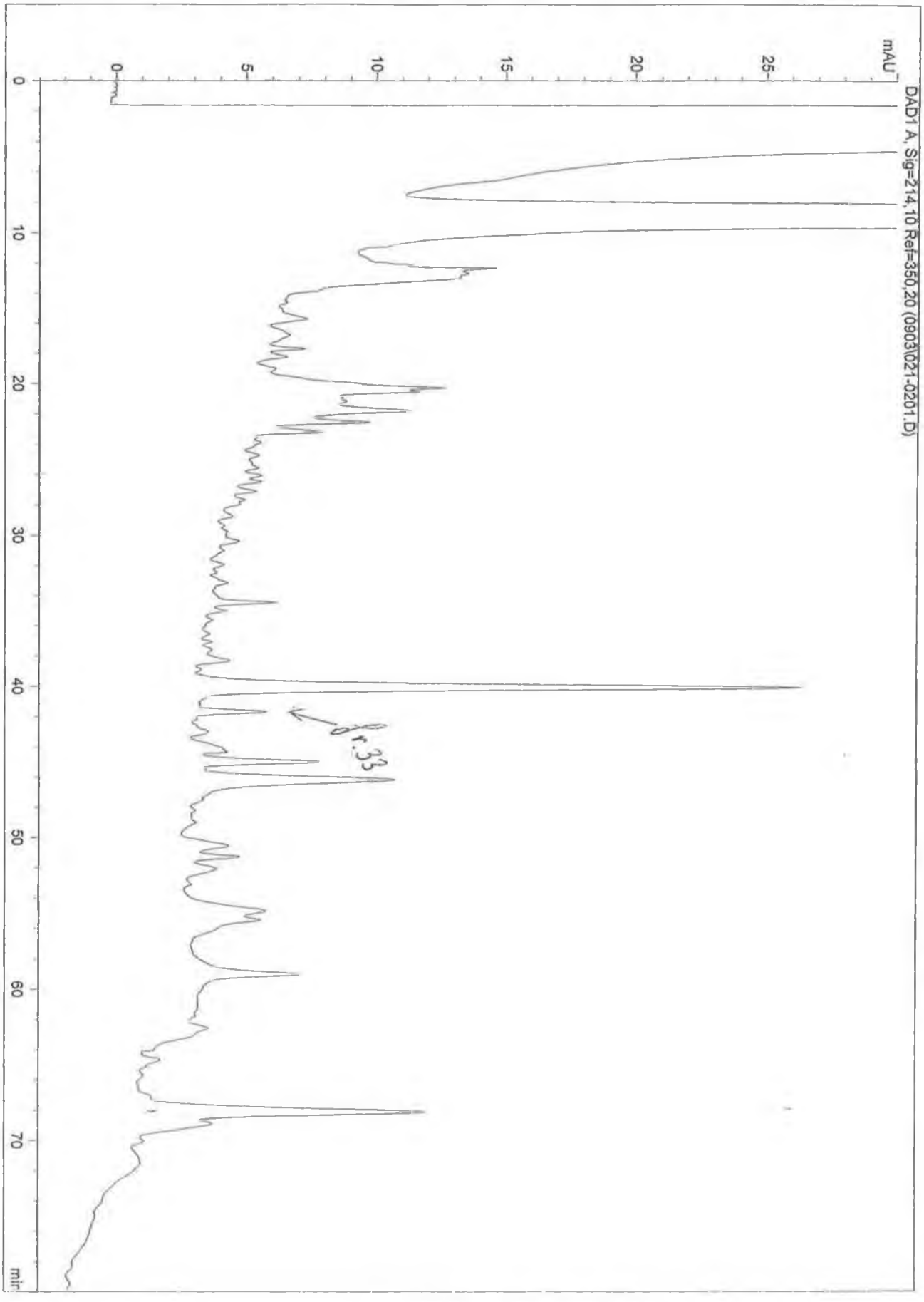
31	16.25	K	c	214	0.10
32	16.52	L	c	1034	0.68



15:Residue 13, Interpolated baseline

Peak No	RT	Peak ID	Type	Height	Pmol Ht
2	4.38	N	c	906	0.39
5	5.02	S	c	1570	1.05
6	5.29	Q	c	1040	0.54
7	5.50	T	c	332	0.25
8	5.79	G	c	3147	1.99
9	6.24	E	c	1309	0.66
12	7.47	H	c	177	0.09
13	8.00	A	c	847	0.56
14	9.45	R	c	74	0.11
15	10.08	Y	c	536	0.36
16	12.24	P	c	339	0.23
17	12.60	M	c	138	0.10
18	13.08	V	c	849	0.61
23	15.34	dpu		608	0.76
24	15.66	F	c	276	0.19
26	16.27	K	c	695	0.33
27	16.52	L	c	982	0.65



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REFERENCE

01E177

*** End of Report ***

Appendix III (010716)

1: P35527. KERATIN, TYPE I C... [gi:547748]


```
LOCUS       K1CI_HUMAN    622 aa                    PRI       20-AUG-2001
DEFINITION  KERATIN, TYPE I CYTOSKELETAL 9 (CYTOKERATIN 9) (K9) (CK 9).
ACCESSION   P35527
PID         g547748
VERSION     P35527  GI:547748
DBSOURCE    swissprot: locus K1CI_HUMAN, accession P35527;
            class: standard.
            extra accessions:Q14665,created: Jun 1, 1994.
            sequence updated: Jun 1, 1994.
            annotation updated: Aug 20, 2001.
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            xrefs (non-sequence databases): HSSP P02876, MIM 144200, InterPro
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            PROSITE PS00226
KEYWORDS    Intermediate filament; Coiled coil; Keratin; Disease mutation.
SOURCE      human.
  ORGANISM  Homo sapiens
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Mammalia; Eutheria; Primates; Catarrhini; Hominidae; Homo.
REFERENCE   1  (residues 1 to 622)
  AUTHORS   Langbein,L., Heid,H.W., Moll,I. and Franke,W.W.
  TITLE     Molecular characterization of the body site-specific human
            epidermal cytokeratin 9: cDNA cloning, amino acid sequence, and
            tissue specificity of gene expression
  JOURNAL   Differentiation 55 (1), 57-71 (1993)
  MEDLINE   94131202
  REMARK    SEQUENCE FROM N.A., AND PARTIAL SEQUENCE.
            TISSUE=Foot sole tissue
REFERENCE   2  (residues 1 to 622)
  AUTHORS   Rosen,E.M., Meromsky,L., Romero,R., Setter,E. and Goldberg,I.
  TITLE     Human placenta contains an epithelial scatter protein
  JOURNAL   Biochem. Biophys. Res. Commun. 168 (3), 1082-1088 (1990)
  MEDLINE   90267446
  REMARK    SEQUENCE OF 449-465.
REFERENCE   3  (residues 1 to 622)
  AUTHORS   Hennies,H.C., Zehender,D., Kunze,J., Kuster,W. and Reis,A.
  TITLE     Keratin 9 gene mutational heterogeneity in patients with
            epidermolytic palmoplantar keratoderma
  JOURNAL   Hum. Genet. 93 (6), 649-654 (1994)
  MEDLINE   94274199
  REMARK    VARIANTS EPPK VAL-156 AND PRO-171.
REFERENCE   4  (residues 1 to 622)
  AUTHORS   Bonifas,J.M., Matsumura,K., Chen,M.A., Berth-Jones,J.,
            Hutchison,P.E., Zloczower,M., Fritsch,P.O. and Epstein,E.H. Jr.
  TITLE     Mutations of keratin 9 in two families with palmoplantar
            epidermolytic hyperkeratosis
  JOURNAL   J. Invest. Dermatol. 103 (4), 474-477 (1994)
  MEDLINE   95015968
  REMARK    VARIANT EPPK SER-160.
REFERENCE   5  (residues 1 to 622)
  AUTHORS   Torchard,D., Blanchet-Bardon,C., Serova,O., Langbein,L., Narod,S.,
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      361 evqssakevt qlrhgvqele ielqsqlskk aaleksledt knrycgqlqm iqeqisnlea
      421 qitdvrqeie cqnqeyslll sikmrlekei etyhnllegg qedfessgag kiglggrggs
      481 ggsygrgsrg gsggsygggg sgggyggggsg srggsggsyg ggsgsgggsg ggyggggsggg
```

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Appendix IV (010716)

1: P04259. KERATIN, TYPE II ... [gi:1346345]


```
LOCUS       K2CB_HUMAN    564 aa                    PRI       20-AUG-2001
DEFINITION  KERATIN, TYPE II CYTOSKELETAL 6B (CYTOKERATIN 6B) (CK 6B) (K6B
            KERATIN).
ACCESSION   P04259
PID         g1346345
VERSION     P04259  GI:1346345
DBSOURCE    swissprot: locus K2CB_HUMAN, accession P04259;
            class: standard.
            created: Mar 20, 1987.
            sequence updated: Feb 1, 1996.
            annotation updated: Aug 20, 2001.
            xrefs: gi: gi: 908788, gi: gi: 908790, gi: gi: 908780, gi: gi:
            908781, gi: gi: 908782, gi: gi: 908783, gi: gi: 908784, gi: gi:
            908785, gi: gi: 908786, gi: gi: 186714, gi: gi: 386849, gi: gi:
            186706, gi: gi: 186707, gi: gi: 186708, gi: gi: 186709, gi: gi:
            186710, gi: gi: 186711, gi: gi: 186712, gi: gi: 186713, gi: gi:
            7428711
            xrefs (non-sequence databases): Aarhus/Ghent-2DPAGE 1418, MIM
            148042, InterPro IPR001664, InterPro IPR003054, Pfam PF00038,
            PRINTS PR01276, PROSITE PS00226
KEYWORDS    Intermediate filament; Coiled coil; Keratin; Multigene family;
            Phosphorylation.
SOURCE      human.
  ORGANISM  Homo sapiens
            Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
            Mammalia; Eutheria; Primates; Catarrhini; Hominidae; Homo.
REFERENCE   1  (residues 1 to 564)
  AUTHORS   Takahashi,K., Paladini,R.D. and Coulombe,P.A.
  TITLE     Cloning and characterization of multiple human genes and cDNAs
            encoding highly related type II keratin 6 isoforms
  JOURNAL   J. Biol. Chem. 270 (31), 18581-18592 (1995)
  MEDLINE   95355491
  REMARK    SEQUENCE FROM N.A.
            TISSUE=Skin
REFERENCE   2  (residues 1 to 564)
  AUTHORS   Tyner,A.L., Eichman,M.J. and Fuchs,E.
  TITLE     The sequence of a type II keratin gene expressed in human skin:
            conservation of structure among all intermediate filament genes
  JOURNAL   Proc. Natl. Acad. Sci. U.S.A. 82 (14), 4683-4687 (1985)
  MEDLINE   85270392
  REMARK    SEQUENCE FROM N.A.
REFERENCE   3  (residues 1 to 564)
  AUTHORS   Rasmussen,H.H., van Damme,J., Puype,M., Gesser,B., Celis,J.E. and
            Vandekerckhove,J.
  TITLE     Microsequences of 145 proteins recorded in the two-dimensional gel
            protein database of normal human epidermal keratinocytes
  JOURNAL   Electrophoresis 13 (12), 960-969 (1992)
  MEDLINE   93162043
  REMARK    SEQUENCE OF 194-202 AND 349-355.
            TISSUE=Keratinocytes
COMMENT     On Jun 1, 1996 this sequence version replaced gi:125107.
            ----------------------------------------------------------------
            This SWISS-PROT entry is copyright. It is produced through a
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ORIGIN
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       61 lyglggskri sigggscais ggygsraggs ygfggagsgf gfgggagigf glgggaglag
      121 gfggpgfpvc ppggiqevtv nqslltplnl qidpaiqrvr aeereqiktl nnkfasfidk
      181 vrfleqqnkv ldtkwtllqe qgtktvrqnl eplfeqyinn lrrqldsivg ergrldselr
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      361 eelqvtagrh gddlrntkqe iaeinrmiqr lrseidhvkk qcanlqaaia daeqrgemal
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      541 svgggsstik ytttssssrk sykh
//
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