The Tumour Suppressor P53 and Apoptotic Regulatory Proteins in Breast Cancer Survival and Response to Therapy

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by

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Dedication

This thesis is dedicated to my wife Grmeen and my kids Peter and Andrew

Abstract

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Many breast cancer patients receive chemotherapy as part of their treatment, but unlike hormonal therapy there are no specific markers for predicting response. Almost all cancer therapies induce cell death by apoptosis. Therefore, factors that control apoptosis such as the tumour suppressor p53 and other regulatory proteins could provide important clues.

Initially breast cell lines (MCF-7, T47-D, ZR-75, MDA-MB-231, MDA-MB-468, HBL-100 and MBA-MB-436) were characterized with regard to proliferation, apoptosis, p53 and phosphorylated p53, $p21^{waf-1}$, ChK2, bcl-2, bax, bcl-x, survivin and XIAP, using immunocytochemistry and western blotting. The cells apart from HBL-100 and MDA-MB-436, were then treated with different doses and durations of doxorubicin and paclitaxel. Proliferation was suppressed and apoptosis induced mainly in cells with wild type p53. P53 was induced after treatment in both ZR-75 and MCF-7 cells. Strong staining for bcl-2 was found in ZR-75 cells and was down regulated with treatment. Bcl-x was strongly expressed in MCF-7, T47-D and MDA-MB-468. $p21^{waf-1}$ was higher in MCF-7 and ZR-75 cells, and was markedly induced after treatment, and to a lesser extent in the other cells. Bax, Survivin and XIAP were detected in all cell lines with some variation. Bax expression increased after treatment, Survivin decreased except in ZR-75 and XIAP decreased in MCF-7.

Breast cancers from patients who had all died from the disease and who had received either neo-adjuvant therapy or adjuvant chemotherapy and / or hormone therapy were examined for the same markers using immunohistochemistry, and related to clinicopathological factors. High proliferation index, the presence of phosphorylated p53, low expression of bcl-2 and over-expression of Survivin were associated with shorter duration of survival, with Survivin expression being independent of other factors. The presence of Survivin and being grade III significantly correlated with shorter survival in patients who received combined adjuvant chemotherapy and hormonal therapy.

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LIST OF ABBREVIATIONS

µL	Microliters
µM	Micromolars
ABC	Avidin Biotin Complex
AIF	Apoptosis Inducing Factor
AJCC	Americant Joint Committee of Cancer
ANOVA	Analysis Of Variance
APAF-1	Apoptosis Protease Activating Factor 1
APS	Ammonium Persulfate
ATCC	American Type of Culture Collection
ATM	Ataxia Telangectasia Mutated
ATR	ATM-Related Polypeptide
Bcl-2	B-cell Leukemia Lymphoma 2
BIR	Baculoviral IAP Repeat
BME	Betamercaptoethanol
BPB	Bromophenol Blue
BrdU	BromodeoxyUridine
BSA	Bovine Serum Albumin
°C	Centigrade
CAF	Cyclophosphamide-Adriamycin-Fluorouracil
CARD	Caspase Recruitment Domain
CDK	Cyclin dependent Kinase
CKII	Casein Kinase II
CMF	Cyclophosphamide-Methotrexate-Fluorouracil
CO_2	Carbon Dioxide
DAB	Diaminobenzidine
DISC	Death Inducing Signalling Complex
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DNA-PK	DNA-dependent Protein Kinase
dPBS	Dulbecco's Phosphate Buffered Saline
DR	Death Receptor
ECL	Enhanced Chemiluminescense
EDTA	Ethylene Diaminetetraacetic Acid
EGFR	Epidermal Growth Factor Receptor
ER	Estrogen Receptor
FADD	Fas Associated Death Domain
FBS	Fetal Bovine Serum
GADD45	Growth Arrest and DNA damage protein
h	Hour
H_2O_2	Hydrogen Peroxide
HCl	Hydrochloric Acid
HRP	Horse Radish Peroxidase

IAPs	Inhibitor of Apoptosis Proteins
IMS	Industrial Methylated Spirit
JNK	c-Jun N Terminal Kinase
KDa	Kilodaltons
LHRH	Leutinizing Hormone Releasing Hormone
mA	Milliampers
MDM2	Murine Double Minute 2
mins	Minutes
mls	Mililiters
mM	Millimolars
NaCl	Sodium Chloride
NAIP	Neuronal Inhibitory Protein
NHS	National Health Service
NHSBSP	National Health Service Breast Screening Program
nM	Nanomolar
NPI	Nottingham Prognostic Index
NRS	Normal Rabbit Serum
NSS	Normal Swine Serum
NST	Non Specific Type
PBS	Phosphate Buffered Saline
PgR	Progesterone Receptor
PHOS	Phosphorylated
PMSF	Phenylmethylsulphonylfluoride
PS	Phosphatidylserine
RING	Really Interesting New Gene
rpm	Revolusion Per Minute
SDS	Sodium Dodecyl Sulphate
TBS	Tris Buffered Saline
TNFR	Tumour Necrosis Factor Receptor
TNFα	Tumour Necrosis Factor Alpha
TNM	Tumour Node Metastasis
TPST	Tris Buffered Saline Tween
Tris	2-amino-2-(hydroxymethyl)-1,3-propandiol
TUNEL	Tdt-mediated bio-dUTP Nick End Labelling
UV	Ultraviolet
v/v	Volume/Volume
WHO	World Health Organization
XAF1	XIAP Associated Factor 1
XIAP	X-chromosome-linked IAP

CHAPTER 1:

INTRODUCTION

Breast Cancer

Breast cancer has been documented for many centuries, as far as the early Egyptians for whom the popular treatment was cautery of the diseased tissue. There have been many developments in management since then, but until more recently these related to surgery (Baum, 1988). However, it is metastatic disease that determines outcome and treatment now takes this into account. There is still though a need to know more about treatment selection and response.

1.1 Epidemiology

Breast cancer comprises 18% of all female cancers and is the most common malignant disease in women (Bray et al., 2004). Each year, one million new cases of breast cancer are diagnosed worldwide. Rates of breast cancer are significantly higher in the developed world than in developing countries (Pecorelli et al., 2003).

In the United Kingdom, breast cancer is the most common cancer for women with more than 40,000 new cases in the year 2000. It is the second highest cause of female mortality after lung cancer. The lifetime risk for breast cancer in women is one in nine. Breast cancer can develop at any age in adulthood. But it is less common under the age of 35 although breast cancers occurring in women under 35 years are usually more aggressive (Walker et al., 1996). Eighty percent of all breast cancers occur in women over the age of 50 years. The 5-year age standardized survival rate has increased significantly over the last three decades, from 52% in the early 1970s to 77% for the period 1996-1999. This relates to early detection and changes in treatment.

1.2 Pathology

1.2.1 Type

In spite of the different classification systems for breast cancer, they all depend on the morphological features described by the World Health Organization (WHO, 1981). Epithelial breast tumours are basically divided into *in situ* and *invasive carcinoma.* Lobular carcinoma in situ is thought to be a risk factor for invasive carcinoma.

Ductal carcinoma in situ

Characterized by growth of tumour cells within the ducts without penetration of the basement membrane. It accounts for 15-20% of breast cancer cases. In the UK, it is classified into high, intermediate and low grades according to the nuclear morphology (NHSBSP, 1995).

Invasive carcinoma- usual types

It denotes tumour penetration of the basement membrane and extension of tumour cell aggregates into the stroma of the mammary gland. In this introduction, only the commonest types of invasive breast carcinoma will be described (table 1.1) (Elston and Ellis, 1998).

Invasive ductal carcinoma-NST

This type is usually diagnosed by exclusion, since the tumour morphological features do not apply for any of the other special types. It is the most common type, comprises 41-75% of breast tumours. The morphological features vary according to the degree of cellularity of the tumour. Cells may be arranged in cords or solid sheets, with or without gland formation. The nuclei may be regular or pleomorphic with multiple nucleoli. In a population-based study, it was associated with lower 5-year relative survival (66.0%) in comparison to breast cancer special types (95 %) (Allemani et al., 2004).

Infiltrating lobular carcinoma

Accounts for 8-14 % of all breast cancers (Borst and Ingold, 1993). Tumour cells are usually regular with round or oval dark nuclei and characteristically they are diffusely infiltrative. According to the histological appearances, they were further sub-classified into classical, alveolar, solid, mixed and tubulo-alveolar subtypes. In spite of the general concept that infiltrating lobular carcinomas are usually associated with favorable prognosis, recent studies showed that they had no survival advantage

comparing with infiltrating ductal carcinomas (Arpino et al., 2004; Molland et al., 2004) Also, it has been shown that infiltrating lobular carcinomas have poorer responsiveness to preoperative chemotherapy than infiltrating ductal carcinomas (Mathieu et al., 2004).

Medullary carcinoma

Usually present as well circumscribed masses soft in consistency composed histologically of large cells with abundant cytoplasm and pleomorphic nuclei. Characteristically the stroma around sheets of tumour cells is infiltrated with many lymphocytes and the tumour border is pushing rather than infiltrative. Although it is apparently associated with favorable prognosis, this is not well established because some studies failed to show any survival advantage over carcinomas of no special type (Ellis et al., 1992, Fisher et al., 1990).

Tubular carcinoma

A tumour is classified as tubular carcinoma if more than 90 % comprises tubular structures and the rest of the tumour has well differentiated morphology. The frequency of symptomatic tubular carcinoma ranges from 1-7% while the frequency of cases detected by mammography is 9-19 % of tumours. Patients with pure tubular carcinoma have a very good prognosis. A study by (Cooper et al., 1978) showed 100 % survival for 15 years and another study showed more than 90 % survival for 10 years (Carstens et al., 1985).

1.2.2 Histological grading

Within the last decade, histologic grading has become one of the powerful indicators of prognosis in breast cancer. Most of grading systems now use nuclear grade (degree of pleomorphism), degree of tubule formation and mitotic rate. Of these methods, (Bloom and Richardson, 1957) was the most common grading system in the United States until recently and in this method, each of the three elements is given a score of 1 to 3 (1 is the best and 3 is the worst); the sum of the three scores gives the grade.

Types	Subtypes
Ductal carcinoma in Situ	High grade Intermediate grade Low grade Mixed types
Microinvasive carcinoma	
Invasive carcinoma- usual types	Ductal NST Lobular Medulary Invasive Cribriform Tubular mixed Mucinous Invasive papillary Metaplastic Mixed types
Invasive carcinoma- rare types	Squamous cell Mucoepidermoid Low-grade adenosquamous Adenocystic Adenomyoepithelioma Malignant myoepithelioma Apocrine Primary oat cell Clear cell Secretory

Table 1.1: Histological classification of epithelial breast tumours. Adapted from Elston and Ellis (1998).

However, the major drawback of this grading system was subjectivity. Therefore, this system has been modified (Elston and Ellis, 1991) (the Nottingham grading system) and is now the preferred system in Europe (table 1.2). It is becoming increasingly popular in US as well (Simpson et al., 2000). This system gave the best results when compared with other methods to investigate the importance of histological grade in breast cancer prognosis (Latinovic et al., 2001).

Tubule formation	Score
>75% of tumour	1
10-75% of tumour	2
<10% of tumour	3

Histologic Grade	Score
Grade I	3-5
Grade II	6-7
Grade III	8-9

Mitotic count	Score

Depending on microscope field area:

	Leitz Ortholux	Nikon Labophot	Leitz Diaplan	
Objective	x25	x40	x40	
Field diameter	0.59	0.44	0.63	
Fields area	0.274	0.152	0.312	
Mitotic count /10 fields				
	0-9	0-5	0-11	1
	10-19	6-10	12-22	2
	≥ 20	≥ 11	≥ 23	3

Nuclear grade	Score
Uniform cells, small and regular	1
Moderate increase in size and variability	2
Marked variation	3

Table 1.2: Nottingham grading system. Adapted from Elston and Ellis (1991).

1.2.3 Staging

Staging of breast cancer is important in relation to the size of the tumour and the extent of the disease. Breast cancer stage is of paramount importance regarding the selection of patient management as well as prognosis. A recent study showed tumour stage to be an independent prognostic factor in breast cancer (Korkolis et al., 2004).

The main system for staging of invasive breast cancer is the tumour node metastasis (TNM) system designated by the American Joint Committee on Cancer (AJCC) and modified by Singletary et al.(2002). This system depends on the tumour size (T), axillary lymph node metastasis (N) and distant metastasis (M) as follows:

TX: Primary tumour cannot be assessed

T0: No evidence of primary tumour.

Tis: In situ carcinoma or Paget's disease of the nipple.

T1: Tumours ≤ 2cm in size, with no skin involvement.

T2: Tumours >2 cm but ≤5 cm in size.

T3: Tumours > 5 cm.

T4: Tumours with of any size with skin or chest wall involvement or peau d'orange.

NX: Lymph nodes cannot be assessed.

N0: No palpable ipsilateral axillary lymph nodes.

N1: Metastasis to movable ipsilateral axillary lymph nodes.

N2: Ipsilateral axillary lymph node metastasis with nodes fused together or fixed to surrounding tissues.

N3: Ipsilateral internal mammary, infraclavicular or supraclavicular node involvement.

MX: Distant metastasis cannot be assessed.

M0: No evidence of distant metastasis.

M1: distant metastasis.

According to the above system, there are 4 stages:

Stage I: T_1, N_0, M_0.

Stage II:

IIA: T0, N1, M0
T1, N1, M0
T2, N0, M0

IIB: T2, N1, M0
T3, N0, M0

Stage III:

IIIA: T0, N2, M0
T1, N2, M0
T2, N2, M0
T3, N1, M0
T3, N2, M0

IIIB: T0, N0, M0
T4, N1, M0
T4, N2, M0

IIIC: Any T, N3, M0

Stage IV: Any T, Any N, M1.

In the recent years, many studies have analyzed the importance of immunohistochemical and genetic markers in breast cancer prognosis. It is expected

that some of these markers will add more information to the current breast cancer staging systems (Singletary et al., 2002).

1.2.4 Nottingham prognostic index

This is a clinicopathological classification system for breast cancer prognostication, categorizes patients into good and poor outcomes according to their combined score of lymph node stage, histological grade and tumour size.

In this index, lymph node stage and histological grade are given a score of 1-3, both scores combined and added to 0.2 X tumour size (cm). Cut off points of 3.4 and 5.4 are used to categorize breast cancer patients into three prognostic groups (good, moderate and poor) (Galea et al., 1992). The 'Nottingham Prognostic Index' (NPI) is used in the selection of the proper management for those patients with operable primary breast carcinoma (Harris et al., 2003). Other prognostic indices include the Cummings Prognostic Index (CMI) and the Mitotic Prognostic Index (MPI) (www.palpath.com/maimpi.htm), and also St. Gallen criteria (Colomer et al., 2004).

1.3 Current methods of breast cancer management

Breast cancer management takes a variety of forms depending on presentation and stage of the cancer as well as the general health of the patient. It includes surgery- wide local excision or mastectomy, with sentinel node, axillary node sampling or axillary dissection; radiotherapy; chemotherapy (adjuvant/neo-adjuvant); and hormone therapy.

Chemotherapy in breast cancer

Cytotoxic chemotherapy for the treatment of breast cancer started in the 1950s. During the 1950s and 1960s, single-agent chemotherapy was used. In early 1970s combination chemotherapy was introduced and subsequently different agents have been employed (Buzdar, 1998). Chemotherapy is used as primary treatment before surgery (neoadjuvant), adjuvant following surgery and for treatment of metastatic disease.

Neoadjuvant chemotherapy

Neo-adjuvant chemotherapy is also called primary or induction chemotherapy and is mainly used for large tumours/ locally advanced cancers, apart from in the elderly. The aim is to reduce tumour size to permit successful surgical removal (Schick et al., 1983) or breast conserving surgery (Bonadonna et al., 1990). Furthermore, the response to the primary treatment may be used as a prognostic indicator (Fisher et al., 1998). Recent studies have shown that neoadjuvant chemotherapy is more effective in patients with breast cancer not expressing steroid hormone receptors (Colleoni et al., 2004).

Adjuvant chemotherapy

Adjuvant chemotherapy is used for those patients with operable breast cancer to increase the cure rate after the primary local therapy (Snyder, 2004). It has been demonstrated that adjuvant chemotherapy reduces relapse and improves survival in all subgroups of women with early breast cancer, but the effect varies between women according to lymph node status, tumour size and other prognostic factors (Lu et al., 2004). Studies have shown that for pre-menopausal women with ER and/or PgR positive breast cancer, adjuvant ovarian ablation (surgical oophorectomy or ovarian irradiation) or ovarian suppression by luteinizing hormone releasing hormone (LHRH) with or without tamoxifen produces equivalent effects to those of combination chemotherapy CMF (Pritchard, 2002, 2003). Accordingly, adjuvant chemotherapy has become common practice worldwide (Gluck, 2001).

Hormonal therapy

Hormonal therapy is important in breast cancer and the main target is estrogen. The role of estrogen in breast cancer is now well established (Jones and Buzdar, 2004). Therefore, adjuvant hormonal therapy is recommended for women with tumours expressing hormone receptors (Gelber et al., 2003) and tamoxifen is the most common adjuvant hormonal therapy. However, the combined hormonal therapy with chemotherapy is more effective than monotherapy in ER-positive cases (Snyder, 2004). Endocrine therapy can also be given as a neoadjuvant treatment especially in

postmenopausal ER-rich cases (The ATAC Trialists Group, 2002). Recently, studies have indicated that anastrozole (aromatase inhibitor) is superior to tamoxifen as adjuvant treatment for ER positive postmenopausal women (Miyoshi et al., 2003).

Commonly used chemotherapeutic drugs

There are several kinds of chemotherapeutic drugs: alkylating agents (cyclophosphamide, thiotepa, L- phenylalanine mustard); antimetabolites (5-fluorouracil and methotrexate); vinca alkaloids (vincristine and vinblastine) and anthracyclines (doxorubicin) and other agents. Treatment with combinations of drugs is more effective than single agents since the maximum dose of a single drug may be insufficient to kill all malignant cells, but in combination, the efficacy may increase to a 100% of cell death. Also, it is known that tumours consist of heterogeneous populations of cells that vary in their resistance and sensitivity to chemotherapy, so that a combination will give a broad effect (Cleator et al., 2002).

The most frequently used combination was cyclophosphamide, methotrexate, 5-fluorouracil (CMF). Subsequently, doxorubicin was incorporated into combinations of cyclophosphamide and 5-fluorouracil (CAF). These combinations were found to be effective for the treatment of both locally advanced and metastatic breast cancers. For women with operable breast cancer, the use of systemic adjuvant therapy has improved the outcome (Garrett et al., 1997).

Since the early 1990s, new agents have been used such as the two taxanes drugs (paclitaxel and docetaxil), the taxanes have been also incorporated into combinations included the anthracyclines (doxorubicin and epirubicin) and drugs from other groups (Hortobagyi, 2000) especially for use with neo-adjuvant treatment (Colleoni et al., 2004). Current evidence suggests that sequential administration of taxane- and anthracycline-based therapy is superior to concomitant administration as a neoadjuvant treatment (Estevez and Gradishar, 2004).

Anthracyclines

This group includes two essential drugs, Doxorubicin and Epirubicin. Doxorubicin is a natural product produced by the Streptomyces species and it is the most commonly used anthracycline. Anthracyclines act by several mechanisms such as interaction with nuclear enzyme topoisomerase II (Smith and Soues, 1994). In the normal resting state of the cell, DNA is tightly coiled into a compact structure. Topoisomerases reduce DNA coiling facilitating engagement of DNA in transcription, replication or repair processes. Anthracyclines also work as DNA intercalators, causing single-stranded or double-stranded DNA breaks (Smith and Soues, 1994).

Taxanes

This group includes Paclitaxel (the first taxane to be identified), which was isolated from the bark of the Pacific Yew (*Taxus Brevifolia*) in 1971. Taxanes act by binding to polymerised microtubules resulting in bundling of microtubules preventing them from performing their role in mitosis (Rowinsky et al., 1988). They became the most active chemotherapeutic agents for the treatment of advanced breast cancer (Piccart et al., 2001).

1.4 Predicting the behavior of breast cancer

Breast cancer is a heterogeneous disease; every single tumour is different in growth rate, pattern of metastasis and other biological features. Accordingly, patients are different in their benefit from breast cancer therapy. With the availability of more treatment modalities, it is important to identify factors that are associated with survival regardless of treatment (prognostic factors) and those identify patients who respond to therapy (predictive factors) (Henderson and Patek, 1998).

1.4.1 Pathological factors

Tumour grade

The role of histopathological features, such as tumour grade in breast cancer prognosis was established some time ago (Bloom and Richardson, 1957) and there is good

evidence that well-differentiated tumours have a good prognosis (Elston and Ellis, 1991). Pinder et al. (1998) showed a significant association between histologic grade and overall survival for both lymph node positive and negative groups. In a recent study by Schneeweiss et al. (2004), it was shown that tumour grade was an independent factor determining survival after treatment with neoadjuvant chemotherapy.

Tumour type

It has been shown that histological classification of breast cancer by type can provide prognostic information (Ellis et al., 1992). Certain types correlated survival but the correlation was not as strong as that with tumour grade (Pereira et al., 1995). A recent study has supported the prognostic power of tumour histology in breast cancer (Korkolis et al., 2004).

Tumour size

Although the clinical evaluation of tumour size is not very accurate with low clinico-pathological agreement (only 54%), pathological size of the primary tumour is considered as a factor for prognosis. Tumour sizes of small tumours should be assessed microscopically and larger tumours macroscopically. Smaller tumours have been shown to have increased survival (Carter et al., 1989; Galea et al., 1992).

Lymph node status

It is now well established that a negative lymph node status is one of the most important favorable prognostic factors for breast cancer survival. Increased mortality is associated with increased number of axillary lymph nodes involved by metastasis; tumours with three or less lymph nodes have a better prognosis than those with four or more lymph nodes (Axelsson et al., 1992; Carter et al., 1989).

More recent studies showed that the ratio of positive to negative nodes is important (Vinh-Hung et al., 2003; Voordeckers et al., 2004), especially for those treated with adjuvant chemotherapy (Megale Costa et al., 2004).

1.4.2 Hormone receptors

Estrogen receptors (ER) and progesterone receptors (PgR) are nuclear steroid hormone receptors. The ER status has been shown to correlate with the patient response to antioestrogen hormonal treatments such as tamoxifen (Robertson et al., 1992; Robertson et al., 1994). The combination of assessment of both ER and PgR increases the prediction of patient response to endocrine therapy. About 50% of ER-positive breast cancers respond to endocrine therapy and this percentage increases to 75% when tumours contain both ER and PgR receptors (Anderson, 2003).

1.4.3 Other biological factors

A range of other factors have been studied, some of which will be discussed in more detail in section 1.9 since they are ones selected for investigation in this thesis.

Proliferation

There has been conflicting data about the relevance of proliferation in breast cancer with some showing that high proliferation is associated with worse prognosis (Veronese et al., 1993) and others showing no difference (MacGrogan et al., 1996). A recent study by van Diest et al. (2004) using different methods for assessment such as mitotic counts, Ki-67/MIB-1 labeling and Cyclin A index, concluded that high proliferation correlates with poor prognosis regardless of method of assessment.

Epidermal growth factor receptor (EGFR)

The epidermal growth factor receptor (EGFR) tyrosine kinase family consists of four members: EGFR, c-erbB-2, c-erbB-3 and c-erbB-4 (Gullick and Srinivasan, 1998). EGFR is a transmembrane protein important for growth of normal breast. High levels of the protein can be detected in about a third of breast cancer cases (Walker et al., 1997) Other studies have shown that over-expression of the receptor is associated with poor prognosis (Abd El-Rehim et al., 2004; Klijn et al., 1992; Tsutsui et al., 2003).

HER-2

HER-2 (c-erbB2) is a proto-oncogene, which encodes for a tyrosine kinase membrane protein. Over-expression of HER2 occurs through gene amplification and mRNA over-expression in approximately 20-30 % of breast cancers (Rampaul et al., 2002). An early study showed that HER2 gene amplification was an independent survival prognostic factor in node-positive breast cancer (Slamon et al., 1987) and this is now well established. Recent studies have shown that coexistence of both HER2 over-expression and p53 protein accumulation are strong prognostic markers associated with short survival in breast cancer (Yamashita et al., 2004) and the over-expression of HER2 can also predict the response to doxorubicin (Campiglio et al., 2003).

P53

There have been many studies investigating the role of the tumour suppressor gene p53 in breast cancer. Some have used immunohistochemistry to detect the stabilized protein, most of these have shown that increased p53 protein expression is associated with poor prognosis (Barnes et al., 1993; Levesque et al., 1998; Stenmark-Askmalm et al., 1994) but others have found no effect (Bianchi et al., 1997) and Gohring et al. (1995) reported an improved survival.

Those studies analyzing mutation status have given more consistent results, with p53 mutations relating to poor prognosis and disease free survival (Hartmann et al., 1997; Pharoah et al., 1999). The location of the mutation can have an impact; mutations confined to the zinc-binding domains are associated with worst overall survival (Borresen et al., 1995) and those affecting DNA binding domains relate to poor prognosis (Berns et al., 1998b). More recently, Lai et al. (2004) reported that mutations in exon 7, silent and missense mixed mutations, mutations in which frame was changed and transcriptional mutations were all associated with poor prognosis.

Bcl-2 family of proteins

The anti-apoptotic protein bcl-2 has been shown to relate to the presence of ER (Teixeira et al., 1995). Loss of bcl-2 is associated with markers of poor prognosis

(Russell et al. 1994) and low expression was found to relate to shorter overall survival (Sjostrom et al., 2002), while overexpression of bcl-2 was associated with a favorable outcome (Berardo et al., 1998; Daidone et al., 1999). There have been conflicting findings for the pro-apoptotic protein bax, with no relationship to prognosis (Veronese et al., 1998) or loss relating to shorter survival (Krajewski et al., 1995; Wu et al., 2000). Bcl-x, which is also anti-apoptotic, was found to relate to longer disease free survival when present in high levels (Krajewski et al., 1995).

1.5 Apoptosis

Apoptosis, also called programmed cell death, represents a physiologic form of cell death involved in every homeostatic and pathologic process in the body (Gastman, 2001). The term apoptosis, involving two Greek words, apo = off from, and ptosis = falling, was described by (Kerr et al., 1972) to denote this form of cell death. Apoptosis involves cleavage of nuclear chromatin between the nucleosomes by specific endonucleases, producing chromatin fragments composed of approximately 200 base pairs (Buja et al., 1993). The inability of damaged cells to undergo apoptosis can contribute to the development of cancer as it allows the persistence of DNA damage (Mooney et al., 2002). The duration of the process of apoptotic cell death depends on the stimulus and the type of cell, but it usually takes from 12-24 hours and changes in cell morphology last for 2-3 hours (Leist and Nicotera, 1997).

1.5.1 Morphology

The process of apoptosis is defined by a series of morphological changes, which can be divided into three phases (figure 1.1).

- Condensation of the nuclear chromatin mainly at the edge of the nucleus, followed by reduction in the nuclear size, reduction of cell volume, but the mitochondria remain morphologically normal.

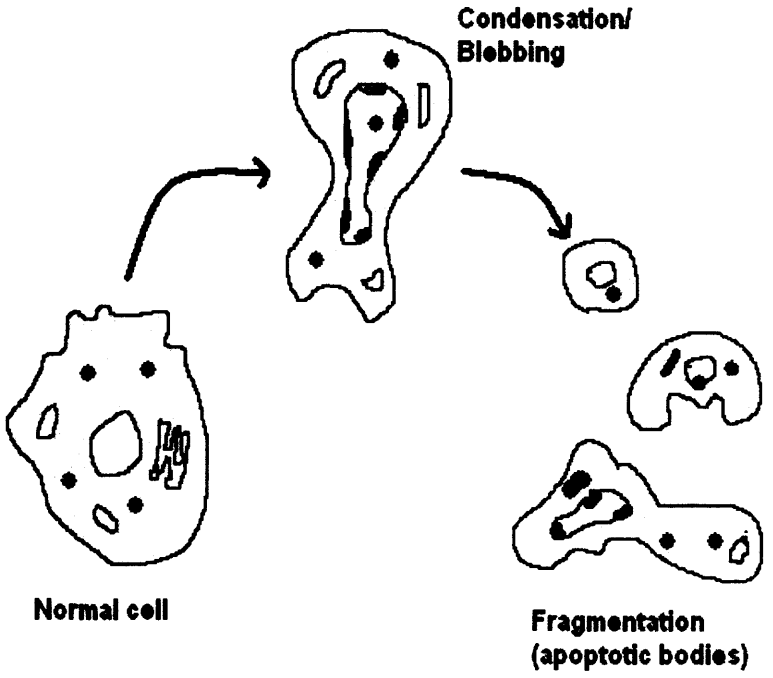

Figure 1.1: Morphological changes of apoptosis, including cell condensation with cytoplasmic blebbing and then fragmentation of the nucleus into small structures named apoptotic bodies.

- Cytoplasmic blebs appear on the cell surface, the cell detaches from the neighbouring cells with fragmentation of both the nucleus and cytoplasm to give multiple small membrane-bound apoptotic bodies.

- Engulfment of apoptotic fragments by macrophages and other malignant cells in addition to degeneration of residual nuclear and cytoplasmic structures, with secondary changes which resemble those of necrosis (Arends et al., 1990; Kerr et al., 1994; Kerr et al., 1972).

1.5.2 Apoptosis regulation

Apoptosis can be activated by exposure of cells to a wide variety of stimuli including developmental signals, cellular stresses, DNA damage and disruption of cell cycle (Dragovich et al., 1998; Yu and Zhang, 2004). More than 200 genes, constituting approximately 0.6 % of genes in the human genome, are involved in the regulation of apoptosis (Reed et al., 2003). Two main apoptotic pathways have been identified, the intrinsic and the extrinsic (Green, 2000) (figure 1.2).

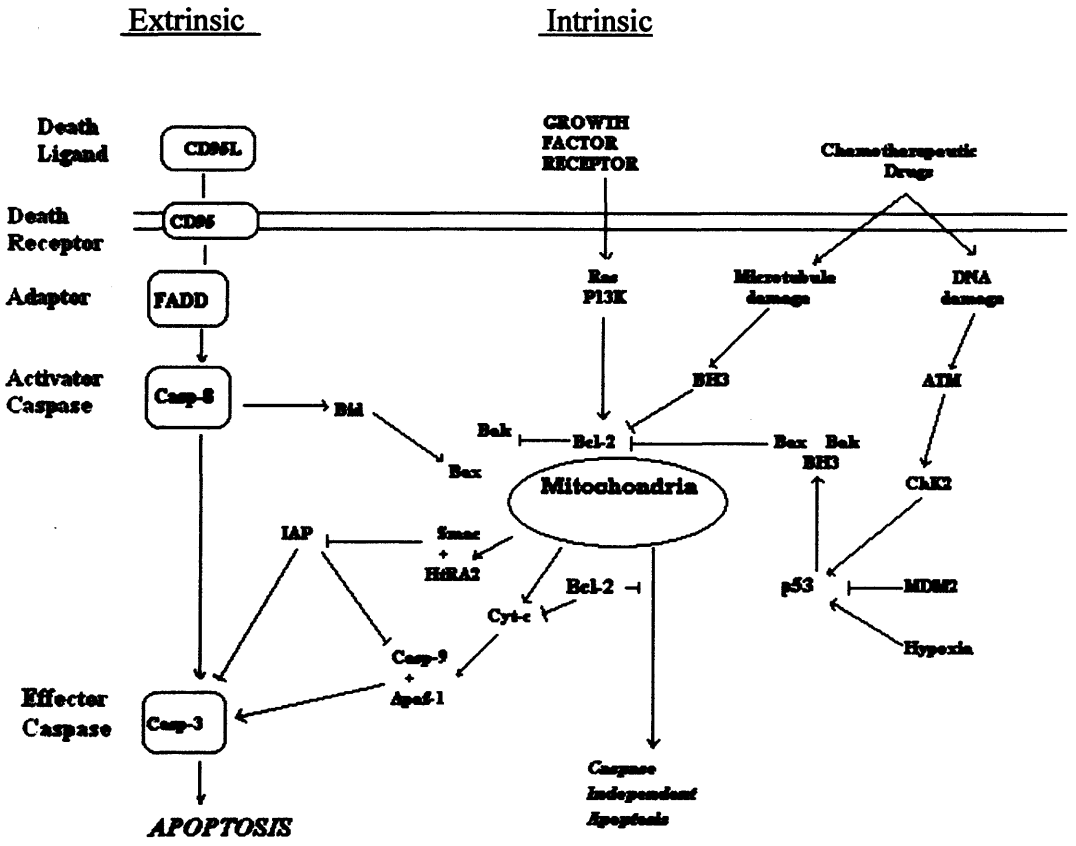

Figure 1.2 the two major pathways of apoptosis in mammalian cells, mitochondrial (intrinsic cell death) pathway and the receptor-mediated (extrinsic) pathway (adapted from (Johnstone et al., 2002)

The extrinsic pathway is critical for immune selection and inflammation whilst various intracellular and environmental stimuli converge on the intrinsic pathway. However,

the two pathways exhibit functional crosstalk. Different apoptotic stimuli signal through different members of the bcl-2 family (see 1.6) (figure 1.3) e.g. bad regulates growth factor induced apoptosis (Motoyama and Hynes, 2003); when activated it inhibits anti-apoptotic bcl-2/bcl-x thus, allowing pro-apoptotic bax to become active.

Intrinsic cell death signaling

The intrinsic cell death pathway is also called the mitochondrial cell death pathway. It is characterized by mitochondrial leakness and collapse of mitochondrial function (Reed and Kroemer, 2000). Upon stimulation by Bax and Bak, changes occur in the mitochondria such as opening of the permeability transition bores, resulting in loss of the membrane potential and influx of water into the mitochondria. This causes rupture of the outer mitochondrial membrane and release of the apoptotic proteins. These include cytochrome-C, SMAC/Diablo, Omi/HtrA2, AIF and endonuclease G into the cytoplasm. Cytochrome-C localizes to the intermembrane space of the mitochondria and when released interacts with Apaf-1 (apoptotic protease-activating factor-1), ATP/dATP, and caspase 9 to form the apoptosome (figure 1.2). This leads to activation of caspase 9 which consequently activates caspases 3 and 7 resulting in cell death by apoptosis (Wang, 2001).

Smac/DIABLO (second mitochondrial activator of caspases/direct IAP-binding protein of low isoelectric point) and Omi/HtrA2 bind to the BIR domains of the inhibitor of apoptosis proteins (IAPs) to inhibit their effect on caspase activity. AIF (apoptosis inducing factor) is the third protein to be released from the mitochondria; it induces partial DNA fragmentation and chromatin condensation independently of caspases (Joza et al., 2001; Wang et al., 2002).The intrinsic cell death pathway is partly regulated by p53 and Ataxia-telangiectasia mutated gene (ATM) (Dragovich et al., 1998).

in mammalian cells inhibited apoptosis, demonstrating that the cell death program is conserved among different species (Deveraux and Reed, 1999).

In humans, seven members of the IAP family have been identified. The first member to be identified was the neuronal apoptosis inhibitory protein (NAIP), followed by the cellular IAP1 (c-IAP1/HIAP-2), cellular IAP2 (cIAP2/HIAP-1), X-chromosome-linked IAP (XIAP/HILP), Survivin, BRUCE, and Livin/ML-IAP. These are located at 5q13.1, 11q22-q23, 11q22-23, Xq24-25, 17q25, 2p21-p22 and 20q13.3 respectively (Liston et al., 2003; Verhagen et al., 2001) (table 1.3).

IAP	Chromosomal Location	Number of Amino acids	Protein Size/KDa	Reference
XIAP	Xq24-25	497	55	(Liston et al., 2003)
Survivin	17q25	142	16.5	(Ambrosini et al., 1998)
cIAP-1	11q22-23	618	70	(Liston et al., 2003)
cIAP-2	11q22-23	604	68	(Duckett et al., 1996)
NAIP	5q13.1	1403	156	(Roy et al., 1995)
LIVIN/ML-IAP	20q13.3	298	31	(Kasof and Gomes, 2001)
Bruce	2p21-p22	2845	528	(Hauser et al., 1998)

Table 1.3: Chromosomal location and molecular characterization of human IAPs.

Structure of IAP family of proteins

The IAP family of proteins is structurally characterized by the presence of 70-80 amino acid domains named Baculoviral IAP Repeat (BIR). Each member of the family contains at least one BIR domain (range 1-3 copies of BIR motifs) (figure 1.5). BIR domains are characterized by an invariant number of amino acids, including three conserved cysteines and one conserved histidine. They are approximately 70 amino acids long, but some of them may contain more than 100 amino acids (Hinds et al., 1999).

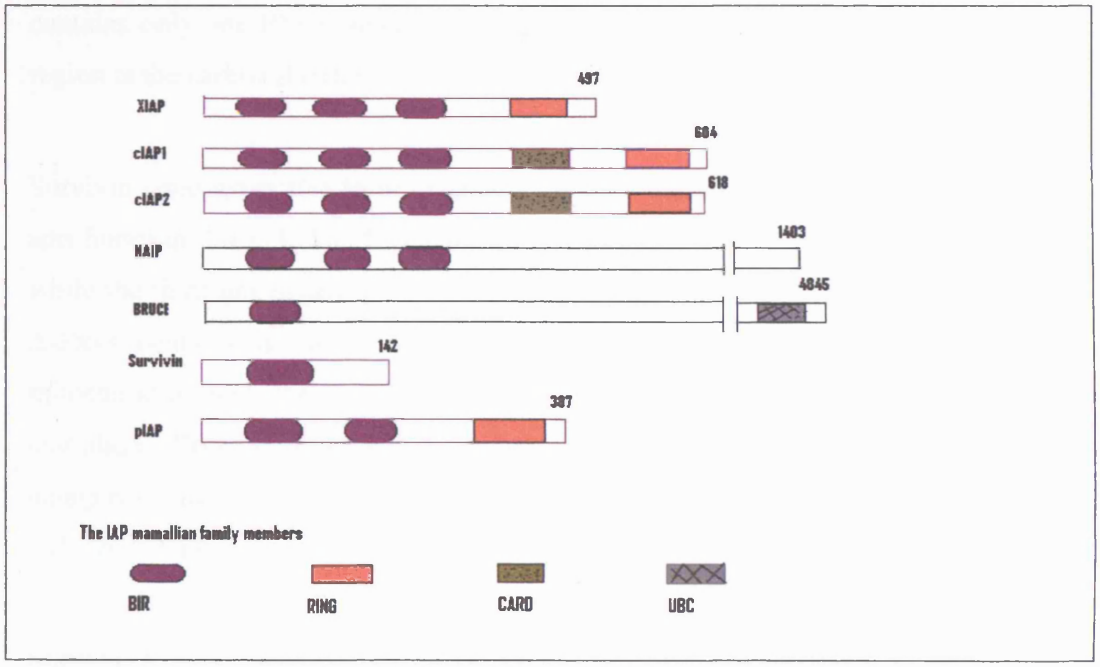

Figure 1.5: Structure of different IAP family members. NAIP contains three BIR domains with a large C-terminus containing a nucleotide-binding olygomerization domain (NOD). C-IAP1, cIAP2 and XIAP contain three BIR domains and a C-terminus RING finger. While Survivin contains single BIR domain and Livin contains single BIR domain in addition to a C-terminus RING finger.

The second motif found in the baculoviral IAPs is the C- terminus RING (Really Interesting New Gene) Zn finger that is present in most IAPs. It is of paramount importance for the baculoviral IAP anti-apoptotic function (Yang et al., 2000). In insect cells, the IAPs require both the amino-terminal BIR domains as well as the carboxyl-terminal RING domains for suppression of apoptosis (Clem and Miller, 1994), while in human IAP family of proteins (c-IAP1, c-IAP2 and XIAP) the anti-apoptotic function can be achieved in the absence of the carboxyl-terminal RING domain (Takahashi et al., 1998). Human c-IAP1 and c-IAP2 proteins contain a caspase recruitment domain (CARD) located between the BIR and the RING domain. This domain is not necessary for apoptosis. The IAP BRUCE contains another domain named Ubiquitin-Conjugated Domain (UBC).

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protein of 142 amino acids with a molecular weight of 16.5 kDa. It has four exons and three introns (Ambrosini et al., 1997). Survivin is the smallest IAP member because it contains only one BIR domain at the amino terminal region with a long alpha-helical region at the carboxyl terminus (Chiou et al., 2003).

Survivin gene gives rise to three splice variants: the wild-type Survivin, Survivin-2B and Survivin Δ-Ex-3. The first two arise from the insertion of an alternative exon -2 while the third one arises from removal of exon-3. The C-terminus region in Survivin Δ-Ex-3 contains a nuclear localization signal that targets this Survivin to the mitochondria and the nucleus, while Survivin and Survivin-2B localize to the cytoplasm. Both Survivin and Δ-Ex-3 are anti-apoptotic proteins, while Survivin-2B antagonizes the other anti-apoptotic variants because it has lost its anti-apoptotic power (Altieri, 2001).

Survivin expression is cell cycle-regulated with major expression in G2/M phase. At mitosis it localizes to different components of the mitotic apparatus, kinetechores, centrosomes, spindle microtubules, central spindle midzone and midbodies. 80% of the total cellular Survivin content in mitotic cells is bound to centrosomes and microtubules of the metaphase and anaphase spindle. In cancer cells, Survivin is located abnormally through the cytoplasm and the nucleus (Reed, 2001) and negatively regulated by wild-type p53 (Mirza et al., 2002).

Survivin function

The exact molecular aspects of Survivin function are not fully known and probably still controversial. In general, Survivin is an anti-apoptotic protein that can interfere with cell cycle progression and microtubule stability. Survivin inhibits apoptosis by either direct or indirect mechanisms. It inhibits the activity of caspase -9 via the BIR domain. Survivin may function to inhibit the caspase activity indirectly through binding to and sequestering smac/DIABLO and consequently preventing smac/DIABLO from binding to other IAPs (Chiou et al., 2003).

Survivin expression in normal and neoplastic tissues

Survivin is strongly expressed in embryonic and fetal organs, but it is undetectable in most normal differentiated adult tissues (Ambrosini et al., 1997). Adult cell types that express Survivin include thymocytes, $CD34^+$ bone-marrow-derived stem cells, endothelial cells, basal epithelial cells of colonic mucosa and epithelial cells of normal uterine cervix. Survivin is also weakly expressed in the placenta, proliferative and secretory endometrium (Frost et al., 2002; Takai et al., 2002b). It has been identified in tumours of the lung, breast, colon, stomach, esophagus, pancreas, bladder, uterus, ovaries, large cell non-Hodgkin's lymphoma, melanoma and non-melanoma skin cancer. Survivin is typically observed in nearly all tumor cells and not just in proliferating cells which suggests Survivin gene deregulation in cancer (Altieri, 2001).

Survivin is also expressed in the pre-neoplastic lesions of the skin, pancreas, uterine cervix and in colonic adenoma. This means that Survivin up-regulation occurs early during tumorigenesis. The expression of Survivin is rare in some tumours such as low-grade non-Hodgkin's lymphoma and even within the same tumor it is sometimes heterogeneous. High-level expression of Survivin has been associated with worse clinical outcome in neuroblastoma, colon and gastric cancers (Ambrosini et al., 1997; Ambrosini et al., 1998).

1.6.2.2 XIAP

XIAP (X-linked IAP), also called MIHA/hILP/BIRC4, is a prototype member of the IAP family. It contains three BIR domains and a RING zinc finger domain near the C terminal (Liston et al., 1996). XIAP is considered to be the most potent member of the IAPs family regarding anti-apoptotic function. It directly inhibits caspases 3, 7, and 9, but does not affect caspases 1, 6, 8, or 10 (Deveraux et al., 1998).

BIR2 of the XIAP protein is responsible for caspase 3 and 7 inhibition activity in addition to some other residues in the linker region that precedes BIR2, while caspase 9

is inhibited by BIR3 alone. The XIAP is cleaved into two fragments, one of which contains BIR1 and BIR2 domains, sufficient to protect cells against fas-induced apoptosis, while the second fragment contains BIR3 and the RING finger domains and protects cells against bax-induced apoptosis (Holcik et al., 2000).

Negative regulation of XIAP

There are two negative regulators of XIAP: the XAF1 (XIAP Associated Factor 1) and Smac/DIABLO. XAF1 is localized in the nucleus; it can bind XIAP and antagonize function by suppressing the caspase activity (Figure 1.6). Upon release of Smac/DIABLO from the mitochondria, it becomes activated, binds XIAP and inhibits its caspase-binding activity (Verhagen et al., 2000).

XIAP expression in cancer

Although, the expression of XIAP has not widely assessed in solid tumours including breast cancer, studies have shown its up-regulation in cell lines and some tumours. High levels were detected in all prostate cell lines at the level of both RNA and protein (McEleny et al., 2002) and in all breast, pancreatic and colon cancer cell lines examined (Yang et al., 2003a). It has been shown to be up-regulated in non small-cell lung cancer cells (Ferreira et al., 2001) and is expressed by immunohistochemistry in 95 % of clear-cell renal cell carcinomas (Ramp et al., 2004).

1.7 Tumour suppressor gene p53

P53 (TP53) was first identified in the late 1970's as an oncogene binding to the large T-antigen in SV40-transformed cells (Lane and Crawford, 1979). Later, it was recognized to be a tumour suppressor gene. Today, p53 has become the most studied tumour suppressor gene in cancer research, probably because abnormalities of the gene are among the most prevalent molecular abnormalities in human cancer (Hainaut et al., 1998). In breast cancer, it is crucial in the development and progression of the disease and alterations to p53 can provide information about many aspects of breast cancer

(Walker et al., 1997). Mechanisms that exert strict control over p53 are tremendously important (Ashcroft and Vousden, 1999).

1.7.1 Structure and function

P53 protein is a member in a small family of proteins that includes two other members, p63 and p73. Although they all have similar structure, p63 and p73 are functionally active in normal development (Irwin and Kaelin, 2001) while p53 is mainly concerned in the prevention of tumour development.

P53 gene is located at chromosome 17p13.1 (McBride et al., 1986; Miller et al., 1986). It encodes a 53-kDa nuclear phosphoprotein that consists of 393 amino acids (Lamb and Crawford, 1986) which can be divided into five functional domains (Figure 1.6): conserved N-terminal trans-activation domain (amino acids 1-95); proline-rich domain; sequence-specific DNA-binding domain (amino acids 102-292); tetramerization domain; and a basic C-terminal tail (amino acids 300-393). These five domains have distinct but inter-dependent functions.

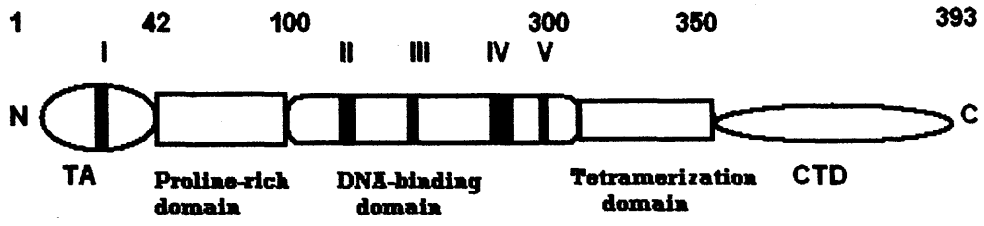


Figure 1.6: the structure of mammalian p53 protein. It contains five highly conserved boxes including five functional regions (I-V) with interdependent functions. TA: transactivation domain, CTD: C-terminal domain.

The central DNA-binding domain of p53 confers its ability to act as a transcription factor. More than 90% of p53 mutations are found in this domain (Hollstein et al., 1994). The tetramerization domain flanks the DNA-binding domain and is important in modulating p53 tumour suppressor function. The N-terminal regulatory region contains

the transactivation domain through which p53 interacts with components of the transcriptional machinery; phosphorylation at the N-terminus of the protein may affect stability of p53. The C-terminus of p53 has other important regulatory functions; it maintains p53 protein in a latent state for specific DNA-binding, acetylation and /or serine phosphorylation (el-Deiry, 1998). The proline-rich region is located between the trans-activation domain and the sequence-specific DNA binding domain and it is required for p53 to suppress cell growth (Walker and Levine, 1996).

1.7.2 Activation

Under normal cellular conditions, p53 is in standby mode, and its activation occurs in response to many cellular stresses such as DNA damage induced by chemotherapeutic drugs, UV irradiation or ionizing irradiation. Other factors that might trigger p53 activation are hypoxia, heat shock, metabolic changes, pH changes, unusual genome instability and over-expression of oncogene products (Levine, 1997).

Several independent pathways act to activate p53; they all depend on specific upstream regulatory kinases (Figure 1.7), which have been shown to phosphorylate residues within the N-terminus of p53. These include members of the PI 3-kinase family such as DNA protein kinase (DNA-PK), Ataxia-Telangiectasia Mutated (ATM), and ATM-related polypeptide (ATR), in addition to other proteins like JNK and CK1.

The DNA protein kinase (DNA-PK) has been shown to phosphorylate p53 on serine 15 and serine 37 in response to DNA strand breaks and disrupts the interaction of p53 with Mdm2 (Woo et al., 1998). The ATM/ChK2 – dependent pathway also becomes activated by DNA double-strand breaks in response to ionizing radiation (Vogelstein et al., 2000). ATM is the gene mutated in ataxia telangiectasia (AT) patients; it phosphorylates p53 at serine 15.

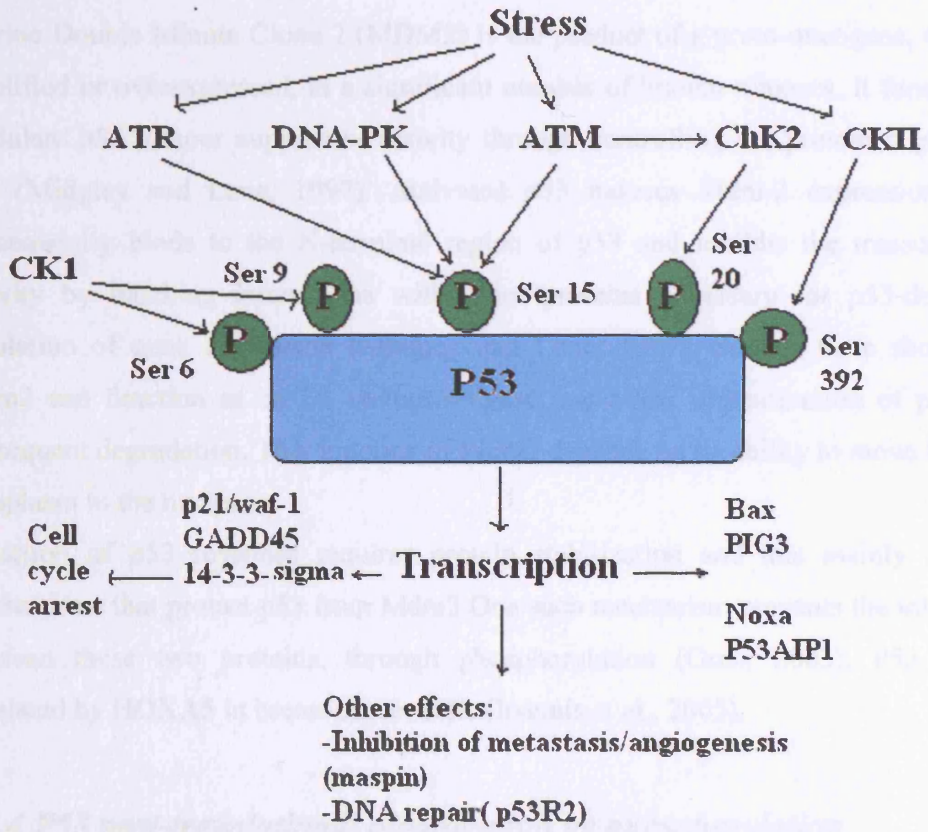


Figure 1.7: the components of p53 signalling, activation occurs in response to a variety of cellular stresses leading to phosphorylation of p53 protein at different sites and consequently activates a group of downstream effector genes that mediate the tumour suppressor activity of p53. Adapted from (Gasco et al., 2002).

1.7.3 P53 and MDM2

Upon activation, p53 protein level increases within 1-12 h after exposure to stress, the increase being due to both increase in translation rate (Fu et al., 1996) and decrease in degradation rate (Maltzman and Czyzyk, 1984).

Murine Double Minute Clone 2 (MDM2) is the product of a proto-oncogene, which is amplified or overexpressed, in a significant number of human tumours. It functions to modulate p53 tumour suppressor activity through controlling the protein degradation rate (Midgley and Lane, 1997). Activated p53 induces Mdm-2 expression which consequently binds to the N-terminal region of p53 and inhibits the transcriptional activity by blocking interactions with other proteins necessary for p53-dependent regulation of gene expression (Midgley and Lane, 1997). Studies have shown that Mdm2 can function as an E3 ubiquitin-ligase and allow ubiquitination of p53 with subsequent degradation. This function of Mdm2 depends on its ability to move from the cytoplasm to the nucleus.

Induction of p53 response requires protein stabilization and this mainly involves mechanisms that protect p53 from Mdm2.One such mechanism prevents the interaction between these two proteins, through phosphorylation (Oren, 2003). P53 is also regulated by HOXA5 in breast cancer cells (Ioannis et al., 2005).

1.7.4 P53 post-translational modification by phosphorylation

In response to stress or DNA-damage, p53 protein has been shown to be phosphorylated at several residues within the N-and C-terminal regions by several cellular kinases. Seven serines (6, 9, 15, 20, 34, 37, and 46) and two threonines (55 and 81) in the amino terminal domain of p53 have been shown to be phosphorylated (Buschmann et al., 2001) and five serines (315, 372, 376, 378, and 392) in the carboxyl-terminal domain (Liu et al., 1999).

Phosphorylation of p53 on serine 392 by purified casein kinase II (CKII) enhances p53 sequence-specific DNA binding which is crucial for transcriptional activation of p53

1.8.1 Cell cycle checkpoints

Cells are constantly exposed to both endogenous and exogenous stresses that may damage the DNA. To maintain the genomic integrity and protect cells against these insults, there is a network of DNA repair pathways named as cell cycle checkpoints (Wood et al., 2001). Cell cycle checkpoints are biochemical-signaling pathways that sense any damage in genomic DNA or in chromosome function. The checkpoint proteins work throughout the cell cycle and lead to delay in cell cycle progression in order to facilitate DNA repair. They maintain arrest of cell cycle until repair is complete, or eliminate hazardous damaged cells through induction of apoptosis (Zhou and Elledge, 2000).

Defects in this checkpoint system may lead to accumulation of damaged and altered genetic information and ultimately to cancer formation (Thompson and Schild, 2002) and can also affect the response of tumours to radiotherapy and chemotherapy (Bartek and Lukas, 2003).

Checkpoints monitor the progress of the cell cycle at three stages, G1 to S, G2 to M, and from metaphase to anaphase (Murray, 1994). P53 plays an important role in cell cycle arrest and is considered as checkpoint regulator. Cells with wild type p53 arrest the cycle at G1 phase, while those with mutant p53 arrest them at S and G2 phases (Eastman, 2004). P53 induces this action through the induction of cyclin-dependent kinase inhibitor p21 (el-Deiry et al., 1993).

1.8.2 P21^{waf-1}

P21 is a 21 KDa nuclear protein encoded by a gene located on chromosome 6p and it is a prototype of a small family of proteins that negatively regulate the cell cycle (el-Deiry et al., 1995). Is also known as Waf-1 (wild-type p53 activated fragment 1), CIP1 (cyclin-dependent kinase interacting protein 1) (Wakasugi et al., 1997), SDI1 (senescent cell-derived inhibitor 1) (Noda et al., 1994) and CAP20 (Lukas et al., 1997).

Induction of p21^{waf-1}

P21^{waf-1} is mainly induced by wild-type p53 in response to DNA damaging agents. It is also induced during terminal differentiation both in vivo and in vitro (Steinman et al., 1994) and has also been linked with cell senescence (Roninson, 2002). P21^{waf-1}can also be induced by other factors independent of p53, such as serum stimulation, treatment with PDGF and FGF, okadaic acid, butyric acid, retinoic acid, cytokines G-CSF and IL-6, in addition to transforming growth factor-beta (Michieli et al., 1994).

Function

P21 has tumour suppressive properties; it inhibits a broad range of cyclin-CDK complexes and therefore plays a pivotal role in cell cycle regulation, cellular senescence and in neoplasia (Noda et al., 1994). It blocks the progression of the cell cycle at the G1/S transition phase. It is also involved in G2/M phase arrest by inhibition of c-myc so keeping cells at the G2 phase after DNA damage (Dulic et al., 1998). It has been suggested that p21^{waf-1} can also localize to the cytoplasm of cancer cells where it plays a different role. It inhibits apoptosis by binding to and consequently inhibiting the apoptosis signal-regulating kinase 1 (Asada et al., 1999).

1.8.3 ChK2

ChK2 (also known as Cds1 or CHEK2) is one of the essential members of genome integrity checkpoints (Bartek and Lukas, 2003). They are serine/threonine kinases activated in response to different genotoxic stresses and function mainly to transduce the checkpoint signals (with help of ChK1) from the upstream kinases such as ATM (ataxia-telangiectasia mutated) and ATR (ataxia-telangiectasia and Rad3-related kinase). ATM and ATR are considered to be sensors of the DNA damage while Chk1 and Chk2 are signal transducers.

ChK2 protein structure

It consists of three functional domains, an SQ/TQ cluster domain (SCD, residues 19-69), a forkhead associated domain (FHA, residues 112-175) and a Ser/Thr kinase domain (residues 220-486) (Ahn et al., 2004) (figure 1.8).

Regulation and activation of ChK2

ChK2 becomes activated after stimulation by any factor that directly or indirectly induces damage to the DNA such as ionizing radiation and chemotherapeutic drugs.

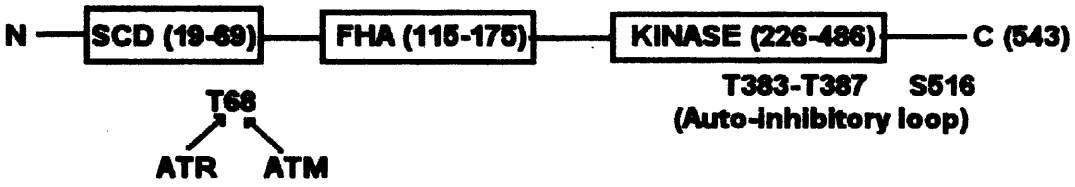


Figure 1.8: ChK2 protein structure.

ChK1 is mainly phosphorylated and activated by the ATR while ChK2 is activated by ATM; this is in addition to the cross talk between the two pathways (Eastman, 2004). Upon activation, ChK2 undergoes dimerization in which the phosphorylated Thr68 in one molecule binds to the FHA domain of the second molecule. Autophosphorylation at Ser516 in the kinase domain is also important for optimal activation of the kinase (Wu and Chen, 2003). After activation, ChK2 starts the downstream activation of other proteins of which two are members of the cell division cycle 25 dual specificity phosphatase family, Cdc25A and Cdc25C. Members of this family are important for the induction of cell cycle progression through activation of cyclin-dependent kinases cdk2 and cdk1 (Brown et al., 1999) (figure 1.9).

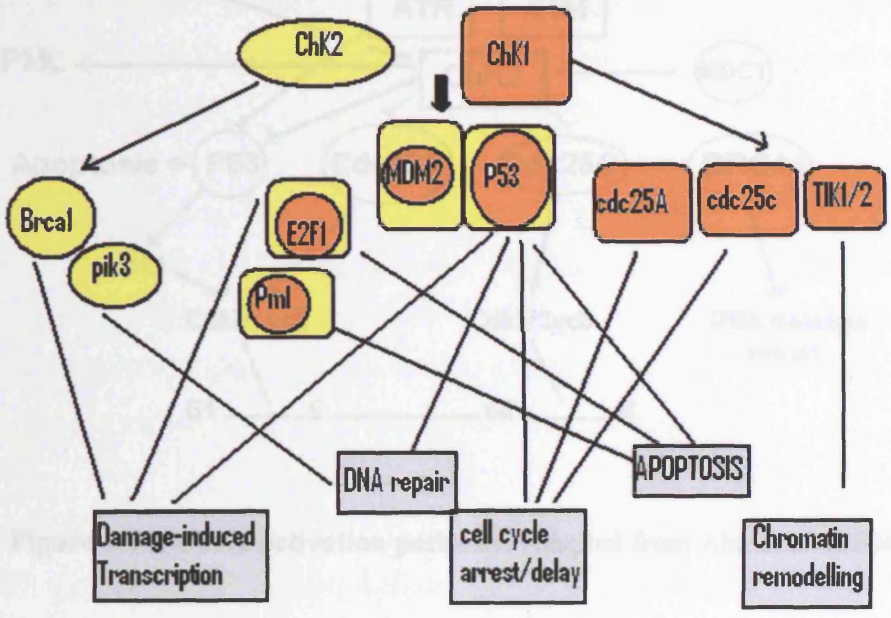


Figure 1.9: ChK1 and ChK2 activation pathways.

Cyclin-dependent kinases (Cdk) are the essential regulators of cell cycle progression. They are maintained in the cell in an inactive phosphorylated form associated with cyclins. Upon activation, Cdc25A dephosphorylates the Cyclin/Cdk complex and the Cdk become activated. Cdc25A acts on cyclin E/cdk2 and stimulates the S phase progression, while Cdc25C acts on cyclin B/cdk1 and stimulates the G2/M progression (figure 1.10).

ChK1 and ChK2 function by phosphorylating and inhibiting Cdc25 proteins. Cdc25A degrades after phosphorylation, while Cdc25C remains inactive in the cytoplasm of the cell (Peng et al., 1997).

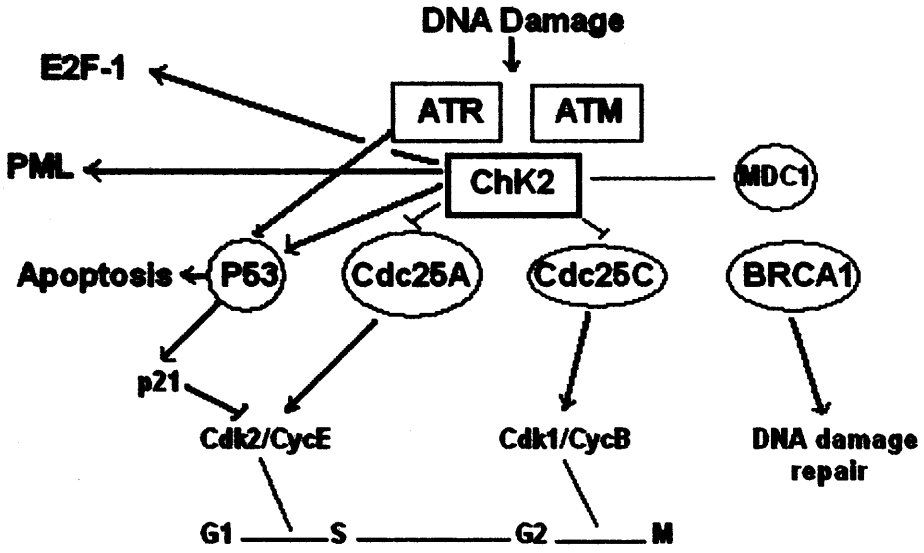


Figure 1.10: ChK2 activation pathway. Adapted from Ahn et al. (2004).

ChK2 also activates E2F-1; its over-expression can induce cell death. Through this mechanism, ChK2 regulates DNA damage-induced apoptosis (DeGregori et al., 1997). After DNA damage, ChK2 also phosphorylates BRCA1 and the Promyelocytic Leukemia (PML). ChK2 is capable of phosphorylating the tumour suppressor gene p53 at N-terminal sites such as ser15, Thr18, ser37 and ser20 with subsequent stabilization of p53 and disruption of the pre-complexed p53 with Mdm-2 (Ahn et al., 2003; Chehab et al., 2000).

1.9 Proliferation/apoptosis and regulatory proteins in breast cancer and relationship to treatment

Proliferation

Each cell is under constant checking mechanisms to maintain genomic integrity. Upon occurrence of any genomic abnormalities, such mechanisms suppress cell proliferation or induce cell death by apoptosis (Evan and Littlewood, 1998). Therefore, cell kinetics

(proliferation and apoptosis) have been shown to be important in breast cancer progression (Bai et al., 2001). The proliferative activity of a tumour is considered to be an important prognostic factor in primary breast cancer. Highly proliferative tumours are generally associated with shorter disease-free survival and overall survival (Ravaioli et al., 1998).

Studies have correlated changes in proliferation with treatment (Makris et al., 1997; Parton et al., 2002) reported that tumours with high Ki-67 levels responded better to chemotherapy, and those with low levels responded better to endocrine therapy. Also, (Archer et al., 2003) showed that pretreatment assessment of proliferation is predictive of response to neo-adjuvant chemotherapy in breast cancer.

Apoptosis

Induction of apoptosis by chemotherapeutics was found to be the key factor in cancer therapy and studies have shown an increase in apoptosis in breast tumours within 24 hours of starting chemotherapy (Ellis et al., 1997). The number of apoptotic cells has shown a significant correlation with prognosis, being increased with poor prognosis (de Jong et al., 2000; Lipponen et al., 1994) and as a predictive marker of drug response (Buchholz et al., 2003). A pilot prospective trial showed that high rates of apoptosis and proliferation at baseline were associated with improved pathological response to doxorubicin or paclitaxil treatment and also increased rate of apoptosis 24-48 h after treatment was associated with improved clinical response (Stearns et al., 2003).

Bcl-2 family

Bcl-2 was the first gene found to block apoptosis. It has been detected, using immunohistochemistry, in about 80% of both node negative and node positive primary breast cancers (Krajewski et al., 1995). Altered expression of bcl-2 proteins was reported to alter drug response in experimental systems *in vitro* and other experiments have shown an inverse correlation between chemosensitivity and bcl-2 levels (Kamesaki et al., 1993; Yang et al., 2003b) showed that bcl-2 negative tumours were more susceptible to anticancer drugs than bcl-2 positive tumours. High bcl-2 expression

may predict a good response to tamoxifen, independent of ER positivity (Elledge et al., 1997). Low bcl-2 levels were found to be associated with shorter overall survival (Sjostrom et al., 2002).

(Krajewski et al., 1995) studied the expression of Bax gene in a group of women with metastatic breast cancer; they showed expression of bax in normal mammary epithelium and most carcinomas in situ but in only one third of breast cancer cases. Reduced expression of bax was associated with a poor response to chemotherapy and poor prognosis in metastatic breast adenocarcinoma. Studies also showed that bax is a determinant of p53-dependent chemo-sensitivity (McCurrach et al., 1997).

Bcl-x is a member of the bcl-2 family. Through alternate splicing, bcl-x can encode several proteins including bcl-x_L (long) protein, which is antiapoptotic, and bcl-x_S (short) protein, which promotes apoptosis by inhibiting bcl-2 and bcl-x_L. Bcl-x expression has been described in a variety of normal and malignant breast tissues. Weak expression of bcl-x was observed in normal epithelial cells while strong staining was observed in about 75% of malignant tissue (Krajewski et al., 1999). Expression of bcl-x was found to be associated with histological grade III tumours (Sierra et al., 2000). and promote resistance to chemotherapy in tumours in vivo (Fujita and Tsuruo, 2000).

Survivin

It has been shown that Survivin, as a member of the IAPs, blocks apoptosis induced by different stimuli through the inhibition of caspase -3 and -7. Although it has an elevated expression in cancers, few studies have assessed the expression of Survivin in breast cancer. (Span et al., 2004; Tanaka et al., 2000; Zhang et al., 2004)) assessed expression of Survivin by immunohistochemistry and have shown that its presence is a significant predictor of worse outcome in breast carcinoma. However, (Kennedy et al., 2003) reported that expression of Survivin protein was favorably associated with both relapse free survival and overall survival in a study of 293 breast cancers. (O'Driscoll et al.,

2003) extended this study and found that the expression of Survivin, Survivin-2B and Survivin Δ-Ex-3 mRNAs were not prognostic indicators of disease outcome.

Survivin protein is involved in tumour cell resistance to some anticancer agents and ionizing radiation (Zaffaroni and Daidone, 2002). Some studies have demonstrated the resistance of Survivin-transfected cells to anticancer drug-induced apoptosis (Tamm et al., 1998). Survivin expression has been shown to resist apoptosis induced by chemotherapeutic drugs in cholangiocarcinoma (Chang et al., 2004).

XIAP

Because of the important role of XIAP in inhibiting apoptosis, it could have a major role in cancer formation, progression and resistance to therapy (LaCasse et al., 1998). There is little published work on the relation of XIAP with either prognosis or response to therapy in breast cancer. Parton et al. (2002) reported that XIAP was not a predictive marker of response to chemotherapy in breast cancer, but that could have been due to the small sample size of the study.

However, in other tumours studies have shown a strong correlation between XIAP levels and patient survival. In acute myeloid leukemia patients with lower levels of XIAP had significant longer survival times than those with increased XIAP levels (Tamm et al., 2000). To study the role of XIAP in the resistance of cancer cells to γ-irradiation, Holcik et al. (2000) used an anti-sense approach. This showed that low dose of γ-irradiation induced an up-regulation of XIAP in non-small cell lung cancer cells and this correlated with enhanced resistance to radiation-induced apoptosis. XIAP anti-sense treatment resulted in the induction of apoptosis in cells with wild-type p53, but not in cells that lacked or had mutant p53.

High levels of XIAP confer the resistance to chemotherapeutic drugs in human lung cancer (Ferreira et al., 2001). Also, reducing the XIAP protein expression increased breast cancer MDA-MB-231 cells susceptibility to chemotherapeutic agents (McManus et al., 2004) and the use of XIAP antagonists has been described as a potential therapy

for cancers over-expressing XIAP (Oost et al., 2004). Down-regulation of XIAP induced apoptosis in chemoresistant ovarian cancer cells (Sasaki et al., 2000).

P53

Several studies have suggested that p53 status could be important in tumour responsiveness to anti-neoplastic agents (Clahsen et al., 1998). Since TP53 is involved in apoptosis, cell cycle control, and repair of DNA damage, loss of p53 function may result in the relative resistance of breast cancers to chemotherapy (Lowe et al., 1993).

Most of the studies performed on the effect of p53 abnormalities on chemosensitivity of human tumours have produced conflicting results, with some studies indicated enhanced sensitivity and others indicated increased resistance to the same compounds (Blandino et al., 1999).

One study revealed that specific mutations correlated with primary resistance to doxorubicin and could be predictive of early relapse (Aas et al., 1996).The response rate to anthracycline-based chemotherapy was low in p53 positive cases (Rahko et al., 2003). In another study, breast cancers with p53 mutations were more likely to respond to paclitaxil (Kandioler-Eckersberger et al., 2000) but conversely, Schmidt et al. (2003) showed that none of the tumours with p53 expression responded to paclitaxil. Geisler et al. (2003) found a significant association between lack of response to 5-fluorouracil and mitomycin and p53 mutations. However, Brown and Wouters (1999); Sjostrom et al.(2000) concluded that neither p53 status nor the ability of the cell to undergo apoptosis appears to play a significant role in the sensitivity of breast cancer tumours to DNA-damaging agents.

P53 status has a role in response of breast cancers to hormonal therapy. The presence of p53 protein predicted poor response to tamoxifen in patients with recurrent breast cancer (Berns et al., 2000; Berns et al., 1998a) and in those with advanced disease.

p21^{waf-1}

There is still some controversy regarding the role of p21^{waf-1} in breast cancer prognosis and response to therapy. Wakasugi et al. (1997) reported the associated p21 expression with favorable prognosis, while Diab et al. (1997) reported the expression of p21^{waf-1} as a nuclear staining in 43% of the studied group of breast cancer tumors, but it was not significantly associated with other prognostic factors or with clinical outcome.

The expression of p21^{waf-1} in breast cancer has been assessed in relation to the response to endocrine therapy; it did not correlate with estrogen receptor (ER) status or with response to endocrine therapy but cases of high p21 expression showed an improved overall survival (McClelland et al., 1999). In the group of patients who were treated with adjuvant systemic therapy, p21+/p53+ tumors were associated with long DFS (disease-free survival) and OS (overall survival), while those with p21-/p53+ had the worst prognosis (Caffo et al., 1996). Sjostrom et al. (2000) concluded that the expression of p21^{waf-1} was not associated with response to chemotherapy in breast cancer.

ChK2

It has also been shown that p53 activation is controlled by some upstream regulatory proteins including the ChK2 protein. Some patients with Li-Fraumeni syndrome were found to have wild type p53 but mutant ChK2, which implies that ChK2 inactivation might be functionally equivalent to p53 mutation (Bell et al., 1999).

Screening of cancer cell lines showed missense mutations in ChK2 in different tumours and somatic mutations of ChK2 have been also found in diverse types of sporadic human malignancies including breast cancer (Sullivan et al., 2002). P53 mutations co-exist in the majority of cases with ChK2 mutation (Sullivan et al., 2002). ChK2 1100delC is a truncating mutation of ChK2 and has a frequency of 1.1% in healthy individuals but present in 5.1% of individuals with breast cancer. People with this type of mutation have a twofold increase of breast cancer risk in women and tenfold increase in men. Thus, ChK2 1100delC is considered a low-penetrance susceptibility gene

(Meijers-Heijboer et al., 2002). ChK2 $^{-/-}$ cells have been shown to be radioresistant with defects in γ-irradiation-induced apoptosis (Takai et al., 2002a). The strong relationship between p53 and ChK2 may support an important role of the latter in breast cancer prognosis and response to therapy, but currently, there are little data on this.

To conclude, proliferation/apoptosis and their regulatory proteins play an essential role in breast cancer, especially in relation to treatment. Although high proliferation is associated with poor prognosis, highly proliferating tumours respond better to treatment. High rates of apoptosis are associated with increased pathological response to therapy. The high expression of bcl-2 is associated with better outcome; tumours expressing bcl-2, bcl-x or reduced expression of bax show variable responses to different types of treatments. Expression of Survivin protein is usually associated with poor outcome in breast cancer, and with resistance to chemotherapeutic agents. Although there is little published work on XIAP in breast cancer, its expression in other tumours was associated with chemoresistance. The role of p53 and $p21^{waf-1}$ is still conflicting, ChK2 may have a role but currently there are no certain data.

1.10 Rational, hypothesis and aims of the work

Chemotherapy is an essential part of treatment in breast cancer. However, chemoresistance is the main obstacle to successful therapy; up to 50% of patients who are given pre- or post-operative cytotoxic therapy do not respond to this treatment. By identifying the biologic predictive factors of response to chemotherapy, clinicians may be better able to accurately identify patients who will be likely to benefit from the treatment and to avoid unnecessary toxicity for patients who may not benefit.

Most cytotoxic agents ultimately kill cancer cells by causing irreparable cellular damage that triggers apoptosis. One of the most important advances in cancer research is the recognition that apoptosis plays a major role in both tumour formation and treatment response (Johnstone, 2002) and the variability in apoptosis regulatory genes found in tumours may contribute in the variability in drug response (Innocenti, 2002). Consequently, the efficacy of cancer treatments depends not only on the cellular damage they cause but also on the cells ability to respond to the damage by activating the apoptotic machinery.

Thus, monitoring expression profiles of apoptosis regulatory genes in tumours in response to specific pharmacological agents may serve to identify predictive markers of drug response (Innocenti, 2002).

The genes most commonly related to apoptosis are:

- P53: the first tumour suppressor gene linked to apoptosis (Voglestein, 2000), p53 mutations occur in the majority of human cancers. The tumour suppressor activity of wild-type p53 is mainly due to its ability to act as a transcription factor and regulates the expression of number of proteins to induce growth arrest ($p21^{waf-1}$) or to regulate apoptosis (bax and bcl-2). The biological functions of p53, as a 'guardian of the genome', in growth arrest, apoptosis and genomic repair make it a potential predictive marker. Studies have found that defects in p53 predict for chemoresistance in some

malignancies, but in breast cancer the predictive value of p53 is still uncertain (Blandino et al., 1999).

- Bcl-2 family proteins: they comprise both anti-apoptotic members (bcl-2 and bcl-x) as well as pro-apoptotic molecules such as bax. Altered expression of bcl-2 family proteins have been reported in various human cancers (Reed, 1999) and may alter the response to treatment and patient survival.

- Inhibitor of Apoptosis Proteins (IAPs): family of endogenous caspase inhibitors that regulate apoptosis. Survivin and XIAP are essential members of this family, expressed at high levels in the majority of human cancers and they may provide important predictive and prognostic clues in breast cancer.

Consequently, my hypothesis is that alterations in the regulation of the apoptotic machinery of breast cancers affect the response to chemotherapy and the duration of patient survival and that one of the main determining factors for this is p53 status of the cancers.

Aims of this study are:

1- Characterization of a group of breast cancer cell lines, with and without p53 mutations, by immunocytochemistry and western blotting regarding the proliferation and apoptosis indices, the expression of p53, ChK2 and $p21^{wafl}$, the expression of the apoptosis regulatory genes (bcl-2, bcl-x and bax) and also the apoptosis inhibitor proteins Survivin and XIAP.

2- Use the previously characterised cell lines as models to study the response of these cells to treatment with two different chemotherapeutic drugs commonly used in breast cancer (Doxorubicin and Paclitaxil) at different concentrations and for different time periods; study the effect of treatment on the expression of the previously characterised biological factors to determine how these factors could affect the response of breast cancer cells to chemotherapy in vitro.

3- Evaluate the expression of selected biological factors found to play a role in the response of breast cancer cells to chemotherapy as revealed in the previous section of the study, on tissues obtained from a group of breast cancer patients who received chemotherapy and/or hormonal therapy as part of their treatment. Then evaluation of the currently used clinico-pathological parameters such as tumour size, grade and lymph node status in relation to different treatment regimes and in relation to the duration of patient survival. Evaluation of the expression of different biological factors in relation to different treatment regimes as well as in relation to the duration of patient survival in attempt to find new factors of prognostic significance in breast cancer.

CHAPTER 2:

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Cell lines

Cell lines were obtained from the American Type of Culture Collection (ATCC, Rockville, Maryland). They were selected on the basis of their p53 mutation status and estrogen receptor (ER) status (table 2.1).

Cell Line	ER Status	P53 Status
MCF-7	+ve	Wild type
T47-D	+ve	Mutation at codon 194 (Between III and IV)
ZR-75	+ve	Wild type
MDA-MB-231	-ve	Mutation at codon 280 (Region V, exon 8)
MDA-MB-436	-ve	No mutation
HBL-100	-ve	Stabilized p53 by SV-40 T-large antigen
MDA-MB-468	-ve	Mutation at codon 273 (Region V, exon 8)

Table 2.1: Breast cell lines with their p53 mutation and ER states.

2.1.2 Tissue culture reagents

Complete Medium: 500mls Dulbecco's Modified Eagle's Medium (DMEM) (Sigma), 5mls L-Glutamine (2mM) (Sigma) and 50 mls (10%) Fetal Bovine Serum (FBS) (Labtech International), to feed cell lines MCF-7, T47-D, HBL-100, and MDA-MB-468.

Medium for 436 cell line: 500mls RPMI-1640 (Sigma) and 50mls FBS (10%).

Medium for ZR-75 cell line: 500mls RPMI, 5mls L-Glutamine (2mM) and 50mls (10%) FBS.

Other materials and reagents

Trypsin-EDTA (Gibco BRL Life Technologies).

Poly-D-Lysine (Sigma).

BrdU (5-Bromo-2-Deoxyuridine) (Sigma).

Doxorubicin hydrochloride (Adriamycin), 10 mg, molecular weight 580.0 (Sigma).

Paclitaxil (Sigma).

Trypan blue (Sigma).

Dimethylsulfoxide (DMSO) (Sigma).

12-well tissue culture plates (Becton Dickinson).

Glass coverslips.

2.1.3 Primary antibodies

P53 protein (DO-7): mouse monoclonal antibody, clone DO-7, IgG2b, raised in mice by immunizing them with recombinant human wild type p53 protein, specific for human p53 protein wild type and mutant forms (Novocastra).

P53 (Phosphospecific) (NCL-p53-PHOS): monoclonal antibody clone FP3-2, IgG1, raised in mice. It detects human p53 protein phosphorylated at serine 392 (Novocastra).

P53 protein (CM1) (NCL-p53-CM1): polyclonal antibody raised in rabbits by immunizing them with a recombinant human p53 protein. It is specific for human p53 protein wild type and mutant forms (amino acids 1-393) (Novocastra).

Polyclonal Rabbit Anti-Human BAX: raised in rabbits by immunizing them with synthetic peptide corresponding to amino acids 43-61 of the human bax protein (DAKO).

Bax (Ab-1): rabbit polyclonal IgG antibody generated by immunizing New Zealand White rabbits with a peptide corresponding to residues 150-165 of the human bax protein (Oncogene).

Bcl-2 Oncoprotein (NCL-bcl-2): clone bcl-2/100/D5, IgG1, raised against human bcl-2 oncoprotein by immunizing mice with synthetic peptide sequence, (Novocastra).

Bcl-2 (Ab-1): monoclonal antibody, clone 100, IgG1κ, derived by immunization of BALB/c mice with a synthetic peptide corresponding to amino acids 41-54 of the bcl-2 protein, it reacts with human bcl-2 but not with murine bcl-2 (Oncogene).

Bcl-x (NCL-bcl-x): mouse monoclonal antibody, clone NC1, IgG2b, raised by immunizing mice with a peptide sequence corresponding to amino acids 46-66 of the human bcl-x protein (Novocastra).

WAF-1 protein (NCL-WAF-1): mouse monoclonal antibody, clone 4D10, IgG1, raised against human WAF-1 protein by immunizing mice with a prokaryotic recombinant fusion protein corresponding to the full length WAF-1 (p21/Cip-1) (Novocastra).

Checkpoint Kinase 2 (NCL-Chk2): mouse monoclonal antibody, clone DCS 270-1, IgG2aκ, raised against human checkpoint kinase 2 by immunizing mice with prokaryotic GST-Chk2 fusion protein (Novocastra).

M30 CytoDEATH: mouse monoclonal antibody, clone M30, IgG2b, raised by immunizing Balb-c-mice with a purified CK18 fragment. It binds to a caspase cleaved formalin-resistant epitope of the human cytokeratin 18 (CK18) cytoskeletal proteins (Roche).

Bromodeoxyuridine (BrdU): mouse monoclonal anti-bromodeoxyuridine, clone Bu20a, IgG1 (DakoCytomation).

Ki67 Antigen (MIB-1) mouse monoclonal IgG1 antibody, clone MM1, raised against recombinant parts of the Ki67 antigen, recognizes native Ki67antigen. It is expressed in all proliferating cells during late G1, S, M, and G2 phases of the cell cycle (Novocastra).

Anti-Survivin, Ab469: Rabbit polyclonal IgG antibody, against the full length of human Survivin protein (Abcam Limited).

XIAP, Affinity-purified goat anti-human XIAP goat IgG antibody, raised against purified E.coli-derived human XIAP, recognizes amino acids 1-497 of human XIAP protein (R&D systems).

Anti-Vinculin monoclonal IgG1 antibody, clone hVIN-1, derived from the hVIN-1 hybridoma produced by the fusion of mouse myeloma cells and splenocytes from immunized BALB/c mice (Sigma).

2.1.4 *Secondary antibodies*

Rabbit anti-mouse immunoglobulins, biotinylated secondary antibody (Dako).
Swine anti-rabbit immunoglobulins, biotinylated secondary antibody (Dako).
Rabbit anti-goat immunoglobulins, biotinylated secondary antibody (Dako).
Anti-rabbit immunoglobulins, with horseradish peroxidase linked whole antibody, raised from Donkey (Amersham Pharmacia Biotech).
Anti-mouse immunoglobulins, with horseradish peroxidase linked whole antibody, raised from sheep, from Amersham Pharmacia Biotech.
Rabbit anti-goat HRP-conjugated (Calbochem).
ABC kit: Streptavidin ABC complex (Dako).

2.1.5 Other reagents and chemicals

Normal rabbit serum (NRS, Gibco BRL Life Technologies).
Normal swine serum (NSS, Gibco BRL Life Technologies).

Diaminobenzidine (DAB) (Vector laboratories), diluted in 1xTBS and 1% hydrogen peroxide.

Mayer's Haematoxylin (BDH Chemicals Ltd).

Aquamount, mounting medium (BDH Chemicals Ltd. Poole).

SeeBlue Pre-Stained Standard and Mark 12 MW standard Protein markers (Invitrogen Life Technologies).

Membranes: nitrocellulose membrane (0.2μm) (Bio-Rad Laboratories).

Marvel, low fat dry milk.

Enhanced Chemiluminescence (ECL) detection reagent (Amersham Pharmacia Biotech).

Hyperfilm-ECL, X-ray films (Amersham Pharmacia Biotech).

Methanol (Fisher Chemicals).

Triton x-100 (Fischer Scientific International).

Tris (Roche).

NaCl (Fischer Scientific).

Glycerol (Sigma).

EDTA (Fischer Scientific).

HEPES (Sigma).

Tween 20 (polyoxyethylene sorbitan monolaurate) (Sigma).

Sodium Orthovandate (Sigma Aldrich).

Leupeptin (Sigma).

PMSF (phenylmethylsulfonylflouride) (Sigma).

Betamercaptoethanol (VWR).

SDS (Sodium dodecyel Sulfate) (Sigma).

BPB (bromophenol blue) (BDH Chemicals Ltd., Poole).

Glycine (Sigma).

BSA (Albumin Bovine Fraction) (ICN Biomedicals).

Dye Reagent Concentrate (BIO-RAD-Protein Assay) (Bio-Rad Laboratories).

Protogel (Polyacrylamide gel) (National Diagnostics).

APS (Ammonium persulfate) (Sigma).

TEMED (Sigma).

Ponceau's solution (Sigma).
Vectabond (Vector Laboratories).
Xylene (Genta Medical).
IMS (Industrial Methylated Spirit, 99%) (Genta Medical).
H_2O_2 (Hydrogen Peroxide 12%) (Fisher Chemicals).
Citric Acid Monohydrate (FISONS Scientific Equipment).
DPX (mountant for microscopy) (BDH).

2.1.6 Tissues

The study involved 165 cases of breast cancer patients who were diagnosed at the University Hospitals of Leicester NHS Trust from the period 1991 to 2002. They had all died from the disease. The cases were identified by the data manager from the breast cancer patient database held in the Department of Surgery under the supervision of Miss A. Stotter. Specimens had been received fresh following surgery and sliced. After fixation in 4% formaldehyde in saline for 18-48 h, blocks were selected, processed through graded alcohols and xyelene, and embedded in paraffin-wax.

The study was approved by the Local Research Ethical Committee (approval number 7117). Information on tumour type, size, lymph node status, grade, estrogen receptor alpha and duration of survival (follow-up period) was available for all cases. Data on progesterone receptors were available for 101 cases and on HER2 were available for 46 cases.

Patients were grouped into two groups according to the method of treatment they received. 18 cases (10.9%) constituted the first group, patients in this group were clinically having an inoperable disease and they received either neo-adjuvant chemotherapy alone or neo-adjuvant chemotherapy followed by adjuvant hormonal therapy. Core biopsies were available from 4 cases; these were taken before the start of treatment. The second group included 147 cases (89.1%); all were clinically having an operable disease. 18 cases (10.9%) received adjuvant chemotherapy alone, 70 cases

(42.4%) received adjuvant chemotherapy followed by adjuvant hormonal therapy by Tamoxifen, and 59 (35.8%) received adjuvant Tamoxifen alone. The NHSBSP Pathology Reporting Guidelines Criteria were used for classification and grading of tumours (NHSBSP, 1995).

2.2 Buffers and washing solutions

TBS (tris-buffered saline): contains 50 mM Tris, 150 mM Sodium Chloride, the pH was adjusted to 7.65 with concentrated HCl.

TBST (1xTBS with 1 % Tween 20).

PBS (phosphate-buffered saline) : contains 2.6 M Sodium Chloride, 60 mM Di-Sodium Hydrogen Orthophosphate Unhydrous, 140 mM Sodium Di-Hydrogen Orthophosphate, and pH was adjusted to 7.4 with concentrated HCl.

dPBS (Dulbecco's Phosphate Buffered Saline) (Gibco BRL Life Technologies).

Citrate Buffer: 0.2 M citrate buffer solution was prepared by dissolving Citric Acid Monohydrate in Sodium Hydroxide pellets.

Lysis buffers: two different lysis buffers were used:

Lysis buffer one (gold)	Lysis buffer two (for bax)
- 1% v/v Triton x-100	- 50 mM HEPES, pH 7.4
- 30 mM Tris pH 8.0	- 50 mM NaCl
- 137 mM NaCl	- 10% Glycerol
- 15% Glycerol	- 0.1% Tween 20
- 5mM EDTA	

Loading buffer, for protein loading samples, made up from

- 1M Tris pH 6.8
- Betamercaptoethanol
- 10% SDS
- BPB (Bromophenol blue)

Running buffer: 25mM Tris, 250 mM Glycine and 0.1% SDS.

Transfer buffers: 25 mM Tris, 192 mM Glycine and +/- 40% methanol.

Blotting buffer for Survivin

100 mM Tris/Hcl, pH 7.5

150 mM NaCl

0.2 % Tween 20

Blotting buffer for XIAP

25 mM Tris Hcl, pH 7.4

0.15 M NaCl

0.1 % Tween 20

Protein stripping buffer (500ml) was prepared as follows:

3.78 g Tris base

20% SDS (50ml)

H_2O (450ml)

2.3 METHODS

2.3.1 Cell culture

Cells were grown in 75ml tissue culture flasks in the appropriate culture medium according to the cell type; ZR-75 cells were grown in RPMI-1640 medium containing L-Glutamine and FBS, while MDA-MB-436 were grown in the same medium but without L-Glutamine. All other cell lines were grown in Dulbecco's Modified Eagle's Medium (DMEM) containing both L-Glutamine and FBS. Cells were maintained in an incubator at 37°C under a humidified atmosphere (5% CO_2/95% air). The medium was changed twice a week and cells were passaged when they become nearly (80%) confluent.

2.3.2 Storing and passaging of cells

Stored cell lines were maintained in a frozen state in liquid nitrogen. An aliquot from each cell line was thawed by warming in water at 37°C for 2 minutes. Cells were washed by centrifugation at 180 g in PBS and plated in 10mls tissue culture flasks containing 7mls of the appropriate medium. The medium was changed the next day and cells left to grow until nearly confluent.

Medium was then removed and cells washed with 5ml Dulbecco's Phosphate Buffered Saline (dPBS). They were trypsinised by incubation with 3ml of trypsin concentrate for 2 minutes. 7mls of medium was then added, they were centrifuged for 2min at 180 g and medium removed. Cells were re-suspended in fresh medium and plated in 1/ 4 or 1/ 5 ratio into new flasks.

For re-freezing, confluent cells were washed once with PBS after removal of the culture medium, trypsinized for 2 minutes in 3 mls of trypsin EDTA, washed in PBS, centrifuged for 5 minutes at 300g and then re-suspended in freezing medium containing 10% FCS, 10% dimethylsulfoxide (DMSO) and 80% culture medium. Cells were first frozen at -80°C overnight and then moved to liquid nitrogen freezer for storage.

2.3.3 Counting of cells

15µl of resuspended cells was mixed with 5µl of trypan blue dye and added to the Haematocytometer. The trypan blue dye differentiates dead from alive cells, dead cells take the dye and appear dark while live cells does not accept the dye and appear shinny. Only alive cells were counted in 4x16 squares of the haemocytometer and the mean value for the cell number in the 16 squares was estimated, multiplied in 10^4 to give the total number of cells in every ml of resuspended cells.

2.3.4 Doubling time

Since the rate of growth for each cell line is different, it was necessary to calculate the doubling time to ensure that a comparable number of cells was used for each cell line in experiments. Cells were plated in 12-well plates at a concentration of $2x10^5$ in 2mls of

medium, in triplicate. Non-adherent cells were removed after 3-4 hours of plating, centrifuged and resuspended in 100 µl of medium of which 15µl was used for cell counting. The number of non-adherent cells was subtracted from the plated number of cells.

New medium was added and cells were cultured for different time periods (24, 48 and 72 hours). After each time period the nonadherent cells were also counted and adherent cells were removed by trypsinization in 500µl of trypsin EDTA, 500µl of fresh medium was added to neutralize trypsin. Then 15µl of which were used for counting. The mean and standard deviation were calculated for each cell line from triplicate assessments. The whole procedure was repeated twice and values used to calculate the doubling time for each cell line

2.3.5 Preparation of cells for immunocytochemistry and western blotting

For immunocytochemistry, cells were plated on coverslips in 12-well plates. Coverslips were coated with poly-D-lysine for 30 minutes, then excess poly-D-lysine was removed and coverslips left to dry for 1 hour. The number of cells plated was adjusted dependent on doubling time scores to give an equivalent number of cells after 48hours. For western blotting, cells were cultured in 10 cm tissue culture dishes and left to grow for 48 hours before treatment.

2.3.6 Drug treatment protocol

Treatment was started 48 hours after cells were put onto coverslips or tissue culture dishes. Old media were removed and cells incubated with a freshly prepared media containing the drug. For negative controls, cells were incubated with fresh media containing the drug vehicle only (methanol for paclitaxil and water for doxorubicin).
Drugs were used at different concentrations: Doxorubicin (0.5, 1and 2µM/ml) and Paclitaxil (10, 50, and 100 nM/ml). Cells were incubated with the drug for different time periods (12, 24 and 48 hours) (figure 2.1). For protein extraction and western blotting, media containing the drug were removed and cells harvested after washing

once with PBS. For immunocytochemistry, cells were fixed immediately after removal of the drug.

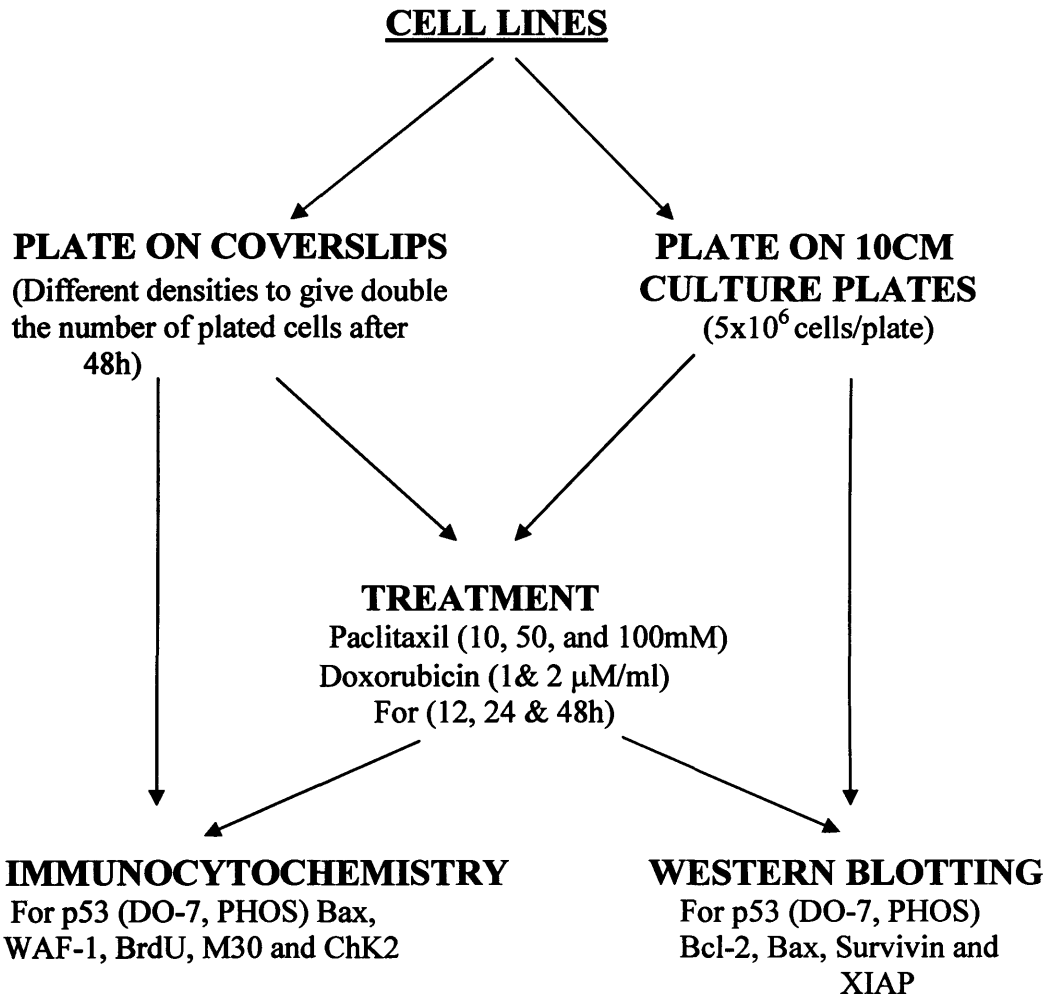


Figure 2.1: summary of cell lines methods: plating, treating cells, different techniques and antibodies used.

2.4 Immunocytochemistry

2.4.1 Optimization of the immunocytochemical technique

Optimization of fixation: five different fixation methods were used to find one that gave the best staining intensity with the least background (table 2.2).

Fixative	Duration of incubation	Temperature at incubation
50% acetone in methanol	7 mins	4°C
Acetone alone	7 mins	4°C
4% freshly prepared paraformaldhyde, followed by: 50% Acetone in Methanol	10 mins 7 mins	4°C -20°C
4% paraformaldhyde, followed by: Methanol	10 mins 7 mins	4°C -20°C
Methanol alone	10 mins	-20°C

Table 2.2: summary of different fixatives tested.

Fixation of cells with Methanol alone at -20°C for 10 mins gave the best staining quality and the least background. Consequently, this fixation method was used in the all subsequent experiments.

Optimization of primary antibodies: different dilutions were used according to manufacturer's data sheets to give the best staining intensity as well as the least extent of non-specific background staining (Table 2.3).

Primary antibody	Company	Optimized concentration
DO-7 (P53)	Novocastra	1:100
P53 PHOS	Novocastra	1:50
CM1 (P53)	Novocastra	1:1000
BCL-2	Novocastra	1:80
BAX	Dako	1:25
BCL-XL	Novocastra	1:40
ChK2	Novocastra	1:25
WAF-1 (P21)	Novocastra	1:20
M30 (apoptosis)	Roche	1:100
BrdU (proliferation)	Dako	1:200

Table 2.3: Different primary antibodies used for immunocytochemistry and their working dilutions

Optimization of secondary antibodies: different concentrations were used; rabbit anti-mouse biotinylated secondary antibody was used with monoclonal primary antibodies and swine anti-rabbit biotinylated secondary antibody with polyclonal primary antibodies. 1:400 concentration (for anti-mouse secondary), and 1:600 (for anti-rabbit) gave the best staining quality.

Optimization of other staining steps: washings for different periods (2x5min, 3x5 mins and 2x10 mins) were tested as well as the duration of incubation of DAB solution and haematoxylin counterstaining.

2.4.2 Immunocytochemical methods

Cells were fixed in 2mls of methanol for 10mins at -20°C, and then washed twice in PBS. Non-specific staining was blocked with normal serum diluted 20% in PBS, species depending on nature of the primary antibody. Cells were incubated for 10 minutes. Primary antibodies were diluted in 20% normal serum to the appropriate concentration (table 4.2) and cells incubated overnight at 4°C. For negative control

staining, the primary antibody was omitted and coverslips were incubated with the normal serum only.

Excess primary antibody was removed and coverslips washed in PBS containing 0.1% BSA, 2x 5 minutes before incubation with the secondary antibody. Cells were incubated with the appropriately diluted secondary antibody for 30 mins at room temperature with agitation on a rocking platform.

The ABC solution was prepared and left for 30 minutes to form the ABC complex. After removal of the secondary antibody, cells were washed 2x5 minutes with PBS/0.1% BSA and then incubated with the ABC solution for 30 minutes at room temperature.

Excess ABC was removed and cells washed with 1xTBS, 2x5 minutes. Cells were incubated with the DAB solution for 10 mins, rinsed in 1xTBS, counterstained with Mayer's hematoxylin, rinsed in tap water and then coverslips were mounted in one drop of Aquamount on a glass slide and left to dry.

2.4.3 Bromodeoxyuridine (BrdU)-labeling method for staining of proliferating cells

Cells were incubated with 1ml of fresh medium containing 1.53 mg/ml of BrdU for three hours at 37 °C prior to termination of culture. After fixation, cells were incubated in 2N HCl solution (prepared by diluting concentrated HCl with RO water 1:4 v/v) for 30 mins at room temperature with agitation. HCl solution was removed and cells were washed in 0.1 M sodium tetraborate, pH 8.5, for 2x5 minutes. Sodium tetraborate solution was removed and cells were washed with TBS containing 0.1% BSA. Immunostaining as described above was then undertaken.

2.4.4 Immunostaining of apoptotic cells by M30

For immunostaining of apoptotic cells by M30 antibody, the standard protocol was used apart from the addition of 0.1% Tween 20 to washing buffer solution (PBS containing 0.1% Tween 20) for all washing steps and the use of an incubation buffer solution (PBS containing 1% BSA and 0.1% Tween 20) for all dilutions of primary and secondary antibodies and for blocking the non-specific staining.

2.4.5 Assessment of the immunostaining

Positive cells showed either nuclear staining as for p53 or cytoplasmic staining as for bax and bcl-2. For the assessments of nuclear staining, 1000 cells were counted for each coverslip and the percentage of positive cells (any degree of staining intensity) were calculated. Assessment of cytoplasmic staining depended on the staining intensity only, regardless of the extent; four different categories were used (no staining, faint, moderate, or strong staining). For evaluation of bax following treatment of cells the H-Score was used (Σ % of cells strong x3; % cells moderate x2; % cells faint x1).

2.5 Western blotting methods

2.5.1 Protein extraction

Cells were cultured in 100mm cell culture dishes. Media either with or without drug were removed and cells washed with PBS. After removal of PBS, 200 µl of lysis buffer with protease inhibitors was added and cells were scraped thoroughly. Lysis buffer containing cells was removed into an eppendorf and cells were disrupted by needling to obtain the total protein extract. Cells and lysates were kept on ice to avoid protein degradation. Cytoplasmic extracts were obtained by centrifugation of lysates at 13000 rpm for 5 mins at 4°C. Supernatants were aliquoted, snap frozen in liquid nitrogen and stored at –80°C.

2.5.2 Measurement of protein (Bradford method)

In order to obtain equal loading of protein for electrophoresis, the amount of protein in each sample was determined. A standard curve was constructed using two solutions of BSA (2μg/ml and 6μg/ml) (table 2.4) and (figure 2.2).

BSA solution	Volume of BSA	Volume of water	BSA concentration
2μg/ml	0 μl	1ml	0(blank)
	100 μl	900 μl	0.2 μg/ml
	200 μl	800 μl	0.4 μg/ml
	400 μl	600 μl	0.8 μg/ml
	600 μl	400 μl	1.2 μg/ml
	800 μl	200 μl	1.6 μg/ml
	1000 μl	0 μl	2.0 μg/ml
6μg/ml	500 μl	500μl	3 μg/ml
	600 μl	400μl	3.6 μg/ml
	700 μl	300μl	4.2 μg/ml
	800 μl	200μl	4.8 μg/ml
	1000 μl	0μl	6.0 μg/ml

Table 2.4: BSA solutions for preparation of protein standards.

One part of Dye Reagent Concentrate was diluted with 4 parts distilled, deionized water and filtered through 0.2 μm Acrodisc Syringe Filter to remove particles. Ten μl of each BSA standard solution was added to 1 ml of the dye reagent, mixed and left at room temperature for 15 mins. For each test, 10 μl of the protein lysate was added to 1 ml of the dye reagent and the absorbance was measured at 595 nm using GENYSIS Spectrophotometer.

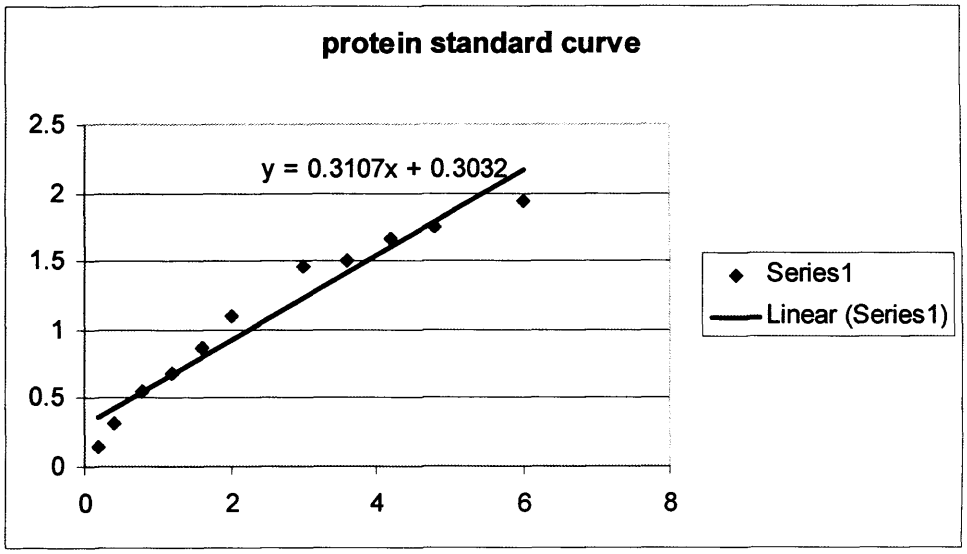


Figure 2.2: protein standard curve constructed by the absorbance values of BSA protein concentrations.

The absorbance values of protein lysates were used to determine the amount of protein in each lysate. Equal amounts of proteins were used after working out the total amount (including protein, loading buffer and the inhibitors) to be loaded in each lane.

2.5.3 Gel electrophoresis

Ten % or 12% separation gels were set up, they were made from:

10%	12%	
6.0ml	5.0 ml	H2O
3.75	3.75 ml	1.5 M Tris-HCl (pH 8.8)
5.0	6.0 ml	30%AA, 0.8% BisAA
150 µl	150µl	10% SDS
150µl	150µl	10% APS
15 µl	15µl	TEMED

Stacking gel was set up on top of separation gel, was made from:

1.4ml	H2O
30% (0.33ml)	Polyacrylamide gel (protogel)

10% (20 µl)	APS
2 µl	TEMED

Protein samples were prepared from: 50µg of protein lysates of known concentrations were mixed with 4x loading buffer (1 M tris pH6.8, Glycerol, Betamercaptoethanol, 10% SDS, and Bromophenol blue) and heated at 100°C for 10 mins.

Protein samples were loaded in the stacking gel; also 5-10 µl of a protein marker was loaded in one lane to indicate the size of the protein bands. Proteins were separated by gel electrophoresis at 100 volts in 1x running buffer at room temperature.

2.5.4 Protein transfer

Separated proteins were transferred, using a semi-dry method, from the gel onto nitrocellulose membrane in the following order:

- Filter paper soaked in transfer buffer with no methanol
- Membrane
- Gel
- Filter paper again soaked in transfer buffer with methanol

Air bubbles were removed, the machine was adjusted to 10-15 volts and the mA estimated according to the gel surface area to not exceed 5 mA /cm^2. Proteins were transferred for 45mins-1hour. After completing the transfer, membranes were stained with Ponceau's solution to confirm protein transfer.

2.5.5 Membrane staining

Non-specific staining was blocked with 5% Marvel skimmed milk solution in TBST for 30 minutes. Primary antibodies were diluted in TBST; membranes were incubated with the primary antibody solution overnight at 4°C, and then washed in TBST, 3x10 mins over the rocking platform (table 2.5).

the primary antibody solution overnight at 4°C, and then washed in TBST, 3x10 mins over the rocking platform (table 2.5).

For XIAP, non-specific staining was blocked by incubating the membrane in a blocking solution containing 2% nonfat dry milk in blotting buffer (25 mM Tris HCl pH 7.4, 0.15 M NaCl, and 0.1 % Tween 20) for one hour. The Goat anti-XIAP antibody was diluted in 1 % nonfat dry milk in the XIAP blotting buffer/ pH 7.4 and membranes were incubated overnight at 4°C.

For Survivin, membranes were blocked for non-specific staining by incubation overnight in blotting buffer containing 100 mM Tris HCl pH 7.4, 0.15 M NaCl, and 0.2 % Tween 20. The anti-Survivin antibody was diluted in 3 % nonfat dry milk in 1 % BSA and applied for one hour at room temperature.

Primary antibody	Working dilution	Source
P53 (DO-7)	0.035 µg/ml	Novocastra
P53 Phosphospecific	0.03 µg/ml	Novocastra
Bax	2-5µg/ml	Oncogene
Bcl-2	2-5 µg/ml	Oncogene
Survivin	2 µg/ml	Abcam
XIAP	1 µg/ml	R&D

Table 2.5: summary of primary antibodies used for western blotting with the working concentration.

Secondary antibodies were diluted in TBST, the anti-rabbit peroxidase linked secondary antibody (1:2,000) was used for polyclonal antibodies and the anti-mouse, peroxidase linked secondary antibody (1:5000) for the monoclonal antibodies. Membranes were incubated with secondary antibody for 1 hour over the rocking platform at room temperature. For XIAP, the anti-goat Ig with HRP-conjugated antibody was used in a concentration of 1:2000 and diluted in the blotting buffer (25 mM Tris HCl, pH 7.4, 0.15 M NaCl And 0.1 % Tween 20).

Membranes were washed in TBST, 3x10 minutes over the rocking platform. For XIAP, membranes were washed in blotting buffer for one hour with frequent change of buffers and containers. For Survivin, membranes were also washed for one hour in blocking buffer with five or more changes of the washing solution. The ECL (Enhanced Chemiluminescence) was used for one minute as a detection solution: membranes were wrapped in a Saran film, exposed to x-ray film for 2-5 minutes and then developed.

2.5.6 Optimisation of the western blotting technique

For p53 protein expression by western blotting, two primary antibodies were used (DO-7 and p53 PHOS) at different concentrations; the optimized dilution for DO-7 was 1:3000 after testing a range of dilutions 1:1000 to 1:5000, and for p53 PHOS was 1:1000 after testing a range of 1:500 to 1: 3000.

The optimized secondary antibody dilution was 1:5000 for monoclonal antibodies and 1:2,000 for polyclonal antibodies, followed testing ranges from 1: 2000 to 1:10,000.

For detection of bax protein, the following were evaluated:

1-Different lysis buffer containing:

- 50 mM HEPES, pH 7.4
- 50 mM NaCl
- 10% Glycerol
- 0.1% Tween 20

2-Different time periods for the protein transfer (30 mins-75 mins).

3-Different concentrations of the primary antibody (1, 2 and 5μg/ml).

Bax peptide (Oncogene) and MCF-7 breast cancer cells treated with doxorubicin were used as positive controls.

2.5.7 Reprobing for vinculin

This was undertaken to check protein loading. 50ml of stripping buffer was mixed with 400 μl of Betamercaptoethanol (BME) and preheated to 60° C in the incubator. The blots were incubated for 45 minutes at 60° C in the incubator; then washed in TBST

three times / 5 minutes each. Membranes were re-blocked for non-specific staining in 10% non-fat dry milk (Marvel) in TBST overnight at 4° C and then washed in TBST five times/ 5mins each.

The primary antibody (mouse anti-alpha vinculin) from Oncogene was applied in a dilution of 1: 1000 for 2 hours at room temperature in 5% non-fat dry milk, followed by washing in TBST five times/ 5mins each. Secondary antibody (Goat anti-mouse) was applied in 1:2000 concentration in 5% non-fat dry milk after washing in TBST five times/ 5mins each. Protein was detected by using ECL.

2.6 Immunohistochemistry

Each case was anonymised. A representative formalin-fixed and paraffin-embedded tissue block from each case was selected by the Pathology Directorate R&D technician K.K. who checked that there were sufficient materials within the blocks, cut sections (4 μm thick) from each block and labeled them with the code. Sections were put on a previously vectabond-coated glass slides and left to dry overnight at 37°C before use.

Sections were dewaxed in xylene with periodic agitation for 5 minutes, rehydrated through graded alcohols with draining in-between each solvent as follows: Xylene/2 minutes, 99% IMS/2x2 minutes, 95% IMS/2 minutes. Sections were washed in running tap water for 5 minutes and then rinsed in distilled water.

2.6.1 Blocking endogenous peroxidase

All sections were immersed in 2% Hydrogen Peroxide (H_2O_2) in water for 10 minutes apart from those sections to be stained with antibodies against Survivin and XIAP. These were blocked directly from alcohol by immersion in 2% Hydrogen Peroxide in Methanol for 10 minutes. Sections were washed in tap water for 2 minutes and then rinsed in distilled water.

2.6.2 Antigen retrieval method

Antigen retrieval was achieved using pressure cooker Prestige, model number 6189. Sections were placed in the pressure cooker containing 1.5 L of 0.2 M citrate buffer solution pH 6.0 and brought to the boil with the lid unlocked. Slides were put in metal slide racks in the boiling buffer and the lid locked, when the large pressure indicator rose. Slides were then gently cooled by filling the pressure cooker with tap water, washed in running tap water for 5 mins, rinsed in distilled water and then equilibrated in TBS for 5 mins.

2.6.3 Optimization of antigen retrieval

Initially, sections were immunostained with and without antigen retrieval by pressure cooking. None of the antigens studied was detected without pressure cooking. Slides were cooked for different time periods 2, 4 and 6 minutes, almost all of monoclonal antigens were detected after 2 minutes of pressure cooking. XIAP antigen was optimally detected after continuous cooking for 4 minutes, while Survivin antigen was detected after 2 subsequent pressure cookings for 2 minutes each (table 2.6).

Antibody	**Duration of pressure cooking**
Survivin	2mins followed by 2mins
XIAP	4 mins continuous
All other antibodies	2 mins

Table 2.6: The optimized duration of pressure cooking for different antigens.

2.6.4 Immunostaining method

Sections were placed in a humid chamber on a rocker platform; non-specific staining was blocked by normal serum diluted 1:5 in TBS; normal swine serum for blocking sections to be stained with polyclonal antibodies and normal rabbit serum for monoclonal antibodies, 100 µl was applied for each section for 20 minutes. Normal

serum was taken off and 100 µl of the appropriately diluted primary antibody was applied to each section and incubated overnight at 4°C (in cold room) (table 2.7).

Antibody	Optimized Concentration
P53 DO7	1:50
P53 PHOS	1:25
WAF-1	1:20
ChK2	1:40
Bcl-2	1:80
Bax	1:50
MIB-1	1:100
M30	1:200
Survivin	1:400
XIAP	1:800

Table 2.7: Primary antibodies for immunohistochemistry

Primary antibody was rinsed off with TBS and then sections were washed in fresh buffer for 5 minutes. Slides were replaced into the humid chamber. 100 µl of the appropriately diluted biotinylated secondary antibody was applied to each section for 30 minutes at room temperature (table 2.8).

The ABC solution was prepared immediately after applying the secondary antibody:

TBS, 1000 µl,

Streptavidin, 1µl,

Biotinylated Horseradish Peroxidase, 1µl.

Secondary Antibody	Working Dilution
Biotinylated Rabbit anti-mouse	1:400
Biotinylated Swine anti-rabbit	1:600
Biotinylated Rabbit anti-goat	1:600

Table 2.8: the optimized working dilution of different secondary antibodies.

After rinsing and washing in fresh buffer for 5 minutes, 100 µl of the ABC solution was applied on each section for 30 minutes followed by further washing. The DAB (diaminobenzidine) solution composed:

TBS, 9.4 mls

An aliquot of Dab (500 µl)

100µl of 3% H_2O_2,

100 µl of the DAB solution was applied for 5 minutes at room temperature. Sections were rinsed off in TBS and then washed in running tap water for 5 minutes, counterstained with Mayer's Haematoxylin for 15 seconds followed by washing in running tap water for 5 minutes to remove excess dye.

Sections were rehydrated with periodic agitation through graded alcohols to xylene as follows: 95%/ 2mins, 99% /2x2mins, and xylene/ 2mins then transferred to fresh xylene and mounted in DPX using the appropriate size coverslips.

2.6.5 Controls

Negative Controls: Sections were incubated with the normal serum overnight at 4° C instead of the primary antibody.

Positive Controls: Sections from breast tumours previously determined to express the antigen were used as positive control with each batch of staining. For bax and bcl-2, sections of normal breast tissue were used as positive controls. Lymphocyte infiltrate and the remains of normal breast epithelium around the tumor were served as internal positive controls.

2.6.6 Immunohistochemical staining for apoptosis by M30

For M30 immunostaining, the same method as mentioned above was used apart from using an incubation buffer (1xPBS containing 1% BSA and 0.1% Tween 20) for blocking the non-specific staining and for dilution of both primary and secondary antibodies. Washing buffer (1xPBS containing 0.1% Tween 20) was also used for all washes.

2.6.7 Analysis of immunohistochemical staining

Antibody	Evaluation method	Reference
Survivin	Semi quantitative according to the percentage of cells expressing distinct nuclear and/or cytoplasmic staining. At least five high power fields x40 were assessed in each slide. For nuclear staining 0 =< 5%, 1= 5-<10%, 2=10 %-< 20%, 3=>20%. For cytoplasmic staining, 0=faint or negative, 1=moderate and 2=strong. Both scores were added together to get the final Survivin score.	Kennedy et al. (2003).
MIB-1	The number of positive cells was counted in 1000 cells in at least 5 high power fields and the percentage was considered as a proliferation index. <20 % positive = low proliferation index 20% or more= high proliferation index	Faneyte et al. (2003).

M30	The number of apoptotic cells was counted in 1000 cells in 5 high power fields and the percentage was estimated and considered as the apoptotic index. The number of apoptotic cells was also counted in 10 high power fields for further assessment of apoptosis.	Koornstra et al.(2004). Kataoka et al. (2004).
Bax	The bax staining was homogenous in all sections, consequently, the intensity of staining was assessed as: 1=negative or faint 2=moderate 3=strong Moderately and strongly stained tumours were classed as positive.	Rochaix e al. (1999).
Bcl-2	Semi-quantitative assessment, each tumour was assigned to one of the following groups: 0(0-4%), 1(5-25%), 2(26-50%), 3(51-75%), 4(76-100). The intensity of staining was determined as 0(negative), 1+ (faint), 2+ (moderate), and 3+ (strong). The intensity was judged relative to lymphocytes or normal breast tissue within tumour which was designated 2+. The overall score was calculated by combining both scores together.	Krajewska et al. (1996).
Waf-1	The number of positive cells was counted in 1000 cells in at least 5 high power fields and the percentage was estimated. Cases were classified as positive (≥5% positive cells) or negative (<5% positive cells).	Modified from Rey et al. (1998).
ChK-2	The number of positive cells was counted in 1000 cells in at least 5 high power fields and the percentage was estimated.	

P53 (DO-7) P53 PHOS	Positive cells were counted in 1000 cells in at least 5 high power fields and the percentage was estimated. Cases considered as Negative= <20% positive cells Positive =20% or more	Walker et al. (1996).
XIAP	The XIAP was assessed according to the intensity of staining as 1: Negative or faint staining 2 : Moderate 3: Strong	

Table 2.9: Methods of assessing the immunocytochemical staining with their corresponding references.

2.7 Statistical analysis

The SPSS version 12.0 for Windows program was used for statistical analysis, data variables were entered either as continuous variables such as (patient age, duration of survival or tumour size) or as grouping variables (high/low or –ve/+ve). The frequency, mean, median, and the range values were calculated for each variable. For grouping of continuous variables, the cutoff points were estimated according to the normal parametric distribution (33.0 and the 66.0 percentiles) or according to the standard classifications (non-parametric).

Chi^2-test was used to compare two or more grouping variables. The Independent Samples T-Test was used to compare grouping variable with two groups against a continuous dependent variable. When the grouping variables contained three or more groups, the One-Way ANOVA test was used to compare this variable against a continuous variable. Alternative to One-Way ANOVA, grouping variables, which does not follow the normal distribution, were analyzed with the non-parametric Kruskall-Wallis Test. The Log Rank Kaplan-Meier Test was used to assess the relation of grouping variable against the duration of survival

.

Cox regression analysis was used to determine the strongest factor that predicts patient survival or response to treatment.

CHAPTER 3:

CHARACTERIZATION OF BREAST CELL LINES FOR P53 AND APOPTOTIC REGULATORY PROTEINS.

Introduction

A large amount of information about breast cancer is based on *in vitro* studies using breast cancer cell lines. One reason is that cell lines can be easily cultured in simple defined media and can provide unlimited, homogenous and self-replicating material for analysis. The breast cancer cell lines used in this thesis were all originally from metastasis to pleura (MCF-7, MDA-MB 231, T47-D, MDA-MB-436 and MDA-MB-468), peritoneum (ZR-75) and had been isolated from pleural and ascetic fluids. HBL-100 cells were established from a breast milk sample obtained from an early lactating healthy woman (Gaffney, 1982). However they were found to carry SV40 genetic information (Caron de Fromentel et al., 1985) and in culture they show features of transformation.

Although there are benefits in using cell lines in cancer biology, there are also drawbacks. One question is the representativeness to primary tumours. In addition long-term culture and culture of cells under different conditions in different laboratories may induce genotype or phenotype alterations (Lacroix and Leclercq, 2004).

Estrogen receptor (ER) status is a prominent criterion for classification of breast cancer cell lines since it plays a crucial role in breast cancer biology and treatment. Cell lines also vary with relation to tumour suppressor gene p53 mutation status. The aim of this part of the study was to characterize a group of breast cancer cell lines with regard to apoptosis, proliferation and the expression of p53 and other apoptotic regulatory proteins prior to using them in *in vitro* model systems.

Methods

This part of the study involved 7 different breast cell lines, classified according to their estrogen receptor (ER) status and p53 mutation status. These were MCF-7 (ER+ve, p53 w.t), ZR-75 (ER+ve, p53 w.t), T47-D (ER +ve, p53 mutant), MDA-MB-468 (ER –ve, P53 mutant), MDA-MB-231 (ER –ve, p53 mutant), MDA-MB-436 (ER –ve, p53 status unknown), HBL-100 (ER –ve, p53 stabilized). All cells were grown on coverslips and

in flasks and cell numbers were adjusted in relation to growth rate (see section 2.1 and 2.8). All were analyzed by immunocytochemistry for proliferation (BrdU), apoptosis (M30), p53 (DO-7, CM-1 and phosphospecfic p53), $p21^{waf-1}$, ChK-2, bcl-x_L, bax, bcl-2. They were also analyzed by western blotting for p53 (DO-7 and phosphospecific p53), bcl-2 and the inhibitor of apoptosis proteins Survivin and XIAP (for western blotting methods see section 2.10).

Results

3.1 Growth regulation

3.1.1 Growth curve

Before using the cell lines in experiments, it was necessary to assess their growth behavior. Experiments were performed in triplicate and cell numbers were counted at 24h, 48h and 72h. Growth curves were constructed to calculate the doubling time for each cell line, so that in subsequent experiments comparable numbers of cells were used for each cell line (see section 2.8.3). Table 3.1 shows the mean number of cells for the three experiments at different time periods. ZR-75 cells showed the maximum number of cells at 48h and 72h, while HBL-100 cells were the slowest growing cell line at the different time points (figure 3.1).

3.1.2 Proliferation-BrdU-labeling

Incorporation of BrdU gives more precise information about the percentage of cells in S phase. All cells were cultured in triplicate and percentages of cells in S phase were assessed at 48 hours. Clear nuclear staining was seen (figure 3.2). The results were obtained by counting a minimum of 1000 cells. HBL-100 cell line showed the highest level of proliferation (48 %) and T47-D the lowest (26.39) with the remainder being between 35 and 42 %.

Cell Line	24 h	48 h	72 h
	Mean ± SD	Mean ± SD	Mean ± SD
MCF-7	24 ± 1.52	50 ± 5.19	69 ± 4.4
T47-D	54 ± 3.78	83 ± 3.02	143 ± 4.72
MDA-MB-231	34 ± 3.90	62 ± 6.37	115 ± 6.8
MDA-MB-468	39 ± 4.64	71 ± 8.18	108 ± 8.61
ZR-75	45 ± 2.70	91 ± 4.78	173 ± 16.75
MDA-MB-436	38 ± 0.75	52 ± 5.57	83 ± 5.10
HBL-100	15 ± 3.51	30 ± 3.51	53 ± 3.78

Table 3.1: mean number of cells at different time points.

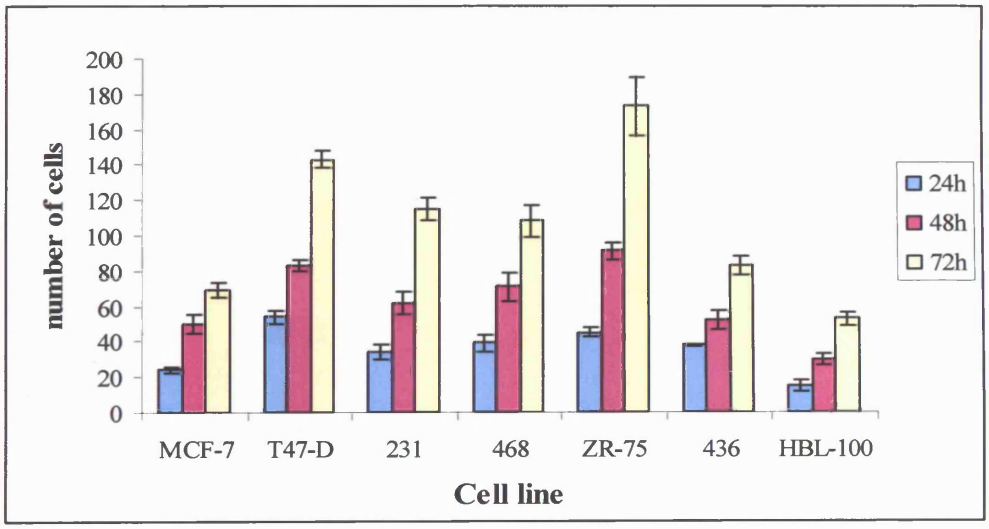


Figure 3.1: growth chart for different cell lines at different time points.

Cell line / Marker	MCF-7	T47-D	231	468	ZR-75	436	HBL-100
Proliferation (BrdU) %±SD	42.3 ±1.13	26.39 ±4.65	42.88 ±2.51	35 ±0.70	41.6 ±5.65	38.1 ±2.96	48 ±1.41

Table 3.2: Proliferation by BrdU for different cell lines.

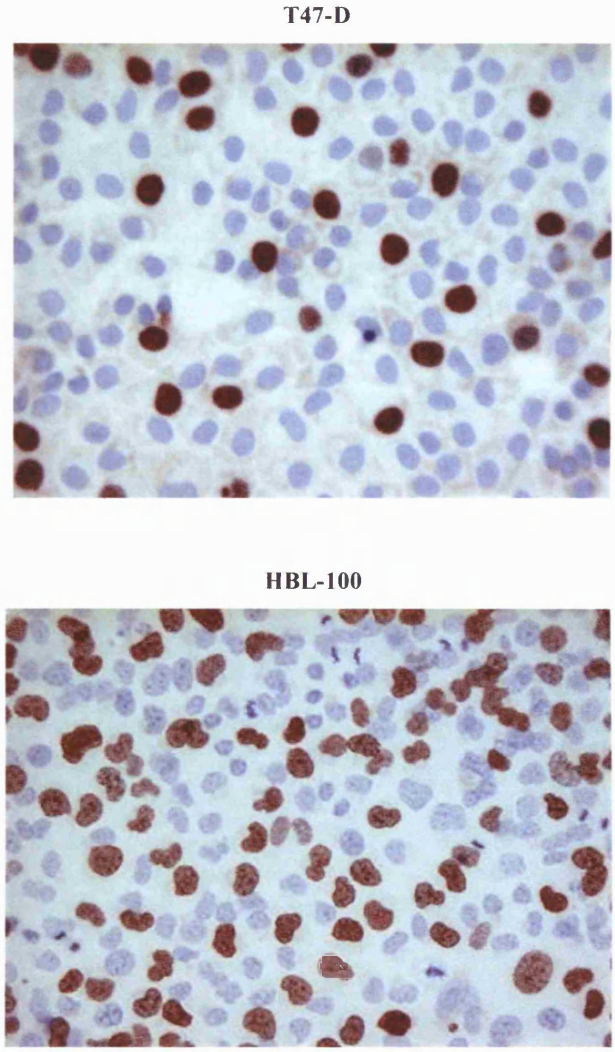


Figure 3.2: proliferation by BrdU in breast cell lines. Nuclear labeling of cells that have incorporated BrdU.

3.1.3 Apoptosis- M30

M30 detects cytokeratin 18 that is cleaved in early apoptosis. All cells were cultured in triplicate for 48 hours prior to immunostaining. Apoptotic cells stained with M30 were usually smaller in size and the nuclei were condensed and dark. The staining was cytoplasmic (figure 3.3). Results were obtained by counting a minimum of 1000 cells and determining the percentage. Generally the number of apoptotic cells was low in all cell lines (range 0.7- 2.0 %) being maximum in T47-D cells and least in MDA-MB-231 cell line (table 3.3).

Cell Line / Marker	*MCF-7*	*T47-D*	*231*	*468*	*ZR-75*	*436*	*HBL-100*
Apoptosis (M30) %±SD	*0.9 ±0.25*	*2.0 ±0.05*	*0.8 ±0.30*	*0.9 ±0.28*	*0.7 ±0.1*	*1.0 ±0.15*	*0.8 ±0.14*

Table 3.3: Apoptosis as assessed by M30 for all cell lines.

3.2 Biological markers

3.2.1 P53

Three different anti-p53 antibodies, two monoclonal (DO-7 and phosphorylated p53) and one polyclonal (CM-1) were used to assess the seven human breast cell lines. All cells were cultured in triplicate for 48 hours prior to immunostaining and protein extraction for western blotting.

3.2.1.1 P53 (DO-7)

Immunocytochemisry

P53 (DO-7) was demonstrated as nuclear (figure 3.4). Cell lines which are known to have wild-type p53 (MCF-7& ZR-75) had less than 10% of cells showing strong nuclear staining and were therefore considered as negative for p53.

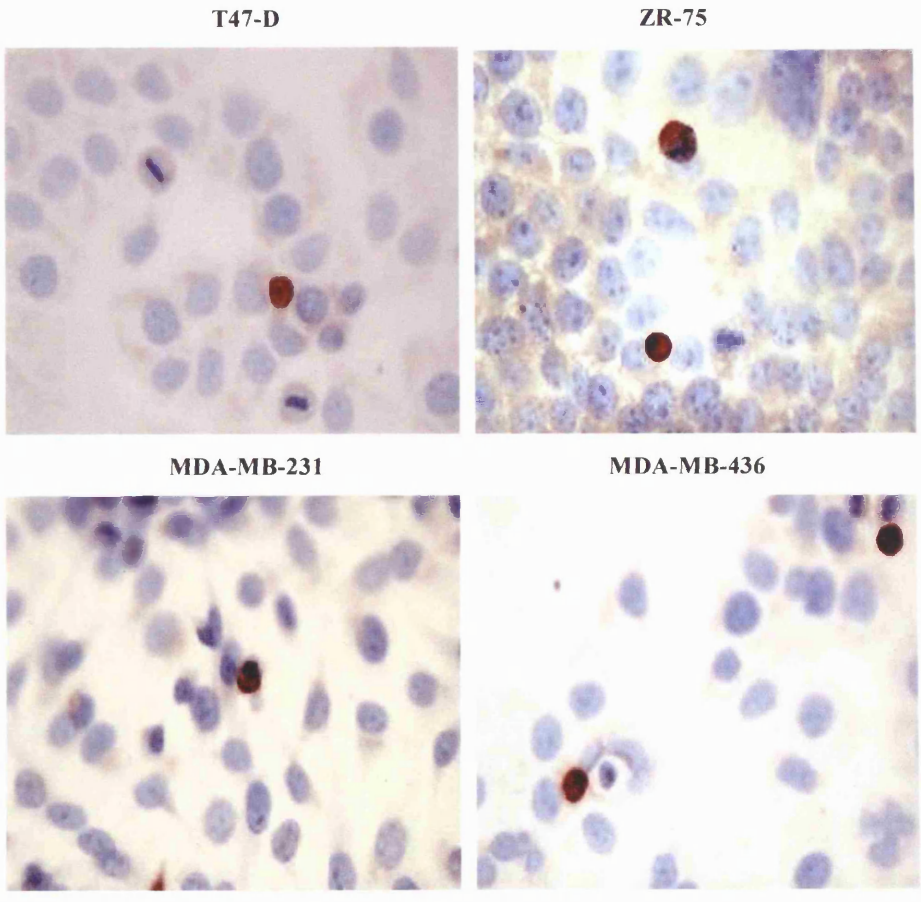


Figure 3.3: Apoptosis by M30 in different cell lines, apoptotic cells showed cytoplasmic staining and dark nuclei.

Those known to have p53 mutations (MDA-MB-231, MDA-MB-468, and T47-D) showed strong staining in a high percentage of cells. The percentage of positive staining was also very high in the HBL-100 cell line, which has a stabilized p53 protein (99.4%). MDA-MB-436, for which p53 mutation status was unknown failed to show any staining with this antibody (table 3.4).

Cell Line / Marker	MCF-7	T47-D	231	468	ZR-75	436	HBL-100
P53 (DO-7) % ±SD	6.0 ±0.7	97.7 ±0.98	96.1 ±1.48	97.0 ±4.24	9.0 ±1.67	0.0 ±0	99.4 ±0.58

Table 3.4: P53 by DO-7 in different cell lines.

Western Blotting

Western blot analysis was performed to confirm p53 protein expression detected by immunocytochemistry. Equal amounts of protein (50 μg) were loaded in each lane, separated by gel electrophoresis and then transferred onto a nitrocellulose membrane. Using the p53 DO-7 antibody, which reacts with both wild-type and mutant p53 proteins, the presence of immunodetectable p53 was shown in 4 cell lines (MDA-MB-231, MDA-MB-468, T47-D and HBL-100). In those with wild-type p53 (MCF-7 and ZR-75) protein bands were either very faint or absent. MDA-MB-436 cells did not express p53 (figure 3.5 A &B).

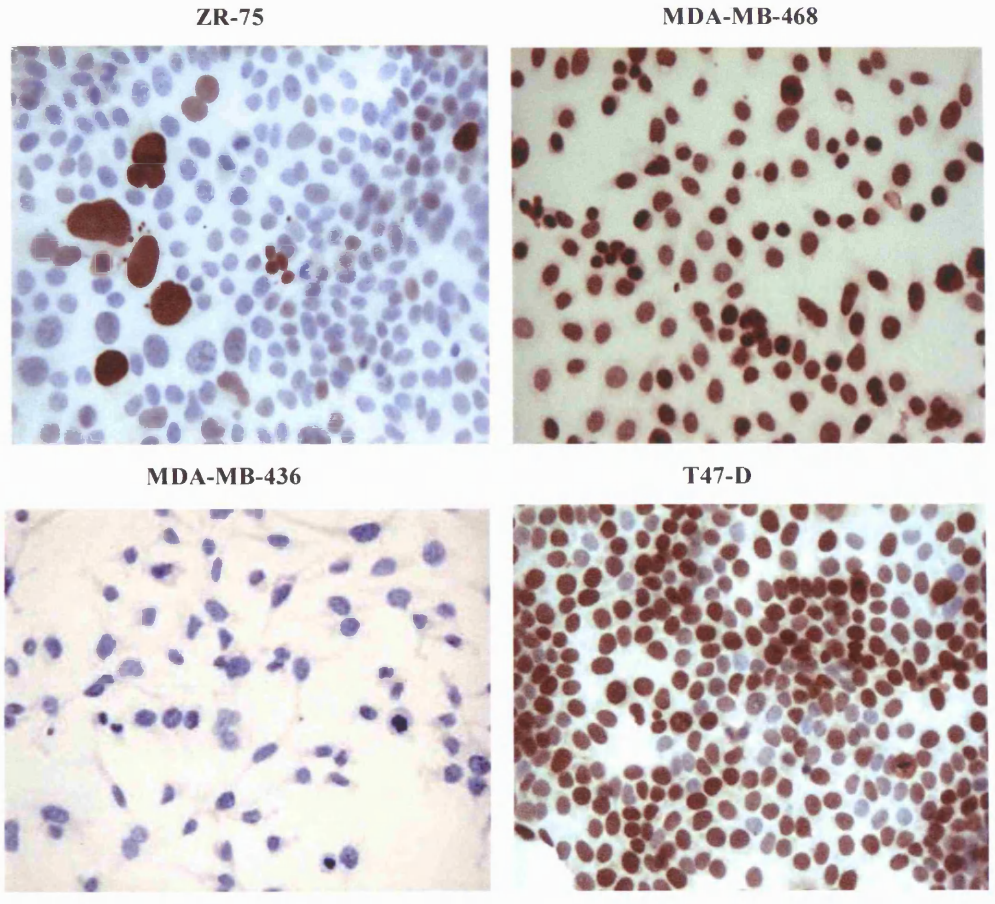


Figure 3.4:P53 (DO-7) immunocytochemistry in different cell lines. Number of positive cells was very low in ZR-75, very high in, MDA-MB-468, and T47-D. No immunoreactivity in MDA-MB-436.

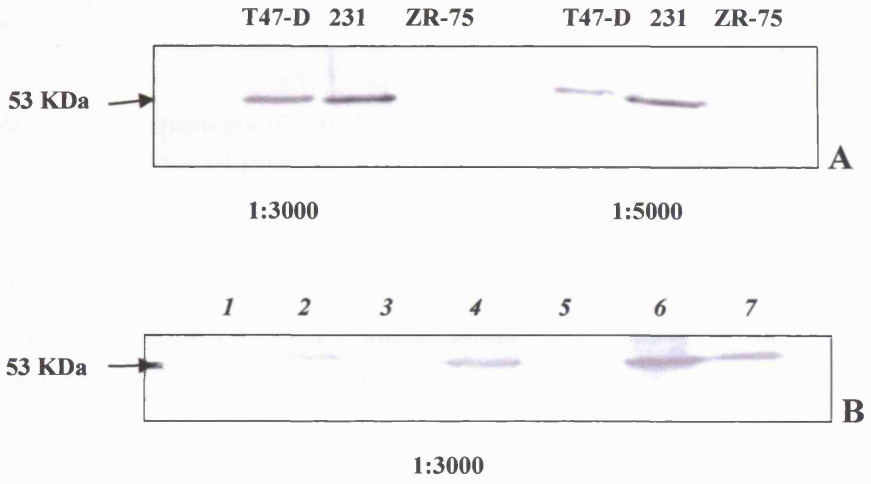


Figure 3.5: Western blotting for p53 by DO-7. 50 µg of protein was loaded in each lane. A, two concentrations of primary antibody, 1:3000 was better than 1:5000. B, shows protein expression in all cell lines. Lane 1: MCF-7, lane 2:T47-D, lane 3: ZR-75, lane 4: MDA-MB-231, lane5: MDA-MB-436, lane 6: HBL-100 and lane7: MDA-MB-468.

3.2.1.2 P53 (Phosphospecific p53)

Immunocytochemistry

The antibody to phosphospecific p53 detects the protein phosphorylated at serine 392. Cells with wild-type p53 had very low levels of reactivity while those with mutant form of p53 had high levels. The extent of reactivity was similar to that with DO-7 for T47-D, MDA-MB-468 and HBL-100, but was less for MDA-MB-231 (48.1 %) (table 3.5) and (figure 3.6). MDA-MB-436 again showed no reactivity.

Cell Line / Marker	MCF-7	T47-D	231	468	ZR-75	436	HBL-100
P53 phosphospecific %±SD	3.93 ±1.68	95.5 ±6.05	45.6 ±2.92	96.9 ±4.31	3.9 ±0.54	0.0 ±0	99.6 ±0.45

Table 3.5: phosphospecific p53 in different cell lines. MCF-7 and ZR-75 showed very low percentage of positive cells in comparison to other cell lines. No staining was detected in MDA-MB-436 cells.

Western blotting

The presence of immunodetectable phosphorylated p53 was shown in 4 cell lines (MDA-MB-231, 468, T47-D and HBL-100), the level in MDA-MB-231 cells being lower than the others. In those with wild-type p53 (MCF-7 and ZR-75) no immunoreactivity was detected, and the same for MDA-MB-436 cells (figure 3.7).

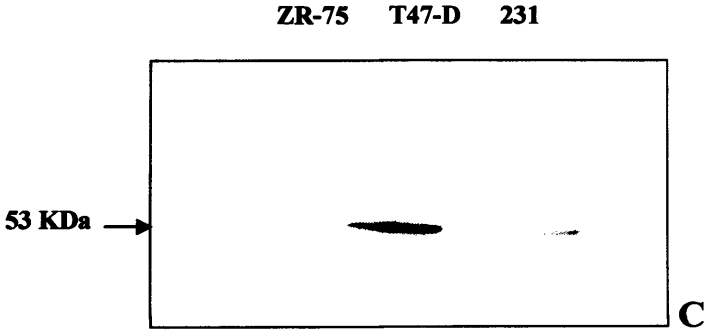


Figure 3.7: western blotting for phosphorylated p53 in some cell lines. 50 µg of protein was loaded in each lane; the primary antibody concentration was 1:1000. 231 showed lower level.

3.2.1.3 P53 (CM-1)

Immunocytochemistry

CM-1 is a polyclonal antibody that detects both wild-type and mutant protein. The results obtained were similar to those with DO-7, in that those cells with mutant or stabilized forms of p53 had higher levels of reactivity while those with wild-type p53 had a very low percentage (table 3.6) and (figure 3.8).

Cell Line / Marker	MCF-7	T47-D	231	468	ZR-75	436	HBL-100
P53 (CM-1) %±	5.9 ±0.0	98.9 ±1.55	98.7 ±0.35	99.5 ±0.70	7.4 ±0.83	0.0 ±0.0	99.9 ±0.14

Table 3.6: P53 (CM-1) in different cell lines.

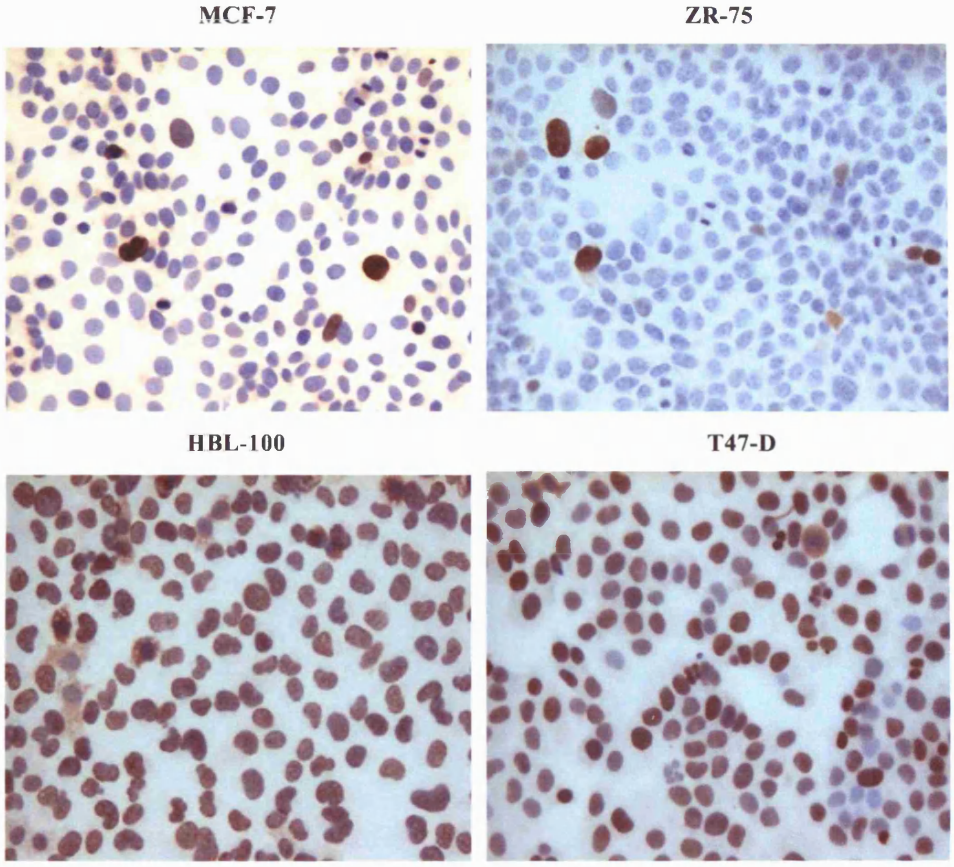


Figure 3.6: Nuclear staining for phosphospecific p53 with high levels in T47-D and HBL-100 cells.

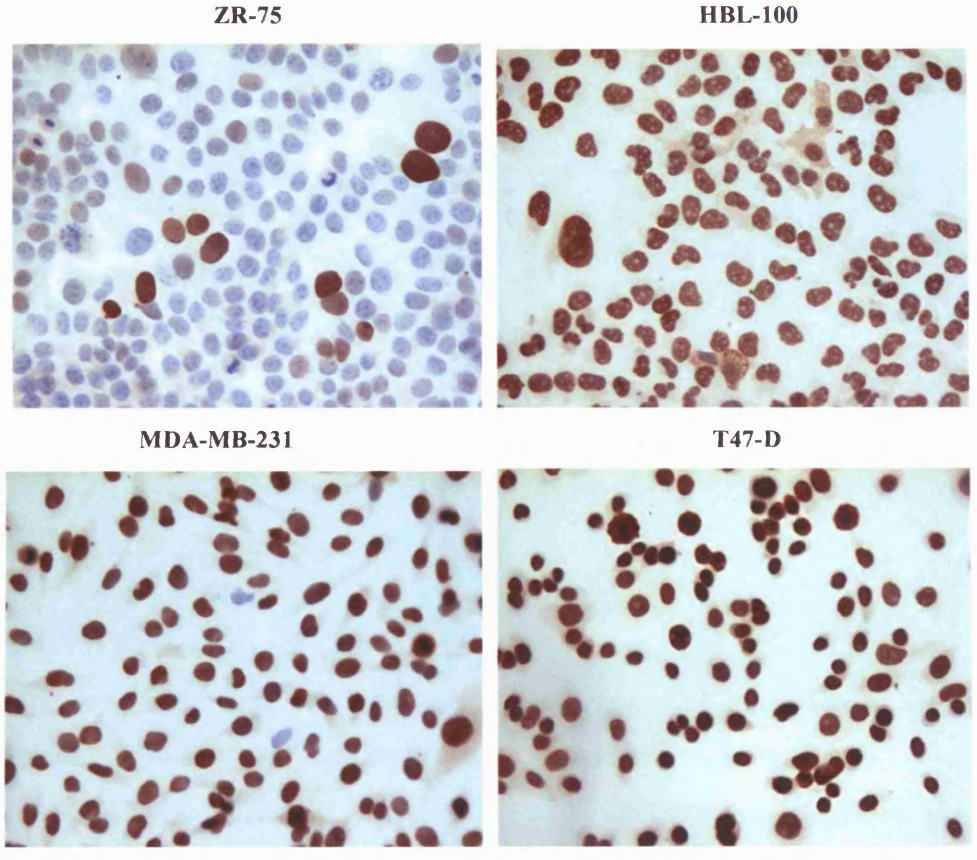


Figure 3.8:P53 (CM-1) immunocytochemistry in different cell lines. Number of positive cells was very low in MCF-7 and ZR-75, very high in MDA-MB-231 and T47-D.

3.2.2 P53 effector proteins

3.2.2.1 P21^{waf-1}

Immunocytochemistry

P21, also called (waf-1/CIP-1) is a cyclin-dependant kinase inhibitor working downstream to p53. It blocks the progression of the cell cycle at the G1/S transition phase and also involved in G2/M phase. The percentage of cells showing reactivity for p21^{waf-1} after 48 hours of culture was generally low but was greater in those that were wild-type for p53 (MCF-7 and ZR-75) (table 3.7) (figure 3.9). As for p53, MDA-MB-436 did not show any staining.

Cell Line / Marker	MCF-7	T47-D	231	468	ZR-75	436	HBL-100
P21^{waf-1} %±SD	7.8 ±2.17	2.8 ±0.75	2.1 ±0.45	1.0 ±0.07	6.6 ±2.1	0.0 ±0	3.9 ±2.07

Table 3.7: P21^{waf-1} in different cell lines.

3.2.2.2 ChK-2

Immunocytochemistry

ChK-2 is an essential upstream regulatory protein of p53. Upon exposure to genotoxic stress, ChK-2 becomes activated and then propagates the signal to the DNA-damage checkpoint cascades of which p53 is the main factor. Consequently, alterations in ChK-2 may disable the action of p53. Immunocytochemical analysis of ChK-2 in breast cell lines cultured for 48 hours revealed nuclear staining, which was homogenous in extent and ranged from moderate to faint. Therefore, assessment of staining was based on intensity rather than on the percentage of positive cells. MCF-7, T47-D and MDA-MB-436 showed moderate staining while in the other cell lines it was faint (table 3.8) (figure 3.10). Similar results were obtained from three experiments.

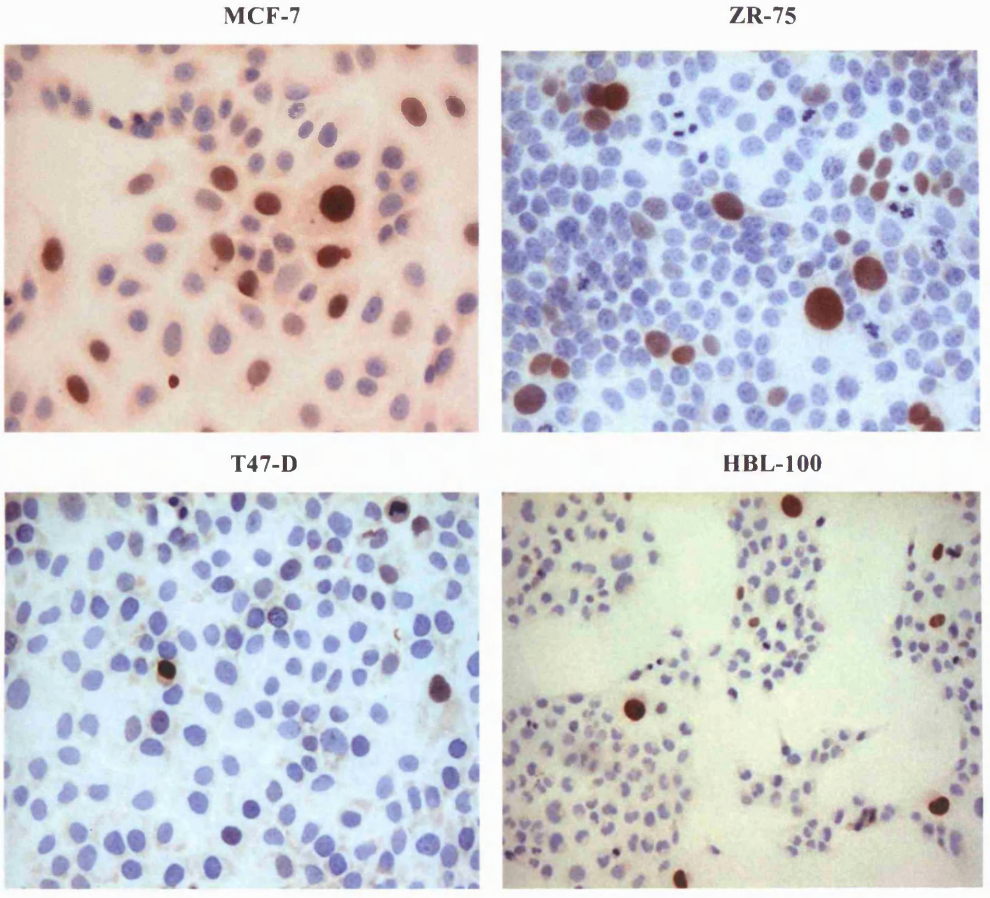


Figure 3.9: $p21^{waf-1}$ nuclear reactivity in different cell lines.

Cell Line / Marker	MCF-7	T47-D	231	468	ZR-75	436	HBL-100
ChK-2	Mod.	Mod.	Faint	Faint	Faint	Mod.	Mod.

Table 3.8: ChK2 immunocytochemistry in different cell lines.

3.2.3 Bcl-2 family proteins

3.2.3.1 Bcl-2

The expression of bcl-2 was assessed by both immunocytochemistry and western blotting.

Immunocytochemistry

Cells were cultured for 48 hours in triplicate. Bcl-2 was detected in 6 out of the seven cell lines with MDA-MB-436 being negative. The staining was homogenous among cells from the same cell line and the intensity ranged from faint to strong; ZR-75 showed the strongest staining in comparison to other cell lines (figure 3.11).

Bcl-2 western blotting

Two different concentrations of bcl-2 primary antibody were used for western blotting (1 and 5 μg/ml). The first concentration revealed a strong band at the level of 26 KDa corresponding to the bcl-2 protein in one cell line only (ZR-75) (figure 3.12A). When more concentrated antibody was used, the same cell line (ZR-75) had the strongest band but bcl-2 was detectable in all but not in T47-D cells (figure 3.12B).

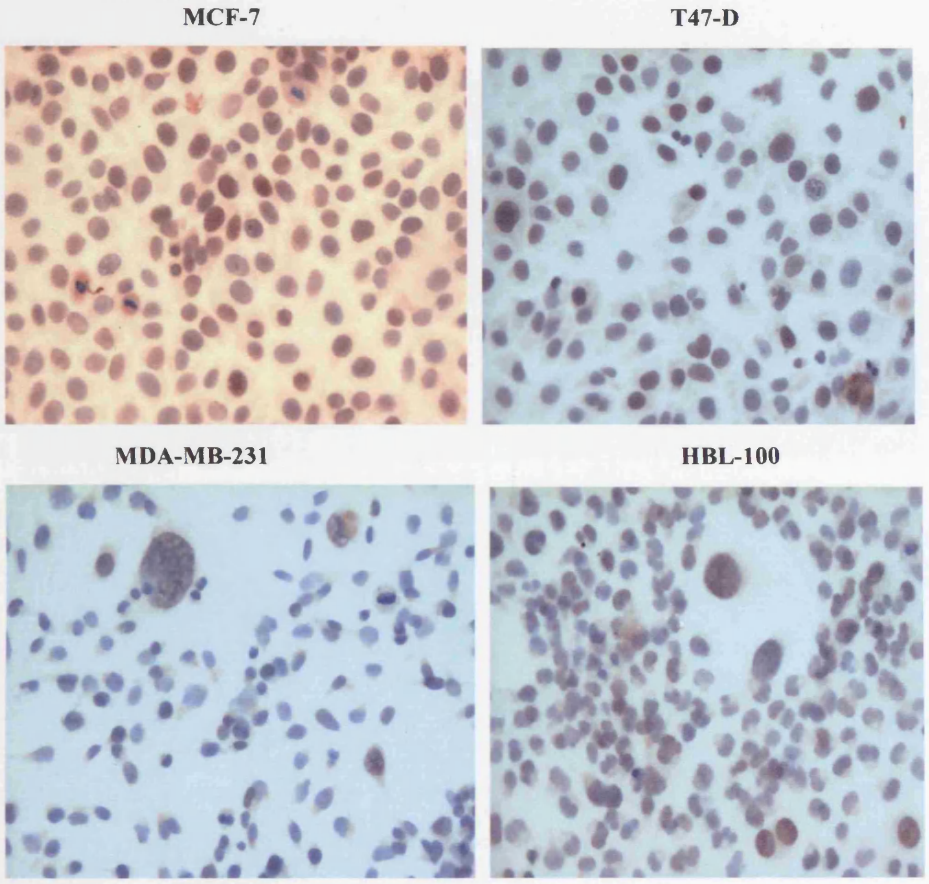


Figure 3.10: ChK2 immunocytochemistry in cell lines.

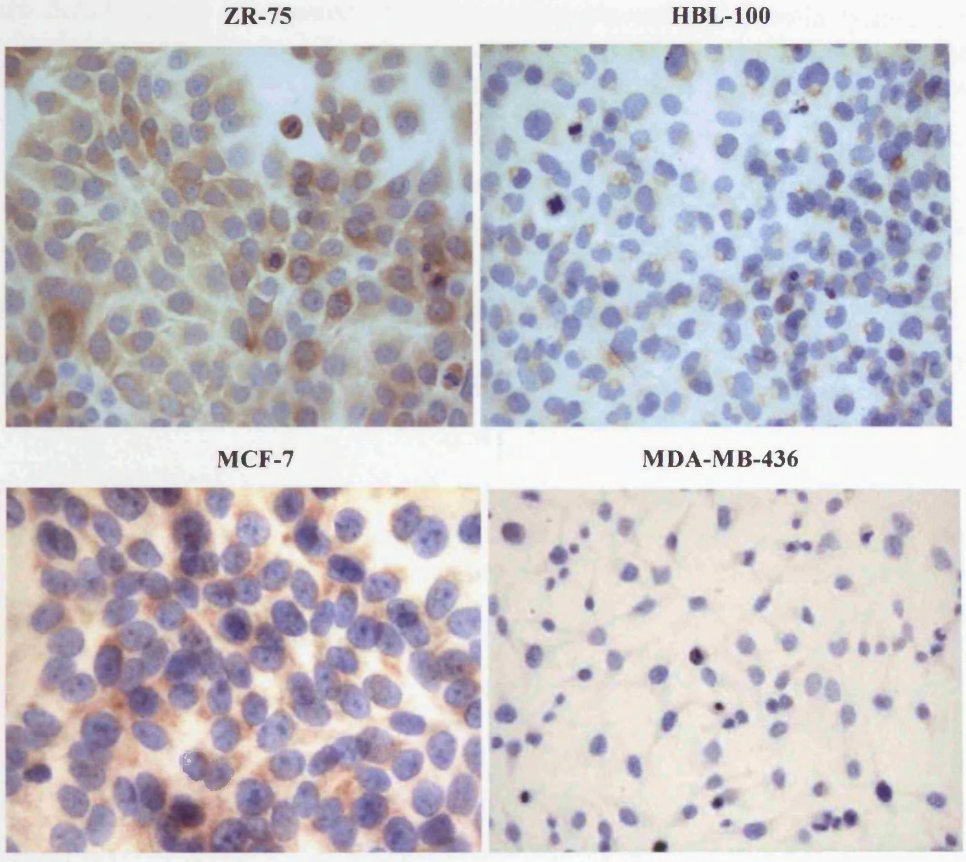


Figure 3.11: **Bcl-2 immunocytochemistry**, the staining ranged from strong (ZR-75), moderate (HBL-100), faint (MCF-7) and totally negative in MDA-MB-436.

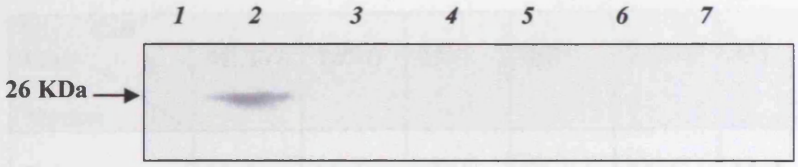


Figure 3.12A: **Bcl-2 by western blotting**, equal amounts of protein lysates (50µg) were loaded in each lane on 12% polyacrylamide gel, then the membranes were blotted for bcl-2, 1µg/ml antibody, lanes 1-7: MCF-7, **ZR-75**, HBL-100, MDA-MB-231, MDA-MB-436, MDA-MB-468 and T47-D.

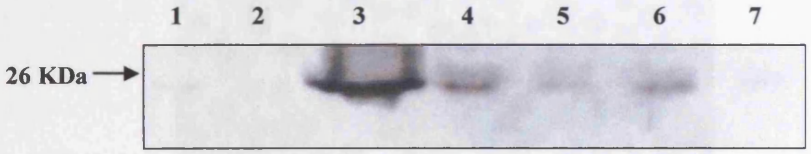


Figure 3.12B: **Bcl-2 by western blotting**, equal amounts of protein lysates (50µg) were loaded in each lane on 12% polyacrylamide gel, then the membranes were blotted for bcl-2, 5µg/ml antibody, lanes 1-7: MCF-7, T47-D, ZR-75, MDA-MB-231, MDA-MB-436, HBL-100 and MDA-MB-468.

3.2.3.2 Bax

Cells were cultured for 48 hours in triplicate and bax protein expression was assessed by immunocytochemistry. Several experiments were undertaken in relation to western blotting.

Immunocytochemistry

Bax protein was expressed as cytoplasmic staining in all cell lines studied. The staining was homogenously expressed by almost all cells but the intensity of staining varied. MDA-MB-468 showed the strongest staining while it was moderate in the others (table 3.9) (figure 3.13).

Cell Line / Marker	MCF-7	T47-D	231	468	ZR-75	436	HBL-100
Bax	Mod.	Mod.	Mod.	Strong	Mod.	Mod.	Mod.

Table 3.9: bax immunocytochemistry in different cell lines.

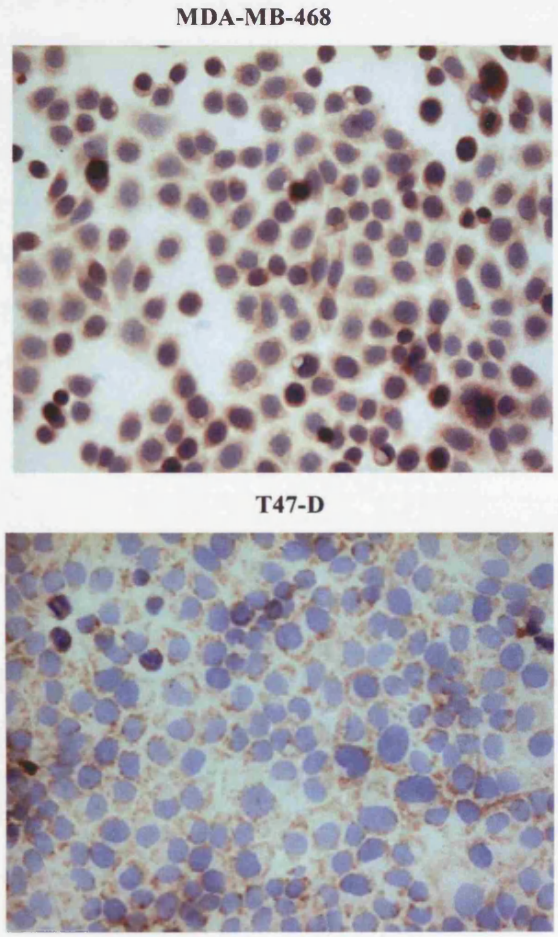


Figure 3.13: Bax expression by immunocytochemistry in breast cell lines.

Bax by western blotting

For bax protein expression by western blotting, several experiments were performed. In the first experiment, the same western blotting technique used for the detection of other antigens such as p53 and bcl-2 was applied and the primary antibody used was the same used as for bax immunocytochemistry (Polyclonal Rabbit Anti-Human BAX from DAKO), but no signal could be detected with different concentrations of the primary antibody. A different primary antibody was used in the second experiment (Bax-Ab-1 from Oncogene) at a range of concentrations including higher and lower than that recommended in the data sheet, but again no signal could be detected. Then a different lysis buffer for protein extraction (see materials and methods 2.2) was used with both primary antibodies. Bax was only detected when the new lysis buffer was used and the membranes were blotted with the bax antibody from Oncogene at a concentration of 1 µg/ml (figure 3.14). The level of signal detected in this experiment was comparable to the intensity of staining detected by immunocytochemistry. However this result was only obtained on one occasion and was not reproducible.

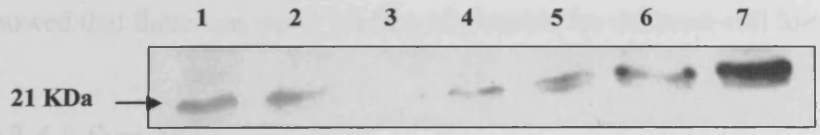


Figure 3.14: Bax by western blotting in different cell lines. 50 µg of protein lysates in each lane, on 12 % polyacrylamide gel), membranes were blocked for non-specific staining in TPST containing 0.3% Tween 20, primary antibody Bax (Oncogene), lanes 1-7 correspond to: MCF-7, ZR-75, T47-D, HBL-100, MDA-MB-231, MDA-MB-436, and MDA-MB-468.

3.2.3.3 Bcl-x_L

Bcl-x is another protein of bcl-2 family members. There are two splice variants of this protein (bcl-x_L and bcl-x_S). The first one has anti-apoptotic functions while the second has pro-apoptotic effect. The antibody used in this experiment detected the bcl-x_L variant, which has an anti-apoptotic function. Bcl-x was assessed by

immunocytochemistry and there was homogenous cytoplasmic staining in 6 out of the 7 cell lines studied. MCF-7, T47-D and MDA-MB-468 cell lines showed strong cytoplasmic staining while in 436 it was moderate and both ZR-75 and HBL-100 showed faint staining (figure 3.15). The only cell line that did not express any bcl-x staining was MDA-MB-231. The antibody was not suitable for immunoblotting.

3.2.4 Inhibitor of apoptosis proteins

Two members of this family are Survivin and XIAP, which are commonly expressed in cancers. Cells were cultured for 48 hours. There were several attempts at trying to detect these proteins by immunocytochemistry, but no definite signal could be detected. Therefore, protein lysates were assessed by western blotting. MDA-MB-436 cells were not assessed.

3.2.4.1 XIAP

All cell lines had an immunodetectable protein band at the level of 55 KDa, which corresponded to the XIAP protein molecular size. These bands were slightly stronger in MDA-MB-468, ZR-75 and HBL-100 (figure 3.16A). Staining for vinculin protein showed that there was equal loading of proteins for different cell lines (figure 3.16C).

3.2.4.2 Survivin

All cell lines revealed immunodetectable protein bands at the level of 16 KDa, which corresponded to Survivin protein. There were stronger bands for MDA-MB-468, T47-D and HBL-100 cells (figure 3.16B), with ZR-75 being low.

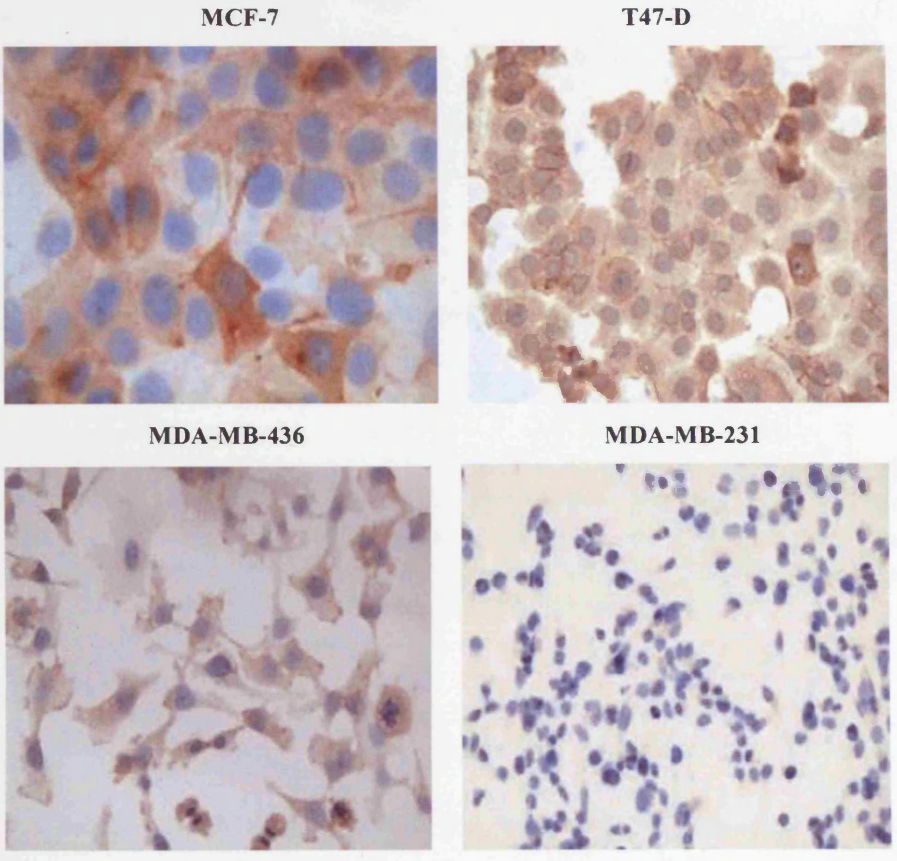


Figure 3.15: Bcl-x immunocytochemisry in different cell lines. Strong staining detected in MCF-7and T47-D, moderate in MDA-MB-436, and no staining in MDA-MB-231 cells.

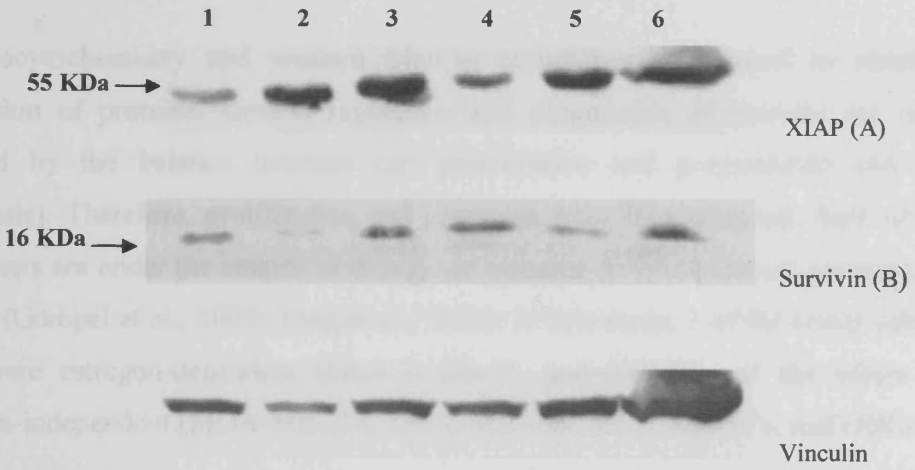


Figure 3.16: Western blotting expression of IAPs. A, XIAP in different cell lines,50 µg of protein in each lane in 10% polyacrylamide gel, the primary antibody concentration 1µg/ml. B, Survivin in the same cell lines, 50 µg/ml protein lysates in each lane in 10% gel, and primary antibody 2µg/ml. vinculin, Lanes1-6 correspond to MCF-7, ZR-75, MDA-MB-468, T47-D, MDA-MB-231 and HBL-100.

Discussion

The experiments performed in this part of the study were to characterize a group of breast cell lines regarding the expression of biological markers that are involved in the regulation of cell proliferation and apoptosis, prior to undertaking experiments considering the effects of chemotherapeutic agents (chapter 4).

Each of the breast cell lines investigated has different characteristics including the rate at which they grow. Initially the doubling rate for each cell line was determined so that cells could be seeded at appropriate numbers to ensure that they achieved the same degree of confluency at the same time point. Whilst there is information about certain features of the cells studied (e.g. ER, EGFR, and p53 status), less is known about some of the other proteins investigated. Also, there can be phenotypic variability between

cells in different laboratories, particularly since some have been maintained in culture for long time periods (e.g. MCF-7) (Lacroix and Leclercq, 2004).

Immunocytochemistry and western blotting techniques were used to check the expression of proteins. Growth regulation and progression of tumours are mainly affected by the balance between cell proliferation and programmed cell death (apoptosis). Therefore, proliferation and apoptosis were first analyzed. Both of these parameters are under the control of estrogenic hormone in breast tissues, amongst other factors (Gompel et al., 2000; Tong et al., 2002). In this study, 3 of the breast cell lines used were estrogen-dependent (MCF-7, ZR-75, and T47-D) and the others were estrogen-independent (MDA-MB-231, MDA-MB-468, MDA-MB-436, and HBL-100).

The proliferation rate in the group of breast cell lines studied ranged from 26.39 % in estrogen responsive T47-D to 48.0 % in the estrogen un-responsive HBL-100. However other cells, which are estrogen responsive (e.g. MCF-7) showed higher proliferation in comparison to T47-D. This may indicate that other regulatory factors for proliferation are important for these breast cancer cells.

Apoptosis was detected by M30, the anti-Cytokeratin 18 antibody which detects cells in early apoptosis (Leers et al., 1999). The rate of apoptosis was generally low (range 0.7 % in ZR-75 to 2.0 % in T47-D). There is lack of literature concerning detection of apoptosis in breast cancer cells by M30, most previous work using other methods such flow cytometry, the TUNEL method and detection of active caspases. (Wang et al., 2004) showed a low level of apoptosis in MCF-7 cells.

I did make some attempt to detect apoptotic cells by flow cytometry after labeling with Annexin-V and propidium iodide (PI), but the results were not consistent. The problem was mainly in the method of harvesting cells. I used trypsin, phenol red-free trypsin and EDTA to detach the monolayer cells in culture prior to labeling them with annexin-V and PI. After several attempts with using different cell lines, it was evident that the apoptotic process is very sensitive to such harvesting procedures and that flipping of the

membrane protein phosphatedylserine can happen as a result, so causing false positive and inconsistent results.

Previous in vitro studies found that 1-3 % of cells had strong nuclear staining with three different antibodies in wild-type p53 cell lines (MCF-7 and ZR-75) (Bartek et al., 1990; Vojtesek and Lane, 1993). In this study, MCF-7 and ZR-75 cells showed strong nuclear staining in 2-9 % with three different antibodies, which is close to previous studies. In contrast to wild-type p53 cells, those with mutated or stabilized forms of p53 (MDA-MB-231, MDA-MB-468, and HBL-100) showed very high level of p53 protein expression. Wild-type p53 is unstable and has a short half-life, therefore, cannot be detected by immunochemistry. Mutant p53 proteins are stable and consequently, easily detected (Finlay et al., 1988). Detection of p53 by immunohistochemistry can reflect the presence of p53 mutations in breast cancer (Schmitt et al., 1998; Vojtesek and Lane, 1993) however some factors may cause discrepancies. These include fixation methods, different epitope specificities of antibodies and the presence of silent mutations (Visscher et al., 1996; Vojtesek and Lane, 1993).

In one cell line (MDA-MB-231) there was a difference in the percentage of positivity of phosphorylated protein on Serine 392 in comparison to the total p53 detected with DO-7 antibody. This shows that some breast cancer cell lines with mutant p53 express less phosphorylated Serine 392 p53, which is supported by a previous study (Yap et al., 2004) who found some tumours with mutant p53 to have unphosphorylated Serine 392 p53 *in vivo*. Western blotting results confirmed the protein expression by immunocytochemistry. Immunodetectable p53 was revealed in 4 cell lines, 3 with mutant p53 (MDA-MB-231, MDA-MB-468, and T47-D) and 1 with stabilized protein (HBL-100). The level of phosphorylated p53 detected by immunoblotting was comparable to that detected by immunocytochemistry, being high in MDA-MB-468, T47-D and HBL-100; lower in MDA-MB-231 and not detected in wild-type p53 cells (MCF-7 and ZR-75).

P53 induces cell cycle arrest, an essential mechanism for regulation of homeostasis, through the transcription of downstream effector proteins such as $p21^{waf-1}$. A way to investigate the functional status of p53 is to evaluate these effector proteins. Higher levels of $p21^{waf-1}$ might indicate less aggressive breast cancer. In this study, cells with functioning (wild-type) p53 showed more staining for $p21^{waf-1}$ than those with mutant p53; 7.8 % and 6.6 % in MCF-7 and ZR-75 respectively, in comparison to other cell lines (usually less than 3.9 %) which is consistent with previous work (Jiang et al., 1998).

ChK-2 is an upstream regulatory protein, regulating the function of p53. It is considered as an essential checkpoint of the cell cycle upon exposure to any DNA damaging agent. Alterations or mutations of ChK2 markedly affect the function of p53. Most of breast cancer cell lines in this study showed either moderate or faint staining with ChK-2.

As mentioned above, cell survival depends on the balance between factors that promote or inhibit apoptosis and one of the critical regulators of apoptosis is the protein encoded by the Bcl-2 gene, which is a member in a family that includes other inhibitors of apoptosis such as Bcl-x and promoters of apoptosis such as Bax. ZR-75 was the single cell line to clearly express bcl-2 by both immunocytochemistry and western blotting, while other cells were either moderate (HBL-100) or faint (other cells). This differs from the findings of (Zapata et al., 1998) who showed the absence of bcl-2 in ZR-75 cells. This may due to the phenotype differences of cell lines between laboratories as discussed before. Bcl-2 was not detected in T47-D cell by western blotting, which is in agreement with a previous report (Mooney et al., 2002).

Bcl-x was expressed in 6 out of the seven cell lines, the staining was strong in MCF-7 and T47-D and faint in other cell lines. (Zapata et al., 1998) detected bcl-x in almost all of breast cancer cell lines studied. Bax protein was detected by immunocytochemistry, but western blotting proved more difficult. On one occasion a band could be detected in most of breast cancer cells being strongest in MDA-MB-468, but this could not be repeated. This could be due to the nature of the antibodies used or the lysis method used

for extraction of the protein. Also, different transfer methods such as wet transfer need to be assessed. Other studies have detected bax with western blotting in almost all breast cancer cells studied (Zapata et al., 1998).

Several proteins that inhibit apoptosis have been identified, of which the inhibitor of apoptosis family (IAPs). Survivin is one member of this newly identified family; it inhibits apoptosis by inhibiting the function of caspase 3 and caspase 7. Previous studies revealed elevated expression of Survivin in fetal tissues as well as various human cancers including breast. In this study, immunodetectable levels of Survivin were shown in all cell lines studied. XIAP, another member of the IAPs family, was also detected by western blotting in all cell lines. Expression was strong in almost all cell lines, being less strong in MCF-7 cells. The immortalized normal breast cell line HBL-100, also expressed strong band of XIAP. This result is in concordance with other studies on breast cell lines (Yang et al., 2003). XIAP has also been found to be highly expressed in almost all of cancer cell lines derived from different human cancers including breast cell lines (Tamm et al., 2000) and also in prostate cancer cell lines (Liu et al., 2004).

MDA-MB-436 cells lacked many of the proteins and so it was decided not to include this in subsequent studies. HBL-100 was used since it was known to have stabilized p53 and was a useful control. However, it was originally derived from normal breast (even if now immortal) (Caron de Fromentel et al., 1985), and so was not used in the subsequent experiments assessing the effects of chemotherapeutic agents on breast cancer cells.

To conclude, p53 protein was detected in T47-D, MDA-MB-231 and MDA-MB-468 breast cancer cells, and HBL-100 cells (known to be stabilized). Strong staining for bcl-2 was found in ZR-75 cells with little reactivity in other cells. Bax was detected in all, and bcl-x was strongly expressed in MCF-7, T47-D, and MDA-MB-468 cells. $P21^{waf-1}$ was higher in MCF-7 and ZR-75 cells. Survivin and XIAP were detected in all cell lines with little variation.

CHAPTER 4:

EFFECT OF CHEMOTHERAPEUTIC AGENTS ON PROLIFERATION, APOPTOSIS AND RELATED PROTEINS IN BREAST CANCER CELL LINES

Introduction

Many genes have been identified that affect the response of cells after exposure to pathological stresses. Because p53 is a key gene involved in tumour response to therapy, tumours with functioning wild-type p53 are thought to respond better to chemotherapeutic drugs than those with mutated gene (Kirsch and Kastan, 1998). Depending on the assumption that apoptosis is the major way of killing cells after treatment with chemotherapeutic drug tumours with mutations in p53, high level of bcl-2 expression or high ratios of bcl-2-bax should be resistant to cancer treatment (Lowe et al., 1993; Reed et al., 1996).

Paclitaxel is used in the treatment of many common human cancers including breast cancer; it inhibits microtubule depolarization in the G_2-M phase of the cell cycle (Rowinsky et al., 1992). Doxorubicin is one of the commonly used chemotherapeutic drugs in the treatment of various cancers including breast cancer (Kroger et al., 1999). It intercalates among DNA resulting in conformational changes in DNA structure and also changes in the activity of topoisomerases.

Methods

In this part of the study, 5 different breast cancer cell lines (MCF-7, ZR-75, T47-D, MDA-MB-231 and MDA-MB-468) were used as models to assess the effect of treatment with chemotherapeutic drugs on the expression of biological factors (see 2.3.6). Two drugs (doxorubicin and paclitaxel) were used for treatment experiments on the basis that those drugs were commonly used in the clinic for treatment of breast cancer patients, and also each of them has different mechanism of action. Doxorubicin induces DNA damage while paclitaxel stabilizes the microtubules but ultimately both drugs kill cancer cells through the induction of apoptosis.

The effect of treatment on the expression of p53 was assessed by two different antibodies (p53 DO-7 and p53 phosphospecific) in the two breast cancer cell lines which harbor a wild-type functioning p53 gene (MCF-7 and ZR-75) while the other cell

lines which have a mutant p53 gene (MDA-MB-231, MDA-MB-468 and T47-D) were not included in these experiments. The effect of treatment on cell proliferation and on the induction of apoptosis was also assessed in the five breast cancer cell lines. Experiments also included the expression of the p53 downstream effector protein $p21^{waf1}$ and the pro-apoptotic protein bax. The effect of treatment on the newly investigated members of inhibitor of apoptosis proteins (IAPs) Survivin and XIAP were also investigated.

The aim of this part of the study was to investigate the effect of treatment by two chemotherapeutic drugs (doxorubicin and paclitaxel) on the expression of different biological factors in breast cancer cell lines (as models) in order to understand the essential biological factors that may affect treatment response or resistance.

Results

4.1 Effect on proliferation

Proliferation was assessed immunocytochemically using BrdU incorporation after treatment of cells with three different concentrations of each drug and for three different time periods (14h, 24h, and 48h) (see 2.3.6).

4.1.1 MCF-7 cells

Proliferation of MCF-7 cells was markedly suppressed by doxorubicin treatment in a time dependent manner, with lowest proliferation at 48h. At 14h and 24h proliferation suppression was slightly greater at lower doses ($p=0.050$), but by 48h the effect was equal with the three different doses (figure 4.1A, 4.2 A & B).

Treatment of MCF-7 cells with paclitaxel also induced suppression of proliferation in a dose-dependent pattern ($p=0.027$ at 48h). Higher doses of treatment were associated with lower percentages of proliferation (figure 4.1B, 4.2 C&D), but not to the same extent as doxorubicin.

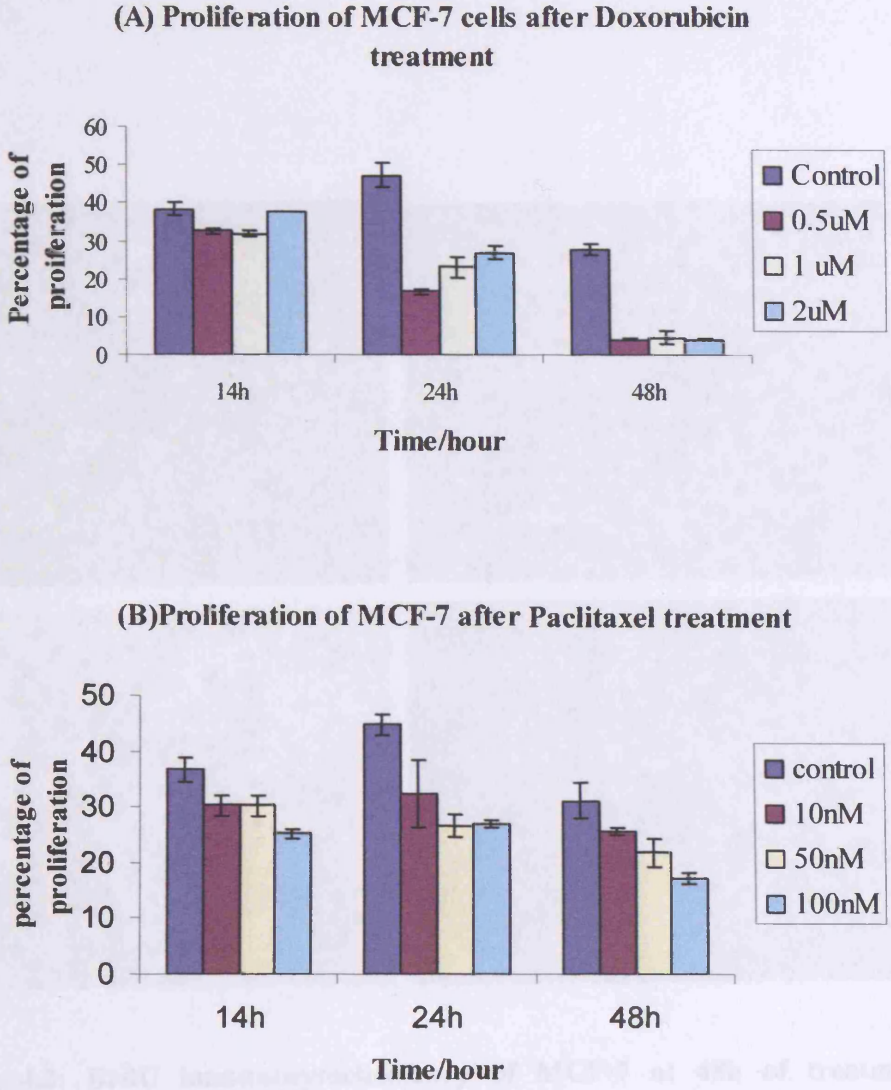


Figure 4.1: BrdU after treatment in MCF-7 cells. A, MCF-7 after doxorubicin treatment. B, MCF-7 after paclitaxel treatment.

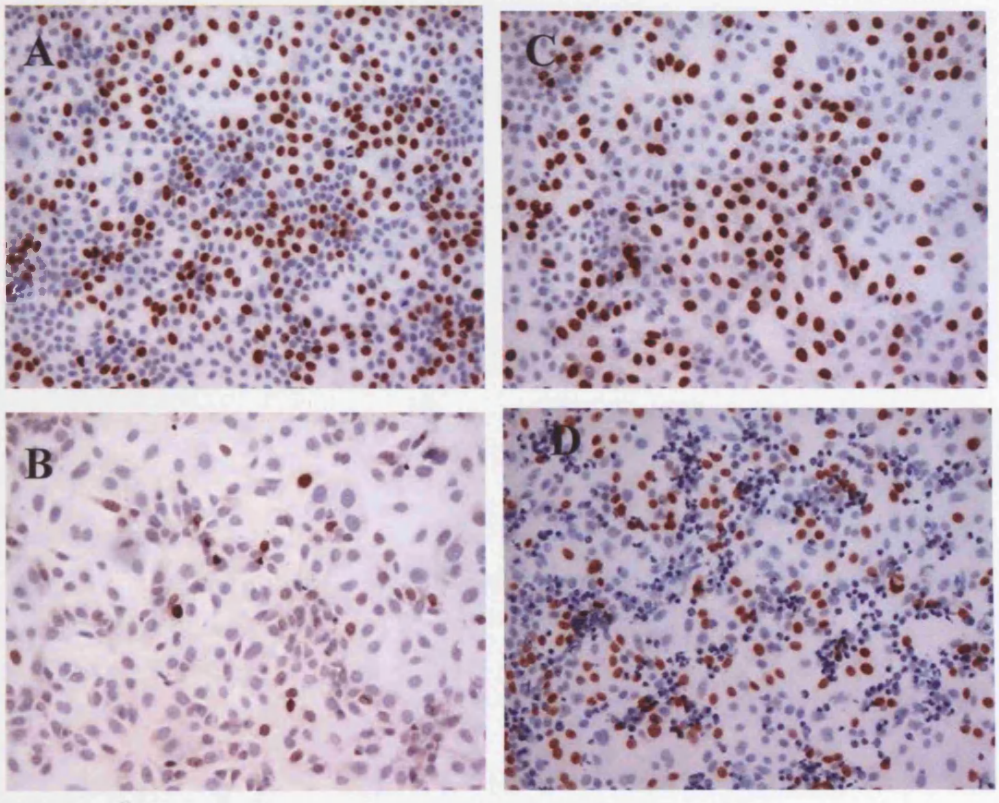
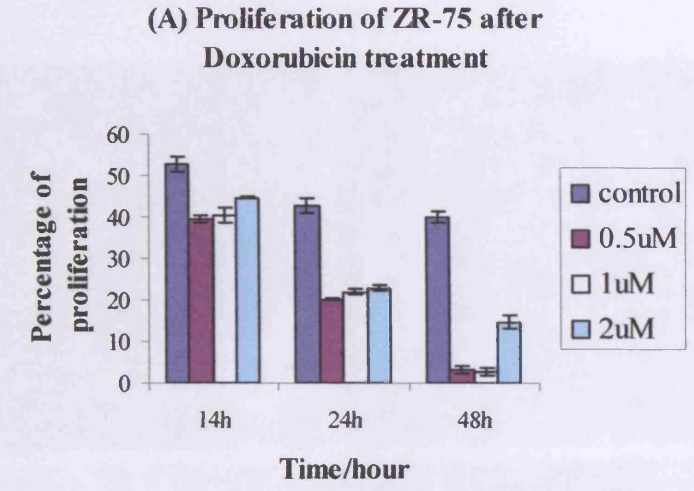


Figure 4.2: BrdU immunocytochemistry of MCF-7 at 48h of treatment. A, doxorubicin control. B, treatment with 1 μM doxorubicin. C, paclitaxel control. D, treatment with 50 nM paclitaxel.

4.1.2 ZR-75 cells

ZR-75 proliferation was markedly suppressed after treatment with both drugs. Doxorubicin induced suppression in a time-dependent manner; the maximum effect was after 48h of treatment. Lower doses of doxorubicin induced greater suppression of proliferation (p=0.05 at 48h) (figure 4.3A, 4.4 A&B). Treatment with paclitaxel also suppressed proliferation markedly and the suppression was dependent on both the dose of the drug and the duration of treatment (figure 4.3B, 4.4 C&D).



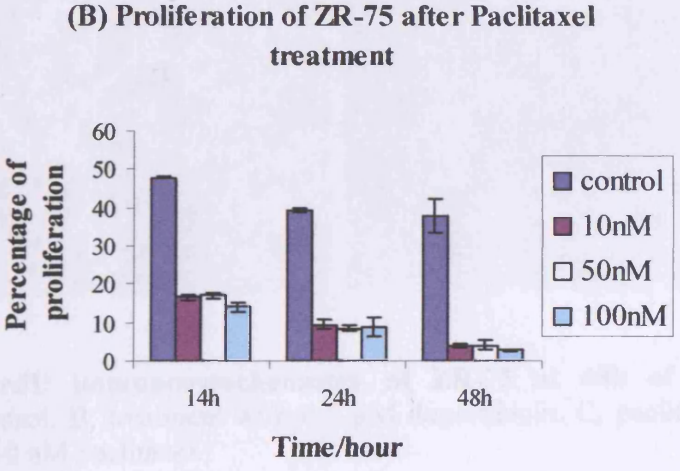


Figure 4.3: BrdU after treatment in ZR-75 cells. A, ZR-75 after doxorubicin treatment. B, ZR-75 after paclitaxel treatment.

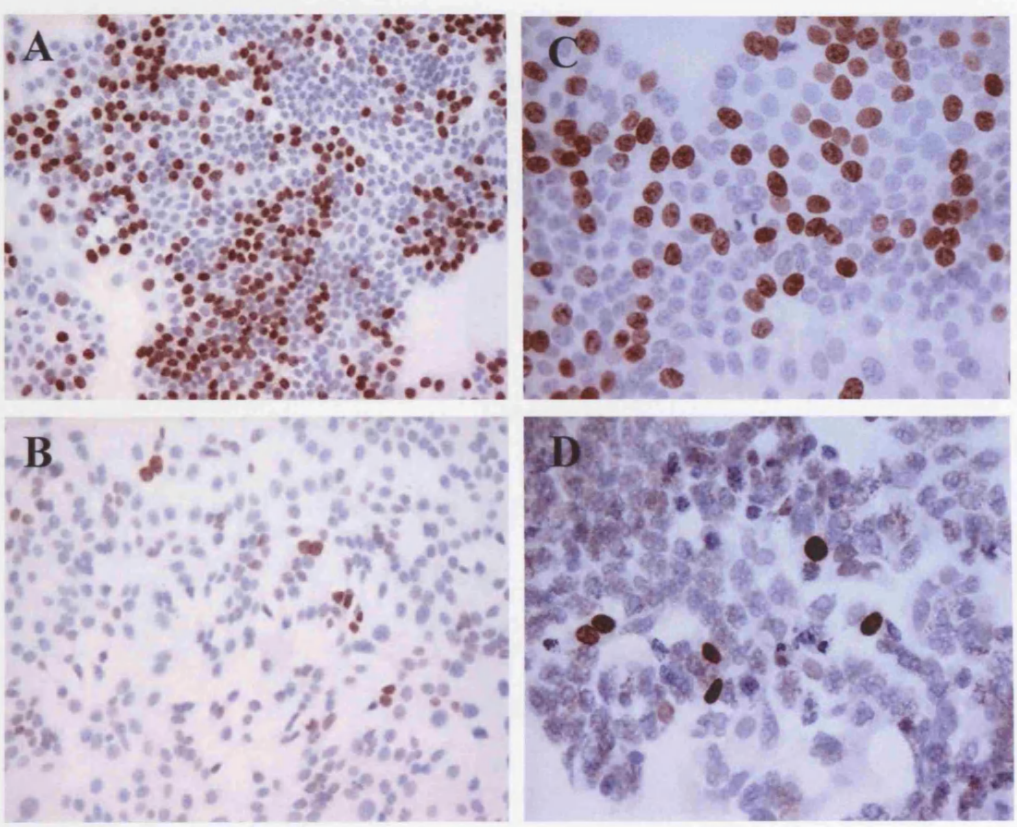


Figure 4.4: BrdU immunocytochemistry of ZR-75 at 48h of treatment. A, doxorubicin control. B, treatment with 0.5 μM doxorubicin. C, paclitaxel control. D, treatment with 10 nM paclitaxel.

4.1.3 T47-D cells

T47-D cells showed growth stimulation with all doses of doxorubicin at 14h and at 24h, greater with 0.5μM and 1μM (p=0.037 at 24h). There was greater stimulation with the lower doses at 48h, but 2.0 μM did show some suppression (figure 4.5A, 4.6 A&B). Paclitaxel had little effect at 14h, some stimulation at 24h and growth suppression at 48h (p=0.046 at 48h) (figure 4.5B, 4.6 C&D). These experiments were repeated several times and each time there was growth stimulation.

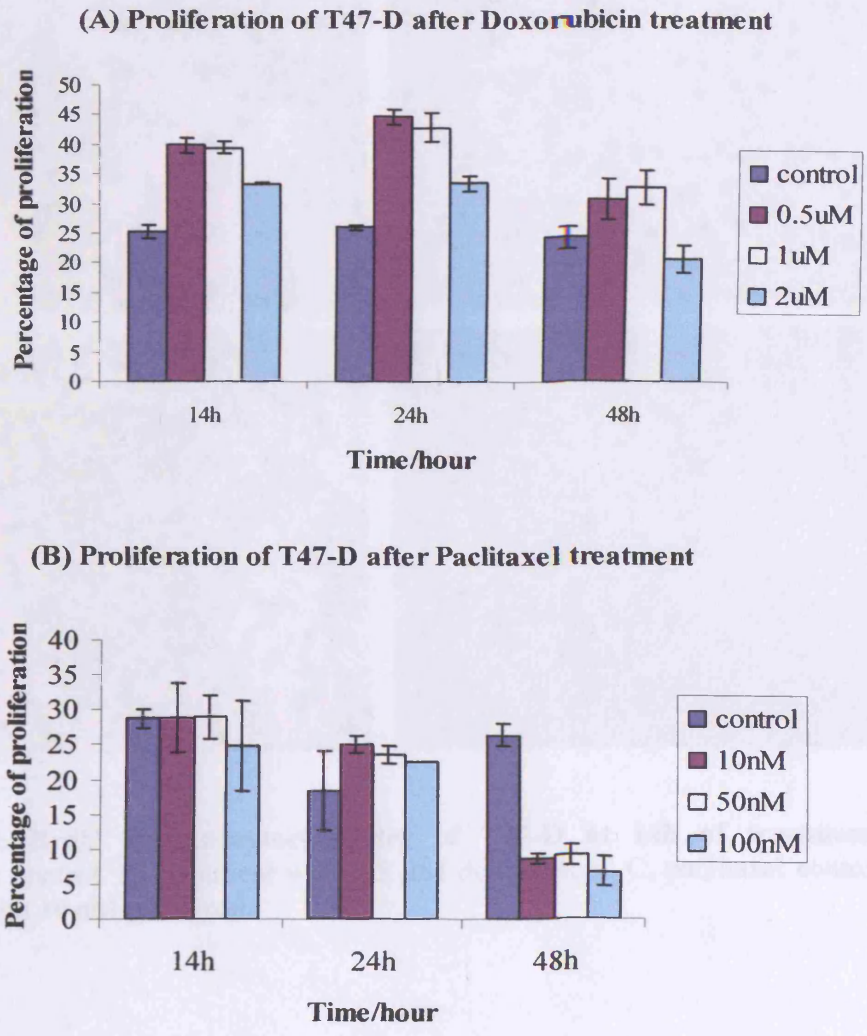


Figure 4.5: BrdU after treatment in T47-D cells. A, cells after doxorubicin treatment. B, cells after paclitaxel treatment.

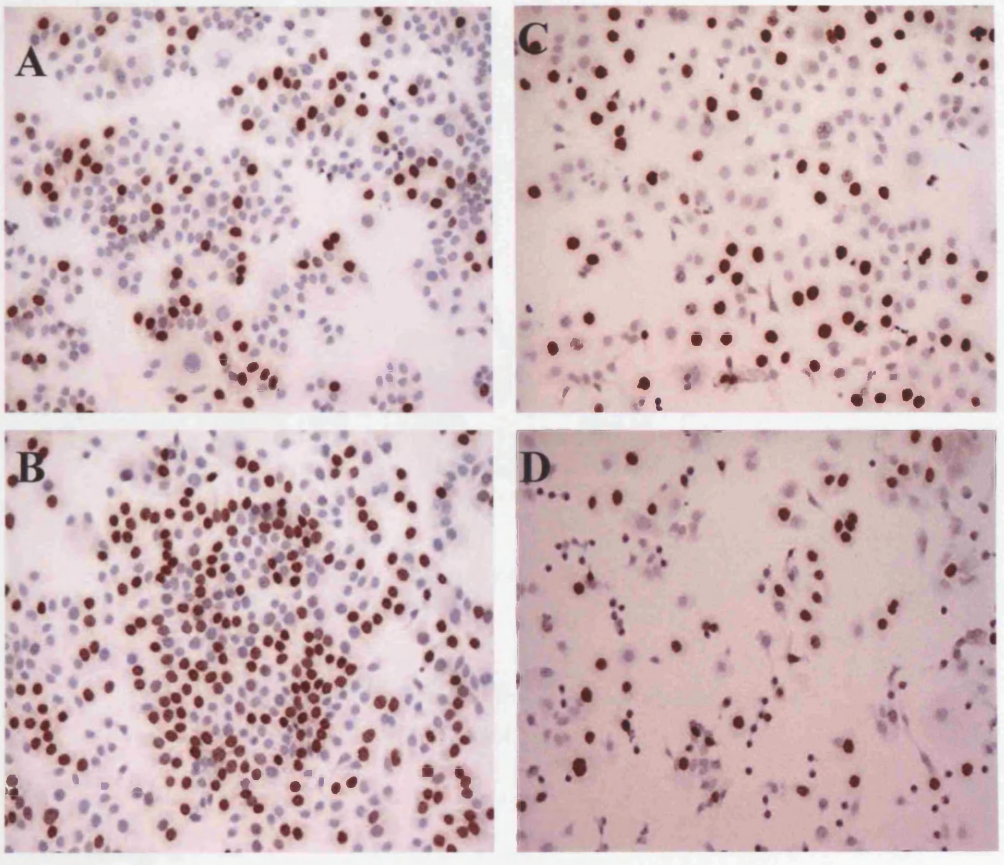
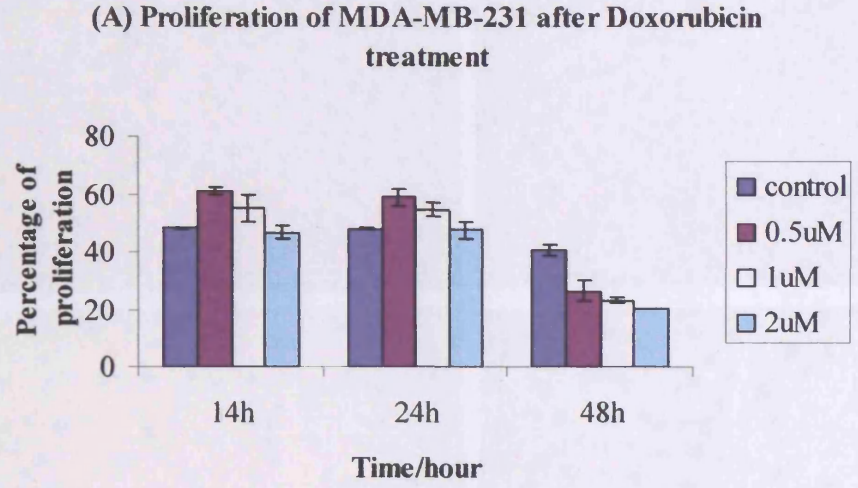


Figure 4.6: BrdU immunocytochemistry of T47-D at 14h of treatment. A, doxorubicin control. B, treatment with 0.5 µM doxorubicin. C, paclitaxel control. D, treatment with 10 nM paclitaxel.

4.1.4 MDA-MB-231 cells

Treatment of MDA-MB-231 cells with doxorubicin at 0.5 and 1.0 μM for 14h and 24h resulted in slight growth stimulation, while treatment with 2.0 μM of the drug had no effect on proliferation. By 48h doxorubicin had a growth suppressing effect in a dose-dependent manner (p=0.021) (figure 4.7A, 4.8 A&B). Treatment with paclitaxel was associated with proliferation suppression; the effect was maximal after 24h of treatment (p=0.046) (figure 4.7 B, 4.8 C&D).



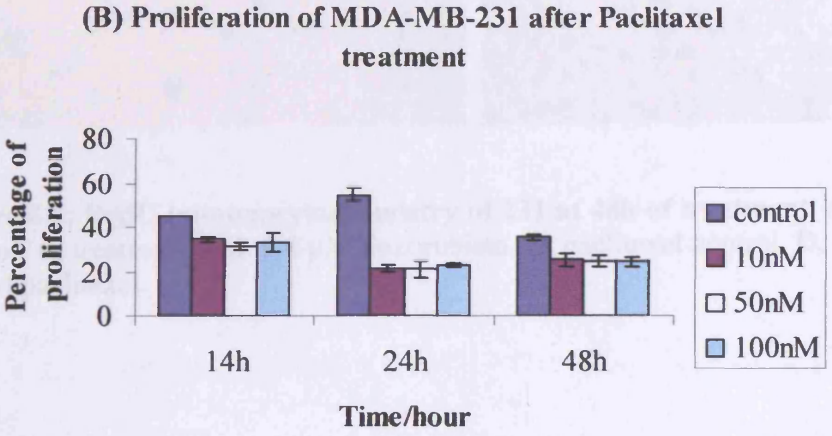


Figure 4.7: BrdU after treatment in 231 cells. A, 231 after doxorubicin treatment. B, 231 after paclitaxel treatment.

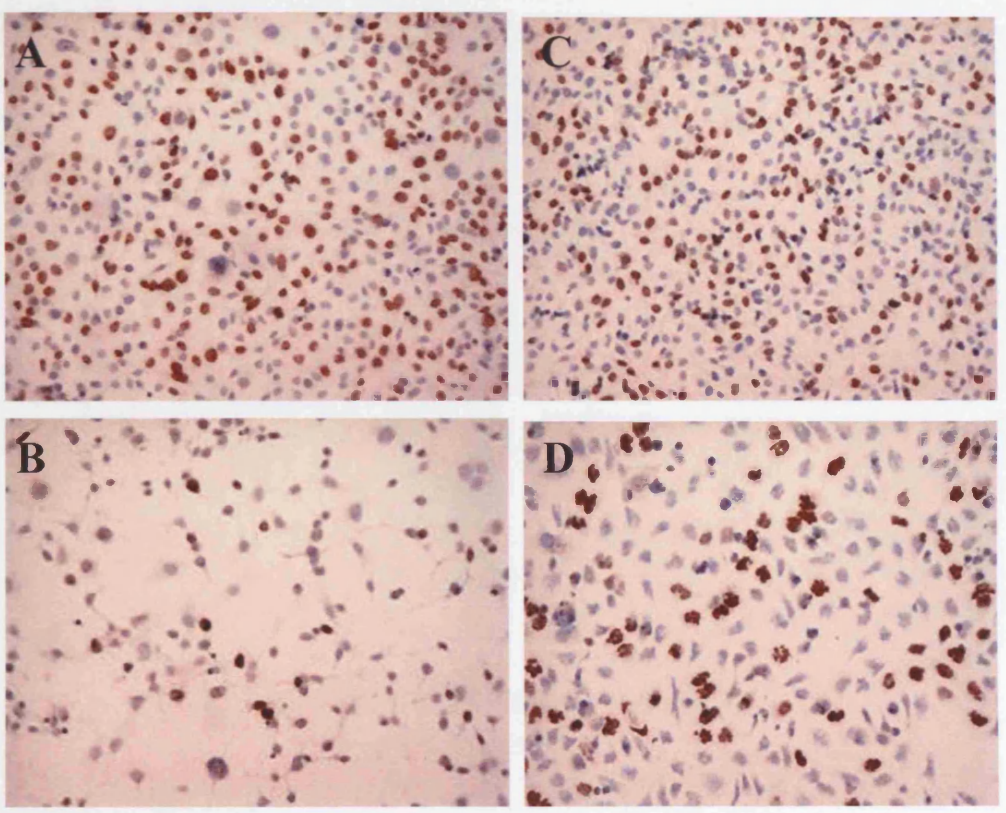


Figure 4.8: BrdU immunocytochemistry of 231 at 48h of treatment. A, doxorubicin control. B, treatment with 0.5 μM doxorubicin. C, paclitaxel control. D, treatment with 10 nM paclitaxel.

4.1.5 MDA-MB-468 cells

Proliferation of MDA-MB-468 cells was not affected by doxorubicin treatment at low doses (0.5 and 1.0 μM) and it was only slightly suppressed by doxorubicin 2.0 μM (figure 4.9 A, 4.10 A&B). Different doses of paclitaxel did not affect proliferation of MDA-MB-468 cells at 14h and 24h of treatment (p=0.199 at 14h), but there was moderate suppression after 48h of treatment with all doses (p=0.046) (figure 4.9 B, 4.10 C&D).

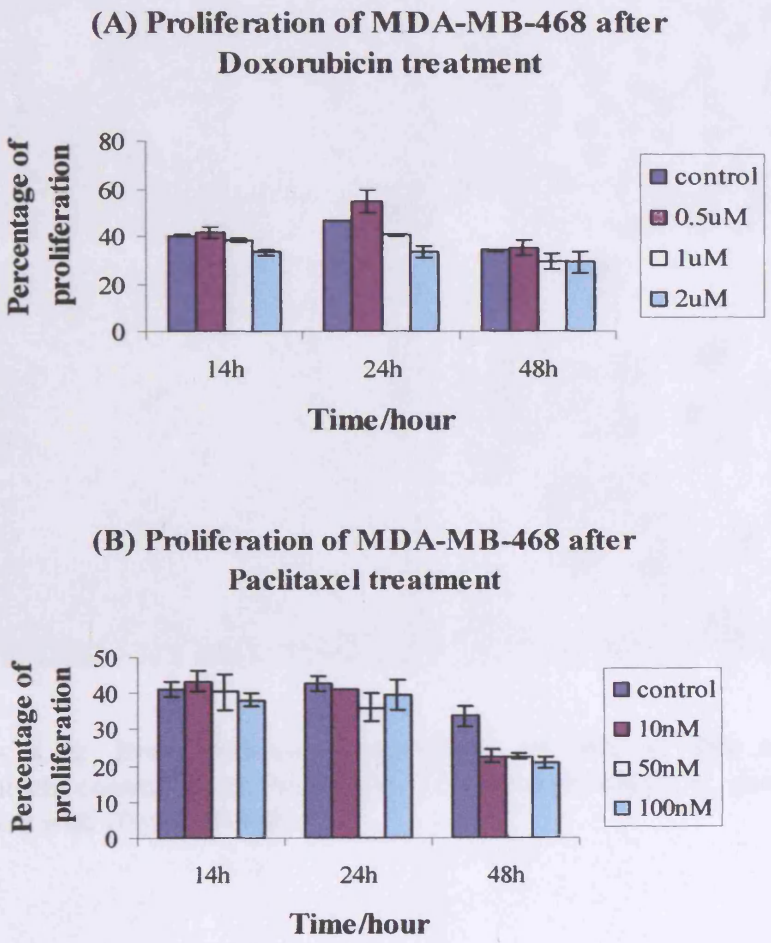


Figure 4.9: BrdU after treatment in 468 cells. A, cells after doxorubicin treatment. B, cells after paclitaxel treatment.

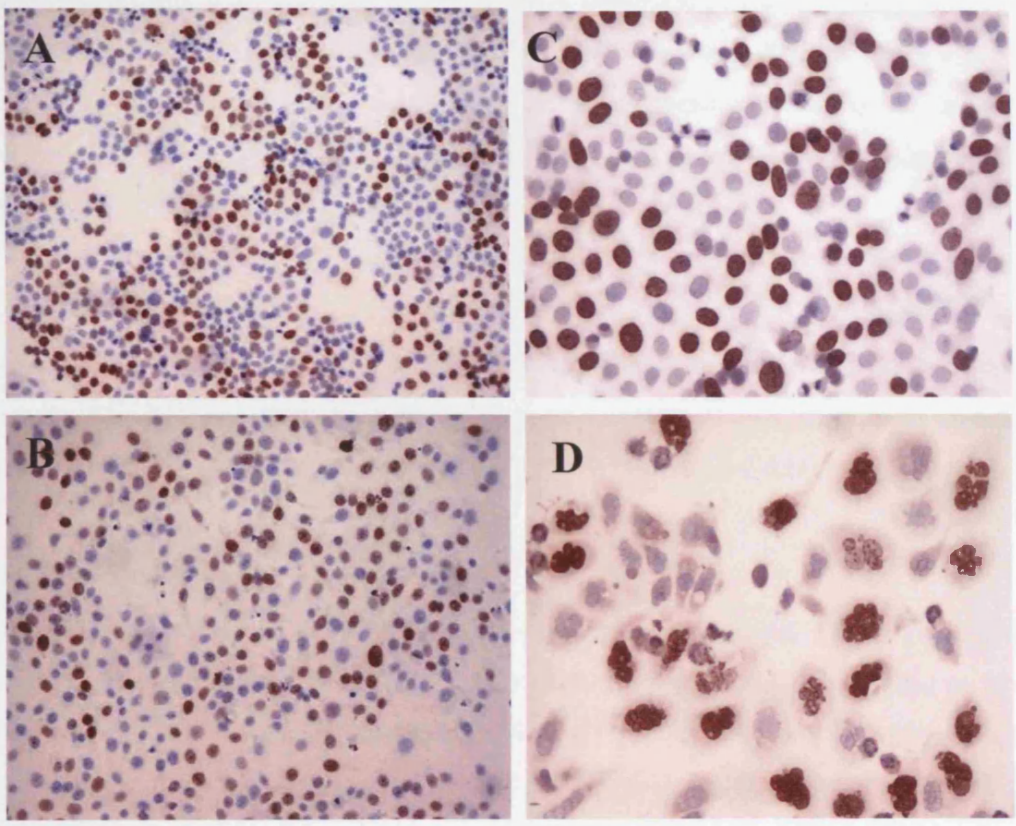


Figure 4.10: BrdU immunocytochemistry of 468 at 48h of treatment. A, doxorubicin control. B, treatment with 1.0 μM doxorubicin. C, paclitaxel control. D, treatment with 10 nM paclitaxel.

4.2 Effect on Apoptosis

Apoptosis was detected by immunocytochemistry using M30 mouse monoclonal antibody.

4.2.1 MCF-7 cells

Apoptosis was markedly induced by doxorubicin treatment at 14h and 24h when cells were treated with higher doses (1.0 and 2.0 μM). Treatment with doxorubicin 0.5 μM at the different time periods had a minimal effect on apoptosis. By 48h of doxorubicin treatment, the percentage of cells in apoptosis had decreased by approximately two-thirds (figure 4.11A, 4.12A& B).

The percentage of apoptotic cells was doubled at 14h of paclitaxel treatment and reached a maximal increase at 24h. At 48h of paclitaxel treatment the percentage of apoptotic cells had decreased back to the level of induction at 14h, apart from with the higher dose (2.0 μM) which remained high (figure 4.11 B, 4.12 C&D).

4.2.2 ZR-75 cells

Apoptosis was induced in ZR-75 cells after doxorubicin treatment. There was a time dependent increase in the induction of apoptosis with about two fold increase at 14h, three fold at 24h and a nine fold increase at 48h of treatment. At 48h, the difference in percentage of apoptosis for different doses of doxorubicin was minimal (figure 4.13 A, 4.14 A&B).

There was more evidence of apoptosis at 14h and 24h with paclitaxel treatment, but there was a massive induction of apoptosis at 48h of treatment (figure 4.13 B, 4.14 C&D).

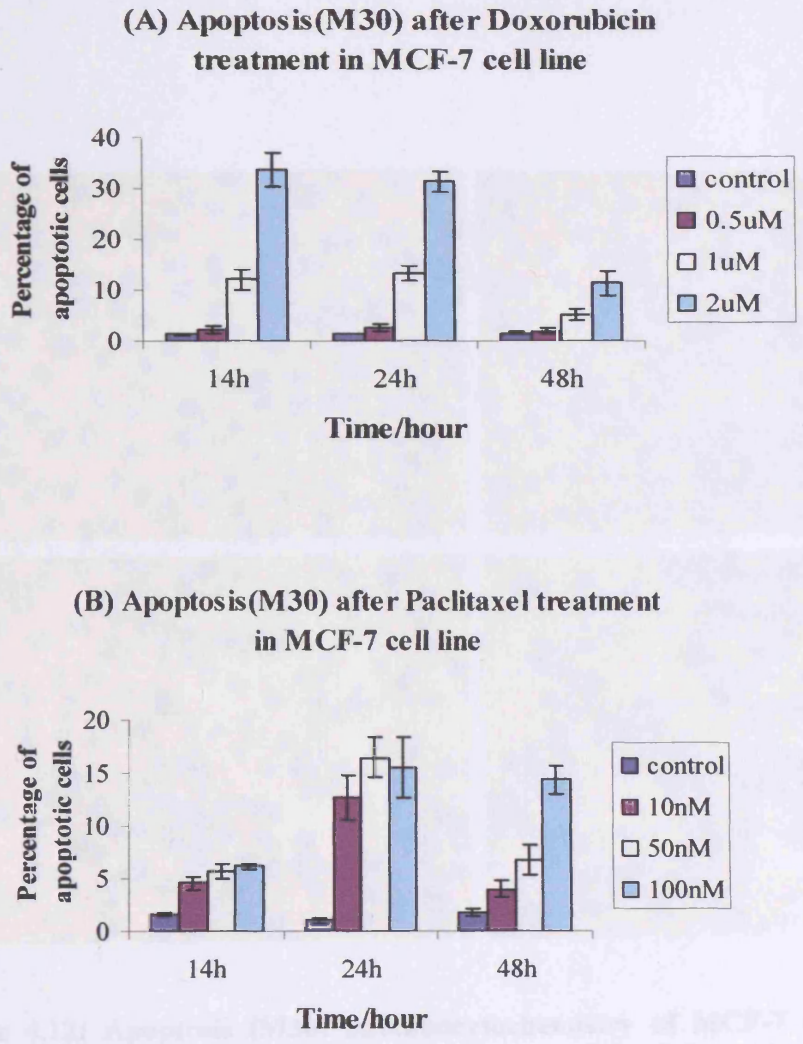


Figure 4.11: Apoptosis (M30) after treatment in MCF-7 cells. A, MCF-7 after doxorubicin treatment. B, MCF-7 after paclitaxel treatment.

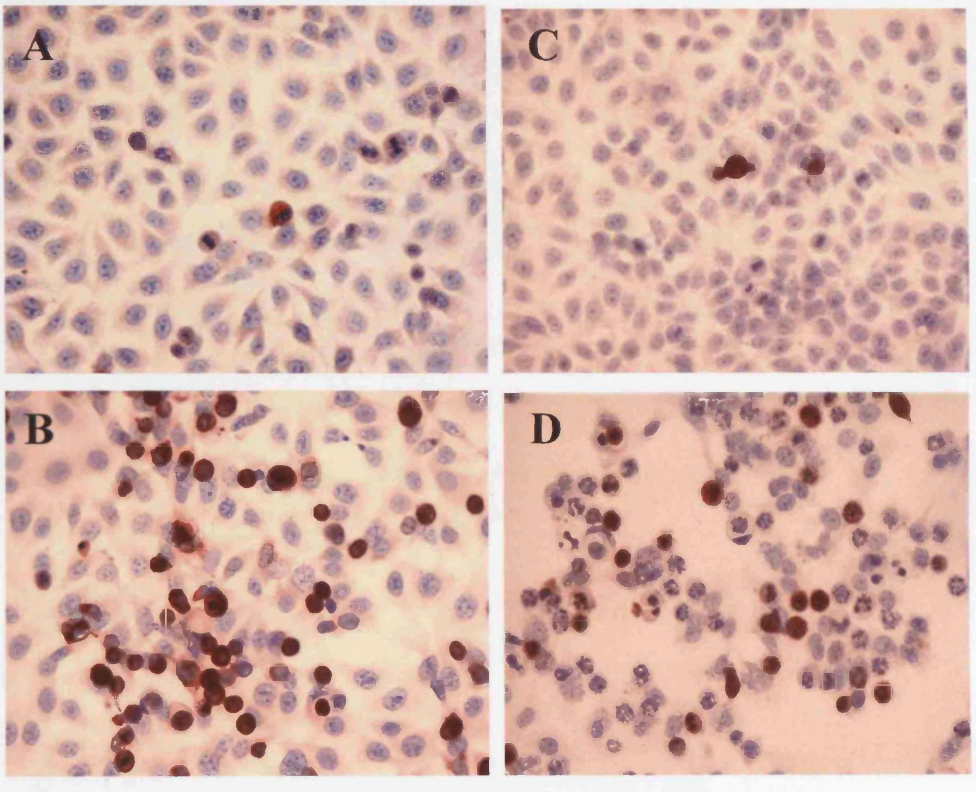


Figure 4.12: Apoptosis (M30) immunocytochemistry of MCF-7 cells at 24h of treatment A, doxorubicin control. B, treatment with 2.0 μM doxorubicin. C, paclitaxel control. D, treatment with 100 nM paclitaxel.

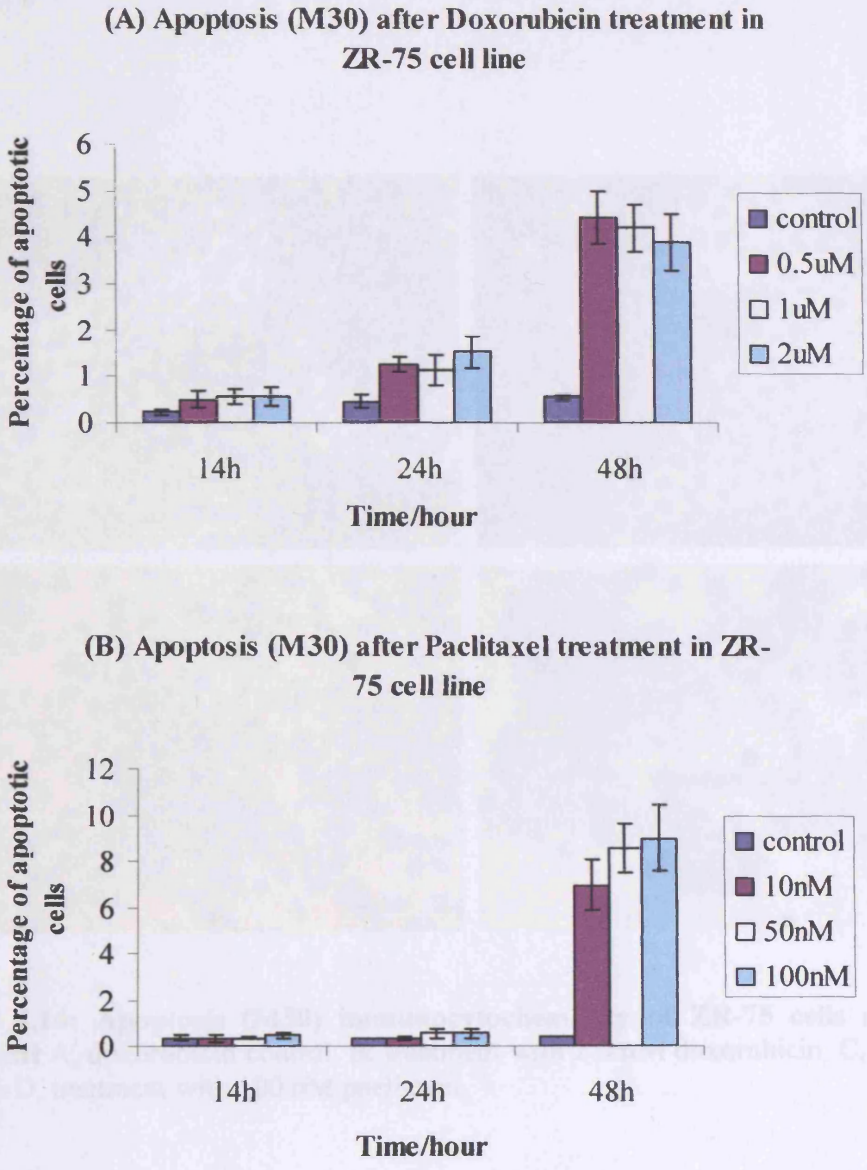


Figure 4.13: Apoptosis (M30) after treatment in ZR-75 cells. A, ZR-75 cells after doxorubicin treatment. B, MCF-7 cells after paclitaxel treatment.

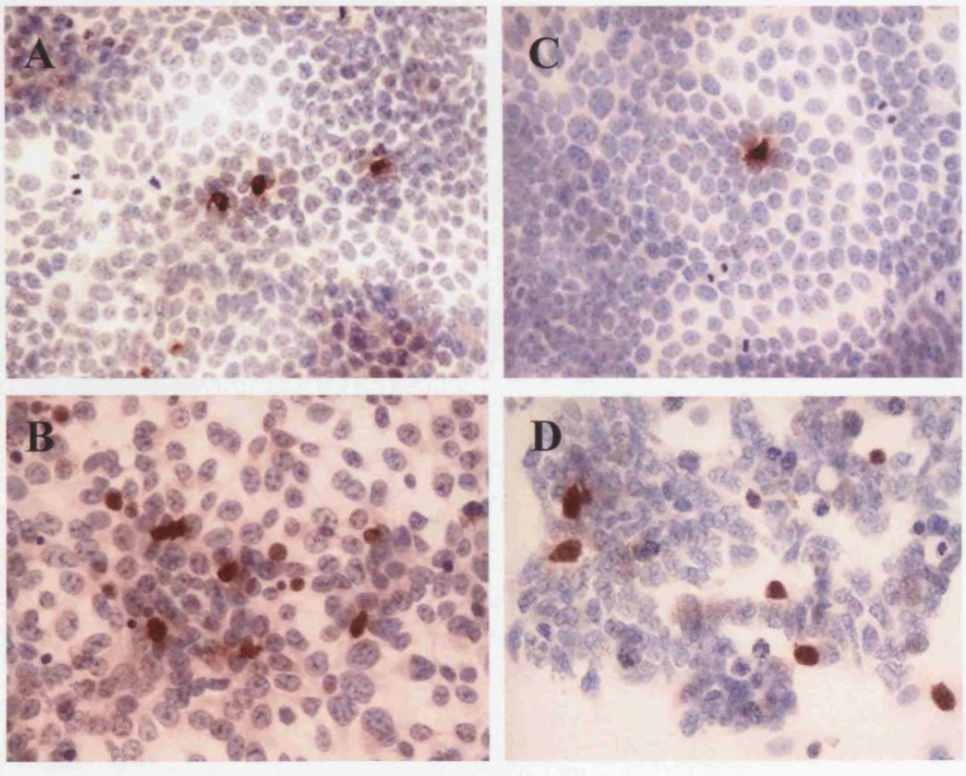
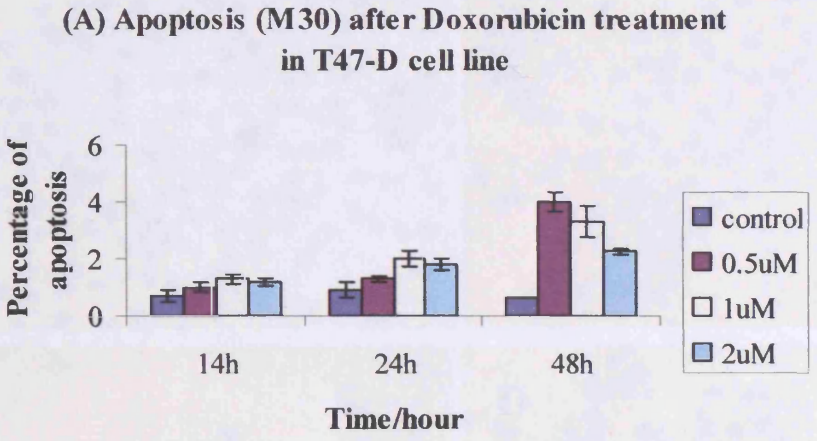


Figure 4.14: Apoptosis (M30) immunocytochemistry of ZR-75 cells at 48h of treatment A, doxorubicin control. B, treatment with 2.0 μM doxorubicin. C, paclitaxel control. D, treatment with 100 nM paclitaxel.

4.2.3 T47-D cells

Both drugs had a similar effect on apoptosis. The percentage of apoptotic cells was approximately doubled at 14h and 24h of treatment. At 48h, both drugs markedly induced apoptosis between 10 and 20 fold, with the effect being greater with lower doses (p=0.026) (figure 4.15 A&B and 4.16 A-D).



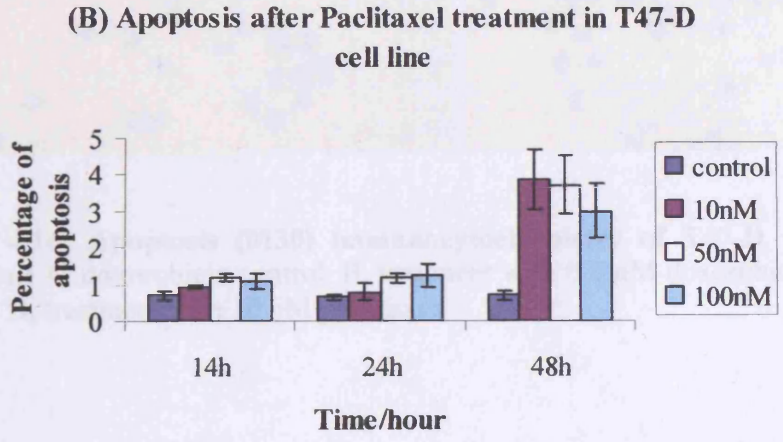


Figure 4.15: Apoptosis (M30) after treatment in T47-D cells. A, cells after doxorubicin treatment. B, cells after paclitaxel treatment.

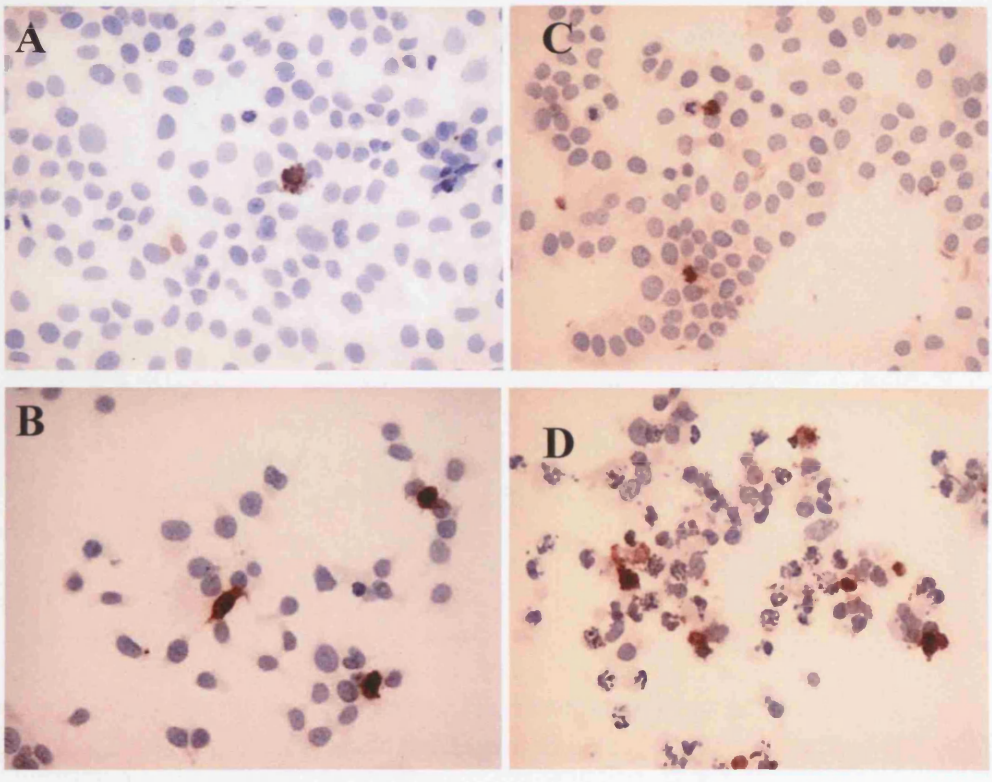


Figure 4.16: Apoptosis (M30) immunocytochemistry of T47-D cells at 48h of treatment A, doxorubicin control. B, treatment with 0.5 μM doxorubicin. C, paclitaxel control. D, treatment with 10 nM paclitaxel.

4.3.4 MDA-MB-231 cells

There was a time and dose-dependent increase in apoptosis with doxorubicin treatment in MDA-MB-321, apart from a slightly lower level with doxorubicin 1.0 μM at 48h. Treatment with 2.0 μM doxorubicin resulted in two fold increase at 14h, 5 fold increase at 24h and 7 fold increase at 48h (figure 4.17 A, 4.18 A&B). Treatment with different doses of paclitaxel resulted in a slight increase in apoptosis at 14h and 24h, but the increase was approximately 3-4 folds at 48h with no difference between doses (p=0.249) (figure 4.17 B, 4.18 C&D).

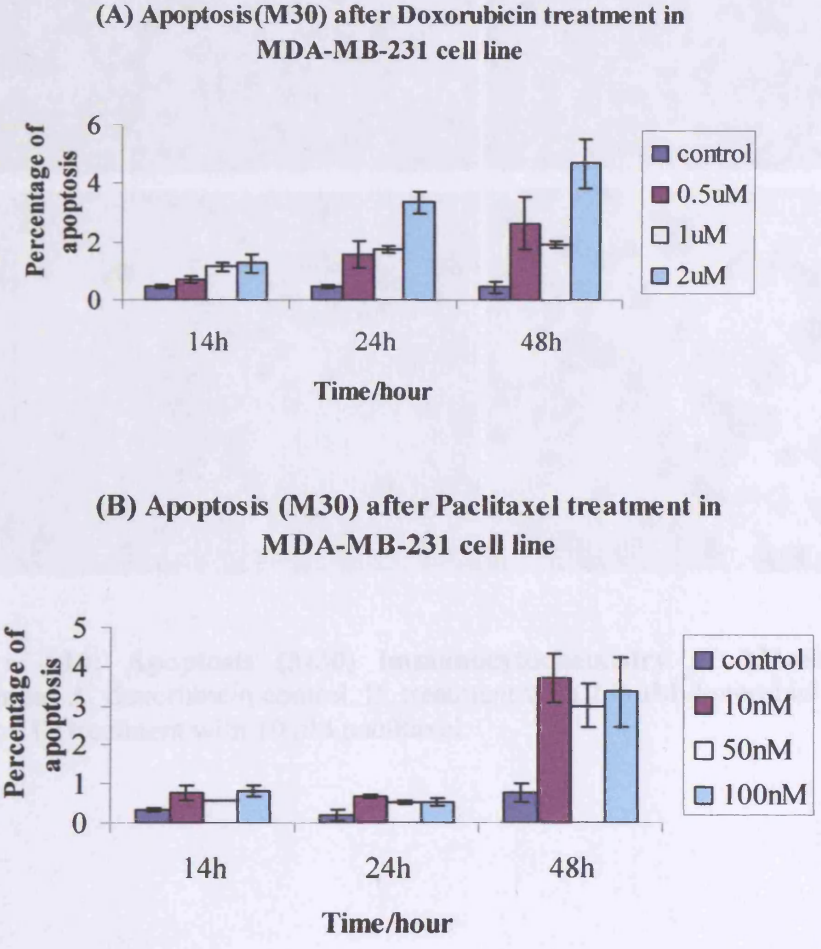


Figure 4.17: Apoptosis (M30) after treatment in 231 cells. A, cells after doxorubicin treatment. B, cells after paclitaxel treatment.

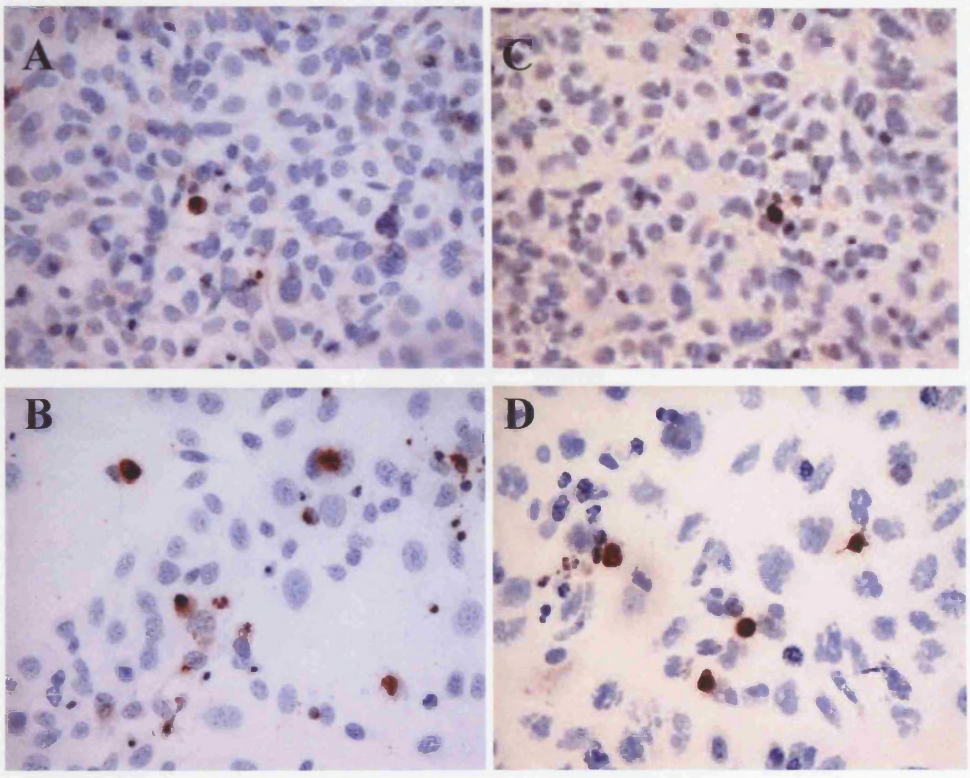


Figure 4.18: Apoptosis (M30) immunocytochemistry of 231cells at 48h of treatment A, doxorubicin control. B, treatment with 2.0 μM doxorubicin. C, paclitaxel control. D, treatment with 10 nM paclitaxel.

4.2.5 MDA-MB-468

Treatment with both doxorubicin and paclitaxel in MDA-MB-468 resulted in a time and dose-dependent increase in the apoptosis percentage at all time points, with greater numbers at higher doses and longer durations. Generally doxorubicin treatment induced more apoptosis (2-5 fold) (figure 4.19A, 4.20 A&B) than paclitaxel (2-2.5 fold) (figure 4.19 B, 4.20 C&D).

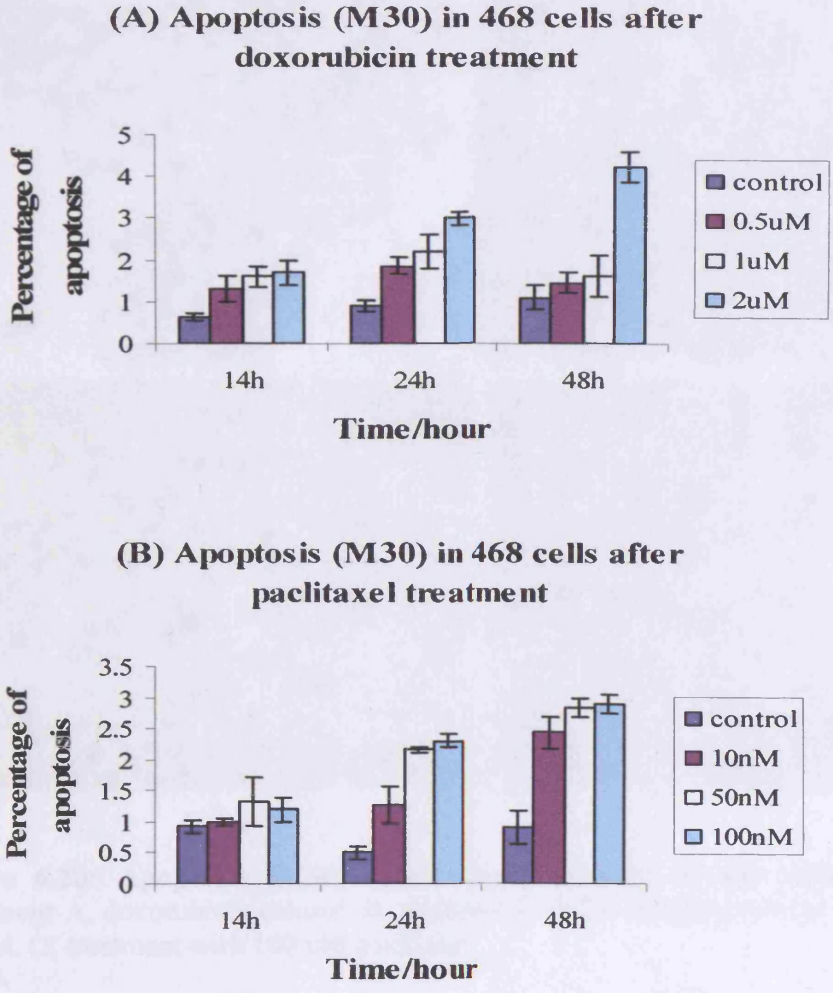


Figure 4.19: Apoptosis (M30) after treatment in 468 cells. A, cells after doxorubicin treatment. B, cells after paclitaxel treatment.

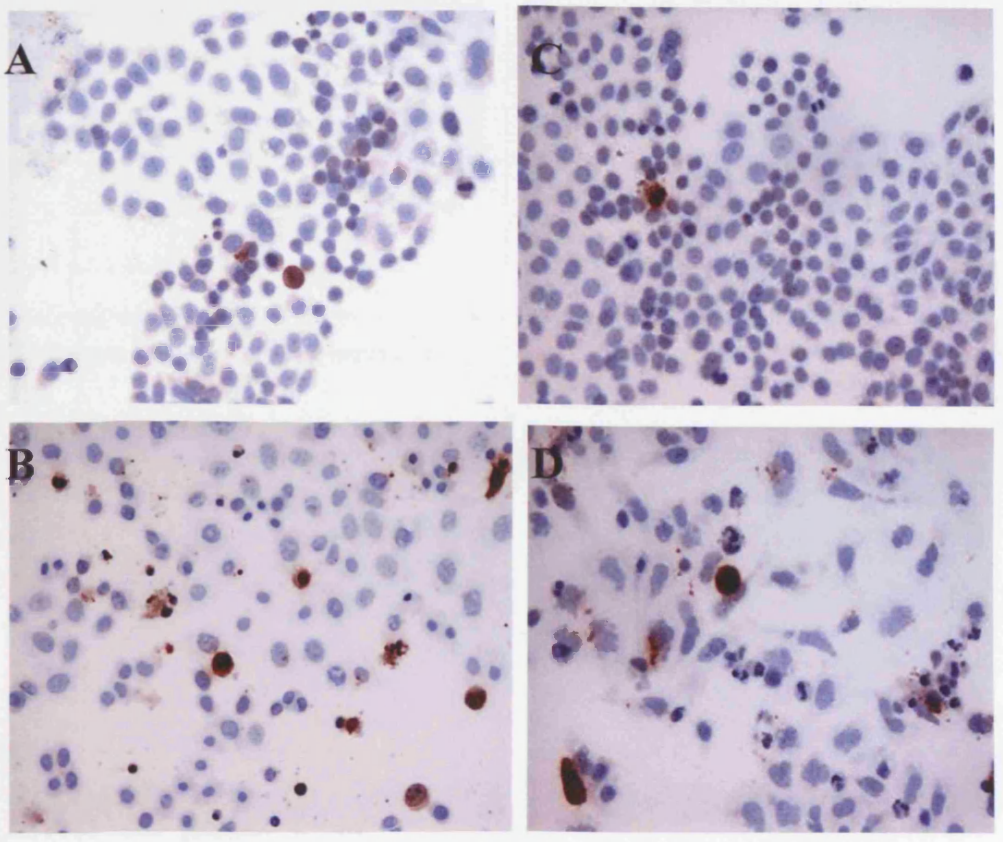


Figure 4.20: Apoptosis (M30) immunocytochemistry of 468 cells at 48h of treatment A, doxorubicin control. B, treatment with 2.0 μM doxorubicin. C, paclitaxel control. D, treatment with 100 nM paclitaxel.

The findings for all cell lines for different doses and time points are summarized in figure 4.21.

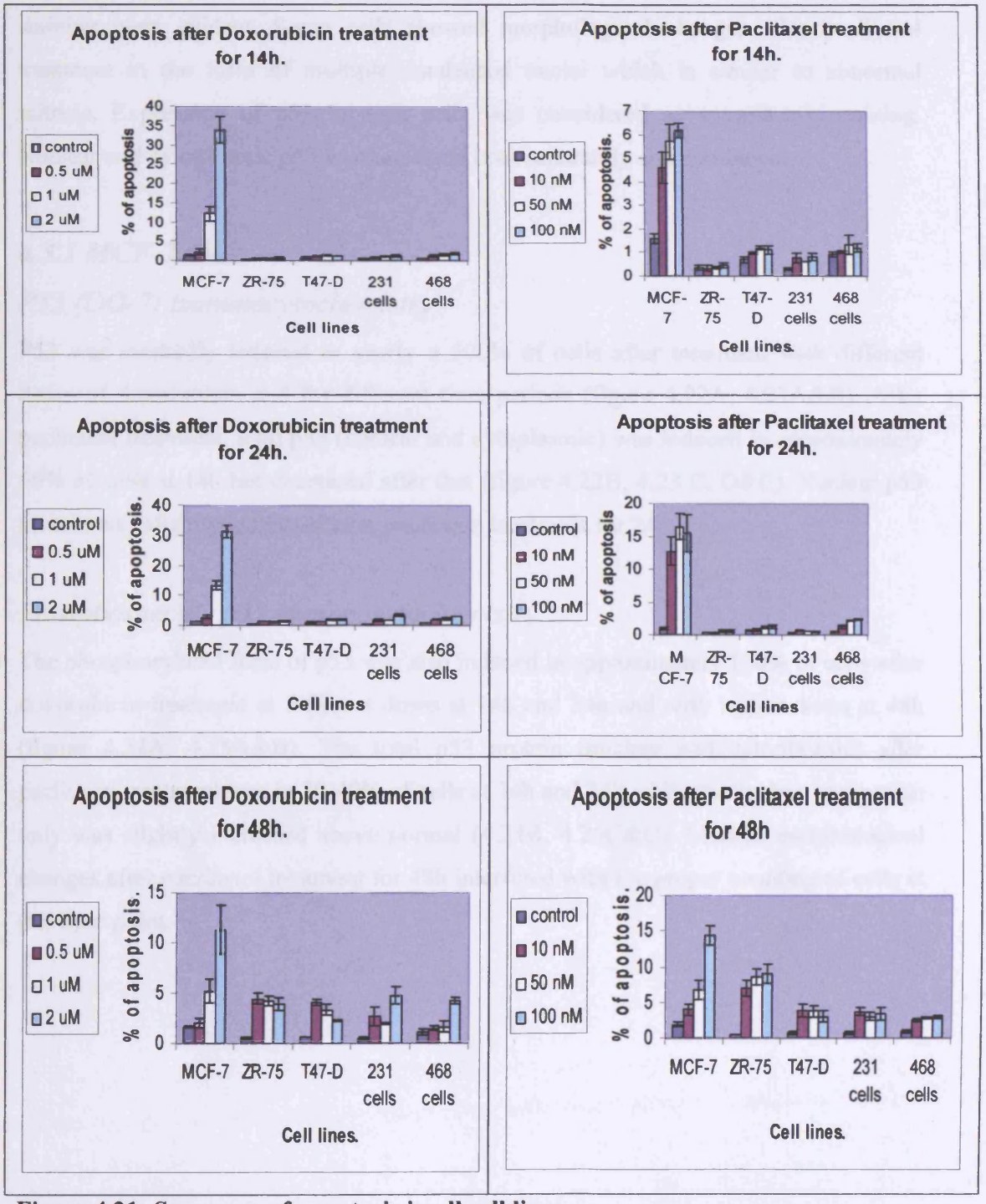


Figure 4.21: Summary of apoptosis in all cell lines.

4.3 Effect on P53

P53 expression by immunocytochemistry appeared as nuclear staining after doxorubicin treatment, while after paclitaxel treatment both nuclear and cytoplasmic staining were evident. Some cells showed morphological changes after paclitaxel treatment in the form of multiple condensed nuclei which is similar to abnormal mitosis. Expression of p53 in such cells was considered as cytoplasmic staining. Nuclear and cytoplasmic p53 were assessed both separately and combined.

4.3.1 MCF-7 cells

P53 (DO-7) immunocytochemistry

P53 was markedly induced in nearly a 100% of cells after treatment with different doses of doxorubicin and for different time periods (figure 4.22A, 4.23A&B). After paclitaxel treatment, total p53 (nuclear and cytoplasmic) was induced in approximately 50% of cells at 14h but decreased after that (figure 4.22B, 4.23 C, D&E). Nuclear p53 protein was slightly increased after paclitaxel treatment for 24h.

Phosphospecific p53 immunocytochemistry

The phosphorylated form of p53 was also induced in approximately 100% of cells after doxorubicin treatment at different doses at 14h and 24h and with higher doses at 48h (figure 4.24A, 4.25A&B). The total p53 protein (nuclear and cytoplasmic) after paclitaxel treatment was in 30-40% of cells at 14h and 24h while the nuclear expression only was slightly increased above normal (4.24B, 4.25C&D). Marked morphological changes after paclitaxel treatment for 48h interfered with the proper counting of cells at this time point.

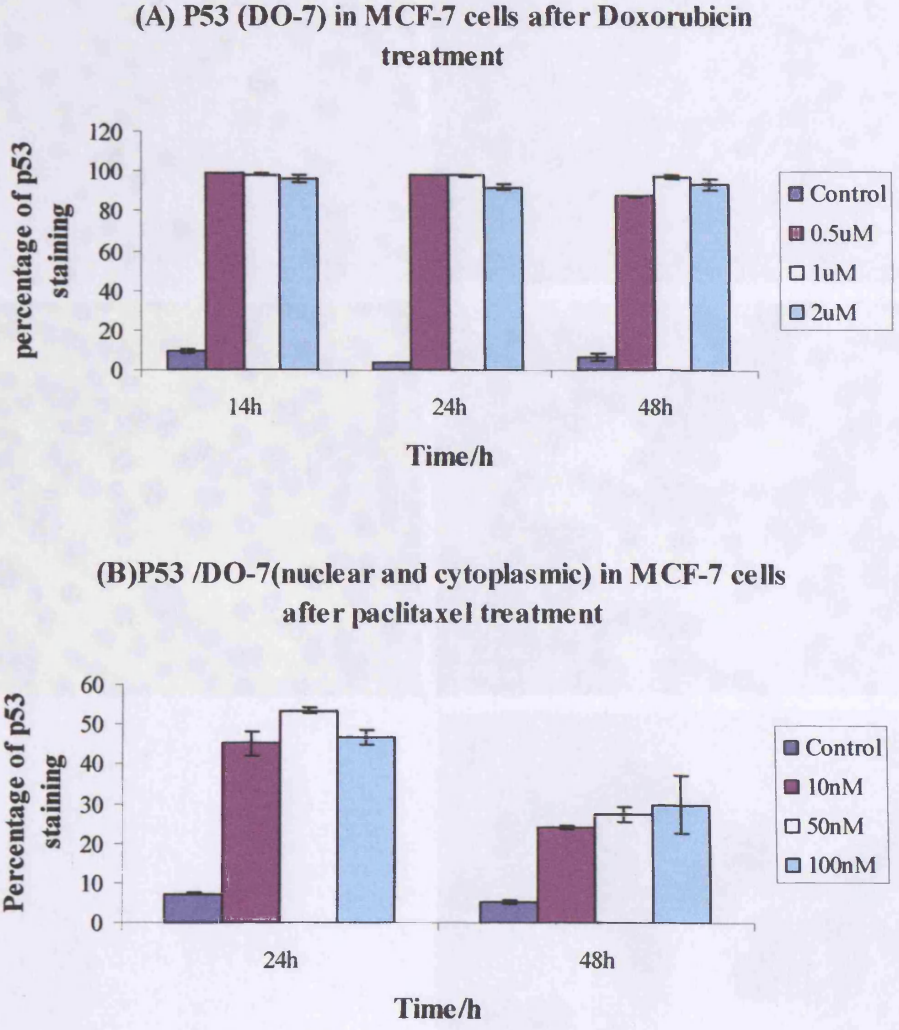


Figure 4.22: P53 (DO-7) in MCF-7 cells. A, cells after doxorubicin treatment. B, cells after paclitaxel treatment.

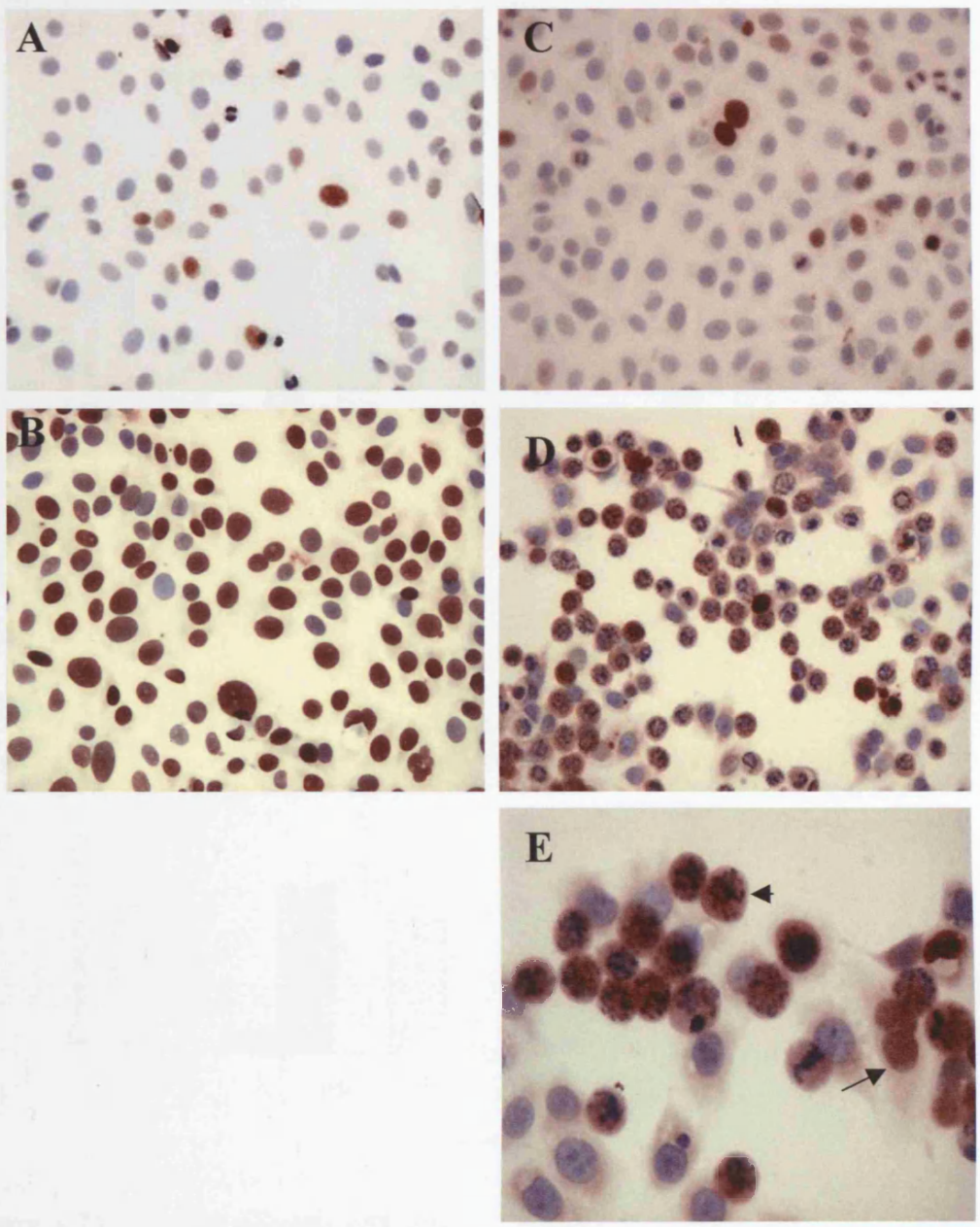


Figure 4.23: P53 (DO-7) immunocytochemistry of MCF-7 cells at 14h of treatment
A, doxorubicin control. B, treatment with 0.5 μM doxorubicin. C, paclitaxel control. D, treatment with 10 nM paclitaxel.E, high power view of D showing cytoplasmic staining in the abnormally mitotic cells (short arrow) and nuclear staining in other cells (long arrow).

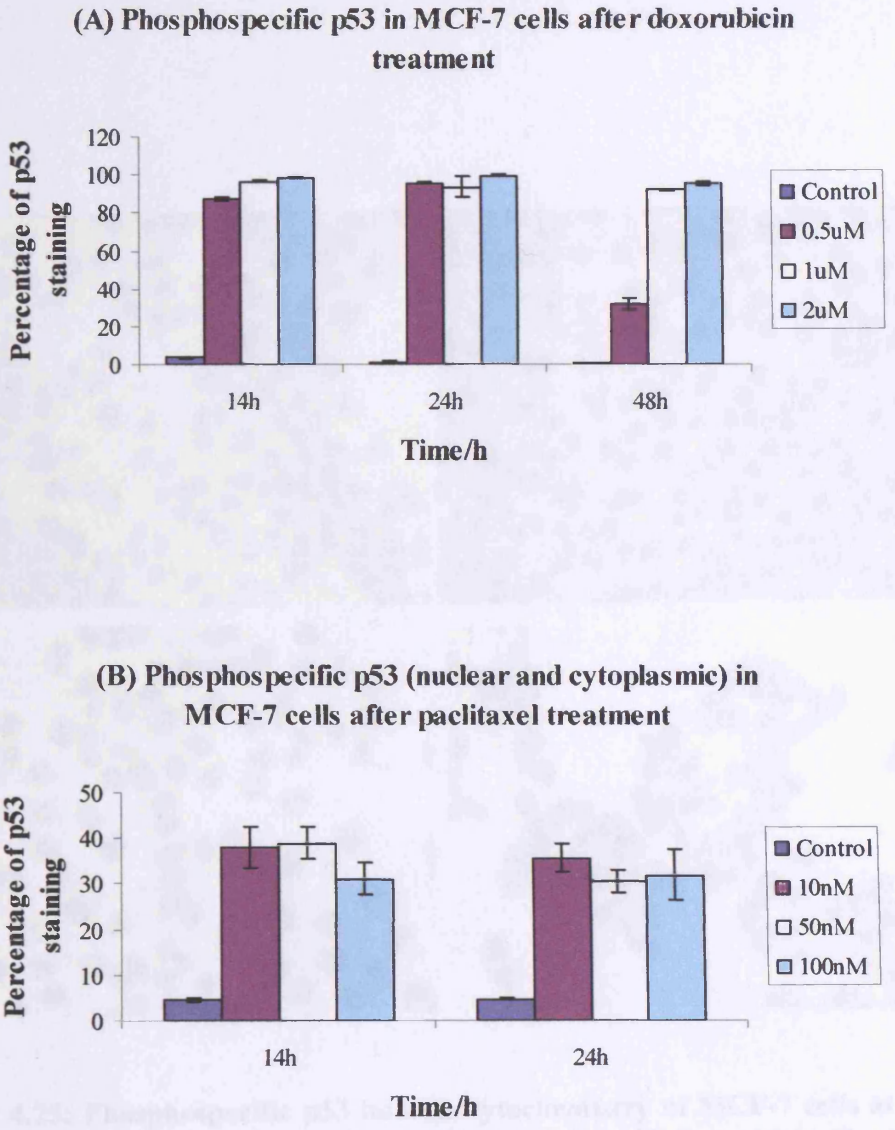


Figure 4.24: Phosphospecific p53 after treatment in MCF-7 cells. A, cells after doxorubicin treatment. B, cells after paclitaxel treatment.

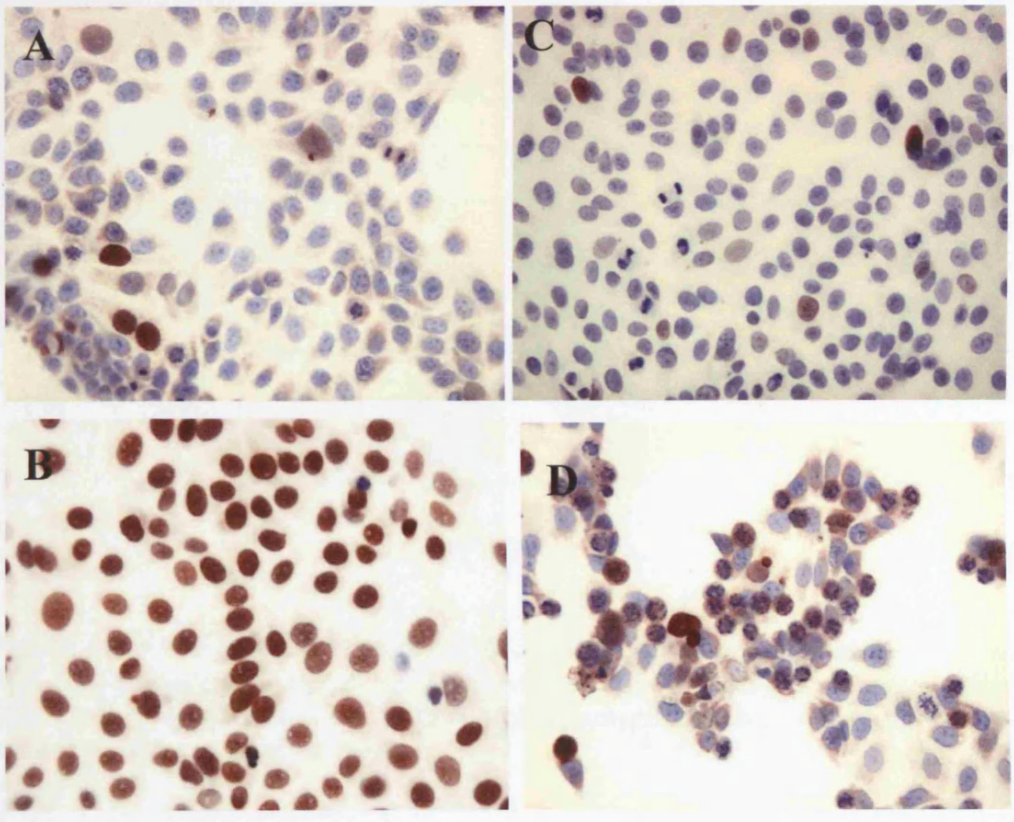


Figure 4.25: Phosphospecific p53 immunocytochemistry of MCF-7 cells at 14h of treatment A, doxorubicin control. B, treatment with 1.0 μM doxorubicin. C, paclitaxel control. D, treatment with 50 nM paclitaxel.

MCF-7 (Western blotting)

Cells were pre-treated with doxorubicin and paclitaxel for 24h and 48h at different doses, and blots assessed for p53 (DO-7) and phosphospecific p53. There was increased expression of p53 protein with both antibodies in MCF-7 at 24h and 48 hours of treatment with doxorubicin (figure 4.26 and 4.32), but it was slightly induced with paclitaxel treatment (as detected with DO-7) but not with phosphospecific p53.

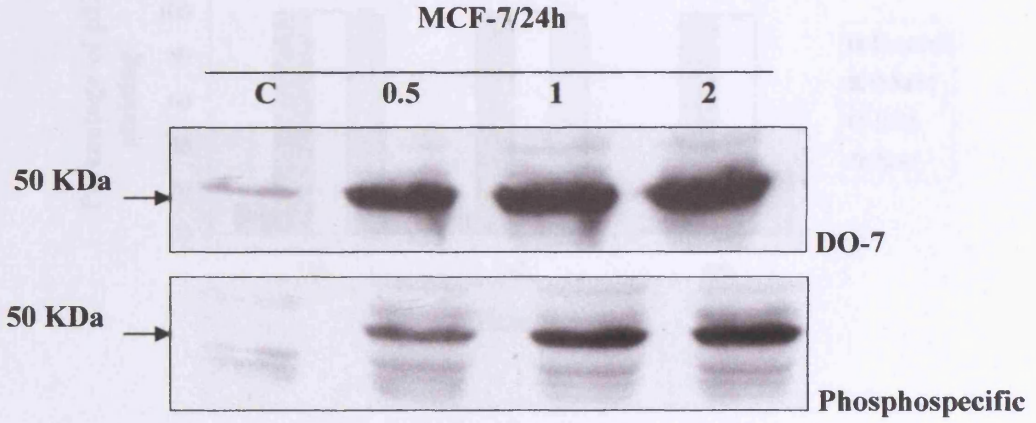


Figure 4.26: P53 by western blotting in MCF-7 cells. 50 µg of proteins in each lane of a 10% polyacrylamide gel, cells treated with 0.5, 1.0 and 2.0 µM doxorubicin for 24h. Do-7 primary antibody 1:3000 and phosphospecific primary antibody 1:1000. C, control protein (no treatment).

4.3.2 ZR-75 cells

P53 (DO-7) immunocytochemistry

P53 was markedly induced in approximately 100% of cells after doxorubicin treatment for 14h and 24h with strong nuclear staining. At 48h with 2.0 µM doxorubicin, the expression decreased to approximately 30 % of cells (figure 4.27A, 4.28A&B). After paclitaxel treatment, the total p53 protein expression increased to 30-40% of cells at 14h and 24h, but the nuclear protein staining was only minimally increased (figure

4.27B, 4.28 C&D). Marked morphological changes after paclitaxel treatment for 48h interfered with the proper counting of cells at this time point.

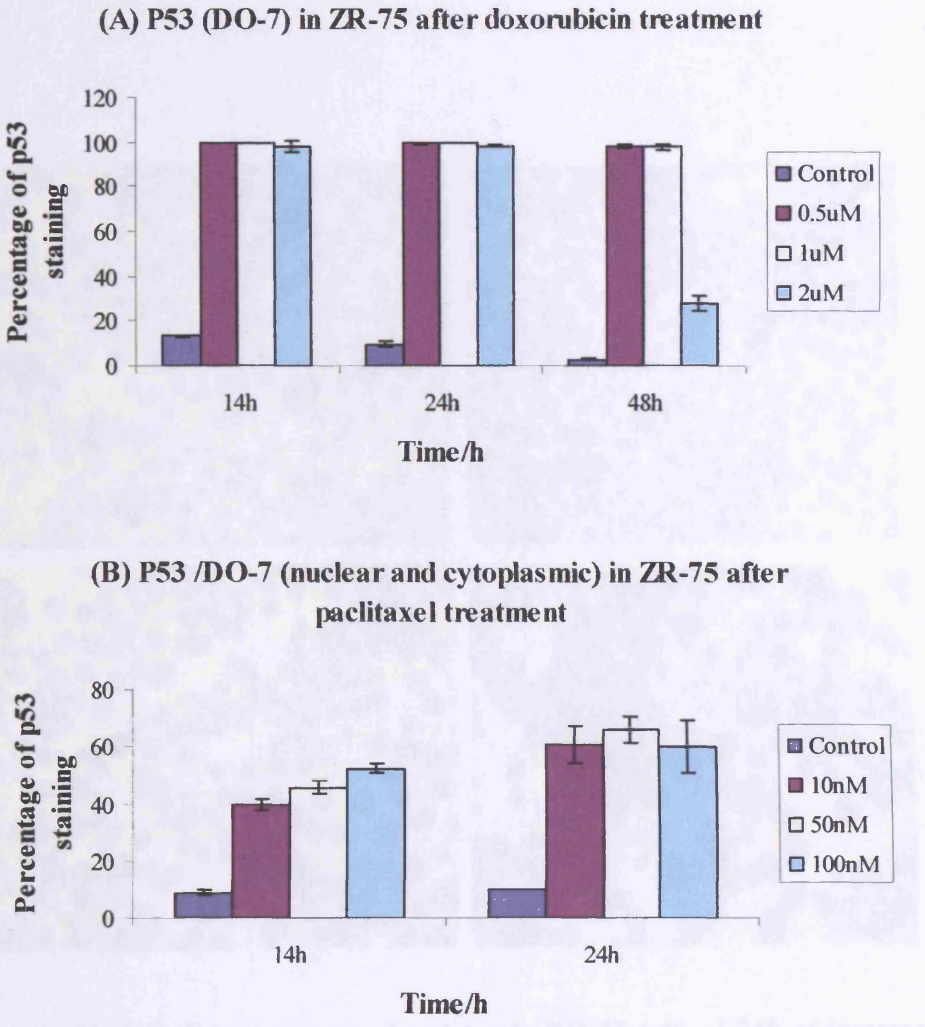


Figure 4.27: P53 (DO-7) after treatment in ZR-75 cells. A, cells after doxorubicin treatment. B, cells after paclitaxel treatment.

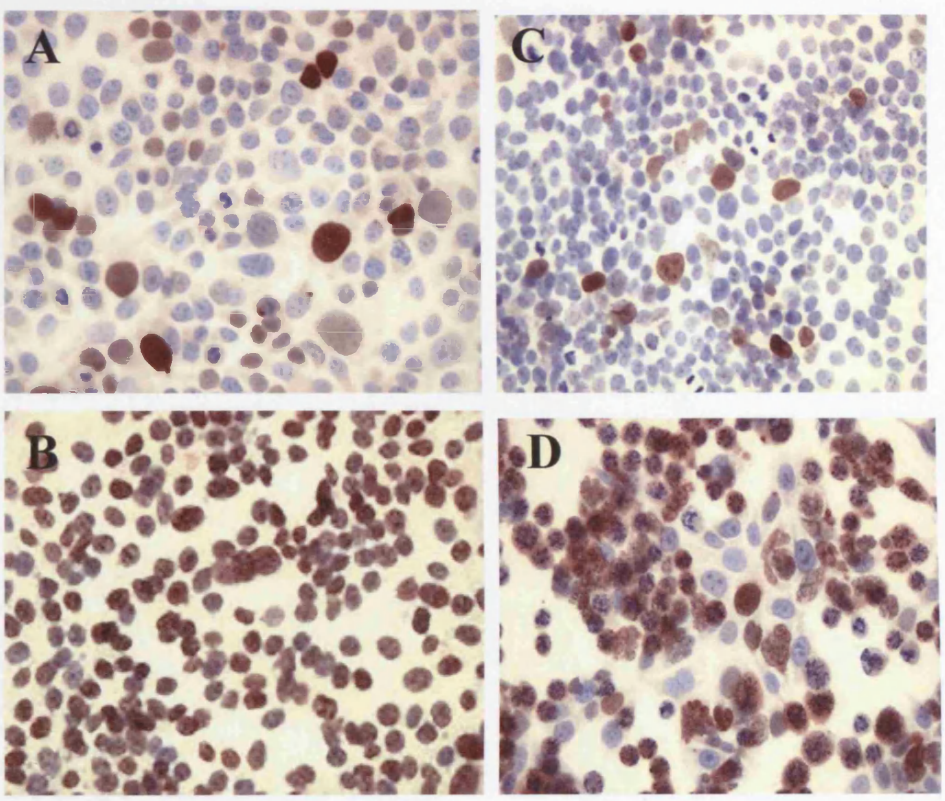


Figure 4.28: P53 (DO-7) immunocytochemistry in ZR-75 cells at 24h of treatment. A, doxorubicin control. B, doxorubicin 1.0 μM treatment. C, paclitaxel control. D, paclitaxel 50nM treatment.

Phosphospecific p53 immunocytochemistry

Phosphorylated p53 was markedly induced after doxorubicin treatment in ZR-75 cells (in about 100% of cells) with all doses at 48h and with higher doses (1.0 and 2.0 μM) at 14h and 24h (figure 4.29A, 4.30 A&B). The total staining was also increased by 4-5 fold with paclitaxel treatment at 14h and 24h. The nuclear staining was slightly increased at 24h of treatment (figure 4.29B, 4.30 C&D).

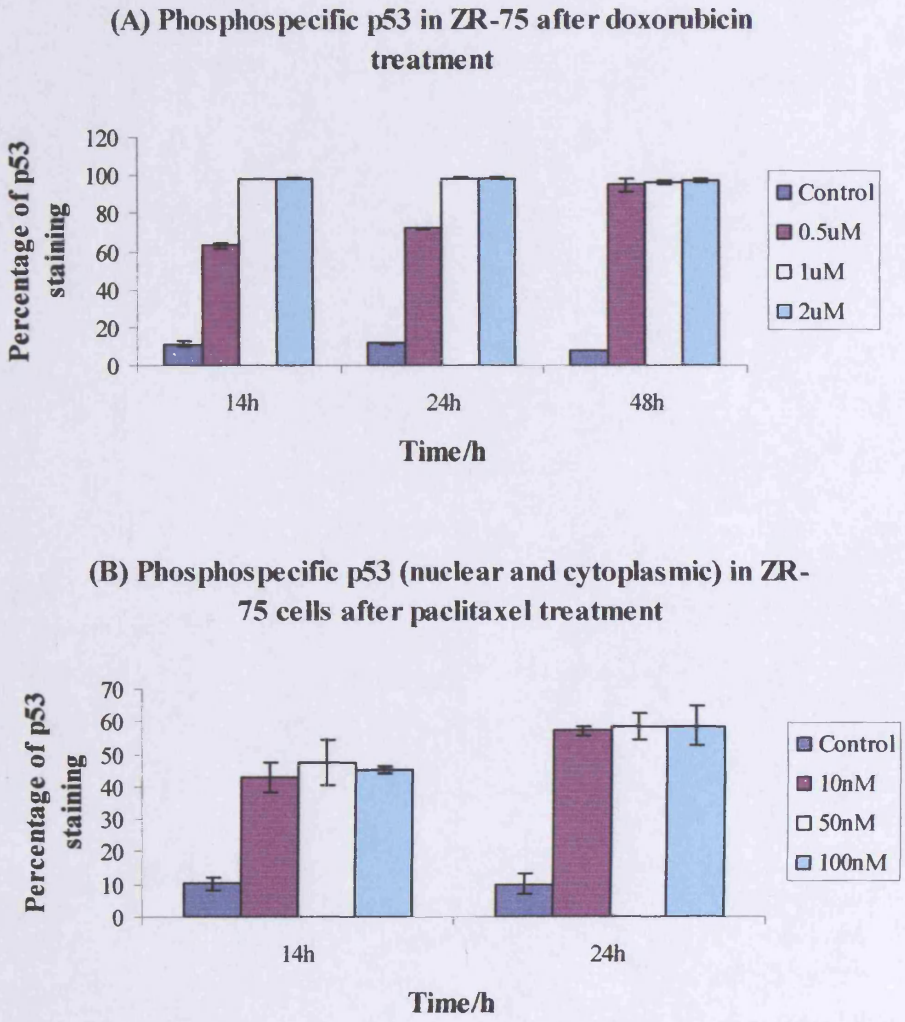


Figure 4.29: Phosphospecific p53 after treatment in ZR-75 cells. A, cells after doxorubicin treatment. B, cells after paclitaxel treatment.

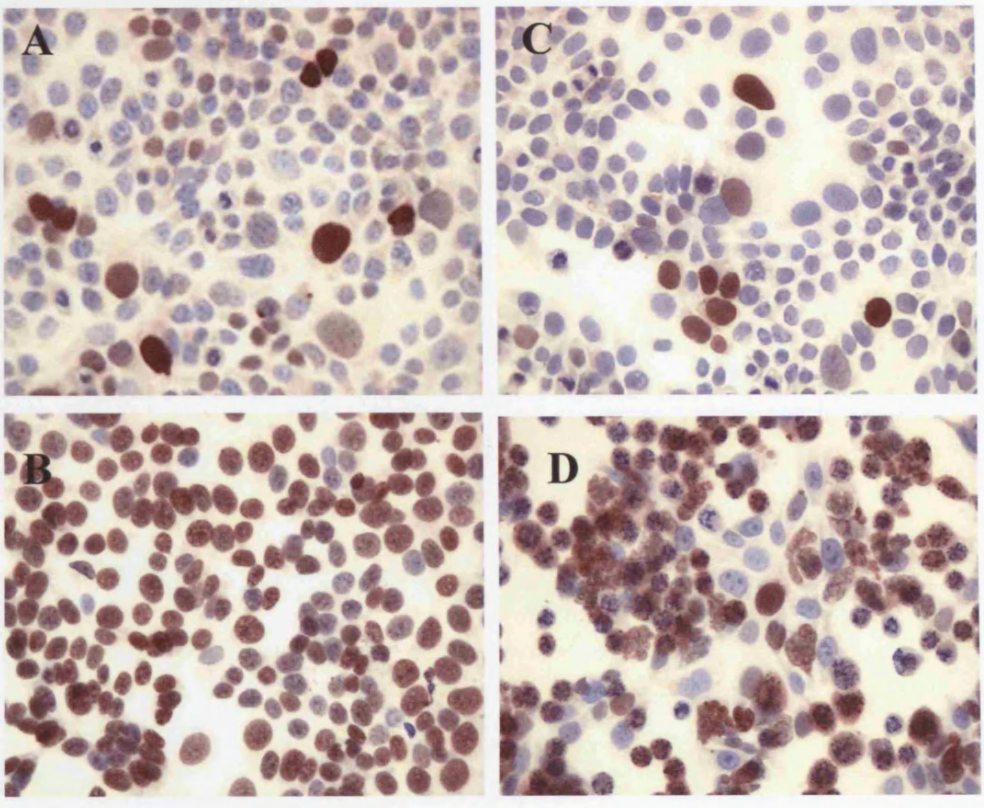


Figure 4.30: Phosphospecific p53 immunocytochemistry in ZR-75 cells at 24h of treatment. A, doxorubicin control. B, doxorubicin 1.0 μM treatment. C, paclitaxel control. D, paclitaxel 50nM treatment.

ZR-75 cells (Western blotting)

P53 with western blotting was assessed by two different antibodies, DO-7 and phosphospecific p53 and cells were pre-treated with doxorubicin and paclitaxel for 24h and 48h in different doses. Experiments revealed increased expression of p53 protein with DO-7 and to a lesser extent with phosphospecific antibody after 24h and 48 hours of treatment with doxorubicin (figure 4.31 and 4.32). Paclitaxel treatment had no immunodetectable effect on phosphospecific p53, but slightly induced p53 as detected with DO-7 antibody.

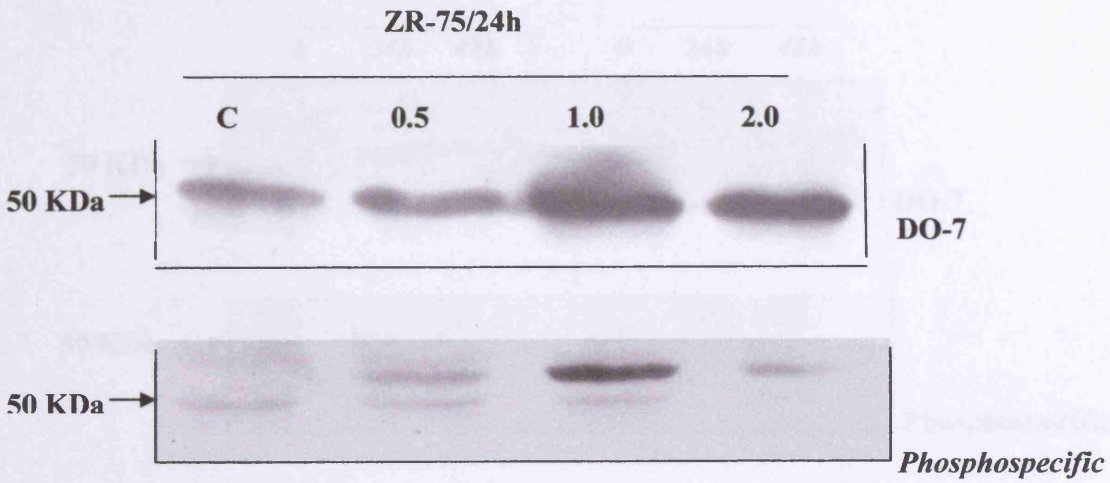


Figure 4.31: P53 by western blotting in ZR-75 cells. 50 µg of proteins in each lane of a 10% polyacrylamide gel, cells treated with 0.5, 1.0 and 2.0 µM doxorubicin for 24h. Do-7 primary antibody 1:3000 and phosphospecific p53 primary antibody 1:1000. C, control protein (no treatment).

Overall comparison

P53 by DO-7 and phosphospecific p53 antibodies was detectable in MCF-7 and ZR-75 after treatment with doxorubicin for 24h and 48h (figure 4.32). With phosphospecific p53, an additional band was seen at a level lower than 53 KDa at 48h of treatment. P53 was also induced after paclitaxel treatment in both cell lines but to a lesser extent as detected by immunocytochemistry and not by western blotting.

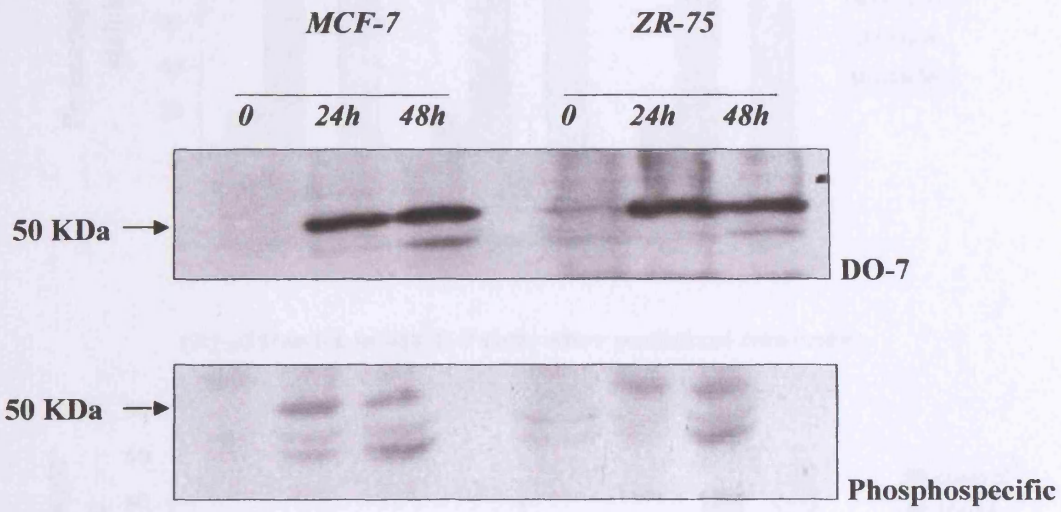


Figure 4.32: Comparison of p53 by western blotting in MCF-7 and ZR-75 cells. 50 µg of proteins in each lane of a 10% polyacrylamide gel, cells treated with 2.0 µM doxorubicin for 24h and 48h. Do-7 primary antibody 1:3000 and phosphospecific p53 primary antibody 1:1000.

4.4 $p21^{wafl}$

4.4.1 MCF-7 cells

$P21^{waf-1}$ was markedly induced in MCF-7 cells after treatment with doxorubicin at different concentration and for different durations (figure 4.33A, 4.34 A&B). Staining was in approximately 100% of cells. There was no increase in the level of $p21^{waf-1}$ at 14h of paclitaxel treatment, but there was slight increase at 24h especially with the moderate dose 50nM (p=0.027) and marked increase at 48h with all doses (figure 4.33B, 4.34 C&D).

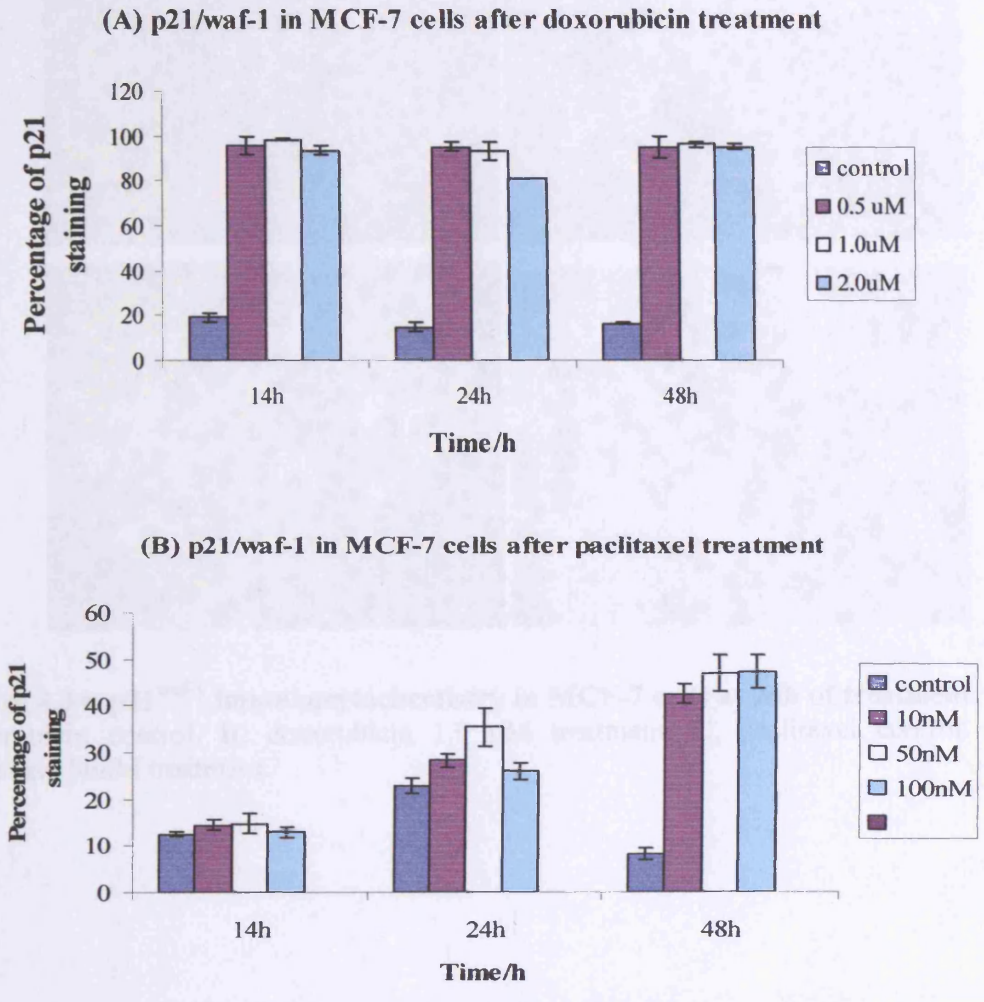


Figure 4.33: $p21^{waf-1}$ in MCF-7 cells. A, after doxorubicin treatment. B, after paclitaxel treatment.

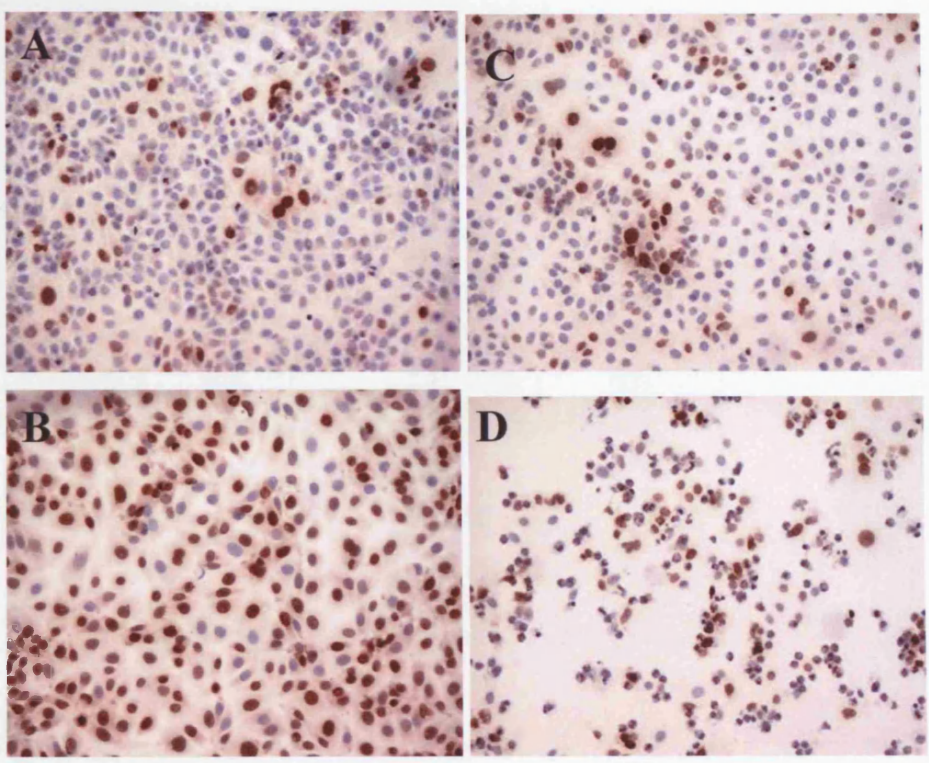


Figure 4.34: $p21^{waf-1}$ immunocytochemistry in MCF-7 cells at 24h of treatment. A, doxorubicin control. B, doxorubicin 1.0 μM treatment. C, paclitaxel control. D, paclitaxel 50nM treatment.

4.4.2 ZR-75 cells

Doxorubicin treatment markedly induced $p21^{waf-1}$ in ZR-75 cells. At 14h different doses of the drug induced the maximum expression, while at 24h and 48h only lower doses (0.5 and 1.0 μM) induced the maximum expression (p=0.027 at 48h) (figure 4.35A, 4.36 A&B). Treatment with paclitaxel had no noticeable increase in $p21^{waf-1}$ expression above control at 14h and 24h (p=0.298 at 24h), but it was markedly increased at 48h (4.25B, 4.36 C&D).

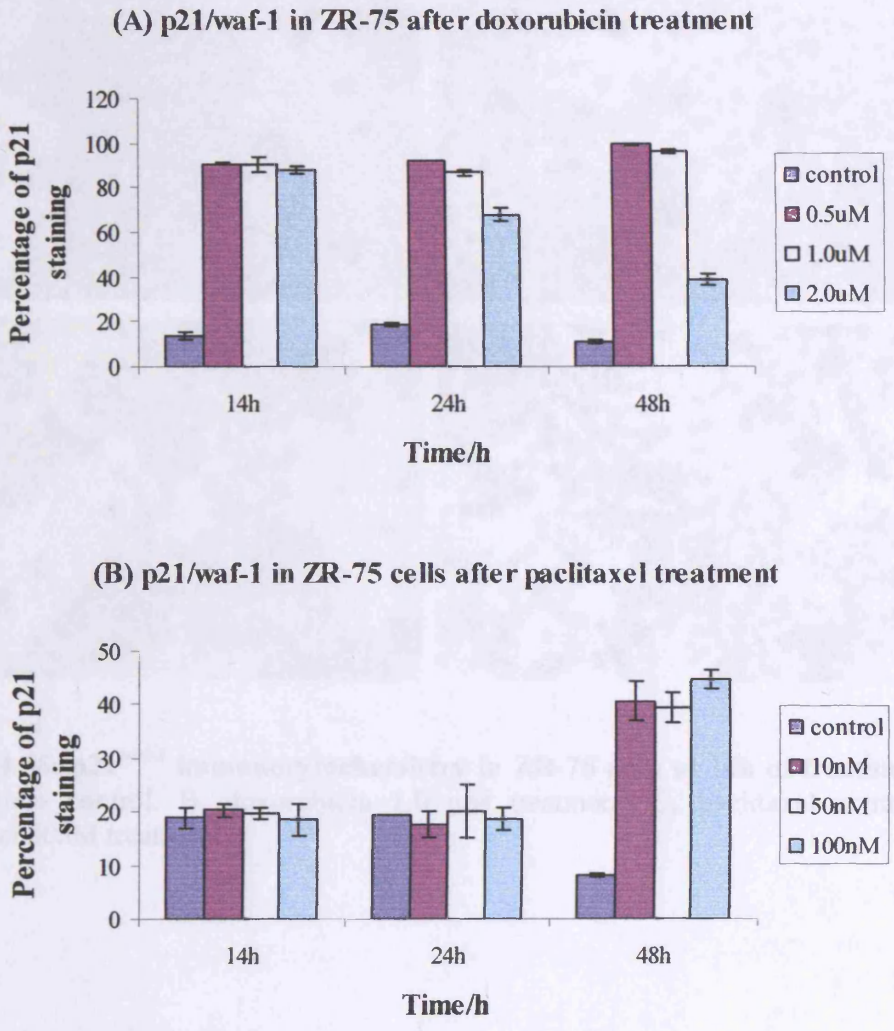


Figure 4.35: $p21^{waf-1}$ in ZR-75 cells. A, after doxorubicin treatment. B, after paclitaxel treatment.

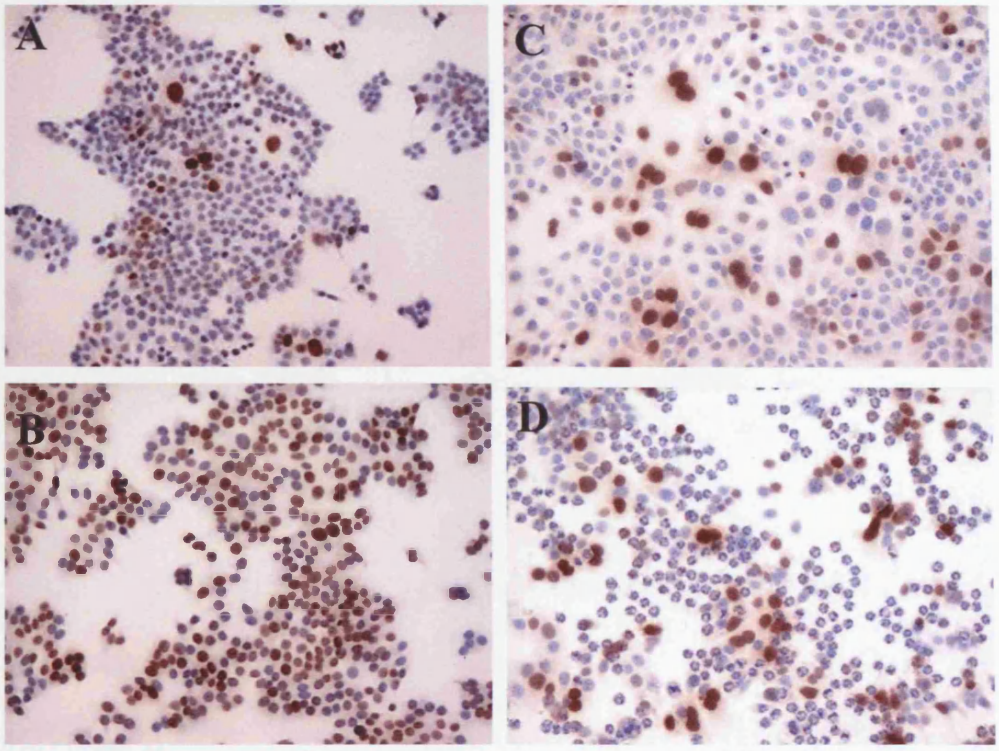


Figure 4.36: p21^{waf-1} immunocytochemistry in ZR-75 cells at 14h of treatment. A, doxorubicin control. B, doxorubicin 1.0 μM treatment. C, paclitaxel control. D, paclitaxel 50nM treatment.

4.4.3 T47-D cells

P21^{waf-1} was induced after doxorubicin treatment in T47-D cells in a time and dose dependent pattern. Lower doses of doxorubicin were associated with greater expression of p21^{waf-1} (p=0.038 at 24h) (figure 4.37A, 4.38 A&B). The expression of p21^{waf-1} was slightly increased at 24h of paclitaxel treatment (p=0.046) and to about 2-3 fold at 48h (figure 4.37B, 4.38 C&D).

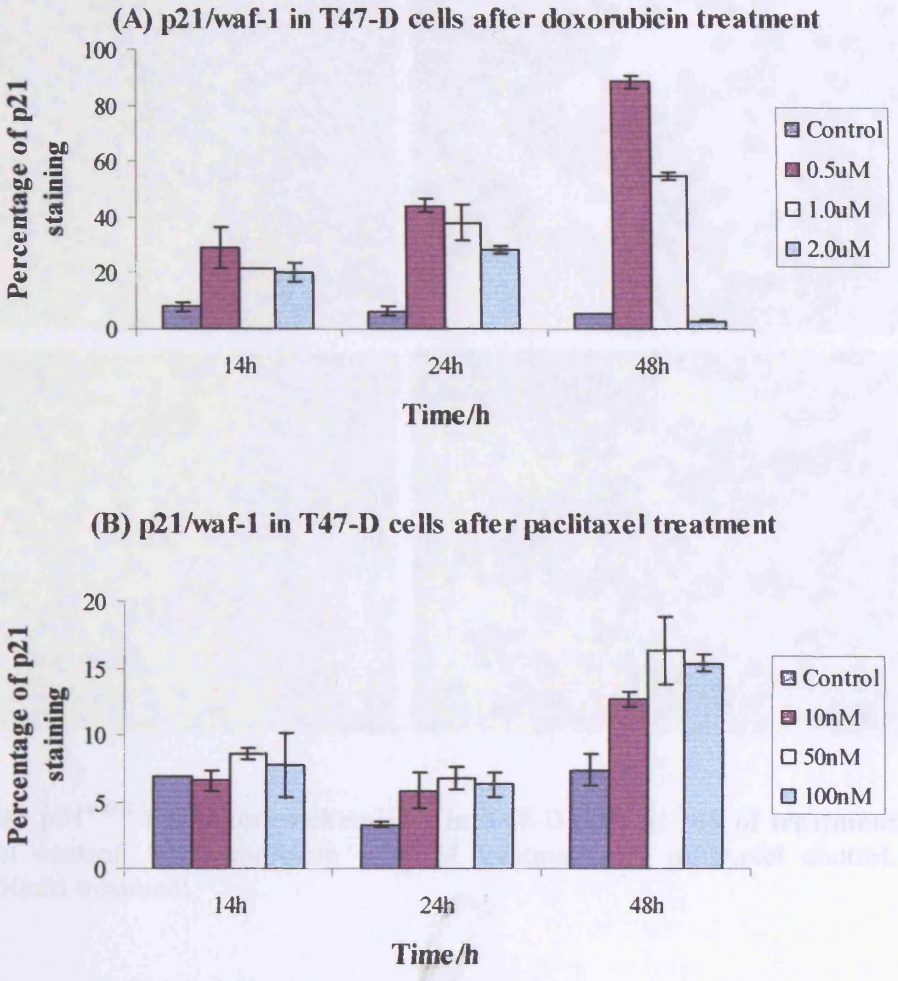


Figure 4.37: p21^{waf-1} in T47-D cells. A, after doxorubicin treatment. B, after paclitaxel treatment.

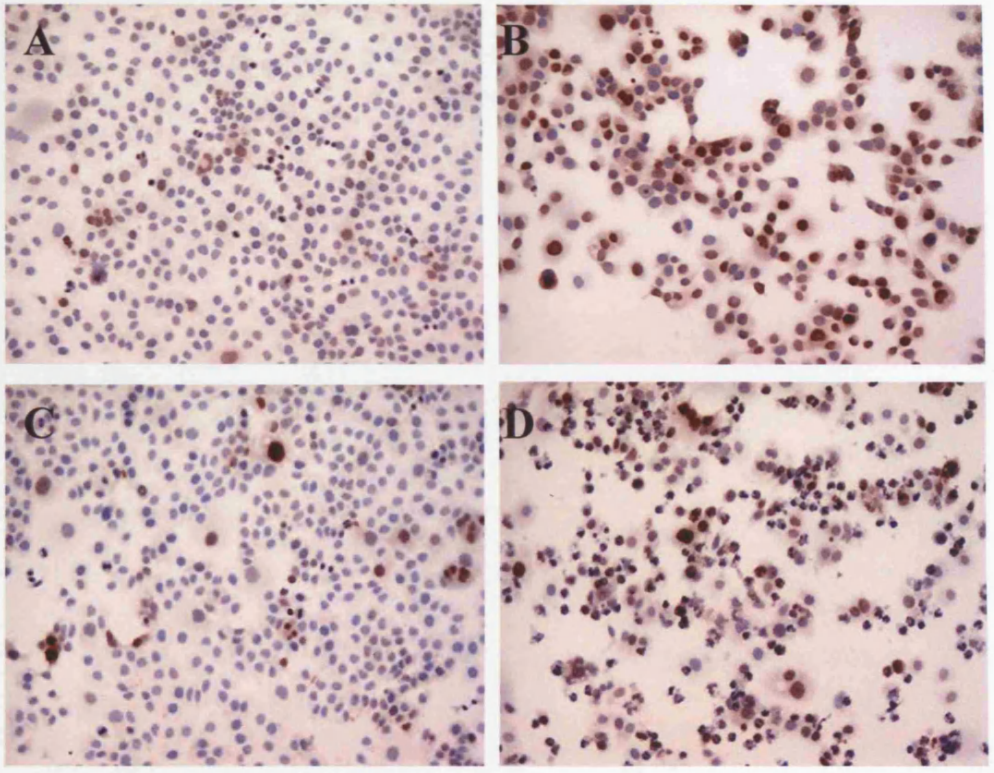


Figure 4.38: p21^{waf-1} immunocytochemistry in T47-D cells at 14h of treatment. A, doxorubicin control. B, doxorubicin 0.5 μM treatment. C, paclitaxel control. D, paclitaxel 50nM treatment.

4.4.4 MDA-MB-231 cells

Treatment with doxorubicin induced the expression of $p21^{waf-1}$ in MDA-MB-231 cells as early as 14h and 24h and reached the maximal expression at 48h (figure 4.39A, 4.40 A&B). At this time point lower doses of doxorubicin (0.5 and 1.0 μM) gave the highest level while treatment with 2.0 μM gave much lower level. $p21^{waf-1}$ was also induced after paclitaxel treatment at 14h and 48h with little effect at 24h (figure 4.39B, 4.40 C&D).

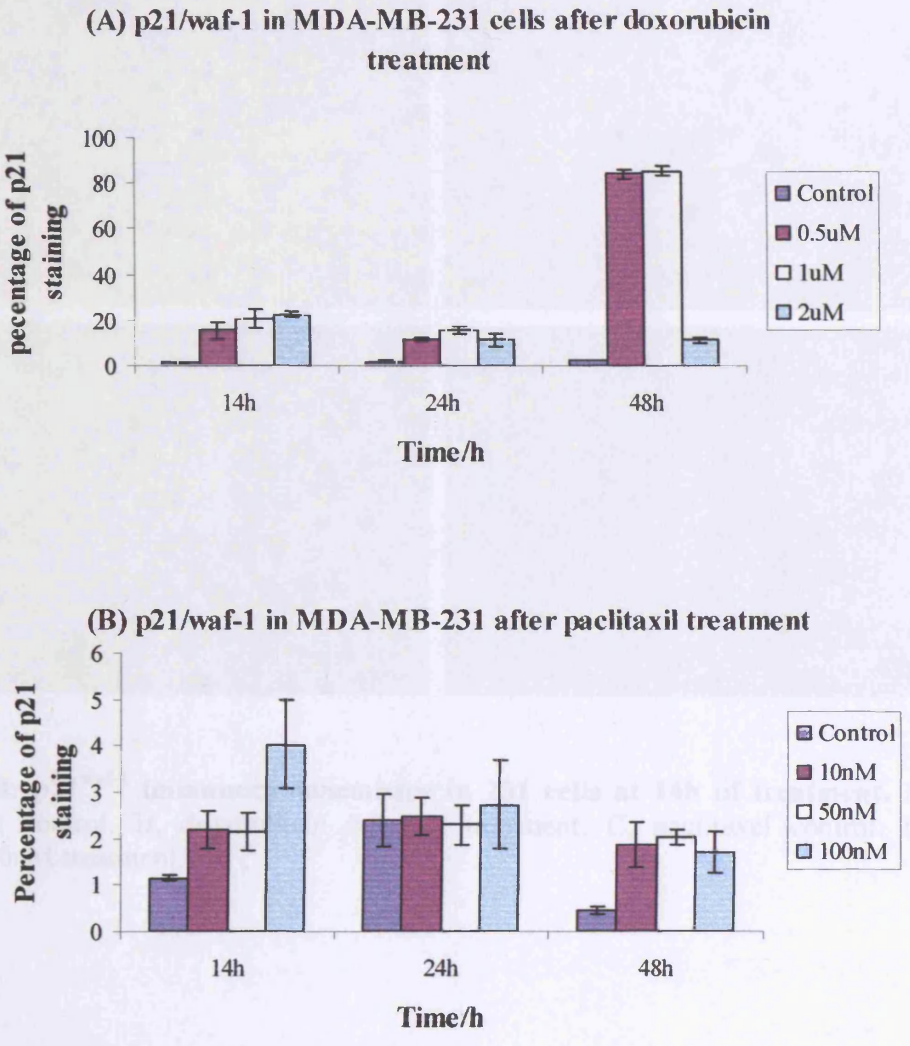


Figure 4.39: $p21^{waf-1}$ in 231cells. A, cells after doxorubicin treatment. B, cells after paclitaxel treatment.

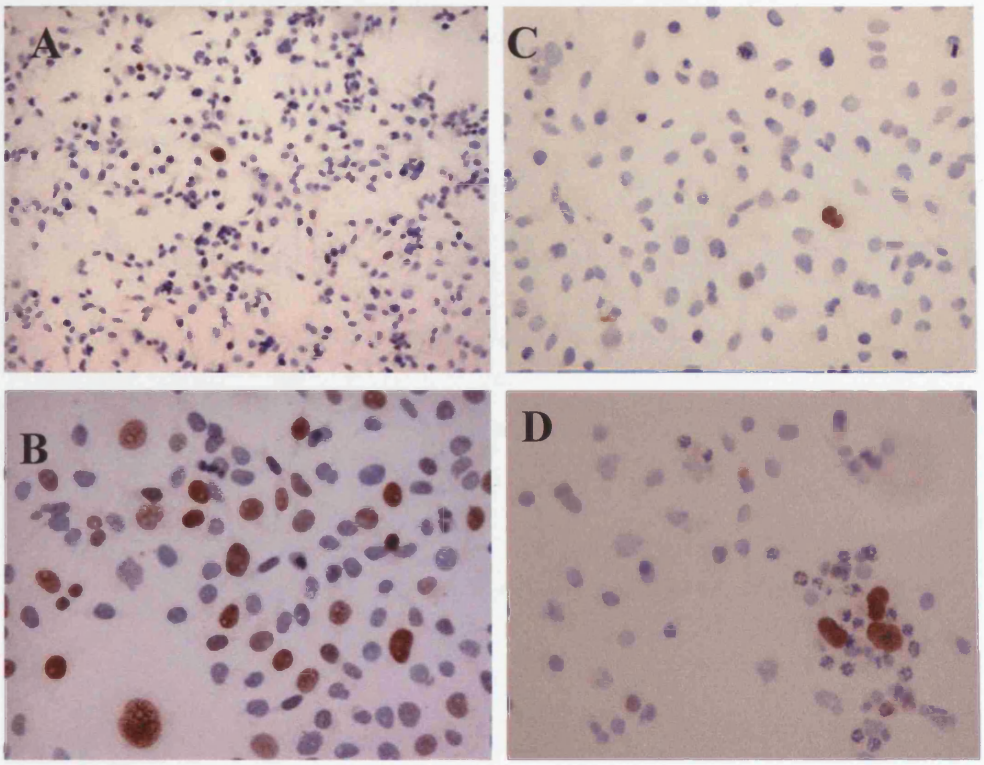


Figure 4.40: p21^{waf-1} immunocytochemistry in 231 cells at 14h of treatment. A, doxorubicin control. B, doxorubicin 0.5 μM treatment. C, paclitaxel control. D, paclitaxel 50nM treatment.

4.4.5 MDA-MB-468 cells

Lower doses of doxorubicin (0.5 and 1.0 μM) induced $p21^{waf-1}$ in MDA-MB-468 at all time points (figure 4.41A). Treatment with paclitaxel failed to induce $p21^{waf-1}$ with different doses and for different time periods (figure 4.41B).

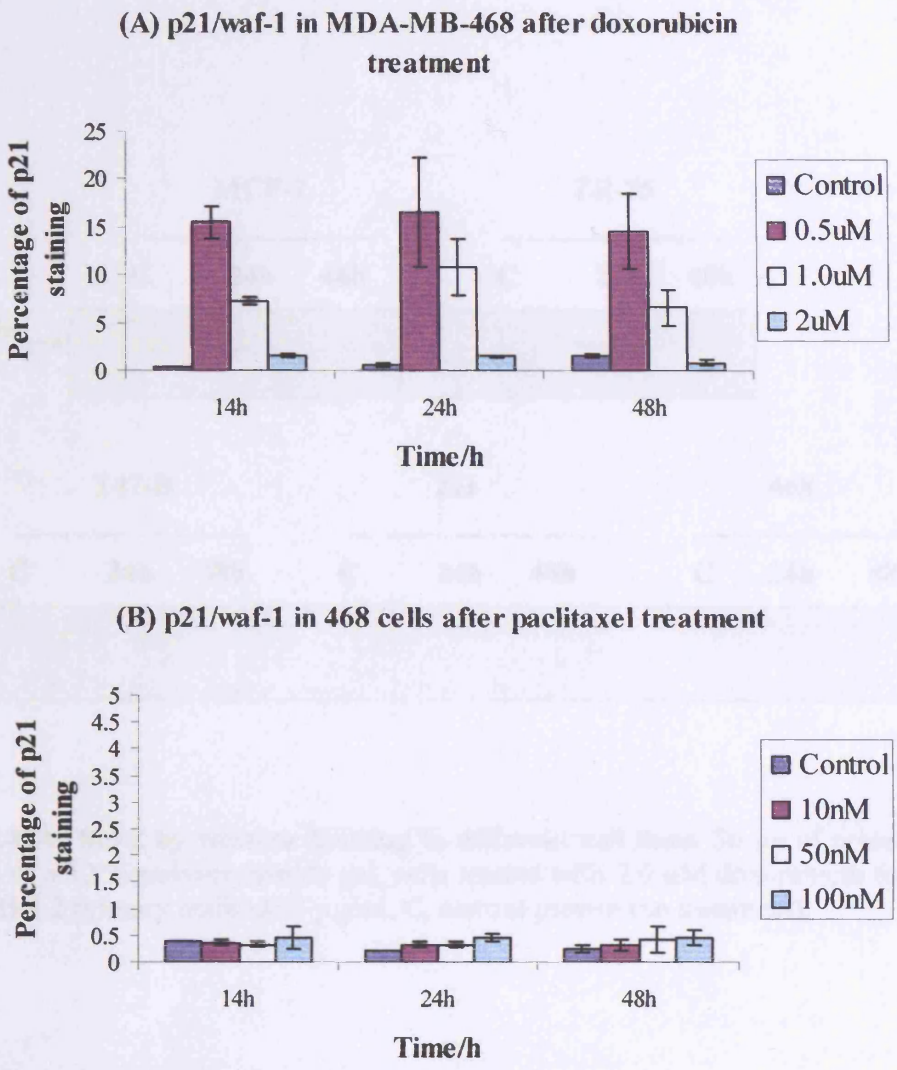


Figure 4.41: $p21^{waf-1}$ after treatment in 468 cells. A, cells after doxorubicin treatment. B, cells after paclitaxel treatment.

4.5 Effect on Bcl-2

Bcl-2 was previously shown in chapter 3 to be strongly expressed in ZR-75 cells with minimal expression in other cells. Treatment with doxorubicin for 24h and 48h resulted in down regulation of bcl-2 protein in a time and dose dependent manner (figure 4.42A, 4.43). With paclitaxel treatment for 24h, down regulation of bcl-2 was associated with band migration, reflecting phosphorylation of the protein (figure 4.42B).

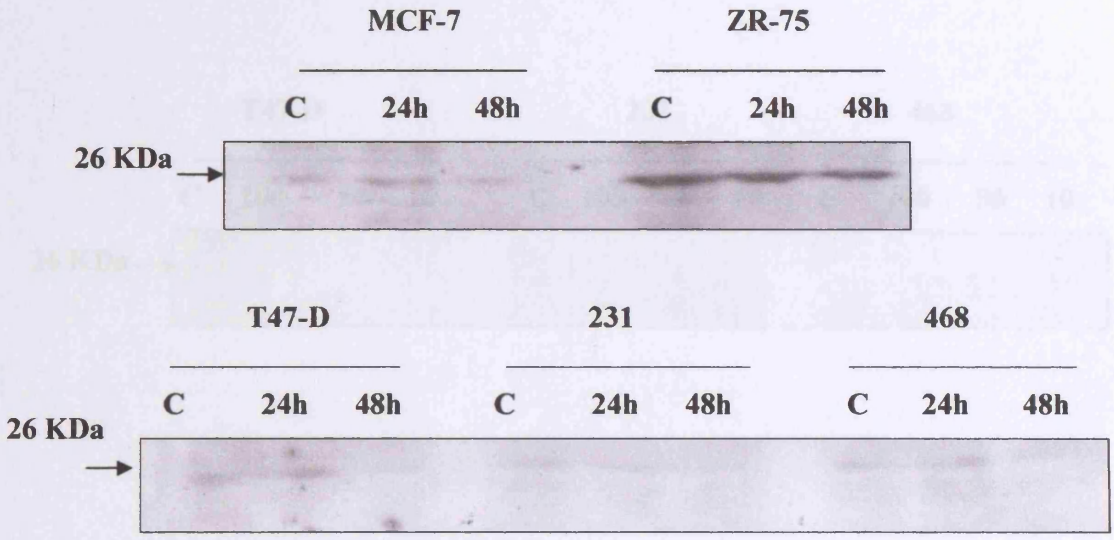


Figure 4.42A: Bcl-2 by western blotting in different cell lines. 50 µg of proteins in each lane of a 12 % polyacrylamide gel, cells treated with 2.0 µM doxorubicin for 24h and 48h. Bcl-2 primary antibody 3 µg/ml. C, control protein (no treatment).

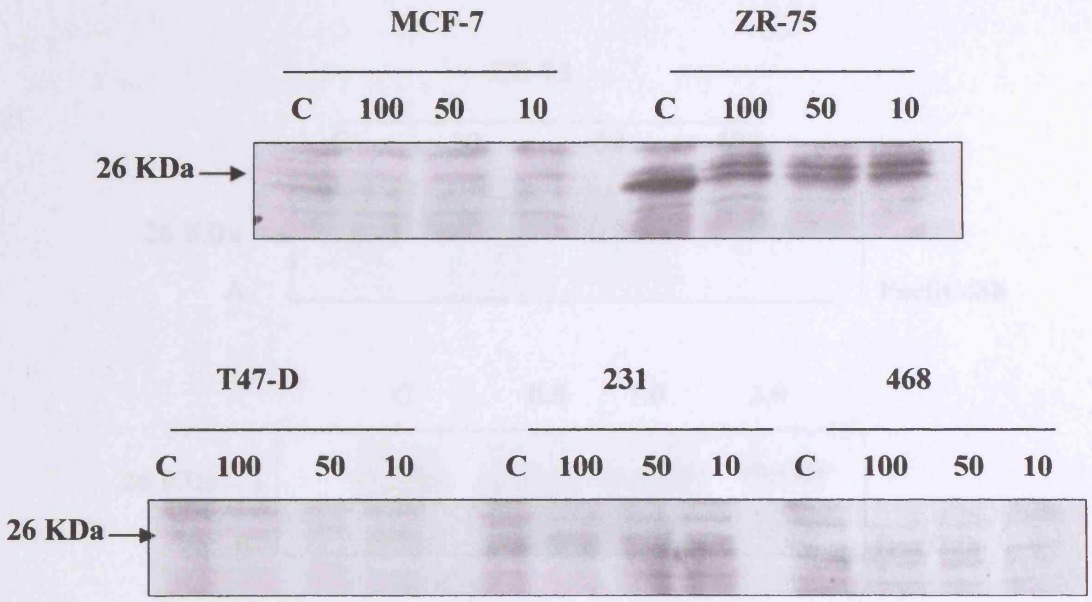


Figure 4.42B: Bcl-2 by western blotting in different cell lines. 50 µg of proteins in each lane of a 12 % polyacrylamide gel, cells treated with 100, 50 and 10 nM paclitaxel for 24h. Bcl-2 primary antibody 3 µg/ml, C, control protein (no treatment).

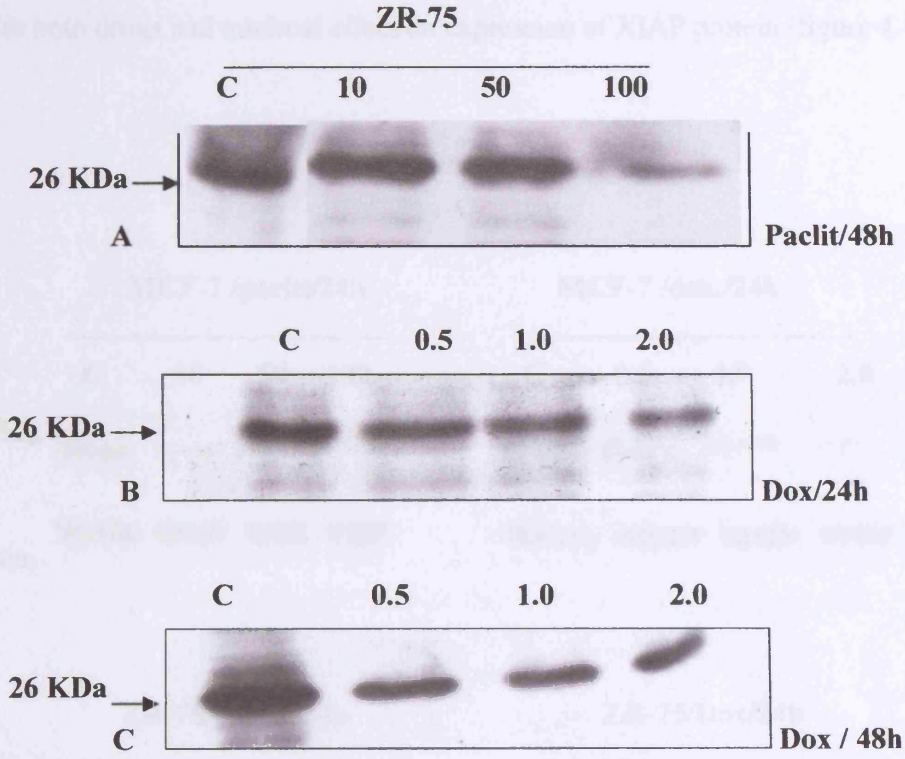


Figure 4.43: Bcl-2 by western blotting in ZR-75 cells. 50 µg of proteins in each lane of a 12 % polyacrylamide gel, cells treated with A, paclitaxel 10, 50 and 100nM for 48h. B, doxorubicin 0.5, 1.0 and 2.0 µM for 24h and C, doxorubicin/48h. Bcl-2 primary antibody 3 µg/ml. C, control protein (no treatment).

4.6 Effect on XIAP

XIAP protein expression was assessed by western blotting. It was down regulated in MCF-7 cells with doxorubicin and paclitaxel treatment in a dose and time dependent manner. There was no effect on ZR-75 cells (figure 4.44A). Treatment of other cell lines with both drugs had minimal effect on expression of XIAP protein (figure 4.44 B).

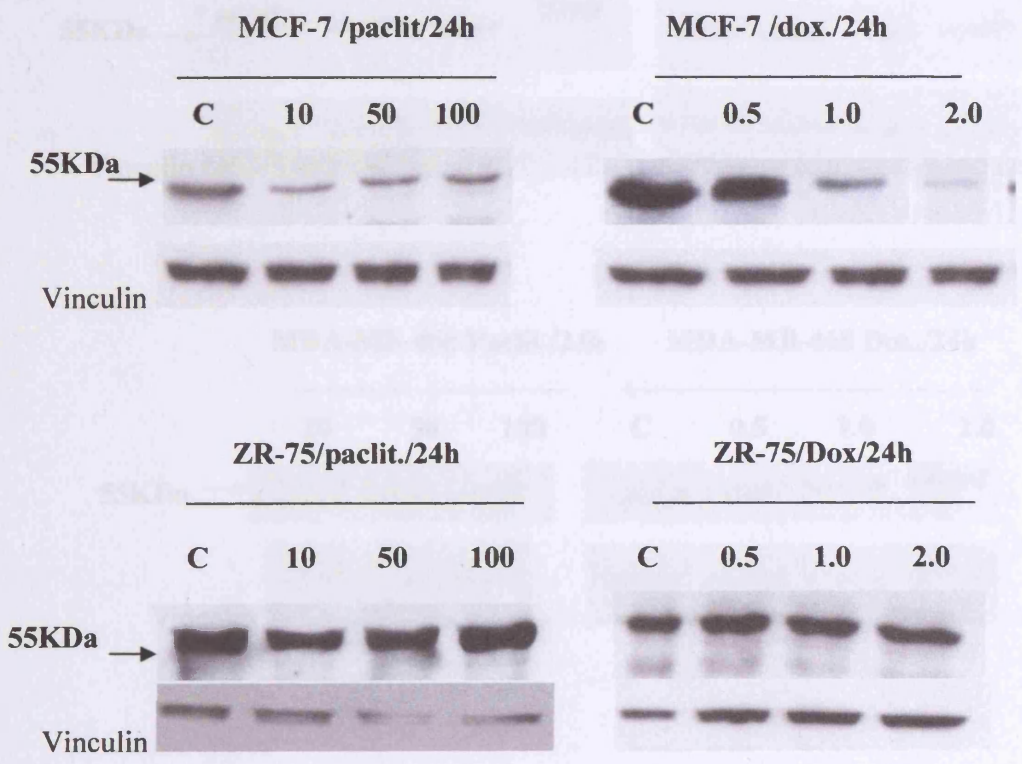


Figure 4.44(A): XIAP by western blotting in MCF-7 and ZR-75 cells. 50 µg of proteins in each lane of a 10 % polyacrylamide gel, cells treated with paclitaxel 10, 50 and 100nM for 24h and doxorubicin 0.5, 1.0 and 2.0 µM for 24h. XIAP primary antibody 1 µg/ml. C, control protein (no treatment).

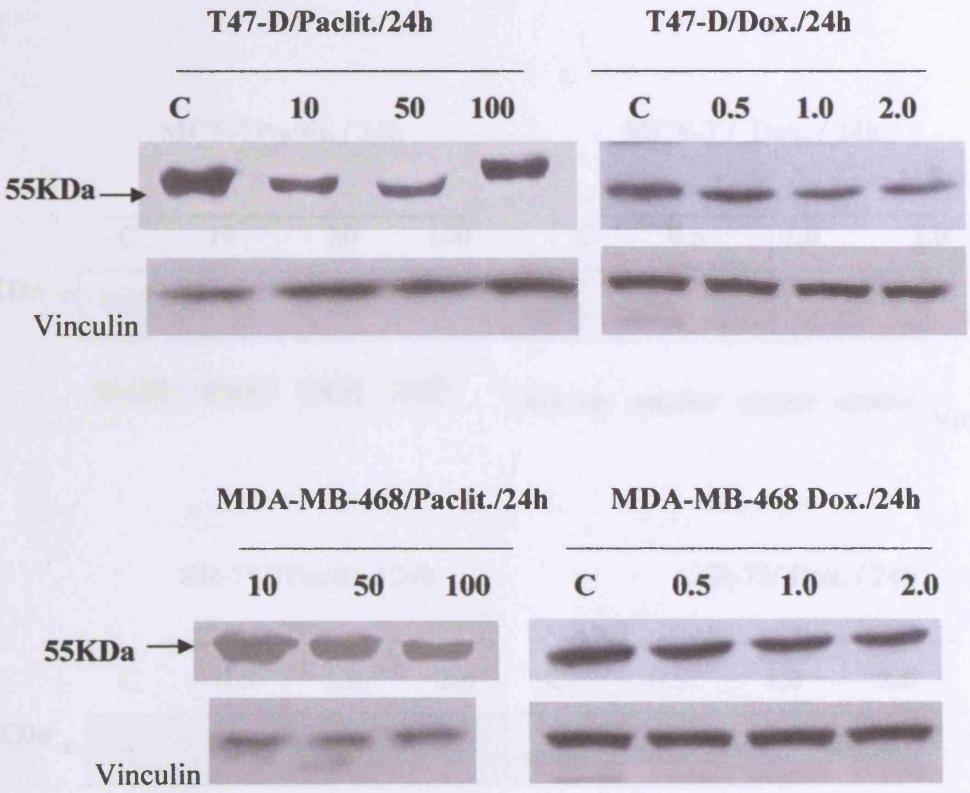


Figure 4.44(B): XIAP by western blotting in T47-D and 468 cells. 50 µg of proteins in each lane of a 10 % polyacrylamide gel, cells treated with paclitaxel 10, 50 and 100nM for 24h and doxorubicin 0.5, 1.0 and 2.0 µM for 24h. XIAP primary antibody 1 µg/ml. C, control protein (no treatment).

4.7 Effect on Survivin

Survivin protein expression was also assessed by western blotting. In MCF-7, it was markedly down regulated with doxorubicin at lower doses (0.5 and 1.0) more than the high dose (2.0 μM) at 24h. Paclitaxel treatment slightly down regulated the expression of Survivin in MCF-7 cells. In ZR-75 cells, it was up-regulated by both drugs in a dose dependent manner at 24h of treatment (figure 4.45 A).

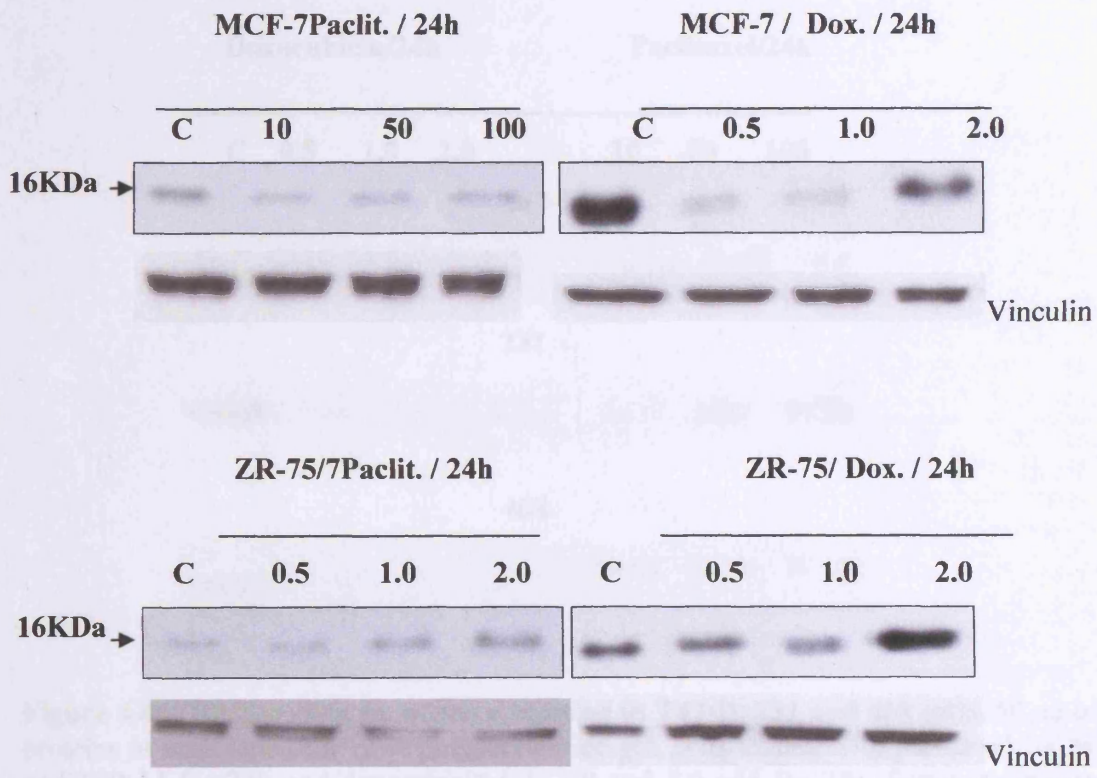


Figure 4.45 (A): Survivin by western blotting in MCF-7 and ZR-75 cells. 50 μg of proteins in each lane of a 12 % polyacrylamide gel, cells treated with paclitaxel 10, 50 and 100nM for 24h and doxorubicin 0.5, 1.0 and 2.0 μM for 24h. Survivin primary antibody 2 μg/ml. C, control protein (no treatment).

In T47-D cells, Survivin was markedly down regulated after doxorubicin treatment for 24h with all doses, while there was no effect with paclitaxel. In 231 cells, Survivin was also down regulated after doxorubicin, but it was only down regulated with smaller dose of paclitaxel (10nM). In 468 cells, it was markedly down regulated after doxorubicin treatment and to a lesser extent with paclitaxel at greater doses (50 and 100nM) (figure 4.45 B).

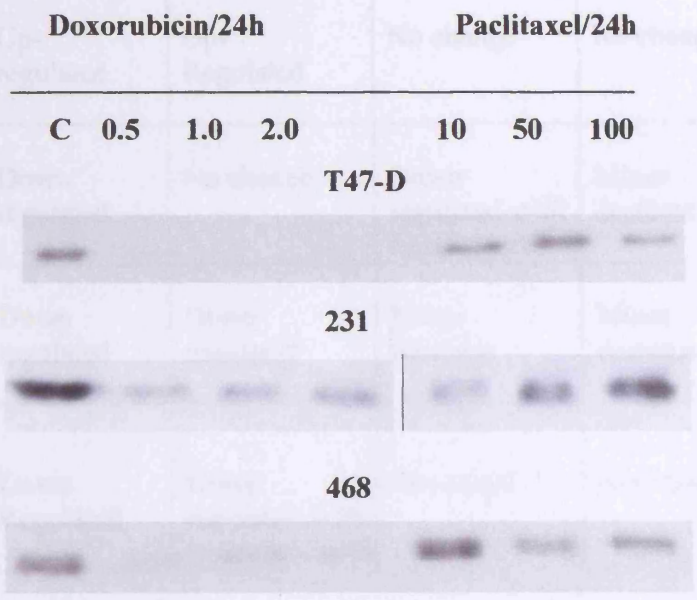


Figure 4.45 (B): Survivin by western blotting in T47-D, 231 and 468 cells. 50 μg of proteins in each lane of a 12 % polyacrylamide gel, cells treated with paclitaxel 10, 50 and 100nM for 24h and doxorubicin 0.5, 1.0 and 2.0 μM for 24h. Survivin primary antibody 2 μg/ml. C, control protein (no treatment).

The effect of treatment on Survivin and XIAP is summarized in table 4.1.

Antigen	*Survivin*		*XIAP*	
	Doxorubicin	*Paclitaxel*	*Doxorubicin*	*Paclitaxel*
MCF-7	Down regulated	Down regulated	Down regulated	Down regulated
ZR-75	Up-regulated	Up-Regulated	No change	No change
T47-D	Down regulated	No change	Down regulated with lower doses	Minor decrease
468	Down regulated	Down regulated	Minor decrease	Minor decrease
231	Down Regulated	Down regulated with lower doses	Not tested	Not tested

Table 4.1: Summary, effect of treatment on Survivin and XIAP protein expression by western blotting.

4.8 Effect on Bax

The semi-quantitative H-Score was used to assess staining.

4.8.1 MCF-7 cells

Bax was expressed in MCF-7 control non-treated cells. With doxorubicin treatment, the level of expression has increased at all time points especially with larger doses (figure 4.46 A and 4.47A&B). With paclitaxel treatment, the expression was also increased being the largest at 48h with all doses (figure 4.46 B).

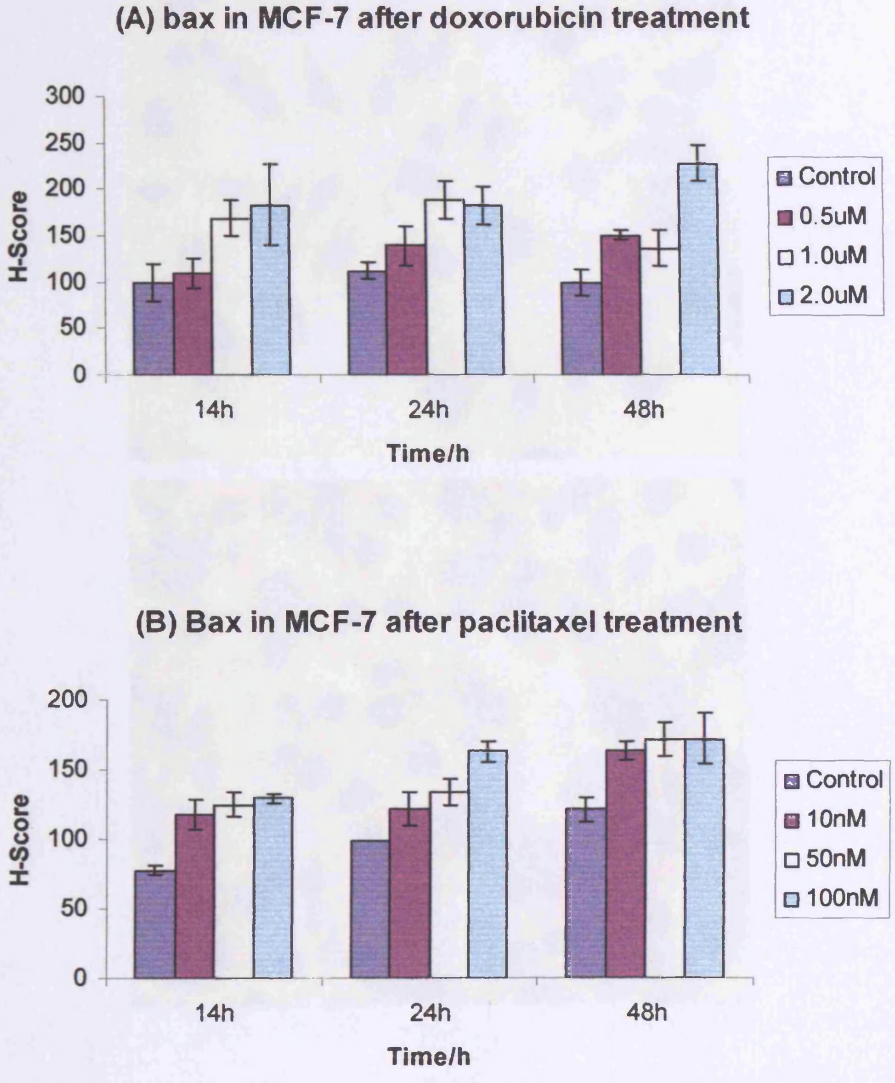


Figure 4.46: Bax in MCF-7 cells. A, after doxorubicin treatment. B, after paclitaxel treatment.

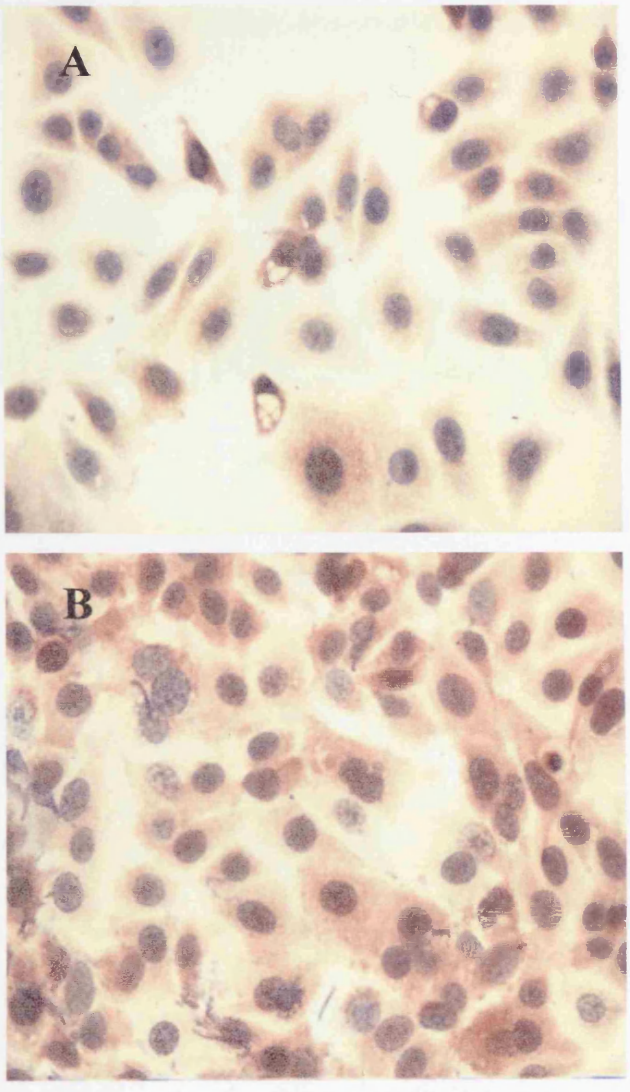


Figure 4.47: Bax immunocytochemistry in MCF-7 cells. A. doxorubicin control 14h. B, doxorubicin 2.0 μM/ 14h.

4.8.2 ZR-75

ZR-75 cells showed high level of bax in control non-treated cells. After doxorubicin treatment, bax expression was markedly increased being highest at 24 and 48h (Figure 4. 48 A). It was also increased after paclitaxel treatment at all time points (Figure 4.48 B).

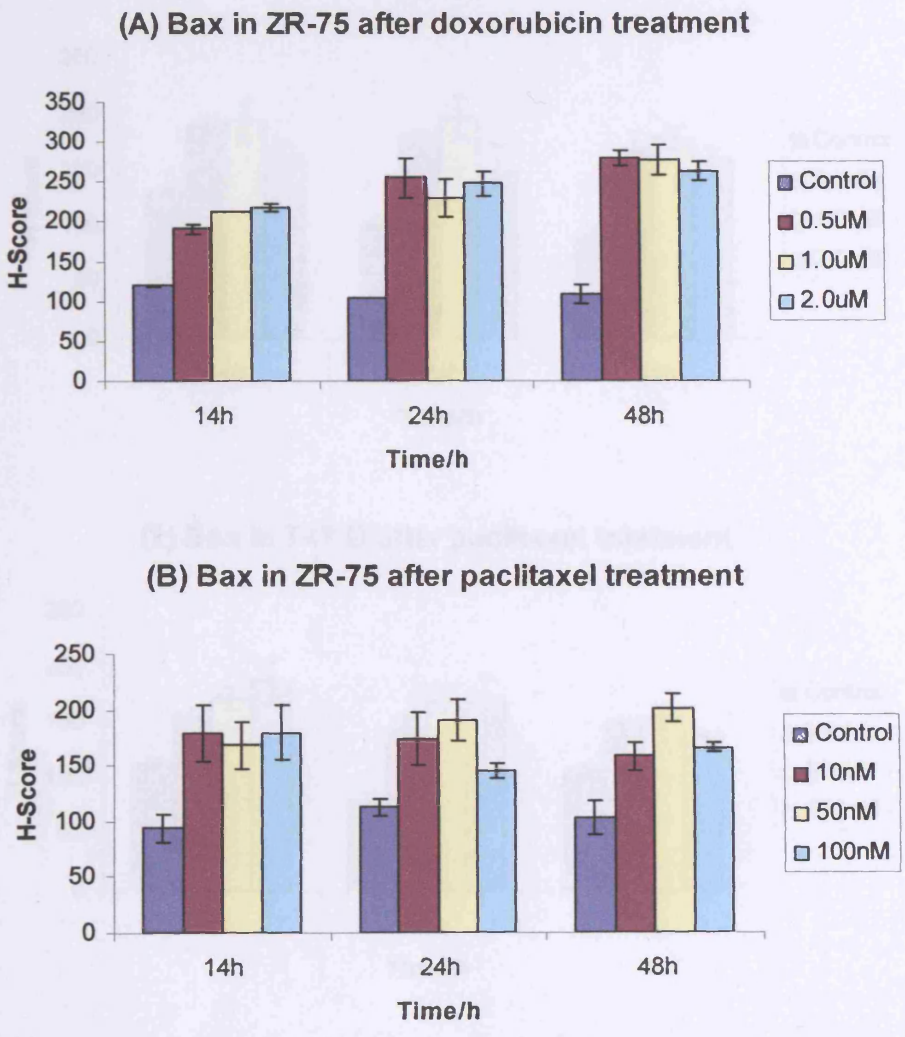


Figure 4.48: Bax in ZR-75 cells. A, after doxorubicin treatment. B, after paclitaxel treatment.

4.8.3T47-D

High level of bax was observed in T47-D control cells. The level of expression was increased with all doses of doxorubicin and paclitaxel treatment and at all time points (Figure 4.49 A and B).

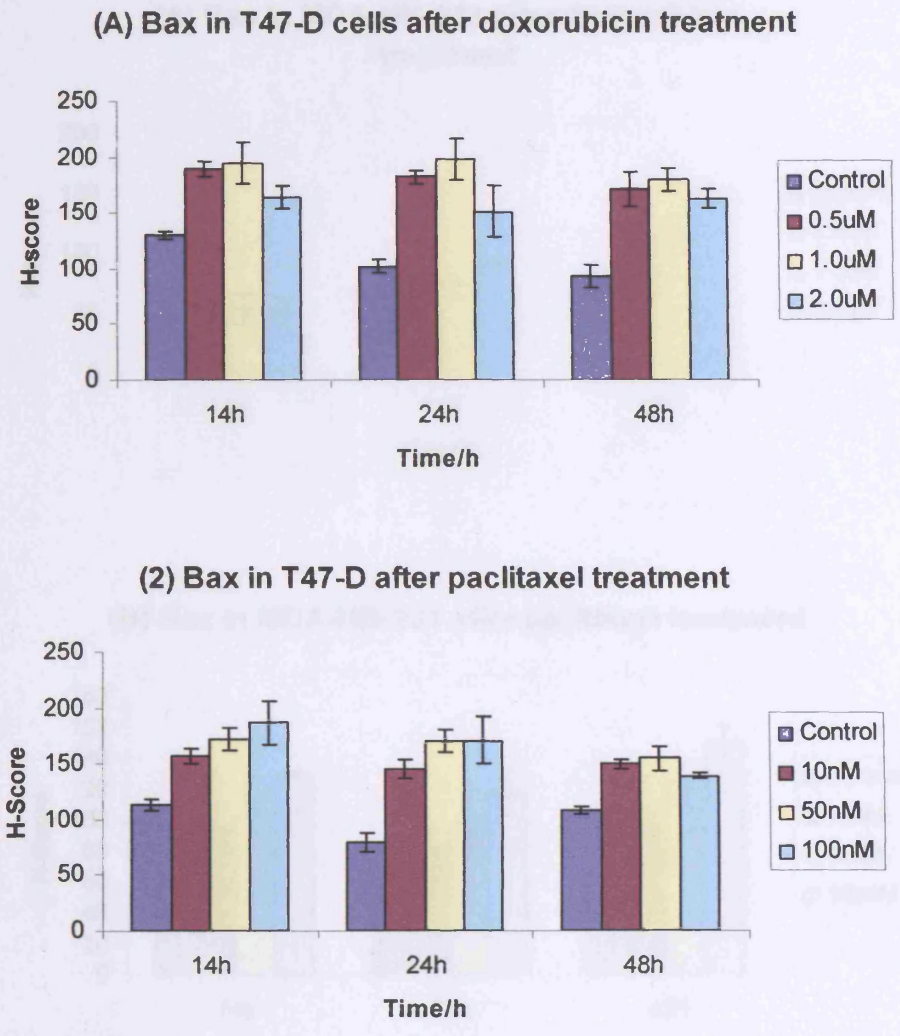


Figure 4.49: Bax in T47-D cells. A, after doxorubicin treatment. B, after paclitaxel treatment.

4.8.4 MDA-MB-231

The expression of bax in control cells was low. It started to increase at 24h and in a dose-dependent manner at 48 h (Figure 4.50 A). Paclitaxel treatment induced bax in MDA-MB-231 with increasing dose at all time points (Figure 4.50 B).

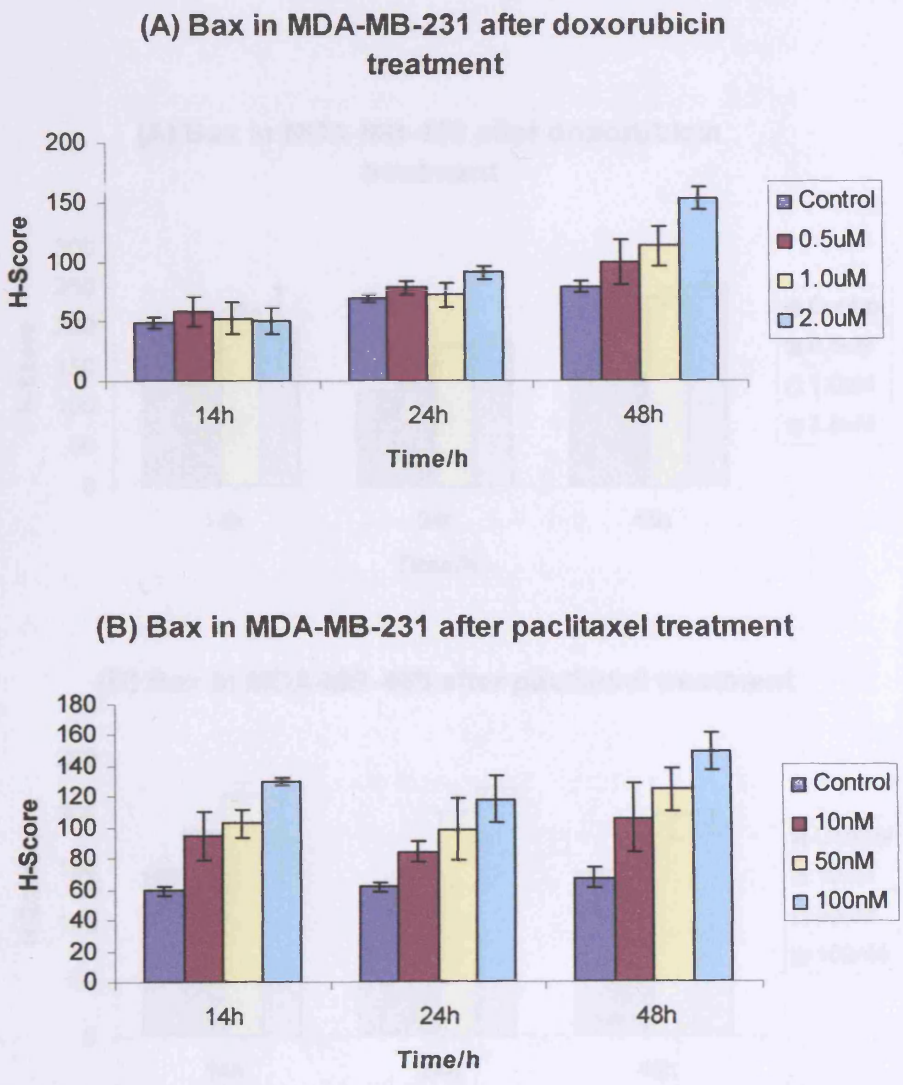


Figure 4.50: Bax in MDA-MB-231 cells. A, after doxorubicin treatment. B, after paclitaxel treatment.

4.8.4 MDA-MB-468

Bax was expressed in MDA-MB-468 cells at high levels in control cells. The expression was increased after doxorubicin treatment, with the highest level being at 48 hours with larger doses (1.0 and 2.0 μM) (Figure 4.51 A). The level of expression was also increased after paclitaxel treatment, being highest with 100 nM at 24h (Figure 4.51 B).

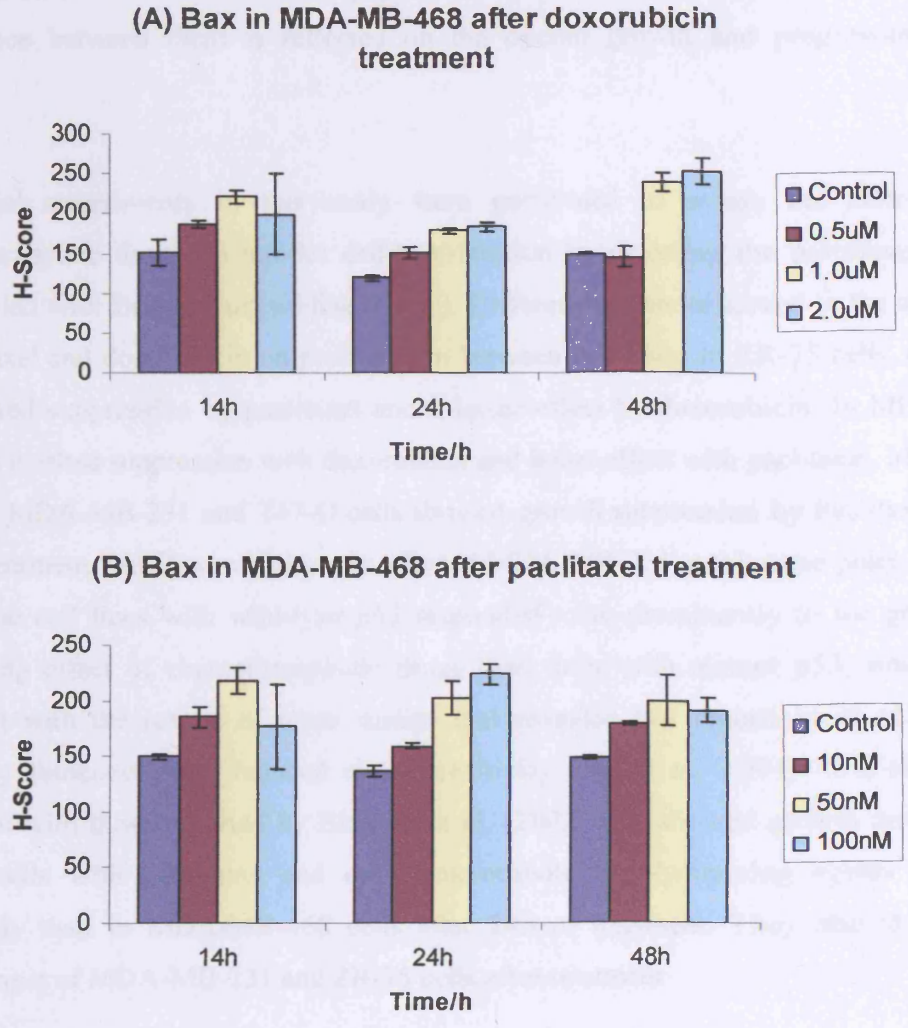


Figure 4.51: Bax in ZR-75 cells. A, after doxorubicin treatment. B, after paclitaxel treatment.

Discussion

Different chemotherapeutic drugs have different mechanisms of action, but all finally induce cell death by the induction of apoptosis. Therefore, mechanisms that regulate this biological event may determine the response or resistance of cancer cells to chemotherapeutic agents. The tumour suppressor gene p53 as 'a guardian of the genome' plays a central role in the regulation of apoptosis. There is an assumption that p53 mutant tumour cells are more susceptible to apoptosis than wild-type cells (Bunz et al., 1999). Studies also revealed that apoptosis and proliferation are linked together and the balance between them is reflected on the overall growth and progression of tumours.

The initial experiments in this study were performed to assess the effect of chemotherapeutic drugs on tumour cell proliferation by detecting the percentage of cells labeled with Bromodeoxyuridine (BrdU). Differences were observed in the effect of paclitaxel and doxorubicin on proliferation between cell lines. In ZR-75 cells, there was marked suppression by paclitaxel and a lesser effect by doxorubicin. In MCF-7, there was marked suppression with doxorubicin and lesser effect with paclitaxel. MDA-MB-468, MDA-MB-231 and T47-D cells showed growth suppression by Paclitaxel at 48h of treatment, but Doxorubicin only affected MDA-MB-231 at this time point. This means that cell lines with wild-type p53 responded more prominently to the growth suppressing effect of chemotherapeutic drugs than those with mutant p53, which is consistent with the results of other studies that revealed that mutations in p53 are frequently associated with reduced chemosensitivity (Fan et al., 1994). It is also in agreement with those reported by Blajeski et al. (2002) who showed growth arrest of MCF-7 cells with paclitaxel and other microtubule depolymerizing agents more extensively than in MDA-MB-468 cells after 24h of treatment. They also showed growth arrest of MDA-MB-231 and ZR-75 cells after treatment.

Paclitaxel as a microtubule disrupting agent has been shown to arrest breast cancer cells in mitosis by initiating the mitotic checkpoint (Blajeski et al., 2002), a mechanism that involves differential activation of MAP kinases (Shtil et al., 1999). After exposure to

this drug, cells exit mitosis and enter an abnormal tetraploid G1-like phase. This results in induction of the $p21^{waf-1}$ (Vidal and Koff, 2000). $p21^{waf-1}$ is a potent inhibitor of cdk2 and cdk4 and responsible for G1/S arrest induced by p53 in response to DNA damage (el-Deiry et al., 1993). It also inhibits the cell cycle progression through direct binding to the proliferating cell nuclear antigen (Waga et al., 1994).

As mentioned above, proliferation is linked with apoptosis and they work together to maintain cell homeostasis. Therefore, experiments were performed to evaluate the process of apoptosis after exposure to chemotherapeutic drugs. Treatment with both doxorubicin and paclitaxel induced apoptosis in breast cancer cell lines. Doxorubicin markedly induced apoptosis in MCF-7 cells as early as 14h and decreased after that, while in other cell lines there was a minimal increase of apoptosis at 14h and 24h, but reached the highest level at 48h. Induction of apoptosis in MCF-7 and ZR-75 was more prominent than that in other cell lines.

Paclitaxel also markedly induced apoptosis in MCF-7 cells as early as 14h and this decreased with prolonged treatment (24h and 48h). As with doxorubicin, paclitaxel induced apoptosis at 14h and 24h but with the highest level at 48h in other cell lines. In ZR-75, apoptosis was markedly induced at 48h. The delay in the induction of apoptosis in ZR-75 to 48h could be attributed the high level of bcl-2 expression found in this cell line, which could be protecting cells from apoptosis. This means that cells with wild-type p53 were more susceptible to apoptosis than those with mutant protein. The effect of paclitaxel on apoptosis was concentration dependent in most of cell lines which is in accordance with previous reports (Woods et al., 1995).

The level of bcl-2 expression and its phosphorylation state modulate the apoptotic response to paclitaxel, and over-expression of bcl-2 is associated with an increased resistance to chemotherapeutic drugs (Teixeira et al., 1995). Studies also showed that the DNA damaging drug doxorubicin induces apoptosis through the activation of the mitochondrial pathway and this was inhibited in bcl-2 over-expressing cells (Ruiz de Almodovar et al., 2001). This may explain why the induction of apoptosis in ZR-75

cells occurred as late as 48h, which is the time required to chemotherapeutic drugs to induce bcl-2 phosphorylation and consequent inactivation, since ZR-75 cells have high level of the anti-apoptotic protein bcl-2. MCF-7 cells were shown to have low levels of apoptosis (chapter 3) (Ruiz de Almodovar et al., 2001) showed the highest level of apoptosis after treatment.

The expression of p53 protein was assessed in two breast cancer cell lines (MCF-7 and ZR-75) known to have wild-type functioning p53 gene, in order to investigate the effect of treatment with chemotherapeutic drugs (as stressful stimuli) on activation and function of p53. Cells were treated in different concentrations and for different time periods and p53 was detected by two antibodies (DO-7 and p53 phosphospecific). Two methods were used, immunocytochemistry and western blotting.

Induction of p53 was seen for MCF-7 and ZR-75 with Doxorubicin treatment with all doses and as early as 14h, but to a lesser extent with paclitaxel treatment. These results are in agreement with those of el-Deiry et al. (1994) who showed the induction of p53 in the nucleus of cells containing wild-type p53 after exposure to DNA-damaging agents including adriamycin (doxorubicin).

Characteristically, cells treated with paclitaxel showed morphological changes in the form of nuclear chromatin fragmentation and condensation similar to the state of abnormal mitosis. In response to DNA damage, there is an increase in phosphorylation of wild-type p53 on serine 392 (Kapoor and Lozano, 1998). A recent study reported that serine 392 phosphorylation is important in regulating the oncogenic function of mutant p53 (Yap et al., 2004). In this study, phosphorylated p53 at this site was induced in wild-type p53 cells (MCF-7 and ZR-75) after treatment with the DNA damaging drug doxorubicin and to a lesser extent after paclitaxel treatment, which is in accordance with previous studies (Yap et al., 2004).

$P21^{waf-1}$ is a downstream effector protein of p53. $p21^{waf1}$ is a cyclin-dependent kinase inhibitor which functions to suppress proliferation. In this part of the study, $p21^{waf1}$

expression was assessed in five different breast cancer cell lines after treatment with both drugs (doxorubicin and paclitaxel) with immunocytochemistry.

$p21^{waf-1}$ was markedly induced in cells with wild-type p53 (MCF-7 and ZR-75) after doxorubicin treatment, but slightly induced after paclitaxel treatment at 14h and 24h with greater increase at 48h. In cells with mutant p53, p21 was markedly induced at 24h and 48h of doxorubicin treatment at lesser doses (T47-D) and slightly induced in MDA-MB-231 and MDA-MB-468 cells. There was either slight increase or no effect after paclitaxel treatment. These results are in agreement with those of el-Deiry et al. (1994) who showed the induction of $p21^{waf-1}$ in the nucleus of cells containing wild-type p53 after exposure to DNA-damaging agents including adriamycin (doxorubicin) whereas cell lines with mutant p53 expressed either low or undetectable levels of $p21^{waf-1.}$ The reason is that p53 transcriptionally activates $p21^{waf-1+}$ expression by directly interacting with its regulatory elements. The induction of p21 in mutated p53 cells (though not extensive) is attributed to the p53-independent mechanism (Sato et al., 2002).

The cytotoxic effect of paclitaxel is mainly through stabilization of microtubules and promoting the microtubule assembly (Schiff and Horwitz, 1980). Studies have shown that paclitaxel induces accumulation of p21 in both p53 wild-type and p53–null cancer cells (Blagosklonny et al., 1995). Barboule et al. (1997) also reported increased expression of $p21^{waf-1}$ in MCF-7 cells from 36h and onwards after treatment with paclitaxel. They observed that about 30% of cells treated with paclitaxel were blocked in an abnormal mitotic stage and other cells have shown condensed chromatin and micronuclei. My results are in agreement with these observations, since I found the appearance of similar abnormally mitotic cells in all breast cancer cell lines assessed, in addition to the induction of $p21^{waf-1}$ after treatment especially at 48h. There was no induction of $p21^{waf-1}$ in MDA-MB-468 cell line. Doxorubicin also induced p21 in wild-type p53 cells (MCF-7 and ZR-75) (Troester et al., 2004).

Bcl-2 expression was assessed by western blotting. In ZR-75 cells, treatment with paclitaxel for 24 hours resulted in phosphorylation of the protein with the appearance of

a band at 26 KDa corresponding to the phosphorylated bcl-2 protein. There was also down-regulation of the protein with doxorubicin treatment for 24h. Both drugs also down regulated the protein at 48 hours. In other cell lines, there was no noticeable effect. This result is supported with those of previous studies (Haldar et al., 1995) who showed that treatment of tumour cell lines with paclitaxel resulted in the increased phosphorylation of bcl-2 with consequent inactivation (most probably through a G2-M phase-dependent activation of a microtubule associated kinase). Doxorubicin induced apoptosis in bcl-2 negative cells but not in bcl-2 positive cells and has been shown to prolong the cell cycle (Knowlton et al., 1998).

Bax is a member of the bcl-2 family of apoptosis regulating proteins. Its expression is regulated by p53 and induced in some types of cells by all forms of genotoxic stress including chemotherapeutic drugs (Zhan et al., 1994). Bax expression was markedly induced in MCF-7 cells as well as ZR-75 cells, both wild-type p53. It was also induced in T47-D and MDA-MB-468 cells and to a lesser extent in MDA-MB-231.

The expression of inhibitor of apoptosis proteins, Survivin and XIAP, was assessed by western blotting in this study. Results showed that Survivin was down regulated with doxorubicin treatment in all cell lines except ZR-75 cells, in which the protein was up regulated at 24h of treatment. It was also down regulated in MCF-7, MDA-MB-468, MDA-MB-231 and to a lesser extent in T47-D, while it was up-regulated in ZR-75 cells after paclitaxel treatment. ZR-75 cells could be protected against the effect of chemotherapeutic drugs on Survivin because of over-expressing the anti-apoptotic protein bcl-2. Previous studies (Hoffman et al., 2002) reported a marked decrease in Survivin level in MCF-7 cells following treatment with doxorubicin 0.5 μg/ml, as early as 12 hours and stated that this effect is restricted to cells with wild-type p53.

Doxorubicin treatment down-regulated XIAP in MCF-7 cells and to a lesser extent T47-D and MDA-MB-468, while ZR-75 cells were not affected. It was also down regulated in MCF-7 cells after paclitaxel treatment, and to a lesser extent T47-D, but there was no effect on ZR-75 or MDA-MB-468. The similarity of the effect of

chemotherapeutic drugs on both Survivin and XIAP may suggest a common regulatory mechanism. Further studies are required to cover the lack of literature on XIAP expression after treatment in breast cancer and other tumours.

To conclude, p53 was induced in MCF-7 and ZR-75 after doxorubicin treatment and to a lesser extent after paclitaxel treatment. Proliferation was markedly suppressed in MCF-7 and ZR-75 cells after treatment with both drugs, while other cell lines with mutant p53 were suppressed after paclitaxel treatment. Cells with wild-type p53 were more susceptible to apoptosis than those with mutant protein. In MCF-7, it was as early as 14h while in ZR-75 it was delayed to 48 hours after treatment. Bcl-2 was phosphorylated in ZR-75 cells at 24h of paclitaxel treatment and down-regulated at 24h with doxorubicin treatment. Both drugs also down regulated bcl-2 protein in ZR-75 at 48h, but there was no effect on other cell lines. Bax was induced in wild-type p53 cells after treatment. Survivin was down regulated after paclitaxel and doxorubicin treatment but in ZR-75 cells, it was up regulated. The effect of treatment on XIAP was similar to that of Survivin.

CHAPTER 5:

ANALYSIS OF CLINICO-PATHOLOGICAL PARAMETERS AND BIOLOGICAL MARKERS IN BREAST CARCINOMA

Introduction

This part of the study involved immunohistochemical analysis of breast cancers in order to assess whether the expression of various biological factors are related to behavior and duration of survival. These included the tumour suppressor gene p53, its upstream regulatory protein ChK2 and downstream regulatory protein Waf-1. It is well known that the progression of cancer is dependent on the balance between cell proliferation and cell death by apoptosis. All chemotherapeutic drugs ultimately perform their action through the induction of apoptosis. Therefore, proliferation and apoptotic indices were assessed along with the expression of apoptotic regulatory proteins Bcl-2, Bax, Survivin and XIAP (see chapter 1).

Currently, the selection of treatments that a patient will receive following surgery depends on standard clinico-pathologic criteria including patient age, tumour size, tumour grade and lymph node status, in addition to the well established relationship between estrogen receptor status and patient response to hormonal therapy. However, there are no specific markers that can predict response to chemotherapy although several have been investigated as discussed in chapter 1. The aim of this part of the study was to compare the standard clinico-pathological factors with immunohistochemical expression of a panel of biological factors to identify those factors that predict duration of survival in relation to therapy and also to stratify patients into high or low risk groups on the basis of these factors.

Methods

Carcinomas from 165 patients were studied. They comprised 18 patients (10.9 %) who received neo-adjuvant chemotherapy before surgery, of which 9 patients had adjuvant hormonal therapy, and 147 patients (89.1 %) who had adjuvant treatment after surgery (wide local excision or mastectomy with axillary dissection). 10.9 % had adjuvant chemotherapy only, 42.4 % had adjuvant chemotherapy followed by hormonal therapy (tamoxifen) and 35.8 % had tamoxifen alone. The chemotherapy regimes were CMF

(Cyclophosphamide, Methotrexate and Fluorouracil), CAF (Cyclophosphamide, Adriamycin and Fluorouracil), AC (Adriamycin and Cyclophosphamide), and AC/Dox/Tax (Adriamycin and Cyclophosphamide/ Doxorubicin/Taxol). Tissues from all cases were analyzed for p53, phosphorylated p53, bcl-2, bax, $p21^{waf-1}$, ChK2, proliferation (MIB-1), apoptosis (M30), Survivin and XIAP using immunohistochemistry (see section 2.11).

Results

5.1 Patients

5.1.1 Age

The 165 patients included in this study were divided into two groups according to the method of treatment they received (a neoadjuvant group and an adjuvant group). Patient's ages at the time of diagnosis ranged from 22-71 years with a median value of 53 years and were grouped into three age groups (22-48, 49-56, and 57-71) to represent pre-menopausal, peri-menopausal and post-menopausal age groups. Table 5.1 shows the distribution of different treatment regimes among the different age groups.

Patient age groups/years	Treatment Regimes				Total
	Neo-adjuvant	Adjuvant Chemo.	Adjuv.Chemo. +Hormonal	Hormonal	
22-48	11(61.1%)	15(83.3%)	25(35.7%)	2(3.4%)	53(32.1%)
49-56	3(16.7%)	2(11.1%)	27(38.6%)	19(32.2%)	51(30.9%)
57-71	4(22.2%)	1(5.6%)	18(25.7%)	38(64.4%)	61(37.0%)
Total	18(100%)	18(100%)	70(100%)	59(100%)	165(100%)

Table 5.1: Distribution of treatment regimes among patient's age groups.

5.1.2 Survival

All patients included in the study had died from the disease. Follow-up of patients after diagnosis of breast cancer showed different durations of survival. The median duration

of survival for the whole group of patients included in the study was 38 months (range was 12-127 months). Survival durations were grouped into three: 12-30 months (35.2%); 31-47 months (33.9%), and 47+ months (30.9%) of the total number of patients. The correlation between age groups and the duration of survival was not statistically significant. Statistical analysis showed a significant difference in relation to treatment regime with the duration of survival. Patients in the neo-adjuvant group had shorter survival than those in the adjuvant group (t-test, p=0.002) (figure 5.1). Within the adjuvant group, there was a significant difference in survival between patients who received adjuvant chemotherapy and hormonal therapy and those who received hormonal therapy alone (p=0.012) (figure 5.2). 8 patients out of 18 (44.4%) of the neo-adjuvant group lived for 12-30months, and another 8 (44.4) lived for 31-47months while only 2 (11.1) lived for >47months. In comparison, survival in the adjuvant group was nearly equally distributed among the different survival groups, 12-30 months (34.0%), 31-47 months (32.7%), and >47 months (33.3%).

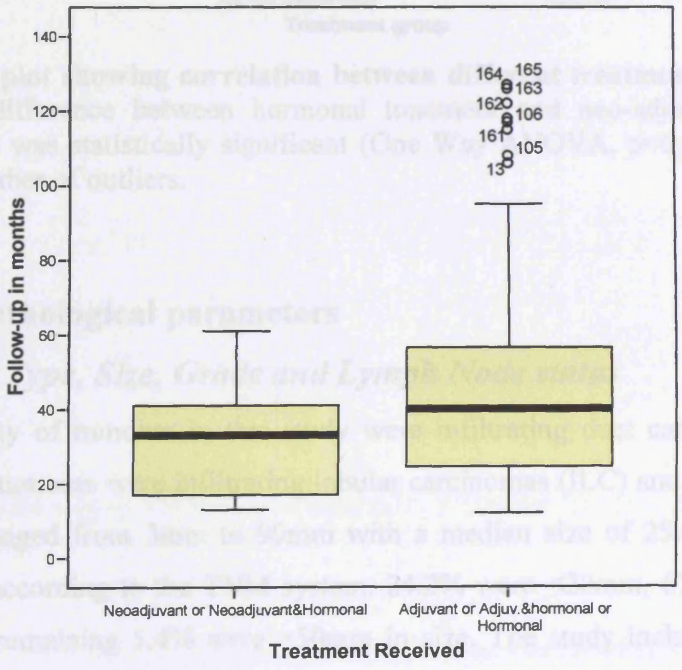


Figure 5.1: Boxplot comparing neo-adjuvant and adjuvant treatment. The second group (adjuvant treatment) was associated with longer duration of survival than the first group (neo-adjuvant treatment) (p=0.002). Numbers refer to case number of outliers.

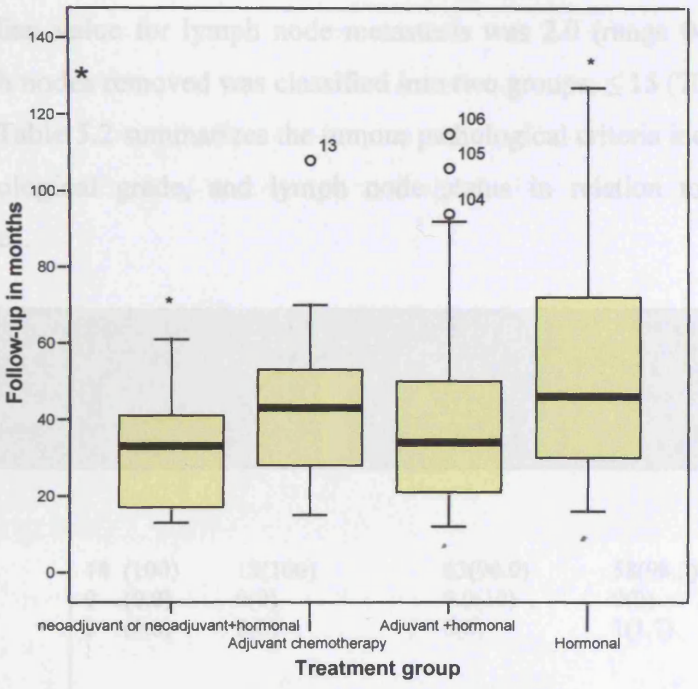


Figure 5.2: Boxplot showing correlation between different treatment regimes and survival. The difference between hormonal treatment and neo-adjuvant treatment (marked with *) was statistically significant (One Way ANOVA, p=0.012). Numbers refer to case number of outliers.

5.2 Clinico-pathological parameters

5.2.1 Tumour Type, Size, Grade and Lymph Node status

The vast majority of tumours in this study were infiltrating duct carcinomas (IDC) (92.2%); only 7 tumours were infiltrating lobular carcinomas (ILC) and one other type. Tumour sizes ranged from 3mm to 90mm with a median size of 25.0mm and were grouped into 3 according to the TNM system. 24.2% were <20mm, 67.8 % were 20-50mm, and the remaining 5.4% were >50mm in size. The study included 3 tumours classified as grade I and 38 tumours classified as grade II; these were combined together and analyzed as one group (25.0%). The other group contained 123 tumours that were grade III (75.0%).

26.0% of tumours had no evidence of lymph node metastasis, while 74.0 % were lymph node metastasis positive, of which 34.7 % had <3 lymph nodes and 39.1 % had >3 nodes. The median value for lymph node metastasis was 2.0 (range 0-30). The total number of lymph nodes removed was classified into two groups, ≤ 15 (70.2%) and > 15 nodes (29.8%). Table 5.2 summarizes the tumour pathological criteria including tumour type, size, histological grade, and lymph node status in relation to the different treatment regimes.

Clinico-pathological parameters	Neo-adjuvant No. (%)	Adjuvant chemotherapy No. (%)	Adjuv. Chem. +Hormonal No. (%)	Hormonal No. (%)	Total (%)
Tumour Type					
IDC	18 (100)	18(100)	63(90.0)	58(98.3)	157(92.2)
ILC	0 (0.0)	0(0)	7.0(10)	0(0)	7(4.2)
Others	0 (0.0)	0(0)	0(0)	1(1.7)	1(0.6)
Tumour Size					
<20 mm	4 (23.5)	4(22.2)	12(17.9)	20(33.9)	40(24.2)
20-50 mm	12 (70.6)	13(72.2)	51(76.1)	36(61.0)	112(67.8)
>50 mm	1 (5.9)	1(5.6)	4(6.0)	3(5.1)	9(5.4)
Tumour Grade					
I+II	2 (11.1)	1(5.6)	14(20.3)	24(40.7)	41(25.0)
III	16 (88.9)	17(94.4)	55(79.7)	35(59.3)	123(75.0)
Lymph nodes					
-ve	4 (23.5)	5(27.8)	7(10.3)	26(44.8)	42(26.0)
1-3	6 (35.3)	7(38.9)	24(35.3)	19(32.8)	56(34.7)
>3	7 (41.2)	6(33.3)	37(54.4)	13(22.4)	63(39.1)

Table 5.2: Pathological criteria in relation to different treatment regimes.

5.2.2 Standard tumour pathological criteria and survival

In the neo-adjuvant group, none of the standard clinico-pathological criteria showed any significant correlation with patient duration of survival. In the adjuvant group, tumour type and grade showed highly significant correlation with survival (p=0.004

and <0.0001) respectively, grade III tumours being associated with shorter duration of survival. Tumour size also showed a significant correlation with survival (p=0.005) in the adjuvant group treated with hormonal therapy but not other groups. There was no correlation with lymph node metastasis status (table 5.3) or with lymph node ratio. However, the total number of lymph nodes removed showed a significant correlation with survival (p=0.017). When all cases were considered, both tumour type and grade also correlated with survival, while tumour size failed to show significant correlation.

Pathological Criteria		*Neo-adjuvant*	*Adjuv. Chemo.*	*Adjuv. +Hormon.*	*Hormo.*	
	Statistical Test	*p*	*p*	*p*	*p*	*Total P value*
Tumour type	One-Way ANOVA	-	-	0.049*	0.020*	0.004**
Tumour size	One-Way ANOVA	0.562	0.614	0.902	0.005**	0.580
Tumour grade	T-Test	0.068	0.001**	0.001**	<0.0001**	<0.0001**
L.N status	Kruskall-Wallis Test	0.739	0.581	0.110	0.695	0.657
Nodes Removed	Kruskall-Wallis Test	0.777	0.825	0.253	0.014*	0.017*

Table 5.3: The relation between pathological criteria and survival. * refers to significance at 0.05 level, ** refers to significance at 0.005 level.

5.3 Estrogen and Progesterone Receptors (ER and PgR)

Not all cases had information on progesterone receptor. 51.2 % of cases showed the presence of estrogen receptors while progesterone receptors were present in 36.5% of cases. Table 5.4 shows the distribution of both receptors among the different treatment regimes and table 5.5 shows the relation between ER and PgR and the different clinico-

pathological parameters. ER and PgR were correlated significantly with tumour grade. Tumours grade III commonly lacked receptors.

Receptors		Neo-adjuvant	Adjuvant chemotherapy	Adjuv. Chem. +Hormonal	Hormonal	
		No. (%)	No. (%)	No. (%)	No. (%)	Total (%)
ER	-ve	9(50.0)	8(44.4)	33(47.8)	30(50.8)	80(48.7)
	+ve	9(50.0)	10(55.6)	36(52.2)	29(49.2)	84(51.2)
PgR	-ve	7(70.0)	6(60.0)	30(65.2)	16(59.3)	59(63.4)
	+ve	3(30.0)	4(40.0)	16(34.8)	11(40.7)	34(36.5)

Table 5.4: ER and PgR receptors in relation to treatment regimes.

Clinico-pathological Parameters		ER			PgR		
		-ve No. (%)	+ve No. (%)	P	-ve No. (%)	+ve No. (%)	P
Type	IDC	79 (98.8)	77 (91.7)	0.105	55 (43.2)	30 (88.2)	0.729
	ILC	1 (1.3)	6 (7.1)		4 (6.8)	3 (8.8)	
	Others	0 (0.0)	1 (1.2)		0 (0.0)	1 (2.9)	
Size/mm							
	<20	21 (27.3)	19 (22.9)	0.699	16 (28.1)	6 (18.8)	0.619
	20-50	51 (66.2)	60 (72.3)		38 (66.7)	24 (75.0)	
	>50	5 (6.5)	4 (4.8)		3 (5.3)	2 (6.3)	
Grade	I+II	4 (5.0)	37 (44.6)	<0.0001**	11 (18.6)	16 (48.5)	0.003**
	III	76 (95.0)	46 (55.4)		48 (81.4)	17 (51.5)	
L.N	-ve	24 (30.8)	18 (22.0)	0.408	12 (20.7)	7 (21.9)	0.729
	1-3	24 (30.8)	31 (37.8)		21 (36.2)	9 (28.1)	
	>3	30 (38.5)	33 (40.2)		25 (43.1)	16 (50.0)	

Table 5.5: ER and PgR receptors in relation to clinico-pathological parameters. Analyzed by Chi-square test, ** refers to significance at 0.005.

Receptors and survival

The presence of both estrogen and progesterone receptor correlated significantly with the duration of patient survival (figure 5.3a and 5.3.b). Table 5.6 shows the relation between ER and PgR and duration of patient survival for the different treatment regimes. There was a significant correlation between the presence of the receptor and the duration of survival only in the group of patients that received hormonal treatment ($p<0.0001$ and $p=0.014$ ER and PgR respectively).

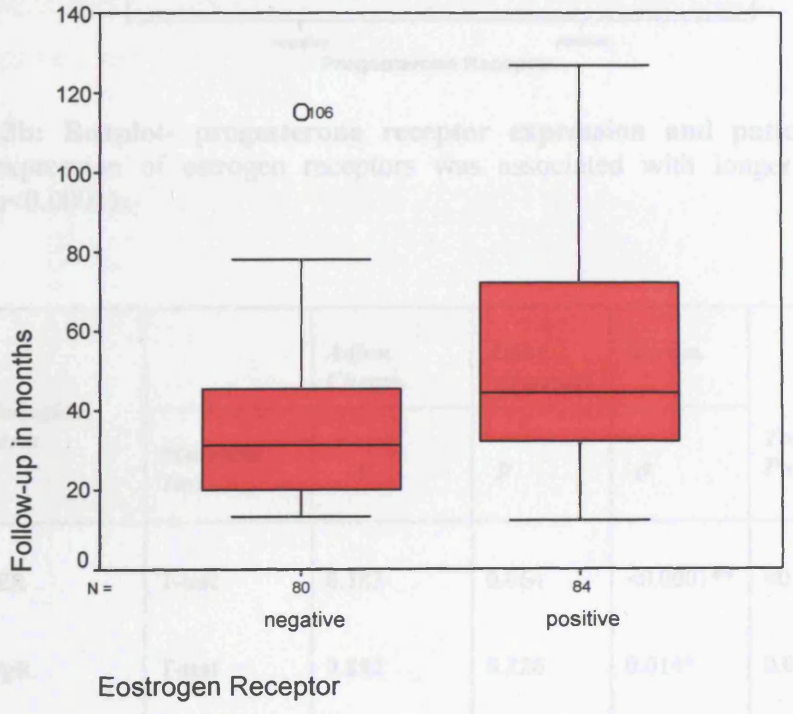


Figure 5.3a: Boxplot- estrogen receptor expression and patient survival. Positive expression of estrogen receptors was associated with longer duration of survival ($p<0.0001$).

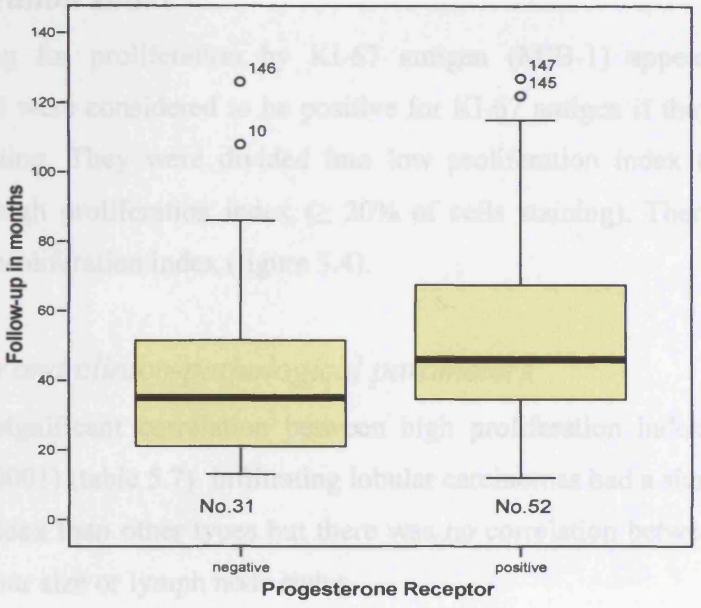


Figure 5.3b: Boxplot- progesterone receptor expression and patient survival. Positive expression of estrogen receptors was associated with longer duration of survival ($p<0.0001$).

Pathological Criteria		*Adjuv. Chemo.*	*Adjuv. +Hormon.*	*Hormo.*	
	Statistical Test	*p*	*p*	*p*	*Total P value*
ER	T-test	0.183	0.064	<0.0001**	<0.0001**
PgR	T-test	0.882	0.326	0.014*	0.010**

Table 5.6: ER and PgR and survival in relation to treatment regimes. * refers to significance at 0.05 level, ** refers to significance at 0.005 level.

5.4 Growth Regulation

5.4.1 Proliferation Index

Immunostaining for proliferation by Ki-67 antigen (MIB-1) appeared as nuclear staining; nuclei were considered to be positive for Ki-67 antigen if they had moderate or strong staining. They were divided into low proliferation index (<20% of cells staining) and high proliferation index (≥ 20% of cells staining). There were 58.2 % having a high proliferation index (figure 5.4).

Proliferation and clinico-pathological parameters

There was a significant correlation between high proliferation index and grade III tumours (p<0.0001) (table 5.7). Infiltrating lobular carcinomas had a significantly lower proliferation index than other types but there was no correlation between proliferation index and tumour size or lymph node status.

Clinico-pathological Parameters.		Proliferation		
		Low No (%)	High No (%)	P
Type	IDC	62 (89.9)	95 (99.0)	0.026*
	ILC	6 (8.7)	1 (1.0)	
	Others	1 (1.4)	0 (0.0)	
Size/mm				
	<20	12 (17.6)	28 (30.1)	0.085
	20-50	50 (73.5)	62 (66.7)	
	>50	6 (8.8)	3 (3.2)	
Grade	I+II	29 (42.0)	12 (12.6)	< 0.0001**
	III	40 (58.0)	83 (87.4)	
L.N	-ve	17 (24.6)	25 (27.2)	0.615
	1-3	22 (31.9)	34 (37.0)	
	>3	30 (43.5)	33 (35.9)	

Table 5.7: Proliferation in relation to clinico-pathological parameters. Analyzed by Chi-square test, p values <0.05 are statistically significant and referred to *, while ** refers to significance at the 0.005 level.

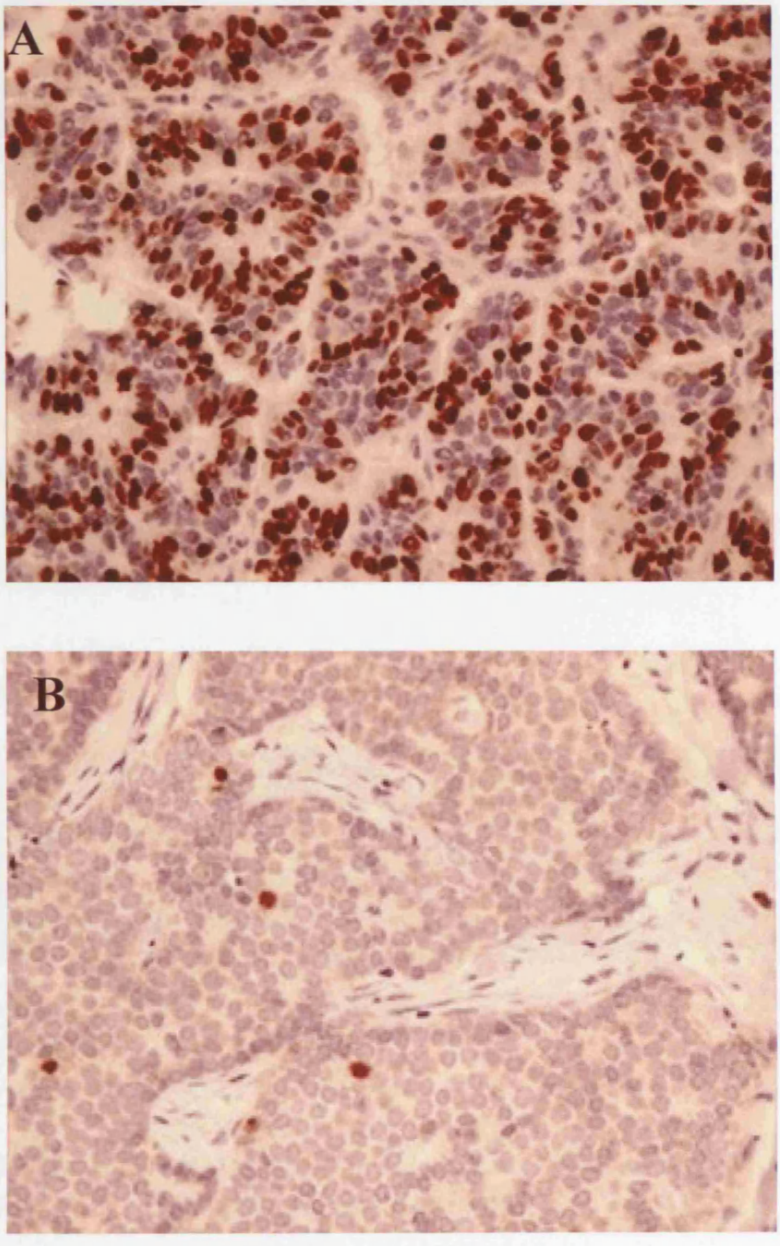


Figure 5.4: Proliferation by MIB-1 immunohistochemistry. A, high proliferation (41.8% of cells positive) B, very low proliferation (0.9 % of cells positive).

Proliferation and other biological factors

High proliferating tumours showed an inverse correlation with the presence of anti-apoptotic protein bcl-2 and ER (p=0.001 both markers). High proliferation index correlated positively with both ChK2 and Survivin (p=0.005 and p<0.0001 respectively) (table 5.9). There was no significant correlation with other biological markers.

Proliferation and Survival

Proliferation index showed significant correlation with survival. High proliferation was associated with shorter duration of survival (p=0.001) (figure 5.5). When analyzed in relation to treatment groups, patients who received adjuvant chemotherapy in addition to adjuvant hormonal therapy showed a significant correlation with proliferation (p=0.026) (table 5.8).

Biological marker	*Statistical Test*	*Neo-Adjuv.*	*Adjuv. Chemo.*	*Adjuv. +Hormon.*	*Hormon.*	*Total P value*
		P	*p*	*p*	*p*	
Proliferation by MIB-1	T-test	0.20	0.059	0.026*	0.083	0.001**

Table 5.8: proliferation and survival in relation to treatment group. * refers to significance at 0.05, ** refers to significance at 0.005 level.

Biological Markers		Proliferation		
		Low	High	P
		No. (%)	No. (%)	
Apoptosis %	<1	37 (54.4)	54 (56.3)	0.939
	1-2	16 (23.5)	23 (24.0)	
	>2	15 (22.1)	19 (19.8)	
Apoptosis/10HPF	<10	29 (35.3)	36 (37.5)	0.664
	10-20	25 (36.8)	29 (30.2)	
	>20	19 (27.9)	31 (32.3)	
P53 (DO-7)	-ve	53 (76.8)	50 (52.1)	0.001**
	+ve	16 (23.2)	46 (47.9)	
P53 phosphospecific	-ve	62 (89.9)	66 (68.7)	0.001**
	+ve	7 (10.1)	30 (31.3)	
Waf-1	-ve	51 (73.9)	69 (71.9)	0.772
	+ve	18 (26.1)	27 (28.1)	
ChK-2	-ve	29 (42.0)	21 (21.9)	0.005**
	+ve	40 (58.0)	75 (78.1)	
Bcl-2	-ve	24 (34.8)	59 (61.5)	0.001**
	+ve	45 (65.2)	37 (38.5)	
Bax	-ve	7 (10.3)	6 (6.3)	0.355
	+ve	61 (89.7)	89 (93.7)	
Survivin	-ve	47 (68.1)	26 (27.4)	< 0.0001**
	+ve	22 (31.9)	69 (72.6)	
XIAP	-ve	9 (13.0)	8 (8.3)	0.326
	+ve	60 (87.0)	88 (91.7)	
ER	-ve	23 (33.3)	57 (60.0)	0.001**
	+ve	46 (66.7)	38 (40.0)	
PgR	-ve	23 (54.8)	36 (70.6)	0.115
	+ve	19 (45.2)	15 (29.4)	

Table 5.9: Relationship between extent of proliferation and biological markers. Analyzed by Chi-square test, ** refers to significance at 0.005.

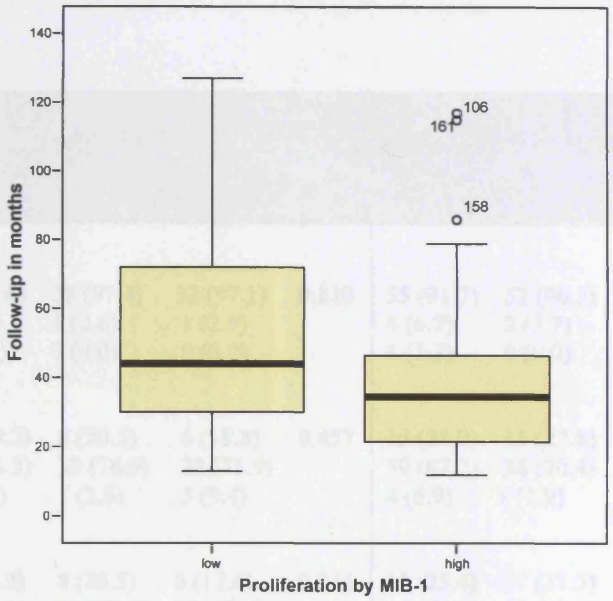


Figure 5.5: Boxplot shows proliferation index and survival. Low proliferation correlated with better survival (p=0.001).

5.4.2 Apoptotic Index

The apoptotic cells were detected by the anti-cytokeratin 18 antibody (M30) which stains cells at different stages of the apoptotic process. Cells in early apoptosis were characterized by cytoplasmic staining and dark condensed nuclei, or clumped chromatin at the inner aspect of the nucleus. Cells in advanced stage were seen as small shrunken cells with darkly stained cytoplasm or as small rounded membrane-bound structures (apoptotic bodies) (figure 5.6). As described in 2.6.7, the apoptotic index was determined by counting the number of apoptotic stained cells in 10 HPF or calculating the percentage of positive cells in a total of 1000 tumour cells.

Apoptosis and clinico-pathological parameters

Assessment of apoptosis by the two different methods of assessment failed to show any significant correlation with any of the clinico-pathological parameters (table 5.10).

Clinicopathological Parameters.	Apoptosis %				Apoptosis /10 HPF			
	<1	1-2	>2	P	<10	10-20	>20	P
Type								
IDC	85 (93.6)	38 (97.4)	33 (97.1)	0.810	55 (91.7)	52 (96.3)	49 (98.0)	0.507
ILC	5 (5.5)	1 (2.6)	1 (2.9)		4 (6.7)	2 (3.7)	1 (2.0)	
Others	1 (1.1)	0 (0.0)	0 (0.0)		1 (1.7)	0 (0.0)	0 (0.0)	
Size/mm								
<20	26 (29.2)	8 (20.5)	6 (18.8)	0.457	15 (25.9)	15 (27.8)	10 (20.8)	0.685
20-50	59 (66.3)	30 (76.9)	23 (71.9)		39 (67.2)	38 (70.4)	35 (72.9)	
>50	4 (4.5)	1 (2.6)	3 (9.4)		4 (6.9)	1 (1.9)	3 (6.3)	
Grade								
I+II	26 (28.9)	8 (20.5)	6 (17.6)	0.344	15 (25.4)	17 (31.5)	8 (16.0)	0.183
III	64 (71.1)	31 (79.5)	28 (82.4)		44 (74.6)	37 (68.5)	42 (84.0)	
L.N								
-ve	28 (31.8)	7 (17.9)	7 (21.2)	0.225	18 (31.0)	14 (26.4)	10 (20.4)	0.321
1-3	30 (34.1)	17 (43.6)	9 (27.3)		17 (29.3)	23 (43.4)	16 (32.7)	
>3	30 (34.1)	15 (38.5)	17 (51.5)		23 (39.7)	16 (30.2)	23 (46.9)	

Table 5.10: Apoptosis in relation to clinicopathological parameters, analyzed by Chi-square test.

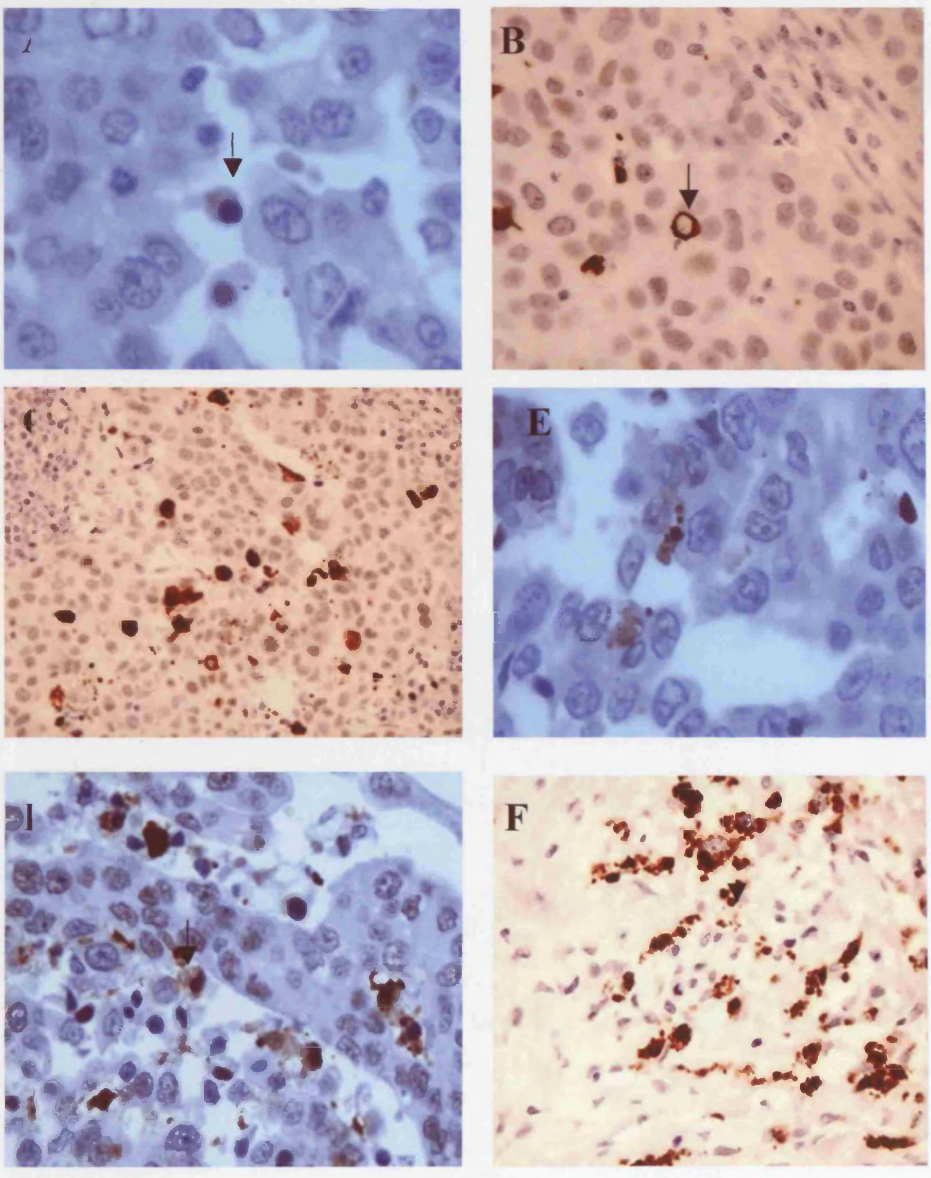


Figure 5.6: Apoptosis detected by M30. A, example for early apoptotic cell with condensed dark nucleus and stained cytoplasm (arrow). B, another early apoptotic cell with condensed chromatin at the inner aspect of the nucleus and stained cytoplasm (arrow). C+D, tumour tissues with high apoptotic index (2.2 % of cells) showing apoptotic cells at different stages of apoptosis. E, example of apoptotic cell showing nuclear fragmentation. F, late apoptosis with frequent apoptotic bodies.

Apoptosis and biological markers

Apoptosis assessed in 10/HPF showed a significant correlation with the inhibitor of apoptosis protein Survivin (p=0.031) while the apoptotic index assessed as a percentage failed to show any correlation. There was no correlation between apoptosis assessed by both methods and any of the other biological markers (table 5.12).

Apoptosis and survival

Overall apoptosis as assessed by the both counting methods showed no correlation with survival (table 5.11). The only significant correlation found was with survival in the neoadjuvant group where tumours with apoptosis 1-2 % had longer survival than other groups (figure 5.7), although numbers were small.

Apoptosis	*Statistical Test*	*Neo-Adjuv.*	*Adjuv. Chemo.*	*Adjuv. +Hormon.*	*Hormon.*	*Total P value*
		P	*p*	*p*	*p*	
Apoptosis %	ANOVA	0.005**	0.274	0.292	0.419	0.383
Apoptosis/10HPF	ANOVA	0.119	0.167	0.888	0.767	0.519

Table 5.11: Apoptosis and survival in relation to treatment group. ** refers to significance at the 0.005 level.

Biological marker	Apoptosis %				Apoptosis /10 HPF			
	<1 No (%)	1-2	>2	P	<10 No (%)	10-20	>20	P
Proliferation								
Low	37 (54.4)	61 (23.5)	15 (22.1)	0.939	29 (35.3)	25 (30.8)	19 (27.9)	0.664
High	54 (56.3)	23 (24.0)	19 (19.7)		36 (37.5)	29 (30.2)	31 (32.3)	
P53 (DO-7)								
-ve	59 (57.3)	26 (25.2)	18 (17.5)	0.401	39 (65.0)	36 (66.7)	28 (56.0)	0.482
+ve	32 (52.5)	13 (21.3)	16 (26.2)		21 (35.0)	18 (33.3)	22 (44.0)	
P53 phosphospecific								
-ve	74 (58.3)	29 (22.8)	24 (18.9)	0.385	48 (80.0)	45 (83.3)	34 (68.0)	0.146
+ve	17 (45.9)	10 (27.0)	10 (27.0)		12 (20.0)	9 (16.7)	16 (32.0)	
Waf-1								
-ve	65 (71.4)	31 (79.5)	23 (67.6)	0.494	42 (70.0)	41 (75.4)	36 (72.0)	0.774
+ve	26 (28.6)	8 (20.5)	11 (32.4)		18 (30.0)	13 (24.1)	14 (28.0)	
ChK-2								
-ve	29 (31.9)	12 (30.8)	8 (23.5)	0.657	22 (36.7)	16 (29.6)	11 (22.0)	0.246
+ve	62 (68.1)	27 (69.2)	26 (76.5)		38 (63.3)	38 (70.4)	39 (78.0)	
Bcl-2 -ve	46 (50.5)	16 (41.0)	20 (58.8)	0.313	29 (48.3)	27 (50.0)	26 (52.0)	0.929
+ve	45 (49.5)	23 (59.0)	14 (41.2)		31 (51.7)	27 (50.0)	24 (48.0)	
Bax -ve	6 (6.6)	4 (10.3)	3 (9.4)	0.743	3 (5.0)	7 (13.0)	3 (6.3)	0.255
+ve	85 (93.4)	35 (89.7)	29 (90.6)		57 (95.0)	47 (87.0)	45 (93.8)	
Survivin -ve	42 (46.2)	19 (50.0)	11 (32.4)	0.273	24 (40.0)	31 (58.5)	17 (34.0)	0.031*
+ve	49 (53.8)	19 (50.0)	23 (67.6)		36 (60.0)	22 (41.5)	33 (66.0)	
XIAP -ve	11 (12.1)	1 (2.6)	5 (14.7)	0.171	8 (13.3)	4 (7.4)	5 (10.0)	0.581
+ve	80 (87.9)	38 (97.4)	29 (85.3)		52 (86.7)	50 (92.6)	45 (90)	
ER -ve	44 (48.4)	15 (39.5)	21 (61.8)	0.164	30 (50.0)	22 (41.5)	28 (56.0)	0.334
+ve	47 (51.6)	23 (60.5)	13 (38.2)		30 (50.0)	31 (58.5)	22 (44.0)	
PgR -ve	31 (62.0)	13 (52.0)	15 (83.3)	0.104	20 (60.6)	18 (56.3)	21 (75.0)	0.295
+ve	19 (38.0)	12 (48.0)	3 (16.7)		13 (39.4)	14 (43.8)	7 (25.0)	

Table 5.12: Apoptosis as determined by the counting methods and relationship to biological markers, analyzed by Chi-square test, * refers to significance at 0.05 levels.

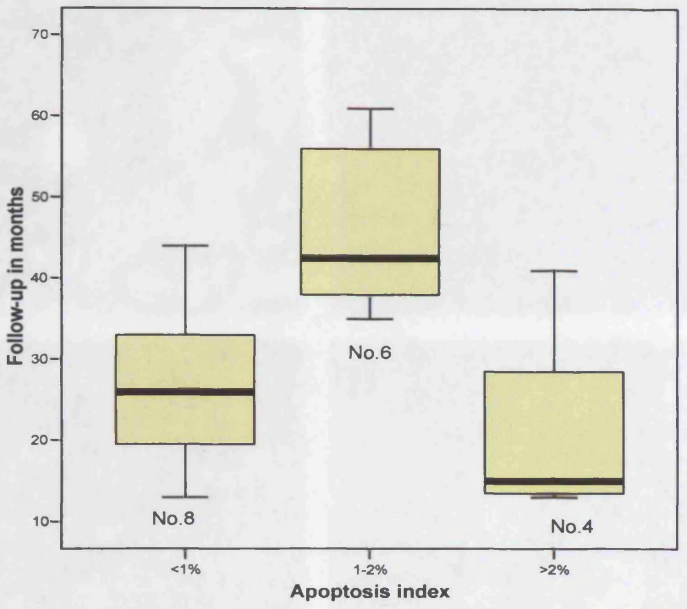


Figure 5.7: Boxplot showing apoptosis index in relation to survival in the neo-adjuvant chemotherapy groups (No. corresponds to the number of cases in each group).

5.5 Biological Markers.

5.5.1 Tumour suppressor gene p53

Sections were stained for the expression of p53 protein by two different antibodies (DO-7 and phosphorylated p53). P53 was detected in 62 of the 165 breast tumours (37.6 %). Phosphospecific p53, which reflects p53 that is phosphorylated at serine 392, was detected in 22.4 % of tumours (figure 5.8). P53 expression as detected by both antibodies showed positive correlation with each other ($p<0.0001$); all tumours positive for phosphospecific p53 were positive with the DO-7 antibody (figure 5.9). The distribution of p53 status in relation to treatment group is shown in table 5.13.

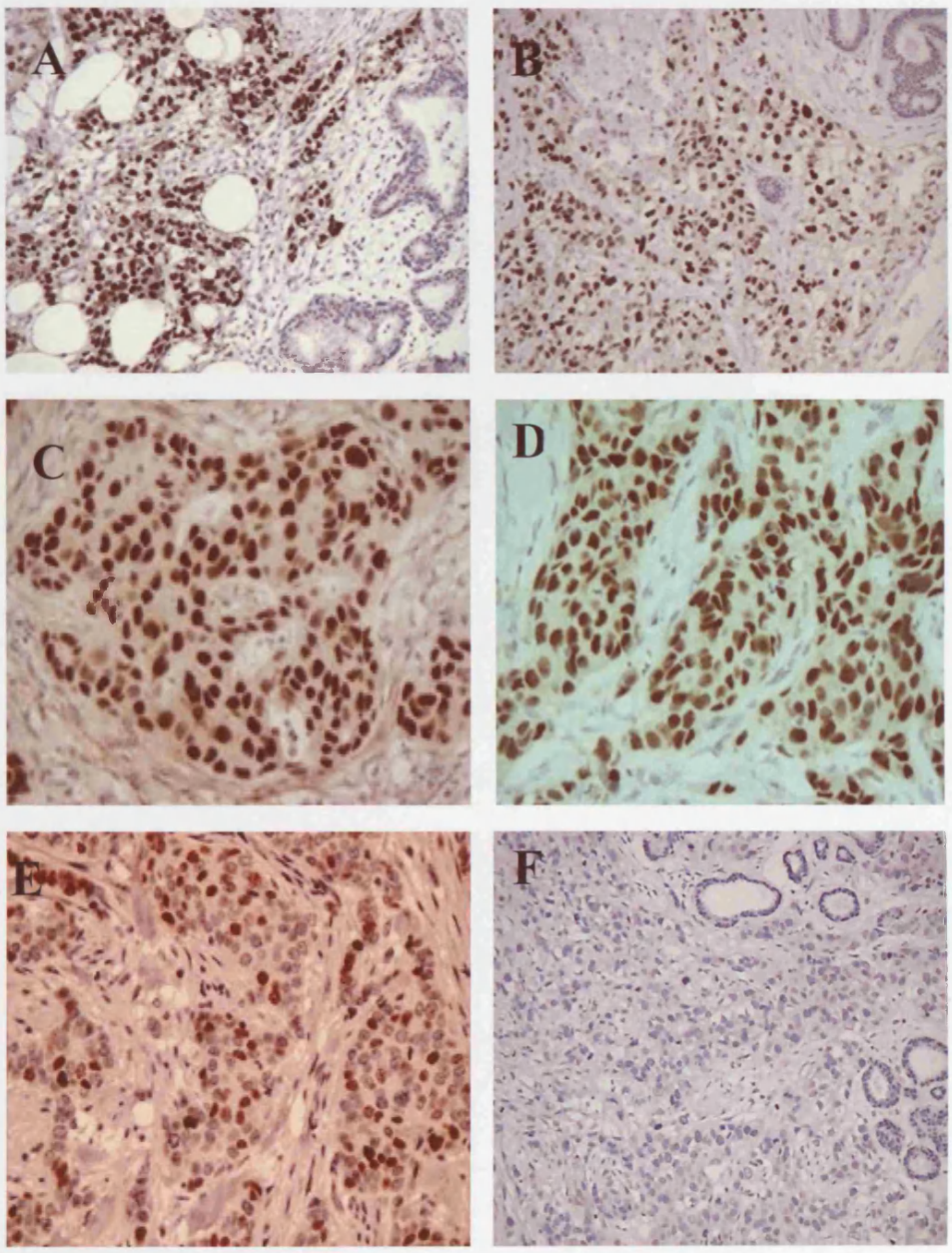


Figure 5.8: P53 immunohistochemistry. A, strong staining for p53 (DO-7) in most tumour cells. B, strong staining for phosphospecific p53 in section from tumour (A). C & D, another tumour stained with DO-7 and phosphorylated p53 respectively. E, 23% of cells staining with DO-7. F, another tumour negative for p53.

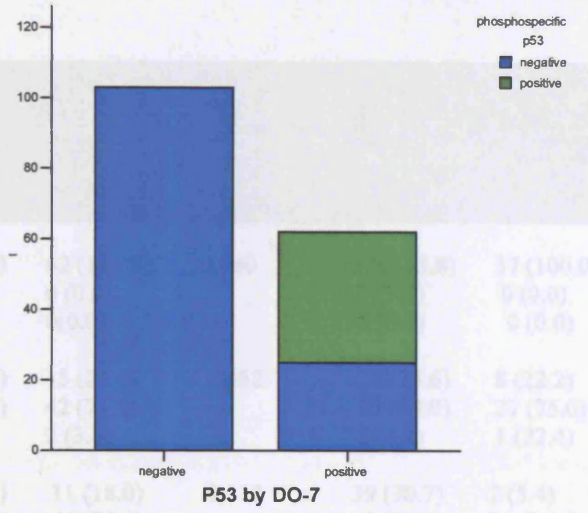


Figure 5.9: P53 DO-7 in relation to phosphospecific p53. All phosphospecific p53 +ve cases were also Do-7 +ve, but none was DO-7 negative.

Table 5.13: Distribution of p53 in relation to treatment group.

P53 antigen		Neo-adjuvant No. (%)	Adjuvant chemotherapy No. (%)	Adjuv. Chem. +Hormonal No. (%)	Hormonal No. (%)	Total (%)
DO-7	-ve	11 (61.1)	14 (77.8)	40 (57.1)	38 (64.4)	103 (62.4)
	+ve	7 (38.9)	4 (22.2)	30 (42.9)	21 (35.6)	62 (37.6)
PHOS	-ve	14 (77.8)	16 (88.9)	49 (70.0)	49 (87.1)	128 (77.6)
	+ve	4 (22.2)	2 (11.1)	21 (30.0)	10 (16.9)	37 (22.4)

P53 and clinico-pathological parameters

The expression of p53 by DO-7 antibody failed to show any significant correlation with tumour type, size, grade, or lymph nodes status. The expression of phosphorylated p53 at serine 392 showed a correlation with tumour grade, in that positive cases were

predominantly grade III tumours (p=0.002). No association between phosphospecific p53 and the other clinico-pathological parameters was found (table 5.14).

Clinico-pathological Parameters		P53 DO-7 -ve No. (%)	DO-7 +ve	DO-7 P	Phosphospecific -ve No. (%)	Phosphospecific +v	Phosphospecific P
Type	IDC	95 (92.2)	62 (100.0)	0.080	120 (93.8)	37 (100.0)	0.297
	ILC	7 (6.8)	0 (0.0)		7 (5.5)	0 (0.0)	
	Others	1 (1.0)	0(0.0)		1 (0.8)	0 (0.0)	
Size/mm	<20	25 (24.5)	15 (25.4)	0.652	32 (25.6)	8 (22.2)	0.612
	20-50	70 (68.6)	42 (71.2)		85 (68.0)	27 (75.0)	
	>50	7 (6.9)	2 (3.4)		8 (6.4)	1 (22.4)	
Grade	I+II	30 (29.1)	11 (18.0)	0.113	39 (30.7)	2 (5.4)	0.002**
	III	73 (70.9)	50 (82.0)		88 (69.3)	35 (94.6)	
L.N	-ve	29 (28.7)	13 (21.7)	0.338	34 (27.4)	8 (21.6)	0.655
	1-3	31 (30.7)	25 (41.7)		41 (33.1)	15 (40.5)	
	>3	41 (40.6)	22 (36.7)		49 (39.5)	14 (37.8)	

Table 5.14: P53 and clinico-pathological parameters. Analyzed by Chi-square test, p values <0.05 are statistically significant, ** refers to significance at the 0.005 level.

P53 and other biological markers

P53 expression by both antibodies (DO-7 and phosphospecific p53) positively correlated with proliferation by MIB-1 (p=0.001) and Survivin expression (p<0.0001). P53 positive tumours were associated with high proliferation (figure 5.10) and with the presence of Survivin. P53 (DO-7) was also positively correlated with the inhibitor of apoptosis protein XIAP (p=0.02). P53 detected by both antibodies was inversely correlated with Bcl-2 expression (p=0.005 and p=0.004) respectively. The presence of $p21^{waf-1}$ and the presence of ER showed an inverse relation with the phosphorylated form of p53 (table 5.16).

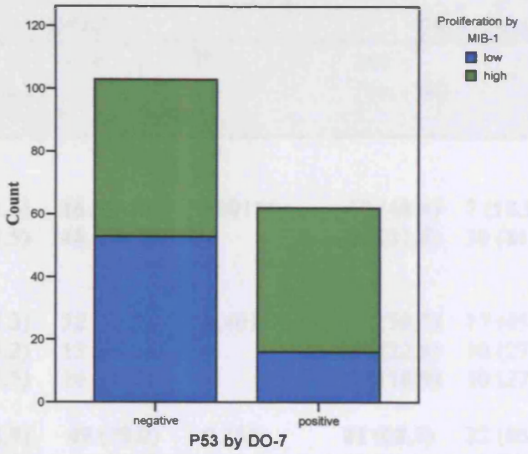


Figure 5.10: P53 DO-7 and proliferation MIB-1. Most of p53 positive cases were highly proliferating.

P53 and survival

Overall there was no relationship between the presence of p53 (DO-7) and the duration of survival. Those cases expressing phosphospecific p53 showed a significant correlation with shorter duration of survival (independent samples t-test, $p<0.0001$) (figure 5.11). When compared with treatment, those patients with phosphospecific p53 positivity who received either hormonal treatment or adjuvant chemotherapy and hormonal showed significant shorter survival than other groups (table 5.15).

P53 antibody	*Statistical Test*	*Neo-Adjuv.*	*Adjuv. Chemo.*	*Adjuv. +Hormon.*	*Hormon.*	*Total P value*
		p	*p*	*p*	*p*	
DO-7	T-test	0.904	0.520	0.405	0.746	0.276
Phosphospecific	T-test	0.873	0.170	0.026*	0.003**	<0.0001**

Table 5.15: Relation between p53 and survival in relation to treatment group.
* refers to significance at 0.05, ** refers to significance at 0.005.

Biological Markers		P53 DO-7 -ve No. (%)	+ve	P	Phosphospecific -ve No. (%)	+v	P
Proliferation (MIB1)							
	Low	53 (51.5)	16 (25.8)	0.001**	62 (48.4)	7 (18.9)	0.001**
	High	50 (48.5)	46 (74.2)		66 (51.6)	30 (81.1)	
Apoptosis %							
	<1	59 (57.3)	32 (52.5)	0.401	74 (58.3)	17 (45.9)	0.385
	1-2	26 (25.2)	13 (21.3)		29 (22.8)	10 (27.0)	
	>2	18 (17.5)	16 (26.2)		24 (18.9)	10 (27.0)	
Waf-1	-ve	71 (68.9)	49 (79.0)	0.158	88 (68.8)	32 (86.5)	0.033*
	+ve	32 (31.1)	13 (21.0)		40 (31.3)	5 (13.5)	
ChK-2	-ve	31 (30.1)	19 (30.6)	0.941	43 (33.6)	7 (18.9)	0.087
	+ve	72 (69.9)	43 (69.4)		85 (66.4)	30 (81.1)	
Bcl-2	-ve	43 (41.7)	40 (64.5)	0.005**	59 (46.1)	24 (64.9)	0.004**
	+ve	60 (58.3)	22 (35.5)		69 (53.9)	13 (35.1)	
Bax	-ve	8 (7.8)	5 (8.2)	0.936	10 (7.9)	3 (8.3)	0.928
	+ve	94 (92.2)	56 (91.8)		117 (92.1)	33 (91.7)	
Survivin	-ve	57 (55.3)	16 (26.2)	<0.0001**	67 (52.3)	6 (16.7)	< 0.0001**
	+ve	46 (44.7)	45 (73.8)		61 (47.7)	30 (83.3)	
XIAP	-ve	15 (14.6)	2 (3.2)	0.020**	16 (12.5)	1 (2.7)	0.084
	+ve	88 (85.4)	60 (96.8)		112 (87.5)	36 (97.3)	
ER	-ve	45 (43.7)	35 (57.4)	0.090	53 (41.4)	27 (75.0)	<0.0001**
	+ve	58 (56.3)	26 (42.6)		75 (58.6)	9 (25.0)	
PgR	-ve	38 (65.5)	21 (60.0)	0.592	43 (61.4)	16 (69.6)	0.082
	+ve	20 (34.5)	14 (40.0)		27 (38.6)	7 (30.4)	

Table 5.16: P53 and biological markers. Analyzed by Chi-square test, * refers to significance at 0.05, ** refers to significance at 0.005.

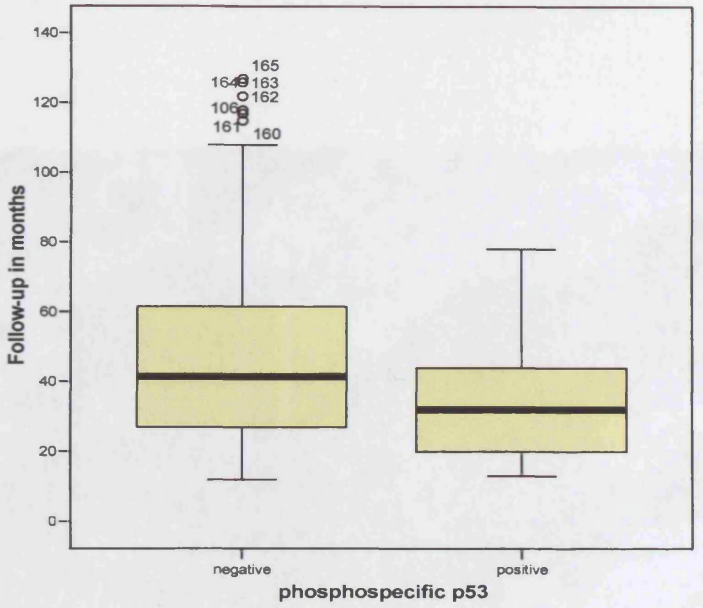


Figure 5.11: Boxplot showing phosphospecific p53 staining and survival. Phosphospecific p53 negative cases lived longer than positive cases ($p<0.0001$).

5.5.2 p21^{waf1}

Staining for p21^{waf-1} was predominantly nuclear with occasional faint cytoplasmic staining. Only nuclear staining was assessed, positive expression was considered when ≥ 5% of cells were stained (moderate/strong or strong) and negative when <5% of cells were stained. Using that cut-off, 27.3 % of tumours were positive for p21^{waf-1} (figure 5.12).

P21^{waf-1} and clinico-pathological parameters

P21^{waf-1} expression showed significant correlation with tumour grade. Grade III tumours were predominantly p21^{waf-1} negative. There was no correlation with other clinico-pathological parameters (table 5.17).

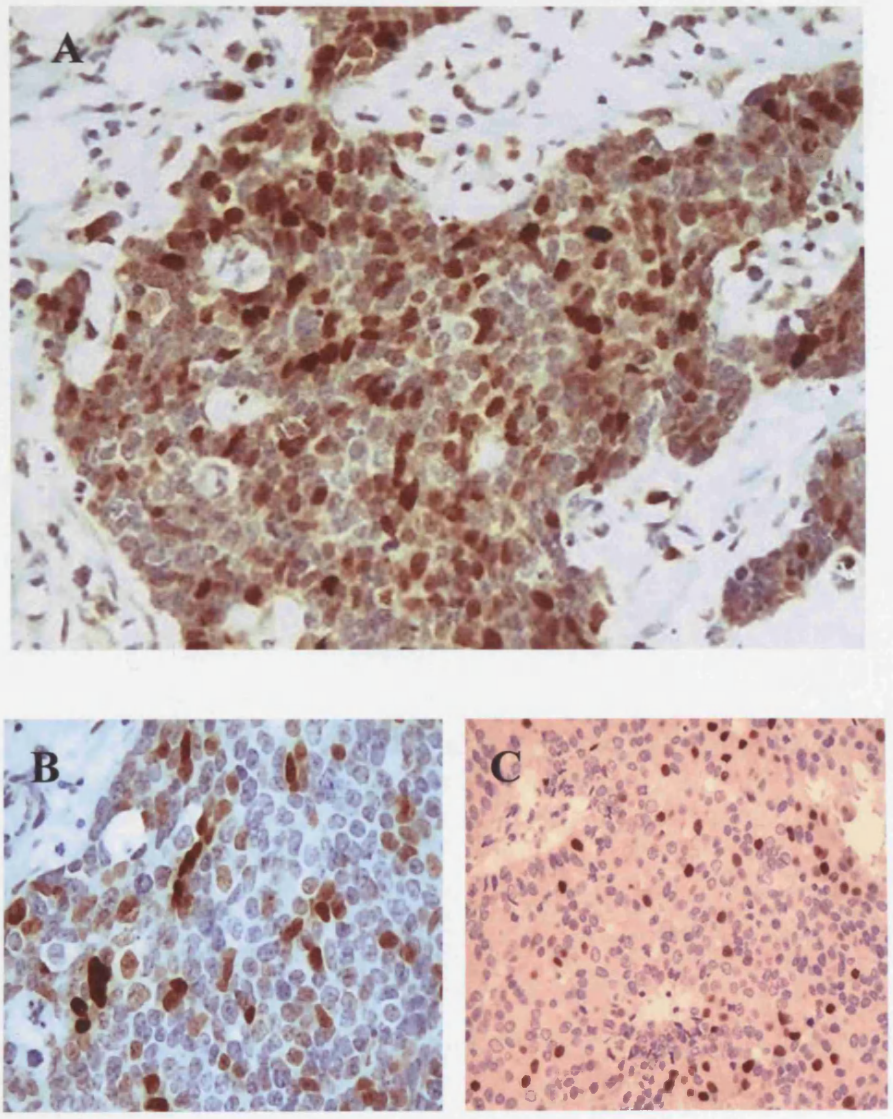


Figure 5.12: p21^{waf-1}immunohistochemistry. A, tumour with high percentage of stained cells (31.6 %). B, high power of A. C, tumour with low number of stained cells (2.2 % for the whole tumour).

Clinico-pathological Parameters.		P21^{waf-1}		
		-ve No (%)	+ve	P
Type	IDC	113 (94.2)	44 (97.8)	0.602
	ILC	6 (5.0)	1 (2.2)	
	Others	1 (0.8)	0 (0.0)	
Size/mm				
	<20	24 (20.3)	16 (37.2)	0.066
	20-50	86 (72.8)	26 (60.5)	
	>50	8 (6.8)	1 (2.3)	
Grade	I+II	24 (20.0)	17 (38.0)	0.015*
	III	96 (80.0)	27 (61.4)	
L.N	-ve	33 (28.0)	9 (2.9)	0.307
	1-3	37 (31.4)	19 (44.2)	
	>3	48 (40.7)	15 (34.9)	

Table 5.17: p21^{waf-1} and relationship to clinico-pathological parameters. Analyzed by Chi-square test, * refers to significance at 0.05 level.

P21^{waf-1} and biological markers

There was a significant correlation between p21^{waf-1} and the presence of ER. P21^{waf-1} positive tumours were predominantly ER positive (p=0.015) (table 5.18). As mentioned in 5.5.1, p21^{waf-1} negative tumours were more likely to express phosphospecific p53. There was no correlation with other biological markers.

Biological Markers		P21waf-1 -ve No (%)	P21waf-1 +ve No (%)	P
Proliferation	Low	51 (73.9)	69 (71.9)	0.772
	High	18 (26.1)	27 (28.1)	
Apoptosis %	<1	65 (71.4)	26 (28.6)	0.494
	1-2	31 (79.5)	8 (20.5)	
	>2	23 (67.6)	11 (32.4)	
Apoptosis/ 10HPF	<10	42 (70.0)	18 (30.0	0.774
	10-20	41 (75.4)	13 (24.1)	
	>20	36 (72.0)	14 (28.0)	
P53 (DO-7)	-ve	71 (68.9)	49 (79.0)	0.158
	+ve	32 (31.1)	13 (21.0)	
Phosphospecific p53	-ve	88 (68.8)	32 (86.5)	0.033*
	+ve	40 (31.3)	5 (13.5)	
ChK-2	-ve	37 (30.8)	13 (28.9)	0.809
	+ve	83 (69.2)	32 (71.1)	
Bcl-2	-ve	61 (49.2)	22 (48.9)	0.824
	+ve	59 (49.2)	23 (51.1)	
Bax	-ve	12 (10.0)	1 (2.3)	0.111
	+ve	108 (90.0)	42 (97.7)	
Survivin	-ve	52 (43.7)	21 (46.7)	0.733
	+ve	67 (56.3)	24 (53.3)	
XIAP	-ve	13 (10.8)	4 (8.9)	0.714
	+ve	107 (89.2)	41 (91.1)	
ER	-ve	65 (54.6)	15 (33.3)	0.015*
	+ve	54 (45.4)	30 (66.7)	
PgR	-ve	39 (60.9)	20 (69.0)	0.456
	+ve	25 (39.1)	9 (31.0)	

Table 5.18: p21waf-1 and relationship to biological markers. Analyzed by Chi-square test, * refers to significance at 0.05.

P21^{waf-1} and survival

The overall relationship between p21^{waf1} and duration of patient survival was of borderline significance (T-Test, p=0.055). When survival was assessed in relation to treatment group, a significant correlation was found between the presence of p21^{waf-1} and longer survival in those patients who received hormonal treatment only (p=0.014) (table 5.19).

Biological marker	*Statistical Test*	*Neo-Adjuv.*	*Adjuv. Chemo.*	*Adjuv. +Hormon.*	*Hormo.*	*Total P value*
		p	*p*	*p*	*p*	
P21^{waf-1}	T-Test	0.252	0.190	0.355	0.014*	0.055

Table 5.19: p21^{waf-1} and survival. * refers to significance at 0.05.

5.5.3 ChK-2

ChK2 protein was immunohistochemically expressed as dense nuclear staining. Tumours were considered positive if ≥20 % of tumour cells were stained and negative if <20 % of cells reacted. Accordingly, 69.7 % of tumours showed positive expression of ChK2. Staining was also seen in normal breast tissue adjacent to carcinomas in some cases (figure 5.13).

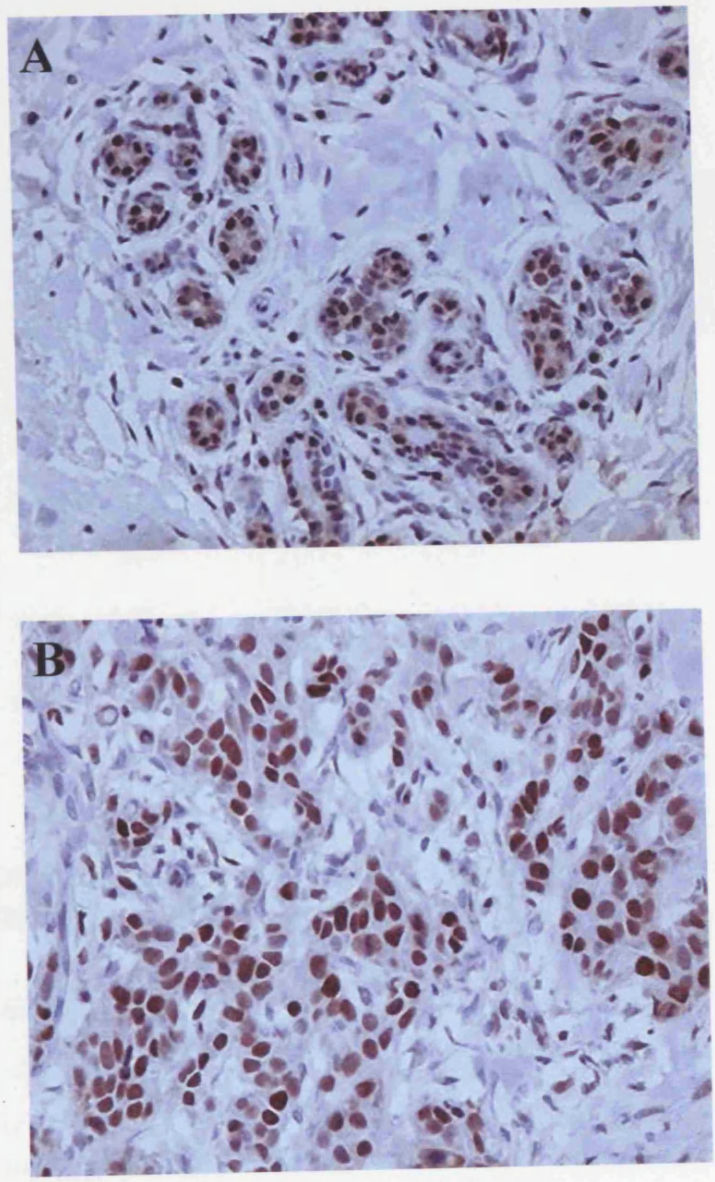


Figure 5.13: ChK2 staining. A: normal breast tissue close to a tumour, B: breast carcinoma.

ChK2 and clinicopathological parameters

ChK2 positive tumours showed a significant correlation with grade III tumours (p=0.003). There was no correlation with other clinicopathological parameters (table 5.20).

Clinicopathological Parameters		ChK2		
		-ve No. (%)	+ve	p
Type	IDC	46 (92.0)	111 (96.5)	0.235
	ILC	4 (8.0)	3 (2.6)	
	Others	0 (0.0)	1 (0.9)	
Size/mm	3-20	8 (16.0)	32 (28.8)	0.218
	21-30	39 (78.0)	73 (65.8)	
	31+	3 (6.0)	6 (5.4)	
Grade	I+II	20 (40.0)	21 (18.4)	0.003**
	III	30 (60.0)	93 (81.6)	
L.N	-ve	12 (24.0)	30 (27.0)	0.835
	1-3	19 (38.0)	37 (33.3)	
	>3	19 (38.0)	44 (39.6)	

Table 5.20: ChK2 and relationship to clinicopathological parameters. Analyzed by Chi-square test, ** refers to significance at 0.005.

ChK2 and biological markers

ChK2 showed a significant correlation with Survivin, ChK2 positive cases were predominantly Survivin positive (p=0.003). Also, the presence of ER was significantly correlated with lack of ChK2 (p=0.012) (table 5.21). Furthermore, as mentioned in 5.4.1 there was a correlation between high proliferation and ChK2 positivity.

Biological Markers		ChK2		
		-ve No. (%)	+ve	P
Proliferation	Low	29 (42.0)	21 (21.9)	0.005**
	High	40 (58.0)	75 (78.1)	
Apoptosis %	<1	29 (31.9)	62 (68.1)	0.657
	1-2	12 (30.8)	27 (69.2	
	>2	8 (23.5)	26 (76.5)	
Apoptosis/10HPF	<10	22 (36.7)	38 (63.3)	0.246
	10-20	16 (29.6)	38 (70.4)	
	>20	11 (22.0)	39 (78.0)	
P53 (DO-7)	-ve	31 (30.1)	19 (30.6)	0.941
	+ve	72 (69.9)	43 (69.4	
P53 phosphospecific	-ve	43 (33.6)	7 (18.9)	0.087
	+ve	85 (66.4)	30 (81.1)	
$P21^{waf-1}$	-ve	37 (30.8)	13 (28.9)	0.809
	+ve	83 (69.2)	32 (71.1)	
Bcl-2	-ve	23 (46.0)	60 (52.2)	0.466
	+ve	27 (54.0)	55 (47.8)	
Bax	-ve	6 (12.0)	7 (6.2)	0.207
	+ve	44 (88.0)	106 (93.8)	
Survivin	-ve	31 (62.0)	42 (36.8)	0.003**
	+ve	19 (38.0)	72 (63.2)	
XIAP	-ve	6 (12.0)	11 (9.6)	0.636
	+ve	44 (88.0)	104 (90.4)	
ER	-ve	17 (34.0)	63 (55.3)	0.012*
	+ve	33 (66.0)	51 (44.7)	
PgR	-ve	16 (59.3)	43 (65.2)	0.592
	+ve	11 (40.7)	23 (34.8)	

Table 5.21: Relationship of ChK2 to biological factors. Analyzed by Chi-square test, * refers to significance at 0.05 level. ** refers to significance at 0.005 level.

ChK2 and survival

There was no relationship between ChK2 expression and duration of patient survival nor was there a correlation with treatment group (table 5.22).

Biological marker	*Statistical Test*	*Neo-Adjuv.*	*Adjuv. Chemo.*	*Adjuv. +Hormon.*	*Hormo.*	*Total P value*
		P	*p*	*p*	*p*	
ChK2	T-Test	0.869	0.534	0.259	0.482	0.309

Table 5.22: ChK2 and survival in relation to treatment group.

5.5.4 Bcl-2

The anti-apoptotic protein bcl-2 was expressed as cytoplasmic staining with different degrees of intensity. It was heterogeneous among tumour cells (figure 5.14). A scoring system was used to assess staining and to classify cases into positive and negative (see 2.6.7). Lymphocytic infiltrate and remains of normal breast tissue around carcinoma always stained positive with bcl-2 and served as internal positive controls. Nearly half of cases (49.7%) were bcl-2 positive.

Bcl-2 and clinico-pathological parameters

There was a significant correlation between bcl-2 and tumour grade. Grade III tumours were predominantly bcl-2 negative ($p<0.0001$) (table 5.23). There was no correlation with other clinico-pathological parameters.

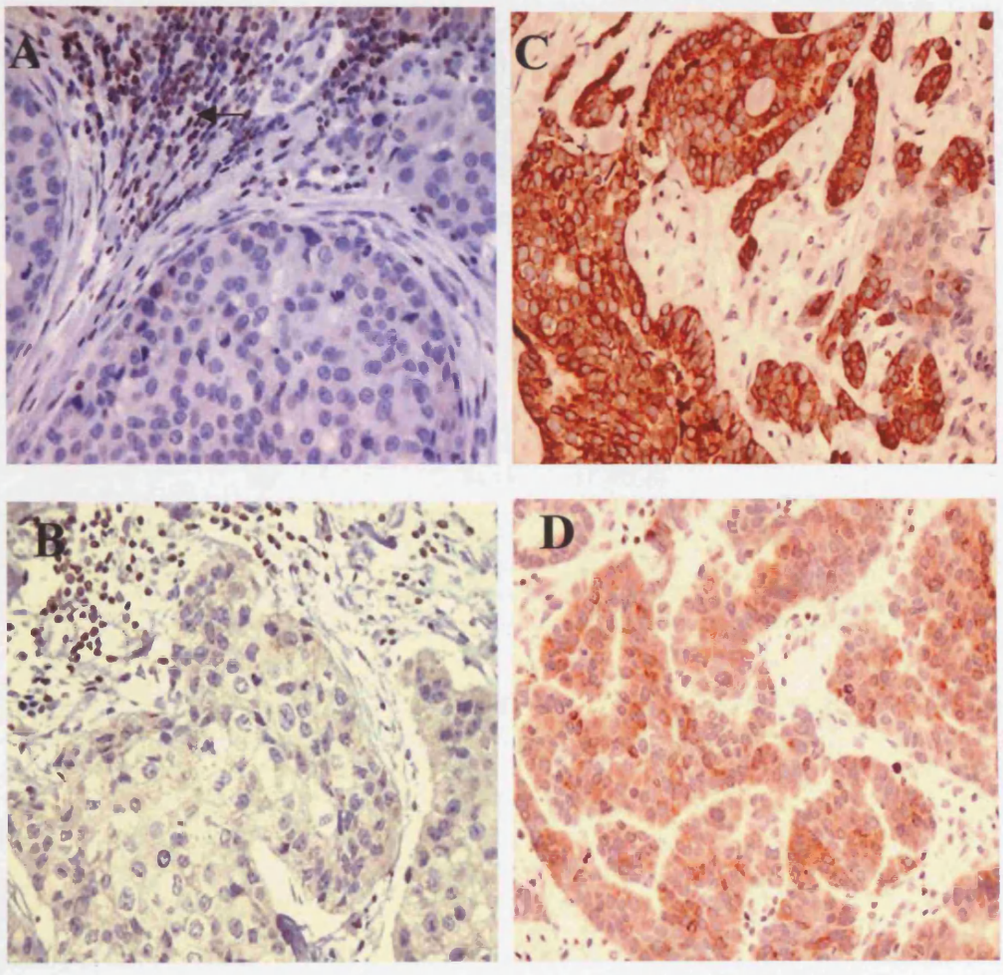


Figure 5.14: Bcl-2 in breast cancer. A, lack of bcl-2 but the lymphocytic infiltrate is positive (arrow). B, faint staining. C, very strong cytoplasmic staining. D, strong staining cells in between moderate staining.

Clinico-pathological Parameters		Bcl-2		
		-ve No. (%)	+ve	p
Type	IDC	80 (96.4)	77 (93.9)	0.550
	ILC	3 (3.6)	4 (4.9)	
	Others	0 (0.0)	1 (1.2)	
Size/mm	3-20	22 (27.2)	18 (22.5)	0.723
	21-30	54 (66.7)	58 (72.5)	
	31+	5 (6.2)	4 (5.0)	
Grade	I+II	11 (13.3)	30 (37.0)	<0.0001**
	III	72 (86.7)	51 (63.0)	
L.N	-ve	20 (24.7)	22 (27.5)	0.891
	1-3	28 (34.6)	28 (35.0)	
	>3	33 (40.7)	30 (37.5)	

Table 5.23: Relationship of bcl-2 reactivity to clinico-pathological parameters. Analyzed by Chi-square test, ** refers to significance at 0.005 level.

Bcl-2 and biological markers

There was an inverse correlation between bcl-2 positivity and the expression of Survivin (p=0.001). The presence of bcl-2 correlated with the presence of both ER and PgR (p<0.0001 for both) (table 5.24). There was an inverse correlation between bcl-2 positivity and high proliferation and p53 (DO-7 and phosphospecific p53) (see tables 12 and 5.9).

Biological Markers		Bcl-2		
		-ve No. (%)	+ve	P
Proliferation	Low	24 (34.8)	59 (61.5)	0.001**
	High	45 (65.2)	37 (38.5)	
Apoptosis %	<1	46 (50.5)	45 (49.5)	0.313
	1-2	16 (41.0)	23 (59.0)	
	>2	20 (58.8)	14 (41.2)	
Apoptosis/10HPF	<10	29 (48.3)	31 (51.7)	0.929
	10-20	27 (50.0)	27 (50.0)	
	>20	26 (52.0)	24 (48.0)	
P53 (DO-7)	-ve	43 (41.7)	40 (64.5)	0.005**
	+ve	60 (58.3)	22 (35.5)	
Phosphospecific p53	-ve	59 (46.1)	24 (64.9)	0.044*
	+ve	69 (53.9)	13 (35.1)	
ChK-2	-ve	23 (46.0)	60 (52.2)	0.466
	+ve	27 (54.0)	55 (47.8)	
Bax	-ve	5 (6.1)	8 (9.9)	0.373
	+ve	77 (93.9)	73 (90.1)	
Survivin	-ve	26 (31.3)	47 (58.0)	0.001**
	+ve	57 (68.7)	34 (42.0)	
XIAP	-ve	10 (12.0)	7 (8.5)	0.458
	+ve	73 (88.0)	75 (91.5)	
ER	-ve	60 (72.3)	20 (24.7)	<0.0001**
	+ve	23 (27.7)	61 (75.3)	
PgR	-ve	38 (88.4)	21 (42.0)	<0.0001**
	+ve	5 (11.6)	29 (58.0)	

Table 5.24: Relationship of bcl-2 to other biological markers.

Bcl-2 and survival

The presence of bcl-2 significantly correlated with longer duration of survival (T-Test p=0.010) (figure 5.15) and there was a trend towards increasing duration of survival relating to higher bcl-2 score (figure 5.16). When individual treatment groups were compared, survival in patients who received hormonal treatment was significantly longer than other treatment groups (table 5.25).

Biological marker	*Statistical Test*	*Neo-Adjuv.*	*Adjuv. Chemo.*	*Adjuv. +Hormon.*	*Hormo.*	*Total P value*
		P	*p*	*p*	*p*	
Bcl-2	T-Test	0.533	0.758	0.146	0.039*	0.010*

Table 5.25: Bcl-2 and survival in relation to treatment group.

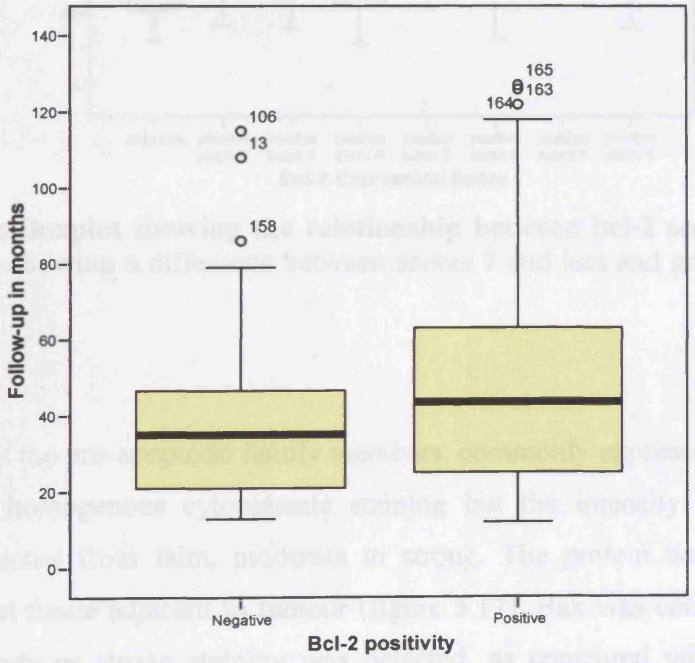


Figure 5.15: Boxplot showing bcl-2 positivity and survival. Positivity is associated with longer duration of survival.

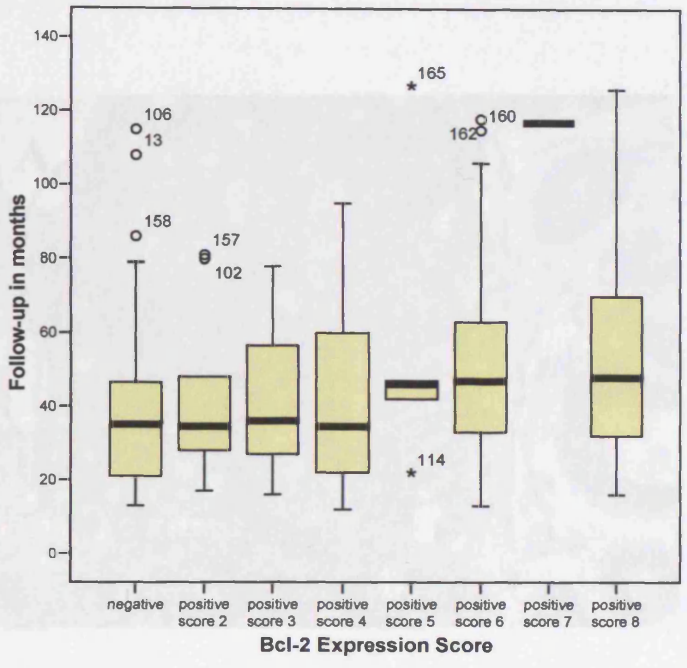


Figure 5.16: Boxplot showing the relationship between bcl-2 scores and survival. Bcl-2 scores showing a difference between scores 7 and less and greater than 7 (t-test, $p<0.0001$).

5.5.5 Bax

Bax is one of the pro-apoptotic family members, commonly expressed in cancer. It was detected as homogenous cytoplasmic staining but the intensity of staining varied between tumours from faint, moderate to strong. The protein was also detected in normal breast tissue adjacent to tumour (figure 5.17). Bax was considered as positive when moderate or strong staining was detected, as compared with the intensity of staining in normal breast tissue (positive control); staining greater than control was considered as strong; the same as normal considered moderate. When less than control, it was classed as negative. 91.0 % of tumours were considered as positive.

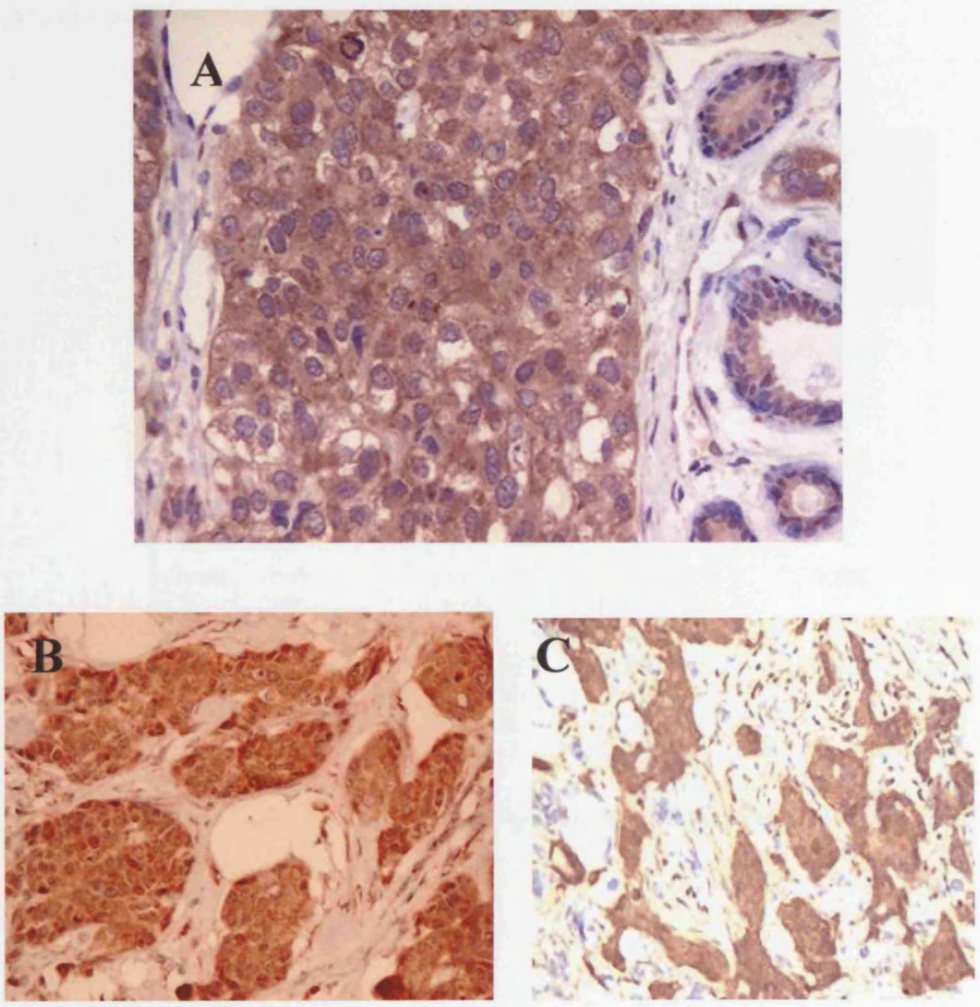


Figure 5.17: Bax immunohistochemical staining. A, strong bax staining in tumour cells with moderate staining in adjacent normal breast. B& C, breast cancer showing strong cytoplasmic staining for bax.

Bax and clinico-pathological parameters

There was a significant correlation between bax expression and tumour type. Infiltrating duct carcinomas were predominantly bax positive (p=0.002) (table 5.26). There was no significant correlation between bax expression and any of the other clinico-pathological parameters.

Clinico-pathological Parameters		Bax		
		-ve No. (%)	+ve	p
Type	IDC	10 (76.9)	145 (96.7)	0.002**
	ILC	3 (23.1)	4 (2.7)	
	Others	0 (0.0)	1 (0.7)	
Size/mm	3-20	1 (7.7)	38 (26.0)	0.128
	21-30	10 (76.9)	101 (69.2)	
	31+	21 (15.4)	7 (4.8)	
Grade	I+II	4 (30.8)	36 (24.2)	0.596
	III	9 (69.2)	113 (75.8)	
L.N	-ve	3 (23.1)	39 (26.7)	0.834
	1-3	4 (30.8)	52 (35.6)	
	>3	6 (46.2)	55 (37.7)	

Table 5.26: Bax and clinico-pathological parameters.

Bax and biological factors

There was no significant correlation between bax expression and any of the biological factors (table 5.27) or duration of survival (table 5.28).

Biological Markers		Bax		
		-ve No. (%)	+ve	P
Proliferation	Low	7 (10.3)	6 (6.3)	0.355
	High	61 (89.7)	89 (93.7)	
Apoptosis %	<1	6 (6.6)	85 (93.4)	0.743
	1-2	4 (10.3)	35 (89.7)	
	>2	3 (9.4)	29 (90.6)	
Apoptosis/10HPF	<10	3 (5.0)	57 (95.0)	0.255
	10-20	7 (13.0)	47 (87.0)	
	>20	3 (6.3)	45 (93.8)	
P53 (DO-7)	-ve	8 (7.8)	5 (8.2)	0.936
	+ve	94 (92.2)	56 (91.8)	
P53 phosphospecific	-ve	10 (7.9)	3 (8.3)	0.928
	+ve	117 (92.1)	33 (91.7)	
$P21^{waf-1}$	-ve	12 (10.0)	1 (2.3)	0.111
	+ve	108 (90.0)	42 (97.7)	
ChK-2	-ve	6 (12.0)	7 (6.2)	0.207
	+ve	44 (88.0)	106 (93.8)	
Bcl-2	-ve	5 (6.1)	8 (9.9)	0.373
	+ve	77 (93.9)	73 (90.1)	
Survivin	-ve	6 (50.0)	67 (44.7)	0.721
	+ve	6 (50.0)	83 (55.3)	
XIAP	-ve	1 (7.7)	16 (10.7)	0.736
	+ve	12 (92.3)	134 (89.3)	
ER	-ve	4 (30.8)	75 (50.3)	0.176
	+ve	9 (69.2)	74 (49.7)	
PgR	-ve	3 (37.5)	54 (65.1)	0.124
	+ve	5 (62.5)	29 (34.9)	
HER2	-ve	2 (60.0)	16 (42.1	0.449
	+ve	2 (40.0)	22 (57.9)	

Table 5.27: Relationship of bax to other biological factors.

Biological factor	*Statistical Test*	*Neo-Adjuv.*	*Adjuv. Chemo.*	*Adjuv. +Hormon.*	*Hormo.*	*Total P value*
		P	*p*	*p*	*p*	
Bax	T-Test	0.757	0.254	0.524	0.789	0.662

Table 5.28: bax and survival in relation to treatment group.

5.5.6 Inhibitor of apoptosis proteins (IAPs)

5.5.6.1 Survivin

Survivin staining was either nuclear, nuclear and/cytoplasmic or cytoplasmic (figure 5.19). The majority of tumours showed combined nuclear and cytoplasmic staining; there was no intratumoural variation in staining pattern. In some tumours, Survivin staining was mainly confined to cells in mitosis (mitotic pattern). A scoring method was used to classify tumours into positive and negative and to give a score for positive cases. Nuclear staining was scored 0-3 according to the percentage of positive nuclei (0 <5%, 1=5-20, 2=21-50, 3 >50%) and cytoplasmic staining scores 0-2 (0=weak or negative, 1=moderate, 2=strong). The overall score was the combination of these. Tumours with score ≤ 1 were considered negative for Survivin and those >1 considered positive. 55.2 % of tumours showed positive expression of Survivin.

Survivin and clinico-pathological parameters

There was a significant correlation between the presence of Survivin and grade III tumours ($p<0.0001$) (table 5.29). There was no correlation with other clinico-pathological parameters.

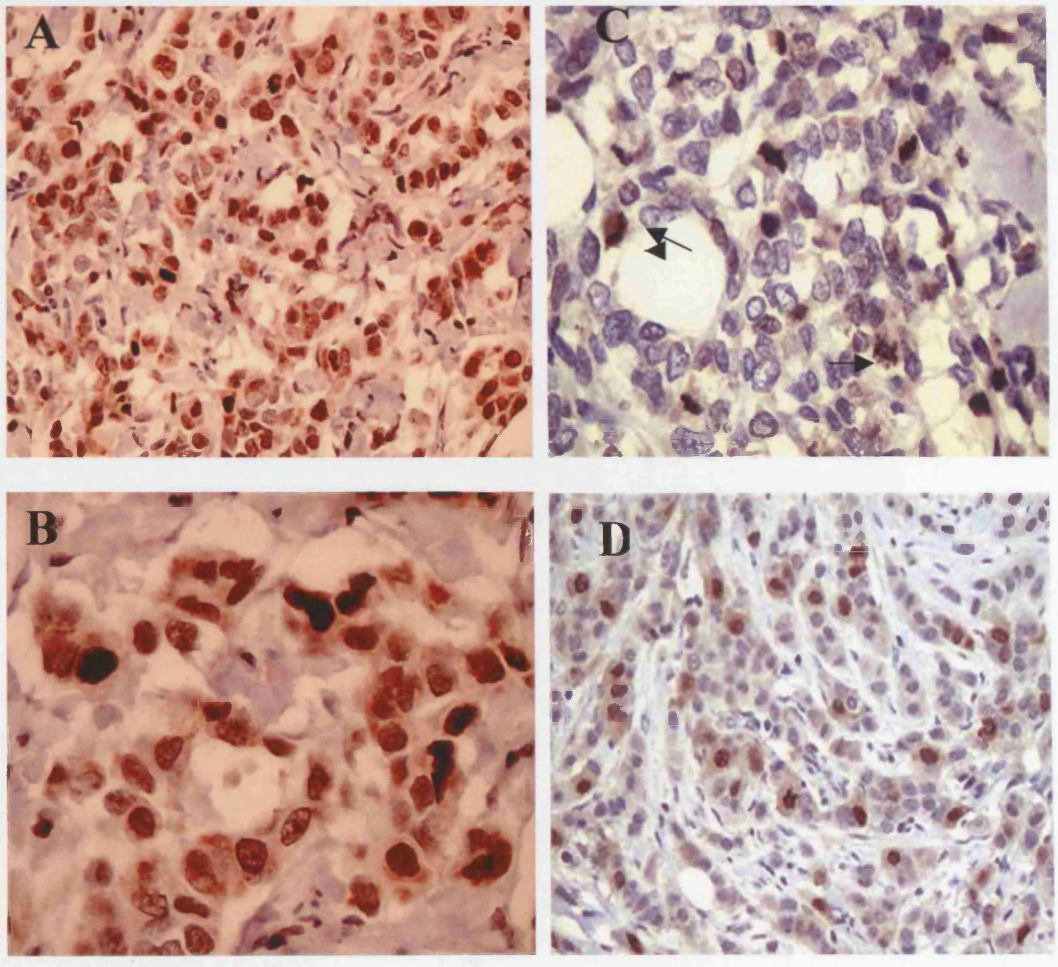


Figure 5.18: Survivin staining in breast cancer. A, both nuclear and cytoplasmic staining is evident in this section. B, high power view of A (40x). C, staining in mitotic cells (mitotic pattern). D, nuclear staining with faint cytoplasmic staining.

Survivin and biological factors

There was an inverse correlation between the presence of Survivin and lack of ER ($p<0.0001$), and PgR ($p=0.023$) (table 5.30). As stated previously, there was a significant correlation between Survivin and bcl-2, ChK-2, p53, apoptosis and proliferation. The presence of Survivin was associated with high proliferation ($p<0.0001$), number of apoptosis >20/10HPF ($p=0.031$), the presence of p53 ($p<0.0001$ with both antibodies DO-7 and phosphospecific p53) and ChK2 ($p=0.003$) and the absence of bcl-2 ($p=0.001$).

Clinico-pathological Parameters		Survivin		
		-ve No. (%)	+ve	p
Type	IDC	67 (91.8)	89 (97.8)	0.178
	ILC	5 (6.8)	2 (2.2)	
	Others	1 (1.4)	0 (0.0)	
Size/mm	<20	17 (23.6)	23 (26.1)	0.398
	20-50	49 (68.1)	62 (70.5)	
	>50	6 (8.3)	3 (3.4)	
Grade	I+II	33 (45.2)	8 (8.9)	<0.0001**
	III	40 (54.8)	82 (91.1)	
L.N	-ve	19 (26.4)	23 (26.1)	0.993
	1-3	25 (34.7)	30 (34.1)	
	>3	28 (38.9)	35 (39.8)	

Table 5.29: Survivin and clinico-pathological parameters.

Biological Markers		Survivin		
		-ve No. (%)	+ve	P
Proliferation	Low	47 (68.1)	26 (27.4)	< 0.0001**
	High	22 (31.9)	69 (72.6)	
Apoptosis %	<1	42 (46.2)	49 (53.8)	0.273
	1-2	19 (50.0)	19 (50.0)	
	>2	11 (32.4)	23 (67)	
Apoptosis/10HPF	<10	24 (40.0)	36 (60.0)	0.031*
	10-20	31 (58.5)	22 (41.5)	
	>20	17 (34.0)	33 (66.0)	
P53 (DO-7)	-ve	57 (55.3)	16 (26.2)	<0.0001**
	+ve	46 (44.7)	45 (73.8)	
P53 phosphospecific	-ve	67 (52.3)	6 (16.7)	< 0.0001**
	+ve	61 (47.7)	30 (83.3)	
$P21^{waf-1}$	-ve	52 (43.7)	21 (46.7)	0.733
	+ve	67 (56.3)	24 (53.3)	
ChK-2	-ve	31 (62.0)	42 (36.8)	0.003**
	+ve	19 (38.0)	72 (63.2)	
Bcl-2	-ve	26 (31.3)	47 (58.0)	0.001**
	+ve	57 (68.7)	34 (42.0)	
XIAP	-ve	9 (12.3)	8 (8.8)	0.460
	+ve	64 (87.7)	83 (91.2)	
ER	-ve	22 (30.1)	58 (64.4)	<0.0001**
	+ve	51 (69.9)	32 (35.6)	
PgR	-ve	18 (50.0)	41 (73.2)	0.023*
	+ve	18 (50.0)	15 (26.8)	
HER2	-ve	6 (28.6)	12 (57.1)	0.061
	+ve	15 (71.4)	9 (42.9)	

Table 5.30: Relationship of Survivin to other biological markers.

Survivin and survival

Patients with Survivin positive tumours had a shorter duration of survival than those that lacked it and this was statistically significant (t-test, $p<0.0001$) (figure 5.19A) (table 5.31). There was also a trend towards shorter survival and increased Survivin score (figure 5.19B). When compared with treatment, the presence of Survivin correlated with shorter survival in patients who received neo-adjuvant therapy and those received both adjuvant chemotherapy and hormonal therapy ($p<0.0001$) in both groups (table 5.31).

Treatment group	*Survivin*	*No*	*Mean survival*	*S.E. 0f Mean*	*p*
Neoadjuvant	-ve	6	47.83	3.47	<0.0001**
	+ve	12	23.83	2.72	
Adjuvant chemo.	-ve	5	58.20	12.63	0.090
	+ve	13	38.54	4.85	
Adjuvant Chemo. + Hormonal	-ve	23	54.96	5.19	<0.0001**
	+ve	46	32.33	2.84	
Adjuvant Hormonal	-ve	39	56.08	4.44	0.288
	+ve	20	47.20	7.17	
Total (all groups)	**-ve**	**73**	**55.19**	**3.07**	**<0.0001****
	+ve	**91**	**35.36**	**2.36**	

Table 5.31: Survivin and survival in relation to treatment group.

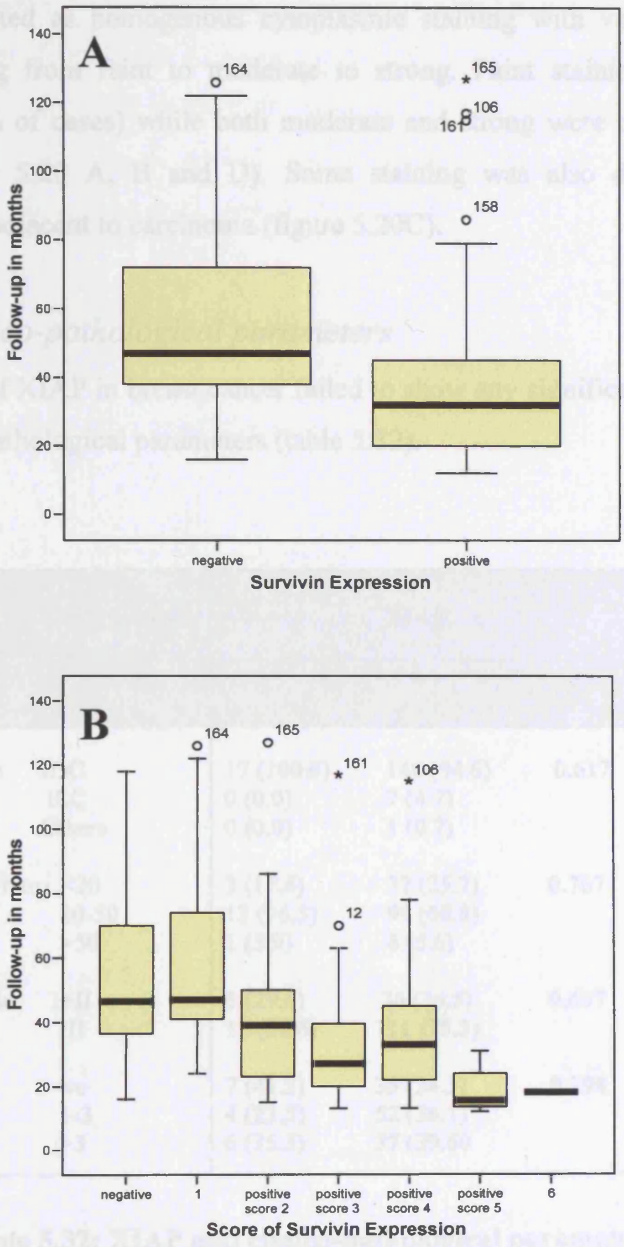


Figure 5.19: Survivin expression and survival. Boxplot showing A, the correlation between both surviving positivity and shorter survival. B. Lower score of Survivin associated with longer duration of survival.

5.5.6.2 XIAP

XIAP was detected as homogenous cytoplasmic staining with variation in staining intensity, ranging from faint to moderate to strong. Faint staining was considered negative (10.3 % of cases) while both moderate and strong were considered positive (89.7%) (figures 5.20 A, B and D). Some staining was also detected in normal epithelial breast adjacent to carcinoma (figure 5.20C).

XIAP and clinico-pathological parameters

The expression of XIAP in breast cancer failed to show any significant correlation with tumour clinico-pathological parameters (table 5.32).

Clinico-pathological Parameters		XIAP		
		-ve No. (%)	+ve	p
Type	IDC	17 (100.0)	140 (94.6)	0.617
	ILC	0 (0.0)	7 (4.7)	
	Others	0 (0.0)	1 (0.7)	
Size/mm	<20	3 (17.6)	37 (25.7)	0.767
	20-50	13 (76.5)	99 (68.8)	
	>50	1 (5.9)	8 (5.6)	
Grade	I+II	5 (29.4)	36 (24.5)	0.657
	III	12 (70.6)	111 (75.5)	
L.N	-ve	7 (41.2)	35 (24.3)	0.298
	1-3	4 (23.5)	52 (36.1)	
	>3	6 (35.3)	37 (39.60	

Table 5.32: XIAP and clinico-pathological parameters.

XIAP and biological markers

There was no correlation between XIAP expression and any of the biological markers (table 5.33), apart from the significant correlation between XIAP and p53 (DO-7) (p=0.020).

5.5.6.2 XIAP

XIAP was detected as homogenous cytoplasmic staining with variation in staining intensity, ranging from faint to moderate to strong. Faint staining was considered negative (10.3 % of cases) while both moderate and strong were considered positive (89.7%) (figures 5.20 A, C and D). Some staining was also detected in normal epithelial breast adjacent to carcinoma (figure 5.20B).

XIAP and clinico-pathological parameters

The expression of XIAP in breast cancer failed to show any significant correlation with tumour clinico-pathological parameters (table 5.32).

Clinico-pathological Parameters		XIAP		
		-ve No. (%)	+ve	p
Type	IDC	17 (100.0)	140 (94.6)	0.617
	ILC	0 (0.0)	7 (4.7)	
	Others	0 (0.0)	1 (0.7)	
Size/mm	<20	3 (17.6)	37 (25.7)	0.767
	20-50	13 (76.5)	99 (68.8)	
	>50	1 (5.9)	8 (5.6)	
Grade	I+II	5 (29.4)	36 (24.5)	0.657
	III	12 (70.6)	111 (75.5)	
L.N	-ve	7 (41.2)	35 (24.3)	0.298
	1-3	4 (23.5)	52 (36.1)	
	>3	6 (35.3)	37 (39.60	

Table 5.32: XIAP and clinico-pathological parameters.

XIAP and biological markers

There was no correlation between XIAP expression and any of the biological markers (table 5.33), apart from the significant correlation between XIAP and p53 (DO-7) (p=0.020).

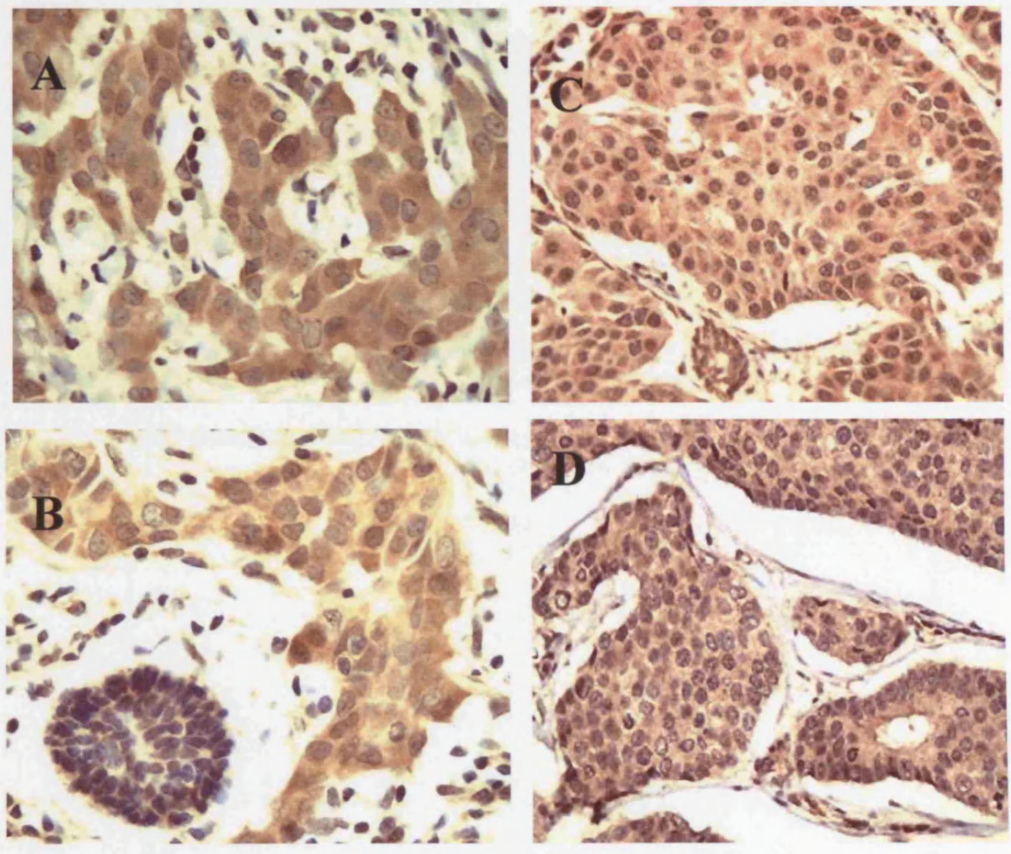


Figure 5.20: XIAP immunohistochemistry in breast cancer. A and C, strong cytoplasmic staining. B, faint staining in normal breast tissue near strong tumour staining. D, moderate cytoplasmic staining.

Biological Markers		XIAP		
		-ve No. (%)	+ve	P
Proliferation	Low	9 (13.0)	8 (8.3)	0.326
	High	60 (87.0)	88 (91.7)	
Apoptosis %	<1	11 (12.1)	80 (87.9)	0.171
	1-2	1 (2.6)	38 (97.4)	
	>2	5 (14.7)	29 (85.3)	
Apoptosis/10HPF	<10	8 (13.3)	52 (86.7)	0.581
	10-20	4 (7.4)	50 (92.6)	
	>20	5 (10.0)	45 (90)	
P53 (DO-7)	-ve	15 (14.6)	2 (3.2)	0.020**
	+ve	88 (85.4)	60 (96.8)	
P53 phosphospecific	-ve	16 (12.5)	1 (2.7)	0.084
	+ve	112 (87.5)	36 (97.3)	
$P21^{waf-1}$	-ve	13 (10.8)	4 (8.9)	0.714
	+ve	107 (89.2)	41 (91.1)	
ChK-2	-ve	6 (12.0)	11 (9.6)	0.636
	+ve	44 (88.0)	104 (90.4)	
Bcl-2	-ve	10 (12.0)	7 (8.5)	0.458
	+ve	73 (88.0)	75 (91.5)	
Bax	-ve	1 (7.7)	16 (10.7)	0.736
	+ve	12 (92.3)	134 (89.3)	
Survivin	-ve	9 (12.3)	8 (8.8)	0.460
	+ve	64 (87.7)	83 (91.2)	
ER	-ve	10 (58.8)	70 (47.6)	0.382
	+ve	7 (41.2)	77 (52.4)	
PgR	-ve	5 (100.0)	54 (61.4)	0.081
	+ve	0 (0.0)	34 (38.6)	
HER2	-ve	1 (33.3)	18 (45.0)	0.695
	+ve	2 (66.7)	22 (55.0)	

Table 5.33: Relationship of XIAP to other biological markers.

XIAP and survival

There was no correlation between XIAP and duration of survival either in total or in relation to treatment group (table 5.34).

Biological factor	*Statistical Test*	*Neo-Adjuv.*	*Adjuv. Chemo.*	*Adjuv. +Hormon.*	*Hormo.*	*Total P value*
		P	*p*	*p*	*p*	
XIAP	T-Test	0.678	0.342	0.930	0.085	0.267

Table 5.34: XIAP and survival in relation to treatment group.

5.6 Predictors of survival in relation to therapy

Assessment of clinico-pathological parameters revealed that tumour grade significantly correlated with survival irrespective of treatment received; therefore tumour grade was considered the most important clinico-pathological parameter for breast cancer outcome. Analysis of the other biological factors, previously shown to play important roles in breast cancer biology, showed that proliferation index, phosphorylated p53, bcl-2, ER and Survivin were significantly related to duration of survival and their risk values were used to stratify patients into high and low risk groups. Analysis of all these factors through Cox regression model showed that both tumour grade and Survivin were the most significant factors in duration of breast cancer survival and could be considered as independent prognostic factors (table 5.35).

When all clinico-pathological and biological factors were assessed by Cox regression analysis in relation to treatment received, Survivin correlated significantly with shorter duration of survival in the neo-adjuvant group (received chemotherapy and/or hormonal therapy) and in the combined adjuvant chemotherapy and hormonal therapy group. In those who received hormonal treatment only in the form of Tamoxifen, tumour size and

ER were correlated with longer duration of survival, but none of the factors correlated with survival for the adjuvant chemotherapy group.

	B	SE	Wald	df	Sig.	Exp(B)
p53phos	.018	.309	.003	1	.953	1.018
MIB1	.134	.294	.207	1	.649	1.143
Bcl2posit	.338	.296	1.305	1	.253	1.402
SurvPosit	.693	.248	7.827	1	.005	2.000
Type	-.041	.337	.015	1	.903	.960
Grade	1.477	.397	13.845	1	.000	4.379
NodeScore	.134	.171	.616	1	.433	1.143
NewSizeG	.228	.160	2.037	1	.153	1.257
ER	.014	.408	.001	1	.974	1.014
PgR	-.196	.360	.297	1	.586	.822
Treatment	-.901	.381	5.598	1	.018	.406

Table 5.35: Cox regression analysis. 'SurvPosit' denotes Survivin positivity.

Discussion

There are many factors that determine outcome from breast cancer that relate to the patient, the nature of the disease and the overall management. This study was undertaken to determine whether factors relating to proliferation and apoptosis could provide additional information about outcome for patients receiving neo-adjuvant therapy and different forms of adjuvant therapy. All patients had died from the disease so duration of survival was the key endpoint. This criterion for selection could affect some of the findings but was chosen because of consent and ethical considerations.

Methodology

Tumour sections from all cases were stained immunohistochemically using the standard ABC method with antigen retrieval by pressure cooking for 2 minutes in citrate buffer. The staining procedure was straight forward with no difficulties in optimization for most of antibodies used. For XIAP and Survivin, optimization of staining proved more difficult and the protocol was modified in certain steps to overcome this. For example,

antigen retrieval period was prolonged for 4 minutes for XIAP staining, and for two subsequent pressure cookings of 2 minutes each for Survivin. For these two antigens, endogenous peroxidase was blocked in hydrogen peroxide in methanol rather than in water.

For detection of apoptosis, M30 antibody was used. This antibody detects cleaved cytokeratin 18, which occurs in epithelial cells early in apoptosis. Studies have shown the usefulness of M30 in the detection of apoptosis in some human cancers, but in breast cancer there was little published work using this antibody. In this work, I found M30 staining an easy and reliable method for the detection of apoptotic cells both in cell lines and in paraffin sections.

Clinico-pathological features

The relationship between patient age and outcome is still an issue of debate. Previous studies have shown that women aged 40-49 years at diagnosis had the best prognosis (Sant et al., 2003; Sant et al., 1998) and that breast cancer patients under the age of 35 had the worst prognosis (Kuru et al., 2003). In this study, there was no significant difference between patient age and survival, which is in concordance with other studies (Ezzat et al., 1998). The lack of correlation between patient age and outcome could be attributed to underlying pathological factors as suggested by (Kollias et al., 1997).

The current clinico-pathological parameters include tumour type, size, grade and lymph node metastasis. Histological tumour type has been shown to relate to breast cancer prognosis (Allemani et al., 2004; Ellis et al., 1992). Most tumour types in this study were infiltrating ductal carcinomas and the number in other types was small, which is probably due to the selection criterion. In this study, tumours classified as infiltrating ductal carcinomas were associated with poor survival in comparison to other types (infiltrating lobular carcinomas) (Mersin et al., 2003). Arpino et al. (2004) showed that there are no survival advantages between these two types. The differences could be attributed to the smaller number of cases in the group of infiltrating lobular carcinomas and other types in comparison to the number of infiltrating ductal carcinomas included

in this study, although infiltrating lobular carcinomas in this study were associated with the presence of good prognosis markers (e.g. low proliferation).

Tumour size constitutes an important criterion for staging of breast cancers (TNM) classification. Tumour sizes were classified into three groups according to the TNM system; these were <20mm, 20-50mm and > 50mm. They were also classified into three groups according to the normal distribution of sizes among tumours, these were 3-20mm, 21-30mm and >30mm. Previous studies have shown that larger tumours are associated with poorer outcome (Fitzgibbons et al., 2000) and this may attributed to the increase in the number of lymph node metastasis with increased tumour sizes (Michaelson et al., 2003). In this study there was a correlation between duration of survival and size only for the group receiving hormonal therapy; smaller size tumours were associated with longer duration of survival.

Tumour histological grade reflects the degree of differentiation. It has been shown in several studies that histological grade predicts overall survival and disease-free survival (Elston and Ellis, 1991; Lundin et al., 2001; Simpson et al., 2000; Zaffaroni and Daidone, 2002) with worse survival in patients with poorly differentiated tumours. The findings in my study are in concordance with the previous reports as there was a highly significant correlation between tumour grade and duration of survival. The relationship was significant for all groups of patients who received adjuvant treatment (adjuvant chemotherapy, adjuvant chemotherapy plus hormonal therapy or hormonal therapy alone), but not for the neo-adjuvant treatment group. There were differences in relationship for the three groups; grade III tumours were associated with shorter survival for all groups; grade I/II were associated with longer duration of survival for hormonal therapy group. (Lundin et al., 2001) found tumour grade to be of value in the selection of patients for adjuvant treatment and (Faneyte et al., 2004) showed that high tumour grade is one of the factors that predict early failure after adjuvant treatment in high risk breast cancer patients.

Lymph node metastasis has been considered the most important factor among the clinico-pathological parameters for breast cancer prognosis; it has shown to predict both overall survival and disease-free survival in many studies (Axelsson et al., 1992; Carter et al., 1989). Not only the presence or absence of lymph node metastasis is important in prognosis but also the number of positive nodes as well; patients with four or more positive axillary nodes have worse prognosis and shorter survival than those with three or less positive nodes (Axelsson et al., 1992) and the relative mortality hazard increase by at least 1.3% with each additional involved node (Vinh-Hung et al., 2003). Recently, studies reported that this cutoff point is not enough and recommended the use of lymph node ratio as a prognostic factor (Voordeckers et al., 2004). I did not find either the lymph node status or the ratio of lymph node positivity to be significant in relation to duration of survival but the total number of lymph nodes removed showed a significant correlation with survival. Tumours with 15 or less lymph nodes were associated with longer survival than those with more than 15 lymph nodes. This is consistent with the results of previous studies (Krag and Single, 2003; van der Wal et al., 2002). However in my group of patients, different axillary surgical procedures were applied by different surgeons over a long time, making the significance of the number of lymph nodes removed more difficult to interpret.

Biological factors

The presence of steroid receptors (ER and PgR) correlated with longer survival. This could be attributed partly to the presence of receptors in tumours of lower grade (I+II) and the lack of receptors in grade III tumours, since I have found better tumour grade to be associated with longer survival. The correlation between the presence of receptors and longer survival was especially in the group of patients who received hormonal treatment. This is in concordance with the well-established role of steroid receptors in the selection of hormonal treatment for breast cancer patients and in the prediction of response to this treatment (Barnes et al., 2004). The probability of death from breast cancer is greater for patients with ER negative than ER positive tumours (Schairer et al., 2004). The role of steroid receptors in predicting the response to chemotherapy is less evident (MacGrogan et al., 1996). Miles et al. (1999) concluded that the greatest

benefit of adjuvant CMF was seen in women with ER negative tumours. Recent studies found that negative ER status is predictive of pathological complete response and consequent overall survival in operable breast cancer patients who received neo-adjuvant chemotherapy (Colleoni et al., 2004; Ring et al., 2004).

Assessment of proliferation-associated antigens such as Ki-67 by immunohistochemistry is an easy way to determine the growth fraction of tumours. In this study, highly proliferating tumours were associated with shorter survival, a finding that is in concordance with previous studies (Thor et al., 1999; van Diest et al., 2004). This could be related to the association of high proliferation with other poor prognostic factors such as high tumour grade and lack of ER.

It has also been shown that proliferation is useful in predicting which tumours would be more likely to respond to therapy. Aas et al. (2003) showed that a high cell proliferation rate was associated with resistance to doxorubicin, especially in tumours with wild-type p53. In my study, high proliferation correlated significantly with shorter survival in the overall group of patients and in those who received adjuvant hormonal and chemotherapy. This could be explained by the theory that tumour growth is regulated by the balance between cell proliferation and cell death; chemotherapeutic drugs perform their action through the induction of apoptosis and tamoxifen has been reported to induce apoptosis (Hickman, 1992) and also to reduce proliferation (Sutherland et al., 1983).

In breast cancer, proliferation and apoptosis are strongly correlated (Cameron et al., 1997). Previous studies have shown that patients with a high apoptotic index have shorter overall survival than those with low apoptotic index (de Jong et al., 2000; Zhang et al., 1998) but others have not shown this (Schondorf et al., 2004). The controversy between studies could be attributed to differences in the method of apoptosis detection. In this study, I assessed apoptotic index by two methods: the percentage of stained apoptotic cells in a total of 1000 tumour cells and the number of apoptotic cells in 10 high power fields (HPF), both methods correlated to each other.

Percentage of apoptosis showed a significant correlation with duration of survival in the group of patients who received neo-adjuvant chemotherapy. This result is supported by those of Takada et al. (2004) who showed the usefulness of the number of apoptotic cells as detected by M30 immunohistochemistry in the response to neo-adjuvant chemotherapy.

Tumour suppressor gene p53 plays a pivotal role in genomic integrity and regulation of tumour growth and progression. Its expression has been shown to play a role in the development of breast cancer, being absent in normal breast epithelium and gradually increase during the development of the disease (Zhang et al., 1997). In this study, all phosphospecific p53 positive tumours were total p53 positive (detected by DO-7). There was a significant correlation between p53 detected by phosphospecific antibody with the duration of survival. The phosphospecific p53 detects p53 protein that is phosphorylated at serine 392; all tumours that over-expressed the phosphorylated form of p53 were associated with shorter survival. Yap et al. (2004) have shown that phosphorylation of p53 at Serine 392 has a biological importance in regulating the oncogenic function of mutant p53 and concluded that the phosphorylation status of mutant p53 at that site plays an important role in determining tumour response to various cancer treatments. This could indicate that phosphorylation of p53 on serine 392 may have other biological functions independent of the DNA binding activity of p53. It has been shown that Serine 392 phosphorylation might activate tetramer formation to promote the dominant-negative effects of mutant form of p53 (Furihata et al., 2002). There was no correlation between total p53 detected by DO-7 antibody, the antibody that detects both wild-type and mutant forms of p53, and duration of survival. This may indicate that the expression of phosphorylated p53 protein reflects the functional status of p53 much better than the total p53 protein (wild-type and mutant). Also in other tumours, over-expression of Serine 392 phosphorylation has been shown to be an independent prognostic factor and associated with poorer prognosis (Matsumoto et al., 2004).

Lack of p53 function in tumours theoretically results in their resistance to chemotherapeutic drugs because of loss of the apoptotic functions of p53. The literature regarding the predictive role of p53 is conflicting. Geisler et al. (2001) showed that p53 mutations predict for resistance to doxorubicin in breast cancer patients. Other reports failed to show any significant relation (Colleoni et al., 1999). In this study, the presence of phosphorylated p53 correlated with shorter survival in those patients who received either hormonal therapy alone or combination of hormonal therapy and chemotherapy.

Another way to investigate the functional status of p53 in tumours is to evaluate its downstream effectors such as $p21^{waf-1}$ (el-Deiry et al., 1994). $p21^{waf-1}$ inhibits cyclin-dependant kinases and arrests the cell at the G1 and G2 phases of the cell cycle and therefore it considered to have tumour suppressor functions (Chen et al., 1995). In this study, the presence of $p21^{waf-1}$ was associated with some factors of good prognosis such as the presence of ER, lower histologic grade, and lack of phosphorylated p53, but there was no correlation with survival in general. There was a correlation with longer survival only in those patients who received hormonal treatment. This could be attributed to the association of $p21^{waf-1}$ with the presence of ER. Previous data on the role of $p21^{waf-1}$ in breast cancer survival is controversial. $p21^{waf-1}$ over-expression has been shown to be associated with shorter disease-free survival (Caffo et al., 1996) while Gohring et al. (2001) and Diab et al. (1997) reported that $p21^{waf-1}$ expression has no prognostic or predictive value in breast cancer. This controversy among studies may be attributed to the use of different cut-off points. Several reports considered the expression of $p21^{waf-1}$ in relation to p53 and recommended evaluating $p21^{waf-1}$ in relation to p53 (positive or negative) (Caffo et al., 1996; McClelland et al., 1999), the combined evaluation providing more accurate p53-dependent functions.

ChK-2 is also related to p53 function, but this works upstream of p53. Upon exposure to DNA-damaging agents, ChK-2 becomes activated and transmits signals to activate p53. It is therefore considered an essential checkpoint control of the cell cycle. There is also increasing evidence of the tumour suppressor functions of ChK-2 (Ahn et al., 2004). I found ChK-2 expression to be associated with higher tumor grade and with

expression of Survivin, but there was no association with the duration of survival, either in general or in relation to certain treatment groups.

P53 also regulates apoptosis through transcription of members of the bcl-2 family such as bcl-2 and bax. Although bcl-2 is an anti-apoptotic protein, most studies have found its expression to be associated with more favorable prognosis (Berardo et al., 1998; Daidone et al., 1999). In my study, the presence of bcl-2 correlated with presence of ER and low proliferation and lack of bcl-2 was associated with lack of Survivin and the presence of p53. There was also a significant correlation between the presence of bcl-2 and longer duration of survival, which is consistent with previous reports (Sjostrom et al., 2002; Yang et al., 2003). This could be attributed to the common association between bcl-2 expression and other factors of good prognosis such as low proliferation and the presence of ER. There was also a significant correlation with survival in patients who were received hormonal therapy; this could relate to the role of estrogen in regulating bcl-2 gene expression (Teixeira et al., 1995). Bax, another member of bcl-2 family, is induced by wild-type p53 and has pro-apoptotic functions. The role of bax as a prognostic factor in breast cancer is conflicting. In this study, bax protein expression by immunohistochemistry failed to show a significant correlation with the duration of overall survival or within treatment groups.

The group of inhibitor of apoptosis proteins (IAPs) is newly discovered and has been shown to play important role in both inhibition of apoptosis and regulation of mitosis (Altieri, 2003). Survivin is a member of this family that is commonly expressed in tumours more than in normal tissues. The presence of Survivin was associated with high proliferation, the presence of p53, ChK2, absence of bcl-2, and high tumour grade. Survivin expression showed a significant correlation with shorter duration of survival which may be attributed to its association with the unfavorable prognostic factors. Cox regression analysis showed that expression of Survivin is significant in survival ($p=0.005$) independent to the other biological markers, thesis suggesting that Survivin expression is an independent factor in breast cancer prognosis. The results of this study support those of previous studies (Span et al., 2004; Tanaka et al., 2000; Zhang et al.,

2004). Furthermore, Span et al. (2004) proposed that Survivin could be useful as a new marker to stratify breast cancer patients for different treatment modalities. Those patients who received either neo-adjuvant treatment or adjuvant chemotherapy and hormonal therapy had shorter duration of survival when their tumours expressed Survivin. Another member of the IAPs family is XIAP which is considered the most potent member in inhibiting apoptosis. In this study, the presence of XIAP was associated with the presence of p53 (DO-7), but failed to show any correlation with other clinico-pathological, other biological factors or treatment groups.

Conclusions

To conclude, results of this part of the study support the view that evaluation of clinico-pathological factors such as tumour size, grade and lymph node status in breast cancer, is not enough to select patients for certain treatment regimes or to predict overall survival and strongly suggests that biological factors should be used. Univariate analysis showed that the expression of certain biological factors were significant in relation to both treatment regimes and overall survival. High proliferation index, the presence of phosphorylated p53, low expression of bcl-2 and over-expression of Survivin were associated with shorter durations of survival and could be considered as poor prognosis factors, selecting for short term survival from breast cancer. This study also supports the role of the previously well-established factors such as high tumour grade as poor prognosis factor in breast cancer, and presence of steroid receptors as a better prognosis factor. With multivariate Cox regression analysis, both tumour grade and Survivin expression showed independent prognostic value ($p<0.0001$ and $p=0.005$) respectively. This provides strong evidence for the importance of Survivin expression as a new prognostic marker in breast cancer. Expression of the phosphorylated form of p53 showed prognostic significance rather than the total p53 detected by DO-7 antibody.

The relevance of these biological factors differed in relation to different treatment groups. Survivin expression showed significant prognostic value in the group of breast cancer patients who received neo-adjuvant treatment (chemotherapy and or hormonal

therapy). Both Survivin positivity and high tumour grade were associated with poor survival for those who received combined adjuvant chemotherapy and hormonal therapy, this means that grade III tumours with positive Survivin expression may not benefit from this form of treatment. For those who received hormonal treatment only, both smaller tumour size and presence of ER showed significantly related to longer survival. Total p53 detected with DO-7 antibody showed no relation to treatment, but the presence of phosphorylated form of p53 is significantly related to shorter survival for those patients who received either adjuvant chemotherapy and hormonal therapy or hormonal therapy alone. This indicates that tumours expressing phosphorylated p53 may not benefit from this treatment. Within the group of patients studied there were no factors could subdivide those who received adjuvant chemotherapy alone into who would have greater or lesser benefit.

In summary, tumour grade, ER, proliferation, phosphorylated p53 and Survivin appeared to be the most important factors, with Survivin a marker worthy of further evaluation.

CHAPTER 6:

GENERAL DISCUSSION

Breast cancer is the most common malignancy among women especially in the western world. Many breast cancer patients receive chemotherapy as part of their treatment. The selection of patients for certain treatments currently rely upon clinico-pathological parameters such as tumour size, lymph node status, grade of malignancy and steroid hormone receptor status. Unfortunately this is not enough since there are no specific markers of response to chemotherapy and patients suffer from the hazardous side effects. The role of the pathologist has now expanded to provide other factors that could predict outcome and in particular response to different treatments.

Most chemotherapeutic drugs induce their action through the induction of apoptosis and the tumour suppressor gene p53 as a 'guardian of genomic integrity' plays a central role in cell cycle checkpoints and regulation of the apoptotic process after DNA damage. Therefore, evaluating the expression of p53 as well as other biological factors involved in the control of apoptosis may provide useful information and add new predictive and prognostic factors for breast cancer patients. Previous studies on the role of p53 and apoptotic proteins have been inconclusive.

The aims of this study were to assess proliferation index, apoptosis and the expression of p53, $p21^{waf-1}$, ChK2, apoptotic regulatory proteins (bcl-2, bax, bcl-x), and the newly recognized inhibitor of apoptosis proteins (IAPs) Survivin and XIAP in breast cancer cell lines; assess how these biological factors related to the ability of the chemotherapeutic drugs to suppress proliferation and induce apoptosis in these cells; and study the expression of these factors in breast tumours in order to clarify their role in prognosis and response to different treatment regimes.

In vitro studies

Proliferation index in breast cancer cells studied ranged from 26.39 % in T47-D cells to 48 % HBL-100 cells. Experiments showed that cell proliferation was markedly suppressed with treatment in those with wild-type p53 and to a lesser extent in other cell lines. Cells treated with paclitaxil showed morphological change in the form of condensed clumped nuclear chromatin, a state resembles abnormal mitosis. This feature was not evident after doxorubicin treatment. The marked suppression of proliferation in

wild type p53 cells suggested the importance of p53 and proliferation in breast cancer response and resistance to treatment.

Although the cell lines studied were different in their p53 mutation status, all showed low levels of apoptosis, but this was markedly induced after treatment. The induction of apoptosis was marked early after treatment for MCF-7, at 48 hours for ZR-75 cells and at different times for the other cell lines. Since both MCF-7 and ZR-75 cells have wild type p53 this suggests that there are both p53-dependent and p53-independent pathways for inducing apoptosis (Lu et al., 2005). There are several methods for detecting apoptosis in cells and tissues, but detection of cleaved cytokeratin 18 with the monoclonal antibody M30 proved to be a reliable method.

P53 was detected in breast cell lines with mutated p53 (T47-D, MDA-MB-231, MDA-MB-468) and in the immortalized normal cell line that has stabilized p53 (HBL-100). It was not detected in cell lines with wild type p53 (MCF-7 and ZR-75). This supports reports that have correlated the detection of p53 by immunohistochemistry with the presence of p53 mutations. After treatment with chemotherapeutic drugs, p53 was induced in cell lines with wild-type p53, more prominently after treatment with the DNA damaging drug doxorubicin. This is in agreement with previous reports (el-Deiry et al., 1994). The phosphorylated form of p53 was also induced after treatment with the DNA-damaging drug doxorubicin but to a lesser extent after paclitaxil treatment.

The down stream effector protein, $p21^{waf-1}$ was expressed in wild type p53 cell lines more than in other cell lines and upon treatment, it was markedly induced in p53 wild-type cells. It was also induced to a lesser extent in other cell lines. This indicates the presence of p53-independent pathway for $p21^{waf-1}$ induction. $P21^{waf-1}$ induces cell cycle arrest and performs a tumour suppressor function.

The anti-apoptotic protein bcl-2 was strongly expressed in one cell line (ZR-75) and its expression was down regulated after treatment. The down regulation of bcl-2 was accompanied by increased induction of apoptosis in this cell line. This was in agreement with those of (Lima et al., 2004) who found that down-regulation of bcl-2 sensitize breast cancer cells MCF-7 to the effect of chemotherapeutic drugs etopozide

and doxorubicin. Bax was expressed in most of breast cancer cell lines in varying degrees. It was induced after treatment mainly in those with wild-type p53.

The inhibitor of apoptosis proteins, Survivin and XIAP were detected in all cell lines with little variation. XIAP was down regulated markedly by treatment in MCF-7 cells, to a slight degree in other cells, but there was no effect on ZR-75 cells. Survivin was down regulated also in MCF-7 and other cells, but it was slightly up regulated in ZR-75 cells. This may indicate a role for p53 and bcl-2 in the regulation of IAPs.

Tissue studies

Clinico-pathological parameters

Evaluation of the current clinico-pathological factors showed significant correlation of tumour grade in relation to prognosis ($p<0.0001$) and response to all forms of adjuvant therapy. Multivariate analysis showed tumour grade as an independent prognostic factor ($p<0.0001$). This is supported by most of studies and now it became clear that tumour grade is important in predicting breast cancer prognosis. Most recent studies showed that grade III tumours are associated with frequent and complex genetic aberrations than those in grade I and tubular breast carcinomas (Simpson et al., 2005). No correlation was found with either tumour size or lymph node metastasis.

Biological markers

Data on the role of proliferation in breast cancer prognosis are conflicting, but the majority of studies concluded that high proliferation is associated with worse prognosis (vanDiest et al., 2004; Veronese et al., 1993). In this study, tumours with high proliferation index were associated with shorter duration of survival ($p=0.001$) and especially in those patients who received combined adjuvant and hormonal therapy ($p=0.26$). This in concordance with most of the previous studies which revealed that high proliferating tumours are more aggressive and more resistant to therapy especially the combined adjuvant chemotherapy and hormonal therapy (tamoxifen).

The role of apoptosis (detected with M30) in breast cancer has rarely been evaluated in relation to outcome or response to therapy. In one study, apoptosis index was correlated with outcome in patients who received neo-adjuvant chemotherapy (Takada et al.,

2004). In my study, two different methods were used to quantitate the apoptotic index in breast tumours and there was no correlation with the duration of survival, but apoptotic index expressed as a percentage of apoptosis significantly correlated with the response to neo-adjuvant chemotherapy ($p=0.005$), which is in concordance with the result of the previously mentioned study.

Total p53 (by DO-7) was not predictive to either duration of patient survival or response to treatment, while the expression of the phosphorylated form of the protein at serine 392 correlated with survival ($p<0.0001$), response to combined adjuvant and hormonal treatment ($p=0.026$) and also the response to hormonal therapy ($p=0.003$). This indicates the importance of the phosphorylated protein rather than the total p53, and detection of the phosphorylated protein by immunohistochemistry may reflects the p53 functional status much better than the total protein.

Expression of the down stream effector protein p21 was associated with better survival (although not to a significant level, $p=0.055$) and with good prognostic factors. It was also correlated with survival in patients who received hormonal treatment ($p=0.014$). The presence of $p21^{waf-1}$ negatively correlated with the presence of phosphorylated p53; there was no relation with total p53. This is further evidence that the phosphorylated form of p53 reflects the functional activity of p53. The positive correlation of $p21^{waf-1}$ with ER may explain the correlation with hormonal treatment and better survival.

Expression of the p53 up-stream regulatory protein, ChK2 was associated with higher tumour grade, high proliferation and the presence of Survivin, but there was no correlation with either duration of survival or response to therapy.

Although bcl-2 is an anti-apoptotic protein, it is usually associated with better prognosis (Yang et al., 2003). In this study, bcl-2 negatively correlated with p53, phosphorylated p53, proliferation and Survivin. It also positively correlated with ER and PgR. High bcl-2 expression was associated with longer duration of survival ($p=0.01$) and correlated with response to hormonal therapy ($p=0.03$). This could be explained by the association of bcl-2 with factors of good prognosis and the presence of hormonal

receptors. The pro-apoptotic protein bax showed no correlation with survival or response to therapy. This is in concordance with other studies (Sirvent et al., 2004).

Members of the inhibitor of apoptosis proteins are showing promising roles in breast cancer prognosis and response to therapy. They are more prominently expressed in tumours and cancer cell lines than in normal tissues. High expression of Survivin was associated with shorter duration of survival ($p<0.0001$) and correlated also with response to treatment with neo-adjuvant chemotherapy and combined adjuvant therapy and hormonal therapy. Multivariate analysis showed the expression of Survivin as an independent predictive factor ($p=0.005$). XIAP showed no significant correlations.

Correlation between in vitro and tissue studies

Suppression of cell proliferation after treatment indicated that assessment of proliferation index in tumours could provide information for response or resistance. In this study, high proliferation was an indication of poor prognosis. Studies have shown that high proliferation is correlated with p53 mutations and resistance to chemotherapy (Aas et al., 2003).

Apoptosis was induced in both wild type and mutant cell lines, but it was marked in p53 wild type cells. This indicates the essential role of p53 in regulating apoptosis. However, the percentage of apoptotic cells detected with M30 failed to show a significant correlation with survival. This may indicate that tumour progression and aggressiveness is attributed not to the number of apoptotic cells alone, but to the balance between apoptosis and proliferation. Therefore, assessment of biological factors controlling both processes is important.

Upon treatment with DNA-damaging agents, p53 was activated and the protein stabilized. Regulation of the oncogenic functions of p53 is mainly through phosphorylation. In this study and in other studies, the presence of the phosphorylated protein reflected the functional status of p53 and associated with poorer prognosis.

The induction of $p21^{waf\text{-}1}$ was marked in wild-type p53 cells and negatively correlated with p53 in cells and tissues. This means that the expression of $p21^{waf\text{-}1}$ reflects the

functional status of p53, although p53-independent pathways for induction of $p21^{waf-1}$exist.

Bcl-2 as an anti-apoptotic member of bcl-2 family resists apoptosis. In ZR-75 cells (bcl-2 highly expressing cells), induction of apoptosis with chemotherapeutic drugs delayed to 48h after treatment; this was accompanied by down regulation of bcl-2.
In tumour tissues, the presence of bcl-2 was associated with factors of better prognosis.

Survivin and XIAP were down regulated in MCF-7 possibly through a p53-dependent mechanism. However, the high expression of bcl-2 in ZR-75 cells (wild type p53) inhibited the effect of bcl-2 on IAPs. This may indicate the important role of bcl-2 in the regulation of IAPs. In tissues, the association of Survivin expression with factors of poor prognosis rendered Survivin as an independent prognostic factors in breast cancer. XIAP was only down regulated in MCF-7 cells and in tissues showed no significant correlations.

Conclusion

My study has shown that phosphorylated p53 rather than total p53 could provide important information about outcome and response to therapy. The more recently described inhibitor of apoptosis protein Survivin, has proved to be a protein of interest for both duration of survival and treatment.

Future work

There are certain areas of this study which merit further investigation to clarify their significance in breast cancer and their applicability.

- Assessment of p53 mutational status of breast cancers and how phosphorylation status related to this, since it is recognized that immunohistochemistry is only a surrogate marker of mutation status.

- Further assessment of the relationship of Survivin and XIAP to bcl-2 and its role in their regulation.

- Application of the prognostic markers to different groups of patients receiving defined treatments (e.g. within clinical trials). The study group used did have limitations.

CHAPTER 7:

REFERENCES

Aas, T., Borresen, A. L., Geisler, S., Smith-Sorensen, B., Johnsen, H., Varhaug, J. E., Akslen, L. A., and Lonning, P. E. (1996). Specific P53 mutations are associated with de novo resistance to doxorubicin in breast cancer patients. *Nat Med* **2**, 811-4.

Aas, T., Geisler, S., Eide, G. E., Haugen, D. F., Varhaug, J. E., Bassoe, A. M., Thorsen, T., Berntsen, H., Borresen-Dale, A. L., Akslen, L. A., and Lonning, P. E. (2003). Predictive value of tumour cell proliferation in locally advanced breast cancer treated with neoadjuvant chemotherapy. *Eur J Cancer* **39**, 438-46.

Abd El-Rehim, D. M., Pinder, S. E., Paish, C. E., Bell, J. A., Rampaul, R. S., Blamey, R. W., Robertson, J. F., Nicholson, R. I., and Ellis, I. O. (2004). Expression and co-expression of the members of the epidermal growth factor receptor (EGFR) family in invasive breast carcinoma. *Br J Cancer* **91**, 1532-42.

Ahn, J., Urist, M., and Prives, C. (2003). Questioning the role of checkpoint kinase 2 in the p53 DNA damage response. *J Biol Chem* **278**, 20480-9.

Ahn, J., Urist, M., and Prives, C. (2004). The Chk2 protein kinase. *DNA Repair (Amst)* **3**, 1039-47.

Alison, M. R. (1999). Identifying and quantifying apoptosis: a growth industry in the face of death. *J Pathol* **188**, 117-8.

Allemani, C., Sant, M., Berrino, F., Aareleid, T., Chaplain, G., Coebergh, J. W., Colonna, M., Contiero, P., Danzon, A., Federico, M., Gafa, L., Grosclaude, P., Hedelin, G., Mace-Lesech, J., Garcia, C. M., Paci, E., Raverdy, N., Tretarre, B., and Williams, E. M. (2004). Prognostic value of morphology and hormone receptor status in breast cancer - a population-based study. *Br J Cancer* **91**, 1263-8.

Altieri, D. C. (2001). The molecular basis and potential role of survivin in cancer diagnosis and therapy. *Trends Mol Med* **7**, 542-7.

Altieri, D. C. (2003). Survivin and apoptosis control. *Adv Cancer Res* **88**, 31-52.

Ambrosini, G., Adida, C., and Altieri, D. C. (1997). A novel anti-apoptosis gene, survivin, expressed in cancer and lymphoma. *Nat Med* **3**, 917-21.

Ambrosini, G., Adida, C., Sirugo, G., and Altieri, D. C. (1998). Induction of apoptosis and inhibition of cell proliferation by survivin gene targeting. *J Biol Chem* **273**, 11177-82.

Anderson, E. (2003). The use of steroid receptors in predicting response to hormonal therapy. *In* "Prognostic and predictive factors in breast cancer" (R. A. Walker, ed.), pp. 135-146. Martin Dunitz, London and New York.

Archer, C. D., Parton, M., Smith, I. E., Ellis, P. A., Salter, J., Ashley, S., Gui, G., Sacks, N., Ebbs, S. R., Allum, W., Nasiri, N., and Dowsett, M. (2003). Early changes in apoptosis and proliferation following primary chemotherapy for breast cancer. *Br J Cancer* **89**, 1035-41.

Arends, M. J., Morris, R. G., and Wyllie, A. H. (1990). Apoptosis. The role of the endonuclease. *Am J Pathol* **136**, 593-608.

Arpino, G., Bardou, V. J., Clark, G. M., and Elledge, R. M. (2004). Infiltrating lobular carcinoma of the breast: tumor characteristics and clinical outcome. *Breast Cancer Res* **6**, R149-56.

Asada, M., Yamada, T., Ichijo, H., Delia, D., Miyazono, K., Fukumuro, K., and Mizutani, S. (1999). Apoptosis inhibitory activity of cytoplasmic p21(Cip1/WAF1) in monocytic differentiation. *Embo J* **18**, 1223-34.

Ashcroft, M., and Vousden, K. H. (1999). Regulation of p53 stability. *Oncogene* **18**, 7637-43.

Ashe, P. C., and Berry, M. D. (2003). Apoptotic signaling cascades. *Prog Neuropsychopharmacol Biol Psychiatry* **27**, 199-214.

Axelsson, C. K., Mouridsen, H. T., and Zedeler, K. (1992). Axillary dissection of level I and II lymph nodes is important in breast cancer classification. The Danish Breast Cancer Cooperative Group (DBCG). *Eur J Cancer* **28A**, 1415-8.

Bai, M., Agnantis, N. J., Kamina, S., Demou, A., Zagorianakou, P., Katsaraki, A., and Kanavaros, P. (2001). In vivo cell kinetics in breast carcinogenesis. *Breast Cancer Res* **3**, 276-83.

Barboule, N., Chadebech, P., Baldin, V., Vidal, S., and Valette, A. (1997). Involvement of p21 in mitotic exit after paclitaxel treatment in MCF-7 breast adenocarcinoma cell line. *Oncogene* **15**, 2867-75.

Barnes, D. M., Dublin, E. A., Fisher, C. J., Levison, D. A., and Millis, R. R. (1993). Immunohistochemical detection of p53 protein in mammary carcinoma: an important new independent indicator of prognosis? *Hum Pathol* **24**, 469-76.

Barnes, D. M., Millis, R. R., Gillett, C. E., Ryder, K., Skilton, D., Fentiman, I. S., and Rubens, R. D. (2004). The interaction of oestrogen receptor status

and pathological features with adjuvant treatment in relation to survival in patients with operable breast cancer: a retrospective study of 2660 patients. *Endocr Relat Cancer* **11**, 85-96.

Bartek, J., Iggo, R., Gannon, J., and Lane, D. P. (1990). Genetic and immunochemical analysis of mutant p53 in human breast cancer cell lines. *Oncogene* **5**, 893-9.

Bartek, J., and Lukas, J. (2003). Chk1 and Chk2 kinases in checkpoint control and cancer. *Cancer Cell* **3**, 421-9.

Bell, D. W., Varley, J. M., Szydlo, T. E., Kang, D. H., Wahrer, D. C., Shannon, K. E., Lubratovich, M., Verselis, S. J., Isselbacher, K. J., Fraumeni, J. F., Birch, J. M., Li, F. P., Garber, J. E., and Haber, D. A. (1999). Heterozygous germ line hCHK2 mutations in Li-Fraumeni syndrome. *Science* **286**, 2528-31.

Berardo, M. D., Elledge, R. M., de Moor, C., Clark, G. M., Osborne, C. K., and Allred, D. C. (1998). bcl-2 and apoptosis in lymph node positive breast carcinoma. *Cancer* **82**, 1296-302.

Berns, E. M., Foekens, J. A., Vossen, R., Look, M. P., Devilee, P., Henzen-Logmans, S. C., van Staveren, I. L., van Putten, W. L., Inganas, M., Meijer-van Gelder, M. E., Cornelisse, C., Claassen, C. J., Portengen, H., Bakker, B., and Klijn, J. G. (2000). Complete sequencing of TP53 predicts poor response to systemic therapy of advanced breast cancer. *Cancer Res* **60**, 2155-62.

Berns, E. M., Klijn, J. G., van Putten, W. L., de Witte, H. H., Look, M. P., Meijer-van Gelder, M. E., Willman, K., Portengen, H., Benraad, T. J., and Foekens, J. A. (1998). p53 protein accumulation predicts poor response to tamoxifen therapy of patients with recurrent breast cancer. *J Clin Oncol* **16**, 121-7.

Berns, E. M., van Staveren, I. L., Look, M. P., Smid, M., Klijn, J. G., and Foekens, J. A. (1998). Mutations in residues of TP53 that directly contact DNA predict poor outcome in human primary breast cancer. *Br J Cancer* **77**, 1130-6.

Bianchi, S., Calzolari, A., Vezzosi, V., Zampi, G., Cardona, G., Cataliotti, L., Bonardi, R., and Ciatto, S. (1997). Lack of prognostic value of p53 protein expression in node-negative breast cancer. *Tumori* **83**, 669-72.

Birnbaum, M. J., Clem, R. J., and Miller, L. K. (1994). An apoptosis-inhibiting gene from a nuclear polyhedrosis virus encoding a polypeptide with Cys/His sequence motifs. *J Virol* **68**, 2521-8.

Blagosklonny, M. V., Schulte, T. W., Nguyen, P., Mimnaugh, E. G., Trepel, J., and Neckers, L. (1995). Taxol induction of p21WAF1 and p53 requires c-raf-1. *Cancer Res* **55**, 4623-6.

Blajeski, A. L., Phan, V. A., Kottke, T. J., and Kaufmann, S. H. (2002). G(1) and G(2) cell-cycle arrest following microtubule depolymerization in human breast cancer cells. *J Clin Invest* **110**, 91-9.

Blandino, G., Levine, A. J., and Oren, M. (1999). Mutant p53 gain of function: differential effects of different p53 mutants on resistance of cultured cells to chemotherapy. *Oncogene* **18**, 477-85.

Bloom, H. J., and Richardson, W. W. (1957). Histological grading and prognosis in breast cancer; a study of 1409 cases of which 359 have been followed for 15 years. *Br J Cancer* **11**, 359-77.

Bonadonna, G., Veronesi, U., Brambilla, C., Ferrari, L., Luini, A., Greco, M., Bartoli, C., Coopmans de Yoldi, G., Zucali, R., Rilke, F., and et al. (1990). Primary chemotherapy to avoid mastectomy in tumors with diameters of three centimeters or more. *J Natl Cancer Inst* **82**, 1539-45.

Borresen, A. L., Andersen, T. I., Eyfjord, J. E., Cornelis, R. S., Thorlacius, S., Borg, A., Johansson, U., Theillet, C., Scherneck, S., Hartman, S., and et al. (1995). TP53 mutations and breast cancer prognosis: particularly poor survival rates for cases with mutations in the zinc-binding domains. *Genes Chromosomes Cancer* **14**, 71-5.

Borst, M. J., and Ingold, J. A. (1993). Metastatic patterns of invasive lobular versus invasive ductal carcinoma of the breast. *Surgery* **114**, 637-41; discussion 641-2.

Bray, F., McCarron, P., and Parkin, D. M. (2004). The changing global patterns of female breast cancer incidence and mortality. *Breast Cancer Res* **6**, 229-39.

Brown, A. L., Lee, C. H., Schwarz, J. K., Mitiku, N., Piwnica-Worms, H., and Chung, J. H. (1999). A human Cds1-related kinase that functions downstream of ATM protein in the cellular response to DNA damage. *Proc Natl Acad Sci U S A* **96**, 3745-50.

Brown, J. M., and Wouters, B. G. (1999). Apoptosis, p53, and tumor cell sensitivity to anticancer agents. *Cancer Res* **59**, 1391-9.

Buchholz, T. A., Davis, D. W., McConkey, D. J., Symmans, W. F., Valero, V., Jhingran, A., Tucker, S. L., Pusztai, L., Cristofanilli, M., Esteva, F. J.,

Hortobagyi, G. N., and Sahin, A. A. (2003). Chemotherapy-induced apoptosis and Bcl-2 levels correlate with breast cancer response to chemotherapy. *Cancer J* **9**, 33-41.

Buja, L. M., Eigenbrodt, M. L., and Eigenbrodt, E. H. (1993). Apoptosis and necrosis. Basic types and mechanisms of cell death. *Arch Pathol Lab Med* **117**, 1208-14.

Bunz, F., Hwang, P. M., Torrance, C., Waldman, T., Zhang, Y., Dillehay, L., Williams, J., Lengauer, C., Kinzler, K. W., and Vogelstein, B. (1999). Disruption of p53 in human cancer cells alters the responses to therapeutic agents. *J Clin Invest* **104**, 263-9.

Buschmann, T., Potapova, O., Bar-Shira, A., Ivanov, V. N., Fuchs, S. Y., Henderson, S., Fried, V. A., Minamoto, T., Alarcon-Vargas, D., Pincus, M. R., Gaarde, W. A., Holbrook, N. J., Shiloh, Y., and Ronai, Z. (2001). Jun NH2-terminal kinase phosphorylation of p53 on Thr-81 is important for p53 stabilization and transcriptional activities in response to stress. *Mol Cell Biol* **21**, 2743-54.

Buzdar, A. (1998). The place of chemotherapy in the treatment of early breast cancer. *Br J Cancer* **78 Suppl 4**, 16-20.

Caffo, O., Doglioni, C., Veronese, S., Bonzanini, M., Marchetti, A., Buttitta, F., Fina, P., Leek, R., Morelli, L., Palma, P. D., Harris, A. L., and Barbareschi, M. (1996). Prognostic value of p21(WAF1) and p53 expression in breast carcinoma: an immunohistochemical study in 261 patients with long-term follow-up. *Clin Cancer Res* **2**, 1591-9.

Cameron, D. A., Ritchie, A. A., Langdon, S., Anderson, T. J., and Miller, W. R. (1997). Tamoxifen induced apoptosis in ZR-75 breast cancer xenografts antedates tumour regression. *Breast Cancer Res Treat* **45**, 99-107.

Campiglio, M., Somenzi, G., Olgiati, C., Beretta, G., Balsari, A., Zaffaroni, N., Valagussa, P., and Menard, S. (2003). Role of proliferation in HER2 status predicted response to doxorubicin. *Int J Cancer* **105**, 568-73.

Caron de Fromentel, C., Nardeux, P. C., Soussi, T., Lavialle, C., Estrade, S., Carloni, G., Chandrasekaran, K., and Cassingena, R. (1985). Epithelial HBL-100 cell line derived from milk of an apparently healthy woman harbours SV40 genetic information. *Exp Cell Res* **160**, 83-94.

Carstens, P. H., Greenberg, R. A., Francis, D., and Lyon, H. (1985). Tubular carcinoma of the breast. A long term follow-up. *Histopathology* **9**, 271-80.

Carter, C. L., Allen, C., and Henson, D. E. (1989). Relation of tumor size, lymph node status, and survival in 24,740 breast cancer cases. *Cancer* **63**, 181-

Chang, Q., Liu, Z. R., Wang, D. Y., Kumar, M., Chen, Y. B., and Qin, R. Y. (2004). Survivin expression induced by doxorubicin in cholangiocarcinoma. *World J Gastroenterol* **10**, 415-8.

Chehab, N. H., Malikzay, A., Appel, M., and Halazonetis, T. D. (2000). Chk2/hCds1 functions as a DNA damage checkpoint in G(1) by stabilizing p53. *Genes Dev* **14**, 278-88.

Chen, Y. Q., Cipriano, S. C., Arenkiel, J. M., and Miller, F. R. (1995). Tumor suppression by p21WAF1. *Cancer Res* **55**, 4536-9.

Chiou, S. K., Jones, M. K., and Tarnawski, A. S. (2003). Survivin - an anti-apoptosis protein: its biological roles and implications for cancer and beyond. *Med Sci Monit* **9**, PI25-9.

Clahsen, P. C., van de Velde, C. J., Duval, C., Pallud, C., Mandard, A. M., Delobelle-Deroide, A., van den Broek, L., Sahmoud, T. M., and van de Vijver, M. J. (1998). p53 protein accumulation and response to adjuvant chemotherapy in premenopausal women with node-negative early breast cancer. *J Clin Oncol* **16**, 470-9.

Cleator, S., Parton, M., and Dowsett, M. (2002). The biology of neoadjuvant chemotherapy for breast cancer. *Endocr Relat Cancer* **9**, 183-95.

Clem, R. J., and Miller, L. K. (1994). Control of programmed cell death by the baculovirus genes p35 and iap. *Mol Cell Biol* **14**, 5212-22.

Colleoni, M., Orvieto, E., Nole, F., Orlando, L., Minchella, I., Viale, G., Peruzzotti, G., Robertson, C., Noberasco, C., Galimberti, V., Sacchini, V., Veronesi, P., Zurrida, S., Orecchia, R., and Goldhirsch, A. (1999). Prediction of response to primary chemotherapy for operable breast cancer. *Eur J Cancer* **35**, 574-9.

Colleoni, M., Viale, G., Zahrieh, D., Pruneri, G., Gentilini, O., Veronesi, P., Gelber, R. D., Curigliano, G., Torrisi, R., Luini, A., Intra, M., Galimberti, V., Renne, G., Nole, F., Peruzzotti, G., and Goldhirsch, A. (2004). Chemotherapy is more effective in patients with breast cancer not expressing steroid hormone receptors: a study of preoperative treatment. *Clin Cancer Res* **10**, 6622-8.

Cooper, H. S., Patchefsky, A. S., and Krall, R. A. (1978). Tubular carcinoma of the breast. *Cancer* **42**, 2334-42.

Crook, N. E., Clem, R. J., and Miller, L. K. (1993). An apoptosis-inhibiting

baculovirus gene with a zinc finger-like motif. *J Virol* **67**, 2168-74.

Daidone, M. G., Luisi, A., Veneroni, S., Benini, E., and Silvestrini, R. (1999). Clinical studies of Bcl-2 and treatment benefit in breast cancer patients. *Endocr Relat Cancer* **6**, 61-8.

de Jong, J. S., van Diest, P. J., and Baak, J. P. (2000). Number of apoptotic cells as a prognostic marker in invasive breast cancer. *Br J Cancer* **82**, 368-73.

DeGregori, J., Leone, G., Miron, A., Jakoi, L., and Nevins, J. R. (1997). Distinct roles for E2F proteins in cell growth control and apoptosis. *Proc Natl Acad Sci U S A* **94**, 7245-50.

Deveraux, Q. L., and Reed, J. C. (1999). IAP family proteins--suppressors of apoptosis. *Genes Dev* **13**, 239-52.

Deveraux, Q. L., Roy, N., Stennicke, H. R., Van Arsdale, T., Zhou, Q., Srinivasula, S. M., Alnemri, E. S., Salvesen, G. S., and Reed, J. C. (1998). IAPs block apoptotic events induced by caspase-8 and cytochrome c by direct inhibition of distinct caspases. *Embo J* **17**, 2215-23.

Diab, S. G., Yu, Y. Y., Hilsenbeck, S. G., Allred, D. C., and Elledge, R. M. (1997). WAF1/CIP1 protein expression in human breast tumors. *Breast Cancer Res Treat* **43**, 99-103.

Dixon, J. M., Anderson, T. J., and Miller, W. R. (2002). Neoadjuvant endocrine therapy of breast cancer: a surgical perspective. *Eur J Cancer* **38**, 2214-21.

Dragovich, T., Rudin, C. M., and Thompson, C. B. (1998). Signal transduction pathways that regulate cell survival and cell death. *Oncogene* **17**, 3207-13.

Duckett, C. S., Nava, V. E., Gedrich, R. W., Clem, R. J., Van Dongen, J. L., Gilfillan, M. C., Shiels, H., Hardwick, J. M., and Thompson, C. B. (1996). A conserved family of cellular genes related to the baculovirus iap gene and encoding apoptosis inhibitors. *Embo J* **15**, 2685-94.

Dulic, V., Stein, G. H., Far, D. F., and Reed, S. I. (1998). Nuclear accumulation of p21Cip1 at the onset of mitosis: a role at the G2/M-phase transition. *Mol Cell Biol* **18**, 546-57.

Earnshaw, W. C., Martins, L. M., and Kaufmann, S. H. (1999). Mammalian caspases: structure, activation, substrates, and functions during

apoptosis. *Annu Rev Biochem* **68**, 383-424.

Eastman, A. (2004). Cell cycle checkpoints and their impact on anticancer therapeutic strategies. *J Cell Biochem* **91**, 223-31.

el-Deiry, W. S. (1998). Regulation of p53 downstream genes. *Semin Cancer Biol* **8**, 345-57.

el-Deiry, W. S., Harper, J. W., O'Connor, P. M., Velculescu, V. E., Canman, C. E., Jackman, J., Pietenpol, J. A., Burrell, M., Hill, D. E., Wang, Y., and et al. (1994). WAF1/CIP1 is induced in p53-mediated G1 arrest and apoptosis. *Cancer Res* **54**, 1169-74.

el-Deiry, W. S., Tokino, T., Velculescu, V. E., Levy, D. B., Parsons, R., Trent, J. M., Lin, D., Mercer, W. E., Kinzler, K. W., and Vogelstein, B. (1993). WAF1, a potential mediator of p53 tumor suppression. *Cell* **75**, 817-25.

el-Deiry, W. S., Tokino, T., Waldman, T., Oliner, J. D., Velculescu, V. E., Burrell, M., Hill, D. E., Healy, E., Rees, J. L., Hamilton, S. R., and et al. (1995). Topological control of p21WAF1/CIP1 expression in normal and neoplastic tissues. *Cancer Res* **55**, 2910-9.

Elledge, R. M., Green, S., Howes, L., Clark, G. M., Berardo, M., Allred, D. C., Pugh, R., Ciocca, D., Ravdin, P., O'Sullivan, J., Rivkin, S., Martino, S., and Osborne, C. K. (1997). bcl-2, p53, and response to tamoxifen in estrogen receptor-positive metastatic breast cancer: a Southwest Oncology Group study. *J Clin Oncol* **15**, 1916-22.

Ellis, I. O., Galea, M., Broughton, N., Locker, A., Blamey, R. W., and Elston, C. W. (1992). Pathological prognostic factors in breast cancer. II. Histological type. Relationship with survival in a large study with long-term follow-up. *Histopathology* **20**, 479-89.

Ellis, P. A., Smith, I. E., McCarthy, K., Detre, S., Salter, J., and Dowsett, M. (1997). Preoperative chemotherapy induces apoptosis in early breast cancer. *Lancet* **349**, 849.

Elston, C. W., and Ellis, I. O. (1991). Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up. *Histopathology* **19**, 403-10.

Elston, C. W., and Ellis, I. O. (1998). Classification of malignant breast disease. *In* "The breast/systemic pathology" (W. S. C. Symmers, ed.), Vol. 13, pp. 239-247.

Estevez, L. G., and Gradishar, W. J. (2004). Evidence-based use of

neoadjuvant taxane in operable and inoperable breast cancer. *Clin Cancer Res* **10**, 3249-61.

Evan, G., and Littlewood, T. (1998). A matter of life and cell death. *Science* **281**, 1317-22.

Ezzat, A., Raja, M. A., Zwaan, F., Brigden, M., Rostom, A., and Bazarbashi, S. (1998). The lack of age as a significant prognostic factor in non-metastatic breast cancer. *Eur J Surg Oncol* **24**, 23-7.

Fan, S., el-Deiry, W. S., Bae, I., Freeman, J., Jondle, D., Bhatia, K., Fornace, A. J., Jr., Magrath, I., Kohn, K. W., and O'Connor, P. M. (1994). p53 gene mutations are associated with decreased sensitivity of human lymphoma cells to DNA damaging agents. *Cancer Res* **54**, 5824-30.

Faneyte, I. F., Peterse, J. L., Van Tinteren, H., Pronk, C., de Vries, E. G., Rodenhuis, S., and Van De Vijver, M. J. (2004). Predicting early failure after adjuvant chemotherapy in high-risk breast cancer patients with extensive lymph node involvement. *Clin Cancer Res* **10**, 4457-63.

Ferreira, C. G., van der Valk, P., Span, S. W., Jonker, J. M., Postmus, P. E., Kruyt, F. A., and Giaccone, G. (2001). Assessment of IAP (inhibitor of apoptosis) proteins as predictors of response to chemotherapy in advanced non-small-cell lung cancer patients. *Ann Oncol* **12**, 799-805.

Finlay, C. A., Hinds, P. W., Tan, T. H., Eliyahu, D., Oren, M., and Levine, A. J. (1988). Activating mutations for transformation by p53 produce a gene product that forms an hsc70-p53 complex with an altered half-life. *Mol Cell Biol* **8**, 531-9.

Fisher, B., Bryant, J., Wolmark, N., Mamounas, E., Brown, A., Fisher, E. R., Wickerham, D. L., Begovic, M., DeCillis, A., Robidoux, A., Margolese, R. G., Cruz, A. B., Jr., Hoehn, J. L., Lees, A. W., Dimitrov, N. V., and Bear, H. D. (1998). Effect of preoperative chemotherapy on the outcome of women with operable breast cancer. *J Clin Oncol* **16**, 2672-85.

Fisher, E. R., Kenny, J. P., Sass, R., Dimitrov, N. V., Siderits, R. H., and Fisher, B. (1990). Medullary cancer of the breast revisited. *Breast Cancer Res Treat* **16**, 215-29.

Fitzgibbons, P. L., Page, D. L., Weaver, D., Thor, A. D., Allred, D. C., Clark, G. M., Ruby, S. G., O'Malley, F., Simpson, J. F., Connolly, J. L., Hayes, D. F., Edge, S. B., Lichter, A., and Schnitt, S. J. (2000). Prognostic factors in breast cancer. College of American Pathologists Consensus Statement 1999. *Arch Pathol Lab Med* **124**, 966-78.

Flores, E. R., Tsai, K. Y., Crowley, D., Sengupta, S., Yang, A., McKeon, F., and Jacks, T. (2002). p63 and p73 are required for p53-dependent apoptosis in response to DNA damage. *Nature* **416**, 560-4.

Fridman, J. S., and Lowe, S. W. (2003). Control of apoptosis by p53. *Oncogene* **22**, 9030-40.

Frost, M., Jarboe, E. A., Orlicky, D., Gianani, R., Thompson, L. C., Enomoto, T., and Shroyer, K. R. (2002). Immunohistochemical localization of survivin in benign cervical mucosa, cervical dysplasia, and invasive squamous cell carcinoma. *Am J Clin Pathol* **117**, 738-44.

Fu, L., Minden, M. D., and Benchimol, S. (1996). Translational regulation of human p53 gene expression. *Embo J* **15**, 4392-401.

Fujita, N., and Tsuruo, T. (2000). In vivo veritas: Bcl-2 and Bcl-X(L)mediate tumor cell resistance to chemotherapy. *Drug Resist Updat* **3**, 149-154.

Furihata, M., Kurabayashi, A., Matsumoto, M., Sonobe, H., Ohtsuki, Y., Terao, N., Kuwahara, M., and Shuin, T. (2002). Frequent phosphorylation at serine 392 in overexpressed p53 protein due to missense mutation in carcinoma of the urinary tract. *J Pathol* **197**, 82-8.

Gaffney, E. V. (1982). A cell line (HBL-100) established from human breast milk. *Cell Tissue Res* **227**, 563-8.

Galea, M. H., Blamey, R. W., Elston, C. E., and Ellis, I. O. (1992). The Nottingham Prognostic Index in primary breast cancer. *Breast Cancer Res Treat* **22**, 207-19.

Garrett, T. J., Vahdat, L. T., and Kinne, D. W. (1997). Systemic adjuvant therapy of breast cancer. *J Surg Oncol* **64**, 167-72.

Gasco, M., Shami, S., and Crook, T. (2002). The p53 pathway in breast cancer. *Breast Cancer Res* **4**, 70-6.

Gastman, B. R. (2001). Apoptosis and its clinical impact. *Head Neck* **23**, 409-25.

Gavrieli, Y., Sherman, Y., and Ben-Sasson, S. A. (1992). Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J Cell Biol* **119**, 493-501.

Geisler, S., Borresen-Dale, A. L., Johnsen, H., Aas, T., Geisler, J., Akslen, L. A., Anker, G., and Lonning, P. E. (2003). TP53 gene mutations predict the response to neoadjuvant treatment with 5-fluorouracil and mitomycin in

locally advanced breast cancer. *Clin Cancer Res* **9**, 5582-8.

Geisler, S., Lonning, P. E., Aas, T., Johnsen, H., Fluge, O., Haugen, D. F., Lillehaug, J. R., Akslen, L. A., and Borresen-Dale, A. L. (2001). Influence of TP53 gene alterations and c-erbB-2 expression on the response to treatment with doxorubicin in locally advanced breast cancer. *Cancer Res* **61**, 2505-12.

Gelber, R. D., Bonetti, M., Castiglione-Gertsch, M., Coates, A. S., and Goldhirsch, A. (2003). Tailoring adjuvant treatments for the individual breast cancer patient. *Breast* **12**, 558-68.

Gluck, S. (2001). The worldwide perspective in the adjuvant treatment of primary lymph node positive breast cancer. *Breast Cancer* **8**, 321-8.

Gohring, U. J., Bersch, A., Becker, M., Neuhaus, W., and Schondorf, T. (2001). p21(waf) correlates with DNA replication but not with prognosis in invasive breast cancer. *J Clin Pathol* **54**, 866-70.

Gohring, U. J., Scharl, A., Heckel, C., Ahr, A., and Crombach, G. (1995). P53 protein in 204 patients with primary breast carcinoma--immunohistochemical detection and clinical value as a prognostic factor. *Arch Gynecol Obstet* **256**, 139-46.

Gompel, A., Somai, S., Chaouat, M., Kazem, A., Kloosterboer, H. J., Beusman, I., Forgez, P., Mimoun, M., and Rostene, W. (2000). Hormonal regulation of apoptosis in breast cells and tissues. *Steroids* **65**, 593-8.

Green, D. R. (1998). Apoptotic pathways: the roads to ruin. *Cell* **94**, 695-8.

Green, D. R. (2000). Apoptotic pathways: paper wraps stone blunts scissors. *Cell* **102**, 1-4.

Greenblatt, M. S., Bennett, W. P., Hollstein, M., and Harris, C. C. (1994). Mutations in the p53 tumor suppressor gene: clues to cancer etiology and molecular pathogenesis. *Cancer Res* **54**, 4855-78.

Gullick, W. J., and Srinivasan, R. (1998). The type 1 growth factor receptor family: new ligands and receptors and their role in breast cancer. *Breast Cancer Res Treat* **52**, 43-53.

Hadjiloucas, I., Gilmore, A. P., Bundred, N. J., and Streuli, C. H. (2001). Assessment of apoptosis in human breast tissue using an antibody against the active form of caspase 3: relation to tumour histopathological characteristics. *Br J Cancer* **85**, 1522-6.

Hainaut, P., Hernandez, T., Robinson, A., Rodriguez-Tome, P., Flores, T., Hollstein, M., Harris, C. C., and Montesano, R. (1998). IARC Database of p53 gene mutations in human tumors and cell lines: updated compilation, revised formats and new visualisation tools. *Nucleic Acids Res* **26**, 205-13.

Haldar, S., Jena, N., and Croce, C. M. (1995). Inactivation of Bcl-2 by phosphorylation. *Proc Natl Acad Sci U S A* **92**, 4507-11.

Harris, G., Pinder SE (2003). The role of the pathologist in assessing prognostic factors for breast cancer. *In* "Prognostic and predictive factors in breast cancer" (R. A. Walker, ed.), pp. 7-21. Martin Dunitz, London and New York.

Hartmann, A., Blaszyk, H., Kovach, J. S., and Sommer, S. S. (1997). The molecular epidemiology of p53 gene mutations in human breast cancer. *Trends Genet* **13**, 27-33.

Haupt, S., Berger, M., Goldberg, Z., and Haupt, Y. (2003). Apoptosis - the p53 network. *J Cell Sci* **116**, 4077-85.

Hauser, H. P., Bardroff, M., Pyrowolakis, G., and Jentsch, S. (1998). A giant ubiquitin-conjugating enzyme related to IAP apoptosis inhibitors. *J Cell Biol* **141**, 1415-22.

Hawkins, C. J., Ekert, P. G., Uren, A. G., Holmgreen, S. P., and Vaux, D. L. (1998). Anti-apoptotic potential of insect cellular and viral IAPs in mammalian cells. *Cell Death Differ* **5**, 569-76.

Henderson, I. C., and Patek, A. J. (1998). The relationship between prognostic and predictive factors in the management of breast cancer. *Breast Cancer Res Treat* **52**, 261-88.

Hickman, J. A. (1992). Apoptosis induced by anticancer drugs. *Cancer Metastasis Rev* **11**, 121-39.

Hinds, M. G., Norton, R. S., Vaux, D. L., and Day, C. L. (1999). Solution structure of a baculoviral inhibitor of apoptosis (IAP) repeat. *Nat Struct Biol* **6**, 648-51.

Hoffman, W. H., Biade, S., Zilfou, J. T., Chen, J., and Murphy, M. (2002). Transcriptional repression of the anti-apoptotic survivin gene by wild type p53. *J Biol Chem* **277**, 3247-57.

Holcik, M., Yeh, C., Korneluk, R. G., and Chow, T. (2000). Translational upregulation of X-linked inhibitor of apoptosis (XIAP) increases

resistance to radiation induced cell death. *Oncogene* **19**, 4174-7.

Hollstein, M., Rice, K., Greenblatt, M. S., Soussi, T., Fuchs, R., Sorlie, T., Hovig, E., Smith-Sorensen, B., Montesano, R., and Harris, C. C. (1994). Database of p53 gene somatic mutations in human tumors and cell lines. *Nucleic Acids Res* **22**, 3551-5.

Hortobagyi, G. N. (2000). Developments in chemotherapy of breast cancer. *Cancer* **88**, 3073-9.

Irwin, M. S., and Kaelin, W. G. (2001). p53 family update: p73 and p63 develop their own identities. *Cell Growth Differ* **12**, 337-49.

Jiang, M., Shao, Z., and Wu, J. (1998). [WAF1/CIP1/p21 gene in wild type p53 and mutant p53 human breast cancer cell lines in relation to its cytobiological features]. *Zhonghua Zhong Liu Za Zhi* **20**, 181-4.

Johnstone, R. W., Ruefli, A. A., and Lowe, S. W. (2002). Apoptosis: a link between cancer genetics and chemotherapy. *Cell* **108**, 153-64.

Jones, K. L., and Buzdar, A. U. (2004). A review of adjuvant hormonal therapy in breast cancer. *Endocr Relat Cancer* **11**, 391-406.

Joza, N., Susin, S. A., Daugas, E., Stanford, W. L., Cho, S. K., Li, C. Y., Sasaki, T., Elia, A. J., Cheng, H. Y., Ravagnan, L., Ferri, K. F., Zamzami, N., Wakeham, A., Hakem, R., Yoshida, H., Kong, Y. Y., Mak, T. W., Zuniga-Pflucker, J. C., Kroemer, G., and Penninger, J. M. (2001). Essential role of the mitochondrial apoptosis-inducing factor in programmed cell death. *Nature* **410**, 549-54.

Kamesaki, S., Kamesaki, H., Jorgensen, T. J., Tanizawa, A., Pommier, Y., and Cossman, J. (1993). bcl-2 protein inhibits etoposide-induced apoptosis through its effects on events subsequent to topoisomerase II-induced DNA strand breaks and their repair. *Cancer Res* **53**, 4251-6.

Kandioler-Eckersberger, D., Ludwig, C., Rudas, M., Kappel, S., Janschek, E., Wenzel, C., Schlagbauer-Wadl, H., Mittlbock, M., Gnant, M., Steger, G., and Jakesz, R. (2000). TP53 mutation and p53 overexpression for prediction of response to neoadjuvant treatment in breast cancer patients. *Clin Cancer Res* **6**, 50-6.

Kapoor, M., and Lozano, G. (1998). Functional activation of p53 via phosphorylation following DNA damage by UV but not gamma radiation. *Proc Natl Acad Sci U S A* **95**, 2834-7.

Kasof, G. M., and Gomes, B. C. (2001). Livin, a novel inhibitor of apoptosis

protein family member. *J Biol Chem* **276**, 3238-46.

Kelekar, A., and Thompson, C. B. (1998). Bcl-2-family proteins: the role of the BH3 domain in apoptosis. *Trends Cell Biol* **8**, 324-30.

Kennedy, S. M., O'Driscoll, L., Purcell, R., Fitz-Simons, N., McDermott, E. W., Hill, A. D., O'Higgins, N. J., Parkinson, M., Linehan, R., and Clynes, M. (2003). Prognostic importance of survivin in breast cancer. *Br J Cancer* **88**, 1077-83.

Kerr, J. F., Winterford, C. M., and Harmon, B. V. (1994). Apoptosis. Its significance in cancer and cancer therapy. *Cancer* **73**, 2013-26.

Kerr, J. F., Wyllie, A. H., and Currie, A. R. (1972). Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer* **26**, 239-57.

Kirsch, D. G., and Kastan, M. B. (1998). Tumor-suppressor p53: implications for tumor development and prognosis. *J Clin Oncol* **16**, 3158-68.

Klijn, J. G., Berns, P. M., Schmitz, P. I., and Foekens, J. A. (1992). The clinical significance of epidermal growth factor receptor (EGF-R) in human breast cancer: a review on 5232 patients. *Endocr Rev* **13**, 3-17.

Knowlton, K., Mancini, M., Creason, S., Morales, C., Hockenbery, D., and Anderson, B. O. (1998). Bcl-2 slows in vitro breast cancer growth despite its antiapoptotic effect. *J Surg Res* **76**, 22-6.

Kohler, C., Orrenius, S., and Zhivotovsky, B. (2002). Evaluation of caspase activity in apoptotic cells. *J Immunol Methods* **265**, 97-110.

Kollias, J., Elston, C. W., Ellis, I. O., Robertson, J. F., and Blamey, R. W. (1997). Early-onset breast cancer--histopathological and prognostic considerations. *Br J Cancer* **75**, 1318-23.

Koopman, G., Reutelingsperger, C. P., Kuijten, G. A., Keehnen, R. M., Pals, S. T., and van Oers, M. H. (1994). Annexin V for flow cytometric detection of phosphatidylserine expression on B cells undergoing apoptosis. *Blood* **84**, 1415-20.

Korkolis, D. P., Tsoli, E., Fouskakis, D., Yiotis, J., Koullias, G. J., Giannopoulos, D., Papalambros, E., Nikiteas, N. I., Spiliopoulou, C. A., Patsouris, E., Asimacopoulos, P., and Gorgoulis, V. G. (2004). Tumor histology and stage but not p53, Her2-neu or cathepsin-D expression are independent prognostic factors in breast cancer patients. *Anticancer Res* **24**, 2061-8.

Krag, D. N., and Single, R. M. (2003). Breast cancer survival according to number of nodes removed. *Ann Surg Oncol* **10**, 1152-9.

Krajewski, S., Blomqvist, C., Franssila, K., Krajewska, M., Wasenius, V. M., Niskanen, E., Nordling, S., and Reed, J. C. (1995). Reduced expression of proapoptotic gene BAX is associated with poor response rates to combination chemotherapy and shorter survival in women with metastatic breast adenocarcinoma. *Cancer Res* **55**, 4471-8.

Krajewski, S., Krajewska, M., Turner, B. C., Pratt, C., Howard, B., Zapata, J. M., Frenkel, V., Robertson, S., Ionov, Y., Yamamoto, H., Perucho, M., Takayama, S., and Reed, J. C. (1999). Prognostic significance of apoptosis regulators in breast cancer. *Endocr Relat Cancer* **6**, 29-40.

Kroger, N., Achterrath, W., Hegewisch-Becker, S., Mross, K., and Zander, A. R. (1999). Current options in treatment of anthracycline-resistant breast cancer. *Cancer Treat Rev* **25**, 279-91.

Kuru, B., Camlibel, M., Ali Gulcelik, M., and Alagol, H. (2003). Prognostic factors affecting survival and disease-free survival in lymph node-negative breast carcinomas. *J Surg Oncol* **83**, 167-72.

LaCasse, E. C., Baird, S., Korneluk, R. G., and MacKenzie, A. E. (1998). The inhibitors of apoptosis (IAPs) and their emerging role in cancer. *Oncogene* **17**, 3247-59.

Lacroix, M., and Leclercq, G. (2004). Relevance of breast cancer cell lines as models for breast tumours: an update. *Breast Cancer Res Treat* **83**, 249-89.

Lai, H., Ma, F., Trapido, E., Meng, L., and Lai, S. (2004). Spectrum of p53 tumor suppressor gene mutations and breast cancer survival. *Breast Cancer Res Treat* **83**, 57-66.

Lamb, P., and Crawford, L. (1986). Characterization of the human p53 gene. *Mol Cell Biol* **6**, 1379-85.

Lane, D. P., and Crawford, L. V. (1979). T antigen is bound to a host protein in SV40-transformed cells. *Nature* **278**, 261-3.

Latinovic, L., Heinze, G., Birner, P., Samonigg, H., Hausmaninger, H., Kubista, E., Kwasny, W., Gnant, M., Jakesz, R., and Oberhuber, G. (2001). Prognostic relevance of three histological grading methods in breast cancer. *Int J Oncol* **19**, 1271-7.

Leers, M. P., Kolgen, W., Bjorklund, V., Bergman, T., Tribbick, G., Persson, B.,

Bjorklund, P., Ramaekers, F. C., Bjorklund, B., Nap, M., Jornvall, H., and Schutte, B. (1999). Immunocytochemical detection and mapping of a cytokeratin 18 neo-epitope exposed during early apoptosis. *J Pathol* **187**, 567-72.

Leist, M., and Nicotera, P. (1997). The shape of cell death. *Biochem Biophys Res Commun* **236**, 1-9.

Levesque, M. A., Yu, H., Clark, G. M., and Diamandis, E. P. (1998). Enzyme-linked immunoabsorbent assay-detected p53 protein accumulation: a prognostic factor in a large breast cancer cohort. *J Clin Oncol* **16**, 2641-50.

Levine, A. J. (1997). p53, the cellular gatekeeper for growth and division. *Cell* **88**, 323-31.

Lima, R. T., Martins, L. M., Guimaraes, J. E., Sambade, C., and Vasconcelos, M. H. (2004). Specific downregulation of bcl-2 and xIAP by RNAi enhances the effects of chemotherapeutic agents in MCF-7 human breast cancer cells. *Cancer Gene Ther* **11**, 309-16.

Lipponen, P., Aaltomaa, S., Kosma, V. M., and Syrjanen, K. (1994). Apoptosis in breast cancer as related to histopathological characteristics and prognosis. *Eur J Cancer* **30A**, 2068-73.

Liston, P., Fong, W. G., and Korneluk, R. G. (2003). The inhibitors of apoptosis: there is more to life than Bcl2. *Oncogene* **22**, 8568-80.

Liston, P., Roy, N., Tamai, K., Lefebvre, C., Baird, S., Cherton-Horvat, G., Farahani, R., McLean, M., Ikeda, J. E., MacKenzie, A., and Korneluk, R. G. (1996). Suppression of apoptosis in mammalian cells by NAIP and a related family of IAP genes. *Nature* **379**, 349-53.

Liu, J., Li, M., and Xia, S. (2004). [Expression and clinical significance of antiapoptosis gene XIAP in prostate cancer]. *Zhonghua Nan Ke Xue* **10**, 832-5.

Liu, L., Scolnick, D. M., Trievel, R. C., Zhang, H. B., Marmorstein, R., Halazonetis, T. D., and Berger, S. L. (1999). p53 sites acetylated in vitro by PCAF and p300 are acetylated in vivo in response to DNA damage. *Mol Cell Biol* **19**, 1202-9.

Lowe, S. W., Ruley, H. E., Jacks, T., and Housman, D. E. (1993). p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. *Cell* **74**, 957-67.

Lowe, S. W., Schmitt, E. M., Smith, S. W., Osborne, B. A., and Jacks, T. (1993). p53 is required for radiation-induced apoptosis in mouse thymocytes. *Nature* **362**, 847-9.

Lu, H. R., Meng, L. H., Huang, M., Zhu, H., Miao, Z. H., and Ding, J. (2005). DNA damage, c-myc suppression and apoptosis induced by the novel topoisomerase II inhibitor, salvicine, in human breast cancer MCF-7 cells. *Cancer Chemother Pharmacol* **55**, 286-294.

Lu, Y. S., Kuo, S. H., and Huang, C. S. (2004). Recent advances in the management of primary breast cancers. *J Formos Med Assoc* **103**, 579-98.

Lukas, J., Groshen, S., Saffari, B., Niu, N., Reles, A., Wen, W. H., Felix, J., Jones, L. A., Hall, F. L., and Press, M. F. (1997). WAF1/Cip1 gene polymorphism and expression in carcinomas of the breast, ovary, and endometrium. *Am J Pathol* **150**, 167-75.

Lundin, J., Lundin, M., Holli, K., Kataja, V., Elomaa, L., Pylkkanen, L., Turpeenniemi-Hujanen, T., and Joensuu, H. (2001). Omission of histologic grading from clinical decision making may result in overuse of adjuvant therapies in breast cancer: results from a nationwide study. *J Clin Oncol* **19**, 28-36.

MacGrogan, G., Mauriac, L., Durand, M., Bonichon, F., Trojani, M., de Mascarel, I., and Coindre, J. M. (1996). Primary chemotherapy in breast invasive carcinoma: predictive value of the immunohistochemical detection of hormonal receptors, p53, c-erbB-2, MiB1, pS2 and GST pi. *Br J Cancer* **74**, 1458-65.

Makris, A., Powles, T. J., Dowsett, M., Osborne, C. K., Trott, P. A., Fernando, I. N., Ashley, S. E., Ormerod, M. G., Titley, J. C., Gregory, R. K., and Allred, D. C. (1997). Prediction of response to neoadjuvant chemoendocrine therapy in primary breast carcinomas. *Clin Cancer Res* **3**, 593-600.

Maltzman, W., and Czyzyk, L. (1984). UV irradiation stimulates levels of p53 cellular tumor antigen in nontransformed mouse cells. *Mol Cell Biol* **4**, 1689-94.

Mathieu, M. C., Rouzier, R., Llombart-Cussac, A., Sideris, L., Koscielny, S., Travagli, J. P., Contesso, G., Delaloge, S., and Spielmann, M. (2004). The poor responsiveness of infiltrating lobular breast carcinomas to neoadjuvant chemotherapy can be explained by their biological profile. *Eur J Cancer* **40**, 342-51.

Matsumoto, M., Furihata, M., Kurabayashi, A., Sasaguri, S., Araki, K., Hayashi, H., and Ohtsuki, Y. (2004). Prognostic significance of serine 392 phosphorylation in overexpressed p53 protein in human esophageal squamous cell carcinoma. *Oncology* **67**, 143-50.

McBride, O. W., Merry, D., and Givol, D. (1986). The gene for human p53 cellular tumor antigen is located on chromosome 17 short arm (17p13). *Proc Natl Acad Sci U S A* **83**, 130-4.

McClelland, R. A., Gee, J. M., O'Sullivan, L., Barnes, D. M., Robertson, J. F., Ellis, I. O., and Nicholson, R. I. (1999). p21(WAF1) expression and endocrine response in breast cancer. *J Pathol* **188**, 126-32.

McCurrach, M. E., Connor, T. M., Knudson, C. M., Korsmeyer, S. J., and Lowe, S. W. (1997). bax-deficiency promotes drug resistance and oncogenic transformation by attenuating p53-dependent apoptosis. *Proc Natl Acad Sci U S A* **94**, 2345-9.

McEleny, K. R., Watson, R. W., Coffey, R. N., O'Neill, A. J., and Fitzpatrick, J. M. (2002). Inhibitors of apoptosis proteins in prostate cancer cell lines. *Prostate* **51**, 133-40.

McManus, D. C., Lefebvre, C. A., Cherton-Horvat, G., St-Jean, M., Kandimalla, E. R., Agrawal, S., Morris, S. J., Durkin, J. P., and Lacasse, E. C. (2004). Loss of XIAP protein expression by RNAi and antisense approaches sensitizes cancer cells to functionally diverse chemotherapeutics. *Oncogene* **23**, 8105-17.

Megale Costa, L. J., Soares, H. P., Gaspar, H. A., Trujillo, L. G., Santi, P. X., Pereira, R. S., de Santana, T. L., Pinto, F. N., and del Giglio, A. (2004). Ratio between positive lymph nodes and total dissected axillaries lymph nodes as an independent prognostic factor for disease-free survival in patients with breast cancer. *Am J Clin Oncol* **27**, 304-6.

Meijers-Heijboer, H., van den Ouweland, A., Klijn, J., Wasielewski, M., de Snoo, A., Oldenburg, R., Hollestelle, A., Houben, M., Crepin, E., van Veghel-Plandsoen, M., Elstrodt, F., van Duijn, C., Bartels, C., Meijers, C., Schutte, M., McGuffog, L., Thompson, D., Easton, D., Sodha, N., Seal, S., Barfoot, R., Mangion, J., Chang-Claude, J., Eccles, D., Eeles, R., Evans, D. G., Houlston, R., Murday, V., Narod, S., Peretz, T., Peto, J., Phelan, C., Zhang, H. X., Szabo, C., Devilee, P., Goldgar, D., Futreal, P. A., Nathanson, K. L., Weber, B., Rahman, N., and Stratton, M. R. (2002). Low-penetrance susceptibility to breast cancer due to CHEK2(*)1100delC in noncarriers of BRCA1 or BRCA2 mutations. *Nat Genet* **31**, 55-9.

Mersin, H., Yildirim, E., Gulben, K., and Berberoglu, U. (2003). Is invasive lobular carcinoma different from invasive ductal carcinoma? *Eur J Surg Oncol* **29**, 390-5.

Michaelson, J. S., Silverstein, M., Sgroi, D., Cheongsiatmoy, J. A., Taghian, A., Powell, S., Hughes, K., Comegno, A., Tanabe, K. K., and Smith, B. (2003). The effect of tumor size and lymph node status on breast carcinoma lethality. *Cancer* **98**, 2133-43.

Michieli, P., Chedid, M., Lin, D., Pierce, J. H., Mercer, W. E., and Givol, D. (1994). Induction of WAF1/CIP1 by a p53-independent pathway. *Cancer Res* **54**, 3391-5.

Midgley, C. A., and Lane, D. P. (1997). p53 protein stability in tumour cells is not determined by mutation but is dependent on Mdm2 binding. *Oncogene* **15**, 1179-89.

Miles, D. W., Harris, W. H., Gillett, C. E., Smith, P., and Barnes, D. M. (1999). Effect of c-erbB(2) and estrogen receptor status on survival of women with primary breast cancer treated with adjuvant cyclophosphamide/methotrexate/fluorouracil. *Int J Cancer* **84**, 354-9.

Miller, C., Mohandas, T., Wolf, D., Prokocimer, M., Rotter, V., and Koeffler, H. P. (1986). Human p53 gene localized to short arm of chromosome 17. *Nature* **319**, 783-4.

Minamoto, T., Buschmann, T., Habelhah, H., Matusevich, E., Tahara, H., Boerresen-Dale, A. L., Harris, C., Sidransky, D., and Ronai, Z. (2001). Distinct pattern of p53 phosphorylation in human tumors. *Oncogene* **20**, 3341-7.

Minn, A. J., Velez, P., Schendel, S. L., Liang, H., Muchmore, S. W., Fesik, S. W., Fill, M., and Thompson, C. B. (1997). Bcl-x(L) forms an ion channel in synthetic lipid membranes. *Nature* **385**, 353-7.

Mirza, A., McGuirk, M., Hockenberry, T., N., Wu, Q., Ashar, H., Black, S., Wen, S., F., Wang, L., Kirschmeier, P., Bishop, W., R., Nielsen, L., L., Pickett, C., B., and Liu, S. (2002). Human survivin is negatively regulated by wild-type p53 and participates in p53-dependent apoptotic pathway. Oncogene **21**, 2613-22.

Miyoshi, Y., Taguchi, T., Tamaki, Y., and Noguchi, S. (2003). Current status of endocrine therapy for breast cancer. *Breast Cancer* **10**, 105-11.

Moll, U. M., Riou, G., and Levine, A. J. (1992). Two distinct mechanisms alter p53 in breast cancer: mutation and nuclear exclusion. *Proc Natl Acad Sci*

U S A **89**, 7262-6.

Molland, J. G., Donnellan, M., Janu, N. C., Carmalt, H. L., Kennedy, C. W., and Gillett, D. J. (2004). Infiltrating lobular carcinoma--a comparison of diagnosis, management and outcome with infiltrating duct carcinoma. *Breast* **13**, 389-96.

Mooney, L. M., Al-Sakkaf, K. A., Brown, B. L., and Dobson, P. R. (2002). Apoptotic mechanisms in T47D and MCF-7 human breast cancer cells. *Br J Cancer* **87**, 909-17.

Motoyama, A. B., and Hynes, N. E. (2003). BAD: a good therapeutic target? *Breast Cancer Res* **5**, 27-30.

Muller, M., Wilder, S., Bannasch, D., Israeli, D., Lehlbach, K., Li-Weber, M., Friedman, S. L., Galle, P. R., Stremmel, W., Oren, M., and Krammer, P. H. (1998). p53 activates the CD95 (APO-1/Fas) gene in response to DNA damage by anticancer drugs. *J Exp Med* **188**, 2033-45.

Murray, A. (1994). Cell cycle checkpoints. *Curr Opin Cell Biol* **6**, 872-6.

Nachmias, B., Ashhab, Y., and Ben-Yehuda, D. (2004). The inhibitor of apoptosis protein family (IAPs): an emerging therapeutic target in cancer. *Semin Cancer Biol* **14**, 231-43.

National Co-ordinating Group for Breast Screening Pathology (1995). Pathology Reporting in Breast Cancer Screening. Second edition. NHSBSP Publications, no3.."

Nicoletti, I., Migliorati, G., Pagliacci, M. C., Grignani, F., and Riccardi, C. (1991). A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. *J Immunol Methods* **139**, 271-9.

Noda, A., Ning, Y., Venable, S. F., Pereira-Smith, O. M., and Smith, J. R. (1994). Cloning of senescent cell-derived inhibitors of DNA synthesis using an expression screen. *Exp Cell Res* **211**, 90-8.

O'Driscoll, L., Linehan, R., S, M. K., Cronin, D., Purcell, R., Glynn, S., E, W. M., A, D. H., N, J. O. H., Parkinson, M., and Clynes, M. (2003). Lack of prognostic significance of survivin, survivin-deltaEx3, survivin-2B, galectin-3, bag-1, bax-alpha and MRP-1 mRNAs in breast cancer. *Cancer Lett* **201**, 225-36.

Oltvai, Z. N., Milliman, C. L., and Korsmeyer, S. J. (1993). Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates

programmed cell death. *Cell* **74**, 609-19.

Oost, T. K., Sun, C., Armstrong, R. C., Al-Assaad, A. S., Betz, S. F., Deckwerth, T. L., Ding, H., Elmore, S. W., Meadows, R. P., Olejniczak, E. T., Oleksijew, A., Oltersdorf, T., Rosenberg, S. H., Shoemaker, A. R., Tomaselli, K. J., Zou, H., and Fesik, S. W. (2004). Discovery of potent antagonists of the antiapoptotic protein XIAP for the treatment of cancer. *J Med Chem* **47**, 4417-26.

Oren, M. (2003). Decision making by p53: life, death and cancer. *Cell Death Differ* **10**, 431-42.

Parton, M., Krajewski, S., Smith, I., Krajewska, M., Archer, C., Naito, M., Ahern, R., Reed, J., and Dowsett, M. (2002). Coordinate expression of apoptosis-associated proteins in human breast cancer before and during chemotherapy. *Clin Cancer Res* **8**, 2100-8.

Pecorelli, S., Favalli, G., Zigliani, L., and Odicino, F. (2003). Cancer in women. *Int J Gynaecol Obstet* **82**, 369-79.

Peng, C. Y., Graves, P. R., Thoma, R. S., Wu, Z., Shaw, A. S., and Piwnica-Worms, H. (1997). Mitotic and G2 checkpoint control: regulation of 14-3-3 protein binding by phosphorylation of Cdc25C on serine-216. *Science* **277**, 1501-5.

Pereira, H., Pinder, S. E., Sibbering, D. M., Galea, M. H., Elston, C. W., Blamey, R. W., Robertson, J. F., and Ellis, I. O. (1995). Pathological prognostic factors in breast cancer. IV: Should you be a typer or a grader? A comparative study of two histological prognostic features in operable breast carcinoma. *Histopathology* **27**, 219-26.

Pharoah, P. D., Day, N. E., and Caldas, C. (1999). Somatic mutations in the p53 gene and prognosis in breast cancer: a meta-analysis. *Br J Cancer* **80**, 1968-73.

Piccart, M. J., Lohrisch, C., Duchateau, L., and Buyse, M. (2001). Taxanes in the adjuvant treatment of breast cancer: why not yet? *J Natl Cancer Inst Monogr*, 88-95.

Pinder, S. E., Elston, C.W., Ellis, I. O., (1998). Invasive carcinoma-usual histological types. *In* "The breast/systemic pathology" (W. S. C. Symmers, ed.), Vol. 13, pp. 283-337.

Pinder, S. E., Murray, S., Ellis, I. O., Trihia, H., Elston, C. W., Gelber, R. D., Goldhirsch, A., Lindtner, J., Cortes-Funes, H., Simoncini, E., Byrne, M. J., Golouh, R., Rudenstam, C. M., Castiglione-Gertsch, M., and

Gusterson, B. A. (1998). The importance of the histologic grade of invasive breast carcinoma and response to chemotherapy. *Cancer* **83**, 1529-39.

Pritchard, K. I. (2002). Adjuvant therapy for premenopausal women with breast cancer: is it time for another paradigm shift? *J Clin Oncol* **20**, 4611-4.

Pritchard, K. I. (2003). The best use of adjuvant endocrine treatments. *Breast* **12**, 497-508.

Rahko, E., Blanco, G., Soini, Y., Bloigu, R., and Jukkola, A. (2003). A mutant TP53 gene status is associated with a poor prognosis and anthracycline-resistance in breast cancer patients. *Eur J Cancer* **39**, 447-53.

Ramp, U., Krieg, T., Caliskan, E., Mahotka, C., Ebert, T., Willers, R., Gabbert, H. E., and Gerharz, C. D. (2004). XIAP expression is an independent prognostic marker in clear-cell renal carcinomas. *Hum Pathol* **35**, 1022-8.

Rampaul, R. S., Pinder, S. E., Gullick, W. J., Robertson, J. F., and Ellis, I. O. (2002). HER-2 in breast cancer--methods of detection, clinical significance and future prospects for treatment. *Crit Rev Oncol Hematol* **43**, 231-44.

Ravaioli, A., Bagli, L., Zucchini, A., and Monti, F. (1998). Prognosis and prediction of response in breast cancer: the current role of the main biological markers. *Cell Prolif* **31**, 113-26.

Reed, J. C. (2000). Mechanisms of apoptosis. *Am J Pathol* **157**, 1415-30.

Reed, J. C. (2001). The Survivin saga goes in vivo. *J Clin Invest* **108**, 965-9.

Reed, J. C., Doctor, K., Rojas, A., Zapata, J. M., Stehlik, C., Fiorentino, L., Damiano, J., Roth, W., Matsuzawa, S., Newman, R., Takayama, S., Marusawa, H., Xu, F., Salvesen, G., and Godzik, A. (2003). Comparative analysis of apoptosis and inflammation genes of mice and humans. *Genome Res* **13**, 1376-88.

Reed, J. C., and Kroemer, G. (2000). Mechanisms of mitochondrial membrane permeabilization. *Cell Death Differ* **7**, 1145.

Reed, J. C., Miyashita, T., Takayama, S., Wang, H. G., Sato, T., Krajewski, S., Aime-Sempe, C., Bodrug, S., Kitada, S., and Hanada, M. (1996). BCL-2 family proteins: regulators of cell death involved in the pathogenesis of cancer and resistance to therapy. *J Cell Biochem* **60**, 23-32.

Ring, A. E., Smith, I. E., Ashley, S., Fulford, L. G., and Lakhani, S. R. (2004).

Oestrogen receptor status, pathological complete response and prognosis in patients receiving neoadjuvant chemotherapy for early breast cancer. *Br J Cancer* **91**, 2012-7.

Robertson, J. F., Bates, K., Pearson, D., Blamey, R. W., and Nicholson, R. I. (1992). Comparison of two oestrogen receptor assays in the prediction of the clinical course of patients with advanced breast cancer. *Br J Cancer* **65**, 727-30.

Robertson, J. F., Ellis, I. O., Pearson, D., Elston, C. W., Nicholson, R. I., and Blamey, R. W. (1994). Biological factors of prognostic significance in locally advanced breast cancer. *Breast Cancer Res Treat* **29**, 259-64.

Roninson, I. B. (2002). Oncogenic functions of tumour suppressor p21(Waf1/Cip1/Sdi1): association with cell senescence and tumour-promoting activities of stromal fibroblasts. *Cancer Lett* **179**, 1-14.

Rowinsky, E. K., Donehower, R. C., Jones, R. J., and Tucker, R. W. (1988). Microtubule changes and cytotoxicity in leukemic cell lines treated with taxol. *Cancer Res* **48**, 4093-100.

Rowinsky, E. K., Onetto, N., Canetta, R. M., and Arbuck, S. G. (1992). Taxol: the first of the taxanes, an important new class of antitumor agents. *Semin Oncol* **19**, 646-62.

Roy, N., Mahadevan, M. S., McLean, M., Shutler, G., Yaraghi, Z., Farahani, R., Baird, S., Besner-Johnston, A., Lefebvre, C., Kang, X., and et al. (1995). The gene for neuronal apoptosis inhibitory protein is partially deleted in individuals with spinal muscular atrophy. *Cell* **80**, 167-78.

Ruiz de Almodovar, C., Ruiz-Ruiz, C., Munoz-Pinedo, C., Robledo, G., and Lopez-Rivas, A. (2001). The differential sensitivity of Bc1-2-overexpressing human breast tumor cells to TRAIL or doxorubicin-induced apoptosis is dependent on Bc1-2 protein levels. *Oncogene* **20**, 7128-33.

Ryan, K. M., Phillips, A. C., and Vousden, K. H. (2001). Regulation and function of the p53 tumor suppressor protein. *Curr Opin Cell Biol* **13**, 332-7.

Sanna, M. G., da Silva Correia, J., Ducrey, O., Lee, J., Nomoto, K., Schrantz, N., Deveraux, Q. L., and Ulevitch, R. J. (2002). IAP suppression of apoptosis involves distinct mechanisms: the TAK1/JNK1 signaling cascade and caspase inhibition. *Mol Cell Biol* **22**, 1754-66.

Sant, M., Allemani, C., Capocaccia, R., Hakulinen, T., Aareleid, T., Coebergh, J. W., Coleman, M. P., Grosclaude, P., Martinez, C., Bell, J., Youngson, J.,

and Berrino, F. (2003). Stage at diagnosis is a key explanation of differences in breast cancer survival across Europe. *Int J Cancer* **106**, 416-22.

Sant, M., Capocaccia, R., Verdecchia, A., Esteve, J., Gatta, G., Micheli, A., Coleman, M. P., and Berrino, F. (1998). Survival of women with breast cancer in Europe: variation with age, year of diagnosis and country. The EUROCARE Working Group. *Int J Cancer* **77**, 679-83.

Sasaki, H., Sheng, Y., Kotsuji, F., and Tsang, B. K. (2000). Down-regulation of X-linked inhibitor of apoptosis protein induces apoptosis in chemoresistant human ovarian cancer cells. *Cancer Res* **60**, 5659-66.

Sato, T., Koseki, T., Yamato, K., Saiki, K., Konishi, K., Yoshikawa, M., Ishikawa, I., and Nishihara, T. (2002). p53-independent expression of p21(CIP1/WAF1) in plasmacytic cells during G(2) cell cycle arrest induced by Actinobacillus actinomycetemcomitans cytolethal distending toxin. *Infect Immun* **70**, 528-34.

Sax, J. K., and El-Deiry, W. S. (2003). p53 downstream targets and chemosensitivity. *Cell Death Differ* **10**, 413-7.

Scaffidi, C., Fulda, S., Srinivasan, A., Friesen, C., Li, F., Tomaselli, K. J., Debatin, K. M., Krammer, P. H., and Peter, M. E. (1998). Two CD95 (APO-1/Fas) signaling pathways. *Embo J* **17**, 1675-87.

Schairer, C., Mink, P. J., Carroll, L., and Devesa, S. S. (2004). Probabilities of death from breast cancer and other causes among female breast cancer patients. *J Natl Cancer Inst* **96**, 1311-21.

Schick, P., Goodstein, J., Moor, J., Butler, J., and Senter, K. L. (1983). Preoperative chemotherapy followed by mastectomy for locally advanced breast cancer. *J Surg Oncol* **22**, 278-82.

Schiff, P. B., and Horwitz, S. B. (1980). Taxol stabilizes microtubules in mouse fibroblast cells. *Proc Natl Acad Sci U S A* **77**, 1561-5.

Schmidt, M., Bachhuber, A., Victor, A., Steiner, E., Mahlke, M., Lehr, H. A., Pilch, H., Weikel, W., and Knapstein, P. G. (2003). p53 expression and resistance against paclitaxel in patients with metastatic breast cancer. *J Cancer Res Clin Oncol* **129**, 295-302.

Schmitt, F. C., Soares, R., Cirnes, L., and Seruca, R. (1998). P53 in breast carcinomas: association between presence of mutation and immunohistochemical expression using a semiquantitative approach. *Pathol Res Pract* **194**, 815-9.

Schneeweiss, A., Katretchko, J., Sinn, H. P., Unnebrink, K., Rudlowski, C., Geberth, M., Beldermann, F., Bastert, G., and Strittmatter, H. J. (2004). Only grading has independent impact on breast cancer survival after adjustment for pathological response to preoperative chemotherapy. *Anticancer Drugs* **15**, 127-35.

Schondorf, T., Gohring, U. J., Becker, M., Hoopmann, M., Schmidt, T., Rutzel, S., Rein, D. T., Ulrich, U., Fechteler, R., Bersch, A., Mallmann, P., and Valter, M. M. (2004). High apoptotic index correlates to p21 and p27 expression indicating a favorable outcome of primary breast cancer patients, but lacking prognostic significance in multivariate analysis. *Pathobiology* **71**, 217-22.

Shtil, A. A., Mandlekar, S., Yu, R., Walter, R. J., Hagen, K., Tan, T. H., Roninson, I. B., and Kong, A. N. (1999). Differential regulation of mitogen-activated protein kinases by microtubule-binding agents in human breast cancer cells. *Oncogene* **18**, 377-84.

Sierra, A., Castellsague, X., Escobedo, A., Lloveras, B., Garcia-Ramirez, M., Moreno, A., and Fabra, A. (2000). Bcl-2 with loss of apoptosis allows accumulation of genetic alterations: a pathway to metastatic progression in human breast cancer. *Int J Cancer* **89**, 142-7.

Simpson, J. F., Gray, R., Dressler, L. G., Cobau, C. D., Falkson, C. I., Gilchrist, K. W., Pandya, K. J., Page, D. L., and Robert, N. J. (2000). Prognostic value of histologic grade and proliferative activity in axillary node-positive breast cancer: results from the Eastern Cooperative Oncology Group Companion Study, EST 4189. *J Clin Oncol* **18**, 2059-69.

Simpson, P. T., Reis-Filho, J. S., Gale, T., and Lakhani, S. R. (2005). Molecular evolution of breast cancer. *J Pathol* **205**, 248-54.

Singletary, S. E., Allred, C., Ashley, P., Bassett, L. W., Berry, D., Bland, K. I., Borgen, P. I., Clark, G., Edge, S. B., Hayes, D. F., Hughes, L. L., Hutter, R. V., Morrow, M., Page, D. L., Recht, A., Theriault, R. L., Thor, A., Weaver, D. L., Wieand, H. S., and Greene, F. L. (2002). Revision of the American Joint Committee on Cancer staging system for breast cancer. *J Clin Oncol* **20**, 3628-36.

Sionov, R. V., and Haupt, Y. (1999). The cellular response to p53: the decision between life and death. *Oncogene* **18**, 6145-57.

Sirvent, J. J., Aguilar, M. C., Olona, M., Pelegri, A., Blazquez, S., and Gutierrez, C. (2004). Prognostic value of apoptosis in breast cancer (pT1-pT2). A TUNEL, p53, bcl-2, bag-1 and Bax immunohistochemical study. *Histol*

Histopathol **19**, 759-70.

Sjostrom, J., Blomqvist, C., Heikkila, P., Boguslawski, K. V., Raisanen-Sokolowski, A., Bengtsson, N. O., Mjaaland, I., Malmstrom, P., Ostenstadt, B., Bergh, J., Wist, E., Valvere, V., and Saksela, E. (2000). Predictive value of p53, mdm-2, p21, and mib-1 for chemotherapy response in advanced breast cancer. *Clin Cancer Res* **6**, 3103-10.

Sjostrom, J., Blomqvist, C., von Boguslawski, K., Bengtsson, N. O., Mjaaland, I., Malmstrom, P., Ostenstadt, B., Wist, E., Valvere, V., Takayama, S., Reed, J. C., and Saksela, E. (2002). The predictive value of bcl-2, bax, bcl-xL, bag-1, fas, and fasL for chemotherapy response in advanced breast cancer. *Clin Cancer Res* **8**, 811-6.

Slamon, D. J., Clark, G. M., Wong, S. G., Levin, W. J., Ullrich, A., and McGuire, W. L. (1987). Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science* **235**, 177-82.

Smith, P. J., and Soues, S. (1994). Multilevel therapeutic targeting by topoisomerase inhibitors. *Br J Cancer Suppl* **23**, S47-51.

Snyder, R. D. (2004). New concepts in breast cancer therapy. *Intern Med J* **34**, 266-9.

Span, P. N., Sweep, F. C., Wiegerinck, E. T., Tjan-Heijnen, V. C., Manders, P., Beex, L. V., and de Kok, J. B. (2004). Survivin is an independent prognostic marker for risk stratification of breast cancer patients. *Clin Chem* **50**, 1986-93.

Stasinopoulost, I., A., Mironchik, Y., Raman, A., Wildest, F., Winnard, P., and Raman, V. (2005). HOXA5-Twist Interaction Alters p53 Homeostasis in Breast Cancer Cells. The J. Biol. Chem **280**, 2294-9.

Stearns, V., Singh, B., Tsangaris, T., Crawford, J. G., Novielli, A., Ellis, M. J., Isaacs, C., Pennanen, M., Tibery, C., Farhad, A., Slack, R., and Hayes, D. F. (2003). A prospective randomized pilot study to evaluate predictors of response in serial core biopsies to single agent neoadjuvant doxorubicin or paclitaxel for patients with locally advanced breast cancer. *Clin Cancer Res* **9**, 124-33.

Stehlik, C., de Martin, R., Kumabashiri, I., Schmid, J. A., Binder, B. R., and Lipp, J. (1998). Nuclear factor (NF)-kappaB-regulated X-chromosome-linked iap gene expression protects endothelial cells from tumor necrosis factor alpha-induced apoptosis. *J Exp Med* **188**, 211-6.

Steinman, R. A., Hoffman, B., Iro, A., Guillouf, C., Liebermann, D. A., and el-

Houseini, M. E. (1994). Induction of p21 (WAF-1/CIP1) during differentiation. *Oncogene* **9**, 3389-96.

Stenmark-Askmalm, M., Stal, O., Sullivan, S., Ferraud, L., Sun, X. F., Carstensen, J., and Nordenskjold, B. (1994). Cellular accumulation of p53 protein: an independent prognostic factor in stage II breast cancer. *Eur J Cancer* **30A**, 175-80.

Storey, A., Thomas, M., Kalita, A., Harwood, C., Gardiol, D., Mantovani, F., Breuer, J., Leigh, I. M., Matlashewski, G., and Banks, L. (1998). Role of a p53 polymorphism in the development of human papillomavirus-associated cancer. *Nature* **393**, 229-34.

Sullivan, A., Yuille, M., Repellin, C., Reddy, A., Reelfs, O., Bell, A., Dunne, B., Gusterson, B. A., Osin, P., Farrell, P. J., Yulug, I., Evans, A., Ozcelik, T., Gasco, M., and Crook, T. (2002). Concomitant inactivation of p53 and Chk2 in breast cancer. *Oncogene* **21**, 1316-24.

Sutherland, R. L., Hall, R. E., and Taylor, I. W. (1983). Cell proliferation kinetics of MCF-7 human mammary carcinoma cells in culture and effects of tamoxifen on exponentially growing and plateau-phase cells. *Cancer Res* **43**, 3998-4006.

Takada, M., Kataoka, A., Toi, M., Bando, H., Toyama, K., Horiguchi, S., Ueno, T., Linder, S., Saji, S., Hayashi, Y., Funata, N., Kinoshita, J., Murakami, S., and Ohono, S. (2004). A close association between alteration in growth kinetics by neoadjuvant chemotherapy and survival outcome in primary breast cancer. *Int J Oncol* **25**, 397-405.

Takahashi, K., and Suzuki, K. (1994). DNA synthesis-associated nuclear exclusion of p53 in normal human breast epithelial cells in culture. *Oncogene* **9**, 183-8.

Takahashi, R., Deveraux, Q., Tamm, I., Welsh, K., Assa-Munt, N., Salvesen, G. S., and Reed, J. C. (1998). A single BIR domain of XIAP sufficient for inhibiting caspases. *J Biol Chem* **273**, 7787-90.

Takai, H., Naka, K., Okada, Y., Watanabe, M., Harada, N., Saito, S., Anderson, C. W., Appella, E., Nakanishi, M., Suzuki, H., Nagashima, K., Sawa, H., Ikeda, K., and Motoyama, N. (2002). Chk2-deficient mice exhibit radioresistance and defective p53-mediated transcription. *Embo J* **21**, 5195-205.

Takai, N., Miyazaki, T., Nishida, M., Nasu, K., and Miyakawa, I. (2002). Survivin expression correlates with clinical stage, histological grade, invasive behavior and survival rate in endometrial carcinoma. *Cancer Lett* **184**,

105-16.

Tamm, I., Kornblau, S. M., Segall, H., Krajewski, S., Welsh, K., Kitada, S., Scudiero, D. A., Tudor, G., Qui, Y. H., Monks, A., Andreeff, M., and Reed, J. C. (2000). Expression and prognostic significance of IAP-family genes in human cancers and myeloid leukemias. *Clin Cancer Res* **6**, 1796-803.

Tamm, I., Wang, Y., Sausville, E., Scudiero, D. A., Vigna, N., Oltersdorf, T., and Reed, J. C. (1998). IAP-family protein survivin inhibits caspase activity and apoptosis induced by Fas (CD95), Bax, caspases, and anticancer drugs. *Cancer Res* **58**, 5315-20.

Tanaka, K., Iwamoto, S., Gon, G., Nohara, T., Iwamoto, M., and Tanigawa, N. (2000). Expression of survivin and its relationship to loss of apoptosis in breast carcinomas. *Clin Cancer Res* **6**, 127-34.

Teixeira, C., Reed, J. C., and Pratt, M. A. (1995). Estrogen promotes chemotherapeutic drug resistance by a mechanism involving Bcl-2 proto-oncogene expression in human breast cancer cells. *Cancer Res* **55**, 3902-7.

The ATAC (Arimidex, Tamoxifen Alone or in Combination) Trialists Group (2002). Anastrozole alone or in combination with tamoxifen versus tamoxifen alone for adjuvant treatment of postmenopausal women with early breast cancer : first results of the ATAC randomized trial. The Lancet **359**, 2131-9.

Thompson, L. H., and Schild, D. (2002). Recombinational DNA repair and human disease. *Mutat Res* **509**, 49-78.

Thor, A. D., Liu, S., Moore, D. H., 2nd, and Edgerton, S. M. (1999). Comparison of mitotic index, in vitro bromodeoxyuridine labeling, and MIB-1 assays to quantitate proliferation in breast cancer. *J Clin Oncol* **17**, 470-7.

Thornberry, N. A., and Lazebnik, Y. (1998). Caspases: enemies within. *Science* **281**, 1312-6.

Tong, D., Schuster, E., Seifert, M., Czerwenka, K., Leodolte, S., and Zeillinger, R. (2002). Expression of estrogen receptor beta isoforms in human breast cancer tissues and cell lines. *Breast Cancer Res Treat* **71**, 249-55.

Troester, M. A., Hoadley, K. A., Sorlie, T., Herbert, B. S., Borresen-Dale, A. L., Lonning, P. E., Shay, J. W., Kaufmann, W. K., and Perou, C. M. (2004). Cell-type-specific responses to chemotherapeutics in breast cancer.

Cancer Res **64**, 4218-26.

Tsutsui, S., Ohno, S., Murakami, S., Kataoka, A., Kinoshita, J., and Hachitanda, Y. (2003). Prognostic value of the combination of epidermal growth factor receptor and c-erbB-2 in breast cancer. *Surgery* **133**, 219-21.

van der Wal, B. C., Butzelaar, R. M., van der Meij, S., and Boermeester, M. A. (2002). Axillary lymph node ratio and total number of removed lymph nodes: predictors of survival in stage I and II breast cancer. *Eur J Surg Oncol* **28**, 481-9.

van Diest, P. J., van der Wall, E., and Baak, J. P. (2004). Prognostic value of proliferation in invasive breast cancer: a review. *J Clin Pathol* **57**, 675-81.

Verhagen, A. M., Coulson, E. J., and Vaux, D. L. (2001). Inhibitor of apoptosis proteins and their relatives: IAPs and other BIRPs. *Genome Biol* **2**, REVIEWS3009.

Verhagen, A. M., Ekert, P. G., Pakusch, M., Silke, J., Connolly, L. M., Reid, G. E., Moritz, R. L., Simpson, R. J., and Vaux, D. L. (2000). Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins. *Cell* **102**, 43-53.

Veronese, S., Mauri, F. A., Caffo, O., Scaioli, M., Aldovini, D., Perrone, G., Galligioni, E., Doglioni, C., Dalla Palma, P., and Barbareschi, M. (1998). Bax immunohistochemical expression in breast carcinoma: a study with long term follow-up. *Int J Cancer* **79**, 13-8.

Veronese, S. M., Gambacorta, M., Gottardi, O., Scanzi, F., Ferrari, M., and Lampertico, P. (1993). Proliferation index as a prognostic marker in breast cancer. *Cancer* **71**, 3926-31.

Vidal, A., and Koff, A. (2000). Cell-cycle inhibitors: three families united by a common cause. *Gene* **247**, 1-15.

Vinh-Hung, V., Burzykowski, T., Cserni, G., Voordeckers, M., Van De Steene, J., and Storme, G. (2003). Functional form of the effect of the numbers of axillary nodes on survival in early breast cancer. *Int J Oncol* **22**, 697-704.

Visscher, D. W., Sarkar, F. H., Shimoyama, R. K., and Crissman, J. D. (1996). Correlation between p53 immunostaining patterns and gene sequence mutations in breast carcinoma. *Diagn Mol Pathol* **5**, 187-93.

Vogelstein, B., Lane, D., and Levine, A. J. (2000). Surfing the p53 network. *Nature* **408**, 307-10.

Vojtesek, B., and Lane, D. P. (1993). Regulation of p53 protein expression in human breast cancer cell lines. *J Cell Sci* **105 (Pt 3)**, 607-12.

Voordeckers, M., Vinh-Hung, V., Van de Steene, J., Lamote, J., and Storme, G. (2004). The lymph node ratio as prognostic factor in node-positive breast cancer. *Radiother Oncol* **70**, 225-30.

Vousden, K. H., and Lu, X. (2002). Live or let die: the cell's response to p53. *Nat Rev Cancer* **2**, 594-604.

Waga, S., Hannon, G. J., Beach, D., and Stillman, B. (1994). The p21 inhibitor of cyclin-dependent kinases controls DNA replication by interaction with PCNA. *Nature* **369**, 574-8.

Wakasugi, E., Kobayashi, T., Tamaki, Y., Ito, Y., Miyashiro, I., Komoike, Y., Takeda, T., Shin, E., Takatsuka, Y., Kikkawa, N., Monden, T., and Monden, M. (1997). p21(Waf1/Cip1) and p53 protein expression in breast cancer. *Am J Clin Pathol* **107**, 684-91.

Walker, K. K., and Levine, A. J. (1996). Identification of a novel p53 functional domain that is necessary for efficient growth suppression. *Proc Natl Acad Sci U S A* **93**, 15335-40.

Walker, R. A., Jones, J. L., Chappell, S., Walsh, T., and Shaw, J. A. (1997). Molecular pathology of breast cancer and its application to clinical management. *Cancer Metastasis Rev* **16**, 5-27.

Walker, R. A., Lees, E., Webb, M. B., and Dearing, S. J. (1996). Breast carcinomas occurring in young women (< 35 years) are different. *Br J Cancer* **74**, 1796-800.

Wang, S., Konorev, E. A., Kotamraju, S., Joseph, J., Kalivendi, S., and Kalyanaraman, B. (2004). Doxorubicin induces apoptosis in normal and tumor cells via distinctly different mechanisms. intermediacy of H(2)O(2)- and p53-dependent pathways. *J Biol Chem* **279**, 25535-43.

Wang, X. (2001). The expanding role of mitochondria in apoptosis. *Genes Dev* **15**, 2922-33.

Wang, X., Yang, C., Chai, J., Shi, Y., and Xue, D. (2002). Mechanisms of AIF-mediated apoptotic DNA degradation in Caenorhabditis elegans. *Science* **298**, 1587-92.

Wolter, K. G., Hsu, Y. T., Smith, C. L., Nechushtan, A., Xi, X. G., and Youle, R. J. (1997). Movement of Bax from the cytosol to mitochondria during apoptosis. *J Cell Biol* **139**, 1281-92.

Woo, R. A., McLure, K. G., Lees-Miller, S. P., Rancourt, D. E., and Lee, P. W. (1998). DNA-dependent protein kinase acts upstream of p53 in response to DNA damage. *Nature* **394**, 700-4.

Wood, R. D., Mitchell, M., Sgouros, J., and Lindahl, T. (2001). Human DNA repair genes. *Science* **291**, 1284-9.

Woods, C. M., Zhu, J., McQueney, P. A., Bollag, D., and Lazarides, E. (1995). Taxol-induced mitotic block triggers rapid onset of a p53-independent apoptotic pathway. *Mol Med* **1**, 506-26.

World Health Organization (1981). International histological classification of tumours. No.2. Histologic typing of breast tumours. pp. 15-16.

Wu, G. S., Burns, T. F., McDonald, E. R., 3rd, Jiang, W., Meng, R., Krantz, I. D., Kao, G., Gan, D. D., Zhou, J. Y., Muschel, R., Hamilton, S. R., Spinner, N. B., Markowitz, S., Wu, G., and el-Deiry, W. S. (1997). KILLER/DR5 is a DNA damage-inducible p53-regulated death receptor gene. *Nat Genet* **17**, 141-3.

Wu, J., Shao, Z. M., Shen, Z. Z., Lu, J. S., Han, Q. X., Fontana, J. A., and Barsky, S. H. (2000). Significance of Apoptosis and Apoptotic-Related Proteins, Bcl-2, and Bax in Primary Breast Cancer. *Breast J* **6**, 44-52.

Wu, X., and Chen, J. (2003). Autophosphorylation of checkpoint kinase 2 at serine 516 is required for radiation-induced apoptosis. *J Biol Chem* **278**, 36163-8.

Yamashita, H., Nishio, M., Toyama, T., Sugiura, H., Zhang, Z., Kobayashi, S., and Iwase, H. (2004). Coexistence of HER2 over-expression and p53 protein accumulation is a strong prognostic molecular marker in breast cancer. *Breast Cancer Res* **6**, R24-30.

Yang, L., Cao, Z., Yan, H., and Wood, W. C. (2003). Coexistence of high levels of apoptotic signaling and inhibitor of apoptosis proteins in human tumor cells: implication for cancer specific therapy. *Cancer Res* **63**, 6815-24.

Yang, Q., Sakurai, T., Yoshimura, G., Suzuma, T., Umemura, T., Nakamura, M., Nakamura, Y., Mori, I., and Kakudo, K. (2003). Prognostic value of Bcl-2 in invasive breast cancer receiving chemotherapy and endocrine therapy. *Oncol Rep* **10**, 121-5.

Yang, Y., Fang, S., Jensen, J. P., Weissman, A. M., and Ashwell, J. D. (2000). Ubiquitin protein ligase activity of IAPs and their degradation in

proteasomes in response to apoptotic stimuli. *Science* **288**, 874-7.

Yap, D. B., Hsieh, J. K., Zhong, S., Heath, V., Gusterson, B., Crook, T., and Lu, X. (2004). Ser392 phosphorylation regulates the oncogenic function of mutant p53. *Cancer Res* **64**, 4749-54.

Yin, X. M., Oltvai, Z. N., and Korsmeyer, S. J. (1994). BH1 and BH2 domains of Bcl-2 are required for inhibition of apoptosis and heterodimerization with Bax. *Nature* **369**, 321-3.

Yu, J., and Zhang, L. (2004). Apoptosis in human cancer cells. *Curr Opin Oncol* **16**, 19-24.

Zaffaroni, N., and Daidone, M. G. (2002). Survivin expression and resistance to anticancer treatments: perspectives for new therapeutic interventions. *Drug Resist Updat* **5**, 65-72.

Zafrani, B., Aubriot, M. H., Mouret, E., De Cremoux, P., De Rycke, Y., Nicolas, A., Boudou, E., Vincent-Salomon, A., Magdelenat, H., and Sastre-Garau, X. (2000). High sensitivity and specificity of immunohistochemistry for the detection of hormone receptors in breast carcinoma: comparison with biochemical determination in a prospective study of 793 cases. *Histopathology* **37**, 536-45.

Zapata, J. M., Krajewska, M., Krajewski, S., Huang, R. P., Takayama, S., Wang, H. G., Adamson, E., and Reed, J. C. (1998). Expression of multiple apoptosis-regulatory genes in human breast cancer cell lines and primary tumors. *Breast Cancer Res Treat* **47**, 129-40.

Zhan, Q., Fan, S., Bae, I., Guillouf, C., Liebermann, D. A., O'Connor, P. M., and Fornace, A. J., Jr. (1994). Induction of bax by genotoxic stress in human cells correlates with normal p53 status and apoptosis. *Oncogene* **9**, 3743-51.

Zhang, G. J., Kimijima, I., Abe, R., Kanno, M., Katagata, N., Hara, K., Watanabe, T., and Tsuchiya, A. (1997). Correlation between the expression of apoptosis-related bcl-2 and p53 oncoproteins and the carcinogenesis and progression of breast carcinomas. *Clin Cancer Res* **3**, 2329-35.

Zhang, G. J., Kimijima, I., Abe, R., Watanabe, T., Kanno, M., Hara, K., and Tsuchiya, A. (1998). Apoptotic index correlates to bcl-2 and p53 protein expression, histological grade and prognosis in invasive breast cancers. *Anticancer Res* **18**, 1989-98.

Zhang, S. Q., Qiang, S. Y., Yang, W. B., Jiang, J. T., and Ji, Z. Z. (2004).

PUBLICATIONS

Abstracts:

- **Role of p53 in determining response of breast cancer cells to chemotherapy.**

  A Hinnis, J Luckett, RA Walker. The Journal of Pathology, September 2003. Volume 201 Supplement, pp: 27^{A}.

- **Study of the potential predictive factors of response of breast cancer to chemotherapy.**

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