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THE EXPRESSION OF P53 AND RELATED PROTEINS IN HUMAN BREAST TUMOURS AND MALIGNANT MELANOMAS

A THESIS SUBMITTED FOR THE DEGREE OF MASTER OF SURGERY

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BABATUNDE JULIAN COKER

MBBS, FRCS (ENGLAND).

ROYAL FREE HOSPITAL & SCHOOL OF MEDICINE

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"Judge a woman not by her tumour but by her Oncogenes"

ANON, ROYAL FREE HOSPITAL, 1996.

This work is dedicated to my father, Okikiolu Oluwajimi Coker, MA, MAO, FRCOG, Emeritus Professor of Obstetics & Gynaecology, whose commitment to education brought us all this far "How can June tell the glory that went with May ?"

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I thank most of all my supervisor Professor Marc Winslet, Head of the Academic Department of Surgery for his unrelenting support through a most character-forming period of my life.

Statement of Originality

All the work produced in this thesis is entirely my own work which was performed in the departments of Histopathology and Surgery of the Royal Free Hospital, London except where indicated in the acknowledgements section. It was carried out between October 1994 and January 1997.

Abstract

Hypothesis

Individuals with the Li-Fraumeni syndrome possess germline mutations in the p53 tumour suppressor gene. These individuals develop tumours at an early age and in various combinations. One combination is breast cancer and malignant melanoma.

Anecdotal reports exist of a greater than expected number of melanomas in patients with sporadic breast cancer and although an association between both tumours has previously been proposed there has been no established molecular, or genetic link. The breast like the skin is developed predominantly from ectoderm and an oestrogenic environment favours the development of both tumours. In fact it has been proposed that the breast is a modified sebaceous gland.

Breast cancer is defined on the basis of epidemiological, cytological and pathological lines, but no single classification reliably predicts clinical behaviour in any subset of tumours with a reliability which exceeds 50%. Similarly, prognosis in melanomas is reliably predicted by the best known Breslow's thickness measurement and Clark's levels of invasion respectively, but a subset of tumours of relatively good prognosis induce early metastasis and death. Both tumours have a poorer prognosis in males.

Are there detectable similar molecular phenotypic differences in breast tumour and melanoma groups related to distinct clinical behaviour and pathological characteristics ?

Is there a link between sporadic breast cancer and malignant melanoma, or are there major similarities in the molecular behaviour of both tumours based on a defect in the p53 tumour suppressor gene's control of the cell cycle ?

The presence of the dysplastic naevus syndrome a.k.a. the atypical mole syndrome increases the risk of the development of melanoma, but the association with breast cancer, although reported has not been established. Is there a link between both conditions based on a defect in the p53 tumour suppressor gene ?

Sporadic breast cancer afflicts 1 in 12 women in the United Kingdom and malignant melanomas affect a young population at their prime, yet we have been unable to

significantly alter the treatment outcomes for these tumours. Have we adopted a simplistic approach to their management ? Should we now examine the immunohistochemical anomalies present in these tumours before we offer treatment advice ?

Aim: (i) To examine the immunohistochemical expression of a set of p53 related oncogenic markers in groups of with patients both breast cancer and malignant melanoma, or breast cancer and the dysplastic naevus syndrome and compare them with control groups of sporadic breast tumours (benign and malignant) of established clinical outcome.

Materials and Methods: Twenty-two malignant breast tumours from patients with either breast cancer and malignant melanoma, or breast cancer and the dysplastic naevus syndrome were examined. The control group consisted of one hundred and thirty-nine archival (139), paraffin-embedded, human breast tumours (93 malignant and 46 benign) that were examined for expression of the p53, tumour suppressor gene protein, MIB-1, the marker of proliferation, Bcl-2, the inhibitor of apoptosis, MDM-2, a zinc finger transcription factor and 2 cyclins (cyclins D and E), candidates for the restriction point protein. The method was the Streptavidin Biotin method with microwave antigen retrieval.

The benign tumours consisted of 2 groups - 30 patients with fibrocystic disease and 16 patients with atypical ductal hyperplasia.

The malignant tumours consisted of two groups, thirty-eight (38) patients with small, low grade, lymph-node negative (T_1N_0) invasive ductal carcinomas (IDC) and 55 patients with high grade breast tumours.

Results: All the patients with breast cancer and malignant melanomas overexpressed the p53 protein. Forty-seven percent of tumours in patients with the dysplastic naevus syndrome overexpressed p53. In the control group, grade III tumours were more likely to overexpress the p53 protein. p53 expression correlated with MIB-1 score in the malignant groups ($p < .0001$ {Grade III} and $p = .0092$ {Grade I-II} respectively). After 2 years grade III cyclin E positive tumours survived longer, but this was not

statistically significant (p = .1). Kaplan-Meier plots showed that patients with T1N0 tumours that were p53 positive were more likely to recur earlier (p = .001).

In the pure ADH group (n = 16), cyclin D expression correlated with cyclin E expression (p = .008), MIB-1 (p = .01) and Bcl-2 (p = .08) respectively. p53 expression correlated with Bcl-2 expression (p = .02) and MIB-1 (p = .09).

In the patients with fibrocystic disease (n = 30) none of the tumours expressed p53 in more than 50% of their nuclei but 28/30 (93 %) expressed Bcl-2.

Conclusion: The overexpression of p53 in all breast tumours in patients with melanomas suggests that these tumours may harbour mutations (either germline or somatic) in the p53 gene but this can only be confirmed by sequencing the p53 gene. Clinical outcome of the distinct unrelated groups of breast tumours may be related to undetected molecular alterations which may modify, both the prognosis and the response to chemotherapeutic interventions. p53 expression in patients with T1N0 tumours may be helpful in predicting early recurrence.

Aim: (ii) To examine the expression of p53 protein MIB-1, Bcl-2 and the anti-melanoma antibody, HMB-45 in a subset of melanomas in patients with p53 positive malignant breast tumours (n = 9) and compare them with a control group of 66 melanomas F = 46, M = 20.

Materials & Methods: Two methods were used, the Alkaline phosphatase and Streptavidin Biotin methods, both with microwave antigen retrieval.

Results: In the malignant melanomas (n =75), all the patients who had malignant breast tumours (n = 9) overexpressed the p53 protein. In the control group Clark's level of invasion correlated with Breslow's thickness (p < .0001), age (p = .03) and the MIB-1 score in the dermo-epidermal junction (p = .05) but not with any of the other oncogenic markers. Bcl-2 expression in the melanomas correlated with Breslow's thickness measurement (p = .08). p53 expression in the melanoma nuclei correlated with over-expression, in the dermo-epidermal junction, of both p53 (p < .0001) and MIB-1 (p = .02), but not the MIB-1 score in the melanomas (p = .21).

Bcl-2 expression in the melanomas correlated with both MIB-1 scores in the melanoma ($p = .008$) and dermo-epidermal junction ($p = .02$) and p53 ($p < .0001$) expression in the melanoma cells.

Conclusion: p53 expression in sporadic melanomas is commoner in individuals who also express the protein in the dermo-epidermal junction. The MIB-1 score in the dermo-epidermal junction and Bcl-2 expression in malignant melanomas may be of prognostic significance and suggests an underlying defect in the apoptotic pathways in these tumours.

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Abbreviations

ABCD - Mnemonic for change

AHML- Atypical melanocytic hyperplasia in a lentiginous epidermal pattern

AMS - Atypical Mole Syndrome

Bcl-2 - B-cell leukaemia/lymphoma-2 gene

CAM - Classic Atypical Mole.

CAMN - Clinically atypical melanocytic naevus

CMM - Cutaneous Malignant Melanoma

DN - Dysplastic naevus

DCIS - Ductal Carcinoma-in-situ

ELND - Elective regional node dissection

ER - Oestrogen Receptor

FAMM - Familial atypical multiple mole and melanoma syndrome

GP - Growth phase

ICE - Interleukin-ß-1-Converting Enzyme

LMM - Lentigo maligna melanoma

MM - Malignant melanoma

NHSSI - Normal Human Serum Heat inactivated

NPI - Nottingham Prognostic index

PCR - Polymerase Chain Reaction

PR - Progesterone receptor

SSMM - Superficial spreading malignant melanoma

TCE - Total cutaneous examination

TCP - Total cutaneous photographs

U-V - Ultraviolet radiation.

wt - Wild type

Introduction

The earliest association between breast cancer and malignant melanoma dates back to 1905 when Parsons reported the presence of a breast cancer and uveal melanoma in four generations of the same family (Jay *et al.,* 1993). Museum specimens from this family have now been traced to the East of London and show evidence of mutations in the p53 tumour-suppressor gene - this is thus believed to be the first report of the Li-Fraumeni syndrome.

Since then there have been numerous anecdotal reports on the association between breast cancer and malignant melanoma (Koh *et al.,* 1987, Abresman *et al.,* 1988, Gutman *et al.,* 1991, Hehir *et al.,* 1992) but only recently has it become accepted that the association is one manifestation of the Li-Fraumeni syndrome (Lynch *et al.,* 1981, Newton *et al.,* 1993). The association between breast cancer and malignant melanoma is still not well known nor well accepted, the latter due to the former to a great extent, yet there is great degree of similarity between both tumours.

Early breast lesions are defined on the basis of histological, cytological and epidemiological analysis. This is despite the multitude of molecular alterations which have been reported in invasive human breast cancer. Knowledge of the molecular basis of breast neoplastic progression is critical to the development of preventative strategies for women at high risk of cancer.

This thesis describes the biological relevance of the p53 tumour suppressor gene and its protein. The role of p53 in regulating the cell cycle is outlined, and the significance of detecting the protein in the nuclei of both malignant and benign tissue is discussed.

Current knowledge of cell growth and replication makes it difficult to draw valid conclusions from studying individual transcription factors, rather they should be considered as part of an array that it is interdependent. The science of molecular biology has enabled previously unavailable avenues to be explored, provoking interest in the events at the cellular level in both benign and malignant tumours. This has placed the focus on the cell cycle and the various factors, both internal and external which affect it.

Disordered "cell cycling" results in anomalies, which if uncorrected may lead to tumour formation. The point at which the disorder is halted by the innate cell cycle "check-points" determines whether a resultant tumour is benign, or malignant.

Research into *in-vitro* cell lines continue to identify various growth and transcription factors necessary for the orderly progression of the cell cycle. One of the most important transcription factors is the p53 tumour suppressor gene product. This protein (without attempting to be simplistic) has two main functions. When anomalies in DNA replication, or damage occur it inhibits progression of the cell cycle and then either allows repair, or if this is deemed impossible compels the cell to undergo apoptosis, (programmed cell death) for the good of the "genome". It has thus been deemed the "guardian of the genome". These functions are carried out in concert with other growth factors, the levels of which rise and fall depending on various positive and negative feedback mechanisms. Ironically, the p53 gene is the most commonly mutated gene in human cancer.

The evolution of multicellular organisms has arisen through mutations, but only mutations which result either in a survival advantage, or non-dominant mutations which do not affect survival. Mutations which are either incompatible with life or normal function are speedily eliminated. It is these tight control mechanisms that has assured survival of individual species. Apoptosis, is a normal physiological process which was first described over 25 years ago (Kerr *et al.,* 1972). It is an aesthetic way of ridding the body of dead and dying cells without the necessity of exciting an inflammatory response. It is thus distinct from necrosis (the other form of cell death) which by definition is accompanied by an inflammatory response. It is seen in the normal breast and other hormone sensitive tissues and in as primitive a creature as the worm *Caenorhabditis elegans.* Most of our recent understanding of the process of apoptosis comes from the study of this worm.

The p53 protein induces apoptosis (in cells which are irreparable) via various mechanisms, the most clearly understood mechanism is via the inhibition of the activity of a

protein, Bcl-2. Overexpression of the Bcl-2 protein leads to the inhibition of apoptosis and vice versa.

The inhibition of apoptosis is physiological in many hormone sensitive organs (such as the breast and the secretory endometrium prior to shedding) but loss of control may lead to the accumulation of cells which would otherwise have "died". This may lead to the formation of a clone of cells which depending on other internal or external factors may become immortal - one mechanism of tumour cell survival.

Mammalian cells arrest at two points in the cell cycle as a result of DNA damage, at the G_1 to S and the G_2 to M transitions. The p53 protein has a central role in the G_1 checkpoint. The timing of protein synthesis is believed to be represent the endpoint of the most critical phase of the cell cycle - critical to the correction or elimination of anomalies in the cell cycle. Entry to this phase is dependent on negotiating one point, the restriction, or "R" point - the final common pathway which if defective leads to deregulated growth with all its consequences. Proteins, called cyclins are believed to be involved in the control of the"R" point. There are multiple cyclins which function in different phases of the cell-cycle - cyclins D and E function in the G_1 phase, cyclin B in the G_2 and the M phase, and cyclin A in the S phase.

The G_1 cyclins (D and E) function downstream from the G_1 checkpoint which p53 controls and control entry and exit from the "R" point.

This control has been described as a "molecular switch" between the various options to continued cell division - temporary cell-cycle arrest, quiescence, differentiation and cell death (Strauss *et al.,* 1995).

The mdm-2 protein, an endogenous protein forms complexes with p53 both *in vivo* and *in-vitro* and when experimentally over-expressed inhibits the transactivating capability of p-53. It is believed to do this by binding directly to the acidic domain of p-53, concealing it from the transcriptional activity of the cell - thus inhibiting the normal function of the p53 protein and indirectly increasing the tumourigenic potential of cells.

Anomalies in the interaction of these five endogenous proteins, - p53, Bcl-2, mdm-2

and the G1 cyclins (D and E), and the genes which control their production is believed to central to the unravelling of the loss of cell-cycle control which produces a cancer cell. This study examines a spectrum of one hundred and thirty-nine (139) archival paraffin-embedded breast tumours (46 benign and 93 malignant) and examined them for each of these factors to aid our understanding of the disorder in the cell cycle which occurs with increasing malignancy - a "paraffin model" of cancer.

Predicting the tumour growth fraction has long been a science belaboured with research tools of unwieldy proportions. In recent times a monoclonal antibody, Ki-67, (a non-histone nucleoprotein of unknown functional significance), has been produced (Gerdes *et al.,* 1983) which is suggested to react with a nuclear antigen present throughout the cell cycle (G1, S, G2, and M phases) of proliferating cells, but is absent in quiescent (Go) cells. This is a novel way of estimating the growth fraction of a tumour which is quick, inexpensive and easily reproducible. MIB-1, a modification of the Ki-67 antibody which was designed for use in paraffin embedded tissue is used in this study.

Twenty-five years ago, it was known that viruses transfected into cell in culture produced changes which resulted in a gain of genetic information i.e. mutations. It was therefore presumed that (i) a similar mutation applied to the cell that had undergone malignant transformation i.e. the malignant cell had more DNA and (ii) this mutation was dominant, hence the inexorable nature of malignancy - this presumption was wrong.

It all began as it often does, as a protracted bout of obstinacy. Harris declared "...if the mutations that generated a tumour cell occurred with a frequency in the usual range and if they acted dominantly it would be unlikely that any human embryo - an immunologically privileged site - would ever reach term without the development of a tumour or at least be well on the way to one before term".

This implied that there were genes that prevented tumours from prevailing! Thus arose the birth of the concept of "tumour suppressor genes" - the rest is history.

In 1992, the study of the p53 gene was recognised as the second most significant scientific trend of the year by *Time* magazine and in 1993 *Science* magazine named p53 the "molecule of the year". The p53 tumour suppressor gene was subsequently located to the short arm of chromosome 17.

Breast Cancer and Malignant Melanoma Exploring the Link

In the United Kingdom there is a higher incidence of both malignant melanoma and breast cancer in females (OPCS, 1992). The oestrogenic hormonal risk factor is implied by this fact and confirmed by phenotypic manifestations of both tumours in an oestrogenic environment. Women with melanomas have a significantly better prognosis than men, with a five year survival of 77% and 64% respectively (OPCS, 1992).

The effects of U-V radiation in the aetiology of malignant melanoma are well known, but only recently are the effects of the sun receiving a high profile in breast cancer. There is a higher incidence of breast cancer in the hotter Southern Europe compared to Northern Europe and the highest incidence of breast cancer is in Hawaii and California respectively (Veronese *et al.,* 1995). Melanomas have been known for a long time to possess oestrogen receptors (Meyskens *et al.,* 1983) and hormonal manipulations have been offered as therapeutic interventions for malignant melanoma (McClay & McClay, 1994).

Pregnancy provokes the appearance of more dysplastic naevi and melanomas in pregnancy are thicker than controls, but their overall prognosis is similar to melanomas of comparable thickness (Mackie *et al.,* 1991). However, breast cancer, carries a worse prognosis in pregnancy. Anecdotal reports exist of a patient developing a melanoma in pregnancy, then developing breast cancer eighteen months later (Koh *et al.,* 1987).

The most common second cancer in patients with melanomas is breast cancer in a large proportion of studies (Bellet *et al.,* 1977, Koh *et al.,* 1987, Guttman *et al.,* 1991) the same is true for the association with the dysplastic naevus syndrome (DNS) (Lynch *et al.,* 1981, 1986).

Cohen proposed that diminished function of the pineal gland (which secretes melatonin) promotes breast cancer and indirectly melanomas (Cohen *et al.,* 1978). Pinealectomy in experimental animals resulted in increased growth of both experimentally induced mammary tumours and transplanted melanomas. Melatonin replacement resulted in a reversal of these effects (Abresman *et al.,* 1987).

Studies of the frequency of multiple primary malignancies in patients with malignant melanoma found more than expected number of patients with breast cancer (Lokich *et al.,* 1975, Lynch *et al.,* 1983, Koh *et al.*, 1987, Gutman *et al.,* 1991). Some have reported a six-fold increase in breast cancer in patients with melanomas (Gutman *et al.,* 1991), others a 4.8 % observed versus 0.6 % age adjusted expected ratio ($p < 0.04$ - .05) (Lynch *et al.,* 1983, Koh *et al.*, 1987). These patients with second non-cutaneous tumours were older than those that just had melanomas and a formula has been developed to calculate the lietime risk (Bellet *et al.*, 1977).

Warren and Gates, after performing 1, 068 autopsies of cancer patients concluded that a patient with one malignancy is more likely to develop another malignancy. Spratt and Hoeg could demonstrate no difference in the incidence of multiple primary malignant neoplasms per patient year at risk between those individuals with multiple malignancies and controls (Spratt & Hoeg, 1966). They concluded that the development of multiple primary malignant tumours was related to numbers of years at risk again this was confirmed by others (Bellet *et al.,* 1977, Gutman *et al.,* 1991). The pathological association appears to be between the superficial spreading malignant melanoma and the invasive ductal carcinoma (Koh *et al.*, 1987).

However, when all this is taken into account the advantage for females persist. We must therefore ask do oestrogens inhibit the growth of melanomas?

Endogenous oestrogens have indeed been shown to inhibit the growth of melanomas. Some tumours which developed in the pre-menopausal state have been seen to metastasize in the menopausal period many years after the initial tumour. In fact 5 year survival is better in pre-menopausal women (Jatoi *et al.,* 1993). The reasons that melanomas are thicker in pregnancy have defied reason, but their overall prognosis is the same as melanomas of identical thickness (Mackie *et al.,* 1991). In breast cancer it has been suggested that the prognosis is similar in both sexes for age matched controls (Willsher *et al.*, 1997). The place of the oral contraceptive appears less clear. Jatoi declares "women who had taken the pill at any time before diagnosis present with

significantly thinner lesions than their counterparts and women who had taken the pill in the year before the melanoma developed, had thinner lesions, than those who had stopped for more than a year. A similar trend is seen among individuals that used the hormone replacement therapy (Jatoi *et al.,* 1993). This seems to contradict a similar study from Australia which seemed to implicate the pill in the aetiology of malignant melanoma with a lag phase of up to ten years quoted (Beral *et al.,* 1984). Melanoma is one of the few tumours to metastasize to the breast and placenta. It may be transferred to the unborn foetus ! Oestrogen receptors have been found in 20% of melanomas and in up to 50% of benign naevi in melanoma patients (Meyskens *et al.,* 1984). This led to a trial of anti-oestrogen therapy for melanomas with a variable response. Tamoxifen as a single agent is minimally active in treating patients with metastatic melanoma, when it is combined with DTIC, BCNU, and DDP a marked improvement in the overall response rate is observed (McClay & McClay, 1994).

A hormonal basis for the development of the DNS phenotype was suggested by the fact that women presented earlier than the men and had a higher frequency of extremity lesions, but survived longer (Reimer *et al.,* 1978). Anecdotal reports exist of four women who developed secondary amenorrhea a year prior to the diagnosis of malignant melanoma and two relatives of melanoma kindred's, without melanomas noted accelerated mole activity following oral contraceptive use.

The rate of histologically proven dysplastic naevi that changed was 2.0 fold higher when women were pregnant, 1.4 fold higher with the use of hormone supplements and 1.1 fold higher with the use of the oral contraceptives (Ellis *et al.*, 1991).

Finally, an anecdotal report of a 35 year old woman with familial DNS who developed six primary cutaneous melanomas during her pregnancy! She had been treated with Clomiphene citrate for secondary infertility. All MMs' developed in pre-existing DN. The authors suggest that Clomiphene, (an anti-oestrogen) may have played a role in the activation, or progression of these "precursor lesions" into malignant melanomas (Kuppens *et al.*, 1992).

Section I

Anatomy and Molecular Biology of the Breast

Chapter 1

Breast Cancer

1.1 Incidence

Carcinoma of the breast was probably the earliest neoplasm for which surgical eradication was attempted. Leonides of Alexandria who preceded Galen was known to have progressively incised and cauterised tumours both to prevent bleeding and destroy the neoplasm. Despite the longstanding knowledge of its existence, breast cancer is still the most common cancer among women in the United Kingdom and North America. One in 9 women in the USA and 1 in 12 in the United Kingdom will develop breast cancer during their lifetime. There were 13 028 female deaths due to breast cancer in England and Wales in 1993 - this represents approximately 1 in 20 of all female deaths in the same period, compared with 80 in males due to the same condition (HPSSS, 1995). In North Thames the standardised mortality ratio (SMR) varies from 76 in Barnet to 117 in South Essex (NTHP, 1996).

In Europe there are an estimated 180 000 new cases per year - it represents 20 % of all malignancies. It is exceptional before the age of 20 years and rare below 30 years (Veronese *et al.*, 1994). The incidence then rises steadily up to the age of fifty years, after which the rate of increase slows down, although the incidence rate continues to rise. The highest incidence rates in the world have been observed among women living in Hawaii, British Columbia and California. In these areas the annual incidence rate is approximately 80-90 per 100 000. The lowest incidence has been observed in some areas of Japan, with an incidence rate approximately 12-15 per 100 000 women. Great Britain has the highest mortality rates - four times the rate of Japanese women (OPCS, 1992). The incidence rates in Europe decrease from the North to the South and from West to East. Studies conducted on migrants have shown the tendency of these populations to reach the incident rates of women living in the host countries within a couple of generations, confirming the importance of the environment and probably life style (Veronese *et al.*, 1995).

1.2 Risk Factors

The aetiology of breast cancer is not known, rather a set of risk factors have been

clearly identified.

1.2.1 Family History

There is a 2-3 fold increase in the risk of breast cancer among first-degree relatives of patients with breast cancer. This is increased in patients with relatives with either pre-menopausal (3 fold), or bilateral breast cancer (5 fold). Lynch and Lynch, have attempted to define the hereditary cases as those with a positive family history of breast cancer, in a distribution that is consistent with an autosomal dominant mode of inheritance (Lynch *et al.* 1986). This is believed to affect 8 % of all patients with breast cancer. The presence of the BRCA1 gene on chromosome 17q12-21, which is also linked to ovarian carcinoma has been found in less than 2 % of women with breast cancer (Miki *et al.,* 1994).

1.2.2 Radiation

Atomic bomb survivors have shown an increased incidence of breast cancer (Tokunaga *et al.* 1984) - this increased risk is also shown in patients who either had multiple fluoroscopies during treatment for pulmonary tuberculosis, or radiotherapy, for post-partum mastitis (Land *et al.* 1980). In these groups sensitivity to radiation is concentrated in the first (RR 2.4), second (RR 2.0) and third (RR 1.6) decades of life (Land *et al.* 1980, Tokunaga *et al.* 1984). There was no increased risk amongst women above the age of thirty. Veronese declares "this is important for it implies that there maybe little risk from repeat mammograms performed for screening" (Veronese *et al.* 1995).

1.2.3 Benign Breast Disease

Fibroadenomas confer an increased risk (1.5) of developing breast cancer. This rises with benign proliferations such as atypical ductal hyperplasia (ADH) and diffuse papillomatosis (McDivitt *et al.*, 1991, Rosen, 1993, Millikan *et al.*, 1995).

1.2.4 Reproductive History & Oestrogenic Theories

Nulliparous women have a greater risk of breast cancer, multiparity and a full term pregnancy before 20 years protect from the disease (MacMahon *et al.*, 1970, Hulka *et*

al., 1994).

Patients undergoing an oophorectomy before the age of 40 years have a reduced risk of breast cancer (Lilienfeld *et al.*, 1956). It is thus ironical that the highest incidence of breast cancer is in post-menopausal women who have long lost their oestrogenic stimuli. There is an increased incidence of breast cancer on prolonged use of the oral contraceptive pill, but a suggested protective effect from use of the progesterone only pill. Oestrogen producing ovarian tumours in post menopausal women are associated with the production of breast cancer. There is a suggestion that there may be an increased risk following prolonged use of hormone replacement therapy, but this is controversial (Pike *et al.,* 1993, Hulka *et al.,* 1994).

1.2.5 Diet

An association between high fat diet and breast cancer was first suggested by experimental studies in which high fat diets containing dimethylbenzanthracene (DMBA) were fed to rats (Carroll and Hopkins, 1979). Breast cancer occurs more commonly in societies which have a high intake of saturated fat and calories. Seventh Day Adventist groups, who have a low intake of dietary fat, have a low incidence of breast cancer, but only in those who adopt the practice at an early age (Philips *et al.*, 1980). Postmenopaysal obesity increases the risk of breast cancer (Pike *et al.,* 1993, Hulka *et al.,* 1994). Japanese immigrants to America acquire the incidence of the home population presumably related to their adoption of to the native diet (OPCS, 1992).

1.2.6 Others

Alcohol, hair-dyes (Nasca *et al.*, 1980) and reverse transcriptase activity in the blood (Al-Sumidaie *et al.*, 1988) have all been suggested as possible risk factors. The presence of reverse transcriptase activity in the monocytes of patients with breast cancer (which suggests the presence of a retrovirus) is particularly interesting since retroviruses cause mammary carcinoma .(Veronese *et al.*, 1995).

1.3 Anatomy of the Breast

The male breast and the immature female breast are similar according to R.J. Last. They both have a fully formed areola and the breast tissue does not extend beyond the margin of the areola, consisting of a few ducts embedded in fibrous tissue. At puberty the female breast enlarges and then retains the female form throughout life.

From a circular base the breast protrudes, or depends, to a degree that varies widely. The size of the base of the breast is fairly constant, it extends from the second to the sixth rib in the midclavicular line, lies over the pectoralis major and extends beyond the border of that muscle to lie on the serratus anterior and external oblique. An axillary tail is sometimes present. Asymmetry of the two breasts is common. The resting (non - lactating) breast consists mainly of fibrous tissue. Glandular tissue is very sparse and consists almost entirely of ducts Fig 1.1. Prior to lactation new alveoli bud off from the ducts into the fibrous tissue and the organ enlarges significantly.

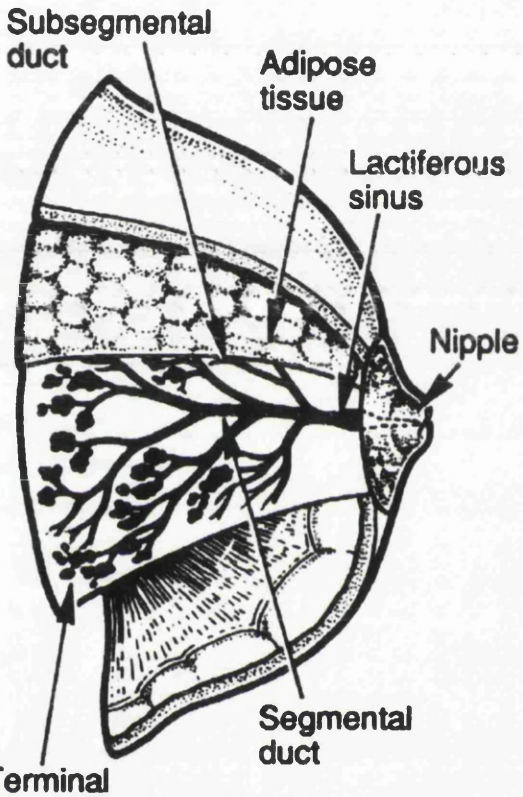

Fig. 1.1 A Schematic diagram of the breast showing the glandular structure in a supporting adipose base. The basic unit is the terminal ductal unit which drains into the segmental ducts and finally into the nipple via a lactiferous sinus.

The whole breast is embedded in the subcutaneous fat which usually obscures the lobules from sight and touch. There is no fat beneath either the nipple, or areola.

Beneath the breast is a condensation of superficial fascia, the continuation upwards of the fascia of Scarpa. Between this fascia and the deep fascia over the pectoralis major is a submammary space in which the lymphatics run.

The axillary tail when present lies in the medial wall of the axilla and may form a discrete mass with little connection with the main duct system. Usually it lies in the subcutaneous fat, which is condensed around it - very rarely it may penetrate the deep fascia of the floor of the axilla. The young breast is protuberant, the older breast pendulous. This is due to support from fibrous strands which connect the deep fascia with the overlying skin. These strands are called the ligaments of *Astley Cooper.* When atrophic they allow the organ to droop, when contracted from the fibrosis around a carcinoma they cause a pitting of the skin - the so called *peau d'orange.*

1.3.2 Lymph Drainage

The lymph capillaries make a rich anastomosing network continuous with the lymph capillaries of neighbouring structures - i.e. those of the opposite side and those of the abdominal wall. Lymph from the breast may thus radiate away to any point on the compass. Only when the lymph leaves the lymphatic capillaries and enters the valved lymphatic vessels is its subsequent course irrevocable. Most of the lymph of the breast drains, in fact, to the axilla. The superficial parts of the breast drain to a subareolar plexus, the deep parts drain to a submammary plexus, both communicate freely through the breast. The submammary plexus lies in the deep fascia over the pectoralis major and serratus anterior.

From these plexuses lymph from most of the breast drains to the pectoral group of axillary nodes (the axillary tail when present drains into the scapular group). The upper convexity drains to the infraclavicular group of lymph nodes. The medial convexity communicates across the midline with the submammary plexus of the opposite breast and cancer may extend, by retrograde permeation, along these pathways. The medial

part of the submammary plexus likewise communicates through the intercostal spaces with anterior intercostal lymphatic vessels. The inferior convexity communicates through the abdominal wall with lymphatic capillaries in the extraperitoneal areolar tissue and so through the diaphragm to mediastinal nodes.

1.3.3 Blood Supply

The blood supply of the breast is derived mainly from the lateral and internal thoracic arteries. It also receives perforating branches from the intercostal arteries. The upper breast is supplied by pectoral branches of the acromio-thoracic artery.

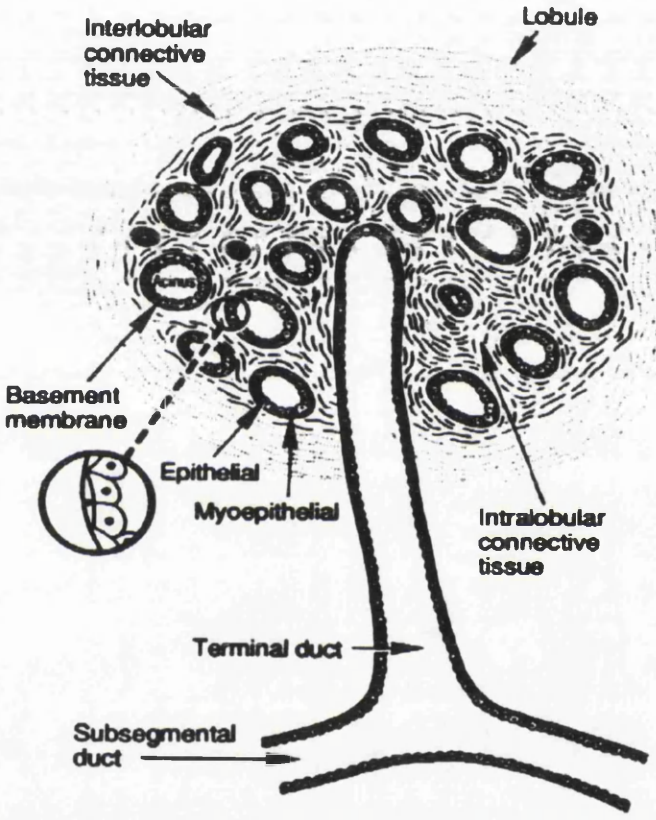

Fig. 2.1 A Schematic diagram of a terminal ductal unit (TDU) the basic functional unit of the breast. The lobule is made up of acini lined by an inner epithelial layer and an outer myoepithelial layer. These acini drain into a subsegmental duct via the terminal duct.

Venous return follows the afore mentioned arteries. The blood is received into large veins that also receive blood also from both the vertebrae and thoracic cage - the spread of malignancy thus involves these bones.

1.4 Histology

The adult female breast is made up of the epithelial elements (responsible for milk formation and transport) namely the acini and ducts, and the supporting tissue muscle,

fascia and fat.

The epithelial elements consist of 15 - 20 or more lobes. Each lobe drains into a mammary duct, each of which ends separately at the nipple as a lactiferous sinus (Fig. 1.1). Each lobe is subdivided into lobules - the functional unit of the breast. A lobule is a collection of between 10 and a hundred acini grouped around and converging on a collecting duct. An acinus is a sphere of cells capable of milk secretion, draining into a terminal duct. It is the confluence of the terminal ducts which give rise to a collecting duct. The terminal duct and its ductules form the terminal ductal lobular unit (Fig. 1.2). The major mammary ducts lie behind the areola. The lobules occupy the more peripheral part of the breast (Fig 1.1). The nipple, areola and the linings of the main lactiferous ducts are covered by stratified squamous epithelium. The major ducts are lined initially by a pseudostratified columnar, and then a double-layered cuboidal epithelium. A flattened layer of cells, the myoepithelial cells are present just beneath the lining epithelium (Fig.s 3.3 & 4.3). These cells contain myofilaments oriented parallel to the long axis of the duct. The ducts and ductules lie on a basement membrane.

1.5 Natural History of Breast Cancer

The transformation from benign to malignant cell occurs in the terminal ductal/lobular unit of the breast according to Veronese (Veronese *et al.*, 1995) (Fig. 1.2). The natural progression from benign to malignant is blurred in breast cancer. Histologically, the *in situ* stage appears to be an early stage but frequently small foci of cancer (< 1 mm) are found which show signs of invasion. Due to the extensive heterogenicity in breast cancer, a uniform histology has not been found within breast carcinomas. The heterogeneity may be explained by a polyclonal origin. The monoclonal origin of cancer (Fialkow, 1979, Millikan *et al.*, 1995) proposes that cancer arises from a single transformed cell. The more likely origin is that an area of the breast is exposed to a carcinogenic agent and as a result of this many normal cells undergo neoplastic transformation simultaneously, the resulting carcinoma is then composed of different clonal subpopulations. Cells that appear to be restricted to the basement membrane by

conventional examination may be shown to have breached it on electron microscopy (Carpenter *et al.,* 1987).

According to Veronese "breast cancer is multicentric in 13% of cases, simultaneous bilateral primary breast tumours occur in about 1% - 4% of cases and occult primaries occur in the opposite breast in 15 % of cases. Furthermore, the incidence of occult carcinomas in the breasts of women over 70 years who died from other causes is 19 times greater than clinical breast cancer, therefore it may well be that occult foci never progress to overt disease. (Veronese *et al.*, 1995). Routine biopsies of the opposite breast at the time of mastectomy produces a 12-15% incidence (Sanderson & Mackie, 1979, Kelsey *et al.,* 1993).

1.5.2 Local Spread

Initially breast carcinoma spreads by infiltrating the surrounding breast tissue.

Intraductal extension of the primary tumour may represent the exclusive or the major component of the proliferative process. The mass, although visible as a mammographic abnormality, is frequently impalpable and may be quite extensive. The presence of an extensive intraductal component often implies that there is carcinoma beyond the resection margins in up to 14% versus 1% of those without an extensive intraductal component (Holland *et al.*, 1990, Veronese *et al.*, 1995).

1.5.3 Growth Rates

There are exponential and gompertzian models of tumour growth in breast cancer which have reported rates between 105-215 days as tumour doubling time. If tumour growth rates are constant these figures are valuable, but if a gompertzian rate of growth is the norm the implications are totally different (Fournier *et al.*, 1980, Lundgen *et al.*, 1983, Galante *et al.*, 1986, Veronese *et al.*, 1995).

1.5.4 Axillary Node Metastases

Secondary deposits in the axillary nodes occur with a frequency which is a function of the tumour size, histological and biological characteristics (Elston & Ellis, 1998, Pinder *et al.* 1998). A higher incidence is found with tumours of the upper and outer quadrants

(Veronese *et al.*, 1995). The axillary nodes are divided into three levels: the first (Level I) includes the nodes lateral to the pectoralis minor; the second includes the nodes posterior to the muscle (Level II) and the third level includes nodes medial to the pectoralis minor (Level III). In a large series of 1446 cases (Veronese *et al.*, 1990) the mean number of nodes was 13.5 at the first level, 4.5 at the second and 2.3 at the third. The spread tends to be orderly - level I involved in 54 %, levels I and II in 23 % and all 3 levels involved in 21 % of cases. An irregular distribution was found in 1.3% of cases, metastases skipping the first level (1.2 %), or the first and second levels (0.1 %). This is used as a predictor of further spread - if level I is clear there is a negligible chance that the other levels harbour secondary disease, but there is a 40 % chance of disease upstream if it is positive.

The fashion of dissecting the internal mammary node faded away many years ago, but it was known that metastases to this node occurred with equal frequency whichever quadrant harboured the primary. The risk of involvement is related to the size of the tumour, the age of the patient and the axillary node status. A formula exists to predict the involvement of this node based on these criteria (Veronese *et al.*, 1995).

1.5.5 Distant Metastases

The most common sites of metastases are the lungs, bones and the liver. Veronese declares that the risk of metastases occurs mainly in the third to fifth year after surgery (Veronese *et al.*, 1995). It is lowest in the first and after the fifth year. The more aggressive the primary tumour the less likely this rule is obeyed. The risk of secondary disease is present up to 20 years after the primary was acquired. Bony secondaries are commonest in the pelvis and spine - this suggests a retrograde spread of tumour through the paravertebral plexus.

1.6 Pathology of Breast Tumours

Breast tumours, in common with all other tumours, can be divided into benign and malignant tumours, but in addition there are intermediate groups the *in-situ* carcinomas. The spectrum of tumours which occur are well documented (Veronese *et al.*,

1995, Elston & Ellis, 1998)

1.6.1 Fibrocystic Change

At least 10% of women develop palpable lumps which spawn the diagnosis of fibrocystic disease. It usually occurs between the ages of 20 to 40 years and rarely develops after the menopause. Faulty end organ hormonal metabolism, or imbalance, (either an excess of oestrogen or, a deficiency of progesterone) is believed to lie behind the development of these lesions. The use of the oral contraceptive decreases the risk of fibrocystic disease.

This term covers a wide spectrum of change within the breast, there are three dominant patterns of morphologic change,

(a) cyst formation and fibrosis,

(b) epithelial hyperplasia (ductal and lobular) and

(c) sclerosing adenosis.

(a) **Simple Fibrocystic change**

This is the commonest form of change, characterized by an increase in fibrous stroma associated with dilatation of ducts and the formation of cysts of various sizes.

(b) **Epithelial Hyperplasia**

The hyperplasias (also termed *epitheliosis)* are important because they confer an increased risk for the development of breast cancer. They may be the predominant change in the breast, or co-exist with fibrocystic change. Microscopically, proliferation results in an increase in the layers of the epithelium lining the duct beyond the usual double layer (Figs. 3.1 & 4.1). The proliferating epithelium sometimes takes the form of solid masses encroaching into the duct lumen, partially but not completely obliterating it, producing irregular lumina called *fenestrations* at the periphery of the of the masses (Fig 1.3).These lesions are usually less than 2-3 mm in diameter. Papillary epithelial projections may occur producing ductal papillomatosis. Architectural atypia of either the papillary, or solid projections produces *atypical ductal hyperplasias.Atypical lobular hyperplasia* is a term used to describe hyperplasias which occur in the terminal

duct and ductules (acini) that have some but not all the features of lobular carcinoma *in-situ.*

(c) **Sclerosing Adenosis**

This is less common than cysts and hyperplasias, produces intralobular fibrosis and proliferation of small ductules, or acini. Macroscopically, the involved area is ill defined and does not have the chalky yellow-white foci and streaks that identify breast carcinoma.

1.6.2 Benign Tumours

Fibroadenomas

The fibroadenoma grows as a spherical nodule, which is usually sharply circumscribed and mobile. These tumours are composed of both fibrous and glandular tissue. Cytogenetic studies have shown that only the fibrous (stromal) component is clonal.

Phylloides Tumours

These tumours, in common with fibroadenomas, arise from the intralobular stroma but, may recur, or be frankly malignant. They vary in size from a few centimetres to massive lesions involving the whole breast. *Phylloides,* is Greek for leaf-like, and sometimes the larger lesions are lobulated due to the presence of nodules of proliferating epithelium lined stroma.

1.6.3 Breast Carcinoma

The histologic range of tumour types based on the WHO classification is,

A. Noninvasive

1a. Intraductal carcinoma

1b. Intraductal carcinoma with Paget's disease

2. Lobular carcinoma in situ

B. Invasive (infiltrating)

1a. Invasive Ductal Carcinoma - not otherwise specified (NOS)

1b. Invasive Ductal Carcinoma with Paget's disease

2. Invasive Lobular Carcinoma

3. Medullary Carcinoma

4. Colloid Carcinoma (Mucinous carcinoma)

5. Tubular Carcinoma

6. Adenoid cystic carcinoma

7. Apocrine carcinoma

8. Invasive papillary carcinoma

1.6.3.1 Ductal Carcinoma *in situ* (DCIS)

This is the most common form of non-invasive carcinoma consisting of 3-4 % of symptomatic and 15 - 20 % of screen detected cancers (Page, 1995, Elston & Ellis 1998). This defines a population of malignant cells that lack the capacity to invade the basement membrane, and therefore, are incapable of distant metastases. However, approximately 2-3 % are associated with axillary node metastases (Fisher *et al.* 1986). Their importance lies in the fact that the cells can spread throughout the ductal system and produce extensive often impalpable lesions involving an entire breast. Extension to the nipple produces Paget's disease of the nipple (Fig.s 10.4 & 11.4).

The subtypes are -

(a) comedocarcinoma or high grade,

(b) intermediate and

(c) non-comedo which consists of cribiform, solid and micropapillary.

Comedocarcinoma is characterized by rapidly proliferating, high grade malignant cells. The centre of the ducts often contain necrotic material, on gross examination a cut section contains cheesy necrotic material - "comedone" like. Mammography shows linear branching calcifications. The comedo type has in general, a greater chance of recurring than the non-comedo type following breast conservation.

A **cribiform** pattern is characterised by duct like structures, within the primary dilated ducts. DCIS can grow to a considerable size without becoming invasive, implying it is a biological entity different from both LCIS and IDC. Furthermore, DCIS is multifocal in 30 % of cases, the foci present mainly in the same breast.

1.6.3.2 Lobular carcinoma *in-situ* (LCIS) defines a proliferative lesion limited to one or more terminal ducts and/or ductules (acini), it is composed of cells that are loosely cohesive, somewhat larger than normal with infrequent mitoses. They are frequently both multifocal and bilateral. The presence of LCIS confers a 30% risk of breast carcinoma in both the ipsilateral and contralateral breasts (McDivitt *et al.*, 1967).

1.6.3.3 Invasive Ductal Carcinoma

This is the most common variety of breast cancer, it accounts for 65 - 80% of all mammary cancers (Elston CW & Ellis, 1998 Pinder *et al.* 1998).

Histologically, most exhibit an increase in fibrous tissue stroma, giving the tumour a hard consistency (scirrhous carcinoma). It imparts a gritty feel on cutting the tumour. The tumour consists of malignant duct lining cells disposed in cords, solid cell nests, tubules, glands and anastomosing masses in various combinations.

Paget's Disease of the nipple is a form of intraductal carcinoma that arises in the main excretory ducts and extends intraepithelially. The diagnostic feature are Paget's cells - which have abundant clear or lightly staining cytoplasm and prominent nucleoli (Fig.s 8.2 & 9.2).

1.6.3.4 Invasive Lobular Carcinoma. These tumours constitute 5 - 10% of breast carcinomas. The tumours arise in the terminal ductules of the breast lobule (Fig. 1.2). In up to 20 % of cases they are bilateral and tend to be multicentric in the same breast. Grossly the tumour is poorly circumscribed and histologically consists of infiltrating tumour cells, often only one cell width (in the form of an "Indian file") loosely dispersed throughout the fibrous matrix (Fig.s 3.4 & 4.4).

1.6.3.5 Inflammatory Breast Carcinoma

This group represents the most aggressive form of breast carcinoma. It accounts for 1 - 4 % of all tumours and is usually but not uniformly fatal. These tumours present as a diffuse infiltration of the breast without a well defined tumour. The diagnosis is usually confirmed by a skin biopsy which shows dermal lymphatic invasion, but this is by no means invariable. Most are poorly differentiated and oestrogen receptor negative. The

WHO report on the histological typing of breast tumours states that inflammatory carcinoma does not constitute a histological type, but rather a clinical entity.

1.7 Breast Cancer Tumour Grade

Grade provides powerful prognostic information. It requires commitment and strict adherence to a recommended protocol (Pinder *et al.*, 1998). Tumours are graded by the Bloom and Richardson modification of Elston & Ellis (Elston & Ellis 1998, Pinder *et al.*, 1998). The method involves the assessment of three components of tumour morphology: tubule formation, frequency of mitoses and nuclear pleomorphism. Each is scored 1-3. Adding the scores gives the overall histological grade.

1.8 Prognostic Factors

The ability to predict the prognosis of an individual patient plays a significant part in planning the treatment of patients with all malignancies and breast cancer in particular. The clinical course for the individual patient and her tumour is highly variable, hence the plethora of prognostic factors. They function as therapeutic decision makers as the emphasis to multiple parameter analysis. Prognostic factors are of value for three main reasons (Miller *et al.*, 1995, Elston CW & Ellis, 1998, Pinder *et al.*, 1998)

- To help select appropriate treatment
- To allow comparisons of treatment between similar groups
- To improve our understanding of the biology of breast cancer

Three prognostic groups are recognised.

1. **Favourable Prognosis** - Malignant tumours with a low risk of death e.g. tubular carcinomas. They require no further treatment.

2. **Intermediate Prognosis** - this includes the majority of breast cancers. The decision to include adjuvant therapy requires further criteria.

3. **Poor prognosis** - highly malignant tumours with a short disease free interval and overall survival they routinely require adjuvant chemotherapy.

Clinico-pathological features are most widely used to predict behaviour, but it is accepted that especially in the intermediate groups, they are of limited prognostic

information.

The list of prognostic factors grows with each year therefore criteria for a "prognostic factor" to achieve have been established (McGuire, 1991).

(a) **Tumour Grade**. The higher the grade the worse the prognosis for the patient - there is 56 % 10 year survival for grade I, versus 33 % for grade III. Mitotic count is the best simple predictor on multivariate analysis followed by tumour size, lymphatic invasion and skin invasion. Peritumoural and vascular invasion both signify a grave prognosis (Elston & Ellis, 1998, Pinder *et al.*, 1998).

(b) **Histological Variety**. Tubular carcinomas, medullary carcinomas and mucoid carcinomas have a better prognosis than the more common IDCs and ILC's (Figueroa *et al.,* 1993, Elston & Ellis, 1998, Pinder *et al.,* 1998).

(c) ***In-situ* carcinoma.** Extensive *in-situ* carcinoma is associated with a greater likelihood of early recurrence (Holland *et al.* 1990).

(d) **Neovascularization.** Angiogenesis, the formation of new vessels has been shown to be essential for the tumour to achieve its full metastatic potential. The density of new vessels correlates with the risk of metastases (Weidner *et al.* 1991, Figueroa *et al.,* 1993, Pinder *et al.*, 1998).

(e) **Tumour size and Lymph Node Status.** Increased tumour diameter and the number of positive lymph nodes have an inverse effect on the prognosis. There is a linear correlation between both parameters (Figueroa *et al.*, 1995, Elston CW & Ellis, 1998, Pinder *et al.* 1998)).

(f) **Tumour Stage.** The tumour, node and metastases (TNM) group of the individual tumour is still widely used and gives an overall flavour of the expected behaviour of the individual tumour, although it is thought ot be unreliable (Elston CW & Ellis, 1998, Pinder *et al.* 1998).

(g) **Oestrogen Receptor.** Oestrogen and progesterone receptor status are cellular markers of endocrine responsiveness (Elston CW & Ellis, 1998, Pinder *et al.* 1998). However, with genetic polymorphisms, variant receptors occur (Veronese et al.,

1995). Both are present in normal mammary tissue, but higher in malignant tumours. There is a higher ER content in obese women, and in European compared to non-European, they are also more likely in the follicular rather than luteal phase. The ER content correlates with the PR status (Hulka, *et al.,* 1994). In post menopausal women high ER content is believed to be due to absence of circulating progesterone to down regulate the oestrogen. There may be a correlation between prolonged HRT and ER negative tumours (Hulka, *et al.,* 1994). There is a positive correlation between ER expression and tumour differentiation and the absence of lymph node metastases, but others have found that it is not of prognostic significance (Elston CW & Ellis, 1998, Pinder *et al.* 1998). Smaller tumours and lobular carcinomas are also more likely to be positive.

(h) **Tumour Doubling time.** Mammography has enabled an estimation of the growth rate - an oft repeated study is that of Lundgren, 139 cancers were diagnosed by mammography, but on a prior mammogram an obvious cancer had been overlooked representing a mean doubling time of 12.4 months; 144 cancers diagnosed between screening (interval cancers) had a mean doubling time of 2.2 months. Patients with a doubling time of less than 3 months had a five year survival of 56 %, whereas all with a doubling time more than three months were alive at 5 years (Fournier *et al.,* 1976, Lundgren *et al.,* 1977, Galante *et al.,* 1986).

(i) **Oncogene and Growth Factor Expression.** Most defined oncogenic events are relatively late in the process. Multiple oncogenes have been reported in breast cancer which affect prognosis (Bartek *et al.,* 1990, Barnes *et al.,* 1992 , Bates *et al.,* 1994, Pinder *et al.,* 1998)

(j) Prognosis has also been linked to **proliferative activity** of a cell population. Various methods have been described to estimate this (see chapter 5). It is an independent prognostic factor in lymph node negative disease. Diploid tumours have a better prognosis than aneuploid which are frequently receptor negative, poorly differentiated and have a high S-phase fraction (Weidner, 1994, Keshgegian *et al.,* 1995, Yu CC *et*

al., 1995). It is of interest that the mitotic count which is used in assesssing tumour grade, may be underestimated by 50 % if there is a delay of more than six hours in fixing the tumour (Pinder *et al.,* 1998).

(k) **Prognostic Index**

Tumour size, grade and lymph node status are traditional prognostic factors which have limitations which restrict their use to the prediction of 5 year survival. Since the introduction of breast screening the number of early breast cancers is rising and traditional prognostic factors have a limited value.

Prognostic indices have thus been developed. Many prognostic indices are available e.g. the Nottingham Prognostic index (NPI). The Nottingham index is a combination of tumour size, stage (the presence and level of histological node involvement) and tumour histological grade (Elston CW & Ellis, 1998, Pinder *et al.,* 1998). Three groups have been defined - the excellent prognosis group (EPG) have a prognosis close that of the age matched controls, the moderate prognosis group (MPG) have a survival of more than 70% but their survival at 15 years is approximately 30% and the poor prognosis group (PPG). The Nottingham index is the only prospectively validated integrated prognostic index so far published.

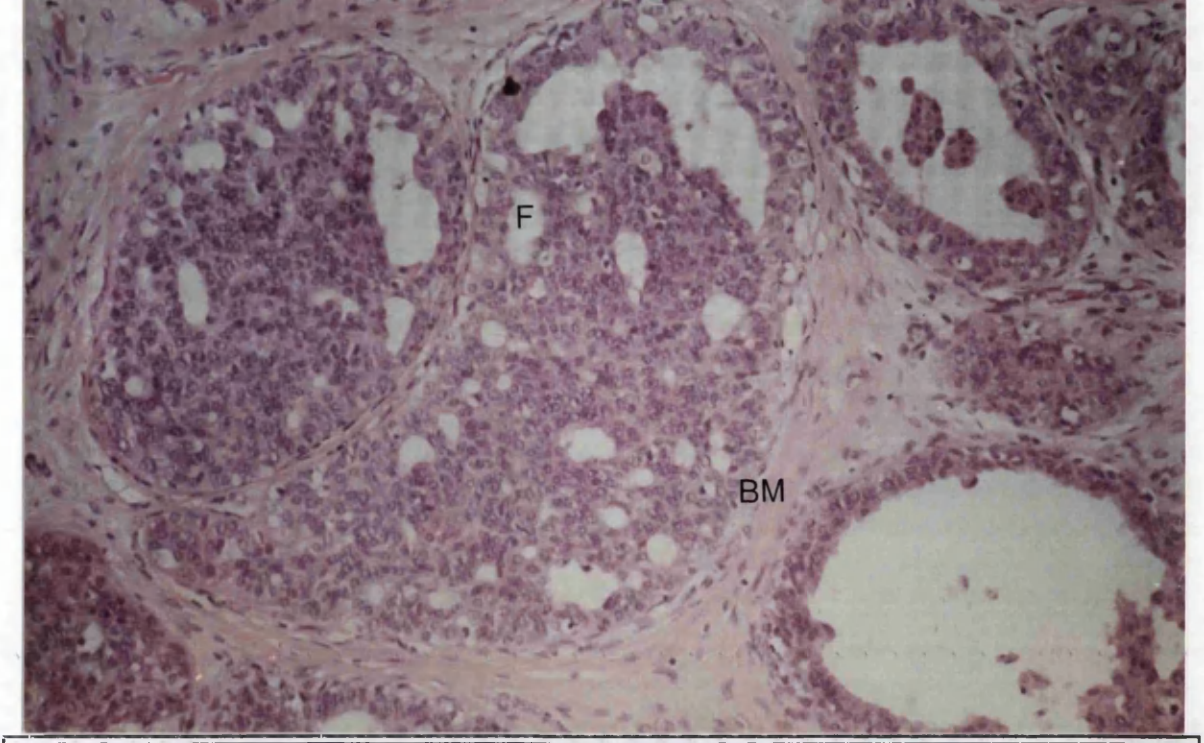

Figure 3.1 Haematoxylin & Eosin section of an area of atypical ductal hyperplasia (ADH). Showing small centrally situated darker cells and larger peripheral paler cells. The nuclei are larger than normal but there are no areas of necrosis. Irregular shaped lumina - fenestrations (F) exist between the abnormal cells. The intact basement membrane (BM) is clearly seen.

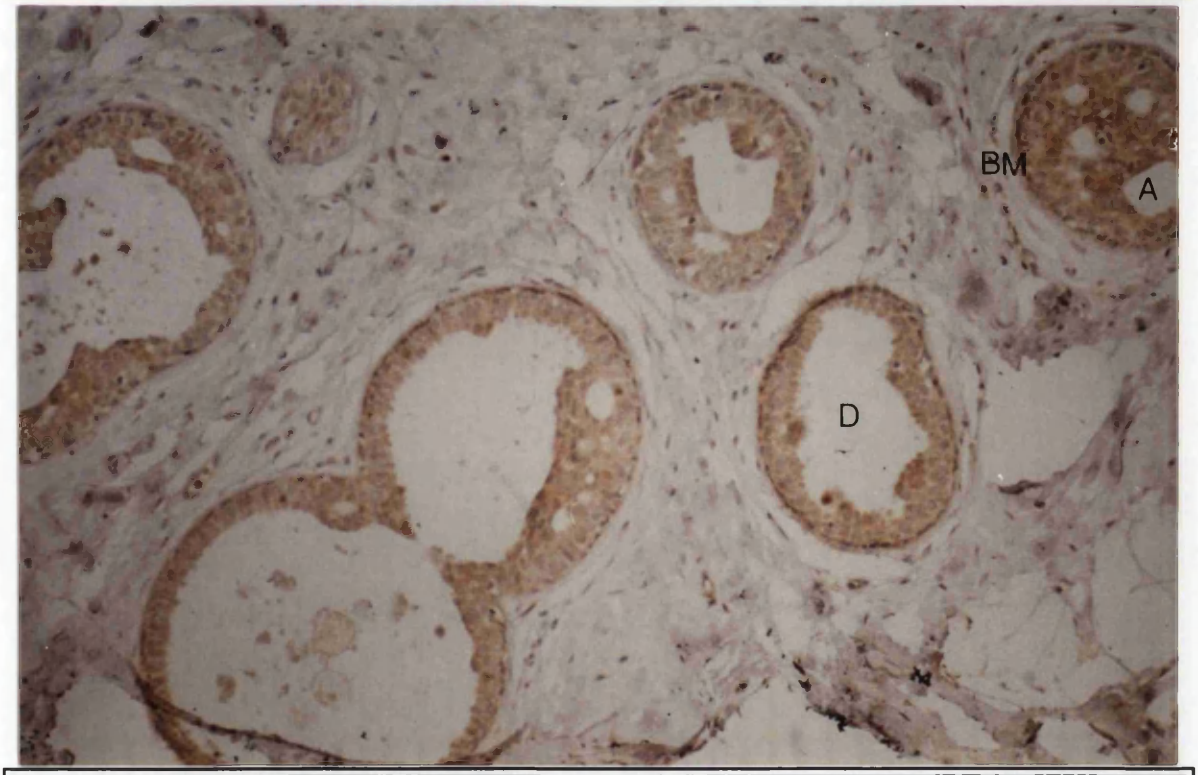

Figure 4.1 Bcl-2 positive area of atypical ductal hyperplasia (A). The intact basement membrane (BM) is clearly seen. Areas of ductal hyperplasia, greater than three cells thick (D) are also positive.

Chapter 2

p53 Tumour Suppressor Gene

2.1 Structure of the p53 molecule

The p53 protein was discovered bound to the Simian Virus 40 (SV40) large tumour (T) antigen (Lane and Crawford, 1979). Immunoprecipitations of large T antigen brought down a nuclear phosphoprotein which weighed 53,000 Daltons. It is encoded by a gene which is 20 kilobases (kb) long, and has 11 exons. It is located on chromosome 17p13.1 (McBride *et al.*, 1986).

There are eleven exons, with 5 highly conserved homologous domains on exons 2, 5,

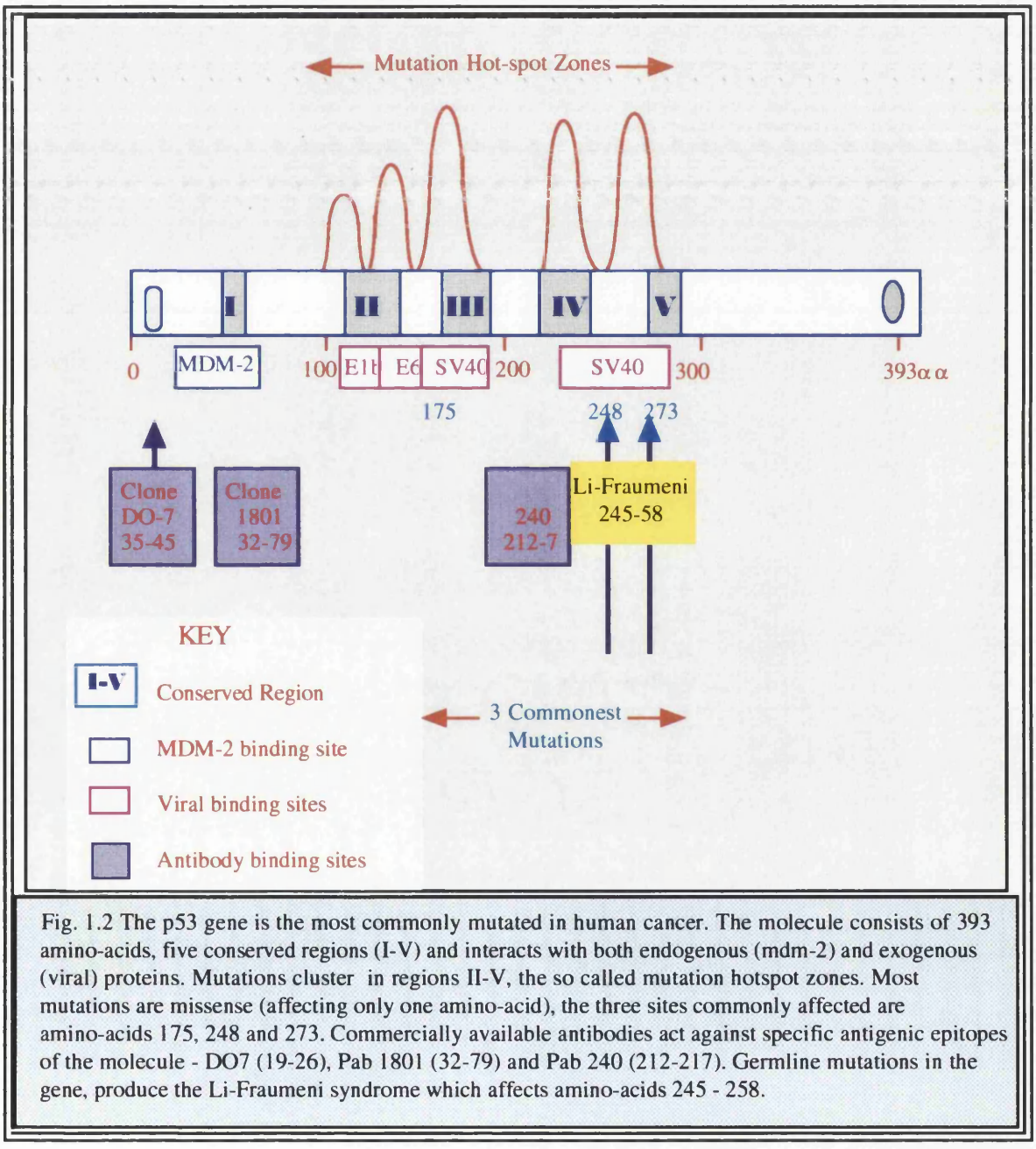

Fig. 1.2 The p53 gene is the most commonly mutated in human cancer. The molecule consists of 393 amino-acids, five conserved regions (I-V) and interacts with both endogenous (mdm-2) and exogenous (viral) proteins. Mutations cluster in regions II-V, the so called mutation hotspot zones. Most mutations are missense (affecting only one amino-acid), the three sites commonly affected are amino-acids 175, 248 and 273. Commercially available antibodies act against specific antigenic epitopes of the molecule - DO7 (19-26), Pab 1801 (32-79) and Pab 240 (212-217). Germline mutations in the gene, produce the Li-Fraumeni syndrome which affects amino-acids 245 - 258.

7 and 8, which specify domains I, III, IV, and V, respectively. Domain II is specified by both exons 4 and 5 (see Fig 2.1) (Harris A, 1992; Harris CC, 1996).

2.2 Functions of the p53 molecule

The p53 protein is essential for the orderly progression of the cell cycle, it screens the events as they occur, and halts the cycle if an anomaly is detected. The protein assists in the repair of the anomaly and then permits the cell cycle to continue, but if repair is deemed impossible it induces programmed cell death, or apoptosis (Harris A, 1992; Levine and Perry, 1993, Harris CC, 1996).

The p53 molecule contains several domains that mediate different biochemical properties of nuclear localization, sequence specific DNA binding and transcriptional activation. It also contains protein binding domains that allow p53 to dimerize with other p53 molecules (homodimers, or tetramers appear to be required for DNA binding and transcriptional activation (Farmer *et al.,* 1992), or with a variety of endogenous (TATA binding protein, mdm-2 gene product (Meltzer *et al.,* 1994), heat shock proteins) and exogenous (adenovirus EIb, human papilloma virus E6, SV40 large T, hepatitis B virus X) proteins (Fig. 1.2) (Harris A, 1992; Levine and Perry, 1993, Harris CC, 1996).

The N-terminus of the p53 protein (encoded by exons 2 - 4) is involved in transcription and multiple point mutations are required to inactivate this function. The carboxy terminus (encoded by exons 9 - 11) is important for nuclear localisation of the protein, recognition of DNA damage and the induction of apoptosis.

The interaction of the p53 protein with viral proteins and the endogenous protein mdm-2 is believed to result in sequestration of the protein shielding it from the transcriptional activity of the cell, abrogating its normal function (Meltzer *et al.*, 1994, Fisher *et al.*, 1994, McCann *et al.*, 1995). However, interestingly, the papillomavirus protein E6 (which is linked to the development of both anal (Barker *et al.,* 1997 unpublished) and cervical carcinoma) has been shown to increase the rate of degradation of p53 (Harris CC, 1996). This could also lead to malignant transformation, if it

resulted in loss of sufficient p53 to inhibit its normal function (Moll *et al.*, 1992).

2.3 Induction of p53 Protein Expression

Cellular insults induce *wild type* p53 expression, both *in-vitro* and *in-vivo* (Kastan *et al.,* 1993). The normal protein has a short half-life of less than 30 minutes and is therefore, not usually detectable in normal tissue by routine immunohistochemistry. Mutations in the gene which encodes the protein result in an abnormal protein, with both altered function and ironically, increased stability. The abnormal protein is detectable by routine immunohistochemistry.

In most cases the mutation results in a loss of function, but gain in function mutations can occur and may transform the protein from a tumour suppressor gene, to a protein with oncogenic potential, a true "*Jekyll and Hyde*" transformation. Some of the mutant p53 proteins can also bind to the wild type protein and inhibit its function by forming complexes that are inactive as transcription factors. This *dominant negative* effect, is one mechanism by which mutation of a single p53 allele can result in a biological effect (gain in function mutation) and it helps reconcile the conflicting concepts that the p53 gene is a tumour-suppressor gene, but that some mutations act in a dominant (oncogene-like) fashion (Levine, 1993).

2.4 Guardian of the Genome, or Orwellian Justice System

The overall effect of p53 induction has been likened to an all powerful, highly-efficient, Orwellian criminal justice system. Ahnen declares "....mutant p53 is like an absent (*deleted*), incompetent (*mutated*), or inattentive (*sequestered*) guardian who fails to see the damage to the victim and the resultant potential for even greater damage to the whole organism" (Ahnen, 1995).

Finally, a corrupt guardian that distracts the well meaning guardians (*dominant negative mutation*) or actively promotes the spread of damage to the rest of the genome (*gain in function mutation*). Mutation is however the basis of both evolution and carcinogenesis, therefore, if p53 were perfect all members of the community could, or would be identical. Homologues of the human p53 gene have been found as far back in the

evolutionary cascade as *Xenopus*.

2.5 Knudson's Two Hit Hypothesis

The p53 tumour suppressor gene is the most frequently mutated gene in human cancer (Holstein *et al.*, 1991, de Fromenthal *et al.*, 1992, Eeles, 1993). Persons heterozygous for germline mutations in tumour suppressor genes are strongly predisposed to one, or more kinds of cancer, and most dominantly inherited cancer is attributable to such heterozygosity. In genetically predisposed individuals virtually all forms of cancer are possible.

Individuals with familial adenomatous polyposis (FAP) develop colon cancers at an earlier than usual age - this is the classic example of genetic susceptibility involving mendelian dominant inheritance with high penetrance (Levi *et al.*, 1993, Icchi *et al.*, 1993, Shirasawa *et al.*, 1993). Here, the heterozygous state of the germline mutation imparts a high risk for just **one** form of cancer, while in the Li-Fraumeni Syndrome it predisposes to several kinds of cancer, although never to all forms. Breast cancer is the most frequent, but, does not afflict all female carriers. This incomplete penetrance of a gene for a particular cancer typifies the heterozygous state for a family cancer gene, its mere presence is not a sufficient condition for cancer. Knudson declares "the simplest explanation for this incomplete penetrance is that oncogenesis requires a somatic mutation in some target cell, an event that may never occur in some heterozygous carriers: two mutations, one germinal and one somatic would be needed. This hypothesis also relates the hereditary and non-hereditary forms of cancer by a common mechanism, the same two mutations would operate in both, the first mutation being germinal in the former and somatic in the latter. In the hereditary case all of the somatic cells would carry a first "hit", whereas in the non-hereditary case only a clone of somatic cells would do so". This constitutes Knudson's two hit hypothesis (Knudson, 1993). Thus, two copies of the tumour suppressor gene would be targets for the two hits. If only one hit occurred either somatic, or germline (producing a mutated product) the other copy of the tumour suppressor gene (the "wild type") functions to prevent the

formation of a tumour or tumours. Individuals with germline mutations, who have a global affectation of one copy of the individual tumour suppressor gene are still more susceptible to tumours. Primary fibroblasts from people with the Li-Fraumeni syndrome have one mutant and one wild type allele (Malkin *et al.*, 1990).

2.6 Tumours and Oncogenes

Cancer arises from disordered, or uncontrolled cell cycling. It has been suggested that at least five different mutations are necessary in order to produce a frankly malignant cell (Yokota *et al.*, 1993, Chang *et al.*, 1995). In familial cancer the first mutation is frequently inherited (Eeles, 1993). The resultant tumour is either benign, or malignant, depending on the degree of disorder in the cell cycle, the worse the disorder the more malignant the tumour.

The genesis and propagation of cancer is dependent on the interaction of three categories of genes *oncogenes, tumour suppressor genes* and the DNA *repair mutator* genes (Frebourg & Friend, 1992, Ahnen, 1995).

O*ncogenes* and *tumour-suppressor genes* are normal cellular genes that when mutated, can induce a biological effect that predisposes the cell to cancer development. According to Eng "Some people carry cancer in their genes" (Eng *et al.*, 1993).

According to Levine "...the major difference between oncogenes and tumour-suppressor genes is that oncogenes are thought to act in a dominant fashion, with an activating mutation in only one allele required for the biological effect, whereas, tumour-suppressor genes act in a recessive fashion and loss of function (inactivation) of both alleles is thought to be required for the biological effect" (Levine, 1993).

The same genes that are in involved in the propagation of hereditary cancer play a part in the development of sporadic cancer (Knudson, 1993, Eng *et al.,* 1993). Clinically, the identification of genetic susceptibility loci may allow premorbid testing, screening of populations, genetic counselling and prevention, including the removal of premalignant lesions.

The concept of the oncogene originated with the discovery of certain viral genetic

elements that are responsible for the tumour forming ability of retroviruses (Harris A, 1992, Harris CC, 1996). *Oncogenes* were first detected as passengers in the chromosomes of retroviruses, they originate from the host genome as *proto-oncogenes*. These genes were actually "stolen" from their host cells. An oncogene has been defined as a gene capable of causing cancer under experimental conditions (Leslie & Howard, 1992).

The p53 tumour suppressor gene was initially classified as an oncogene until it was revealed that cDNA cloned from cell lines contained mutations and the wild type was shown to suppress growth (Eliyahu *et al.*, 1990, Baker *et al.*, 1990).

Four characteristics of oncogenes have been described (Krontiris, 1995, Harris A, 1992). First, many different species of vertebrates and invertebrates have the same genes of this class, some, are conserved by evolution in organisms as primitive as yeasts. Second, the genes are activated in common non-viral cancers affecting humans. Third, the effects of activated oncogenes on cells are dominant, that is, there is a positive effect on growth even in the presence of the normal, or inactivated, versions of the same genes. The fourth and probably the most significant characteristic of *oncogenes*, is that each one encodes a protein that is implicated in signal transduction, the orderly and specific transmission of growth regulatory messages from outside the cell to the machinery controlling replication inside the cell's nucleus. Thus, arose the idea that the mutation of cellular signal transduction genes (now designated "*proto-oncogenes*") would permanently activate a message to make cells keep dividing (Krontiris, 1995).

2.7 p53 Mutations

Mutations in the p53 gene are the commonest abnormalities in human cancer (Holstein *et al.*, 1991, de Fromenthal *et al.*, 1992).

Many mutant p53 genes have now been sequenced and the mutations cluster in the four conserved domains. Human, mouse, rat, chicken, the rainbow trout and *Xenophus* p53 genes have similar sequences in the conserved domains (Harris A, 1992, Finlay, 1995,

Harris CC, 1996).

The most common combination of genetic alterations in the p53 gene in cancers is mutation of one allele and deletion of the other. About 80-90% of all the mutations found in the p53 gene are mis-sense mutations, that result in a single amino-acid change in the protein. Some of the mutations affect the transcriptional activity of the protein, others affect the protein binding ability (Harris CC, 1996). The loss of function mutations may however, confer an *oncogenic function* by changing the repertoire of genes which the new protein controls.

About 35 to 61 % of breast cancers harbour p53 mutations (Bartek *at al.,* 1990a, b, Yokota *et al.*, 1993). The great majority of point mutations (93%) are distributed over the large hydrophobic midportion, between amino acids 120-290 in the DNA binding domain (Holstein *et al.*, 1991, Levine *et al.*, 1991, Greenblatt *et al.*, 1994).

These somatic mutations of the p53 gene in human cancer occur over a large area of the gene, but are most commonly restricted to exons 5-8, which contain the conserved region. It is of interest that most of the mutations in the p53 gene (80%) result in conformational changes that stabilize the protein and increase its concentration within the cell. The frequently mutated sites (conserved regions) also represent the areas where the normal protein interacts with viral proteins (Fig 1.2) (Harris A, 1992).

The reasons for the frequent mutations of the p53 gene have been suggested as (a) the large size of the conserved region, allowing many different point mutations to exert a common conformational effect and (b) the mutations are dominant negatives (i.e. mutant p53 can inactivate *wild type* p53) ensuring that a single allele can abrogate normal p53 function.

Constitutional, or germline mutations of the p53 gene have been detected in a patient with an ependymoma, in 4% of patients with osteogenic sarcomas (Toguchida *et al.*, 1992) and in some families with the Li-Fraumeni syndrome (Barnes *et al.*, 1992, Malkin *et al.*, 1990). These mutations have been found in exons 4-9. Point mutations of the p53 gene does not invariably result in overexpression of the protein, Malkin

failed to find elevated expression in fibroblasts from the Li-Fraumeni syndrome with constitutional p53 mutations (Malkin *et al.*, 1990).

The **Li-Fraumeni syndrome** is defined as the association of sarcoma at a young age with sarcoma, or other defined tumours at a young age in two close relatives. The spectrum of cancers that is involved in the syndrome include osteosarcomas (Toguchida *et al.*, 1992, Iavarone *et al.*, 1992 Eeles, 1993), brain tumours, leukaemia, adrenocortical tumours, early onset breast cancer (Sidransky *et al.*, 1992), melanomas (Alsken *et al.*, 1992, Albino *et al.*, 1994), lung, pancreatic and prostatic cancer. The overall penetrance of gene carriers in the LFS is 90 % by the age of 50 and the majority of the tumours are early onset breast cancer (EOBC) (Eeles, 1993).

Some mutations are common to all tissues (175, 248, 273, 282) and others are unique to a specific tissue type e.g. lung tumours almost uniquely have residue 157 mutations, while 249 mutations are most common in hepatocellular carcinomas (Harris A, 1992, Finlay, 1995, Harris CC, 1996).

Some mutations in the gene arise from the action of tissue specific mutagens in the environment (e.g. hepatocellular carcinoma) while other mutations reflect a strong selection pressures, to produce the types of mutations that eliminate p53 functions (loss of DNA binding) in cancer cells.

As the p53 protein normally regulates passage through the cell cycle, disruption of its normal function may lead to mutagenic errors in replication and division resulting in the accumulation of genetic damage, including loss of the wild type allele, or loss of other tumour suppressor loci such as the retinoblastoma gene (Harris A, 1992, Levine and Perry, 1993).

2.8 The cell-cycle and p53

The p53 protein is expressed in all cells of the body, but is at its highest levels in the thymus, testis and ovary, organs that have recombination systems (Levine, 1995). p53 transcriptional activity is low, or absent in normal cells. However, expression of *wild type* p53 in normal cells of a cancer prone family has been documented (Barnes *et al.*,

1992).

It is now recognised that p53 is induced in response to chemotherapeutic agents (Harris A, 1992, Harris CC, 1996) and U-V, or γ–radiation (Kastan *et al.*, 1992) - events which induce DNA damage and that it is a key component of the physiologic check-point pathway, that governs the transition of cells from the G1 to S phase in the cell cycle (Perry and Levine, 1993). In the absence of normal p53 function, cells may progress through the cell cycle despite genomic injury, producing daughter cells with an increased rate of aneuploidy and gene amplification. Biochemically, p53 exerts its regulatory effects as a transcription factor via binding to a specific recognition sequence in target genes (Meltzer *et al.*, 1994). It is the failure of repair in the cell which harbours a mutated, sequestered, or absent p53 protein that leads to an accumulation of mutations and chromosomal re-arrangements which lead to a genetically unstable cell (Shay *et al.*, 1993). Despite its induction in response to DNA damage it appears to play no part in the normal embryological development of the organism (Vogelstein *et al.*, 1992). Thus, individuals with germline mutations in the p53 gene develop normally, as do mice with homologous deletions of the gene (Chang *et al.*, 1995). It has been suggested that exposure of cells to "stress" which occurs in a tumour confers a survival advantage to cells with p53 mutations (Vogelstein *et al.*, 1992), because without the mutations wild type p53 would be induced, stopping the cell cycle and either allow repair, or induce apoptosis. The frequency of allele loss on chromosome 17p is so high in breast cancer that it is likely to confer a survival advantage that is selected for during breast tumour formation (Davidoff *et al.*, 1991).

Mammalian cells arrest at two points in the cell cycle as a result of DNA damage, at the G1 to S and the G2 to M transitions (Pardee, 1982, Chang *et al.*, 1995). The p53 tumour suppressor gene product has a central role in the G1 checkpoint. p53 protein levels increase in response to gamma-irradiation (Kastan *et al.*, 1992). This appears to be post-transcriptional modifications and correlates with inhibition of DNA synthesis. Cell lines containing mutant p53 genes do not arrest in G1 after gamma-irradiation,

progressing through the S-phase normally. Transfection of wild-type p53 into these cells partially restores the G1 arrest. p53 induces the expression of growth arrest and DNA damage-inducible (GADD) genes specifically GADD-45 and $p21^{CIP1}$.(Kastan *et al.*, 1992, Chang *et al.*, 1995). According to Chang "since $p21^{CIP1}$ is a cyclin dependent kinase and proliferating cell nuclear antigen (PCNA) inhibitor (Chang *et al.*, 1995), p53 mediated induction of $p21^{CIP1}$ could be responsible for DNA damage induced G1 arrest. Furthermore, GADD45 binds PCNA *in vivo* after ionizing radiation induced DNA damage and has been shown to stimulate DNA repair *in-vitro*. In addition, overexpression of GADD45 appears to inhibit DNA synthesis. Therefore, p53 induction after DNA damage could eventually result in the inhibition of DNA replication and the induction of DNA repair by the activation of the expression of the $p21^{CIP1}$ and GADD45 genes. In this scenario, cells with no, or defective p53 could let DNA replication proceed with a damaged template, which in turn could result in gene mutation and amplification".

2.9 Methods to Detect p53 in Human Cancer

Methods for detecting p53 include

(a) sequencing mRNA,

(b) testing for serum antibodies against the protein (Angelopoulou *et al.*, 1995a, 1995b),

(c) the polymerase chain reaction (PCR),

(d) immunohistochemistry and

(e) Single strand conformational polymorphism (SSCP).

Sequencing from mRNA should reveal most mutations, but not if the mutations lead to loss of expression. Genomic sequencing by polymerase chain reaction (PCR) from DNA may give similar information, with less degradation than mRNA in tumours. However, since normal tissues make up at least 50% of the tumour mass, representative samples may not be taken. Microdissection from slides can overcome this problem, if necessary.

SSCP (single strand conformational polymorphism) of DNA is the most sensitive method and has picked up mutations that were not detected by sequencing, probably because the latter method was contaminated with normal tissue and SSCP may be sensitive enough to detect 10% mutant sequences among wild-type cDNA. Touch preparations are used to obtain relatively pure sources of cancer cells for PCR (Hall and Lane , 1994).

2.10 Serum Antibodies against p53

Antibodies have been found in the sera of up to 10 % of patients with breast carcinoma (Harris CC, 1991). Caron de Fromenthal found similar antibodies in the sera of 12-14 % of children with a wide variety of cancers. This rose to 20% in patients with Burkitt's lymphoma. The antigenic response is dependent upon p53/HSP70 complexes (Davidoff *et al.,* 1992). There have been reports of associations between p53 antibodies and high histological grade and absence of steroid receptors. Antibodies have also been found in the serum of patients with hepatomas, colon and ovarian cancer (Angelopoulou *et al* 1995a, 1995b).

Mutant proteins are produced in increased amounts in tumours either in the cell, or on its membrane. The host immune system mounts a humoral response to the high levels of the mutant protein and produces antibodies against them (Harris A, 1992, Angelopoulou *et al.,* 1995a, 1995b).

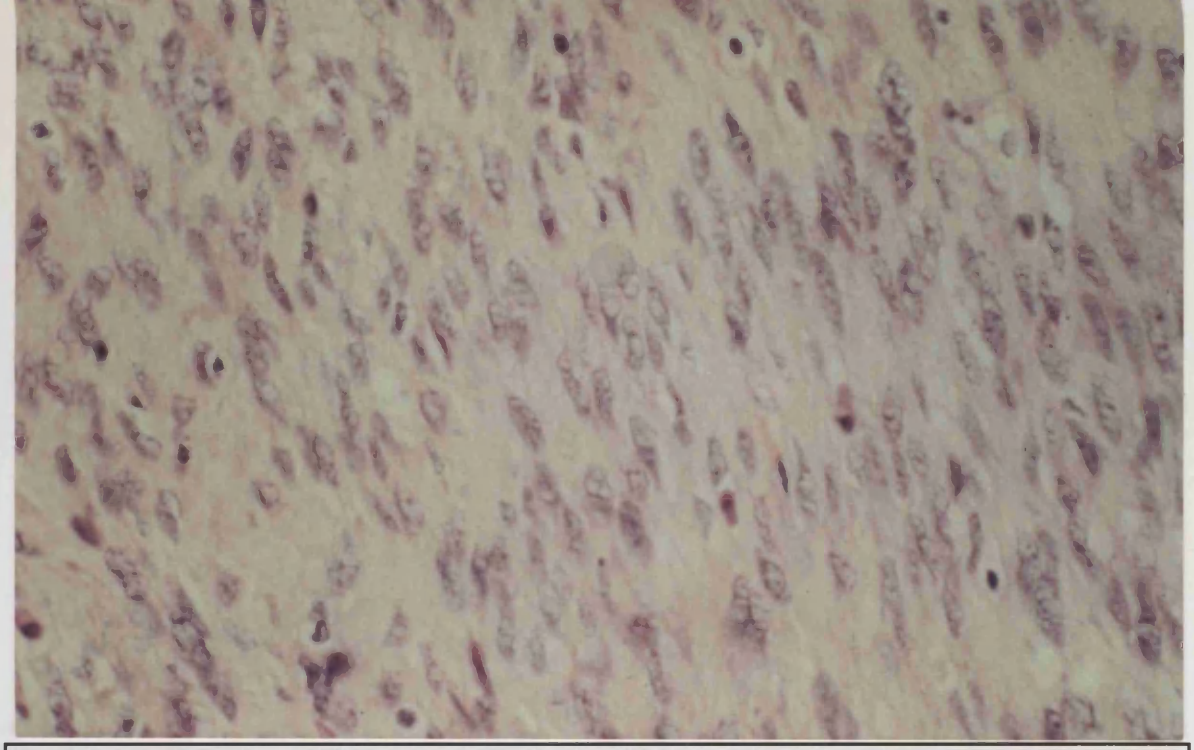

Fig. 2.2 Haematoxylin & Eosin section of a Sarcoma. A spindle celled tumour with large pleomorphic nuclei (cigar-shaped), tumour giant cells and numerous mitotic figures. There is no tumour necrosis.

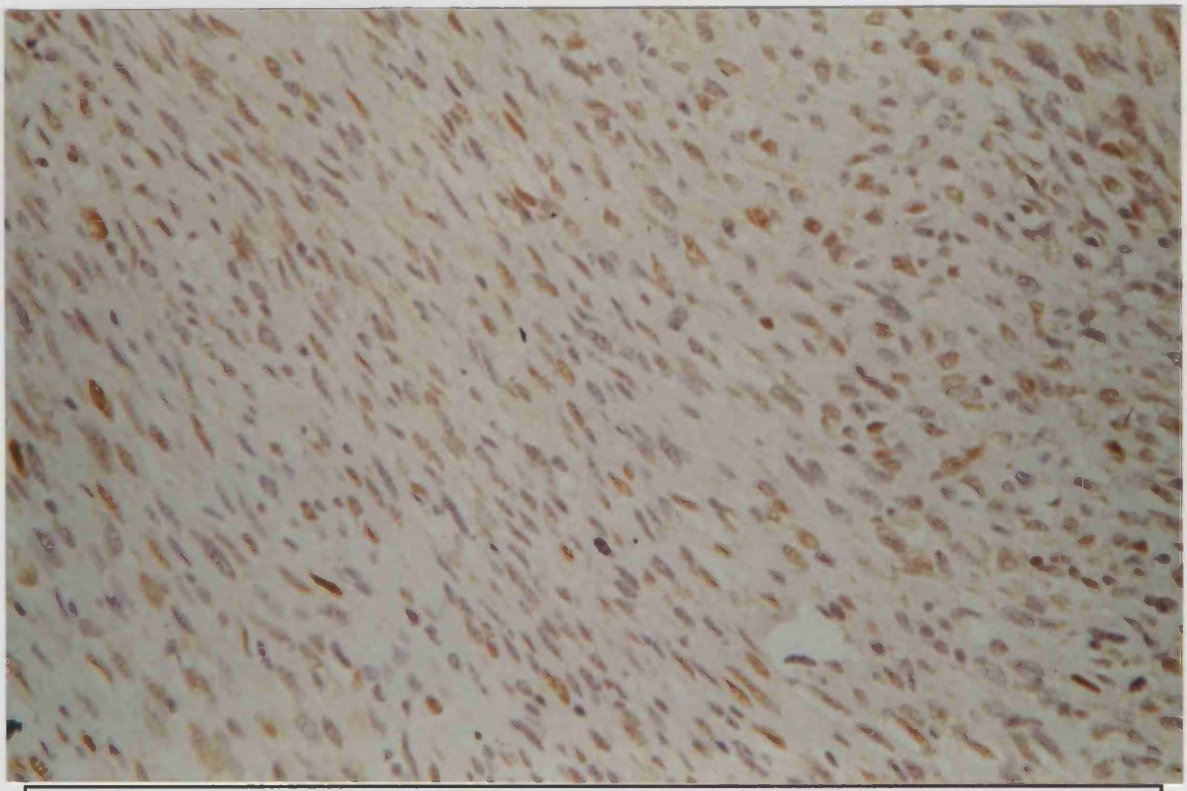

Fig. 3.2 p53 positive Sarcoma of the Breast. Greater than 50% of the tumour nuclei are overexpressing the p53 protein.

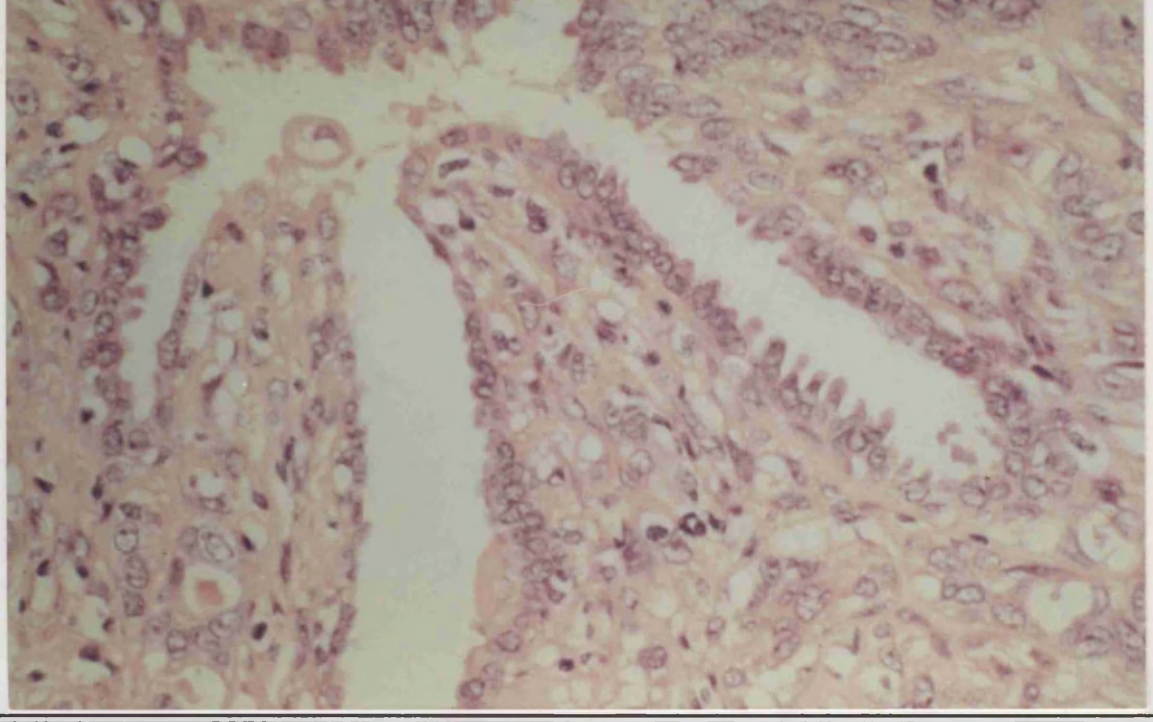

Fig. 4.2 Haematoxylin & Eosin Section of the area adjacent to the sarcoma in Fig. 2.2 showing a hypercellular stroma accompanied by a benign reactive ductal proliferation with leaf like projections.

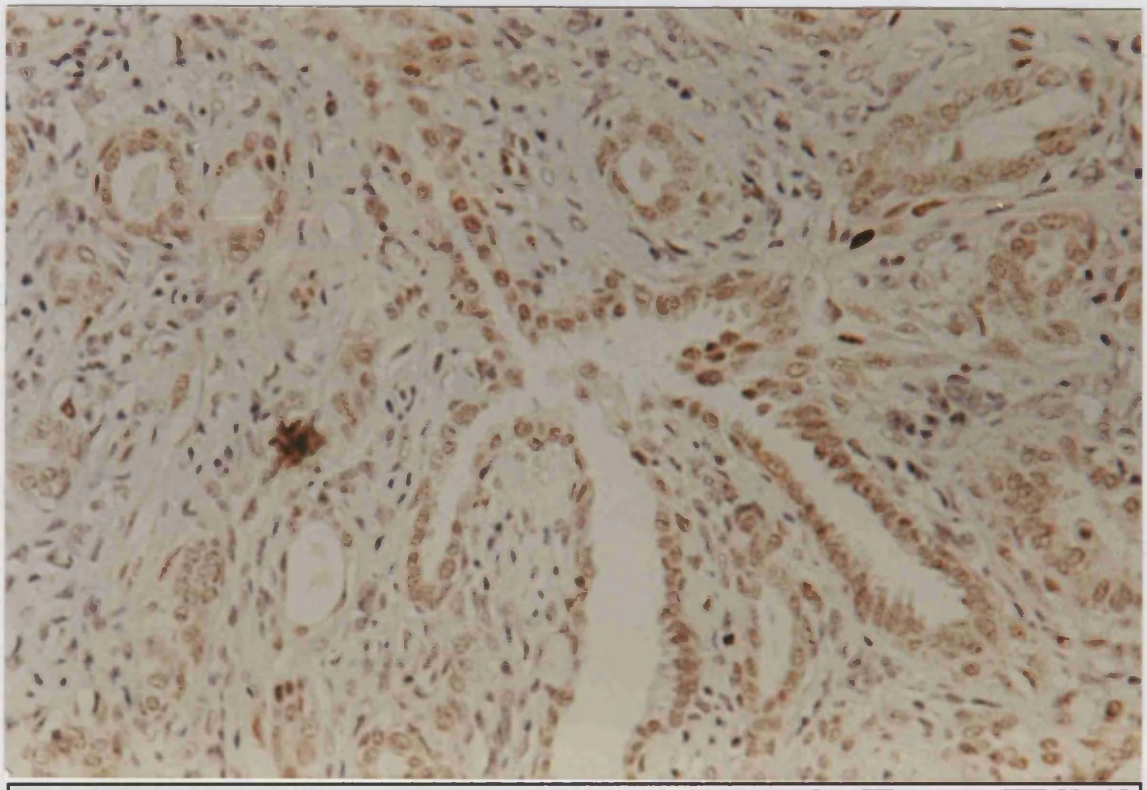

Fig. 5.2 p53 positive benign hyperproliferations, leaf like projections adjacent to the sarcomatous nodule

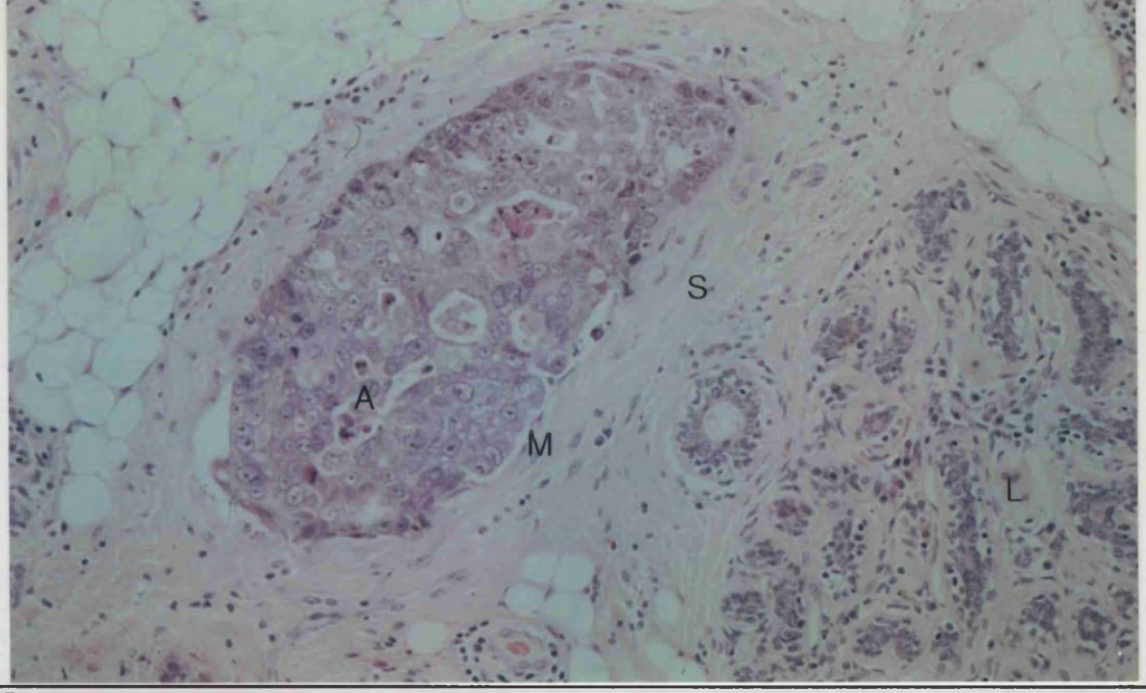

Figure. 6.2 Histological Section of a high grade *Ductal carcinoma in-situ* (DCIS), the breast duct is filled by a population of large pleomorphic cells which are confined within the basement membrane (M) in surrounding fibrous stroma (S). On the right there is a normal lobule (L). An area of apoptosis (A) - showing an area of nuclear condensation is clearly visible. A normal lobule (L) with normal sized nuclei is shown for comparison.

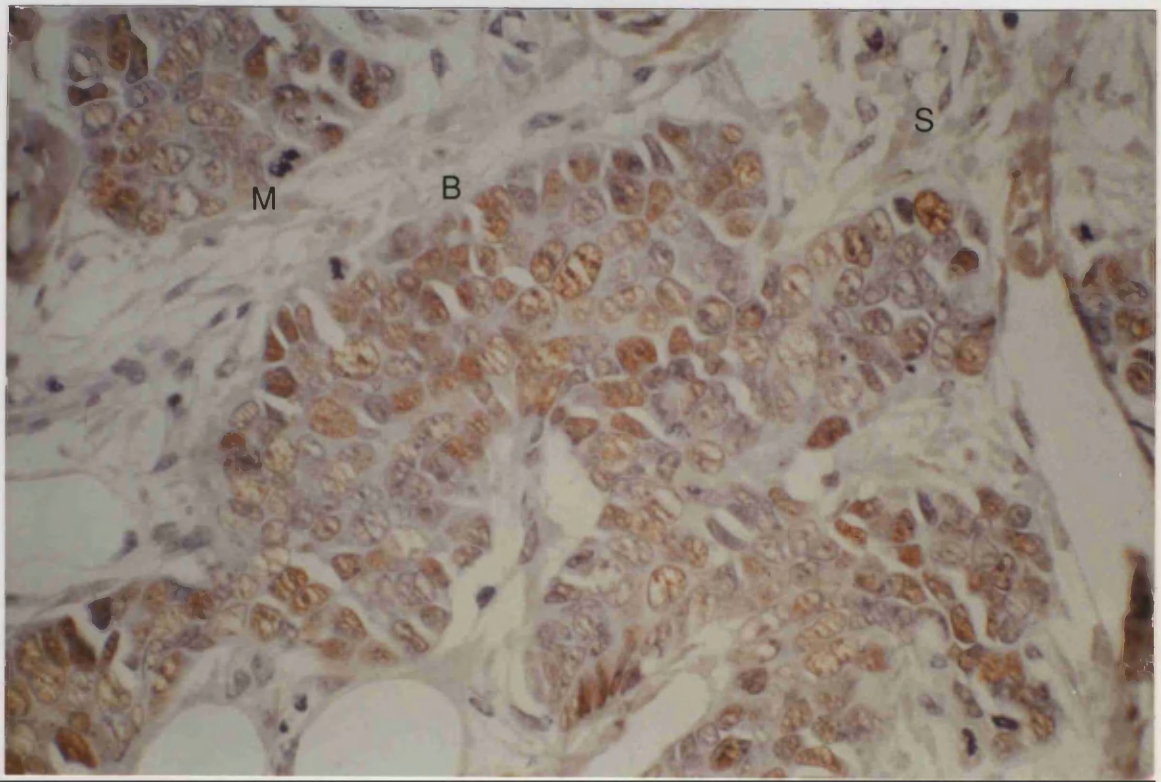

Fig. 7.2 An area of *ductal carcinoma-in-situ* (DCIS) showing showing nuclear pleomorphism, surrounded by an intact basement membrane (B). Greater than 50% of the nuclei are overexpressing the p53 protein (brown nuclei) and these are interspersed with negative cells (blue nuclei) embedded in a fibrous stroma (S). Some abnormal mitoses (M) are visible in the lower right hand corner.

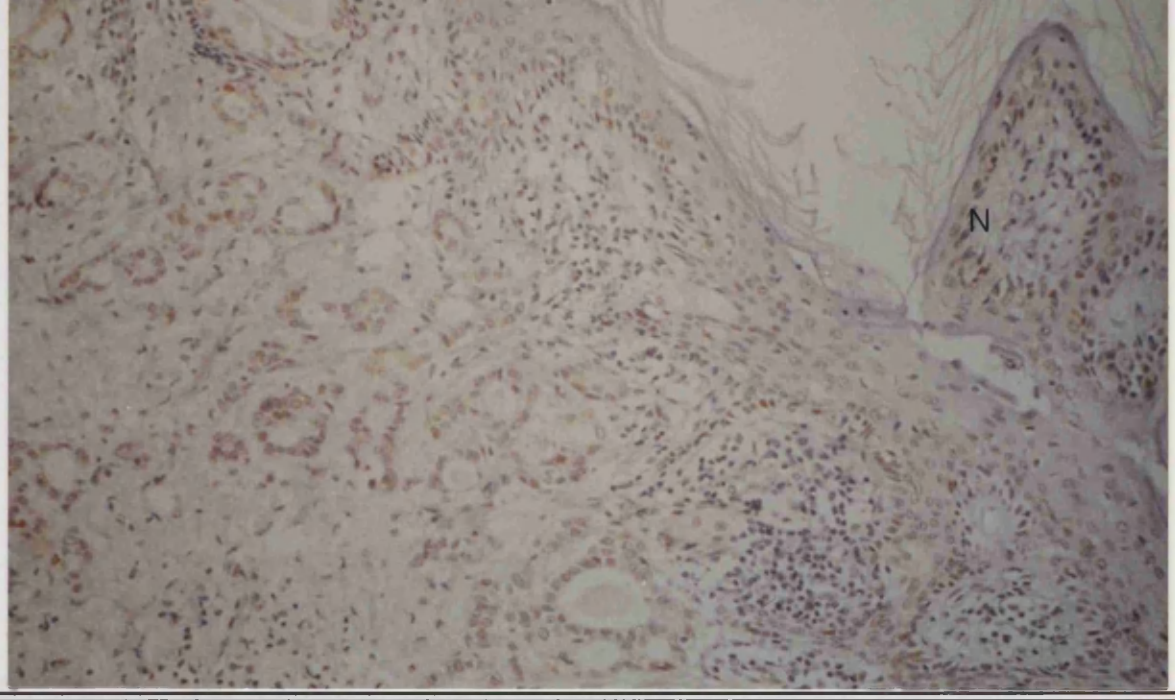

Fig. 8.2 Paget's Disease of the Nipple. The p53 positive nuclei (brown cells) are larger than the normal keratinocytes and possess cytoplasmic vacuoles - these are the Pagetoid cells. There is infiltration of these neoplastic cells into the epidermis of nipple (N) with only occasional nuclei expressing the p53 protein. In the lower left hand corner there is a well differentiated Grade I Invasive Ductal Carcinoma.

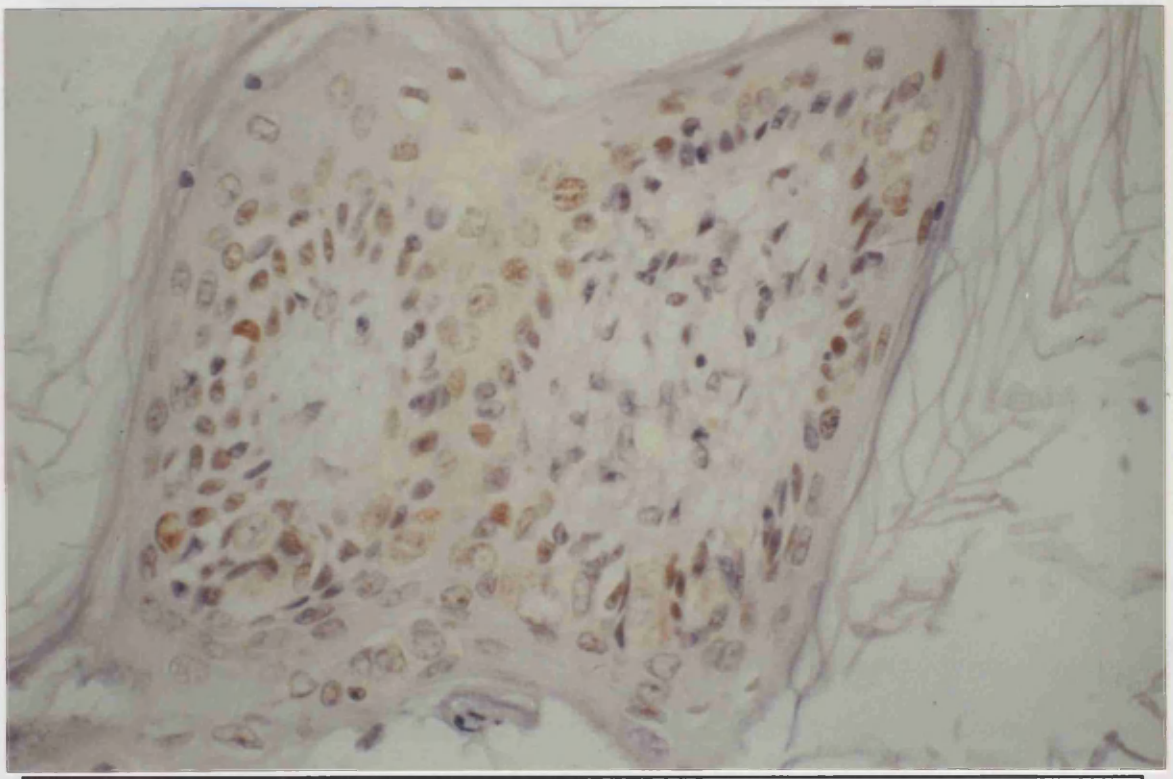

Fig. 9.2 Nipple in Paget's Disease. At higher magnification x 40. There is infiltration of the p53 positive neoplastic cells (stained brown) into the epidermis of the nipple.

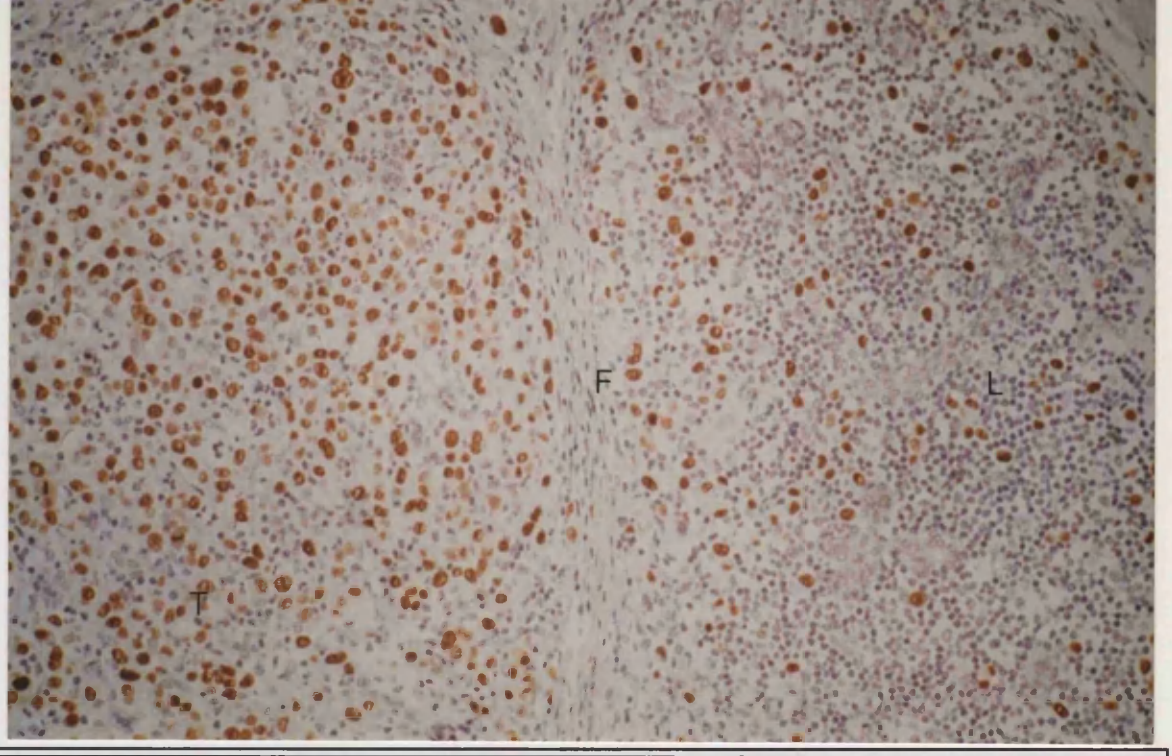

Figure 10.2. High grade breast tumour (T) (grade III) with greater than 75% of the tumour nuclei positive for the p53 protein (brown nuclei). There is a florid lymphocytic response (L) and obvious nuclear pleomorphism. The two regions are separated by stromal elements - fibroblasts (F).

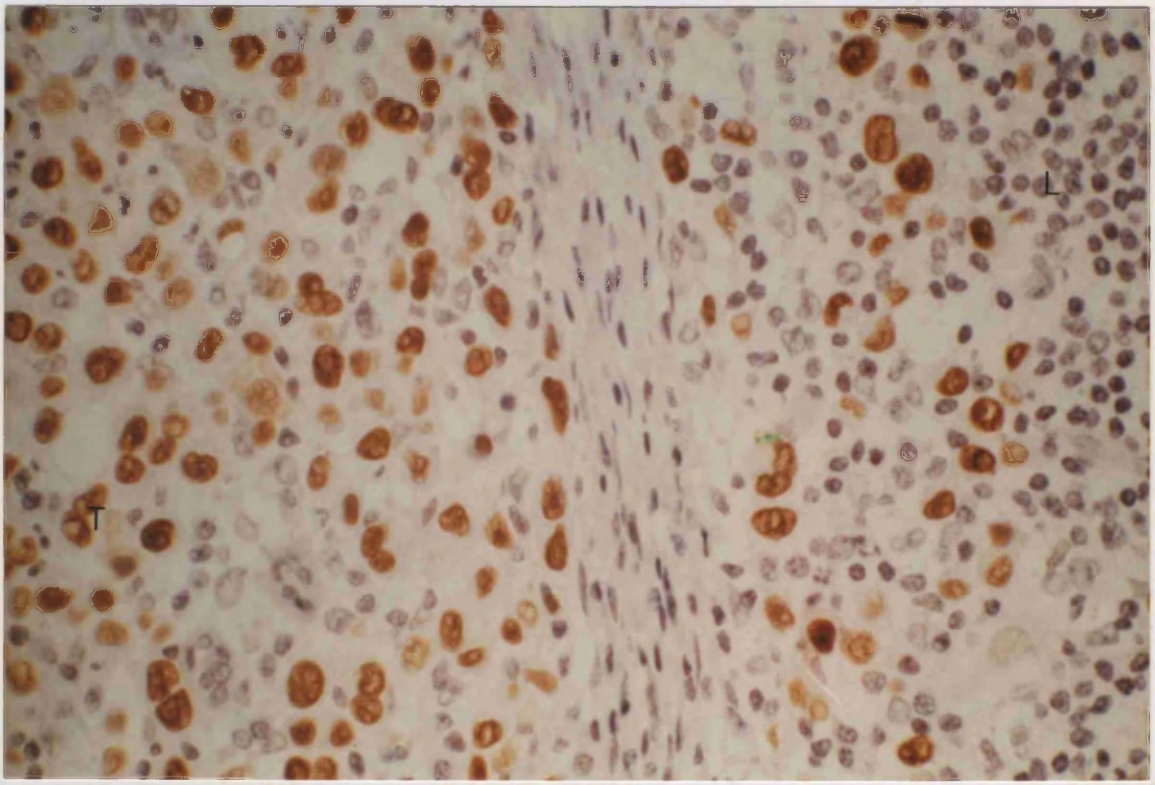

Fig. 11.2 Higher magnification showing the tumour nuclei (T) which are positive for the p53 protein separated by a fibrous stroma, there is a profuse lymphocytic response (L). The lymphocytes are negative.

Chapter 3

Apoptosis and Bcl-2

3.1 Definition: The intercellular connections that characterize advanced forms of life would not be possible without a mechanism to remove individual cells that are no longer needed, or that function abnormally. Such physiological cell death, in the absence of inflammation, is achieved by apoptosis, a structurally distinct programmed cell death pathway (Carson & Ribiero, 1993).

3.2 History

The term "apoptosis" has its origins steeped in the mystique of scientific discovery. It was suggested in 1972 by a professor of Greek at the university of Aberdeen on the prompting of three pathologists excited by their new and exciting discovery (Kerr *et al.*, 1972). It is a Greek word used to describe "dropping off" or "falling off" of petals from flowers, or leaves from trees. The authors propose that it be pronounced apo-ptosis - the second half of the word pronounced like "ptosis" (with the "p" silent) - which comes from the same root "to fall" the term used to describe the drooping upper eyelid.

The discovery of a distinctly different mode of cellular death with ultrastructural features that are consistent with an active "inherently controlled" phenomenon was a major advance.

3.3 Morphology

In a seminal account the electron microscopic structural changes in apoptosis were described over 25 years ago (Kerr *et al.*, 1972). Apoptosis characteristically affects scattered single cells and is manifested histologically by the formation of small roughly spherical, or ovoid cytoplasmic fragments, some of which contain pyknotic remnants of nuclei. These are termed **apoptotic bodies**. It takes place in two stages, the first comprises the formation of apoptotic bodies, the second their phagocytosis and degradation by other cells. The formation of apoptotic bodies involves marked condensation of both nucleus and cytoplasm, nuclear fragmentation, and separation of protuberances that form on the cell surface to produce many membrane bound, compact, but otherwise well preserved cell remnants of greatly varying size (Kerr *et al.*,

1972, 1994). Cells that are undergoing apoptosis are recognised as having condensed and separated from their neighbours and the nuclear chromatin is aggregated in dense masses beneath the nuclear envelope. Fully developed apoptotic bodies show closely packed organelles, which may themselves be condensed, but which are apparently intact, both chemically and structurally.

Apoptosis can always be detected in untreated malignant neoplasms and it participates in the regression that follows at least some forms of therapy (Kerr *et al.*, 1972, Rasbridge *et al.*, 1993, Harris CC, 1996, Pinder *et al.*, 1998). Its focal appearance at specific times during normal ontogenesis indicates that it is implicated in the fashioning of developing organs and digits and the involution of phylogenetic vestiges in the embryo. It often appears spontaneously or in response to physiological stimuli and can be triggered by both noxious stimuli and ischaemia. *"It seems possible to us that focal hyperplasia in tissues subject to cyclical hormonal stimulation, such as the breast, might be due to failure of clones of cells to respond in the normal way to falling hormonal concentrations by undergoing apoptosis. In certain circumstances apoptosis is an inherently programmed event, determined by intrinsic clocks specific for the cell type involved"* (Kerr *et al.*, 1972). These prophetic words uttered over 25 years ago have now been proven.

3.4 Biochemical Mechanisms

The end results of apoptosis are clearly visible in the routine histological section (Kerr *et al.*, 1972, Ellis & Pinder, 1998). However, the biochemical events which precede it are complex. There is no invariable sequence of events that occurs during apoptosis (Wyllie *et al.*, 1980, Oberhammer *et al.*, 1993, Hetts, 1998).

It is established that a series of enzymatic steps precede apoptosis which may or may not be preceded by protein synthesis. A rise in calcium is believed to provoke endonucleases which cleave DNA - forming fragments of DNA base pairs.

The detection of DNA fragments is one widely used *in-vitro* method of proving that apoptosis has occurred (Wyllie *et al.*, 1980). These fragments are detected readily by

agar-rose gel electrophoresis. However, this fragmentation is not always immediate, nor does it occur in all forms of apoptosis (Oberhammer *et al.*, 1993).

3.5 Physiological and Pathological States Associated with Apoptosis

Apoptosis can be provoked either by physiological, or pathological stimuli. Generally, anything that produces necrosis by direct cell destruction can induce apoptosis - this includes both radiotherapy and chemotherapy, **if**, the cell initially survives (Harris A, 1993, Kerr *et al.*, 1994, Wyllie *et al.*, 1994). It thus represents a co-ordinated cellular response to noxious stimuli that are not immediately lethal.

Embryogenesis
Normal Tissue Turnover
Shedding of the intestinal lining
Regression of the lactating breast after weaning
Atrophy of the prostate after castration
Withdrawal of a trophic hormone

TABLE 3.1 PHYSIOLOGICAL STATES ASSOCIATED WITH APOPTOSIS

(Carson & Ribiero, 1993, Kerr *et al.*, 1972, 1994, Hetts, 1998).

The cytotoxic effects of glucocorticoids on immature thymocytes, and of tumour necrosis factors on susceptible malignant cells, are due to apoptosis (Clark *et al.*, 1993, Kerr *et al.*, 1994). It plays an important role for clonal selection of lymphocytes in the thymus, clearance of antigens, and immune-mediated cytotoxicity (Carson & Ribiero, 1993, Hetts, 1998).

3.5.1 The Apoptotic Sequence

It is now clear that apoptosis is the end result of a rather aesthetic process of ridding the body of dead and dying cells. Four steps precede it (Fig 3.1) but multiple substances interact to produce the end result (Carson & Ribiero, 1993, Hetts, 1998).

- Commitment to death by intracellular or extracellular signals
- Cell killing or execution, intracellular protease activation

- Engulfment of cell corpse
- Degradation of cell corpse by phagocytes

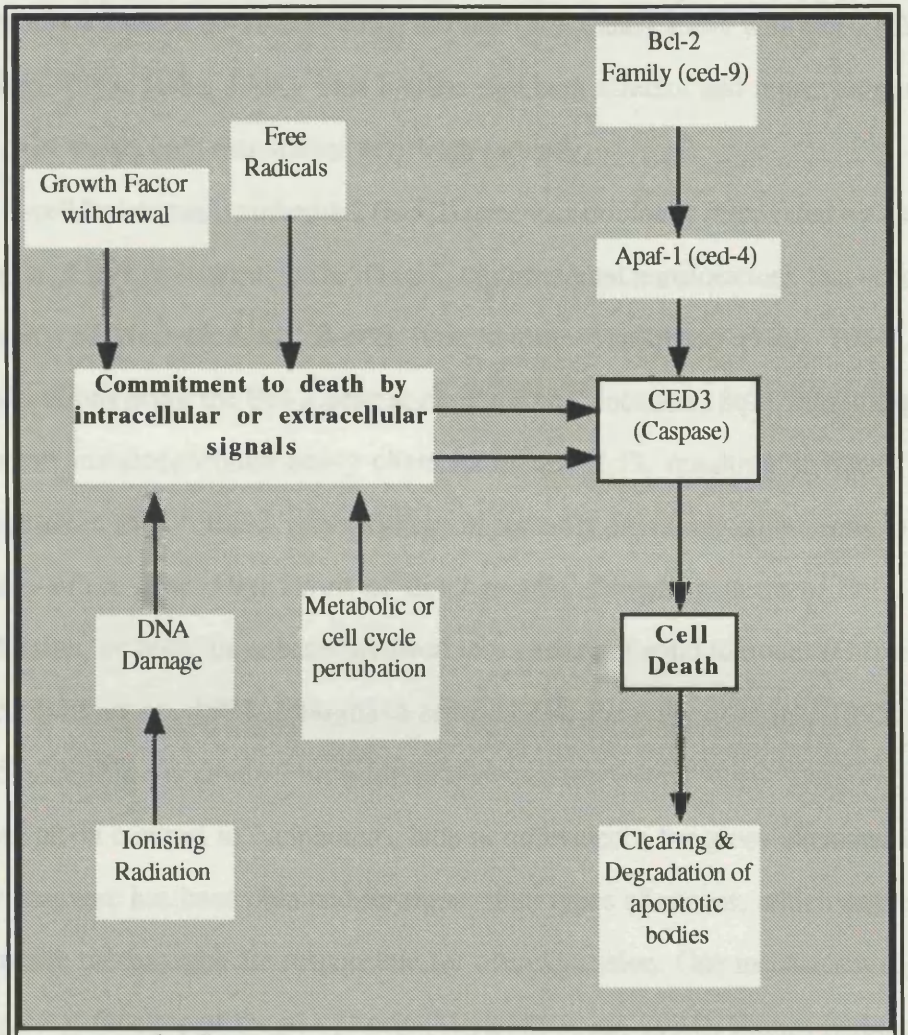

FIGURE 3.1 SCHEMATIC DIAGRAM SHOWING THE STAGES OF APOPTOSIS (HETTS, 1998). *Apaf-1 Apoptosis protease activating factor 1

3.6 Bcl-2 and the control of Apoptosis

Abnormal apoptosis promotes tumour formation by allowing the accumulation of dividing cells and obstructing the removal of genetic variants (Carson & Ribiero, 1993). The decision to die is often made actively by cells, initiating the process of apoptosis in response to local cytokines, signals from viral genes, or injury. The best defined genetic pathway of cell death exists in the nematode *Caenorhabditis elegans.* Two autosomal recessive death effector genes, ced-3 and ced-4, are required for the

death of all 131 cells destined to die during worm development (Ellis *et al.*, 1991). One autosomal dominant repressor gene, ced-9, can save those cells in its gain of function form. The products of both ced-3 and ced-9 have mammalian homologues: CED-3 with the protease interleukin-1ß-converting enzyme (ICE), and CED-9 with Bcl-2 (Carson & Ribiero, 1993, Hetts, 1998). This implies that both effector and repressor genes will also exist within each mammalian cell death pathway.

The B-cell leukaemia/lymphoma-2 (Bcl-2) gene was originally discovered by Tsujimoto because of its involvement in the t(14;18) chromosomal translocations that occur in the majority of Non Hodgkin B-cell lymphomas (Tsujimoto *et al.*, 1984). These translocations bring the Bcl-2 gene at chromosomal location 18q21 into juxtaposition with the immunoglobulin heavy chain locus at 14q32, resulting in transcriptional deregulation of the Bcl-2 gene without necessarily involving alterations to coding regions of the gene. High levels of Bcl-2 protein, aberrant patterns of Bcl-2 protein production, or both, have been involved in a variety of solid tumours (Grover *et al.*, 1993, Leek *et al.*, 1994, Bharghava *et al.*, 1994, Piettenberg *et al.*, 1995, Norris, 1995).

However, in contrast to lymphomas, little or no evidence for gross alteration in Bcl-2 gene structure has been obtained for these other types of cancer, which suggests that alternative mechanisms are responsible for overexpression. One mechanism suggested is a gain of function of the p53 protein (Miyashita *et al.,* 1994). Clones harbouring the oncogenic t(14;18) chromosomal translocation are commonly present in normal humans, that such clones are long-lived and that they rise in frequency with age (Liu *et al.*, 1994).

Bcl-2 blocks death following a variety of stimuli, it confers a death-sparing effect to certain haemopoetic cell lines following withdrawal of growth factor, it protects primary neuronal cultures from nerve growth factor withdrawal death (Oltavi *et al.*, 1993, Hockenberry *et al.*, 1990, 1993, Vaux *et al.*, 1993). In complex epithelium such as the skin and gut it is restricted to stem cell and proliferation zones (Hockenberry *et*

al., 1991, 1993, Rodriguez-Villanueva *et al.*, 1995, Piettenberg *et al.*, 1995, Norris, 1995). In the adult nervous system it is more prominent in the regeneration competent peripheral nervous system than the central nervous system (Hockenberry *et al.*, 1991, 1993).

Although the process of cell death controlled by Bcl-2 can occur in many cell types, there appears to be more than one physiological cell-death mechanism (Carson & Ribiero, 1993, Hetts, 1998). Targets of cytotoxic T-cells and cells deprived of growth factor both exhibit changes characteristic of apoptosis, such as DNA degradation. However, Bcl-2 expression protects cells from factor withdrawal, but fails to prevent cytotoxic T-cell killing. DNA degradation is, thus, not specific for any one cell-death mechanism. The ability of Bcl-2 to protect cells from a wide variety of pathological, as well as physiological stimuli indicates that many triggers can serve to activate the same suicide pathway, even some thought to cause necrosis and not physiological cell death (Vaux *et al.*, 1993). It has been demonstrated *in-vitro* that oncogene expression determines intrinsic apoptotic rates and in this way may significantly influence the net growth rate and extent of necrosis in tumours (Arends *et al.*, 1994).

3.7 Other Oncogenes in Apoptosis

It is now accepted that one mechanism that essentially all chemotherapeutic agents ultimately kill cancer cells is by activating endogenous cellular pathways for apoptosis (Harris A 1992, Lowe *et al.*, 1993, Harris CC, 1996, Hetts, 1998). DNA-damaging agents such as ionizing radiation (IR) activate the tumour suppressor p53 and in some cases can cause apoptosis. Loss of p53 also contributes to cytotoxic chemotherapy treatment failures, by making malignant cells more resistant to both the drugs and gamma radiation (Daidone *et al.*, 1995, Harris A 1992, Harris CC, 1996). M1 cells, which do not express the endogenous tumour suppressor gene p53, undergo apoptosis following activation of a temperature sensitive p53 transgene, where it has been shown that Bax, an important mediator of apoptosis, is a p53 target gene (Selvakumaran *et al.*, 1994). One mechanism proposed for p53 induced apoptosis is due to down-regulation

of Bcl-2 (Miyashita *et al.*, 1994, Haldar *et al.*, 1994).

It now clear that Bcl-2 belongs to a family of proteins (Carson & Ribiero, 1993, Ueda & Shah, 1994, Hetts, 1998). There are both apoptosis promoting (Bax and Bad) and inhibiting (Bcl and Bclxl) proteins (Oltavi *et al.*, 1993, Selvakumaran *et al.*, 1994, Pietenpol *et al.*, 1994, Hetts, 1998). Bax appears to be an unique p53-regulated gene in that its induction by IR not only requires functional p53 but also requires that the cells be apoptosis "proficient" (Zhan *et al.*, 1994). **C-myc** is classified as a nuclear putative transcription factor. It has been implicated in cell transformation, differentiation and cell-cycle progression but also has a central role in some forms of apoptosis. These opposing roles of myc in cell growth and death require that other gene products dictate the outcome of c-myc expression on a cell. Interaction between **R-Ras** and Bcl-2 genes have also been identified which indicate that both genes has an ability to suppress apoptosis (Ueda & Shah, 1994, Sakai *et al.*, 1994). The **APO-1** antigen was defined during studies of monoclonal antibodies raised against human B-lymphoblast cell line. One of the antibodies was found to induce apoptosis of activated human B and T-lymphocytes and of cells of a variety of human lymphoid tumour-derived cell lines. The cell membrane antigen which this antibody attaches was designated APO-1 (Carson & Ribiero 1993, Ueda & Shah, 1994, Wyllie, 1994).

3.8 Bcl-2 and Related Genes

The protein Bcl-2 has a carboxy terminal membrane anchor and is localized to the outer mitochondrial, nuclear and endoplasmic reticulum membranes (Hockenberry *et al.*, 1991, 1993). The **Bcl-x** gene is a Bcl-2 related gene which can function as an independent regulator of apoptosis. Alternative splicing resulted in two distinct Bcl-x mRNAs. The protein product of the larger mRNA, Bcl-xL, is similar in size and predicted structure to Bcl-2. It inhibits cell death upon growth factor withdrawal as well as Bcl-2 when stably transfected into an IL-3 dependent cell line. The second mRNA species, Bcl-xs, encodes a protein that inhibits the ability of Bcl-2 to enhance the survival of growth factor deprived cells. In *vivo*, Bcl-xs mRNA is expressed at high

levels in cells that undergo a high rate of cell turnover, such as developing lymphocytes. In contrast, Bcl-xL is found in tissues containing long-lived post mitotic cells such as adult neural tissue (Bois *et al.*, 1993, Ueda & Shah, 1994, Krajewski *et al.*, 1994).

The proteins Bcl-2 and Bax are detectable immunohistochemically in a variety of normal tissue and malignant tumours including skin, the bone marrow, uterine endometrium, melanomas and both benign and malignant breast tumours (Grover *et al.*, 1993, Leek *et al.*, 1994, Krajewski *et al.*, 1994).

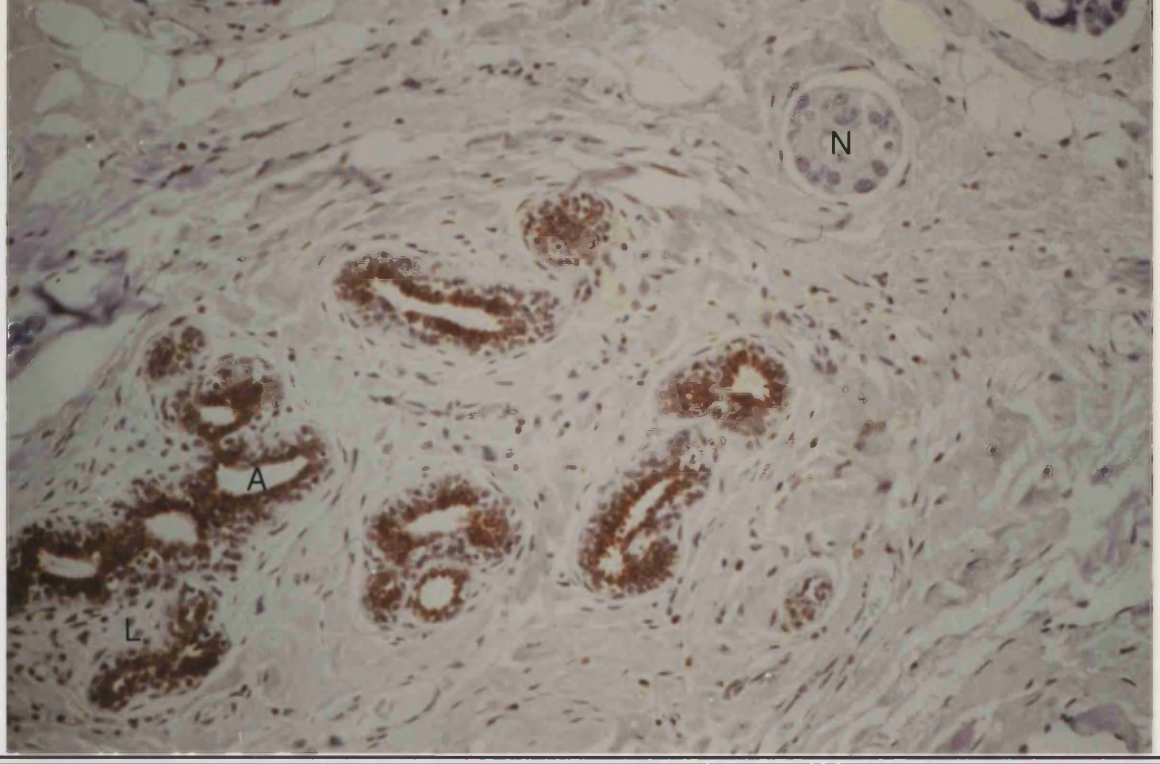

Fig. 1.3 Tumour nodule (N) in a lymphatic or perineural space which is negative for the Bcl-2 protein. There is one lobule (L) made up of several acini (A) which is positive for the Bcl-2 protein. The periluminal epithelium is positive but the myoepithelial elements are negative.

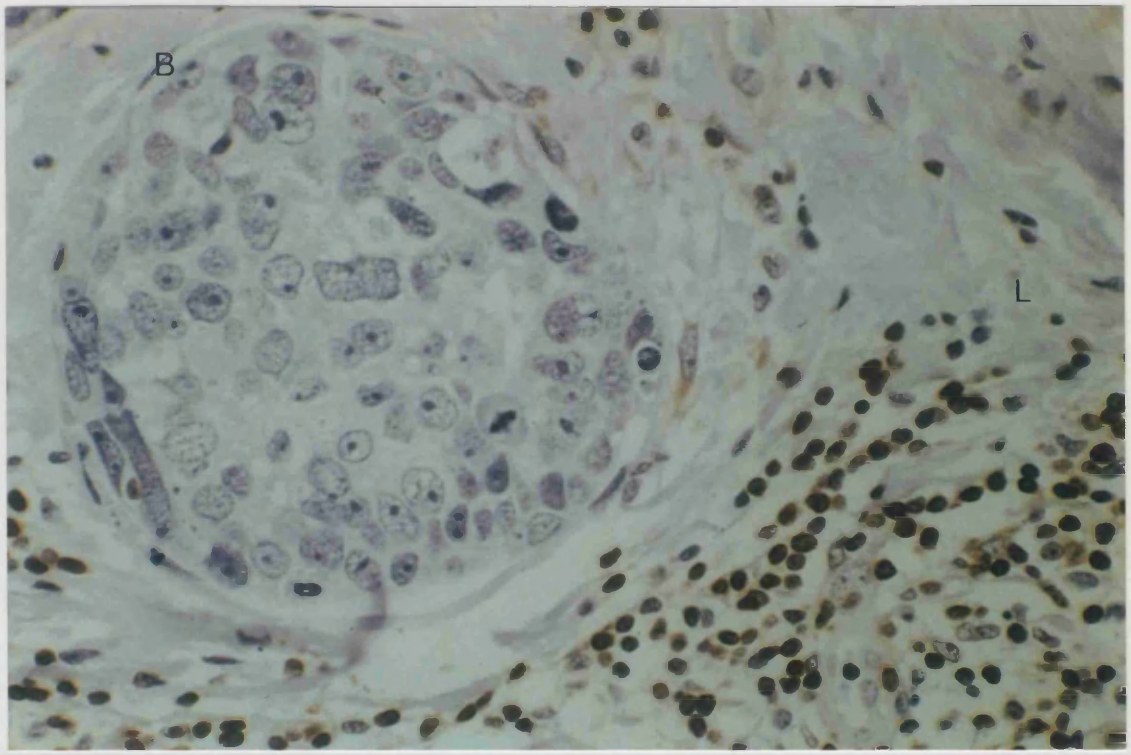

Fig. 2.3 High grade DCIS which is negative for the Bcl-2 protein. Showing nuclear pleomorphism within an intact basement membrane (B), the lymphocytes (L) which surround the area of DCIS are positive for the Bcl-2 protein and serve as an internal control.

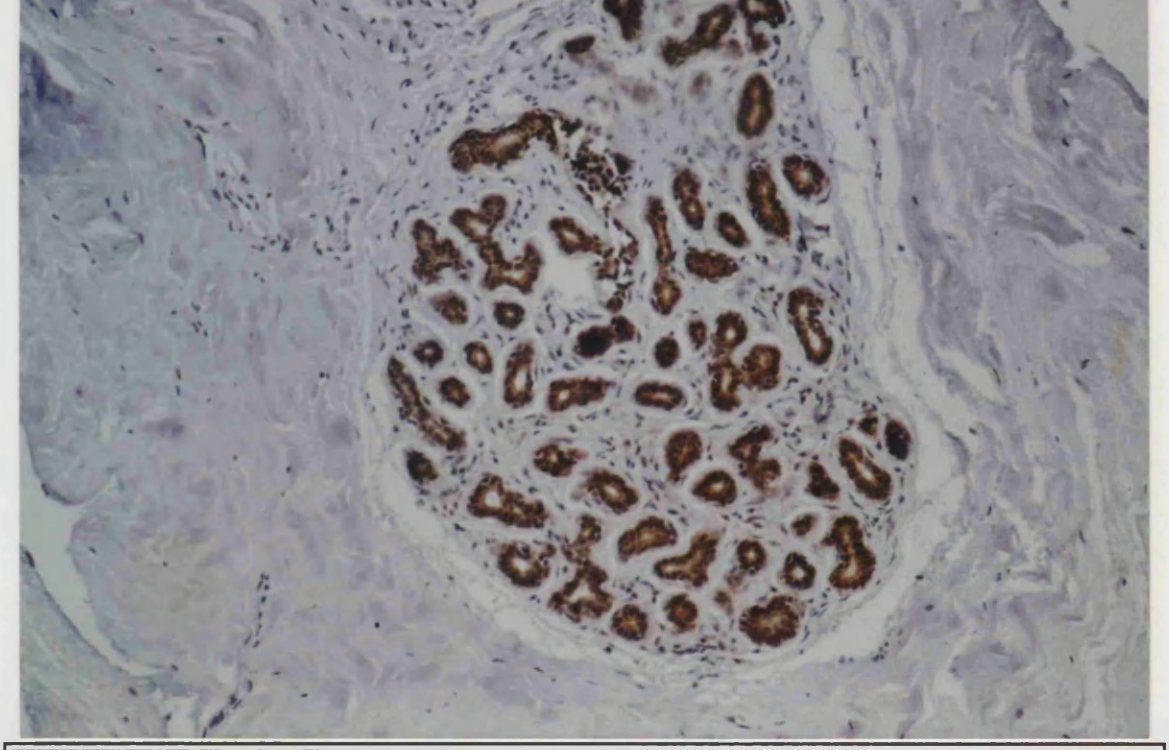

Fig. 3.3 Acini of a normal breast lobule showing overexpression of the Bcl-2 protein in the periluminal epithelial cells (brown), the outer layer of myoepithelial cells are negative. Mag. x 4.

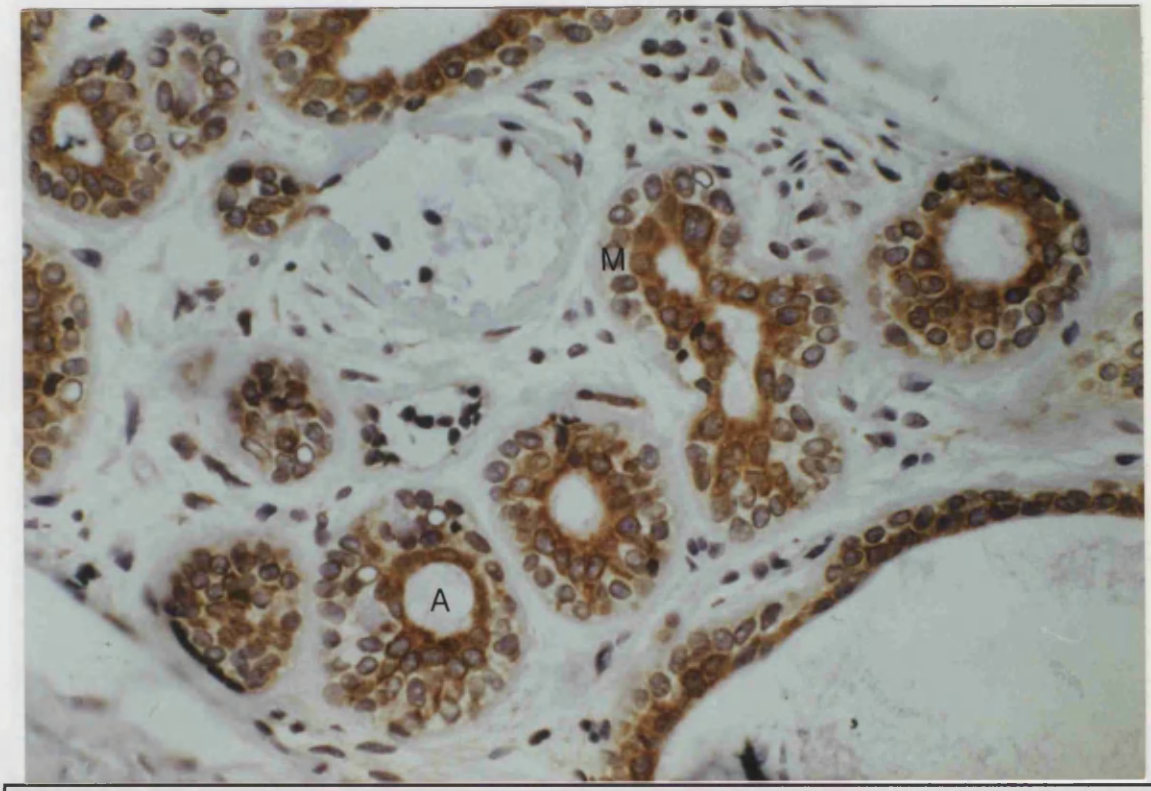

Fig. 4.3 Acini (A) of a normal breast lobule showing overexpression of the Bcl-2 protein in the periluminal epithelial cells (brown), the outer layer of myoepithelial cells (M) are negative. Mag x 40.

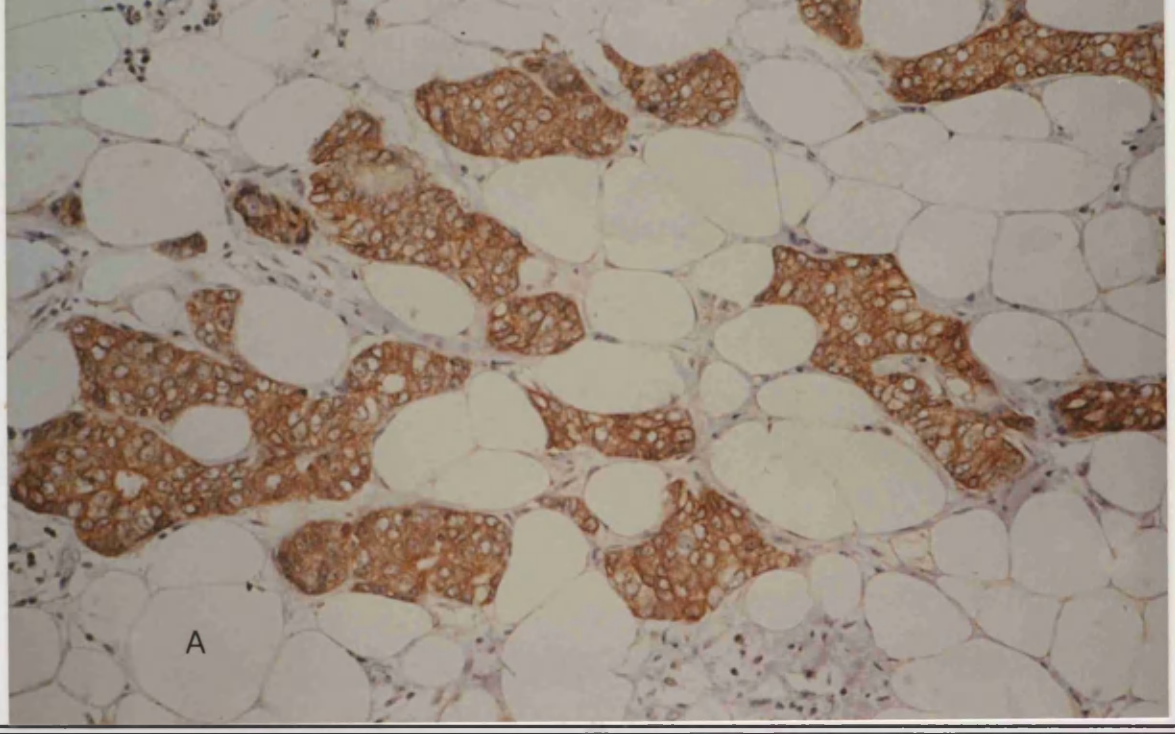

Fig. 5.3 Bcl-2 positive irregular nests of high grade invasive ductal carcinoma (IDC) infiltrating adipose tissue (A). The cells are overexpressing the Bcl-2 protein in the cytoplasm (brown) but the nuclei are negative (blue). There is minimal lymphocytic response.

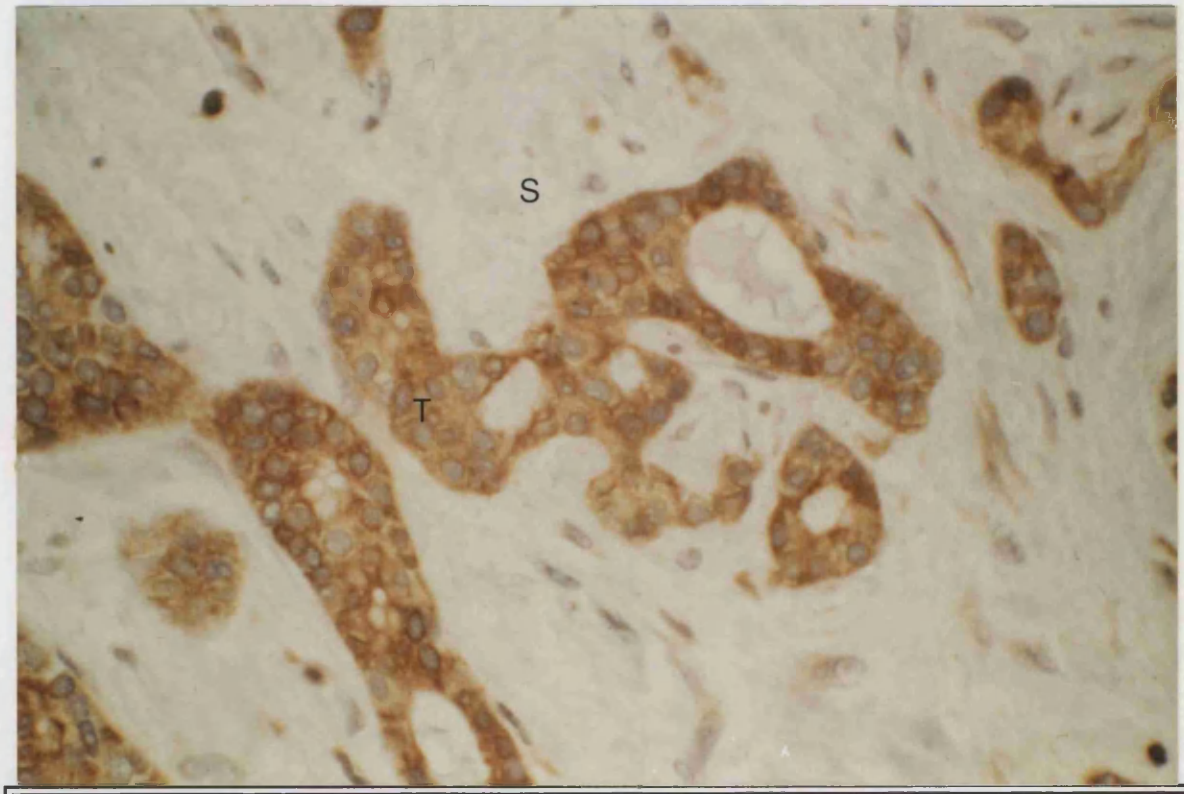

Fig. 6.3 Bcl-2 positive invasive ductal carcinoma (T) within fibrous stroma (S). The cells are overexpressing the Bcl-2 protein in the cytoplasm (brown) but the nuclei are negative (blue).

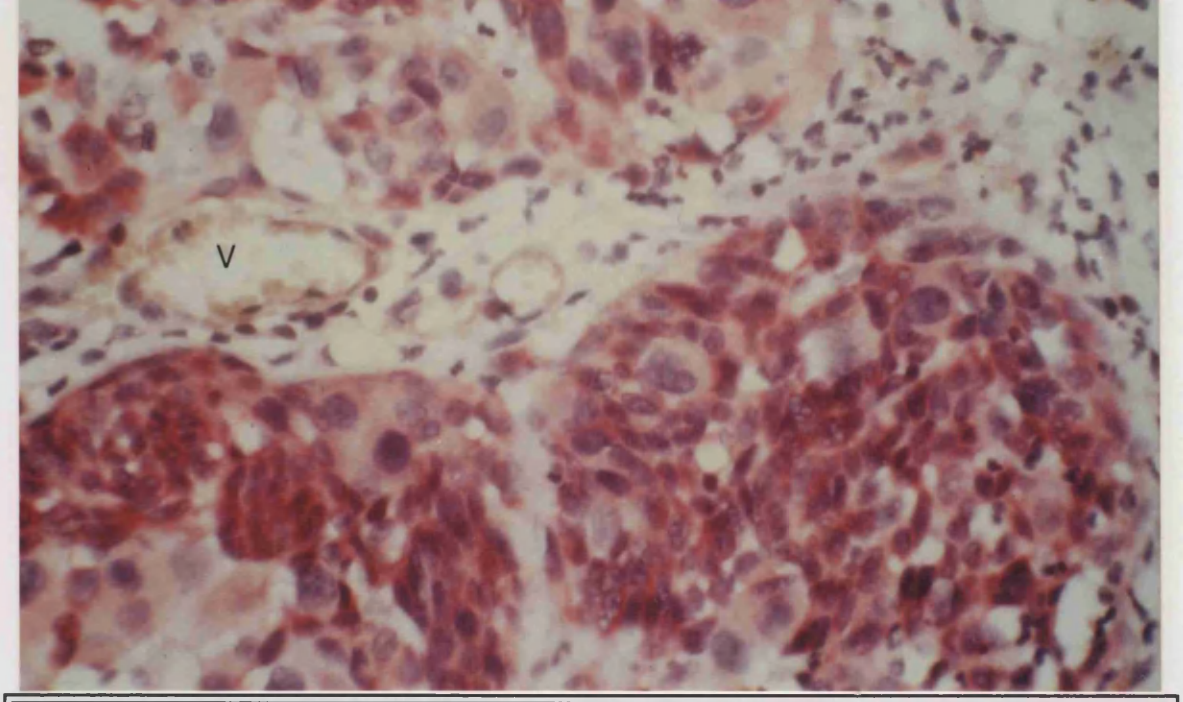

Fig. 7.3 Bcl-2 positive malignant melanoma stained by the Alkaline Phosphatase method. There is diffuse cytoplasmic staining in the melanoma cells but the lymphocytic positivity is less evident (c.f. Fig. 9.3). Two vascular (V) spaces are clearly seen.

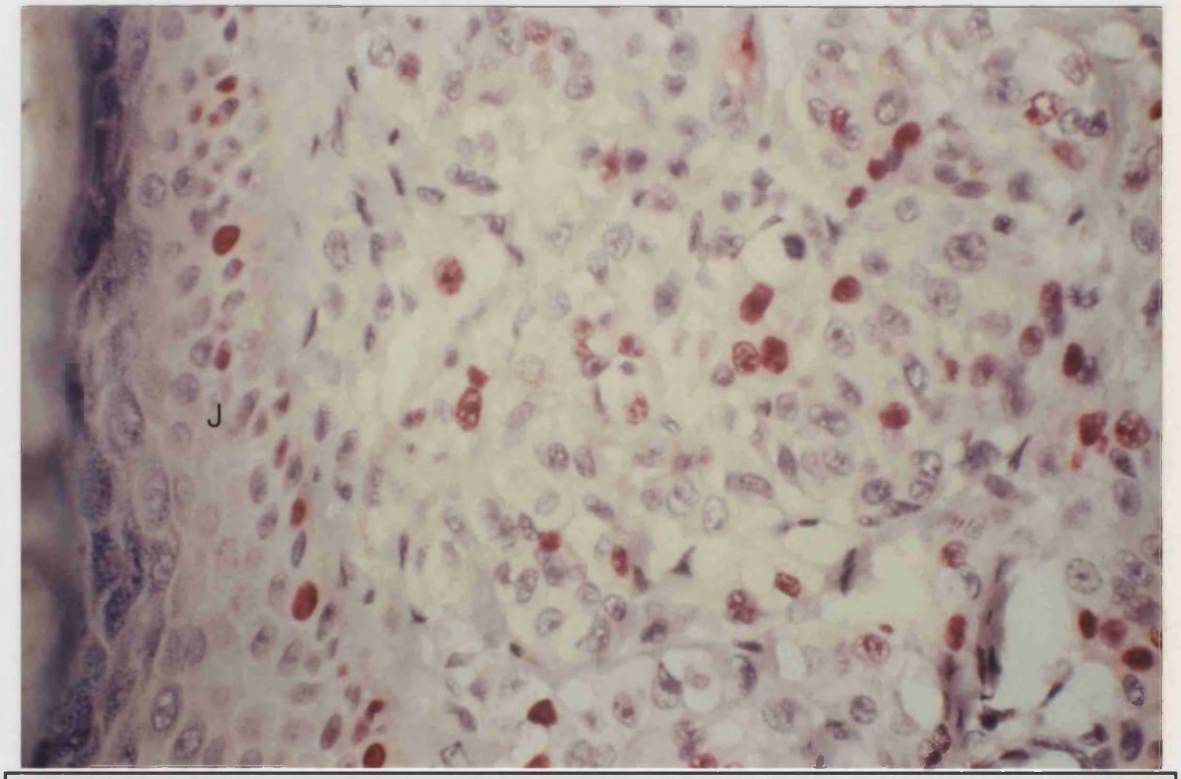

Fig. 8.3 MIB-1 positive malignant melanoma stained by the Alkaline Phosphatase method. Showing melanoma cells with a moderate amount of cytoplasm, pleomorphic nuclei and prominent nucleoli. The positive cells i.e. those in cycle are stained red. Cells in the dermo-epidermal junction (J) in cycle are also stained.

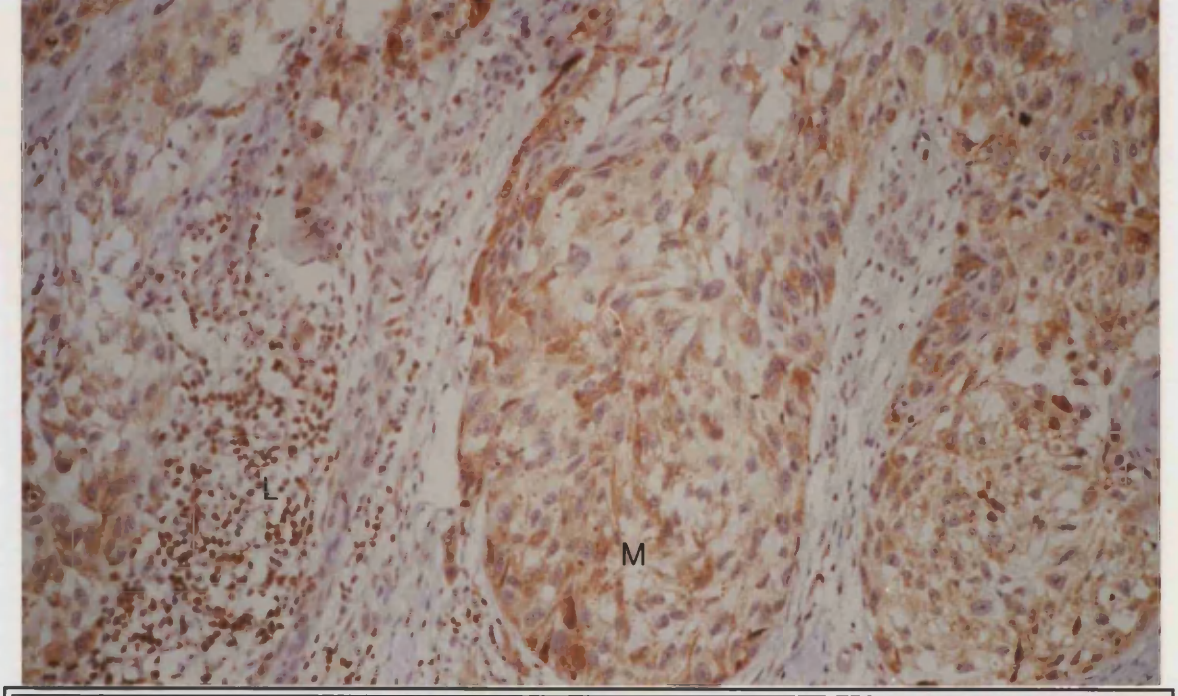

Fig. 9.3 Bcl-2 positive malignant melanoma stained by the Streptavidin Biotin Method. There is infiltration and expansion of the epidermal rete pegs by neoplastic melanocytes (M). There are positive reactive lymphocytes (L) with diffuse cytoplasmic staining in both the melanoma cells and the lymphocytes.

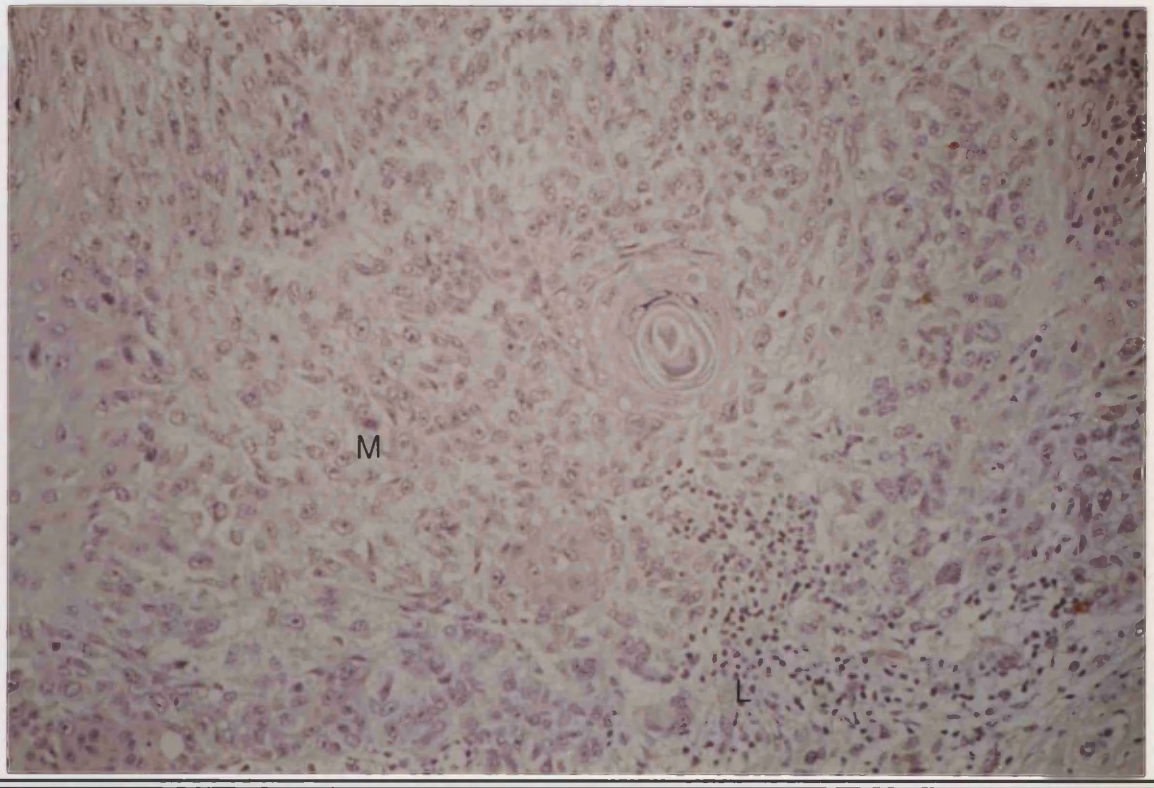

Fig. 10.3 Malignant Melanoma negative control. This section of the melanoma did not have the primary antibody Bcl-2 applied to it. It shows infiltration and expansion of the epidermal rete pegs by neoplastic melanocytes (M). There are reactive lymphocytes (L) which are also negative.

Chapter 4

Cyclins and the Cell cycle

4.1 The Cell cycle

Most of our understanding as to how cells grow and divide comes from the study of cells grown *in vitro*. The time required for a cell to go through one division *in vitro*, varies from one cell line to the other and in most cases lies between 10 and 48 hours (Graña & Reddy, 1995).

The cycle is typically divided into four phases. The periods associated with DNA synthesis (S phase) and mitosis (M phase) are separated by gaps of varying length called G1 and G2. The signalling pathways that regulate cell cycle progression seem to be primarily associated with the G1 phase of the cell cycle (Hall & Coates, 1995).

All multicellular organisms (including humans) have evolved a tight control mechanism over cellular proliferation as a prerequisite for their survival. This control is a kind of molecular switch between the alternative routes towards cell division, temporary cell-cycle arrest, quiescence, differentiation and cell death (Strauss *et al.,* 1995, Hall & Coates, 1995).

Reflecting its crucial role in proliferation control, this switch mechanism has been appropriately called the "restriction point" or "R point" in mammalian cells (Pardee, *et al.,* 1974, Pardee, *et al.,* 1982, Saslow-Weinstat *et al.,* 1995). Occurring during the G1 phase of the cell cycle. This checkpoint may, if defective, lead to deregulated growth with devastating consequences for the organism.

Loss of the restriction point control was found to be associated with malignant transformation and was proposed as one of the hallmarks of cancer by A. Pardee two decades ago (Pardee, *et al.,* 1974, Pardee, *et al.,* 1982).

The "R" point was originally defined as the time in G1 when cells commit to entering the S phase and complete the round of cell division. Whereas, the initial phase of G1 is strictly dependent on the presence of mitogenic stimuli, progression through the "R" point results in a reduced requirement for growth factors and appears to serve as a switch to an intrinsic cell-cycle machinery that ensures an ordered completion of the cell division cycle. Biologically, the restriction point enables a cell to withdraw into a state

of quiescence, either to survive nutritional, or growth factor deprivation, allow for repair of damaged DNA, or to conform with conditions that induce differentiation. The "R" protein is postulated to have the following properties (1) synthesis during G1 (2) instability and (3) stabilization and/or overproduction in tumour cells (Pardee, 1974, 1982).

The eukaryotic cell cycle engine is composed of protein complexes that are activated in an ordered fashion and trigger the initiation of specific events such as DNA replication, nuclear envelope breakdown, spindle formation and chromosome segregation. This protein engine is subject to a careful control signalling transduction pathways and feedback loops that ensure that each event is performed correctly and in the proper sequence. Specific transduction pathways connect the cell cycle machinery with extracellular signals. Mitogenic growth factors bind their specific receptors and initiate a cascade of events that culminates in the expression and assembly of different kinase holoenzymes composed of a regulatory subunit, named cyclin dependent kinase (CDK) (Hall & Coates, 1995).

Proteins which are essential for the control of the progress of a cell through the cell cycle are known as cyclins, because their concentrations increase, or decrease at discrete times or stages in the cell cycle. The cyclins show remarkable conservation over evolutionary time scales and it is possible to demonstrate that human cyclins can function in yeast cells. The cyclins are regulatory subunits for the cyclin dependent protein kinases (cdk) which, in turn phosphorylate critical target proteins that regulate transitions in the cell cycle.

Mammalian cells have multiple cyclins (cyclins D and E for the G1 phase, cyclin A for the S phase and cyclin B for the G2 and M phases) (Levine *et al.*, 1995). Cyclin regulate multiple kinases (cdk1, 2, 3, 4 and 5, or cdc2) at transition points in the cell cycle.

Cyclins D1, D2 and D3 are thought to function in the G1 phase of the cell cycle by regulating the activity of cyclin dependent protein kinases. All three D-type cyclins can be shown to associate with two specific kinases, cdk4 and cdk6, providing at least six

possible combinations (Bates *et al.*,1994).

Functional retinoblastoma as well as acting as a potential substrate for D-cyclin kinases contributes to the formation, or stability of the complexes at least in human cells (Bates *et al.*, 1994, Muller *et al.*, 1994). The Rb-cyclin D1 pathway represents a frequent target of oncogenic abnormalities in breast cancer.

Overlying this cyclin regulated positive control of the cell cycle are a series of checkpoints that stop progression in response to external signals or conditions (negative control). These checkpoints react to cell size, nutrient levels, hormonal responses, or the presence of DNA damage in the genome of the cell to be duplicated. If conditions are not favourable for cell division, a checkpoint will activate and stop progression through the cycle (Levine *et al.*,1995, Grana *et al.*,1995). The level of nutrients, or hormonal status in the cell environment are measured by receptors that then use the signal transduction pathways to communicate with the cyclins and components of these signal transduction pathways are often *oncogenes*. Functions, that negatively regulate these pathways or negatively regulate the cell cycle, are often encoded by *tumour suppressor genes.*

4.2 Cyclin D1

The controlled sequential expression of a family of proteins, the cyclins, governs the progression from one part of the cell cycle to the next. Cyclin D1 is the first of the family of cyclins to be expressed in the cell cycle and in both normal and tumour cells it reaches a maximum in the late G1 phase of the cell cycle. It is required to be degraded to a low levels in the nucleus before progression into the S-phase. During this time the cyclin E protein increases with concomitant cyclin E/cdk complex activity which may thus indirectly depend on cyclin D1. The maintenance of the cyclin E/cdk complex promotes exit from G1 to the S phase by phosphorylating and inactivating the retinoblastoma gene product (McIntosh *et al.*, 1995).

Amplification of chromosome 11q13 has been observed in about 10-20% of breast cancers (Gillette *et al.*, 1994, Zuckerburg *et al.*, 1995, Dickson *et al.*, 1995, Peters *et*

al., 1995) and numerous other tumours including squamous cell carcinomas of the head and neck, oesophagus, transitional cell carcinomas of the bladder and epithelial ovarian tumours. Cyclin D1/PRAD 1 is a likely "driver" gene for this amplification because of its role in cell cycle control and it has been specifically implicated as an oncogene in parathyroid adenomas and B-cell lymphomas with 11q13 translocation breakpoints (Peters *et al.,* 1995).

4.3 Cyclin E

Cyclin E was identified as a protein which would complement G1 cyclin mutations in yeast and subsequent work highlighted a similar role in mammalian cells. It has become apparent that overexpression of cyclin E shortens the length of the G1 phase accelerating progression to the S phase. It is a protein which is 395 amino-acids long. It has been suggested as a candidate for the restriction point protein (Keyomarsi *et al.,* 1993, Qing-Ping Dou *et al.,* 1993). Expression does not correlate with other markers of proliferation such as Ki-67 nor does it correlate with cyclin D (Sasano *et al.,* 1997).

It is overexpressed in tumour cell lines in multiple formations both in complexes with cdk 2 and alone. Multi-isoforms may arise due to altered post-transcriptional or translation regulation (Keyomarsi *et al.,* 1993). When wild type cyclin E is overexpressed in normal cells the length of G1 is decreased, but cells are not transformed. Overexpression increases with tumour grade and stage (Qing-Ping Dou *et al.,* 1996) but this has been refuted (Sasano *et al.,* 1997).

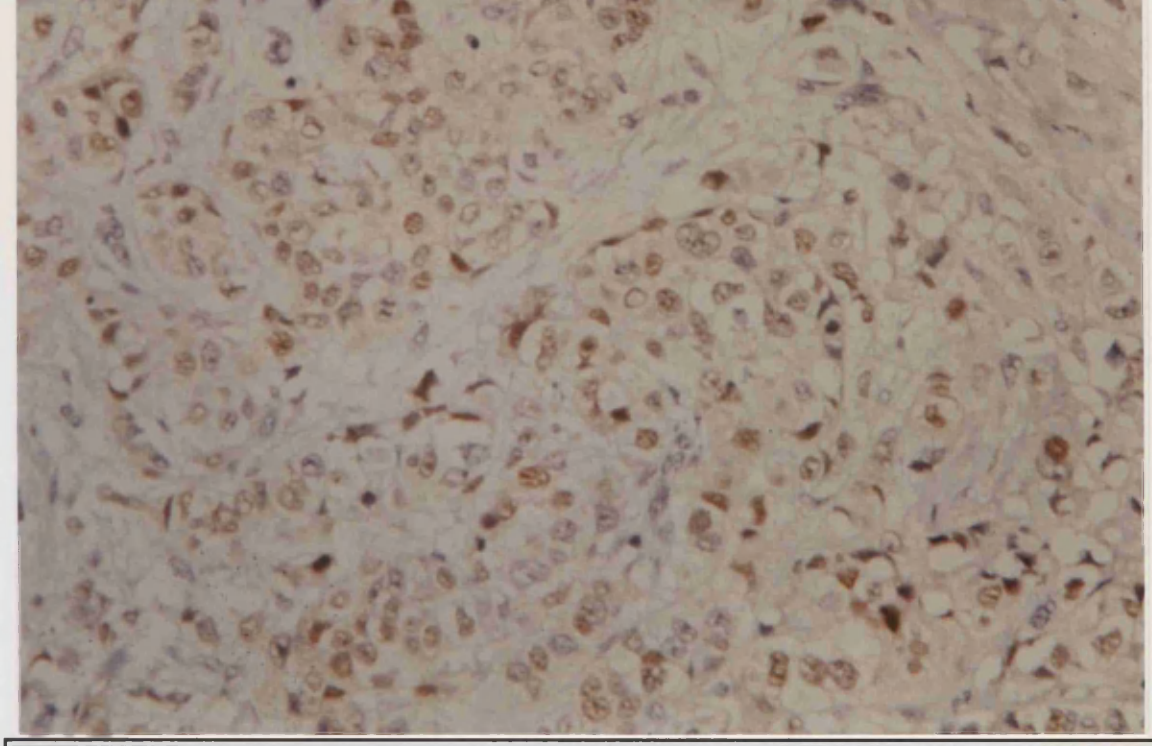

Fig. 1.4 Invasive Ductal Carcinoma showing overexpression of the Cyclin D protein in about 50% of the tumour nuclei.

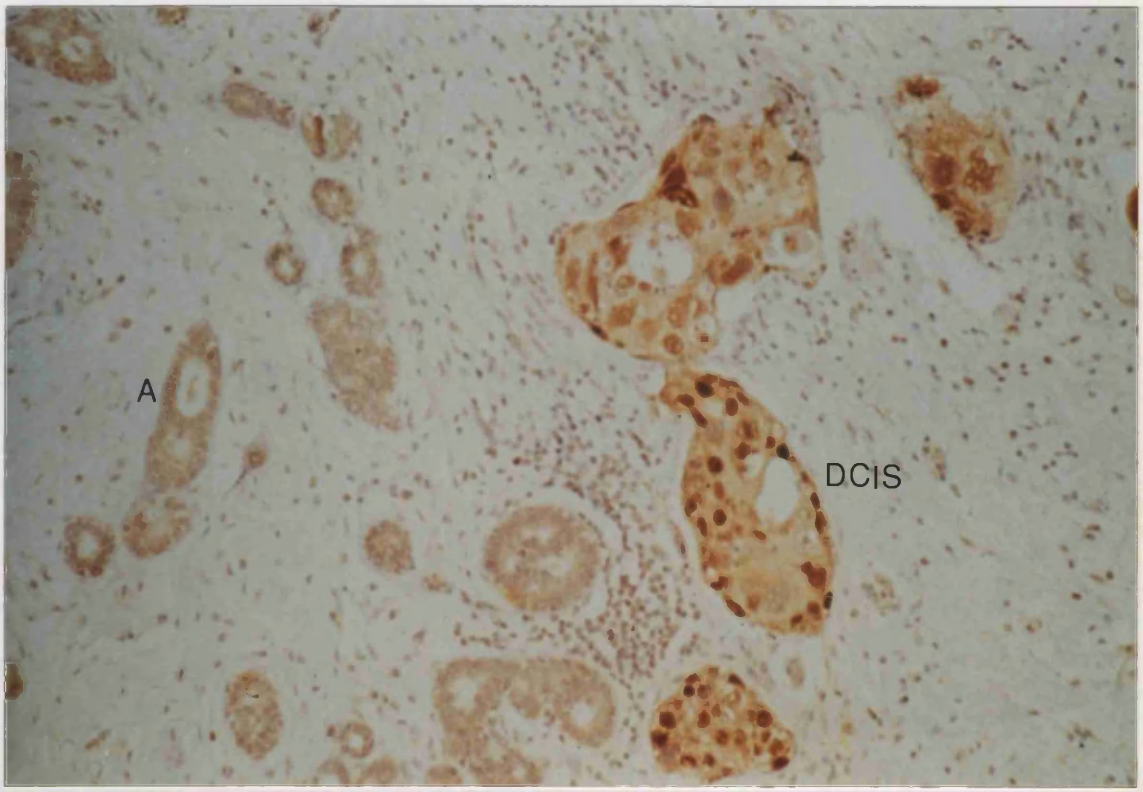

Fig. 2.4 Cyclin D positive area of *ductal carcinoma in situ* DCIS. Section shows benign hyperplastic acini (A) on the right which only show a weak cytoplasmic positivity to cyclin D. On the left the area of *ductal carcinoma in situ* is strongly positive for the cyclin D protein.

4.4 MDM-2

MDM-2 is an evolutionary conserved gene originally identified as a highly amplified gene present on double minutes in a spontaneously transformed tumourigenic derivative of a Balb/c cell line called 3T3DM (Fakharzadeh *et al.,* 1991). It has now been localized to chromosome 12q 13-14 (Oliner *et al.,* 1992).

Analysis of the predicted amino-acid sequence of the MDM-2 protein suggests that it may be a DNA binding protein, or transcription factor. It is also thought to function as a regulator of p53 function. This stems from evidence that the MDM-2 protein forms oligomeric complexes with the p53 protein *in-vivo* and *in-vitro* and when experimentally overexpressed inhibits the transactivating capability of p-53 (Vogelstein & Kinzler, 1992). This inhibition is thought to result from the MDM-2 protein binding directly to the acidic activation domain of p-53, concealing it from the transcriptional machinery (Oliner *et al.,* 1992, Momand *et al.,* 1992, McCann *et al.,* 1994).

The product of the Murine Double Minute-2 gene forms a complex with both mutant and wild type p53 protein. It is a cellular phoshoprotein with a molecular mass of 90 kd. The mdm-2 gene enhances the tumourigenic potential of cells when it is overexpressed and encodes a putative transcription factor (Hall *et al.,* 1997).

It has been postulated that there is a p53-mdm-2 autoregulatory loop, which may be concentration dependent (Oliner *et al.,* 1992). Inactivation of wild type p53/MDM-2 during entry of the cells from the resting to the growing phase would provide an explanation for the seemingly contradictory roles of p53 in cellular proliferation. The conformational hypothesis proposed by Milner implies p53 can adopt either a growth inhibitory (G_0), or a growth promoting role (G_1), depending on p53 conformation or levels. Recent *in vitro* work, however, has shown that p53 and MDM-2 expression during proliferation may not be functionally related and MDM-2 may not be required for proliferation (Mosner *et al.,* 1994). MDM-2 amplification in non-epithelial tumours has been found to be between 4 to 70 fold in 14% of osteogenic sarcomas, 10% of primary brain tumours and up to 36% of soft tissue sarcomas (Martinez *et al.,* 1995).

None of the gastrointestinal tumours, or breast cell lines investigated have shown evidence of aberrant MDM-2 gene copy number. Expression has been shown in proliferative zones of non-malignant tissue (Martinez *et al.,* 1995). It is expressed in 7 - 97 % of breast carcinomas and been associated with both good and bad prognostic factors (McCann *et al.,* 1994, Buenos-Ramos *et al.,* 1996).

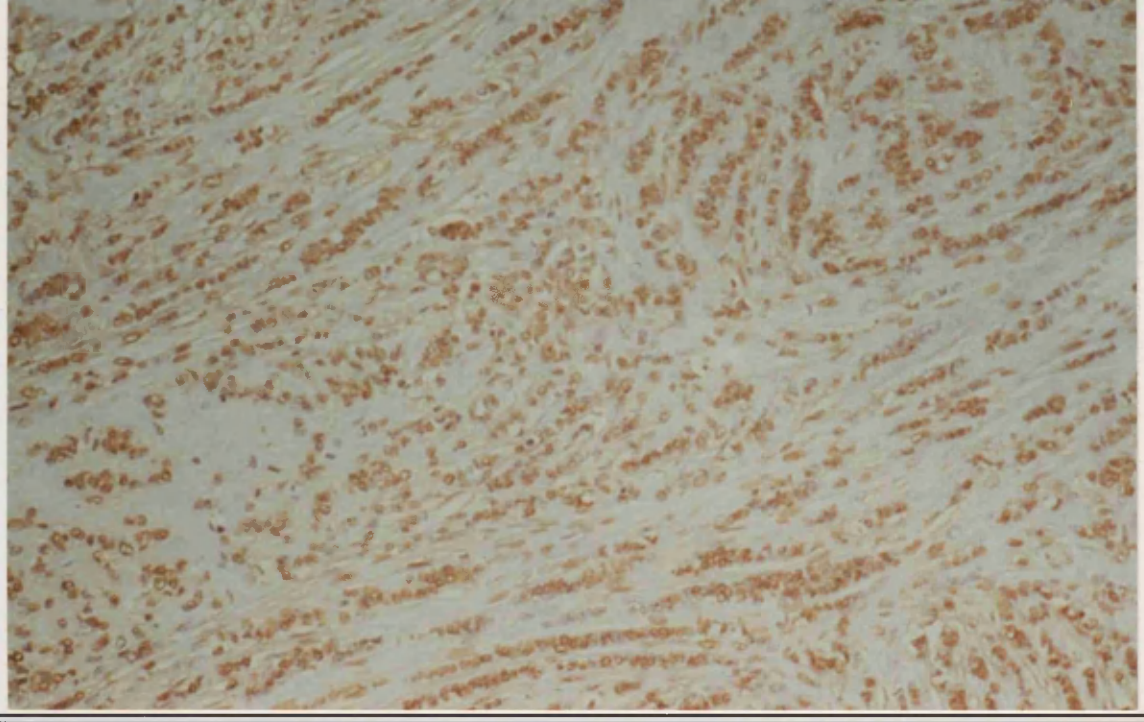

Figure. 3.4 MDM-2 positive Lobular carcinoma. Mag x 4. The cells are arranged in a single line the so called "Indian - file" pattern.

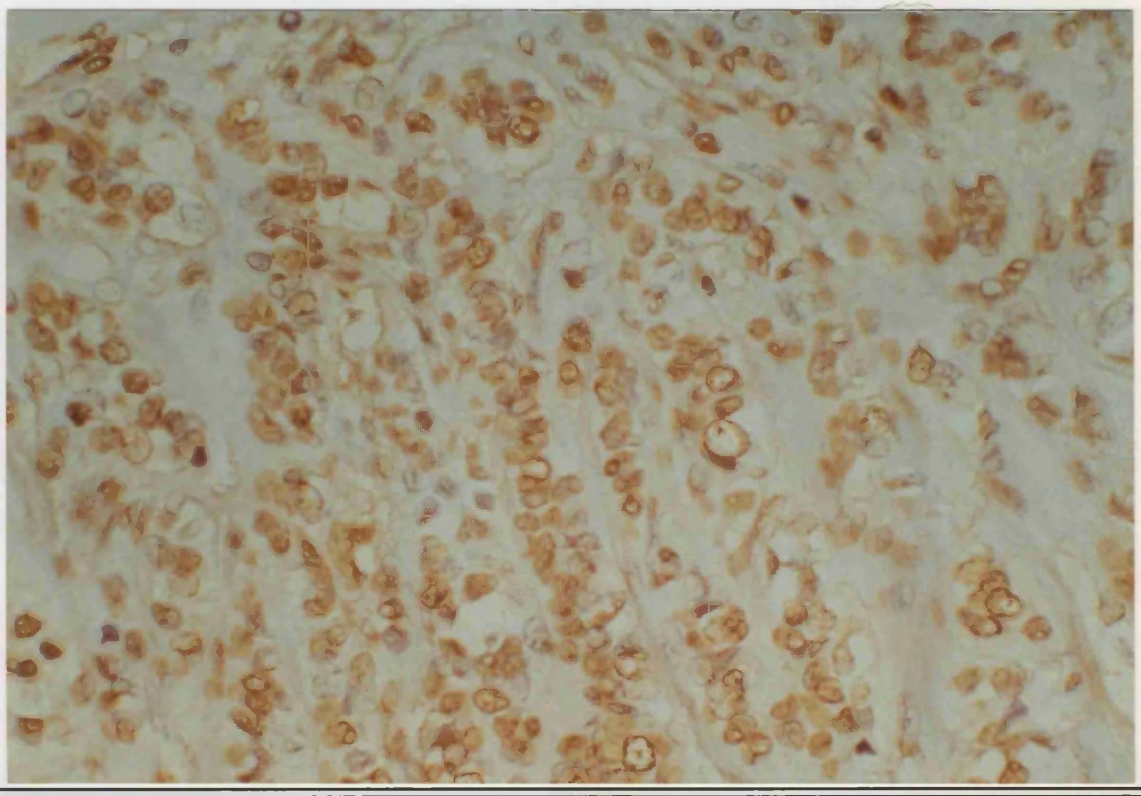

Figure. 4.4 MDM-2 positive Lobular carcinoma. Mag x 40. Widespread nuclear positivity (brown nuclei). The cells are arranged in a single line the so called - "Indian File" pattern.

Chapter 5

MIB-1 and Markers of Proliferation

Cell proliferation is regulated to a greater, or lesser degree by the p53 tumour suppressor gene in concert with other growth factors and proteins (Hall & Coates, 1995). Anomalies in the control of the cycle, produce an increase in the subset of cells in cycle, over and above the normal percentage - this "growth fraction" is highly variable. Numerous methods exist to measure the growth fraction of the tumour which is a reflection of the importance of the estimation in the overall prediction of prognosis (Hall & Coates, 1995, Elston & Ellis 1998, Pinder *et al.,* 1998).

(a) The **mitotic index** has been described as "the oldest, easiest, fastest and cheapest way of assessing proliferation" (Harris, 1995) and needs no explanation.

(b) The **Thymidine labelling index** is determined by counting the number of labelled nuclei after a period of incubation of a tumour with tritiated Thymidine.

(c) **Flow cytometry** (which measures the proportion of cells in the synthesis [S] phase fraction of the cell), produces a histogram of the DNA content of a tumour and can be used for fresh, frozen, needle aspirates and paraffin embedded tissue. These procedures are time consuming, complex procedures and not appropriate in the routine laboratory setting (Lee *et al.,* 1992, Elston & Ellis 1998, Pinder *et al.,* 1998).

(d) **Proliferating cell nuclear antigen** (PCNA), is a nuclear antigen related to the DNA-polymerase δ which is present throughout the cell cycle in proliferating cells. It is detectable by routine immunohistochemistry but has only a weak correlation with traditional prognostic factors.

(e) A monoclonal antibody, **Ki-67**, has been produced which is suggested to react with a nuclear antigen present throughout the cell cycle (G_1, S, G_2, and M phases) of proliferating cells, but is absent in quiescent (Go) cells. Ki-67, is a non-histone nucleoprotein, of unknown functional significance and is expressed in cycling, non-resting cells (Gerdes *et al.,* 1983). This antibody is suitable for use on both frozen sections and paraffin-embedded sections. Both PCNA and Ki-67 are selectively expressed in proliferating cells (Miettinen *et al.,* 1993, Nicholson *et al.,* 1993) but PCNALI does not correlate with Ki67LI (Nicholson *et al.,* 1993). Observations on

tumour groups such as breast carcinomas and melanoma have shown a correlation between Ki-67 and PCNA scores and disease aggressiveness (Pavelic *et al.*, 1992) and that these antigens have a parallel distribution (Miettinen *et al.*, 1993). For RFS Ki-67 had a significant prognostic value (Palma *et al.*, 1996, Nicholson *et al.*, 1993).

MARKER	Go	G1	M	G2	M
MITOTIC INDEX	–	–	–	–	+
*BR-DU (SASAKO *et al.*, 1992) (REW *et al.*, 1992)	–	–	+	+	+
S-PHASE FRACTION	–	–	+	–	–
KI-67/MIB-1	–	±	+	+	+
MITOSIN	–	–	–	±	+
HISTONES	–	–	+	±	–
TOPOISOMERASE II	–	–	±	+	
DNA POLYMERASE (A, ß)	–	±	+	+	±
CYCLINS (**PCNA, A, D, E)	–	±	+	±	±

***Bromodeoxyuridine ** Proliferating cell nuclear antigen**

TABLE 5.1. CORRELATION OF MARKERS OF PROLIFERATION WITH STAGES OF THE CELL CYCLE

(f) The monoclonal **MIB-1™** (Immunotech S.A.) antibody reacts with the Ki-67 nuclear antigen (345 and 395 kD double band in Western blot analysis of proliferating cells) associated with cell proliferation and found throughout the cell cycle (G_1, S, G_2, M phases) and absent in resting (Go) cells. This antibody recognizes native Ki-67 antigen and recombinant fragments of the Ki-67 molecule. It gives an immunocytochemical staining pattern identical to Ki-67 on paraffin embedded tissue sections, frozen sections and cytological samples (smears/imprints/cytospins) and is more specific for paraffin embedded tissue because the Ki-67 antigen is rapidly catabolized at the end of the M phase (McCormick *et al.*, 1993)

Tumours with a high Ki-67 labelling index (LI) have a poor prognosis, are associated with p53 positivity (Marchetti *et al.*, 1993, Nicholson *et al.*, 1993) and high grade (Leonardi *et al.*, 1992, Kennedy *et al.*, 1993). Ki-67 status as a measure of prolifera-

tion has been correlated with other markers of proliferation namely, pAgNOR counts (Stump *et al.,* 1992), Bromodeoxyuridine labelling indices (Sasako *et al.,* 1992), Ki-S1 (Bevilacqua *et al.,* 1996), flow cytometry (Lee *et al.,* 1992) and mitotic counts (Lee *et al.,* 1992). Other immunohistochemical markers of poor prognosis have shown a correlation with the Ki-67 antigen for example c-myc protein expression (Pavelic *et al.,* 1992). Others have found a greater degree of Ki-67 positivity in lymph node positive tumours, and tumours that are poorly differentiated (Bouzubar *et al.,* 1989, Pavelic *et al.,* 1992). Ki-67 expression however, is independent of tumour size, lymph-node status and ER expression (Bouzubar *et al.,* 1989, Railo *et al.,* 1993, Bevilacqua *et al.,* 1996). Conversely a correlation between low Ki-67 labelling index, oestrogen positivity and greater differentiation has been documented (Locker *et al.,* 1992, Nicholson *et al.,* 1993).

EXPRESSED IN ALL PHASES OF THE CELL CYCLE EXCEPT GO
GOOD CORRELATION WITH FLOW-CYTOMETRY
CORRELATION WITH THYMIDINE LABELLING
CORRELATION WITH BROMO-DEOXYURIDINE INCORPORATION
MAY OVER-ESTIMATE THE GROWTH FRACTION OF TUMOURS
LOW INDEX ASSOCIATED WITH ER
ASSOCIATION WITH HISTOLOGICAL GRADE.
ASSOCIATION WITH P53 STATUS
ASSOCIATION WITH EARLY DISEASE RECURRENCE

TABLE 5.2 ADVANTAGES OF MIB-1 ASSESSMENT

MIB-1 has been shown to be of independent prognostic value in low-risk breast cancer - defined as negative axillary nodes, tumour diameter < 50 mm and no histological evidence of invasion of skin, or deep fascia (Jensen *et al.,* 1995).

An excellent correlation exists between the histological grade of malignancy of breast tumours and their Ki-67 status (Locker *et al.,* 1992). This trend is reflected in the

average number of Ki-67 positive tumour cells within the individual grades of malignancy. There is a correlation between mitotic figure counts (MFC), MIB-1 positivity and all proliferation markers including the S phase fraction in paraffin processed tissue sections of breast carcinomas (Weidner, 1994, Keshgegian *et al.*, 1995, Yu CC *et al.*, 1995) except Ki-67. MIB-1 positivity has been associated strongly with disease-free survival, up to 46 months in female (Keshgegian *et al.*, 1995, Pinder *et al.*, 1995) and male breast carcinoma (Pich *et al.*, 1994). Patients with tumours having a high proportion of MIB-1 positive cells show a higher 5-year probability of relapse of disease and death than those with a low one (Veronese *et al.*, 1995, Pichet *et al.*, 1996). p53 correlations with both the presence of MIB-1 and the histological grade (Beck *et al.*, 1995). It has been suggested that the MIB-1, or Ki-67 status may replace histological grade as the former is reproducible without special histological expertise (Veronese *et al.*, 1995).

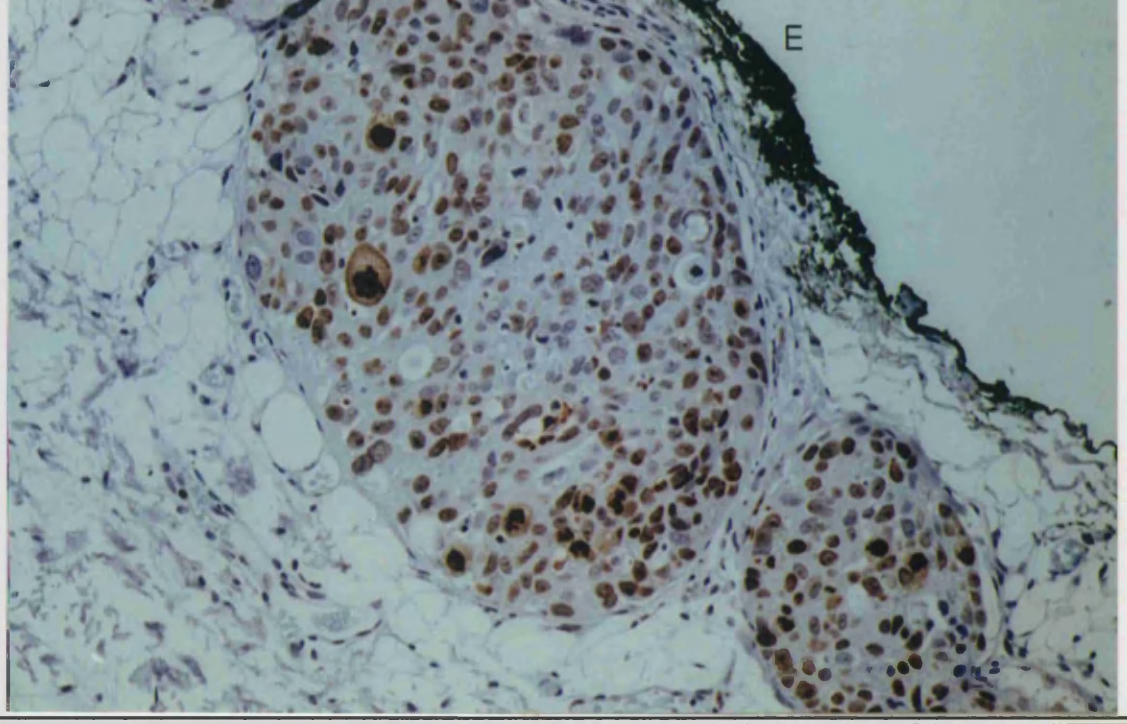

Fig. 1.5 MIB-1 positive Ductal Carcinoma in-situ. The two areas are close to the excision margin are marked with Indian ink (E). The DCIS is contained within the basement membrane and the nuclei of cells in G1, G2, M and the S phases are stained brown. An abnormal mitoses is clearly shown at 9 O' clock.

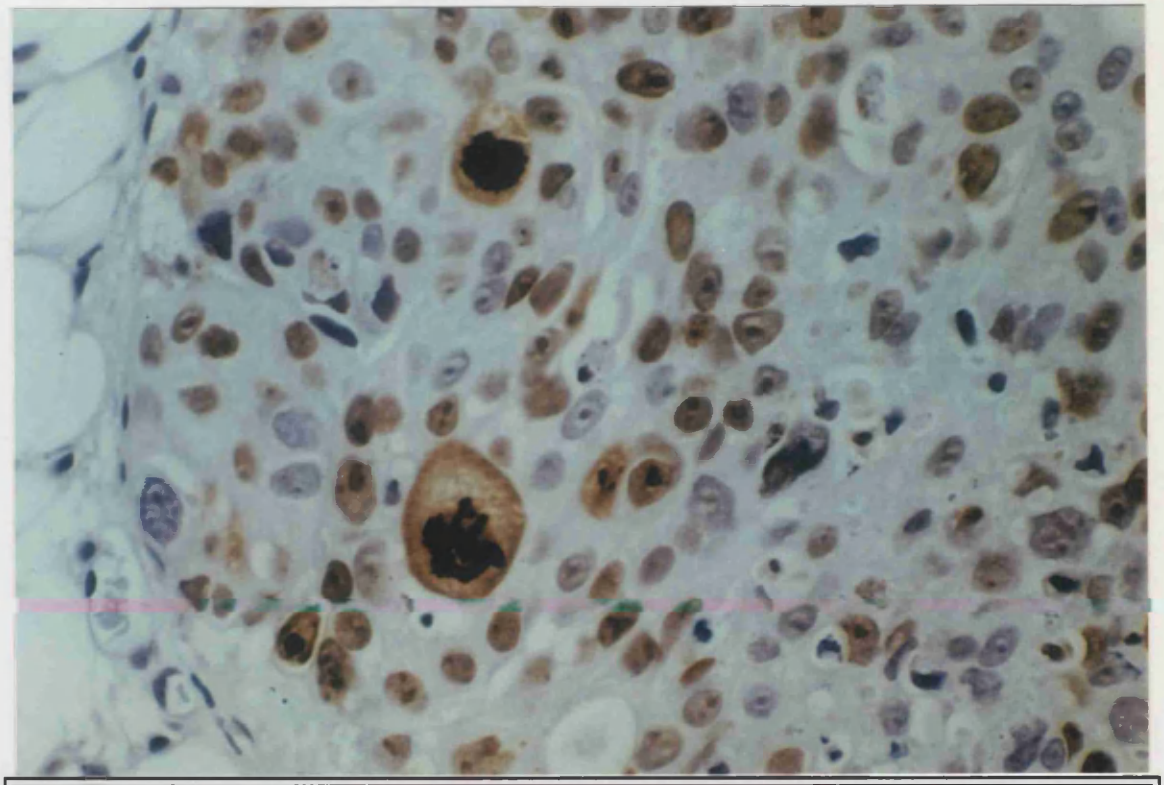

Fig. 2.5 MIB-1 positive Ductal Carcinoma in-situ. Mag x 40. The abnormal mitoses are clearly shown the largest at 8 O' clock.

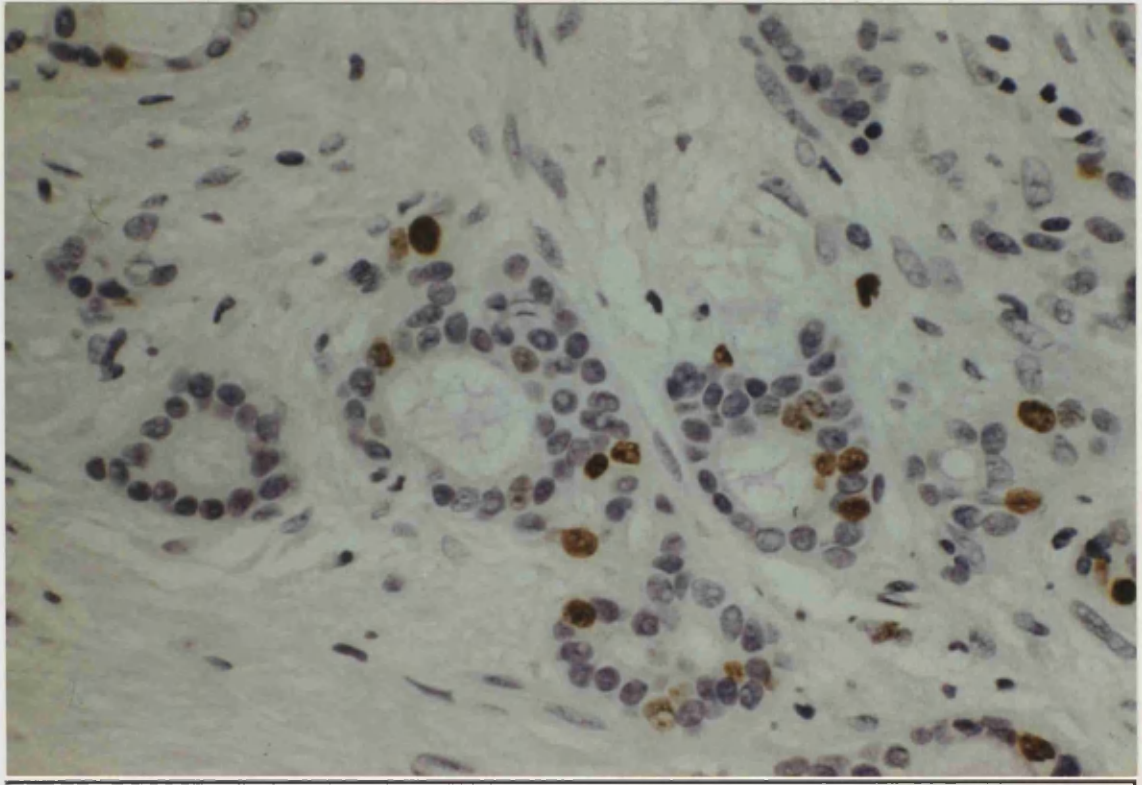

Fig. 3.5 A low grade invasive ductal carcinoma showing MIB-1 positive cells (brown). These represent the cells in cycle. There is a correlation between tumour grade and MIB-1 score in this subset of tumours ($p > 0.0001$).

Chapter 6

Molecular Biology of Breast Tumours

The treatment of breast cancer has since remained in the domain of the surgeon since the time of Leonides of Alexandria, who preceded Galen.

Surgical treatment over the years has varied from the radical mastectomies of Halstead, to the breast conserving surgery of the 80's. This is a reflection of the fact that surgery has not significantly altered the overall survival from the disease. It is therefore obvious, that combined modalities of treatment are necessary to significantly alter the clinical outcome from the disease (Pinder *et al.,* 1998, Elston & Ellis 1998).

Multiple molecular alterations have been reported for invasive human breast cancer, but early breast lesions are still defined on the basis of cytologic, histologic and epidemiological analysis (Pinder *et al.,* 1998). Epidemiological studies, clearly indicate that practically any breast lesion confers an increased risk for the development of breast cancer, over and above the normal population. This rises from 1.5 to 8.0 as we move from the purely fibrocystic change in the breast, to the atypical ductal hyperplasias (Harris, 1995).

The histopathologist, has furnished us with tools, which enable us to predict the prognosis correctly in 75% of patients, with small, low-grade tumours, that are lymph-node negative. However, in tumours of intermediate grade, the prognosis is only predictable in less than 50 % of the cases. This has forced us to examine molecular anomalies in the individual patient, as a more reliable way of both improving our predictive ability, treatment outcomes and to enable us to compare treatment results from centre to centre. The development of prospectively validated prognostic indices has clearly shown that the traditional prognostic indices although valuable can be clearly improved (Pinder *et al.,* 1998, Elston & Ellis 1998) It is now clear that a prognostic index is both possible and desirable.

Colonic carcinoma is frequently described as having many similarities to breast carcinoma and it has been proposed that based on this, that a speciality defined as *colomastology,* will emerge (Taylor, 1994). However, in colonic carcinoma while the *adenoma-carcinoma* sequence is well defined, as benign lesions increase in size they

clearly become more malignant, this does not occur in the breast. This is in direct contrast with the breast where lesions as small as 0.5 cm, may already show invasion. Furthermore, the lesion described as a *ductal carcinoma-in-situ* may frequently achieve considerable size without any signs of invasion of the basement membrane and ironically, in 2-3% of cases may be associated with lymph-node metastases (Veronese *et al.,* 1995). This clearly implies that there are significant limitations of conventional pathology in predicting the clinical outcome of a large group of patients.

Molecular biologists and cell kineticists describe cancer as disorder in the cell cycle, the greater the degree of disorder the less well differentiated the tumour which results (Shay *et al.,* 1993). Every variety of breast tumour from benign, to malignant represents a possible end result of disorder in the cell cycle. The larger and less well differentiated tumours, probably possess more disorder than the benign tumours.

Three groups of genes control the cell cycle - the tumour suppressor genes, the oncogenes and the DNA repair mutator genes. It is mutations in these genes which contribute the molecular insults which build up over the lifetime of both the individual and the tumour.

The G1 phase of the cell cycle varies between 10 - 48 hours, which implies at least twice a day, cells in various parts of the body are prone to develop anomalies, which if uncorrected will produce tumours. This clearly puts the importance of the cell cycle and its control mechanisms into context. The p53 protein and its negative feedback interaction with the Bcl-2 protein, represents only the tip of the iceberg. Aberrations in either protein, or the genes which encode them, therefore, have dire consequences for the survival of maimed cells harbouring DNA mutations. It must be emphasized that overexpression of the Bcl-2 protein itself is not associated with genetic anomalies in the encoding gene, in either breast tumours, or melanomas.

Since p53 inactivation occurs in over half of the human cancers, it is possible that loss of p53 mediated repression of Bcl-2 gene expression, accounts at least in part, for the frequent abnormalities in Bcl-2 protein production seen in tumours (Miyashita *et al.,*

1994).

Failure of the p53/Bcl-2 pathway does not inevitably lead to tumour formation, as there are other non-p53 dependent pathways, which may induce apoptosis of maimed cells (Kerr, 1994, Ueda & Shah, 1994, Hetts, 1998). Further down the cell cycle there is a checkpoint, or "R" point, manned by the cyclins, which control entry to and from a point which controls the final common pathway, after which protein synthesis and all its implications will follow (Pardee, 1972, 1992). Aberrations in these genes are clearly of dire consequences to the organism. It is therefore not difficult to imagine that tumours which harbour mutations, in both sets of genes acquire a significant growth advantage.

In between the overtly malignant lesions, are a group of intermediate lesions which although not frankly malignant confer an increase risk on the individual both for the development of further malignant tumours in both the ipsilateral and contralateral breast - these are areas of atypical ductal hyperplasia and diffuse papillomatosis. These risk factors assume increasing importance if a family history of breast cancer exists (Harris, 1995, Elston & Ellis, 1998).

Up to 5% of breast biopsies contain atypical ductal hyperplasia (Fig 1.4), a lesion with cytological features of early noninvasive cancer, but limited in extent. The clinical importance of ADH is indicated by epidemiological studies in which a biopsy of ADH conferred a four, to fivefold increased risk for later breast carcinoma generalized over both breasts at any site, suggesting that the presence of the lesion is a risk factor (Veronese *et al.,* 1991, Saslow-Weinstat *et al.,* 1995).

Patients with biopsies containing small, non-comedo ductal carcinomas *in situ* (DCIS), in which neoplastic cells are confined to the breast duct, have an increased risk for breast cancer at the same site in the breast. Although potentially malignant, non-comedo DCIS lesions can remain uncommitted to invasion and metastasis. More advanced comedo forms of DCIS, distinguished by cell necrosis, frequent mitoses and a high degree of nuclear *atypia* are generally thought to represent committed precursors of

invasive breast cancer. Little is known, however, of their evolutionary lesions. Knowledge of the molecular basis of breast neoplastic progression will is critical to the development of preventative strategies for women at high risk of cancer.

6.2 Biological Implications of Immunohistochemical Detection of the p53 Protein

The detection of the p53 protein in the nucleus, or cytoplasm must be viewed in the context of the biological functions of the protein (Wynford-Thomas, 1992).

Overexpression of the protein in the nucleus of the cell occurs for three reasons

(a) Missense mutations of the p53 tumour suppressor gene (Davidoff *et al.,* 1991, Andersen *et al.,* 1993),

(b) Overexpression of the normal p53 protein (Barnes *et al.,* 1992, Fisher *et al.,* 1994) and

(c) Hypothetically, a failure of degradation of the normal protein (Harris A, 1992, Harris CC, 1996).

The best example of altered cellular environment leading to increased p53 protein expression is the extensive overexpression of p53 in nasopharyngeal carcinoma associated with EB virus infection in the absence of p53 gene mutation (Harris CC, 1996).

Cytoplasmic positivity occurs because,

(a) the method of fixation leads to leakage due to nuclear damage (Harris A, 1992), or

(b) sequestration of the protein in the cytoplasm by both exogenous (Moll *et al.,* 1992, Riou *et al.,* 1993) and endogenous proteins (McCann *et al.,* 1994) has occurred.

Failure to detect the protein in a malignant cell occurs because,

(a) There is a null mutation in the p53 tumour suppressor gene, which results in the production of no protein at all (Bartek *et al.,* 1990, Barnes *et al.,* 1995),

(b) The normal minute amounts are being expressed, which are usually, not detectable,

(c) Rapid degradation of the protein as occurs with the human papilloma virus (HPV) or,

(d) Technical error in the detection system (Fisher *et al.,* 1994, Bourne, 1995).

The wide variation in percentage positivity has been attributed to loss of antigen by the various methods of fixation (Harris CC, 1992, Fisher *et al.,* 1994).

6.3 p53, Apoptosis and Chemotherapy

The response of the individual tumour to chemotherapeutic interventions is dependent on the histological grade, tumour type and on the particular chemotherapeutic agent. Cells in cycle are more susceptible to these interventions, which by definition, also affect other groups of cells with a rapid turnover, for example, the haemopoetic system and the gastrointestinal tract.

There are two methods a chemotherapeutic agent may affect a tumour, either by a dose dependent nuclear disruption, which then excites an inflammatory response i.e. necrosis, or apoptosis. Apoptosis occurs either via a p53 dependent pathway, or a non p53 dependent pathway, and usually requires a lower dose that required to achieve necrosis (Harris A, 1992, Rasbridge *et al.,* 1993, Ueda & Shah, 1994, Harris CC 1996).

Every malignant tumour contains non malignant cells namely, blood vessels, tissue stroma and various host cells including lymphocytes, neutrophils and macrophages.

Conventionally, a tumour consists of three compartments (Calman and Giles, 1988),

(a) the growth fraction,

(b) the *clonogenic* fraction - a non-proliferating compartment composed of cells not in active division, but capable of doing so if appropriately stimulated and

(c) the third compartment is composed of dead cells, or cells with only a limited capacity for division.

Growth is related to a decrease in cell loss, rather than cell growth. A Gompertzian model of growth is presumed to predominate in tumours i.e. small tumours grow rapidly and logarithmically and as the tumour becomes larger the growth rate slows down. In addition smaller tumours tend to have a larger growth fraction, implying they may be more susceptible to chemotherapy.

Tumour size is determined by the balance between cell gain (proliferation) and cell loss (cell death and differentiation). Cell loss is sometimes considerable and apoptotic cell death is a critical in the equation which predicts tumour size and thus progression. Measurement of apoptotic cells in tumours is difficult to quantify with accuracy, since the half-life of histologically recognizable apoptosis is short and cell samples are often heterogeneous (Dive, 1991, Elston & Ellis, 1998).

The exploration and understanding of the process of programmed cell death, or apoptosis, has forced a reconsideration of the mechanism whereby tumour cells can acquire, or lose sensitivity to cytotoxic treatments.

Non-physiological extremes in the external environment of the cell (e.g. hyperthermia and hypoxia) and high concentrations of noxic substances cause a loss of membrane integrity, collapse of cellular homeostasis and a depletion of ATP levels. The cell ruptures, releasing degradative lysosomal enzymes which mediate an inflammatory reaction in the immediate locality. This is not genetically influenced and it appears uncontrollable in terms of possible drug intervention, with the possible exception of drugs which alter tumour vasculature (Baillie *et al.,* 1995).

It has been proposed that there are cellular hierarchies which determine the propensity of a cell to undergo apoptosis e.g. in the intestine and the testis. Dive declares ".....the relative promiscuity of the apoptotic response in haemopoetic cells, which amplify in numbers as they proliferate and differentiate, might be important to prevent inheritance of damage and its amplification during development" (Dive *et al.,* 1991).

In other cells where division and differentiation is not associated with a significant amplification of cell numbers, apoptosis may be more difficult to trigger because the cells are programmed with a greater survival potential.

The ability of tumour cells to detect cellular damage and activate the apoptotic response may determine the response to chemotherapeutic intervention. Oncogenic expression can sensitize cells to apoptosis. p53 is required for the efficient activation of apoptosis following irradiation, or treatment with chemotherapeutic agents (Harris AL, 1991,

Harris CC, 1996). Thus, the absence of wild type p53 expression leads to a dramatic increase in cellular resistance to these agents, implying that tumour cells can acquire drug and radiation resistance through mutations that interfere with apoptosis. In particular, the status of the p53 gene, which is mutated in a high percentage of tumours, may be an important determinant of the efficacy of many treatment protocols (Dive *et al.,* 1991, Lowe *et al.,* 1993).

Treatment with chemotherapeutic agents and/or radiotherapy has been shown to change the oncogenic expression of tumours (Rasbridge *et al.,* 1993, Chang *et al.,* 1995, Linn *et al.,* 1996). At lower doses the cytotoxic action of many anti-cancer agents is largely determined by the genotype of the cell, rather than the genotoxicity of the agent. It has been demonstrated that not all forms of apoptosis are p53 dependent, but cells which undergo apoptosis via non-p53 mechanisms usually require higher doses of radiation (Wyllie *et al.,* 1990, Harris AL, 1991, Kerr *et al.,* 1994, Harris CC, 1996). Thus, cells that sustain sufficient damage may undergo apoptosis regardless of their p53 status. Since p53 can suppress immortalization, it would not be surprising if many established cell lines acquire defects in p53-dependent apoptosis. This would be a logical reason why many established cell lines require higher concentrations of chemotherapeutic agents to induce apoptosis (Lowe *et al.,* 1993). In the absence of p53, oncogene expressing cells are unable to trigger the death programme in response to environmental signals. These signals can arise from drugs that interfere with cellular homeostasis, or cause DNA damage, from mitogen deprivation, high cell density, or changes in cellular microenvironment that might accompany metastases.

It has been suggested that the cytotoxicity of many anticancer agents involves a common genetic pathway, therefore, lesions in apoptotic pathways could generate a resistant phenotype (Kerr *et al.,* 1994). Hemizygous cells showed an intermediate level of resistance to anti-cancer agents. Point mutations leading to the expression of dominant negative p53 alleles may have a similar effect and their intrinsic ability to inhibit wild-type p53 function may contribute to variability in cellular response to

anticancer agents. These factors include the mdm-2 oncogene, the human papilloma virus E6 protein, or the adenovirus E1B gene (Farmer *et al.,* 1992, Levine & Perry, 1993). Conversely, mutations that activate genes that normally suppress apoptosis might also contribute to drug resistance. Indeed over expression of the Bcl-2 oncogene rescues myc-expressing cells from apoptosis occurring upon serum withdrawal and etoposide treatment (Wagner *et al.,* 1993).

According to Levine ".....the relationship between p53 expression in a particular tumour and response to chemotherapy is reflected in three types of clinical patterns" (Levine, 1995). First, there are a number of tumour types in which a high percentage of primary tumours have acquired p53 mutations. These include malignant melanoma and cancers of the lung, colon, prostate, bladder and cervix. In general patients with these tumours respond poorly to treatment with either radiation, or chemotherapy. Furthermore, in many of these tumour types, the presence of p53 mutations corresponds with poor prognosis.

Second, there are tumours that rarely exhibit p53 mutations at presentation. Included in this group are testicular cancer, Wilm's tumour and childhood acute lymphoblastic leukaemia. In these forms of cancer, chemotherapeutic intervention is extremely effective.

Third, upon relapse of acute lymphoblastic leukaemia, failure of therapy correlates with the occurrences of mutations in the p53 gene. Furthermore, in several tumour types, p53 mutations have been identified in relapse specimens that were not present in the primary tumour.

6.4 Clinical Implications of p53 Expression in Breast Tumours

The overexpression of the p53 protein has been proposed as an independent prognostic factor in breast cancer and linked with traditional prognostic factors. Expression varies with the histological type of breast cancer. Mutations of the p53 gene are frequent in medullary (39%) and ductal (26 - 50 %) (Bartek *et al.*,1990a, b, Davidoff *et al.,* 1991, Marchetti *et al.,* 1993, Moll *et al.,* 1993, Singh *et al.,* 1993), less common in lobular

(12%) and absent in papillary and mucinous carcinomas (Marchetti *et al.,* 1993). A strong correlation has been found between p53 mutations and nuclear accumulation of the protein (Davidoff *et al.,* 1991, Andersen *et al.,* 1993). There is also a significant association between mutations in the p53 gene and high proliferative activity as shown by the antibody Ki-67 (Bouzubar *et al.,* 1989, Leonardi *et al.,* 1992).

Of interest is that up to seventy percent of colonic carcinomas harbour mutations in the p53 tumour suppressor gene (Moll *et al.,* 1992). However, while 90 % of colon cancers which have only one allele (reduced to homozygosity) have the mutant p53 allele, 60 % of breast tumours retain the wild type allele (Davidoff *et al.,* 1991).

Purely benign lesions fail to express this protein (Milikan *et al.,* 1995), nor is it seen in non-malignant epithelium adjacent to tumours, but it is sometimes seen in the dysplastic epithelium adjacent to p53 positive carcinomas (Barnes *et al.,* 1995). Positivity has also been reported in *in-situ* carcinoma (Davidoff *et al.,* 1991).

Similar staining patterns have been observed in cell cultures of malignant breast epithelium (Bartek *et al.,* 1990a, b).

Statistically significant associations of p53 protein accumulation with oestrogen (Thor *et al.,* 1992, Barnes *et al.,* 1995) and progesterone (Barnes *et al.,* 1995) receptor negativity and with high nuclear grade abound (Thor *et al.,* 1992, Barnes *et al.,* 1995 Michalides *et al.,* 1996). Earlier reports failed to find a relationship with either PR (Ostrowski *et al.,* 1991), or ER status (Singh *et al.,* 1993). There were also statistically significant associations independent of other prognostic factors between p53 protein accumulation and metastases-free and overall survival, for randomly accrued and both sporadic and familial tumours, but not for grade III tumours (Thor *et al.,* 1992, Al Sawan *et al.,* 1992, Barnes *et al.,* 1995). The association is reported for both node-negative and positive groups, invasive ductal carcinomas and lobular (Barnes *et al.,* 1995). Furthermore, expression correlates with survival after relapse (Barnes *et al.,* 1995) in some studies, but not others (Hanzal *et al.,* 1992).

6.5 Breast Cancer and Bcl-2

The normal biological mechanism of action of Bcl-2 is as yet unknown. It does appear, however, that it may be under hormonal control, acting as a regulator of cellular events until it is switched off. At this point in normal tissue, apoptosis would occur. In neoplastic tissues the reduction in expression of Bcl-2 may require alternative survival pathways. If such cells are relatively more resistant to apoptosis, this may contribute to their resistance to therapy and environmental stress, and hence a poorer prognosis. Bcl-2 confers survival benefit on cells *in-vitro* and these cells after a latent period undergo malignant transformation - this is the true test of an oncogene, the transformation from clonicity to overt malignancy. It is attractive to hypothesize that this is the case in the breast. It is tempting to hypothesize, that the transformation of a group of cells from clone to tumour is, initially at least, under a gene that suppresses apoptosis and remains so long enough for other genes, or environmental factors to induce tumourigenicity.

In continuously renewed systems, such as those found in the epithelium of the gut, skin and breast, cells lose Bcl-2 expression prior to apoptotic cell death (Carson & Ribiero, 1993, Ueda & Shah, 1994, Hetts, 1998). Bcl-2 is often demonstrated in glandular cells in which regulation of hyperplasia and involution is controlled by hormones and growth factors (breast), complex differentiating epithelium with long-lived stem cells (skin, intestine) and fully differentiated long lived non-cycling cells (neurons) (Kerr, 1994). This group of cells were shown over 25 years ago to show histological evidence of apoptosis (Kerr *et al.,* 1972).

In the normal breast, a continuous renewal system is in operation, which is partly under hormonal control. This process progresses at a much slower rate than for example, that seen in the gut. Other glandular organs with slow cell turnover rates for example the thyroid also show Bcl-2 expression (Carson & Ribiero, 1993, Ueda & Shah, 1994, Hetts, 1998).

Bcl-2 is universally expressed in normal breast epithelium, and a subset of tumours

lose expression at a later stage in their progression, this group is associated with the established molecular markers of poor prognosis (Leek *et al.,* 1994, Bharghava *et al.,* 1994, Joensu *et al.,* 1994). Expression of the Bcl-2 protein in the breast tumours as detected immunohistochemistry varies between 32-79% (Gee *et al.,* 1994, Joensu *et al.,* 1995) and correlation with ER and PR status appears to be the norm (Leek *et al.,* 1994, Bharghava *et al.,* 1994, Doglioni *et al.,* 1994, Nathan *et al.,* 1994, Sierra *et al.,* 1995). There appears to be no correlation with lymph-node status (Nathan *et al.,* 1994, Gee *et al.,* 1994), although others found that individuals with Bcl-2 positivity were 3.6 times more likely to have positive lymph-nodes (Sierra *et al.,* 1995). An inverse relationship between cerb-2 (Nathan *et al.,* 1994, Gee *et al.,* 1994) and EGRF (Doglioni *et al.,* 1994, Gee *et al.,* 1994) has also been documented. However, there is no convincing evidence that Bcl-2 is an independent prognostic factor on multivariate analysis. Moderate to "strong" expression is commoner in the lobular than the ductal carcinoma and in older women. It is associated with several favourable prognostic factors such as low cell proliferation rate estimated either by the mitotic count or the S-phase fraction size, high histological grade of differentiation and lack of p53 expression (Leek *et al.,* 1994, Bharghava *et al.,* 1994, Doglioni *et al.,* 1994, Nathan *et al.,* 1994, Sierra *et al.,* 1995). It was also favourably associated with lack of tumour necrosis, low stromal Cathepsin D expression, and DNA diploidy, but not with the primary tumour size, or the axillary node status. However, Bcl-2 again had no independent prognostic significance on multivariate analysis.

The expression of Bcl-2 has been used to predict response to hormonal intervention, or chemotherapy, as many cytotoxics drugs do induce apoptosis (Rasbridge *et al.,* 1993, Joensu *et al.,* 1994, Harris CC, 1996).

The expression of the Bcl-2 proto-oncogene, was investigated prior to and following endocrine therapy with the anti-oestrogen, Tamoxifen. In that study, the immunohistochemical expression of Bcl-2 was observed in 32% of invasive breast cancers, but in 65% of tumours treated with Tamoxifen. Treatment with the drug resulted in an

increase in tumours that expressed the Bcl-2 protein and a lowering of Ki-67 score (Johnston *et al.,* 1994, Daidone *et al.,* 1995). This suggests that the cellular proliferation rate is indirectly linked to apoptosis suppression and implies that one mechanism of action of Tamoxifen is to downgrade the tumour, thereby possibly rendering the cells more susceptible to apoptosis (Johnston *et al.,* 1994, Daidone *et al.,* 1995).

It has been shown that the Bcl-2 oncoprotein blocks chemotherapy induced apoptosis in a human leukaemia cell line. The fraction of p53-positive cells was reduced by primary chemotherapy in about 50% of previously p53-positive tumours, whereas Bcl-2 expression was only marginally affected (Miyashita *et al.,* 1993). DNA ploidy and hormone receptor status did not change in about 75% of cases, regardless of the chemotherapeutic regimen (Daidone *et al.,* 1995).

6.6 Cyclins

The amplification of chromosome 11q13 has been observed in about 10-20% of breast cancers (Gillette *et al.,* 1994, Zuckerburg *et al.,* 1995, Dickson *et al.,* 1995, Peters *et al.,* 1995) and numerous other tumours. Cyclin D1/PRAD 1 is a likely "driver" gene for this amplification because of its role in cell cycle control and it has been specifically implicated as an oncogene in parathyroid adenomas and B-cell lymphomas with 11q13 translocation breakpoints (Peters *et al.,* 1995).

It is overexpressed at the protein level in two to four times as many cases of breast cancer (Bartkova *et al.,* 1994, Gillette *et al.,* 1994, Dickson *et al.,* 1995, McIntosh *et al.,* 1995, Michalides *et al.,* 1996, Marsh *et al.,* 1998). Heterogeneity of expression in a tumour nodule has been suggested to be due to the fact that cyclin D1 is expressed in a cyclical manner even in cancer cells (McIntosh *et al.,* 1995), but this is not always the case (Michalides *et al.,* 1996).

Cyclin D1 plays a critical role in the timing of the initiation of DNA synthesis in the normal cell cycle of mammalian cells. Deregulated expression of this protein has been seen in a variety of tumours, either as a result of gene amplification, or chromosomal translocation (Bates *et al.,* 1994, 1995, McIntosh *et al.,* 1995, Dickson *et al.,* 1995)

A comparison of the expression patterns in matched lesions at different stages of breast tumour progression appear to suggests that Cyclin D1 aberrations appear to be a relatively early event and that when acquired by a tumour, is maintained throughout breast cancer progression including metastatic spread (Bartkova *et al.,* 1994, Muller *et al.,* 1995). The intensity of the staining has been correlated to DNA amplification of the gene (Gillette *et al.,* 1994, Saslow-Weinstat *et al.,* 1995). Cyclin D is not expressed in several human tissues such as lymph-nodes, spleen and tonsils (Bartkova *et al.,* 1994). Overexpression of the cyclin D1 protein has also been found in breast cancer cell lines, both in the absence and presence of gene amplification, but at lower levels than in breast tumour specimens (Bartkova *et al.,* 1994). Conversely, amplification does not invariably lead to overexpression of cyclin D (Buckley *et al.,* 1993, Zhang *et al.,* 1994, Oyama *et al.,* 1998). It has been implicated in cell cycle progression in breast cancer cell lines, but its expression can be inhibited by both anti-oestrogens and anti-progestins (Musgrove *et al.,* 1993). There is a correlation between Western blotting and immunostaining data (Saslow-Weinstat *et al.,* 1995). Single strand conformational polymorphism (SSCP), has failed to reveal any tumour specific mutations in the coding region of exons 1 - 5 (Hosokawa *et al.,* 1995).

Benign breast lesions are negative for the protein (Millikan *et al.,* 1995, Zhang *et al.,* 1994), although expression has been reported in areas of atypical ductal hyperplasia (Alle *et al.,* 1998, Gillett *et al.,* 1998). Up to 80 % of lobular carcinomas express the cyclin D protein (Oyama *et al.,* 1998).

Anti-cyclin antibodies have been developed (Bartkova *et al.,* 1994, Michalides *et al.,* 1996) which can be correlated with proliferation indices in breast tumours (Dutta *et al.,* 1995, Gelb *et al.,* 1992). Tumours which are cyclin D1 positive have been correlated with both oestrogen and progesterone receptor positivity (Michalides *et al.,* 1996, Dickson *et al.,* 1995), the retinoblastoma protein (de Jong *et al.,* 1998), p21 (Rey *et al.,* 1998) but this is not a uniform finding (McIntosh *et al.,* 1995). Tumours that are positive tend to be of higher grade (Zhang *et al.,* 1994) and it is associated with

apoptotic body counts in breast tumours (de Jong *et al.,* 1998). There is no correlation with mdm-2, c-jun and p53 expression, neither do any confer prognostic significance when co-expressed with cyclin D1 (McIntosh *et al.,* 1995). Furthermore, co-expression with EGFR (epidermal growth factor receptor), or the retinoblastoma protein have a significantly poorer prognosis in comparison to those expressing cyclin D1 alone (McIntosh *et al.,* 1995).

6.7 MDM-2

The MDM-2 protein is detectable by immunohistochemistry either when there has been an alteration in gene copy i.e. gene amplification, or as a result of interactions with other cell-cycle proteins at cellular level specifically, the p53 protein. It is usually detectable in the nucleus (McCann *et al.,* 1994).

Simultaneous expression of p53 and MDM-2 has been proposed to be due to the normal activity of growth control mechanisms (Martinez *et al.,* 1995, Harris CC, 1996) but has been associated with a poorer prognosis in breast cancer (Buenos-Ramos *et al.,* 1996). MDM-2 positive tumours tend to be of low grade and stage (Meltzer *et al.,* 1994) although associations with high grade and lymph-node secondaries exist (Buenos-Ramos *et al.,* 1996, Jiang *et al.,* 1997). However, it is not clear whether overexpression of MDM-2 in the absence of gene amplification is the same as amplification (Meltzer *et al.,* 1994).

In glioblastoma cell lines it has been found that MDM-2 amplification is only found in tumours with a normal p53 gene i.e. in tumours expressing wild type p53 (He *et al.,* 1994).

In breast carcinoma 7 - 97 % positivity has been reported on paraffin embedded sections - predominantly nuclear with little cytoplasmic staining (Meltzer *et al.,* 1994, McCann *et al.,* 1994, Jiang *et al.,* 1997, Buenos-Ramos *et al.,* 1996). MDM-2 protein positivity was significantly associated with low levels of p53 (McCann *et al.,* 1994) but this has been contested (Buenos-Ramos *et al.,* 1996). However, MDM-2 status was not associated with age, tumour grade, lymph node status or tumour size (McCann *et*

al., 1994) nor oestrogen receptor status (Buenos-Ramos *et al.,* 1996).

There is also no correlation between p53 expression and mdm-2 gene amplification in human lymphoid tumours (Cesarmanet *et al.,* 1994, Martinez *et al.,* 1995).

Section II

Anatomy and Molecular Biology of the Skin and Malignant Melanomas

Chapter 1

Malignant Melanoma

Definition A malignant melanoma is a tumour arising from the epidermal melanocyte.

1.1 Incidence

There has been a steady rise in the incidence of malignant melanoma of the skin in all parts of the world from which reliable data can be obtained since 1950 (Swerdlow *et al.,* 1986, Mackie, 1995, 1998). According to Mackie... "in Norway and Australia the incidence of melanoma doubled over the 10 year period 1970-80 (Mackie, 1995, 1998) and since that time similar rates of increase have continued to be recorded for both Scandinavia and Australia (Balch *et al.,* 1983). The story is similar in parts of North America, Scotland (Mackie *et al.*, 1982) and Germany (Garbe *et al.*, 1994). In Germany the incidence has doubled every 12-15 years (Garbe *et al.*, 1994).

The incidence of most cancers rises with age, but with melanoma there is little increase in the rates after the age of 45 years. The highest incidence rates in the world are from Australia and New Zealand with annual incidence figures of about 40 new cases per 100,000 population. The current rate of increase is more rapid than for any other malignancy with the exception of lung cancer in women. In Europe, the sex incidence is unequal with a ratio of 2:1 in females:males and annual incidence figures of around 12/100,000 for women and 6/100,000 for men. This difference is not seen in higher incidence areas, where both sexes are affected equally and has been interpreted as indicating a minor hormonal effect which is overcome in areas of greater exposure to intense ultra-violet radiation. Unfortunately, the diagnosis is often delayed for a number of reasons and the overall 5-year survival rate in England and Wales in 1981 was 25% for men and 75% for women (CRC, 1989). The better prognosis for women is likely to be due to earlier diagnosis of the disease.

There were approximately 32,000 new cases diagnosed in America in 1992, with 6,700 deaths expected due to the tumour. If the present rate of increase continues, melanoma by the year 2000 will affect 1% of all Americans (Mackie, 1995, 1998).

Melanoma is very rare prior to puberty, but when it does occur, about 50% arise in giant congenital naevi (Cellabos *et al.*, 1995). Melanoma can also develop as a result of

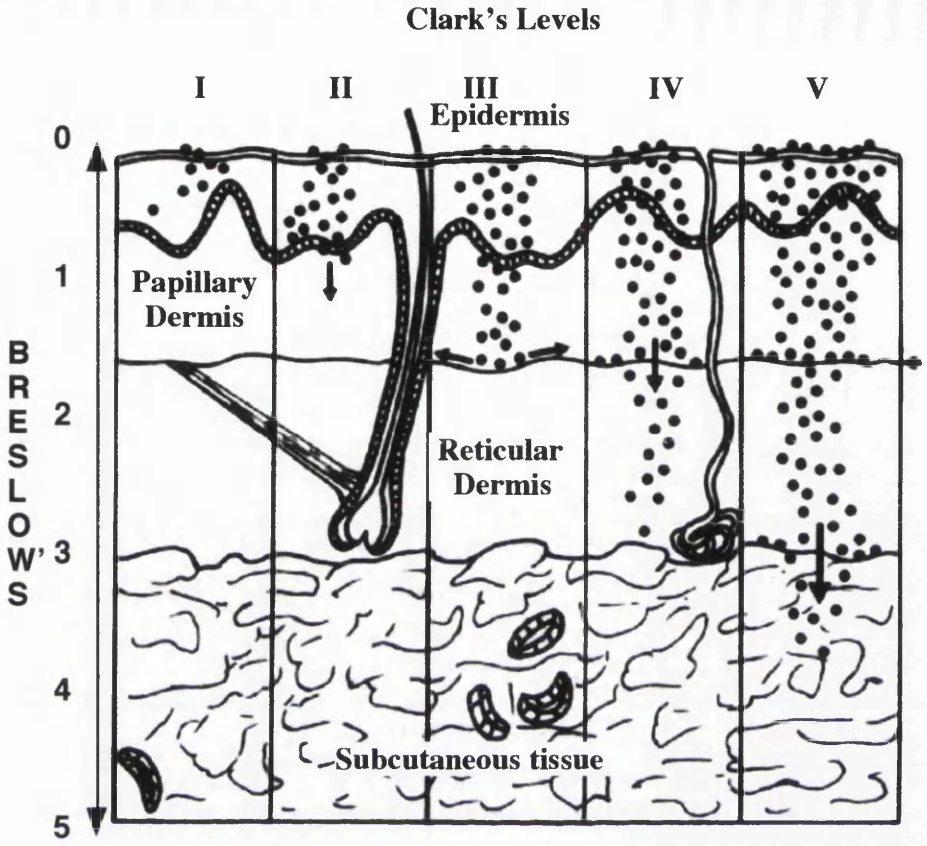

Figure 2.i Schematic diagram of the layers of the skin. Clark's Levels are shown from I to V and Breslow's thickness measurement is shown from 0 - 5 mm in thickness.

transplacental spread from an affected mother to foetus. From the second decade onwards there is a steady rise in the age specific incidence. The mean age at presentation is early in the sixth decade.

Malignant melanoma is extremely rare in childhood (Cellabos *et al.*, 1995), but it is one of the few cancers to have a significant impact on young adults. Twenty-two percent of malignant melanoma cases occur in people aged less than 40, whereas, only 4% of all malignant neoplasms occur in this age group (CRC, 1989). In the age 15-34 years, melanoma is the fourth commonest cancer in women and the seventh commonest cancer in men (OPCS, 1992).

Patients overall have a good prognosis. Survival of treated cases when their melanoma

is at an early stage of its development, before metastasis, is very good and prognosis is also related to the thickness of the tumour, known as the Breslow thickness *vide infra* (Breslow, 1970). Patients with "thin" tumours have a better overall prognosis (OPCS, 1992).

The highest incidence areas are those with long hours of sunlight throughout most of the year. In North America and other similar regions there is an inverse relationship between melanoma incidence and distance from the equator (Mackie, 1995, 1998). Pigment is protective, as black-skinned individuals living a similar lifestyle to white people in the same geographic area have a lower incidence of malignant melanomas. Mackie declares "in Northern Europe the incidence is higher than in the south. Figures are higher in northern countries, such as Norway. This may be partly due to habits of sun exposure, with Scandinavians flying regularly to the Mediterranean countries on holiday, and partly due to phenotypic differences, with for example, a noticeably fairer-skinned population in northern Italy than in southern Italy" (Mackie, 1995, 1998). Intense intermittent exposure of non-acclimatised skin is considered a greater risk factor, rather than continual, occupational sun exposure (Mackie *et al.*, 1982). Reviews from Denmark and Canada have incriminated outdoor activities such as sailing and swimming as risk factors for melanoma.

"The typical melanoma patient is an indoor office worker who is not exposed to regular daily sunlight, but who enjoys one, or two holidays each year in an area of high UV intensity" (Lee *et al.*, 1980, Cook *et al.*, 1984).

Age at migration studies from Europe to sunnier climates such as Israel, or Australia indicate that exposure to intense UV radiation at a young age may be critical (Cellabos *et al.*, 1995, Mackie, 1995, 1998). There is a higher than expected incidence of melanoma among employees at the Livermore laboratory, an atomic energy establishment in North America (Schneider & Sagebiel, 1987). There is a possible weak link between the use of the oral contraceptive and malignant melanoma (Beral *et al.*, 1984) but this is refuted (Jatoi, 1993).

1.2 Anatomy of the Skin

According to R.J. Last ..... "the skin is the largest organ in the body. It functions to protect the individual from adverse external conditions and mirrors our state of general health."

The essential skin is the dermis, or *corium*, a strong and tough fibrous tissue rich in blood vessels, lymphatics and nerves. It is covered by a surface layer of cells the epidermis. The epidermis is covered by a special epithelium which becomes flatter towards the surface - *stratified squamous epithelium.* The thickness of the skin depends on two factors, namely the thickness of the horny layer and the thickness of the dermis itself. The colour of the skin in blondes depends on the amount of blood in the capillaries of the dermis and how much the keratin obscures this. Where keratin is thin the skin is red (e.g. the red margin of the lips); where keratin is thick the skin is white (e.g. palms). Pigmentation is produced by mobile cells containing pigment - the melanocytes. They lie between the cells of the epidermis, usually in the deepest layers. Some occur also in the dermis. Some of these cells are colourless, but they show a positive DOPA reaction i.e. they convert **D**ihyd**O**xy**P**henyl**A**lanine into melanin.

Yellow elastic fibres occur in the dermis and impart to it an elasticity that gradually diminishes with advancing age, as the elastic fibres progressively atrophy.

1.2.1 Histology

The epidermis is divided into four easily recognizable layers. (Fig 2.1)

1. **Stratum Basale** (Stratum Germinatum)

The stratum basale is the most basal layer of cells in the epidermis and it rests on a thick basement membrane. The cells of the stratum basale are highly proliferative, undergoing repeated mitosis. This division results in the production of a proliferative stem cell layer that remains on the basement membrane and gives rise to the next generation of cells as well as cells that differentiate into keratinocytes.

The layering seen in the epidermis represents the progressive accumulation of keratin within the progeny of the proliferative cells of the stratum basale.

2. **Stratum Spinosum**

This varies from two to six cell layers in thickness. Small spine like projections appear to join this layer on light microscopy. These are desmosomes.

The two preceding layers together are known as the *Stratum Malphigii.*

3. **Stratum Granulosum.**

This consists of three to five layers of flattened, irregularly shaped cells loaded with keratohyalin granules. These granules stain intensely with various basic dyes including Haematoxylin.

4. **Stratum Corneum.**

This is the most apical layer of the epidermis, ranging in the thickness from as few as five to as many as several hundred cell layers. Where the stratum corneum is particularly thick, there is a thin transitional zone between the stratum granulosum and the stratum corneum known as the *stratum lucidum.*

1.3 Embryology of the Skin

The skin develops from ectoderm. The appendages of the skin are the sweat glands, nails, hair follicles and the sebaceous glands. All these are formed from downwards growth of from the surface epithelium. This implies the skin and its appendages are of ectodermal origin. The fibrous tissue (collagen) and the yellow elastic fibres of the dermis are derived from mesoderm.

1.4 Pathology of Malignant Melanoma

According to Mackie..... "WH Clark [Jnr] suggested in 1969 that using a combination of clinical and pathological features, malignant melanoma could be divided into three main subsets, the superficial spreading malignant melanoma, the nodular melanoma and the lentigo maligna melanoma (Mackie, 1995, 1998). In 1975, Reed added a fourth group the *acral lentiginous,* or *palmoplantar mucosal* melanoma.

1.4.1 Superficial Spreading Malignant Melanoma

The superficial spreading malignant melanoma comprises over 50% of melanomas on white-skinned people. The essential pathological features are the presence of a focus of

malignant melanoma cells invading the dermis, with areas of *in situ* malignant change in the adjacent epidermis. This consists of the presence of cytologically atypical melanocytes in the suprabasal layers of the epidermis, both singly and in clumps. On Haematoxylin and Eosin sections, the pattern may be very similar to that seen in extramammary Paget's disease, and the term pagetoid melanoma is therefore also used to describe this lesion.

1.4.2 Nodular Melanoma

The pathological features of a nodular melanoma are a focus of invasive melanoma cells in the dermis in direct contact with the immediately overlying epidermis, and no morphological abnormality apparent in the adjacent epidermis on either side of the invasive nodule. It is therefore a primary malignant melanoma with no recognizable adjacent *in situ* disease, or radial growth phase. According to Mackie... "There is still a tendency to use the term nodular melanoma for any primary melanoma which has a visible nodule. This is incorrect - SSMM, LMM and acral malignant melanomas, can all in the course of later growth, develop elevated nodular areas".

1.4.3 Acral Lentiginous or Palmoplantar Mucosal Melanoma

This describes lesions which arise in non-hair-bearing areas of the palm, or sole. The essential pathological features are the presence of an extensive area of lentiginous change in the epidermis, around the focus of invasive primary melanoma. An important feature is the presence of skip areas, with foci of relatively normal epidermis in areas of gross lentiginous change. This feature makes it particularly important to examine the excision specimen thoroughly, to determine whether, or not the lesion has been completely excised.

Primary melanomas arising on the mucosal surface of the oral cavity, the vulvo-vaginal and the rectal areas have some features in common with acral lentiginous lesions and the term *palmoplantar mucosal melanoma* is sometimes used to describe the entire group. In this particular variety the focus of invasion may be very difficult to find even in the presence of metastases.

1.4.4 Lentigo Maligna Melanoma

This variant is found on chronically light exposed skin, usually the face, and has a long pre-invasive period during which there is striking lentiginous replacement of the basal keratinocytes by atypical melanocytes, but no downward invasion into the underlying dermis which generally shows actinic damage of the dermal collagen.

1.4.5 Desmoplastic and Neurotrophic Variants

The desmoplastic variant is frequently seen in association with chronically sun-damaged skin and overlying lentigo maligna melanoma. It is important because it can be difficult to be absolutely certain that excision is complete and local recurrence is a problem. The neurotrophic melanoma is characterized by metastatic spread along the nerve trunks. This commonly occurs in lesions on the head and neck area and is quite painful.

1.4.6 Radial Growth Phase Melanoma.

Early melanomas may have a slightly elevated and palpable border. On microscopic examination, large epitheloid melanocytes are dispersed in nests and as individual cells throughout the entire thickness of the epidermis, with focal extension into the papillary dermis the rule. The enlargement of such circular lesions is at the periphery, hence, the term radial growth phase. These lesions do not metastasize (Clark *et al.*, 1984).

1.4.7 Vertical Growth Phase Melanoma

This occurs after about 1 to 2 years, the character of the radial growth phase changes focally. Melanocytes exhibit focal mitotic activity and grow as spheroidal nodules, in a manner similar to the growth of metastatic nodules. The nodules expand more rapidly than the rest of the tumour in the surrounding papillary dermis. The net direction of growth tends to be perpendicular to that of the radial growth phase, hence the term vertical growth phase (Clark *et al.*, 1984, Mackie, 1995, 1998).

1.4.8 Metastatic Melanoma

Metastatic melanoma, the final phase in tumour progression, arises from the melanocytes of the vertical growth phase. Initial metastases in malignant melanoma

usually involves the regional lymph nodes, but spread through the blood stream is also common. Haematogenous metastases are usually widespread in comparison with other neoplasms, and virtually every organ may be involved.

1.5 Theory of the Evolution of Malignant Melanoma -The Dual Pathway of Mishima.

Mackie declares..... "an interesting concept on the differing evolutionary pathways of lentigo maligna melanoma and the SSMM was proposed by Mishima. It is based on the clinical observation of very different prognoses for the more slowly evolving (LMM), by comparison with the nodular (Mishima, 1967). LMM is radio-sensitive, does not develop an amelanotic form, occurs later in life and is almost entirely confined to exposed sites. The naevocytic malignant melanoma, on the other hand, is found on both exposed and non-exposed sites, affects a younger age group, may be found in an amelanotic form and is relatively radio-resistant. The melanocytes in LMM are intensely DOPA sensitive and have a dendritic outline, whereas naevocytic melanoma cells are more rounded in outline, tend to occur in clumps or theques and tend to lose DOPA positivity in the deeper parts of the lesion. Also, ultrastructurally the melanosomes in both groups differ in shape and size".

1.6 Risk Factors

Multiple risk factors have been proposed and refuted (Table 2.1), but most are in agreement that the AMS, the absolute number of moles, sun exposure and fair skinned individuals with little tendency to tan are the major risk factors (Mackie *et al.,* 1982, Beval *et al.,* 1984, Swerdlow *et al.,* 1986, Carey *et al.*, 1994, Garbe *et al.,* 1994). It is also of interest that sun exposure has also been linked to the development of intraocular melanomas (Tinker *et al.,* 1994).

1.6.1 Association of Malignant Melanoma with Pre-existing naevi

A clinical history of a pre-existing pigmented lesion at the site of primary cutaneous melanoma has been determined in 18% to 85% of cases (Swerdlow *et al.,* 1986, Mackie, 1995, 1998) and a residual component of a melanocytic naevus in contiguity

SPECIFIC MM RISK FACTORS	RELATIVE RISK
AMS	2-92 TO 85-1269*
> 50 MN	2-64
CONGENITAL MN	17-21
PHENOTYPIC TRAITS	
FRECKLES	3-20
FAIR COMPLEXION	2-18
BLOND HAIR	2-10
RED HAIR	2-6
TENDENCY TO SUNBURN	1-5
INABILITY TO TAN	2-5
BLUE EYES	2-3
SUN EXPOSURE	
CONSTANT	2-5
INTERMITTENT	2-3
IMMUNOSUPPRESSION	2-8
HX. OF NON-MELANOMA SKIN CANCER	3-1

FIG. 2.1 RISK FACTORS FOR MM (Slade *et al.*, 1982)

*Depending on Kraemer or Rigel class.

with melanoma has been observed in 18% to 72% of cases. DN have been found in histological continuity of up to 21.8% of melanomas (Rhodes *et al.*, 1983, Clark *et al.*, 1984, Barnhill *et al.*, 1985, Ackerman *et al.*, 1985).

1.7 Prognostic Factors

A number of factors (Table 2.2) have been introduced as prognostic factors, the best established is tumour thickness, reported by **Alexander Breslow** in 1970 (Breslow, 1970). Appropriate blocks are cut from the apparently thickest area of the primary melanoma and examined with an ocular micrometer. This measures the distance between the overlying epidermal granular layer and the deepest invasive area of the primary lesion. This figure in millimetres is the *Breslow thickness* measurement.

Patients with tumours less than 0.76 mm thick almost all survive. In tumours between 0.76 - 1.5 mm the 5 year survival is over 80%. As the thickness increases the prognosis worsens. For tumours thicker than 3.5 mm it is under 40%, irrespective of the type or extent of surgery, provided that the primary tumour is completely excised.

William H. Clark (Jr.) introduced the *Clark's levels* in 1975, as levels of invasion into the dermis as a way of predicting prognosis (see Table 2.3). Five year survival figures fall steadily with deeper levels. Ulceration even if only microscopic, is an independent poor prognostic sign (Balch *et al.* 1983). The mitotic count and the presence of tumour cells in vessels are poor prognostic signs associated with both local and distant recurrence (Mackie, 1995, 1998).

BRESLOW THICKNESS (BRESLOW, 1970)
CLARK'S LEVEL (CLARK, 1975)
REGRESSION
ULCERATION
MITOTIC COUNT
SEX
SITE
MEAN VESSEL NUMBER

***TABLE 2.2 PROGNOSTIC FACTORS IN MM**

*(MACKIE 1992, 1998)

1. INTAEPIDERMAL OR *IN SITU.*
2. A FEW CELLS IN THE PAPILLARY DERMIS.
3. OCCUPATION AND EXPANSION OF THE PAPILLARY DERMIS BY MELANOMA CELLS.
4. INVASION OF THE RETICULAR DERMIS.
5. INVASION INTO THE FAT.

TABLE 2.3. CLARK'S LEVELS OF INVASION

Regression in and around a primary melanoma is a sign of disputed prognostic significance - this may be due to the difficulty in defining histological regression. Regression in thin melanomas is associated with a poorer than expected 5 year survival, and with mean vessel number (MVN) has recently been associated with early death and metastases (Graham *et al.*, 1994). A prognostic index based on the tumour thickness measured by the Breslow method and the number of mitotic figures in a primary melanoma has been suggested and this is more accurate than the thickness measurement

alone (Mackie, 1992, 1998).

1.7.2 Sex and Site

Melanomas on the extremities have a better prognosis than those of the head, neck or trunk (axial). However, melanomas on the sole of the foot or the subungual region have a prognosis similar, or worse than axial lesions (OPCS, 1992), but this has been disputed (Ball *et al.,* 1995). For every site and thickness, women have a better prognosis than men (OPCS, 1992).

1.8 Diagnosis

The essence in making the diagnosis of a melanoma lies in observing a "change" in a

MAJOR FEATURES
CHANGE IN SIZE.
CHANGE IN SHAPE
CHANGE IN COLOUR
MINOR FEATURES
DIAMETER >7 MM.
INFLAMMATION.
OOZING OR BLEEDING.
MILD ITCH

TABLE 2.4. SEVEN POINT CHECKLIST

A - ASYMMETRY.
B - IRREGULAR BORDER
C - COLOUR VARIATION.
D - DIAMETER > 6 MM.

TABLE 2.5. MNEMONIC FOR CHANGE

pigmented lesion. The seven point check list is commonly used (Table 2.4), or in North America the mnemonic ABCD (Table 2.5) (Mackie, 1992, 1998). Sometimes, melanomas are amelanotic, giving rise to difficulty in making the diagnosis, both clinically and pathologically. This has resulted in the use of monoclonal antibodies to differentiate amelanotic lesions from other non-melanotic skin tumours - the antibodies which been used are NKI-C3, HMB-45 and antibodies to the S-100 protein (Gown *et al.,* 1986, Mackie, 1992, 1998).

1.9 Staging of Malignant Melanoma

Melanomas are classified as shown in Tables 2.6 and 2.7.

STANDARD SYSTEM	
I	LOCALIZED PRIMARY MELANOMA
II	REGIONAL LYMPH NODE METASTASES
III	DISSEMINATED DISEASE

TABLE 2.6 STAGING OF MM

TNM CLASSIFICATION		
I	<1.5 MM	(LEVEL I - III)
II	> 1.5 - 4 MM	(LEVEL IV)
III	> 4 MM SATELLITES	(LEVEL V)
IV	DISTANT DISEASE	

TABLE 2.7 STAGING OF MM

TUMOURS < 1 MM 1 CM EXCISION MARGIN
TUMOURS 1-4 MM 2 CM EXCISION MARGIN
PRIMARY CLOSURE PREFERABLE TO STSG*
STSG* FROM THE IPSILATERAL LIMB
AXILLARY & INGUINAL BLOCK DISSECTION FOR SECONDARIES
ELECTIVE BLOCK DISSECTION ONLY FOR CLINICAL TRIALS
SELECTIVE LYMPHADENECTOMY UNDER EVALUATION
ISOLATED LIMB PERFUSION FOR PALLIATION
? CYTOKINE INFUSION IN THE FUTURE
LASER VAPOURISATION FOR MULTIPLE CUTANEOUS LESIONS

TABLE 2.8. SURGICAL TREATMENT OF MM (Ball *et al.*, 1995).

*Split thickness skin graft

1.10 Surgical Treatment of Malignant Melanoma

Excision biopsy remains the standard way of diagnosing malignant melanoma, providing the histopathologist with a complete specimen for microstaging.

A margin of 1-2 mm of normal skin is taken using an elliptical incision with its long axis in the line of the natural skin creases. A discussion of the different modalities for the treatment of malignant melanomas is beyond the scope of this thesis but a summary is shown in Table 2.8.

1.11 Familial Melanoma and the Genetics of Malignant Melanoma

Among common cancers, melanoma exhibits one of the strongest tendencies to cluster in families. It has been estimated that 8% to 12% of melanomas occur in the familial setting (Lynch *et al.,* 1978, 1983, Green *et al.,* 1983, Mackie *et al.,* 1989, Carey *et al.,* 1994, Piepkorn *et al.,* 1994). The familial basis of individual cases of melanoma may be obscured by incomplete ascertainment, late, or variable age of disease onset, or variable penetrance of the gene in family members, thus this is probably a low estimate. Furthermore, the gene or genes that confer hereditary susceptibility to melanoma may also be involved pathophysiologically in sporadic cases through random, somatic (postzygotic) mutational events that alter normal molecular function at those loci. The increasing incidence of malignant melanoma underscores the importance of determining the molecular mechanisms that lead to its development. The concept of tumour suppressor genes is a fundamental tenet in the understanding of malignant transformation.

The mean age of onset of familial melanoma is younger than that of sporadic melanoma and approximately 12% of patients with familial melanoma have multiple primaries. It is accepted that most familial melanomas are hereditary, although the mode of genetic transmission may be heterogeneous amongst families (Greene *et al.*, 1983, Fountain *et al.,* 1990).

For multiple melanomas diagnosed sequentially, mean tumour thickness from the first to the second melanoma decreased significantly (Carey *et al.*, 1994, Kang *et al.*, 1994). Melanoma is rare in childhood and it is not known whether the genetic and the environmental factors incriminated in the development of malignant melanoma in adults play a role in childhood melanomas in childhood (Mehregan *et al.*, 1993, Cellabos *et al.*, 1995).

The original strategy used to circumvent difficulties in identifying those who harbour the genetic defect exploited a proposed melanoma precursor, the dysplastic naevus, or atypical mole, as the phenotypic marker from which the presence of the of the

melanoma-associated genotype was inferred. In part because the criteria for the dysplastic naevus have neither been well defined nor generally agreed upon, multiple independent attempts to confirm the assignment of a gene to that location have failed. (Fountain *et al.,* 1990, Piepkorn *et al.,* 1994). It has been proposed that the FAMM has an autosomal dominant mode of inheritance (Lynch *et al.,* 1983).

The goal of genetic analysis of malignant melanoma is to identify genes involved in the transformation of melanocytes and melanoma tumour progression.

These studies provide strong evidence that genes on chromosomes 1,6,7 and 9 are involved in the aetiology of human melanoma (Sober & Burstein, 1995).

1.12 Association with other Malignancies

The incidence of a second, noncutaneous invasive cancer in patients with malignant melanoma varies from 1.5% to 20% (Guttman *et al.,* 1991).

The most common second cancer is breast cancer in a large proportion of studies (Bellet *et al.,* 1977, Koh *et al.,* 1987, Guttman *et al.,* 1991) the same is true for the association with the dysplastic naevus syndrome (DNS) (Lynch *et al.,* 1981, 1983).

Two cases of malignant melanoma associated with neurofibromatosis in two first-degree female relatives from a family with the dysplastic naevus syndrome have been documented (Stokel *et al.,* 1993). Three close relatives of an individual with the FAMM syndrome were discovered to have pancreatic carcinoma in Omaha, the link is postulated via an as yet unidentified endocrine mechanism (Lynch *et al.,* 1984).

In 207 consecutive patients with eye melanoma referred to Moorfields Hospital in London, five patients were seen who had primary melanomas of both the eye and the skin. In three of these patients, the cutaneous melanomas were discovered only as a result of their study. The number of cutaneous melanomas expected in this cohort of patients with eye melanomas was no more than 0.4 on the basis of the United Kingdom incidence of both tumours (Tobal *et al.,* 1993, Bataille *et al.,* 1993). This difference was highly significant and confirms previous reports of a proposed link between both tumours (Lynch *et al.,* 1981, 1984). Three of the five patients also had the "atypical

mole syndrome phenotype" (Newton *et al.,* 1993), suggesting that the atypical mole syndrome predisposes to both types of melanoma. Reports of associations between breast cancer, carcinoma of the lung and larynx and tumours of the gastrointestinal tract have also been published (Lynch *et al.*, 1984).

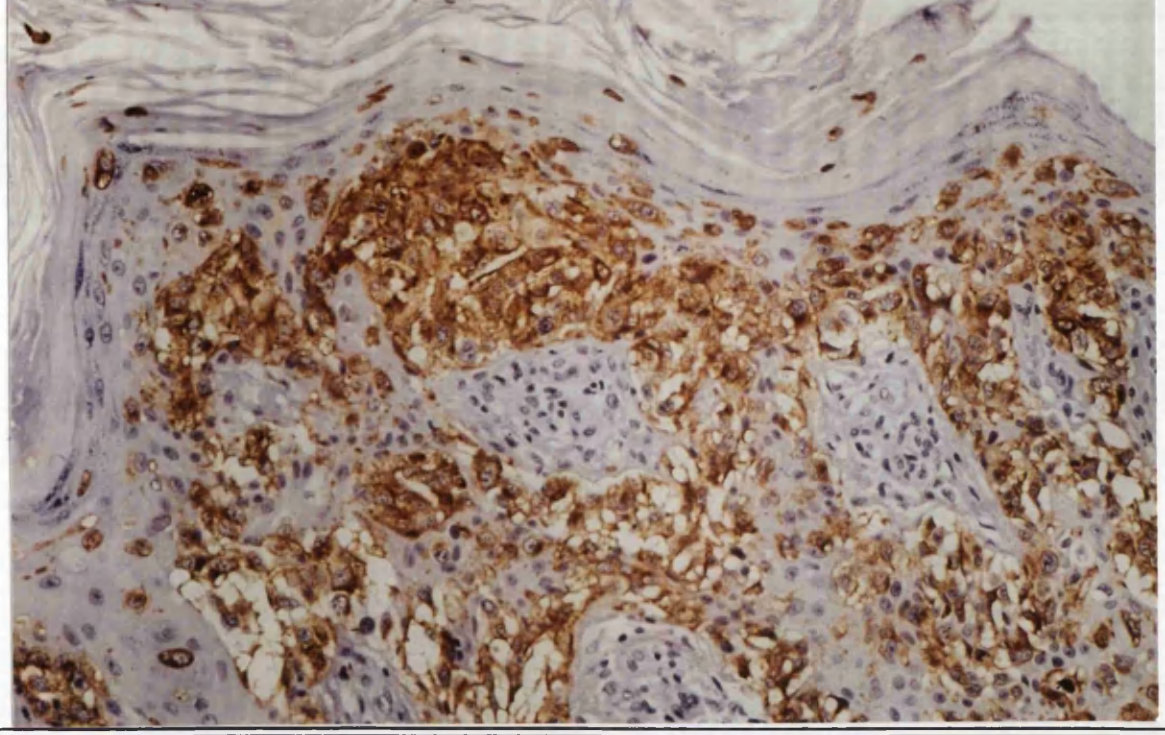

Fig. 2.2 HMB-45 positive Malignant Melanoma. The anti melanoma antibody clearly stains the melanocytes (brown.) There is infiltration of the melanocytes into the epidermis.

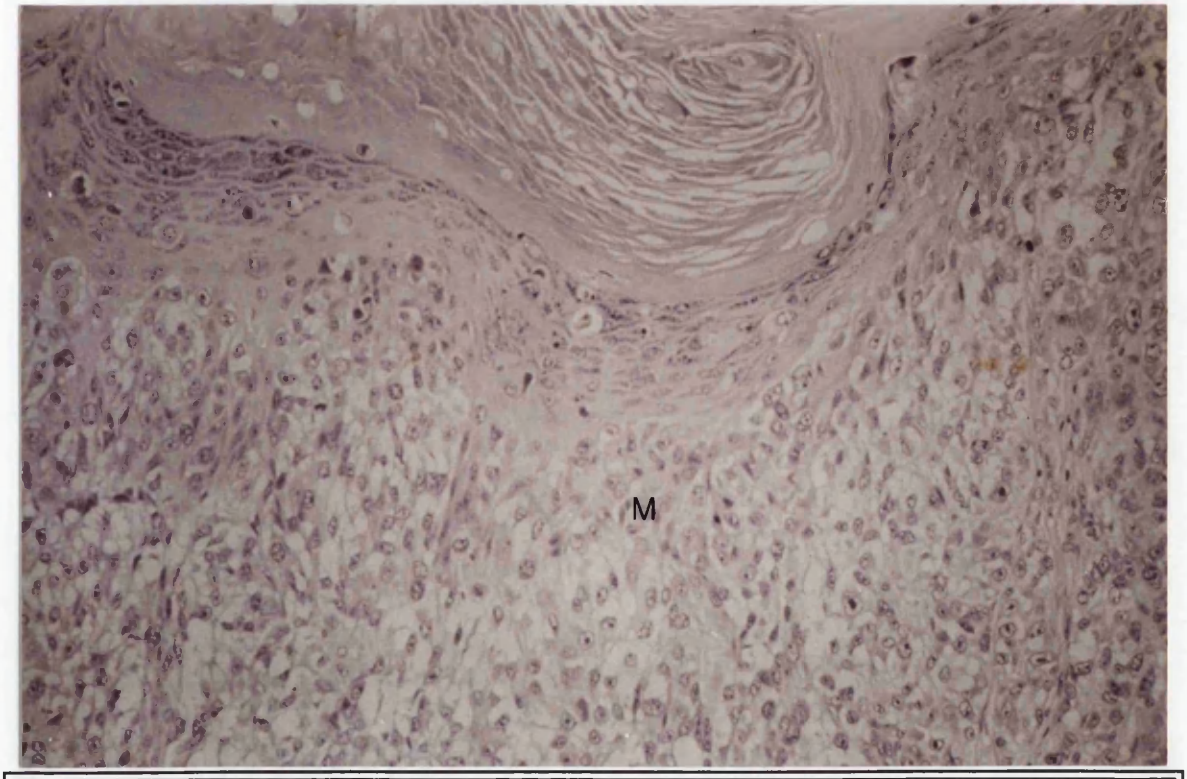

Fig. 2.3 Negative control. This section did not have the primary antibody HMB-45 applied. Nests of melanocytes and single melanoma cells within the epidermis.

Chapter 2

Dysplastic Naevus Syndrome

2.1 Definition

The dysplastic naevus syndrome defines an individual with multiple moles, usually between ten to a 100, occasionally less. The moles are classically located in what has been termed horse-collar area of the trunk, on sun-shielded skin and on other areas not normally affected by banal naevi, such as the feet, scalp, buttocks and the vulval area. The clinical appearance of these moles is classical - they vary in size between 5 millimetres and 2 centimetres, are of irregular outline, variegated in colour, and they appear macular but frequently have a palpable component. Occasionally, they show a degree of surrounding inflammation (Greene *et al.*, 1980, 1985, Slade *et al.*, 1995). Histologically, they are identified by the presence of architectural atypia (lentiginous melanocytic hyperplasia, bridging of junctional nests and focal elongation of epidermal rete ridges) and also cytological atypia of naevus cells. There are two recognised forms of the syndrome, the familial, which is believed to have an autosomal dominant mode of inheritance and the sporadic (Lynch *et al.*, 1984) The histological features have spawned much controversy and at a recent consensus conference (NIH, 1992) it was agreed to substitute the term Dysplastic naevus syndrome (DNS) with the Atypical mole syndrome (AMS) - thus shifting the onus from a histological diagnosis to a clinical diagnosis. The dysplastic naevus is important because of persuasive epidemiological evidence that they are markers of increased melanoma risk. The term dysplastic naevus is retained through much of this thesis as it is still widely used in recent literature despite the consensus conference.

2.2 Historical Perspectives

According to Clark..... "the first description of familial cutaneous malignant melanoma (CMM) was in 1820 by Sir William Norris, who reported an affected father and son. One hundred and thirty years later, E.P. Crawley reported CMM in three members of a single family". In 1978, the clinical and histological features of a melanocytic precursor to CMM, were described in seven families. The disorder was designated the B-K mole syndrome (named after two young patients, members of the

first two kindred's studied, who together contributed seven melanomas to that study) (Clark *et al.*, 1978, Reimer *et al.*, 1978). Affected individuals had morphologically atypical moles that were larger and more irregular in outline and more numerous than common acquired naevi, variably pigmented, and tended to occur on sun-shielded skin (e.g. the scalp and bathing trunk area), unusual sites for ordinary moles (Greene *et al.*, 1983, 1985). Histologically, these lesions were characterized by nuclear atypia and a disorderly growth pattern of melanocytes, leading to their designation as "dysplastic naevi" (DN). They have subsequently been described at various times as large atypical mole syndrome, familial atypical mole and melanoma syndrome, "classic atypical mole syndrome" (CAMS), Clark's naevus syndrome (Slade *et al.*, 1995). The term Dysplastic naevus syndrome was first suggested by Greene, to unify the terminology, but unfortunately it failed (Greene *et al.*, 1980). Most of these terms are gradually slipping out of common parlance and despite the consensus conference *vide supra* DNS is still widely used.

2.3 Prevalence

The highest single risk factor identified for the development of malignant melanoma is the combination of a positive family history of malignant melanoma in a first degree relative, combined the presence of dysplastic naevi in the *familial* DNS - such patients have a 500-fold increase in melanoma risk (Carey *et al.*, 1994, Kang *et al.*, 1994, Halpern *et al.*, 1994, Marghoob *et al.*, 1994, Schneider *et al.*, 1994, Sober & Burstein, 1995). It is estimated that individuals - with this syndrome make up about 5% of the total MM incidence (Greene *et al.*, 1984, Crutcher *et al.*, 1986, Mackie *et al.*, 1989). The prevalence of DN ranges from 5% to 17%, in populations from Australia, the United States and Scotland respectively. In Sweden 18% have clinical DN but as many as 22% of the population have 100, or more common acquired naevi (Augusston *et al.*, 1991). The Swedes had an average of 67 moles per person and the DN groups had more common naevi and more sun sensitive skin (Augusston *et al.*, 1991). It is estimated that most adults have between 15-20 moles (Browse, *et al.*, 1992). Further-

more, dysplastic naevi have been described both clinically and histologically in 25% to 35% of unselected patients with melanoma (Elder *et al.*, 1982, Rhodes *et al.*, 1983). Dysplastic naevi have been associated with invasive melanoma in normal and immunosuppressed individuals, regardless of a family history of melanoma (Greene *et al.*, 1981, Baird *et al.*, 1992) but others have suggested that it is actually a chromosomal instability disorder (Caporaso *et al.*, 1987).

Classifications of individuals with DN and their melanoma risk, based on personal history of melanoma and family of DN, have been devised (Kraemer *et al.*, 1983, Rigel *et al.*, 1989).

PATIENT		PERSONAL HISTORY OF MM	FAMILY HISTORY OF MULTIPLE NAEVI	FAMILY HISTORY OF MM
A	MULTIPLE DYSPLASTIC NAEVI	NO	NO	NO
B	MULTIPLE DYSPLASTIC NAEVI	NO	YES	NO
C	MULTIPLE DYSPLASTIC NAEVI	YES	NO	NO
D*	MULTIPLE DYSPLASTIC NAEVI	YES	YES	YES

TABLE 2.1. CLASSIFICATION OF DYSPLASTIC NAEVUS PATIENTS Kraemer *et al.*, 1983. The number 1, 2, 3, etc., after D denotes the number of affected relatives.

Thus, they proposed "DNS kindreds" namely, non familial (sporadic) and familial DNS with, or without melanomas - labelled A to D in order of presumed increasing melanoma risk. Type A comprises non-familial and type B comprises familial (i.e. with more than one family member having dysplastic naevi) DNS kindred's in which neither the patient, nor any blood relatives have a MM. Type C is a kindred with only one individual who has both DN and CMM and no other blood relatives with dysplastic naevi, or MM. Type D is familial DNS (with at least two family members having DN with melanoma) is further divided into D-1 (with only one family member having melanoma plus dysplastic naevi) and D-2 (with at least two blood relatives having melanoma plus DN, synonymous with the B-K Mole syndrome). The importance of this table is that it attempts to put the relative risk of MM in each group into perspective.

It is accepted that whilst such a table will be unable to predict accurately the exact risk of individuals groups (because of the influence of other risk factors such as common naevus count, sun burn episodes, hormones etc.), it helps decide which individuals are at low risk, and who therefore can be followed up less frequently.

Newton at the Royal London Hospital devised an AMS scoring system based on three characteristics (Newton *et al.,* 1993) (Table 2.2).

CHARACTERISTIC	SCORE
TWO OR MORE CLINICALLY ATYPICAL NAEVI	1
MORE THAN 100 NAEVI >2 MM OR >50 NAEVI IF <20 YR OR > 50 YR	1
NAEVI ABNORMALLY DISTRIBUTED (I.E.≥1 ON BUTTOCKS OR DORSUM FOOT) (>2)	1
NAEVI ON ANTERIOR SCALP	1
IRIS PIGMENTED LESIONS	1

Table2.2. AMS - A scoring system. (Newton *et al.* 1993).

A score of 3 was chosen as the AMS phenotype, because it was found in the family cancer clinic at the Royal London Hospital to occur frequently in patients with melanoma but rarely in the general population. They identified 15% of individuals with their "London" AMS phenotype and 2.1% in their controls, this suggests that a substantial percentage of patients with MM in the UK have the AMS phenotype. They found that more men than women had the AMS phenotype. They, however, failed to show any difference in Breslow thickness between the AMS and non AMS groups, this is in marked distinction from the large studies from North America (Newton *et al.* 1993, Carey *et al.*, 1994, Kang *et al.*, 1994). This last observation it is suggested may be due to different tumour biology, poor patient compliance, or a higher biopsy threshold in the UK (Newton *et al.* 1993).

Other prospective studies are difficult to compare because different statistical methods are applied to the data, and the definition of the DNS phenotype although similar is not the same. They all, however, present unequivocal evidence that DNS leads to MM at an earlier age (Carey *et al.*, 1994, Kang *et al.*, 1994, Halpern *et al.*, 1994, Marghoob *et al.*, 1994, Schneider *et al.*, 1994).

2.4 Histological Diagnosis

Melanocytes are unique in that they are capable of pigment synthesis. Their proliferative lesions, therefore, are readily apparent. Pigment synthesis and the epidermal location of much of the melanocytic system have permitted observers to record detailed and comprehensive features of the evident steps of tumour progression as it affects melanocytes. Each step in both the precursor lesions and in established melanomas, is characterized by the acquisition of properties not manifested in the preceding lesions. The common acquired melanocytic naevus is the only proliferation of melanocytes that maybe found in histological association with the entire spectrum of melanocytic neoplasia (Baird *et al.*, 1992, Sober & Burstein, 1995). The problem however, is predicting which common acquired naevus will "change", this represents the importance of the DN for either in the familial setting, or as a sporadic DN it heightens clinical awareness as to the increased risks of MM.

In the words of Clark ".....the common acquired melanocytic naevus is viewed as a focal proliferation of melanocytes, destined in most instances to follow a programmed pathway of differentiation that leads to disappearance of the naevus. If the pathway of differentiation is not followed, characteristic lesions result, and such lesions are regarded as the formal histogenic precursors of malignant melanoma. Such a developmental flaw is termed aberrant differentiation, and the resultant precursor lesions designated melanocytic dysplasia. The vast majority of melanocytic naevi showing melanocytic dysplasia are terminal lesions that do not progress to melanoma.

If melanoma is to develop via a precursor lesion, however, the naevus with melanocytic dysplasia is that precursor. When melanomas do develop they develop focally within the precursor (Clark *et al.*, 1984)."

A WHO consensus to resolve matters established two major criteria and four minor criteria for the diagnosis of DNS.

The **Major** criteria are,

(1) basilar proliferation of atypical melanocytes (extending at least three rete ridges or

"pegs" beyond any dermal naevo-cellular component) and

(2) organization of this proliferation in a lentiginous or epitheloid-cell pattern.

Minor criteria are

(1) the presence of lamellar fibrosis, or concentric eosinophilic fibrosis,

(2) neovascularization, (3) inflammatory response and

(4) fusion of rete ridges.

The diagnosis requires presence of both major criteria and at least two minor criteria.

2.5 Clinical Features

The clinical features are listed below in table 3.3.

BETWEEN 10 - 100 MOLES, OCCASIONALLY LESS
HETEROGENEOUS - NEIGHBOURING NAEVI DIFFER FROM EACH OTHER
MULTI-COLOURED
SHOULDERING - PERIPHERAL MACULAR TAN ZONE
SIZE BETWEEN 0.5 CM - 2.0 CM
ELEVATION - MINIMAL IN RELATION TO SIZE

TABLE 2.3 CLINICAL FEATURES OF DYSPLASTIC NAEVI

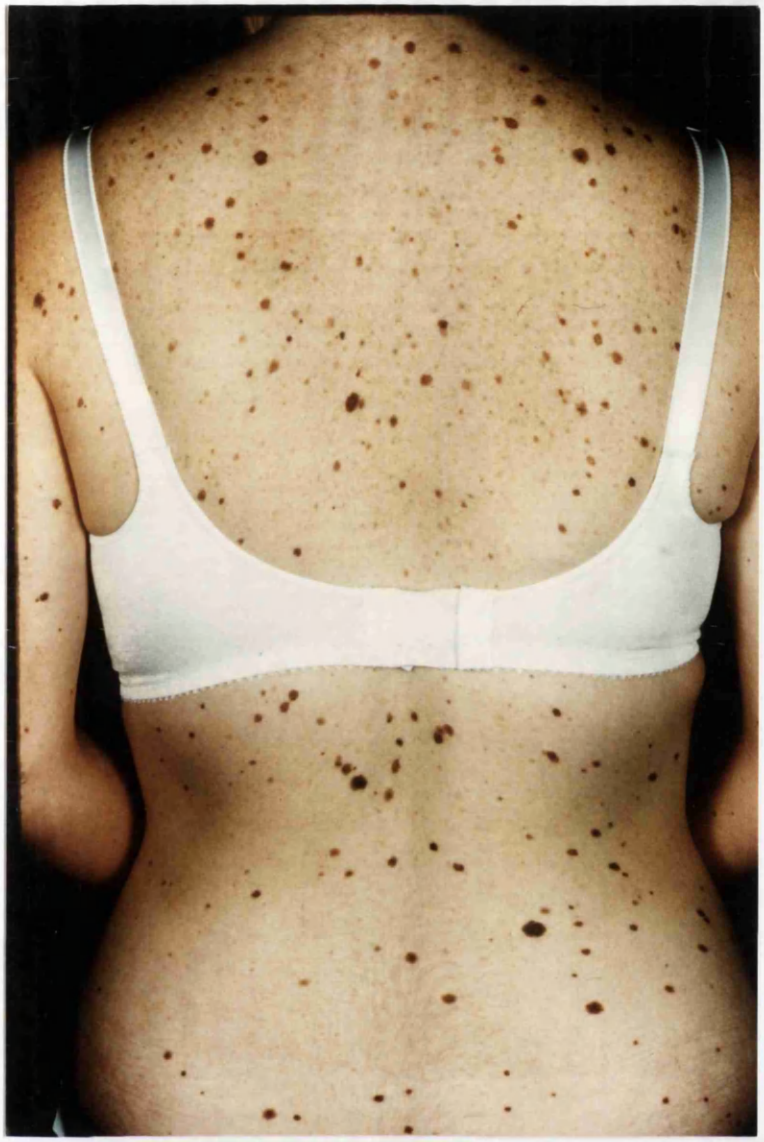

FIG. 2.4 THE DYSPLASTIC NAEVUS SYNDROME DEFINES AN INDIVIDUAL WITH MULTIPLE MOLES, USUALLY BETWEEN TEN TO A 100, OCCASIONALLY LESS. THE MOLES ARE CLASSICALLY LOCATED IN WHAT HAS BEEN TERMED HORSECOLLAR AREA OF THE TRUNK, ON SUNSHIELDED SKIN AND ON OTHER AREAS NOT NORMALLY AFFECTED BY BANAL NAEVI, SUCH AS THE FEET, SCALP, BUTTOCKS AND THE VULVA AREA. THE CLINICAL APPEARANCE OF THESE MOLES IS CHARACTERISTIC THEY VARY IN SIZE BETWEEN 5 MILLIMETRES AND 2 CENTIMETRES, ARE OF IRREGULAR OUTLINE, VARIEGATED IN COLOUR AND THEY APPEAR MACULAR BUT FREQUENTLY HAVE A PALPABLE COMPONENT. OCCASIONALLY, THEY SHOW A DEGREE OF SURROUNDING INFLAMMATION.

HISTOLOGICALLY, THEY ARE IDENTIFIED BY THE PRESENCE OF ARCHITECTURAL ATYPIA, LENTIGINOUS MELANOCYTIC HYPERPLASIA, BRIDGING OF JUNCTIONAL NESTS, FOCAL ELONGATION OF EPIDERMAL RETE RIDGES AND ALSO CYTOLOGICAL ATYPIA OF NAEVUS CELLS. THERE ARE TWO RECOGNISED FORMS OF THE SYNDROME, THE FAMILIAL WHICH IS BELIEVED TO HAVE AN AUTOSOMAL DOMINANT MODE OF INHERITANCE AND THE SPORADIC. THE HISTOLOGICAL FEATURES HAVE SPAWNED MUCH CONTROVERSY AND AT A RECENT CONSENSUS CONFERENCE IT WAS AGREED TO SUBSTITUTE THE TERM DYSPLASTIC NAEVUS SYNDROME (DNS) WITH THE TERM ATYPICAL MOLE SYNDROME (AMS), THUS SHIFTING THE ONUS FROM A HISTOLOGICAL, TO A CLINICAL DIAGNOSIS.

Chapter 3

Applied Anatomy & Molecular Biology of the Skin & Malignant Melanomas

3.1 The skin, p53 & Malignant Melanomas

Individual organ systems undergo renewal via various mechanisms. According to RJ Last.... "the skin consists of specialized elements which over time renew the outer covering epithelium replacing it with a stratified squamous keratinized layer of varying thickness". The thickness varies depending on its location. The soles and palms possess the thickest coverings. Most of the activity occurs at the *stratum basale* which rests on a thick basement membrane. The cells of the *stratum basale* are highly proliferative, undergoing repeated mitosis. This division results in the production of a proliferative stem cell layer that gives rise to the next generation of cells as well as cells that differentiate into keratinocytes.

The layering seen in the epidermis represents the progressive accumulation of keratin within the progeny of the proliferative cells of the *stratum basale*. The appearance of the skin is dependent on multiple factors including nutritional status, age, race, degree of oil and sebum production, lifestyle and not least sun-exposure. Childhood sun - exposure has been shown to increase the number of naevi which appear and the phenotypic expression of the dysplastic naevus syndrome and therefore, indirectly skin tumour formation (Kopf *et al.,* 1985, Barnhill *et al.,* 1990). This phenotypic expression and histological atypia can also be modified by topical agents, specifically topical retinoic acid can reduce, or in fact cause the disappearance of the DN (Halpern *et al.,* 1994). The rate of appearance of naevi is highest below the age of 30 years. Halos have been observed around the naevi before they disappear (Clark *et al.,* 1975, 1978, 1984, Barnhill *et al.,* 1990).

Melanocytes are derived from the neural crest and hence ectoderm. They rest on the apical side of the basement membrane, between cells of the *stratum basale* and they produce long processes that are pushed around and between the basal cells of the epidermis. They synthesize melanin in membrane bound melanin granules, which are then passed into the cells of the *stratum basale,* presumably by exocytosis from the melanocyte, followed by phagocytosis, by keratinocytes. Melanocytes have an enzyme

called *tyrosinase,* which catalyses one step in the reaction that converts tyrosine into melanin. Moles (the common naevocellular naevus) so called because of the granular melanin pigment within them are dynamic regions within the skin.

According to Clark... "the common naevocellular naevus tend to become less *atypical* over time. Two phenomena have been put forward to explain this - cohort effect and disappearance of naevi over time" (Clark *et al.,* 1984).

The cohort effect denotes increasing naevus counts in successive birth cohorts leading to a higher naevus count in younger persons. The postulated mechanisms of disappearance of naevi is by (a) a *senescence-differentiation* pathway that includes evolution into flesh coloured dermal papules and subsequent regression with a histology of "**Schwannian**" differentiation and (b) by immunologic mechanisms. This is of interest for an immunologic mechanism was postulated as a cause for the dysplastic naevus syndrome when it was first described in 1978 (Clark *et al.,* 1975, 1978). A higher than expected number of DN have been found in both renal transplant patients and children on maintenance chemotherapy, up to three years after maintenance chemotherapy for haematological malignancies (the median increase being 66 naevi per child) (Greene *et al.,* 1981, Baird *et al.,* 1992). Sun exposure, or particularly exposure to UV-radiation induces clinically detectable changes in the appearance of skin - this is due to a DOPA like reaction which results in the skin becoming darker. Removal of the exposure over time results in a loss of pigmentation. According to Mackie .... "pigmentation is protective and dark races tend to have a lower incidence of skin tumours" (Mackie, 1992, 1998).

It is of interest that the proliferative index of the normal epithelium has been estimated to be in the order of 3 - 18.5 % depending on the techniques employed. It is therefore not surprising that in common with other continuously renewed systems the skin is prone to the development of cell cycle anomalies and therefore, tumours. In such systems the control of the cell cycle via its various checkpoints is important in limiting tumour formation. The natural history of the common naevus has been well document-

ed (Clark *et al.,* 1984, Barnhill *et al.,* 1992, Baird *et al.,* 1992). It develops from a small 2 millimetre macule and may end up as a malignant melanoma, or indeed overtime may disappear by a process which has been described as *"senescence-differentiation* pathway". The fact that up to half of all melanomas arise within a pre-existing naevus suggests that these are islands of unstable, or potentially active epithelium and mnemonics have been developed which help us to describe the possible symptoms of change. The classical prognostic factors (Clark's level and Breslow's thickness measurement) both indirectly measure the degree of growth at the time of clinical diagnosis and the smaller either value, the better the prognosis. Thus, simply halting abnormal growth early improves prognosis. This translates in clinical terms into "earlier diagnosis improves prognosis". This is generally true apart from a subset of thin melanomas that have a poor prognosis (Graham *et al.,* 1994). In addition, the location of the individual tumour is thought to have a bearing on prognosis and the so-called "BANS" area is still considered a poor prognosis group (Rosin, 1991) although prospective studies have disproved this (Ball, 1995). Exposure to ultraviolet light has long been associated with an increased risk for the development of skin cancer and it has now been shown that exposure of the normal epidermis to doses of radiation that produce mild sunburn rapidly results in the appearance of the p53 protein, both in the epidermis and the superficial dermal fibroblasts (Hall *et al.,* 1993, Service *et al.,* 1994). It is believed that it is the slow repair of the induced DNA damage that results in skin tumour formation (Service *et al.,* 1994). This has been supported by the reports of p53 expression in both Bowen's disease and frank squamous cell carcinoma. (Hughes *et al.,* 1995). Immunohistochemistry of archival paraffin embedded tissue enables us to produce snap-shots of tissue sections at the time of biopsy. Recent studies on the role of the p53 gene in human melanoma have largely been contradictory. The results of immunohistochemical staining vary from the complete absence of staining to very high levels (Mcgregor *et al.,* 1993, Cristofolioni *et al.,* 1993, Lassam *et al.,* 1993, Albino *et al.,* 1994). Some have found a correlation with disease free interval while others

positivity only in lesions over 1.5 mm in thickness (Florenes *et al.,* 1994).

The general consensus is difficult to define, but appears to be that p53 aberrations may not be helpful in predicting prognosis. p53 anomalies may be present only at a late stage in the great majority, or possibly involved in only a specific subset of melanomas (Albino *et al.,* 1992). It is obvious that in view of the wide variety of results, positivity needs to be interpreted with caution. Point mutations have been found in 0 - 25% of cultured melanomas (Albino *et al.,* 1994, Lubbe *et al.,* 1994) and 0% in 34 fresh melanoma biopsies (Albino *et al.,* 1994, Montano *et al.,* 1994). p53 overexpression was found in 5 - 92% of paraffin-embedded melanoma specimens (Mcgregor *et al.,* 1993, Cristofolioni *et al.,* 1993, Lassam *et al.,* 1993, Albino *et al.,* 1994). Up to 15% of benign lesions show p53 immunoreactive nuclei (Mcgregor *et al.,* 1993, Cristofolioni *et al.,* 1993, Lassam *et al.,* 1993, Albino *et al.,* 1994). The low frequency of p53 point mutations and variable frequency of p53 expression suggests that derangement of the p53 gene by point mutations is not a common perturbation in the majority of melanoma cells and that overexpression of p53 in this tumour type is due to a mechanism other than point mutation (Albino *et al.,* 1994).

p-53 positivity in naevi and in the normal epidermis may be related to mechanisms of DNA repair, apoptosis, or to a specific phase of the cell cycle.

Population-based differences, or differences in case selection and sample handling may account for the above widely differing results. The demonstration of p53 positivity in benign skin lesions greatly hinders the possibility of a diagnostic use of p53 immunostaining in dermatopathology (Cristofolioni *et al.,* 1993). A significant correlation has been found between strong p53 immunoreactivity and malignant melanomas associated with a poor prognosis (Mcgregor *et al.,* 1993) and the highest proportion of reactive cells was observed in metastatic melanomas (Lassam *et al.,* 1993, Yamamoto *et al.,* 1993, Strech *et al.,* 1994). p53 overexpression has been found to be localized to the nodular portion of the same melanoma (Lassam *et al.,* 1993, Hashimoto *et al.,* 1993).

In choroidal melanomas the story is less clear with p53 expression present in the malignant but not the benign form (Tobal *et al.,* 1992). These observations suggest that acquisition of abnormalities of the p53 gene may be an important step in the development of choroidal malignant melanoma.

p53 expression shows a correlation with the marker of proliferation MIB-1 in both forms of melanoma (Moy *et al.,* 1995, Gelsleischter *et al.,* 1995) suggesting a similar biological behaviour to breast carcinoma.

3.2 Implications of Bcl-2 Positivity in the Skin and Malignant Melanomas

In normal skin it has been shown that the distribution of the Bcl-2 protein is related to "active" regions of the skin i.e. the basal keratinocytes and the melanocytes (Rodriguez-Villanueva *et al.,* 1995, Plittenberg *et al.,* 1995, Norris, 1995). Others have suggested that melanocytes are the only cells that constitutively express the protein (Plittenberg *et al.,* 1995, Tron *et al.,* 1995). The expression of Bcl-2 in melanomas may therefore simply be a reflection of the protein in melanocytes and not related to malignancy (Morales-Ducret *et al.,* 1995) on the other hand it may confer an increased resistance to malignancy (Cerroni *et al.,* 1995, Morales-Ducret *et al.,* 1995) and the loss of expression may be related to naevus resolution (Norris, 1995). Ironically, expression may prove to be a vehicle for *antisense* therapy (Cerroni *et al.,* 1995).

High levels of expression of the Bcl-2 protein have been reported up to 100% of melanocytic naevi in and 65 - 93 % of malignant melanomas (Ramsay *et al.,* 1995, Morales-Ducret *et al.,* 1995, Cerroni *et al.,* 1995). This expression disappears with thicker melanomas and metastatic lesions (Saenz-Santamaria *et al.,* 1994, Ramsay *et al.,* 1995). The results indicate that Bcl-2 protein expression may be a common finding in cutaneous melanocytic lesions regardless of their biologic behaviour, but does not correlate with the site of histologic localisation of stem cell compartments (Cerroni *et al.,* 1995, Rodriguez-Villanueva *et al.,* 1995).

Expression of the protein in the great majority of MM seems to exclude a prognostic

significance of Bcl-2 in MM of the skin (Cerroni *et al.,* 1995). However, others report decreased expression with increasing malignancy, but when expressed in lymph node metastasis may be associated with shorter survival (Tron *et al.,* 1995, Grover *et al.,* 1996).

There is a correlation between Bcl-2 expression and the marker of proliferation, MIB-1, in both uveal (Mooy *et al.,* 1995) and cutaneous melanomas (Gelsleischter *et al.,* 1995).

The control of apoptosis is complex, but it is logical to presume that all the cells that overexpress the Bcl-2 protein are capable of undergoing a transformation from clonicity to oncogenicity.

Ultra-violet radiation has long been implicated in the pathogenesis of melanomas and now it has been shown that this form of radiation induces IL-10 production in melanocytes (Plittenberg *et al.,* 1995). IL-10, in turn induces the expression of Bcl-2 lending itself to a possible mechanism of malignant change. It has been shown *in-vitro* that melanocytes generate free radicals under a variety of conditions including exposure to UV-radiation. Studies in Bcl-2 deficient transgenic mice have shown that Bcl-2 is important in melanocyte function and survival (Vies *et al.,* 1993). The expression of Bcl-2 has been suggested as a mechanism which these melanocytes survive under these conditions (Plittenberg *et al.,* 1995).

It is clear that apoptosis is part of the normal program of keratinocyte differentiation in the epidermis and cycling hair follicles (both in the matrix and root sheath) (Norris, 1995). However, in the cycling follicle the control of apoptosis is related to transforming growth factor-ß and not p53 activation which controls other pathways which are related to UV-radiation. There is also evidence that naevi that are undergoing neurotization fail to express the protein (Norris, 1995).

In common with breast cancer more advanced melanomas tend not to express the protein (Ramsay *et al.,* 1995, Tron *et al.,* 1995), ironically, there is an association with poor prognosis in metastatic melanomas (Grover *et al.,* 1995). Loss of expression with

increasing malignancy maybe an epiphenomenon (Ramsay *et al.,* 1995), or reflects the activity of another as yet unidentified form of Bcl-2. It has been suggested that cells with a lower proliferative rate that express Bcl-2 may accumulate genetic alterations at a slower rate than those with higher growth fractions (Ramsay *et al.,* 1995). Furthermore, in common in many non-haemopoetic malignancies overexpression due to the 14:18 translocation has not been detected (Norris, 1995, Morales-Ducret *et al.,* 1995). There is an inverse relationship between p53 expression and Bcl-2 expression in metastatic melanoma and it is believed that in the case of p53 aberrations found late in the disease these tumours were less likely to express the Bcl-2 protein (Tron *et al.,* 1995).

Section III

Methods, Results & Discussion

Chapter 1 Materials and Methods

1.1 Patient selection

Patient names were obtained from the computerized pathology records of the department of pathology at the Royal Free Hospital, London. The SNOMED™ computer programme allowed easy retrieval of records from 1978 to date. All patients had been treated in the Academic Department of Surgery in the Hospital. Three main groups of patients were chosen for the study - patients with benign breast disease, malignant breast disease and malignant melanomas.

1.2 Benign Breast Disease

The benign group (n = 46) were subdivided into two groups - fibrocystic disease (n = 30) and atypical ductal hyperplasias (n = 16). The fibrocystic group was chosen to represent a series of breast tumours with the least risk of breast cancer in the long term. They had their tumours excised between 1985 and 1987 and since then had no evidence of malignancy either of epithelial, or connective tissue i.e. neither the breast, nor other system. This fact was confirmed by reviewing all pathology records and medical notes on these patients. The patients with atypical ductal hyperplasia represent a group with an uncommon group of lesions (up to 3 % of all biopsies) which although benign, confer an increased risk (RR = 5.5) of breast carcinoma in both the ipsilateral and contralateral breasts (RR = 8 with a family history of breast carcinoma).

1.3 Malignant Breast Disease

There were two groups of patients with malignant breast disease

Group A - patients with good prognosis breast cancer i.e. T1N0 tumours. They were low grade tumours (grade I and II Elston & Ellis tumour grading system), less than 2 cm in diameter, with no histological positive lymph-nodes. This group of patients had their tumours excised after 1991, which coincided with the introduction of both the comprehensive history sheets for all patients in the breast clinic and the commencement of the computerized histology reports (n = 38). Eight of these patients had areas of atypical ductal hyperplasia in the same section and one had paget's disease of the nipple (Fig.s 8.2 & 9.2).

Group B - patients with poor prognosis breast cancer. This group all had grade III tumours and had been seen, treated and collected in a database by one surgeon SP (n = 55).

1.3.1 Breast Tumours in patients with Malignant Melanomas or the DNS

As a continuation of the work initially performed in this department and to explore further the aberrations of the p53 protein we examined the same markers in an unrelated group of patients with malignant breast tumours and either malignant melanomas, or the dysplastic naevus syndrome (a.k.a. atypical mole syndrome). This group of 22 included 20 patients with pure invasive ductal carcinoma (IDC), one patient with lobular carcinoma and one sarcoma of the breast. Only seventeen tumours were studied, 5 tumours had previously been shown by other workers in our department (Crow *et al.,* 1994 unpublished) to overexpress the p53 protein- these were unavailable for further study.

This subgroup had all been treated for breast cancer in the academic department of surgery and nine of them had malignant melanomas either preceding, or following their breast tumours. Three patients had their melanomas treated elsewhere, including one who had an enucleation at Moorfield's Eye hospital in London for a conjunctival melanoma. In these three patients, archival material on the tumours was obtained from the respective departments of pathology, namely the department of pathology at Roehampton university hospital, Institute of Ophthalmology (Moorfield's' hospital) and the St. Stephen's Hospital for skin diseases. The patients with the dysplastic naevus syndrome were jointly managed by the clinical geneticist at the Royal Free Hospital School of Medicine, London, Dr. Kay McDermott. She kindly supplied the picture of the patient with the dysplastic naevus syndrome on page 143. Incidentally, this patients' sister had breast cancer but not the full phenotypic manifestations of DNS.

1.4 Malignant Melanomas

This group consisted of 75 patients (55 female and 20 male) seen and treated for malignant melanoma at the Royal Free Hospital, London. Nine patients had breast

cancer as a second tumour. Sixty-six formed a control group for the melanomas in the patients with breast cancer and were randomly selected from an alphabetical list of 162 melanomas (F:M ratio was 2:1), generated by the computer of melanomas seen between 1986 and 1995. Only patients who had excision biopsies were included in the study. These patients had no evidence of either breast cancer, or benign breast disease at the time of diagnosis. Data on the site, Breslow's thickness, Clark's level of invasion and the presence of other tumours was collected from the pathology report and the clinical notes.

1.5 Clinical Details

All patients with malignant breast tumours (except 10 in the poor prognosis grade III group) had primary excision followed by a wide excision and axillary clearance up to the posterior border of the pectoralis minor muscle (level II clearance). Patients with tumour cells histologically evident in any node were classified as lymph node positive. The size of the tumour was measured as the maximum dimension of the tumour in the bisected specimen. All patients had tumours less than 5 cm in diameter and none had evidence of distant metastases at the time of presentation. Post-operatively each patient received either radiotherapy, or chemotherapy, or both depending on TNM status and patient wishes. They were reviewed 2 weeks after surgery when treatment decisions were taken. Thereafter, reviews took place at 3 monthly intervals for 6 months, then 6 monthly for one year; then yearly. All patients were offered regular mammograms each year after diagnosis.

Data was obtained on for 5 year survival/disease recurrence in the medical records department. Baseline investigations at the time of diagnosis included a full blood count, urea and electrolytes, and liver function tests. A chest X-ray, bone scan and ultrasound scan of the liver were also performed. A detailed family history was taken via a standard *questionnaire* in patients seen after 1991. Metastatic disease was confirmed by clinical and radiological examination.

1.6 Immunoperoxidase Staining Methods *

The detection of an antigen-antibody reaction in tissue on a laboratory bench involves producing an easily detectable and reproducible colour coded positive reaction.

The most widely used method is the immunoperoxidase method. It involves the interaction of antibodies and the enzyme peroxidase. Peroxidase is used because it is inexpensive, easily obtainable in highly purified form (minimizing the risk of contamination) and is stable. Furthermore, only small amounts are present in tissue specimens (this endogenous peroxidase activity is easily "quenched") and there are widely available substances (chromagens) which produce a coloured precipitate at the site of the antigen-antibody reaction. There are four methods employed to detect this reaction - the direct method, indirect method, the PAP and the Avidin Biotin method. The last two methods are the most sensitive and were used throughout this thesis.

1.7 Substrate Solutions

In the immunoperoxidase staining method, the enzyme, horseradish peroxidase (HRP) forms a complex with hydrogen peroxide (the substrate) and then reacts with an electron donor (DAB) to produce a coloured (brown) end product. The HRP is then available to interact with more hydrogen peroxide and thus form more coloured molecules.

1.8 Chromagens

The chromagens, produce coloured end-products, as well as donating electrons to the enzyme (HRP) hydrogen peroxide reaction. In this study two chromagens were used

(a) 3, 3 diaminobenzidine tetrahydrochloride (DAB), this produces a brown colour that is insoluble in alcohol. The insolubility of the end-product implies that conventional mounting media can be used. The main disadvantage of DAB is that it is a potential carcinogen, therefore, all handling was carried out in a fume cupboard.

(b) 3 - amino - 9 - ethylcarbazole, or AEC, forms a red end product that is soluble in alcohol. This is a major disadvantage, not only because conventional mounting media cannot be used, but also because the specimens cannot be dehydrated by conventional

methods (which involve the use of alcohol), and must therefore be air dried. Furthermore, counterstaining with Mayer's haemalum, (which is blue) frequently results in loss of positive cells. and the red product fades with time. This method was used predominantly to stain melanomas to prevent confusion with the melanin pigment, which is brown.

1.9 Monoclonal Antibodies

Monoclonal antibodies against the different antigenic epitopes of the p53 molecule have been developed. Clones DO7 and DO-1 react with both wild and mutant p53, PAb 240 reacts preferentially with mutant proteins. The epitope map for the most common antibodies is shown in Fig 1.2.

Differences have been noted in the same patient groups for different antibodies (Callaghan *et al.,* 1992). It is important to point out that the antibodies that have been assessed against the mutant p53 have been shown to differentiate in immunoprecipitation assays, which depend on the mutant protein being in its native conformation (Harris, 1992).

There is evidence that different epitopes may be detected during the cell cycle, possibly relating to different functions of p53 (Milner *et al,* 1993).

1.10 Section Preparation

Paraffin blocks of representative tumours were obtained from the block store of the department of Histopathology in the Royal Free Hospital, London. Haematoxylin and Eosin sections of each tumour were reviewed by two pathologists (J.C. - consultant pathologist and J.R. senior registrar in pathology), both with a special interest in breast pathology, to confirm the histological diagnosis.

The blocks were placed on a freezing tray for ten minutes before sections were cut. Each block was "shaved" to align it with the angle of a *Reichart-Jung* microtome.

The microtome blade was cleaned with xylene before insertion onto the microtome to remove the grease and prevent curling of the section. Three micron (3μm) sections of each tumour block were then cut on a *Reichart-Jung* microtome. The sections were

floated out on industrial methylated spirit 740P to get rid of the folds in the individual sections, before being transferred to a water bath at 40 °C. This smoothed out any remaining folds in the section. The section was then picked up on 3-aminopropyltriethoxysilane (APES™) coated slides and immediately placed in a hot room at 37 °C for forty-eight hours before staining. The slides were coated with APES™ as follows - plain slides with frosted edges were placed in slide racks and degreased by soaking for 5 minutes in industrial detergent and then rinsed in running tap water. The slides were then soaked in double distilled water, at room temperature, obtained from a still, for 5 minutes, to remove any charged particles and finally, rinsed once in industrial alcohol 740P. They were then placed in a hot room, at a temperature of 37 °C, for 20 minutes to dry. A solution of fresh 3-Aminopropyltriethoxysilane (APES™ Sigma A3648) was then prepared. The preparation of 3-Aminopropyltriethoxysilane solution was carried out in a fume cupboard, with the protection of goggles and gloves in the department of histopathology. APES™ stored at - 4 °C, was removed from the refrigerator, where it was stored in a plastic bottle placed in a metal tin. Six millilitres of 3-Aminopropyltriethoxysilane was placed in a 400 ml glass staining tray in a fume cupboard, 284 mls of Acetone (BDH AnalaR) was added, and gently mixed to create a 3 % APES™ solution. The dried slides in racks (previously degreased) were then placed in the 3 % APES™ solution at room temperature, in the fume cupboard, for 3 minutes. They were then rinsed once in a separate 400 ml staining jar containing double distilled water, in the fume cupboard. The slides were then placed on absorbent paper towels, in the fume cupboard, for one minute, before transfer to a hot room at 37 °C for 48 hours. The staining jar which contained the APES™ solution was then emptied into designated bottles for storage of waste APES™, the staining jar was then rinsed with acetone, which was also poured into the waste container. The gloves and the absorbent towels were discarded into the colour coded yellow bins designated for clinical waste and marked for incineration.

After 48 hours in the hot room the sections were either stained, or stored at room

temperature in slide racks, protected from the dust. Twelve serial 3μm sections of each tumour were made, they were numbered sequentially, by one individual B.J.C. Immunohistochemical staining with a series of monoclonal antibodies followed.

1.11 Preparation of Normal Human Serum in Immunohistochemistry

Fifty millilitres of blood was taken from healthy volunteers and transferred to a glass tube(s) until half-full. The tubes were placed flat at 37 °C for 1 hour and then stored at 4 °C overnight to shrink the clot. They were then spun at 2000 revs per minute, for 20 minutes, and the supernatant eluted off. The eluent was heated at 56 °C for 1 hour to deactivate complement. The resulting fluid was filtered through a 0.2 mm filter (to ensure depletion of infectious agents) and 1 ml aliquots were stored at - 70 °C.

An immunohistochemical session, with positive and negative controls, using a polyclonal antibody in parallel with previously tested NHS is performed. If there is no background the new NHS may be used in all appropriate procedures.

1.12 Immunohistochemistry of p53 Avidin Biotin (ABC) method

Prior to commencing the immunohistochemical session all sections were de-paraffinised as described above. The sections were soaked in three serial baths of fresh xylene (mixture of isomers) EEC label EEC No:215-535-7 (CHEMIX), each soaking lasting for exactly five minutes. They were then soaked in three serial baths of fresh methanol (BDH AnalaR), each soaking lasting for two minutes. Following this they were rehydrated by soaking once in double distilled water (collected from a still at room temperature) for five minutes and then in phosphate buffered saline at a pH of 7.4 (made up as described above). Next, endogenous peroxidase activity was blocked by "quenching" in 3 % hydrogen peroxide for 20 minutes. The solution of 3 % hydrogen peroxide was made just before use. Twenty millilitres of 1M hydrogen peroxide (30 % proof BDH AnalaR) was placed a conical flask containing 180 millilitres of double distilled water at room temperature and stirred continuously with a magnetic stirrer for 2 minutes before use. The slides were soaked in the 3 % hydrogen peroxide solution for 20 minutes. The reaction is then terminated by rinsing in double distilled water for 5

minutes, followed by PBS at a pH of 7.4, for five minutes.

1.13 Antigen Retrieval Solution

The antigen retrieval method was based that described by Shi (Shi *et al.*, 1991). Fresh citric acid buffer (0.01 M) the standard antigen retrieval solution at a pH of 6.0 is made up each day. This consists of 2.1 g citric acid monohydrate [AnalarR BDH Merck 10081] dissolved in 1.0 litre of double distilled water, at room temperature, in a Pyrex™ beaker, to which is added 13 millilitres of 2 M sodium hydroxide [AnalarR Volumetric solution]. The pH of the solution is brought up to 6.0, at room temperature, with buffer components.

Two-hundred and fifty millilitre plastic staining dishes containing citrate buffer at a pH of 6.0 was placed in a Solvadex T463 750 watt microwave oven (with a revolving plate) set at full power (mark 10) and heated for three minutes, till the solution was visibly boiling. The slides after they had been de-waxed and degreased as described above were mounted in plastic slide racks and soaked in PBS at a pH of 7.4 while the buffer heated. The slides (mounted in racks) were then placed in the boiling citrate buffer solution, (in 250 ml slide containers), covered, and heated for a further 10 minutes. The same protocol was followed throughout. Following, this period of heating the slides were left to cool in the citrate buffer for 20 minutes. They were then rinsed in PBS at a pH of 7.4, for 5 minutes, before being transferred to a humidity chamber at room temperature.

Each specimen was then placed flat in a humidity chamber at room temperature, excess fluid (PBS) was tapped off and the specimens outlined with a grease pen (DAKO™) - this ensures that the specimens were easily located, prevents both the loss of antibody and cross contamination from one section to the other when two sections are placed on the same slide.

The primary antibody p53, clone DO7 (DAKO™) was then applied at room temperature in a humidity chamber. The **p53 DO-7** (DAKO™) monoclonal mouse antibody was supplied in liquid form as tissue culture supernatant (RPMI 1640 medium containing

foetal calf serum) dialyzed against 0.05 M TRIS/HCl, at a pH of 7.2 containing 15 mM NaN_3. The mouse immunoglobulin concentration was 355 mg per litre and the isotype was IgG2b, kappa. Ten microlitres of p53 DO-7 (DAKO™) was added to a solution of 10% Bovine serum albumin diluted in PBS. The antibody was thus used at a concentration of 1:100 (this concentration was achieved after titration of progressively increasing concentrations against known controls - see Appendix I). The slides were incubated with the antibody for 60 minutes, following which they were washed for five minutes with a continuous flow of PBS from a wash bottle. The secondary antibody consisted of the biotinylated antibody of the StreptABComplex/HRP Duet, Mouse /Rabbit (DAKO™). Ten microlitres of heat inactivated normal human serum was placed in 1 millilitre centrifuge tubes, to this was added 900 microlitres of phosphate buffered saline at room temperature - this produced 10% heat inactivated normal human serum (NHSHI) in 1 ml aliquots. Remove 5µl of 10% NHS (at room temperature) from a 1 millilitre solution and replacing it with 5µl of biotinylated, affinity-isolated goat antibody to mouse/rabbit immunoglobulins in 0.01 M phosphate buffered saline and 15 mM NaN_3 at a pH of 7.2.

The secondary antibody was ready for use straight after constitution. The total protein concentration was 14.5 grams per litre and it was stored at 4 °C. This was applied for forty-five minutes. The secondary biotinylated antibody was then washed off with PBS from a wash bottle. Each slide was washed three times.

The tertiary reagent ABC complex of the StreptABComplex/HRP Duet, Mouse /Rabbit (DAKO™) was the applied for forty-five minutes. Reagent A, consists of 1 ml streptavidin, in 0.01 M phosphate-buffered saline (PBS) and 15 mM NaN_3, at a pH of 7.2. Reagent B, consists of 1 ml biotinylated horseradish peroxidase, in 0.01 M phosphate-buffered saline and 15 mM NaN_3, at a pH 7.2. Twenty-five micolitres of reagent A was added to 5 millilitres of 10 % heat inactivated normal human serum, in a *Bijout* bottle, at room temperature, this is followed immediately by 25 micolitres of reagent B. The solution was gently mixed and allowed to stand at room temperature for

20 minutes before use. The ABC complex was then applied for 45 minutes, then washed off three times with a PBS solution.

At the commencement of each immunohistochemical staining session, 3, 3 diaminobenzidine tetrahydrochloride (DAB) which is stored at - 4 °C, is removed from the freezer and allowed to attain room temperature.

Just after the tertiary reagent is applied the DAB is measured out. Sixty milligrammes of DAB at room temperature is measured out in a Sartorius microbalance (model no. B 120S) which is placed permanently in a fume cupboard. The fume cupboard was switched on before measuring the DAB. One millilitre of TRIS at pH 7.6 (prepared as described above) in a liquipipette is introduced into the measuring boat containing the DAB, the liquipipette is then used to transfer the DAB, partially dissolved in the TRIS into a beaker of 99 mls of TRIS, at room temperature, mounted on a Gallenhamp magnetic hotplate stirrer set to revolution position 5, (outside the fume cupboard). Just before use 1 millilitre of 0.1 M Imidazole (glyoxaline BDH Gurr R GPR™) solution is added to the TRIS. The solution of Imidazole is made up by measuring out 68.08 milligrammes of Imidazole (glyoxaline BDH Gurr R GPR™) on a Sartorius balance, at room temperature and dissolving it in 100 millilitres of double distilled water (obtained from a still) placed in a glass beaker, on a Gallenhamp magnetic stirrer thermostat hot plate, set to revolution position 5. Forty microlitres of 30 % hydrogen peroxide (BDH AnalaR), at room temperature was added to the solution of DAB. If the DAB failed to dissolve completely the solution was filtered before use. The TRIS for DAB was then tested on the ABC mixture as follows, 200µl of ABC mixture is placed in a 1 millilitre microfuge tube to which is added 200µl of fresh DAB solution. The solution changed colour to black/brown, which confirmed the activity of the ABC.

The slides were then covered with DAB solution till the control slide showed a positive reaction, or for a maximum of five minutes. The reaction was then terminated by washing in double distilled water for five minutes. The slides were then counterstained with Mayer's haemalum (BDH Gurr) for one to four minutes, depending on the age of

the Haemalum, the older Haemalum tended to take longer to produce an adequate counterstain. The slides were then rinsed in double distilled water three times and dehydrated by soaking in 3 sequential baths of Methanol (BDH AnalaR Gurr R) each for 10 minutes, followed by sequential baths of Xylene (mixture of isomers) EEC label EEC No:215-535-7 (CHEMIX), each for ten minutes. DePex™ mounting medium BDH Gurr was applied on to a cover slip and placed over the section, which was then allowed to dry overnight.

1.14 Immunohistochemistry of p53 by the Alkaline Phosphatase Method

This method was used for the malignant melanomas because the final product,3 - amino - 9 - ethylcarbazole, or AEC, forms a red end product and helps to distinguish positive cells from the brown of the melanin pigment.

Sections are de-paraffinised as described above by inserting them in serial baths of xylene (mixture of isomers) EEC label EEC No:215-535-7 (CHEMIX) and methanol (BDH AnalaR) respectively. They were then rehydrated by inserting in baths of double distilled water obtained from a still. Two hundred and fifty millilitre containers of freshly prepared citrate buffer, at a pH of 6.0 was placed in a Solvadex T463 750 watt microwave oven (with a revolving plate) set at full power (mark 10) and heated for three minutes, till the solution was visibly boiling. The slides after they had been de-waxed and degreased as described above, were mounted in plastic slide racks and then placed in the boiling citrate buffer solution, (in 250 ml slide containers), covered, and heated for a further 10 minutes. The same protocol was followed throughout. Following, this period of heating the slides were left to cool in the citrate buffer for 20 minutes. They were then rinsed in TRIS for 5 minutes, before being transferred to a humidity chamber at room temperature.The specimens were outlined with a DAKO™ grease pen and the primary antibody p53, clone DO7 (DAKO™) was then applied at room temperature in a humidity chamber. The p53 DO-7 (DAKO™) monoclonal mouse antibody was supplied in liquid form as tissue culture supernatant (RPMI 1640 medium containing foetal calf serum) dialyzed against 0.05 M TRIS/HCl, at a pH of

7.2 containing 15 mM NaN_3. The mouse immunoglobulin concentration was 355 mg per litre and the isotype was IgG2b, kappa. Ten microlitres of p53 DO-7 (DAKO™) was added to a solution of 10% Bovine serum albumin diluted in PBS. The antibody was thus used at a concentration of 1:100 (this concentration was achieved after titration of progressively increasing concentrations against known controls - see Appendix I). The slides were incubated with the antibody for 60 minutes, following which they were washed for five minutes with a continuous flow of TRIS from a wash bottle.

Biotinylated rabbit anti mouse immunoglobulins (DAKO™) (RAMB) diluted in 10% normal human serum was placed on the slides for forty-five minutes at room temperature. This was used at a concentration of 1:200. Following this the slides were rinsed with TRIS from a wash bottle at room temperature for five minutes. The ABComplex was then applied.

ABComplex/AP consisted of reagents A and B. Reagent A consists of streptavidin in 0.01 M phosphate buffer, 0.15 M NaCl in 15mM NaN_3, at a pH of 7.2. The streptavidin had an isoelectric point at about neutral pH, thus nonspecific binding due to ionic interaction was avoided. Reagent B consisted of biotinylated alkaline phosphatase in 0.05M TRIS/HCl, 0.1M NaCl, 1mM $MgCl_2$ and 15mM NaN_3 at a pH of 7.2. The ABC solution was made up fresh at each session. It consists of five millilitres of TRIS/HCl, at a pH of 7.6, placed in a *Bijout* bottle, at a room temperature to which was added one drop of reagent A and one drop of reagent B. The cap of the *Bijout* bottle was then replaced and the mixture shaken. The solution was then left to stand for thirty minutes before use.

Following this the slides were rinsed with TRIS from a wash bottle at room temperature for five minutes. The fast red chromagen was made up as described by the manufacturers. One fast red tablet (DAKO™) was added to 3 mls of supplied buffer to give a red solution. The solution was applied for 15 to 30 minutes, or until the positive control changed colour. Following this the slides were rinsed with double distilled water at room temperature for five minutes.

The slides were then soaked in Meyer's Haemalum for 60 seconds. Following this the slides were rinsed with double distilled water at room temperature for five minutes and allowed to dry at room temperature overnight.

A coverslip was applied with Locktite™ and placed under Ultra-Violet light and allowed to bond overnight.

1.15 Scoring in Breast Tumours

A system to quantify the results of immunohistochemistry is essential in order to compare the results both with other studies and other systems of classification. All specimens were evaluated with an Nikon optiphot-2 microscope using an ocular magnification of x 40, ten to twenty fields per tumour were examined depending on its cellularity (minimum 1,000 cells). Mitoses were counted as the number per 10 high power fields, this method does not take into account the cellularity, or ratio of parenchyma to stroma of a given tumour. Tumours were classified in three ways (1) the intensity of the staining, (2) the proportion of the cells staining and (3) a combination of the two (score 1 plus score 2).

Each variable was given a score of 0 - 3, and an average of the two scores was taken as the final score. The score for percentage of positive cells was 1, 2 and 3 for 1 - 10 %, between 10 - 50% and greater than 50% positive cells respectively. All slides were scored blind by two independent observers, with scores entered on the a score sheet designed for the purpose and the average score of both individuals taken as the final score and then randomly checked by a third individual.

1.16 Scoring in Malignant Melanomas

All specimens were evaluated with an Nikon optiphot-2 microscope using an ocular magnification of x 40, serial sections of all melanomas were examined with each oncogenic stain compared to the HMB-45 stain which localized the site of the melanoma cells (Fig. 2.2 & 2.3) (Gown *et al.,* 1986). The number of positive cells in the melanoma were scored for both intensity and percentage of positive cells and an average of 0 -3 was obtained as the final score. In addition the dermo-epidermal

junction was scored by a similar method when it became obvious that a significant number of cells, i.e. non-malignant cells were expressing the various proteins. The melanoma sections always included some histologically normal skin and the normal dermo-epidermal junction was scored 0-3 based on the percentage of positive cells in this region. The score for percentage of positive cells was 1, 2 and 3 for 1-10 %, between 10 - 50% and greater than 50% positive cells respectively. All slides were scored blind by two independent observers, with scores entered on the a score sheet designed for the purpose and the average score of both individuals taken as the final score and then randomly checked by a third individual. This method was used for both the p53 protein and the MIB-1 protein respectively and extended to the pilosebaceous units for the p53 protein. For the Bcl-2 protein a score was obtained for the tumour and then for lymphocyte positivity or negativity.

The staining pattern in the melanomas for each oncogenic marker was similar to that obtained in the breast tumours. The p53 protein and MIB-1 produced a nuclear stain in the melanoma cells, the dermoepidermal junction (Fig 8.3) and the pilosebaceous units. The Bcl-2 protein and the HMB-45 anti-melanoma antibody produced a cytoplasmic stain (Figs. 2.2, 7.3 & 9.3). The Bcl-2 protein also stained the lymphocytes even when the tumour was possible and this served as an internal control (Fig. 1.3).

1.17 Immunohistochemistry of Bcl-2

A monoclonal mouse antibody (clone124) supplied in liquid form as tissue culture supernatant (RPMI 1640 medium containing fetal calf serum) dialyzed against 0.05 M TRIS/HCl, pH 7.2 containing 15mM NaN_3 was used. The mouse immunoglobulin concentration was 230 mg/ L, with a total protein concentration of 15.0 g/L. It was stored at 2 - 8 °C.

The immunogen was a synthetic peptide comprising amino-acids 41-54 of human Bcl-2 protein. It was used at an optimal staining concentration 1:80, a concentration obtained after titration against known positive controls, which consisted of sections of human tonsil and breast. Two staining methods were used as described above, the streptavidin

biotin method (for both breast carcinomas and melanomas) and the alkaline phosphatase method (for melanomas) both with microwave-antigen retrieval.

The staining pattern was similar for both sets of tumours i.e. breast and melanomas. They both produced a cytoplasmic stain with nuclear sparing (Fig.s 3.3 & 4.3) and stained any reactive lymphocytes. The lymphocytes served as reliable and reproducible internal positive control (Fig. 2.3).

The sections were cut, mounted on APES slides and dewaxed as described above. This was followed by a period of rehydration and microwave antigen retrieval for 15 minutes, rinsing and cooling as described above. The primary antibody was prepared fresh at the commencement of each staining session.

1.18 Immunohistochemistry of Cyclin E

A monoclonal antibody specific for the human cyclin E protein Clone13A3™ Novocastra Laboratories was used. It was of IgG Class IgG2a, kappa. The antigen used for immunization was a recombinant protein corresponding to a site on the full length cyclin E molecule. The hybridoma partner was mouse myeloma (p3-NS1-Ag4-1). The preparation consisted of lyophilized tissue culture supernatant containing 15mM sodium azide. It was reconstituted with 1 millilitre of sterile distilled water. It produced a nuclear staining pattern in the positive control which was placenta it was used at a concentration of 1:40 which was obtained after titrating increasing concentrations of the fresh antibody against known positive controls.

The sections were cut, mounted on APES slides and dewaxed as described above. This was followed by a period of rehydration and microwave antigen retrieval for 15 minutes, rinsing and cooling as described above. The primary antibody was prepared fresh at the commencement of each staining session.

1.19 Immunohistochemistry of MDM-2

The monoclonal antibody was specific for the murine double minute (MDM-2) gene product. The clone 1B10™ Novocastra Laboratories of the IgM class of immunoglobulins was used. The antigen used for immunization was a recombinant

protein corresponding to a site on the carboxy-terminal portion of the MDM-2 molecule. The hybridoma partner was mouse myeloma (p3-NS1-Ag4-1), which was prepared in tissue culture supernatant containing 15mM sodium azide. It produced a nuclear staining pattern and a sarcoma was the positive control.

The sections were cut, mounted on APES slides and dewaxed as described above. This was followed by a period of rehydration and microwave antigen retrieval for 15 minutes, rinsing and cooling as described above. The primary antibody was prepared fresh at the commencement of each staining session.

1.20 Immunohistochemistry of Cyclin D1

A monoclonal antibody specific for the human, monkey, rat and mouse cyclin D protein Clone DCS-6™ Novocastra Laboratories was used. The antigen used for immunization was recombinant human cyclin D1 protein of immunoglobulin class IgG2a, the hybridoma partner was mouse myeloma (NS-2).

The preparation consisted of tissue culture supernatant containing 15mM sodium azide, it was reconstituted with 1 ml of sterile distilled water.

The positive control was a breast carcinoma. The staining pattern produced both cytoplasmic and nuclear positivity.

The sections were cut, mounted on APES slides and dewaxed as described above. This was followed by a period of rehydration and microwave antigen retrieval for 15 minutes, rinsing and cooling as described above. The primary antibody was prepared fresh at the commencement of each staining session.

1.21 Immunohistochemistry of the Oestrogen Receptor

Two oestrogen receptor antibodies were evaluated

(1) The oestrogen receptor clone CC4-5, specific for NCL-ER-LH2™ Novocastra Laboratories was of IgG Class IgG1. The antigen used for immunization was the recombinant ER fusion protein and the hybridoma partner was mouse myeloma (p3-NS1-Ag4-1). It was prepared in tissue culture supernatant containing 15mM sodium azide and reconstituted with 1 ml of sterile distilled water.

The positive controls were lobular and tubular breast carcinomas and it produced a nuclear staining pattern.

(2) The oestrogen receptor monoclonal mouse antibody was in liquid form as tissue culture supernatant (RPMI 1640 medium containing foetal calf serum) dialyzed against 0.05 M TRIS/HCl, pH 7.2 containing 15 mM NaN_3. The mouse Ig concentration was 180 mg/L, of isotype IgG_1, kappa. The total protein concentration was 10.3 g/L and it was stored between 2 - 8 °C. The clone was ID5 and the immunogen was recombinant human oestrogen receptor protein. This was specific for the N-terminal domain (A/B region) of the receptor. The staining was predominantly localized to the nuclei with no cytoplasmic staining. It was used at a concentration of 1:75 which was obtained after titrating increasing concentrations of the fresh antibody against known positive controls. The sections were cut, mounted on APES slides and dewaxed as described above. This was followed by a period of rehydration and microwave antigen retrieval for 20 minutes, rinsing and cooling as described above. The primary antibody was prepared fresh at the commencement of each staining session.

1.22 Immunohistochemistry of HMB-45

The HMB-45 reacts with a melanoma specific antigen present in melanocytic cells and junctional naevi. The antibody does not react with normal melanocytes, or intradermal naevus cells. It is non-reactive with other neoplasms, including sarcomas, carcinomas and glial tumours (Gown *et al.,* 1986). The anti-melanoma antibody HMB-45 was used to characterize the nature of the cells which were positive for the individual antibodies. The species was the mouse IgG_1, affinity purified monoclonal antibody in phosphate-buffered saline containing 1% BSA and 15mM sodium azide.

Two staining methods were used as described above, the streptavidin biotin method and the alkaline phosphatase method, both with microwave-antigen retrieval.

The sections were cut, mounted on APES slides and dewaxed as described above. This was followed by a period of rehydration and microwave antigen retrieval for 15 minutes, rinsing and cooling as described above. The primary antibody was prepared

fresh at the commencement of each staining session.

1.23 Immunohistochemistry of MIB-1™

Sections are prepared as described above. The MIB-1™ antibody (Immunotech S.A.), is a recombinant peptide corresponding to a 1002 bp Ki-67 cDNA fragment.

The hybridoma consisted of myeloma cells combined with 63Ag8.6453 and Balb/c spleen cells. The IgG1 (Mouse) isotype was used.

The monoclonal antibody MIB-1™ specifically reacts with the Ki-67 nuclear antigen (345 and 395 kD double band in Western blot analysis of proliferating cells) associated with cell proliferation and found throughout the cell cycle (G_1, S, G_2, M phases) and absent in resting (G_0) cells (Gerdes *et al.*, 1983). This antibody recognizes native Ki-67 antigen and recombinant fragments of the Ki-67 molecule. The MIB-1 monoclonal antibody gives an immunocytochemical staining pattern identical to Ki-67 on paraffin embedded tissue sections, frozen sections and cytological samples (smears/imprints /cytospins). It was used in a concentration of 1:50 after titration as described above. The staining pattern was nuclear.

The sections were cut, mounted on APES slides and dewaxed as described above. This was followed by a period of rehydration and microwave antigen retrieval for 15 minutes, rinsing and cooling as described above. The primary antibody was prepared fresh at the commencement of each staining session. Two staining methods were used as described above, the streptavidin biotin method (for both breast carcinomas and melanomas) and the alkaline phosphatase method (for melanomas) both with micro-wave-antigen retrieval. The staining pattern was nuclear both sets of tumours i.e. breast and melanomas.

Chapter 2

Results

In the breast tumour patients clinical data on dates of tumour recurrence and death were collected from the clinical notes of the individual patients. Data was also collected on age of menarche, breast feeding, the use of the contraceptive pill, family history of breast carcinoma, or other malignancy.

For patients with malignant melanomas data was collected on tumour thickness, site, Clark's level, Breslow thickness and a family history of the dysplastic naevus syndrome, malignant melanomas or other malignancy.

Statistical analysis was performed on a ***PowerPC*** 7200/90MHz with the statistical package Statview 4.5. Spearman's rank correlation coefficient was used to compare continuous variables and the Mann-Whitney test to compare the nominal with continuous variables. Traditional prognostic factors were compared to oncogenic markers. Analysis was performed for the two groups of breast tumours separately and together.

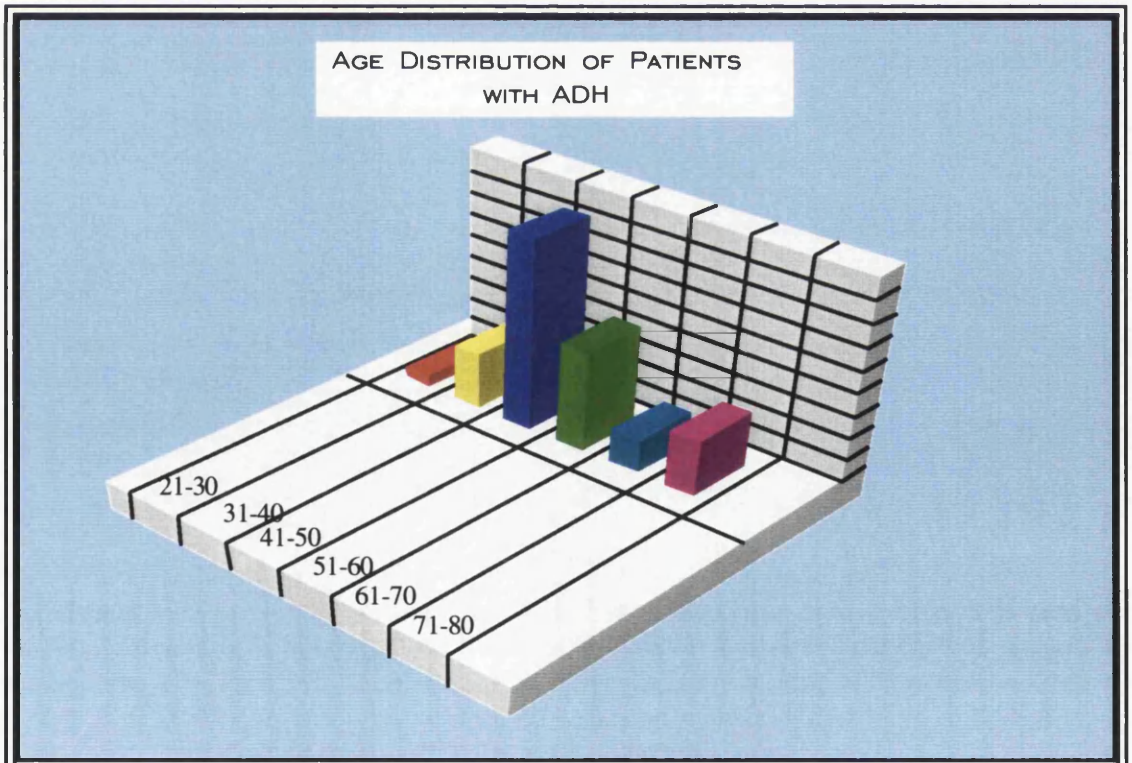

Figure 1.2 Bar-chart showing the age distribution of patients with Atypical Ductal Hyperplasia. Most were between the ages of 41 to 50 years.

2.2 Benign Breast Disease

There were forty-six patients, 30 with pure fibrocystic disease of the breast (and no evidence of other epithelial tumours) and sixteen patients had atypical ductal hyperplasia. In the patients with fibrocystic disease none were p53 positive i.e. scored either 2 or 3 but 93 % were Bcl-2 positive. The age range was 21 to 80 years. Most (39.1 %) of the patients with ADH were between the ages of 41 to 50 years (Fig. 2.1). In the pure ADH group 1 overexpressed p53 in greater than 50% of their tumour nuclei, 5 Bcl-2, 3 cyclin D, 2 MDM-2 and 2 were cyclin E positive. p53 expression correlated with Bcl-2 expression (p = .02) and MIB-1 (p = .09) respectively.

2.3 Low Grade Tumours

There were thirty-eight low-grade tumours, eight had an area, or areas of atypical ductal hyperplasia and 12 had DCIS on the same section respectively. The age range was 40

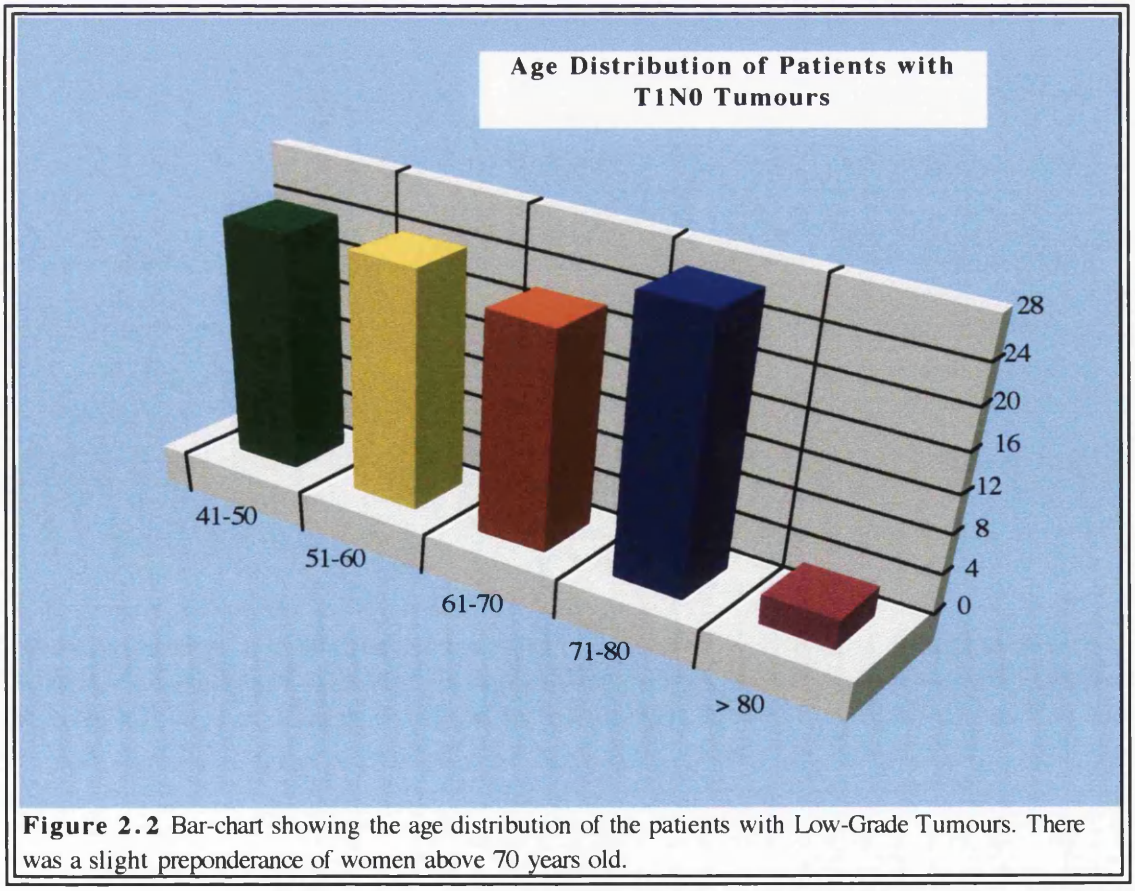

Figure 2.2 Bar-chart showing the age distribution of the patients with Low-Grade Tumours. There was a slight preponderance of women above 70 years old.

to 80 years with a mean of 61 years. Twenty-seven percent were above the age of seventy years and therefore postmenopausal (Fig. 2.2). Fifty-seven percent had grade II tumours and the rest were grade I. Twelve patients had evidence of DCIS on the same section.

Sixty-four percent were ER positive as determined by the clone ID5 (Table 4.2). Sixty-five percent were post-menopausal tumours. Fifty-percent had a family history of breast cancer.

2.3.1 Oncogene Scores

Thirty-seven percent of the low-grade tumours were negative for the p53 protein and 14 % were strongly positive (Table 1.2).

Thirty-eight percent of grade I and II tumours had less than 10 % of their cells in cycle i.e. MIB-1 scores of 1, but 47 % had greater than 50% of their cells in cycle (Table 2.2). Fifty-nine percent had a score of 3 for Bcl-2 and only 16 % were completely negative (Table 3.2). Thirty-one percent and 6 % scored 3 for cyclins D and E respectively (Table 5.2 & 6.2).

In the ADH negative low-grade tumours 10/30 (33%) overexpressed cyclin D, 2/30 (6%) cyclin E and 21/30 (70%) were oestrogen receptor positive as determined by the monoclonal antibody, clone ID5. In the ADH positive group (n = 8) 4/8 (50%) were oestrogen receptor positive as determined by the monoclonal antibody, clone ID5. None, were either cyclin D, or E positive.

In the ADH negative group there was a correlation between p53 expression and the Bcl-2 protein ($p = .005$). Furthermore, Bcl-2 showed a strong correlation with cyclin E ($p = .0099$), grade ($p = .005$) and MIB-1 ($p = .0092$) score respectively but a weaker correlation with oestrogen receptor status ($p = .11$). When the ADH group was added to the analysis, the correlation between Bcl-2 and p53 ($p = .001$), grade ($p = .01$), MDM-2 ($p = .06$) and MIB-1 ($p = .009$) respectively persisted. MIB-1 also correlated with grade ($p = .02$), cyclins D ($p = .03$) and E ($p = .001$), respectively. MDM-2 expression correlated with cyclin D ($p = .09$).

Frequency Distribution for p53	Grade I-II		
From (≥)	To (<)	Count	Percent
0.000	1.000	13	37.143
1.000	2.000	10	28.571
2.000	3.000	7	20.000
3.000	4.000	5	14.286
	Total	35	100.000

TABLE 1.2 RESULTS OF P53 EXPRESSION IN GRADE I-II TUMOURS

Frequency Distribution for MIB-1	Grade I-II		
From (≥)	To (<)	Count	Percent
0.000	1.000	1	2.778
1.000	2.000	13	36.111
2.000	3.000	5	13.889
3.000	4.000	17	47.222
	Total	36	100.000

TABLE 2.2 RESULTS OF MIB-1 EXPRESSION IN GRADE I-II TUMOURS

Frequency Distribution for Bcl-2	Grade I-II		
From (≥)	To (<)	Count	Percent
0.000	1.000	6	16.216
1.000	2.000	2	5.405
2.000	3.000	7	18.919
3.000	4.000	22	59.459
	Total	37	100.000

TABLE 3.2 RESULTS OF BCL-2 EXPRESSION IN GRADE I-II TUMOURS

Frequency Distribution for ER	Grade I-II	
	Count	Percent
pos	25	67.567
neg	12	32.432
Total	37	100.000

TABLE 4.2 RESULTS OF ER STATUS IN GRADE I-II TUMOURS

Frequency Distribution for CD	Grade I-II		
From (≥)	To (<)	Count	Percent
0.000	1.000	8	22.857
1.000	2.000	8	22.857
2.000	3.000	8	22.857
3.000	4.000	11	31.429
	Total	35	100.000

TABLE 5.2 RESULTS OF CYCLIN D EXPRESSION IN GRADE I-II TUMOURS

Frequency Distribution for CE	Grade I-II		
From (≥)	To (<)	Count	Percent
0.000	1.000	9	27.273
1.000	2.000	14	42.424
2.000	3.000	8	24.242
3.000	4.000	2	6.061
	Total	33	100.000

TABLE 6.2 RESULTS OF CYCLIN E EXPRESSION IN GRADE I-II TUMOURS

2.4 Grade III Tumours

There were 55 grade III tumours, the age range was from 20 - 86, with a mean of 52 years. Thirty-one percent were between 41 to 50 years old (Fig. 3.2). Only 13 % were alive at the time of this study and 37 % were dead within two years. Thirty-six had tumour in their lymph-nodes and nine did not (ten patients did not have formal lymph-node dissections). Sixty-three percent had a p53 score of 2-3 and only 5 (8 %) had less than 10 % of their tumour nuclei positive (Table 7.2). Forty-percent, 36 % and 20 % of patients scored 3, 2 and 1 respectively for the MIB-1 protein (Table 8.2). Fifty-two percent were negative for Bcl-2 (Table 9.2) but similar numbers 24 % and 27 %

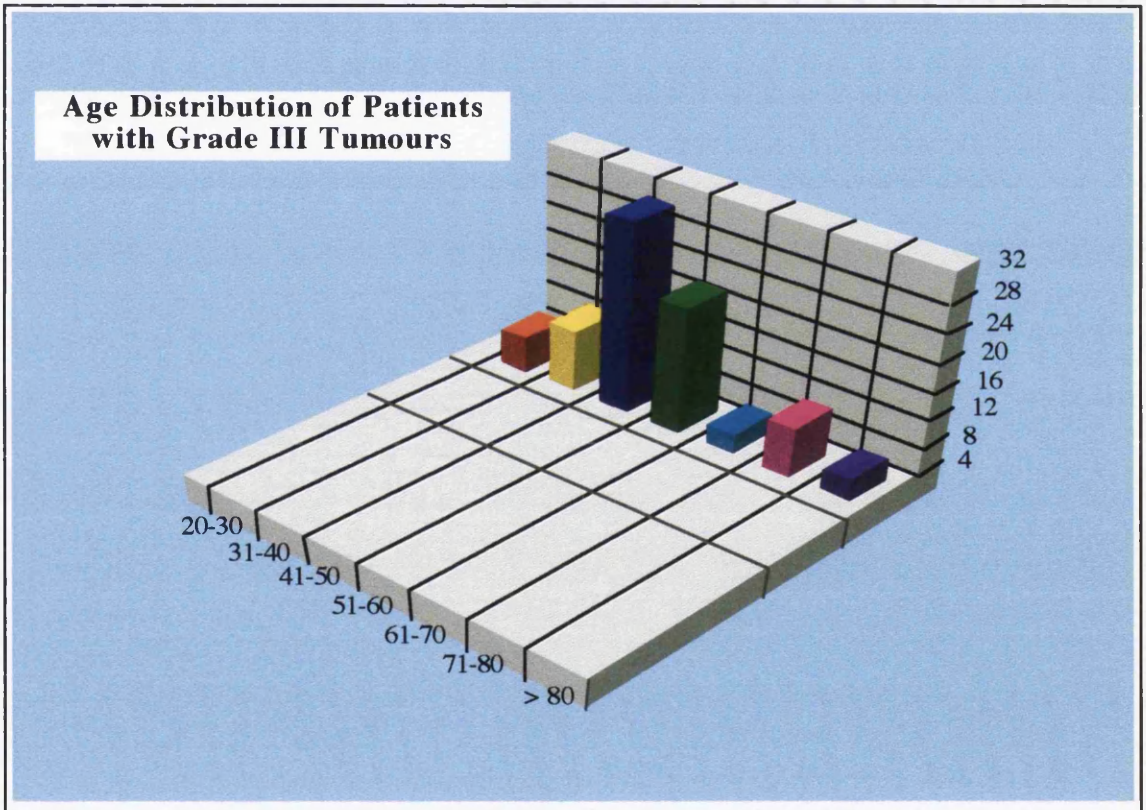

Figure 3.2 Bar-chart showing the age distribution of patients with Grade III Breast Tumours. Most were between the ages of 41 to 50.

respectively scored 3 for cyclins D and E (Table 11.2 & 12.2).

Seventy-three percent of tumours were strongly positive (score = 3) for the MDM-2 protein (Table 13.2).

There was a correlation between p53 and MIB-1 ($p < .0001$) (Spearman Rank

Correlation), but neither lymph-node status (p = .3) (Mann-Whitney U), Bcl-2 (p = .2) (Spearman Rank Correlation), cyclin D (p = .4) nor cyclin E (p = .2) (Spearman Rank Correlation).

There was no correlation between Bcl-2 and the oestrogen receptor status (p = .2) (Mann-Whitney U), or MIB-1 (p = .26) (Spearman Rank Correlation).

There was also a correlation between MDM-2 and both p53 (p = .01) and MIB-1 respectively (p = .001) (Spearman Rank Correlation).

However, there was no correlation between MIB-1 and Bcl-2 (p = .2), cyclin D (p = .8), cyclin E (p = .2) (Spearman Rank Correlation), or either the oestrogen receptor (p = .01) (Mann-Whitney U), or lymph-node status (p = .6) (Mann-Whitney U).

There was a weak correlation between Bcl-2 and cyclin D (p = .1) but not cyclin E (p = .8) (Spearman Rank Correlation). Furthermore, MDM-2 showed a correlation with both cyclin D (p = .07) and cyclin E (p = .008) respectively (Spearman Rank Correlation).

Overall lymph-node status did not show a correlation with any of the oncogenic proteins but grade III tumours that were lymph-node positive, were more likely to be p53 positive (Fig. 4.2). Only one protein cyclin E (p = .08) showed a correlation with the oestrogen receptor status (Mann-Whitney U) with Bcl-2 (p = .2) and MIB-1 (p = .2) showing non-significant correlations respectively.

Frequency Distribution for p53	Grade III		
From (≥)	To (<)	Count	Percent
0.000	1.000	16	28.571
1.000	2.000	5	8.929
2.000	3.000	12	21.429
3.000	4.000	23	41.071
	Total	56	100.000

TABLE 7.2 RESULTS OF P53 EXPRESSION IN GRADE III TUMOURS

Frequency Distribution for MIB-1	Grade III		
From (≥)	To (<)	Count	Percent
0.000	1.000	2	3.636
1.000	2.000	11	20.000
2.000	3.000	20	36.364
3.000	4.000	22	40.000
	Total	55	100.000

TABLE 8.2 RESULTS OF MIB-1 EXPRESSION IN GRADE III TUMOURS

Frequency Distribution for Bcl-2	Grade III		
From (≥)	To (<)	Count	Percent
0.000	1.000	29	52.727
1.000	2.000	8	14.545
2.000	3.000	9	16.364
3.000	4.000	9	16.364
	Total	55	100.000

TABLE 9.2 RESULTS OF BCL-2 EXPRESSION IN GRADE III TUMOURS

Frequency Distribution for LN	Grade III	
	Count	Percent
neg	9	20.000
pos	36	80.000
Total	45	100.000

TABLE 10.2 SHOWING THE LYMPH-NODE STATUS IN PATIENTS WITH GRADE III TUMOURS - THE STATUS WAS NOT KNOWN IN TEN PATIENTS

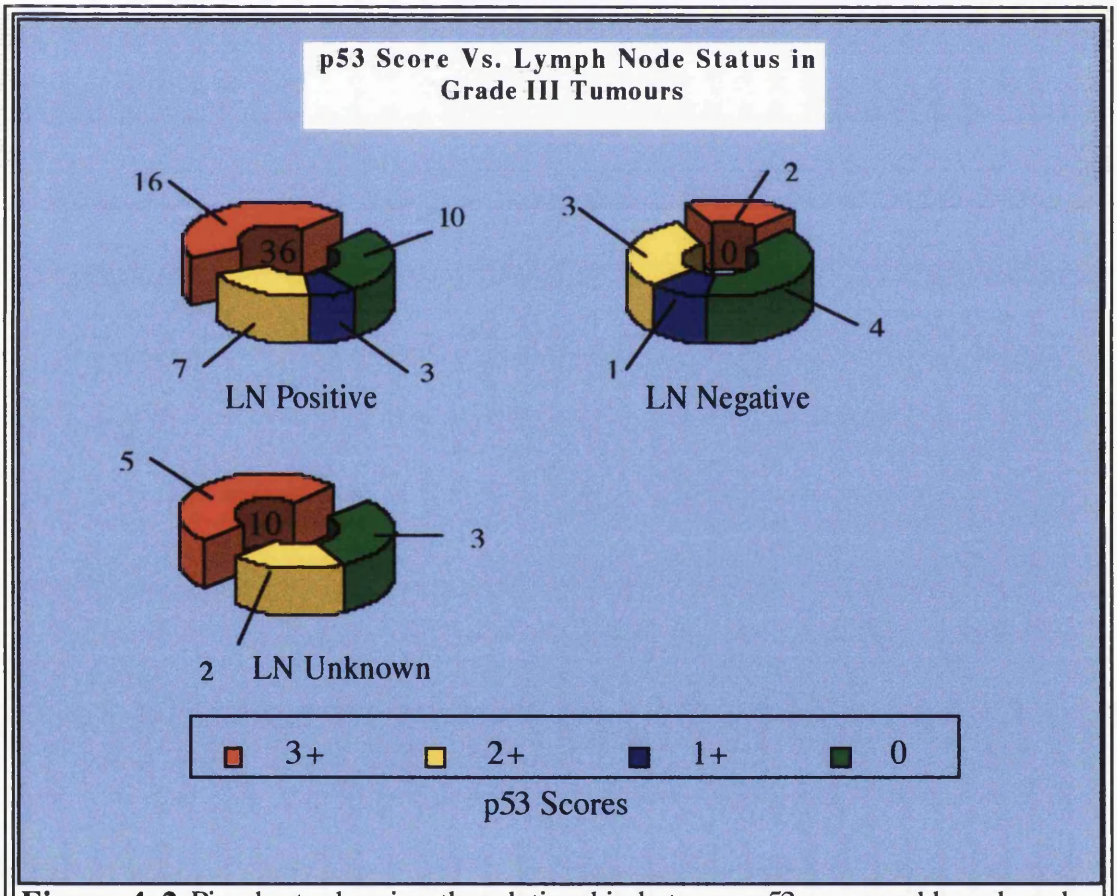

Figure 4.2 Pie-charts showing the relationship between p53 score and lymph-node status in Grade III Tumours. Only two out of ten lymph-node negative tumours were p53 positive. Forty-four percent of tumours that were lymph-node positive were p53 positive.

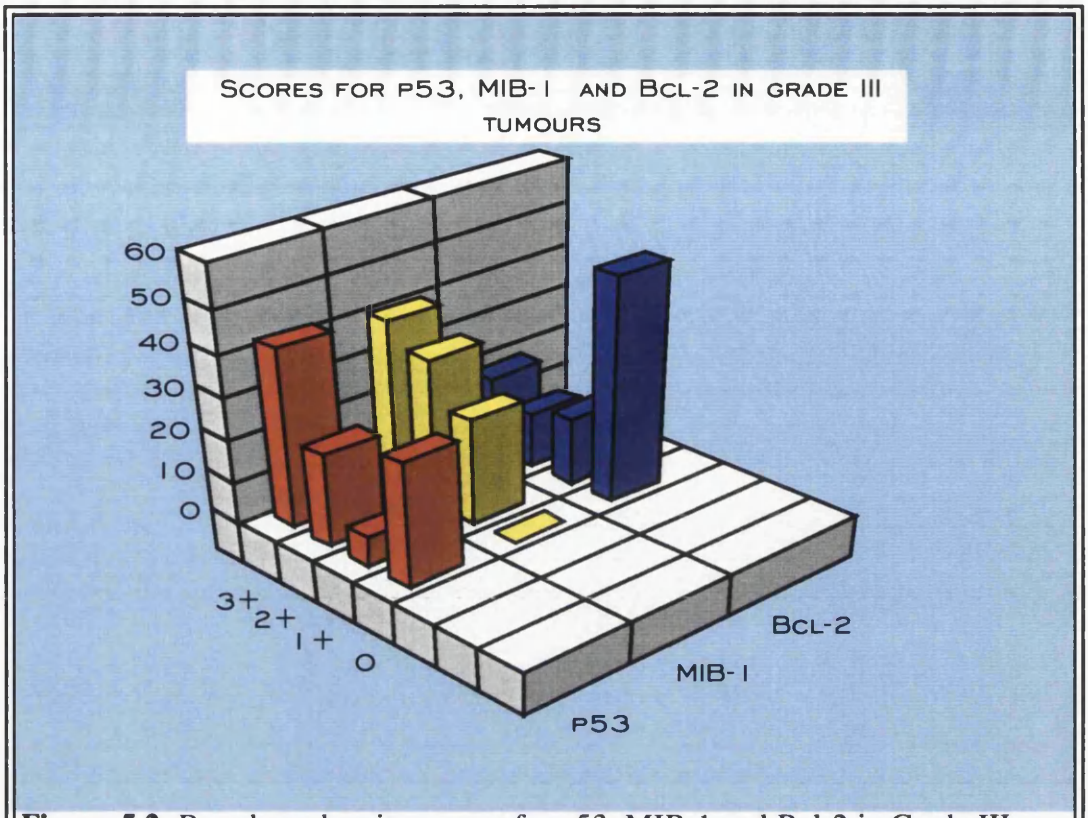

Figure 5.2 Bar-chart showing scores for p53, MIB-1 and Bcl-2 in Grade III Tumours. There was a correlation between p53 and MIB-1 ($p < .0001$). Most grade III tumours were Bcl-2 negative, implying that the inhibition of apoptosis is not the main method of survival. However, there was no correlation between Bcl-2 and p53 ($p = .2$)

Frequency Distribution for CE	Grade III		
From (≥)	To (<)	Count	Percent
0.000	1.000	9	17.647
1.000	2.000	14	27.451
2.000	3.000	14	27.451
3.000	4.000	14	27.451
	Total	51	100.000

TABLE 11.2 RESULTS OF CYCLIN E EXPRESSION IN GRADE III TUMOURS

Frequency Distribution for CD	Grade III		
From (≥)	To (<)	Count	Percent
0.000	1.000	20	37.736
1.000	2.000	10	18.868
2.000	3.000	10	18.868
3.000	4.000	13	24.528
	Total	53	100.000

TABLE 12.2 RESULTS OF CYCLIN D EXPRESSION IN GRADE III TUMOURS

Frequency Distribution for MDM-2	Grade III		
From (≥)	To (<)	Count	Percent
0.000	1.000	3	5.769
1.000	2.000	1	1.923
2.000	3.000	10	19.231
3.000	4.000	38	73.077
	Total	52	100.000

TABLE 13.2 RESULTS OF MDM-2 EXPRESSION IN GRADE III TUMOURS

2.5 Patients with Breast Cancer and Malignant Melanoma or the Dysplastic Naevus Syndrome

In the tumours from patients with breast cancer and malignant melanoma or the

dysplastic naevus syndrome (DNS) there was a correlation between p53 and MIB-1 ($p = .001$) (Spearman Rank Correlation) and oestrogen receptor status ($p = .04$) respectively (Mann-Whitney U). Bcl-2 correlated with cyclin D ($p = .06$) (Spearman Rank Correlation) but none of the other oncogenic markers .

In addition MIB-1 correlated with oestrogen receptor status ($p = .02$) (Mann-Whitney U) and MDM-2 ($p = .05$) (Spearman Rank Correlation) respectively. There were no other statistically significant associations (Results in Appendix I).

2.6 Analysis for all Breast Tumours

The recurrence free interval for breast cancer patients was determined by life table analysis on patients who had been followed up for greater than 3 months after surgery. Statview 4.5 for power Macintosh was used for basic statistics, the log-rank test and lifetables. The data was analyzed for the individual groups separately and then all the malignant tumours were grouped together and the analysis repeated. The malignant melanomas were analyzed separately. The grouping variables for the Mann-Whitney tests were the oestrogen receptor status for all malignant breast tumours and in addition the lymph-node status for grade III tumours. For the malignant melanomas the grouping variable was the lymphocytic response as revealed by the Bcl-2 positivity of the lymphocytes. Spearman rank correlation coefficient was used to compare continuous numeric variables. Kaplan Meier plots were generated for traditional prognostic factors and the individual oncogenes with censored variables taken into account. Multivariate analysis was performed to detect potential independent prognostic factors - Cox's proportional hazards model was used.A statistical analysis was performed for all the malignant breast tumours - this confirmed the correlation between p53 and MIB-1 ($p < .0001$) and grade respectively ($p < .0001$) (Spearman Rank Correlation, Table 15.2). Furthermore, oestrogen receptor status correlated with p53 ($p = .02$) grade ($p = .002$) and MIB-1 ($p = .08$) respectively but not age ($p = .11$) (Mann-Whitney U). The correlation between MIB-1 and age persisted ($p < .0001$) (Spearman Rank Correlation, Tables 14.2 & 15.2). Bcl-2 did not correlate with any of the oncogenic markers.

ONCOGENE/SCORE	0	1	2	3	TOTAL
P53	31.5 %	17.3 %	20.6 %	30.4 %	100 %
MIB-1	2.1 %	26.3 %	47.2 %	24.1%	100 %
BCL-2	38.0 %	36.9 %	10.8 %	14.1 %	100 %
GRADE	0.0 %	14.6 %	20.2 %	65.1 %	100 %
MDM-2	9.5 %	3.1 %	20.6 %	66.6 %	100 %
CYCLIN D	29.6 %	19.7%	22.2 %	28.3%	100 %
CYCLIN E	19.4 %	31.1 %	28.5 %	20.7 %	100 %

TABLE 14.2 SHOWING THE PERCENTAGE OF TUMOURS POSITIVE FOR EACH ONCOGENIC PROTEIN.

	AGE	P53	MIB-1	BCL-2	MDM	ER	CYCLIN D	CYCLIN E
AGE	*							
P53	.19	*						
MIB-1	.73	<.0001	*					
BCL-2	.51	.08	.29	*				
MDM	.71	.01	.0007	.82	*			
ER	.11	.03	.10	.20	.36	*		
CYCLIN D	.05	.78	.76	.13	.05	.25	*	
CYCLIN E	.60	.28	.01	.96	.0003	.04	.39	*
GRADE	.18	.0005	<.0001	.35	<.0001	.0016	.82	.001

TABLE 15.2 RESULTS OF STATISTICAL ANALYSES FOR ALL BREAST TUMOURS IN EACH ONCOGENIC GROUP

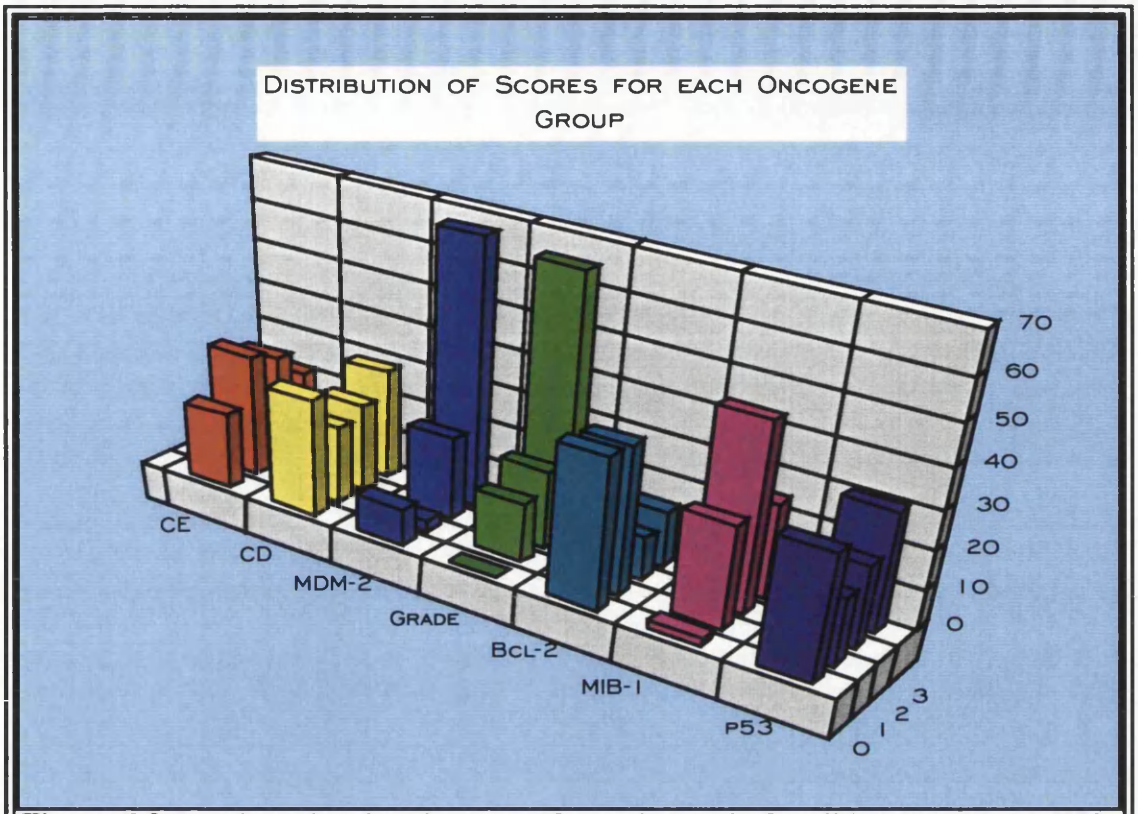

Figure 6.2 Bar chart showing the scores for each protein for all breast tumours. P53 correlated with MIB-1, the marker of proliferation. MDM-2 ($p = .01$) and MIB-1 ($p < .0001$) both correlated with grade. Bcl-2 correlated with cyclin D.

2.5 Malignant Melanomas

There were seventy-four melanomas. The mean age was 51 years (range 1 - 87). Fifteen percent were between the ages of 20 39 years which is slightly below the national average of 22 percent of melanomas for this age group, but still well above the expected percentage of all tumours at this age group which is 4 %. (OPCS, 1992). Of the sixty-six control melanomas over half (51 %) were located on a limb. Eleven and 13 % were located on the back and chest respectively (Fig. 8.2). A similar distribution was found in the nine patients with both malignant melanomas and breast cancer with 70 % having tumours on either the lower or upper limb. Forty percent were either level I or II melanomas, with 21 % and 33 % in level III or IV respectively (Fig. 10.2). Only 6 % were level V. Thirty-nine percent were relative good prognosis melanomas with a

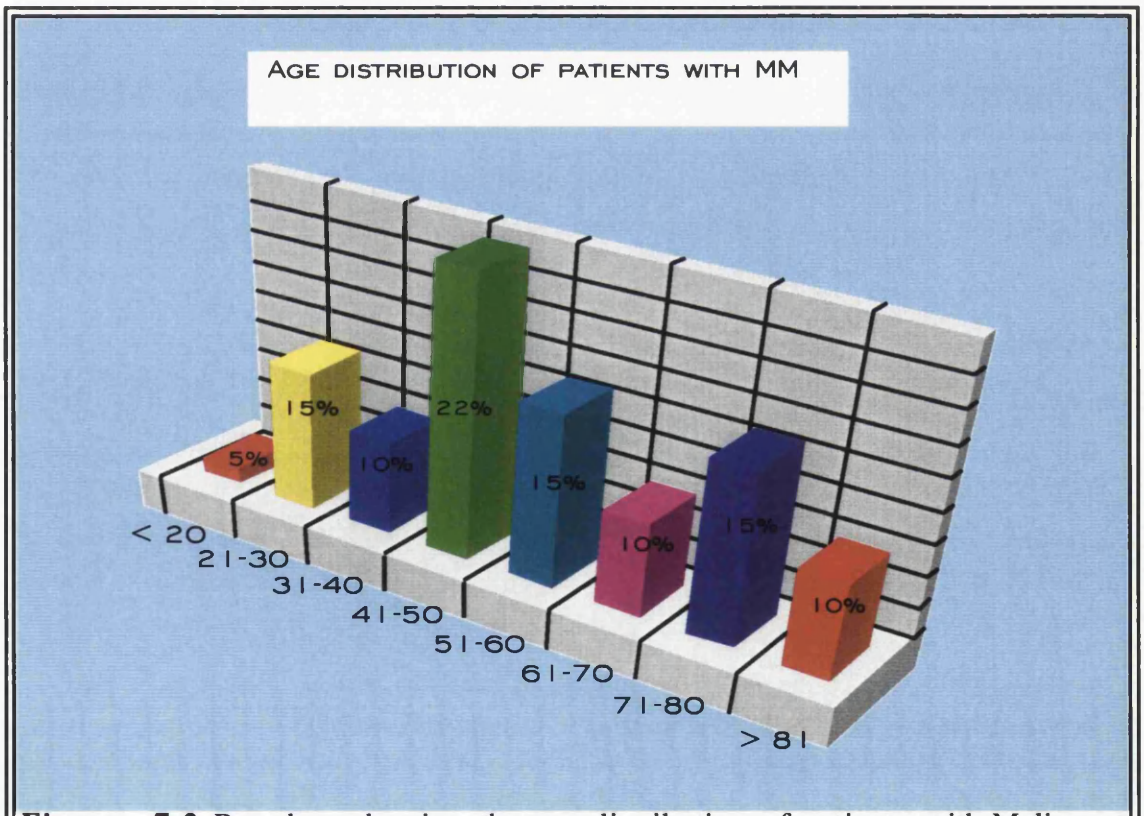

Figure 7.2 Bar-chart showing the age distribution of patients with Malignant Melanomas. Most were between 41 to 50 years old.

Breslow's thickness measurement less than 0.7 millimetre, but 36 % were over 1

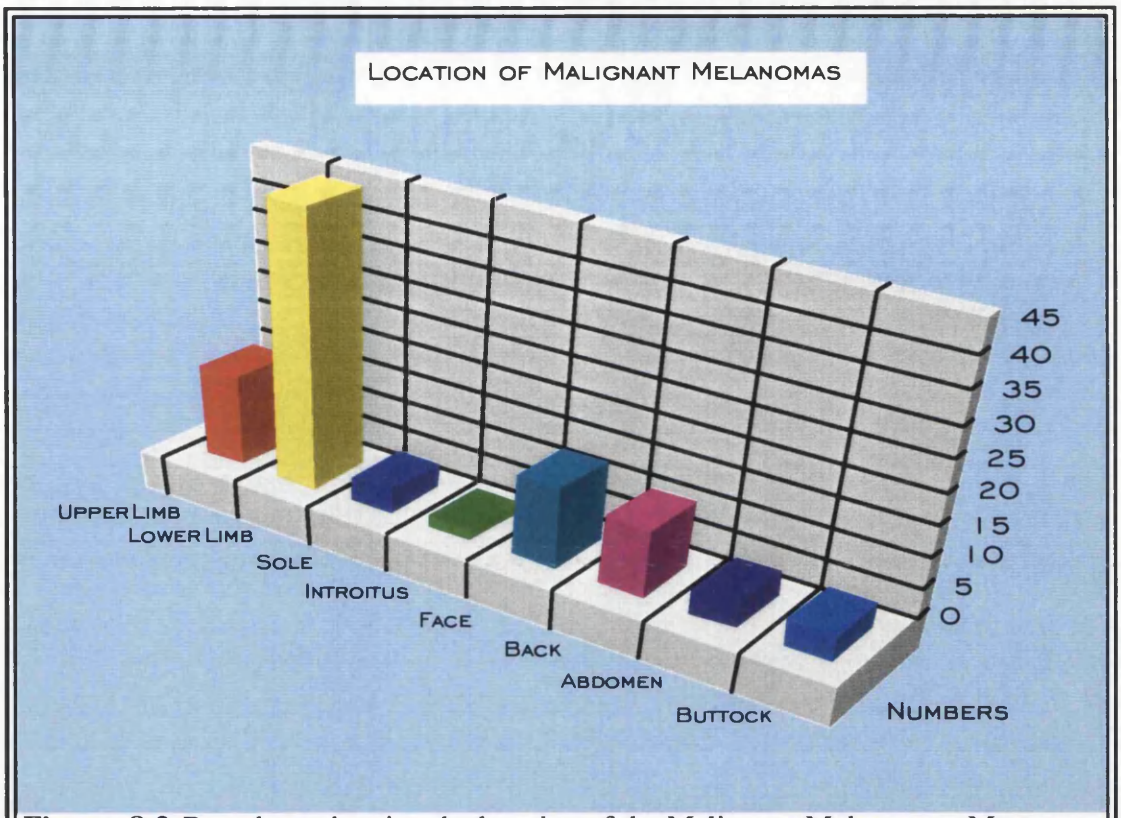

Figure 8.2 Bar-chart showing the location of the Malignant Melanomas. Most were on either the upper, or lower limb.

millimetre thick (Fig. 9.2). There was a correlation between p53 in the melanoma cells and p53 ($p < .0001$) and MIB-1 ($p = .02$) in the dermo-epidermal junction (Spearman Rank Correlation) respectively. However, p53 did not correlate with MIB-1 ($p = .2$) in the melanoma cells (Spearman Rank Correlation) nor Bcl-2 in the lymphocytes ($p = .5$) (Mann-Whitney U). There was a correlation between Bcl-2 and p53 ($p < .0001$) and MIB-1 ($p = .008$) (Spearman Rank Correlation) in the melanoma cells respectively and p53 expression in the dermo-epidermal junction ($p = .008$). Furthermore, MIB-1 in the dermo-epidermal junction correlated with Clark's level ($p = .05$) and Bcl-2 ($p = .02$) (Spearmann Rank Correlation). Clark's level correlated with Breslow's thickness measurement ($p < .0001$) (Spearman Rank Correlation). There was a weak insignificant correlation between Bcl-2 positivity in the lymphocytes and MIB-1 ($p = .14$) but none of the other oncogenic markers showed any relationship. However, p53

in the tumour ($p = .07$) and DEJ ($p = .002$) correlated with p53 expression in the sweat glands and pilosebaceous units respectively.

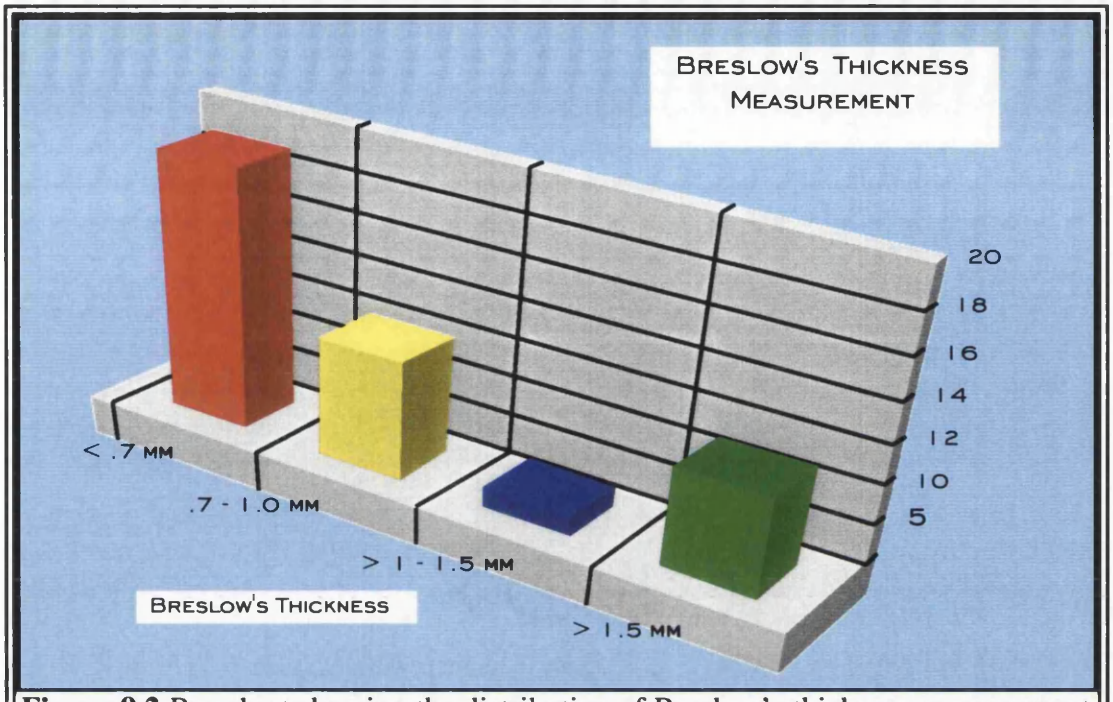

Figure 9.2 Bar-chart showing the distribution of Breslow's thickness measurement in patients with Malignant Melanomas. Most were relatively good prognosis melanomas < 0.7 mm thick.

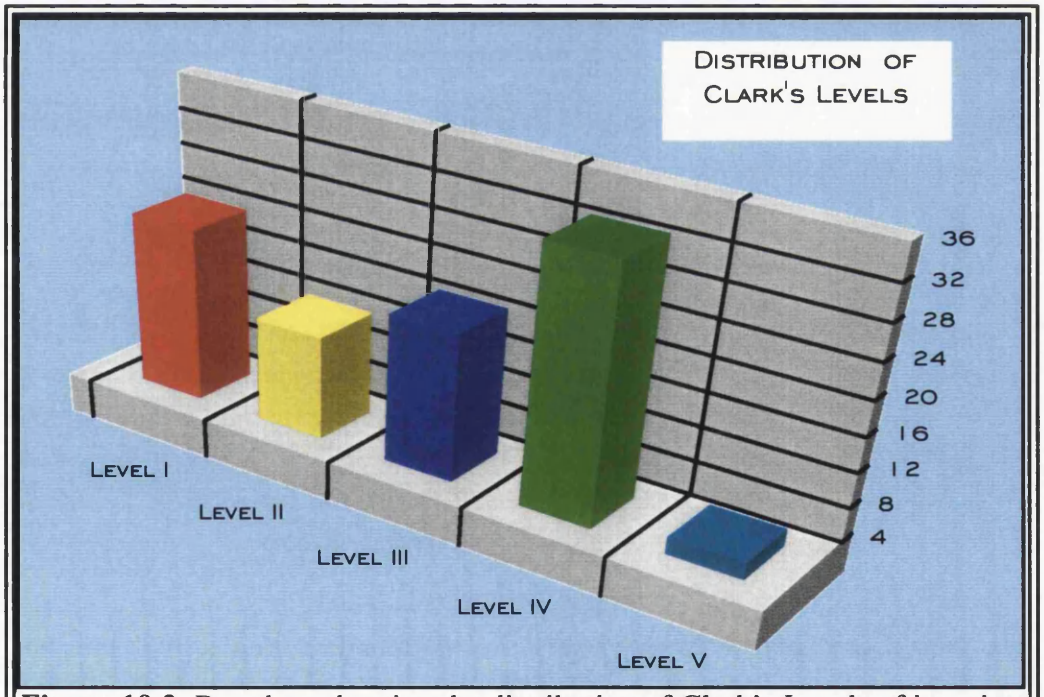

Figure 10.2 Bar-chart showing the distribution of Clark's Levels of invasion in patients with Malignant Melanomas. There was a slight preponderance of Level IV Melanomas.

Frequency Distribution for p53 Tumour	MM	
From (≥)	To (<)	Percent
0.000	1.000	21.667
1.000	2.000	18.333
2.000	3.000	13.333
3.000	4.000	46.667
	Total	100.000

TABLE 16.2 RESULTS OF P53 EXPRESSION IN MALIGNANT MELANOMAS

Frequency Distribution for p53 DEJ	MM	
From (≥)	To (<)	Percent
0.000	1.000	42.373
1.000	2.000	25.424
2.000	3.000	8.475
3.000	4.000	23.729
	Total	100.000

TABLE 17.2 RESULTS OF P53 EXPRESSION IN THE DERMO-EPIDERMAL JUNCTION OF MALIGNANT MELANOMAS

Frequency Distribution for MIB-1 Tumour	MM	
From (≥)	To (<)	Percent
0.000	1.000	8.929
1.000	2.000	39.286
2.000	3.000	28.571
3.000	4.000	23.214
	Total	100.000

TABLE 18.2 RESULTS OF MIB-1 EXPRESSION IN MALIGNANT MELANOMAS

Frequency Distribution for MIB-1 DEJ	MM	
From (≥)	To (<)	Percent
0.000	1.000	22.727
1.000	2.000	40.909
2.000	3.000	27.273
3.000	4.000	9.091
	Total	100.000

TABLE 19.2 RESULTS OF MIB-1 EXPRESSION IN THE DERMO-EPIDERMAL JUNCTION OF MALIGNANT MELANOMAS

Frequency Distribution for Bcl-2 Tumour	MM	
From (≥)	To (<)	Percent
0.000	1.000	16.949
1.000	2.000	28.814
2.000	3.000	15.254
3.000	4.000	38.983
	Total	100.000

TABLE 20.2 RESULTS OF BCL-2 EXPRESSION IN THE MALIGNANT MELANOMAS

Frequency Distribution for Bcl-2 LN	MM	
	Count	Percent
neg	35	53.84
pos	30	46.15
Total	65	100.00

TABLE 21.2 RESULTS OF BCL-2 EXPRESSION IN THE LYMPHOCYTES OF MALIGNANT MELANOMAS

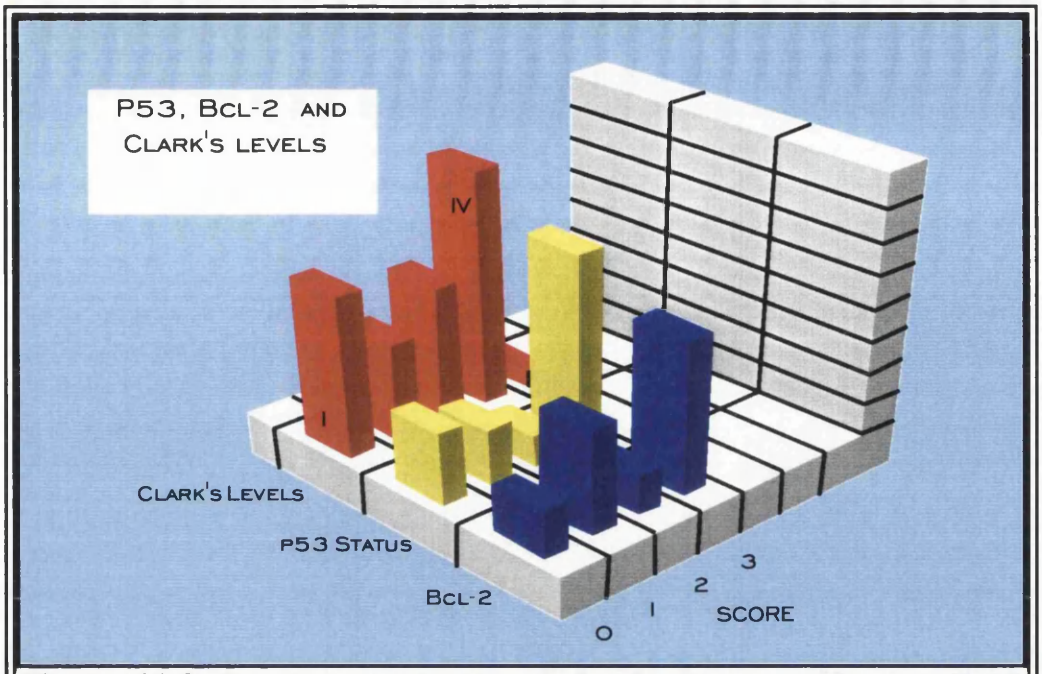

Figure 11.2 Bar-chart showing the relationship between p53 status, Bcl-2 score and Clarke's levels in the Malignant Melanomas.

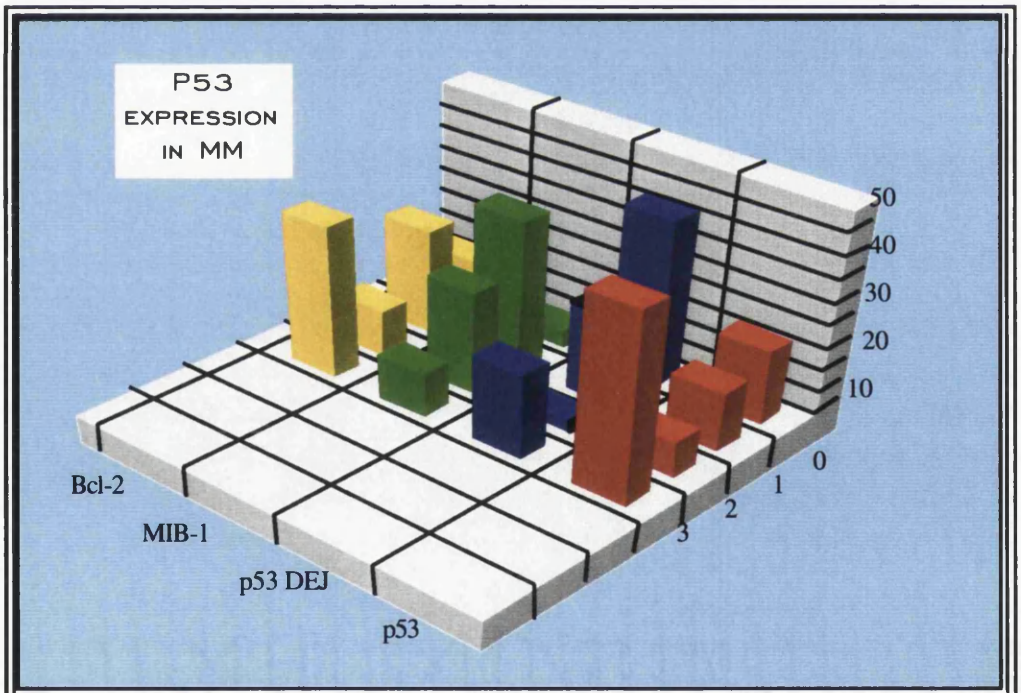

Figure 12.2 Bar-chart showing relationship between p53 expression in the Melanoma cells with, MIB-1 - the marker of proliferation, Bcl-2 - the inhibitor of apoptosis and p53 expression in the dermo-epidermal junction (DEJ). p53 & MIB-1 correlated with p53 expression in the DEJ. The correlation between p53 and Bcl-2 is clearly demonstrated ($p = .001$).

Chapter 3

DISCUSSION

3.1 Breast Tumours, Oncogenic markers and Prognosis

The transformation of a normal to a malignant cell is believed to require at least four or five mutations (Yokota *et al.,* 1993, Chang *et al,* 1995), mutations in genes which have variously been called DNA repair mutator genes, tumour suppressor genes and oncogenes. The most important of these is the p53 tumour suppressor gene which is mutated in 50 % of human cancers (Harris 1992, 1996). The most frequent mutations are missense mutations which result in the production, ironically of both a more stable and dysfunctional protein. The dysfunctional protein may result both in loss of normal function and gain in function of other oncogenes, notably Bcl-2, which then results in an impaired ability to induce apoptosis. This, the ideal setting for the creation of clones of immortal cells. In addition a dysfunctional p53 becomes unavailable for the normal interaction with MDM-2, which if itself is abnormal increases the disorder in the cell cycle and confers oncogenic properties which are sufficient to induce tumourigenesis. This would occur frequently if not for the restriction, or "R" point manned by as yet an unquantified protein believed to be either cyclins D, or E. If this point is breached in the presence of mutation, apoptosis is overcome and tumourigenesis occurs.

Germline mutations in the p53 gene produce the "Li-Fraumeni" or "Li-Fraumeni like syndromes" - these individuals are prone to multiple tumours at various combinations at an early age. A subset of the study group may indeed belong to this group. However, individuals with multiple tumours may themselves possess somatic mutations in the gene which also increases the likelihood of a second malignancy - further supporting Knudson's two hit hypothesis.

The breast is the ideal tissue to explore the interactions of the p53 protein, it is widely available, the pathological characteristics and classifications have been standardised and clinical outcomes are well documented. Serial sections of histologically abnormal tumours were examined to determine if similar cell groups within the same tumour exhibited predictable interactions. At the present state of our knowledge of breast pathology we are unable to define a so called *adenoma-carcinoma* sequence which has

been so eloquently documented in colorectal pathology. This is one of the reasons we are unable to reliably predict clinical prognosis in breast cancer. The *ductal carcinoma in-situ* does not seamlessly evolve into pure carcinoma although up to 2 % have been associated with lymph-node metastasis (Veronese *et al.,* 1995). Atypical ductal hyperplasias and diffuse papillomatosis are benign proliferations which have been epidemiologically linked with a greater risk for the development of breast cancer, but again do not neatly evolve into overt carcinomas. *Ductal carcinoma in-situ* frequently involves the whole breast, but even then the malignant cells do not show invasion of the basement membrane, neutralizing the "size is important" theory which is so useful in colorectal carcinoma. In some areas of DCIS the electron microscope reveals a breach of the basement membrane which is not visible on routine histological examination (Carpenter *et al.,* 1987).

In an attempt to examine the p53 interactions at various stages in this study subgroups based on established histological criteria were chosen. This is the setting for the two groups of breast tumours (benign and malignant) each one further subdivided into good and poor prognosis based on the conventional pathological criteria. In an attempt to examine the "field" there was an overlap between the good prognosis malignant breast tumours and the poor prognosis benign tumours i.e. eight good prognosis breast tumours contained histological evidence of atypical ductal hyperplasia on the same section. The presence or absence of DCIS was added to the analysis but did not seem to adversely influence prognosis. The p53 dependent apoptotic pathway in a series of melanomas from patients that had known "p53 positive" breast tumours was examined and compared to a control group with only melanomas.

Numerous studies have shown that oncogenic markers in malignant tumours may be related to clinical outcome based on various devised scoring systems (Barnes *et al.,* 1994, Fisher *et al.,* 1994, Saslow-Weinstat *et al.,* 1996). While these markers may aid our treatment of established tumours, it is preventative strategies, or tests which fulfil the criteria for a screening test which are, or would be of greater value. No single

marker has achieved this status. This tells us a frequently reinforced fact that tumourigenesis itself is disorder in the cell cycle and no single aberration will always be present. According to Elston and Ellis .... "most breast cancer is chronic and analysis too early has led to many misleading articles on the value of individual prognostic factors" (Elston & Ellis, 1998). What is desirable is to identify a common initiating event in these tumours and this is where archival studies are important in immunohisto-chemistry. The presence of large banks of tumours in departments of pathology combined with designated breast clinics allows us to initiate these studies.

The breast tumour staging system has established a mode of standardizing reports which with careful audit will improve comparisons of treatment outcomes from various centres.

The aim of this study was to evaluate the expression of these markers in tumours of defined pathological characteristics and known clinical outcome and at the same time explore the interactions of the p53 protein in serial sections of archival paraffin embedded tumours. In the breast it is difficult to define the natural history of tumours because the lesions are neither visible, nor available for endoscopic examination Therefore, archival studies are extremely important in providing information on the "fossils" of cellular interactions. In 1991 Dr. William McGuire discussed the guidelines for the evaluation of potential prognostic factors (Table I). This involves establishing a biological hypothesis to justify the study of the particular factor, followed by a small pilot study.

BIOLOGICAL HYPOTHESIS
PILOT STUDY VS. DEFINITIVE STUDY
SAMPLE SIZE CALCULATION
PATIENT POPULATION BIAS
METHODOLOGICAL VALIDATION
OPTIMIZED CUT-OFF VALUES
REPRODUCIBILITY

TABLE 1. MCGUIRE PROGNOSTIC FACTORS

The biological hypothesis is established and now more definitive studies are required to validate the above findings.

Although the strongest predisposition to breast cancer remains genetic susceptibility (Rosen, 1993), cellular oncoproteins and environmental factors play a significant part in the aetiology and pathogenesis of these common tumours.

3.2 Advantages & Disadvantages of Immunohistochemistry

Immunohistochemistry with microwave antigen retrieval allows us to examine the archaeological remnants of past cellular interactions. It provides a freeze-frame of the last molecular anomalies that occurred prior to surgery and allows us to piece together the growth characteristics of the individual tumour. The staining patterns of the individual oncogenic protein are a reflection of both normal and abnormal reactions. It is the localisation of the proteins combined with our knowledge of their individual activities which enables us to piece together the natural history of the individual tumour. Immunohistochemistry takes advantage of the two unique properties of antigens, namely their immunogenicity (the ability to induce antibody formation) and specific reactivity (the ability to react with the antibodies they induced).

Cytologic specimens, exfoliated cells, needle biopsy specimens and fixed tissues including archival tissue can all be examined for the expression of the p53 protein (Koutselini *et al.,* 1991, Harris CC, 1996). One of the main advantages of this method of study is the ability to examine archival tissue for the expression of various oncogenic proteins and correlate this with known clinical outcomes. There is a correlation between expression of the p53 protein in both paraffin embedded material and frozen tissue specimens (Kerns *et al.,* 1992, Gretarsdottir *et al.,* 1996). Overexpression of the protein in breast tumours has been shown to correlate with mutations in the gene which encodes for the p53 protein (Davidoff *et al.,* 1991, Singh *et al.,* 1993, Andersen *et al.,* 1993).

Population bias is minimized in immunohistochemical assays on archival material as only two very thin sections are needed for a positive test and a negative control (Barnes

et al., 1991, 1992). It also allows the investigator to examine the distribution of the antigen in the malignant and benign tissue in the section and to study its relationship to surrounding normal tissue. Nuclear staining of the tumour cells with the absence of reactivity in surrounding uninvolved tissue, or stroma is considered positive (Fig.s 7.2 - 11.2). The advantages of immunohistochemistry are, of course demonstration of high expression of presumptively mutated protein within the tumour, heterogeneity of expression, co-expression with other tumour markers and ready availability. If only some epitopes are detected with each antibody, a panel may be necessary (Harris H, 1993, Harris CC, 1993, 1996, Baas *et al.,* 1994).

The classification of immunohistochemical staining is subjective and there are differences in the way results are described. Most groups use schemes which divide tumours into four categories, those with no staining and those with low, moderate, and high levels of staining; thus the moderate and high levels are likely to relate to p53 point mutations (Davidoff *et al.,* 1991, Singh *et al.,* 1993, Andersen *et al.,* 1993). Generally, either the slides are scored by individuals, or a computer-aided scoring system based on colour intensity is used. Several distinct patterns of expression have been defined. In some tumours the majority of cells and sometimes all, show p53 expression (Fig.s 10.2 & 11.2), this is often at a very high level giving very intense staining. In contrast, other tumours show variable levels of expression and sometimes only occasional cells stain (Fig. 8.2). These contrasting phenotypes may have a very different biological basis. For example, occurrence of just occasional strongly positive cells in a tumour does not seem to correlate with obvious molecular abnormality of p53 (Barnes *et al.,* 1991, 1992). They may represent the normal working of the p53 system in which the wild type protein accumulates in response to spontaneous genetic errors occurring at a higher frequency in the tumour than in the normal surrounding tissue. In contrast, the presence of strong staining in the majority of cells is frequently associated with mutation (Marchetti *et al.,* 1994, Hall & Lane, 1994, Barnes *et al.,* 1991, 1992).

In recent years, the results of breast cancer research directed at the identification of

prognostic factors has become an increasingly controversial topic. The crux of the problem is the difficulty in comparing results of studies from different laboratories. This is due to the absence of guidelines for methodology (including follow-up time of patients), the probes and immunological reagents used, and the size and characteristics of the tumour panel surveyed. The main disadvantages of immunohistochemistry relate to our embryonic scientific behaviour. It is an almost pure science and the introduction of both a positive and negative control into each session is absolutely essential. Random checks and external validation processes have to be built into the system to ensure continued reliability and reproducibility. Strict controls are essential and once established must be adhered to. If a single stage of the whole process is incorrectly performed it results in the complete loss of valuable histological sections, which are then irretrievable. If the procedure is carried out manually each session may last up to six hours, which ties up members of the laboratory preventing them from participating in other activities. In recent times automated machines which are able to perform the process have become available and these may remove this bottle-neck.

Although the immunohistochemical overexpression of a protein may give us an idea of the genetic lesion, only sequencing the individual gene will confirm, or refute our deductions. This is a major limitation when attempting to prove a genetic link between individuals but it remains useful as a screening tool before the expensive methods of gene sequencing are used.

3.3 Staining Patterns of the Oncoproteins in the Breast

3.3.1 Staining Patterns of the p53 Protein in the Breast

This p53 protein was predominantly detected in the nucleus and reflects the main site of activity of the p53 protein. The protein was predominantly found in the nuclei of malignant breast tumours (Fig.s 9.2 - 11.2), in areas of adjacent *in-situ* disease (Fig.s 7.2 & 8.2) but only infrequent cells stained in the benign glandular structures. Other workers have demonstrated that in a subset of tumours when the protein is localized to the cytoplasm the p53 gene is usually intact (Moll *et al.* 1992). This particular

characteristic pattern was not demonstrated in this study.

In benign tumours less than 1 % of tumours expressed the protein and this was in only a few cells - this occasional cell expressing the protein is probably a reflection of the normal functional p53 which has been induced in response to an anomaly in the cell cycle. These findings appear to be similar to other studies (Bartek *et al.,* 1990, Barnes *et al.,* 1995, Millikan *et al.,* 1995).

3.3.2 Staining Patterns of the MIB-1 Protein in the Breast

The cells that expressed this protein were predominantly malignant and the stain was always nuclear. Examination under a higher magnification frequently revealed an abnormal mitoses within the tumour (Fig.s 1.5 & 2.5). Benign structures tended to have less than 1 % of the cells positive and the presence of the protein tended to mirror known proliferative zones. Cells such as lymphocytes failed to express the protein.

3.3.3 Bcl-2 Localisation in the Breast

The protein was mainly localized to the cytoplasm of the positive cells. There were three distinct staining patterns - (a) in the breast tumours this usually involved the whole tumour staining with the same intensity (Fig.s 3.3 & 4.3) (b) in the benign lobules the cells close to the luminal border stained strongly while the surrounding myoepithelial cells were negative (Fig.s 1.3 & 2.3) - this supports the theory that Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death (Hockenberry *et al.,* 1990) and (c) in the areas of atypical ductal hyperplasia there was generally less intense staining, this probably implies a smaller amount of the protein is required for the inhibition of apoptosis in these tumours, but this needs to be validated (Fig. 4.1). In the high grade tumours the profuse lymphocytic response was highlighted by the positive stain for Bcl-2 protein, while many of the actual tumours were negative (Fig.s 1.3 & 2.3). This maybe explained by the fact that high-grade tumours tended to survive due to a higher proliferative rate and it is well recognised that large tumours frequently do undergo spontaneous necrosis for the same reason (Harris CC & Holstein 1993, Kerr *et al.,* 1994). The inhibition of apoptosis therefore is probably not

the main method of tumour cell survival.

In the patients with fibrocystic disease (n = 30) none of the tumours expressed p53 in more than 50% of their nuclei but 28/30 (93 %) expressed Bcl-2.

3.3.4 Cyclin Localisation in the Breast

The cyclins showed two distinct staining patterns. The cyclin D positive tumours stained both the nucleus and cytoplasm of the positive breast tumours (Fig.s 3.4 & 4.4). The cyclin E predominantly stained the nucleus, although occasionally the vascular endothelium stained positive.

3.3.5 MDM-2 localisation in the Breast

MDM-2 predominantly stained the nucleus and this again was reflection of the proteins site of activity (Fig.s 3.4 & 4.4).

3.4 Clinical Implications of Immunohistochemical Results

3.4.1 Atypical Ductal Hyperplasia and Fibrocystic Disease

Three patients with ADH had a family history of breast cancer (only one had both IDC and ADH). Most patients at diagnosis were between the ages of 41 - 50 (50%), followed by 6 (25 %) between 51 and 60 years of age. Only one was below 30 years of age. This group is by definition a high-risk group and indeed during our period of study two patients developed carcinomas. Atypical ductal hyperplasias and areas of fibrocystic disease have one thing in common they consist of a monoclonal group of cells (Millikan *et al.,* 1995). The predominant finding was that these lesions almost invariably overexpressed the Bcl-2 protein which reinforces the clonal theory of the origin of areas of ADH (Millikan *et al.,* 1995). The staining sessions did not quantify the amount of protein that these tumours expressed. However, it was generally agreed that they never stained with the intensity found in malignant tumours, or indeed in benign lesions adjacent to tumours (Fig.s 3.3, 4.1 & 4.3). This may imply that a smaller quantity of protein is required to inhibit apoptosis in normal and benign structures compared to malignant tissue. These tumours tended to show only a few cells which expressed both the p53 protein and MIB-1 but there was a statistically

significant correlation between this group of tumours (p = .09). This group revealed a subset that overexpressed both the cyclins D and E proteins respectively and the association was statistically significant (p = .008). Furthermore, the cyclin D positive tumours tended to have more cells in cycle as shown by their MIB-1 scores (p = .01). A weaker correlation existed between the cyclin D positive tumours and Bcl-2 (p = .02).

Most areas of ADH expressed p53 in less than 10 % of their tumour nuclei (score = 1). This implies that the nature of the p53 protein that was expressed in the group was the *wild type* protein and these tumours survive by the inhibition of apoptosis, in the presence of an intact apoptotic pathway. This is further reinforced by the Bcl-2 scores with 31 % expressing the protein in all the cells.

The presence of MDM-2 is not unexpected for in some instances it acts in concert with p53 and anomalies are bound to appear as disorder progresses.

Recent reports suggest that area of ADH are completely negative for the cyclin D protein and anomalies in its expression have been proposed as one of the early changes in the malignant transformation involving breast tumours (Saslow-Weinstat *et al.,* 1996). This study has revealed that indeed 2 areas of ADH were positive for both cyclins D and E which is similar to recent reports of one out of eight areas of ADH expressing cyclin D (Gillett *et al.,* 1998), although reports of up to 36 % of areas of ADH expressing the protein exist (Alle *et al.,* 1998). This demonstrates that there are multiple pathways to cell cycle disorder and raises doubts about the candidature of either protein in its present form as the restriction point (or "R") protein. On the other hand, it may reflect the pathological difficulties that arise in distinguishing areas of atypical ductal hyperplasia from *ductal carcinoma-in-situ.* It has been shown that tumour areas that are obvious *ductal carcinoma-in-situ*s on light microscopy have been shown to breach the basement membrane when examined by the electron microscope (Carpenter *et al.,* 1987).

3.4.2 Low Grade Tumours

In this group (n = 38), 17 patients were pre-menopausal, 6 had a family history of breast cancer, 4 were nulliparous and 5 had tumour recurrence within 5 years. The follow-up period before recurrence varied between 20-53 months. This subset of tumours were chosen for their histological and clinical features of good prognosis i.e. tumours less than 2 cm. of low grade and with no positive nodes in the axilla. They were therefore expected to contain fewer detectable anomalies than the poorer prognosis grade III, lymph-node positive tumours. Eight patients had atypical ductal hyperplasia (ADH) on the same section, of these 3 were post menopausal breast tumours and three had DCIS on the same section. One patient had atypical lobular hyperplasia on the same section.

The most striking difference between the 2 subgroups i.e. ADH positive and negative was revealed in the results for the apoptotic proteins - p53 and Bcl-2. In the ADH negative group only two tumours out of thirty (6 %) expressed p53 in greater than 50 % of their tumour nuclei, this is similar to other studies on low grade tumours (Millikan *et al.,* 1995) but three (37.5 %) of the eight tumours that had histological evidence of both carcinoma and ADH on the same section were p53 positive. The presence of ADH increases the risk of subsequent development of breast cancer from between 5.5 to 8 depending on the family history - these immunohistochemical findings therefore support the theory that these patients overall have a more unstable "field" and may therefore require more radical therapy. It is tempting to speculate that (a) if biopsies had been taken a few years prior to their treatment for breast cancer evidence of ADH would have been found and (b) some areas of ADH may have progressed to invasive carcinoma - or indeed one cell deviated from the clonal growth pattern and developed into a tumour. Furthermore, this supports evidence that p53 mutations confer a growth advantage to the breast tumour a late stage in their development. Areas of DCIS were noted on the same section in twelve patients but this did not affect early recurrence on statistical analysis. p53 expression in the areas of DCIS tended to mirror the expression

in the tumour (Fig. 7.2).

Similarly, for the Bcl-2 protein, only one out of eight (12.5 %) of ADH positive tumours scored 3, but 18 (60 %) of ADH negative tumours were positive.

Tumour cells by definition are parasitic and have autonomous, more advanced methods of survival. At least three mechanisms have been documented - the inhibition of apoptosis (Kerr *et al.,* 1972, 1994), increased proliferation (Gilles, 1988) and the production of angiogenetic factors (Baille *et al.,* 1995). Benign tumours which overexpress Bcl-2 survive by the inhibition of apoptosis, as they have lower proliferation indices as measured both by the traditional mitotic count and in recent times the MIB-1 score. It has been well known for many years that there is evidence on the routine histological section of apoptotic bodies further supporting this fact (Kerr *et al.,* 1972) (Fig. 6.2) The Bcl-2 protein was on the luminal side of benign ductal structures (Fig.s 3.3 & 4.3), implying that the higher concentration of the protein was in the cells most likely to undergo apoptosis. The fact that the predominant p53 score in these low-grade tumours (ADH negative) was 1 and 60 % were Bcl-2 positive suggests that the p53 pathway is intact and these tumours may be more amenable to treatment with chemotherapy. Such tumours are more likely to undergo apoptosis in response to the chemotherapeutic agent at a lower dose (Lowe *et al.,* 1993, Fisher *et al.,* 1994, Harris, 1996).

Previous studies have suggested a relationship between Bcl-2 expression and both the ER and PR expression (Gee *et al.,* 1994, Joensu *et al.,* 1995) and linked the activity of the protein to hormonal regulation. Seventy percent of the tumours were ER positive and the high degree of Bcl-2 positive tumours was similar to that found in other studies (Leek *et al.,* 1994, Bharghava *et al.,* 1994, Senna *et al.,* 1995), but this study did not demonstrate the correlation between both markers which had been so widely reported ($p = .11$). This may be due to our smaller sample size, differing patient populations and a different method of scoring oestrogen receptor status. Most T1N0 tumours expressed the p53 protein less in less than 10 % of the tumour nuclei ($n = 10$) and this suggests

that the protein has been induced in response to DNA damage, the response of an intact gene. This is further supported by the fact that p53 expression correlated with ER expression (p = .01 Mann-Whitney). The oestrogen receptor is a transcription factor which has a growth regulatory function and has been shown to interact with p53 in *vivo* (Yu *et al.,* 1997).

There was no correlation of p53 expression with MIB-1, the marker of proliferation. However, MIB-1 expression did correlate with tumour grade (p = .0006) and it has been suggested that this estimation may replace tumour grade in the long term (Veronese *et al.,* 1992, 1995). MIB-1 showed a correlation with grade in both groups of patients with T1N0 tumours i.e. those that were ADH positive (p = .009) and those that were negative (p = .0092) respectively. The expression of the MIB-1 protein is easy to quantify and takes less time than a routine histological section. It is cheap, easily reproducible and permanent records may be kept in the form of both the original slide and a picture of the result which may be attached to the clinical notes (Fig.s 1.5 - 3.5). Only half of the ADH positive IDC's were Bcl-2 positive - this is quite interesting because areas of ADH are believed to arise from anomalies that arise in a single cell and as such a clone of cells would be expected to survive by inhibiting apoptosis (Millikan *et al.,* 1995). A tumour that is derived from breast epithelial cells might also be expected to express Bcl-2 and benign lobular structures on the same section as patients with tumours, overexpressed the Bcl-2 protein (Fig. 3.3). Furthermore, at higher magnification it was obvious that the protein was present only in the cells close to the luminal region of the lobule, the myoepithelial cells around them failing to overexpress the protein (Fig. 3.3 & 4.3). The localization of the protein clearly implies that the inhibition of apoptosis is one of its main functions in these benign structures in the breast lobules. Apoptosis is a feature commonly seen in tumours and presumably the ability to resist apoptosis may seem to offer an advantage to a rapidly growing tumour, by slowing down the cell rate loss. The expression of the protein in the breast tumours was not at the periphery where it is considered is the most active site, nor was the

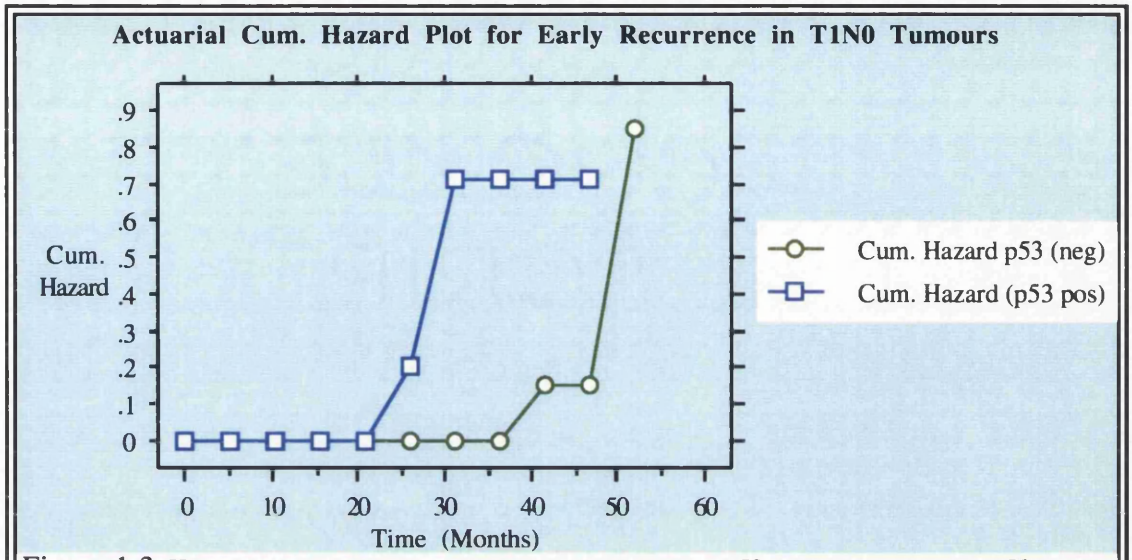

Figure 1.3 HAZARD PLOT FOR RISK OF EARLY RECURRENCE IN P53 POSITIVE TUMOURS. P53 TUMOURS WERE MORE LIKELY TO RECUR EARLIER, THIS WAS STATISTICALLY SIGNIFICANT P = .01.

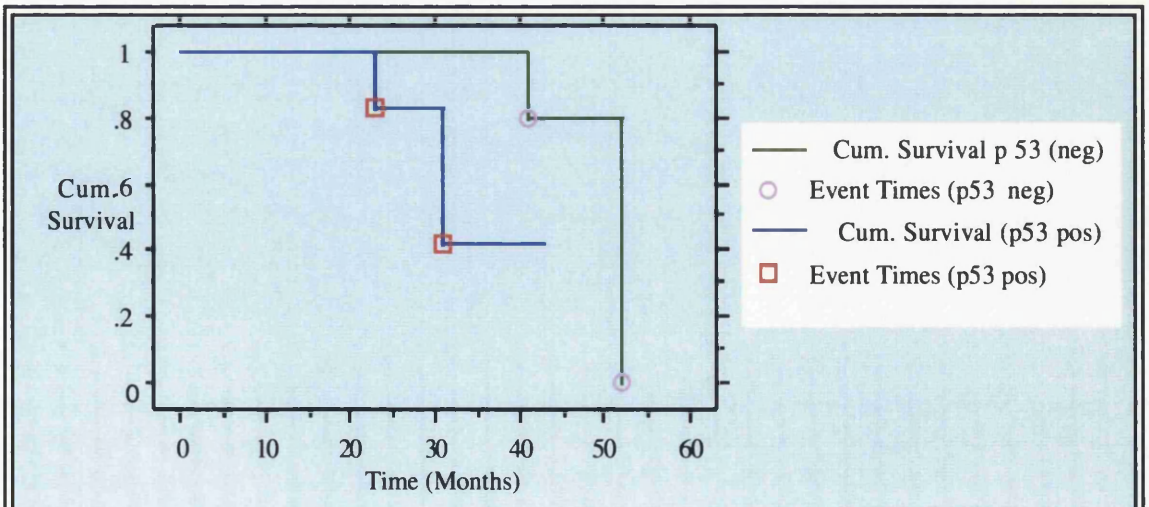

FIGURE 2.3 KAPLAN-MEIER PLOT SHOWING RELATIONSHIP BETWEEN P53 STATUS AND EARLY RECURRENCE FOR LOW-GRADE TUMOURS. P53 POSITIVE TUMOURS WERE MORE LIKELY TO RECUR EARLIER P = .01.

protein present only in the centre of the tumour, rather it was present in all tumour cells (both those in cycle [MIB-1 positive] and those not in cycle [MIB-1 negative]) in Bcl-2 positive T1N0 tumours, implying that each cell is capable of survival by the inhibition of apoptosis. What is more difficult to explain is the correlation between cyclin E and Bcl-2 ($p = .0099$). It must be stated therefore that a subset of tumours which are histologically of good prognosis may possess other genetic lesions which may confer properties of growth survival which cannot be explained simply by the inhibition of apoptosis and may explain their different biological behaviour in the long term.

The importance of immunohistochemical assessment of oncogenic proteins lies in the ability to predict prognosis and therefore, modify clinical treatment modalities.

p53 has been proposed as an independent prognostic factor in node-negative breast tumours (Bartek *et al.*, 1990, Thor *et al.*, 1995). Kaplan-Meier plots showed that patients with T1N0 tumours that were p53 positive were more likely to recur earlier ($p = .01$) (Fig.s 1.3 & 2.3) but Bcl-2 status had no independent prognostic significance (Fig. 3.3). However, patients that were p53 negative and Bcl-2 positive recurred later (Fig. 4.3) but this was not statistically significant. Bcl-2 has not been shown to of independent prognostic significance (Leek *et al.*, 1994, Senna *et al.*, 1995), and this is unequivocally confirmed.

MIB-1 and the oestrogen receptor status were not of prognostic significance nor were any of the other oncogenic markers.

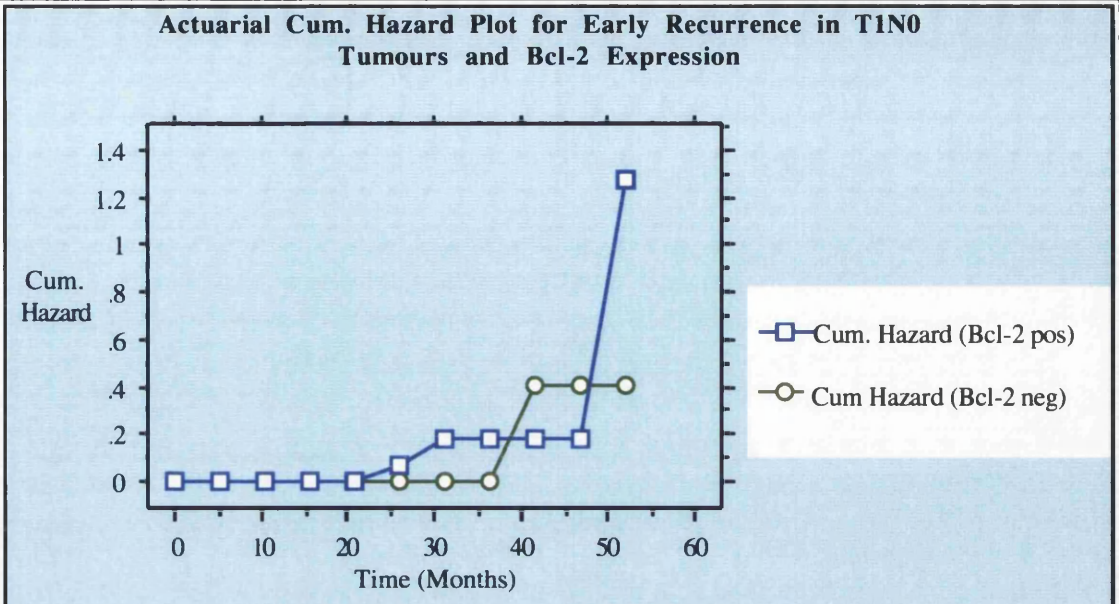

Figure 3.3 HAZARD-PLOT SHOWING RELATIONSHIP BETWEEN BCL-2 POSITIVE AND NEGATIVE TUMOURS AND EARLY RECURRENCE. THERE WAS NO STATISTICAL DIFFERENCE BETWEEN EITHER GROUP.

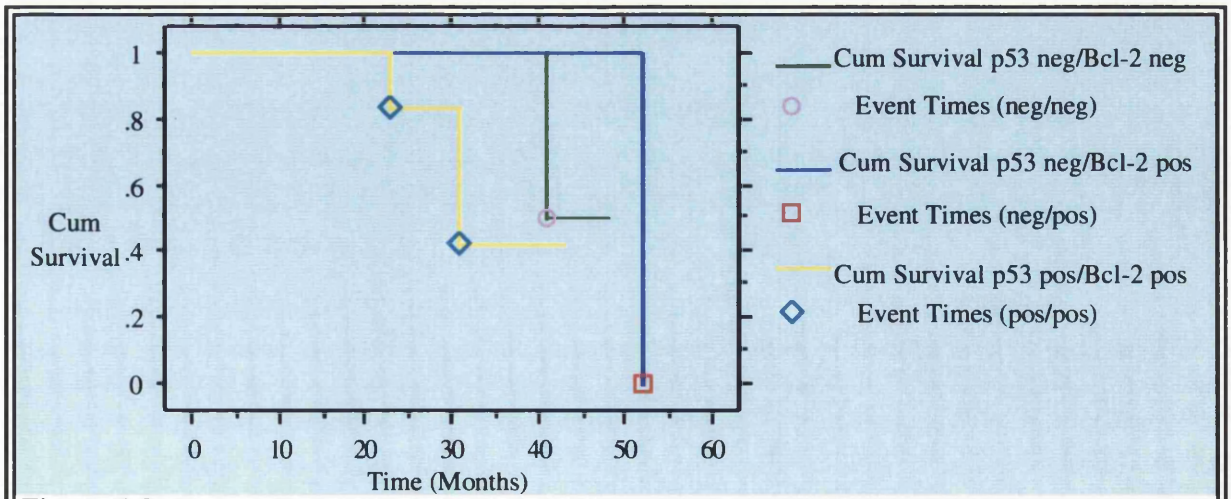

Figure 4.3 KAPLAN MEIER PLOT SHOWING RELATIONSHIP BETWEEN P53 AND BCL-2 STATUS AND EARLY RECURRENCE IN PATIENTS WITH T1N0 TUMOURS. PATIENTS THAT WERE POSITIVE FOR BOTH MARKERS RECURRED EARLIER AND THOSE THAT WERE P53 NEGATIVE AND BCL-2 POSITIVE RECURRED LATER.

3.5 Grade III Tumours

These high grade tumours by definition have a high mitotic count, significant nuclear pleomorphism and very few if any tubules - these are the elements of the routine grading system. In this group, 40 were lymph-node positive, 10 were negative and the status was unknown in 5. The mean age of these patients was 52 years (range 27 - 86). The expression of the 53 protein closely mirrored the histological anomalies, which is strong evidence for the theory that as the complexity of the tumour increases, so do both the malignancy and molecular anomalies increase. Twenty-three (41%) over expressed the p53 protein in greater than 50% of the tumour nuclei (score = 3) and 28 % were completely negative. This is similar to other studies (Thor *et al.,* 1992, Barnes *et al.,* 1995, Michaelides *et al.,* 1996) but direct comparisons are difficult because of different scoring systems. Within the group of p53 negative tumours it is generally accepted that a subset may have null mutations which result in the production of no protein at all (Barnes *et al.,* 1992). Only 8 % expressed the protein in less than 10 % (score = 1) of their nuclei (implying that they probably have intact genes). This group may therefore still have an intact apoptotic pathway and be more responsive to chemotherapy (Harris A, 1992, Harris CC 1996).

The intermediate group is more difficult to define but probably reflect patients with missense mutations, which can only be proven by sequencing the p53 gene.

In this group p53 expression correlated with both MIB-1 ($p < .0001$) and MDM-2 ($p = .014$) respectively. Twenty-two (40%) tumours scored 3 for the MIB-1 protein, with overall 76 % having greater than 10 % of their tumour nuclei in cycle. The protein was frequently note in abnormal mitoses (Fig.s 1.5 & 2.5). This was not an unexpected finding and is in general agreement with most other studies showing a correlation with p53 (Marchetti *et al.,* 1993, Nicholson *et al.,* 1994) high grade and proliferation (Leonardi *et al.,* 1992, Kennedy *et al.,* 1993). Seventy percent were oestrogen receptor negative, as determined by the monoclonal antibody, clone ID5. However, the oestrogen receptor status was not of prognostic significance when combined with other

traditional prognostic factors on actuarial analysis (Fig. 5.3) and it has been suggested that in a subset of positive tumours the receptor itself may be mutated (Veronese *et al.*, 1992, Hulka *et al.*, 1993).

In the breast, the commonest lesions are simple cysts and areas of fibrocystic change which represent the frontline clinical abnormalities. As the cell cycle anomalies acquire increasing complexity, overt tumours result and a spectrum from the well, to the poorly differentiated, aptly represent the increasing genetic insults, which represent frank malignancy. This is clearly shown by the progressive increase in the percentage of tumours which overexpressed the p53 protein, as we moved from the pure fibrocystic disease (< 5 %) to the grade three tumours (41 %).

Areas of DCIS adjacent to frankly invasive tumours overexpressed the p53 protein (Fig. 7.2) this tended to mirror the expression in the invasive ductal carcinoma, but there was no correlation between the presence of DCIS and any of the oncogenic markers.

The Bcl-2 protein is not overexpressed due to a mutation in the gene which controls its production but rather as a consequence of p53 malfunction (Miyashita *et al.*, 1994). The expression of the protein is therefore an indication of the method of cell survival. This is supported by the fact that the protein was present in the luminal cells of the benign glandular structures, but not the supporting myoepithelial elements (Fig.s 3.3 & 4.3) and predominantly in most benign tissue (93 % of fibrocystic tumours). It is logical to assume that it is more likely to be found in mature or well differentiated tissue rather than the poorly differentiated tissues of the grade III tumours. This is supported by other studies (Leek *et al.*, 1994, Bharghava *et al.*, 1994, Senna *et al.*, 1995). Hence, fifty-two percent

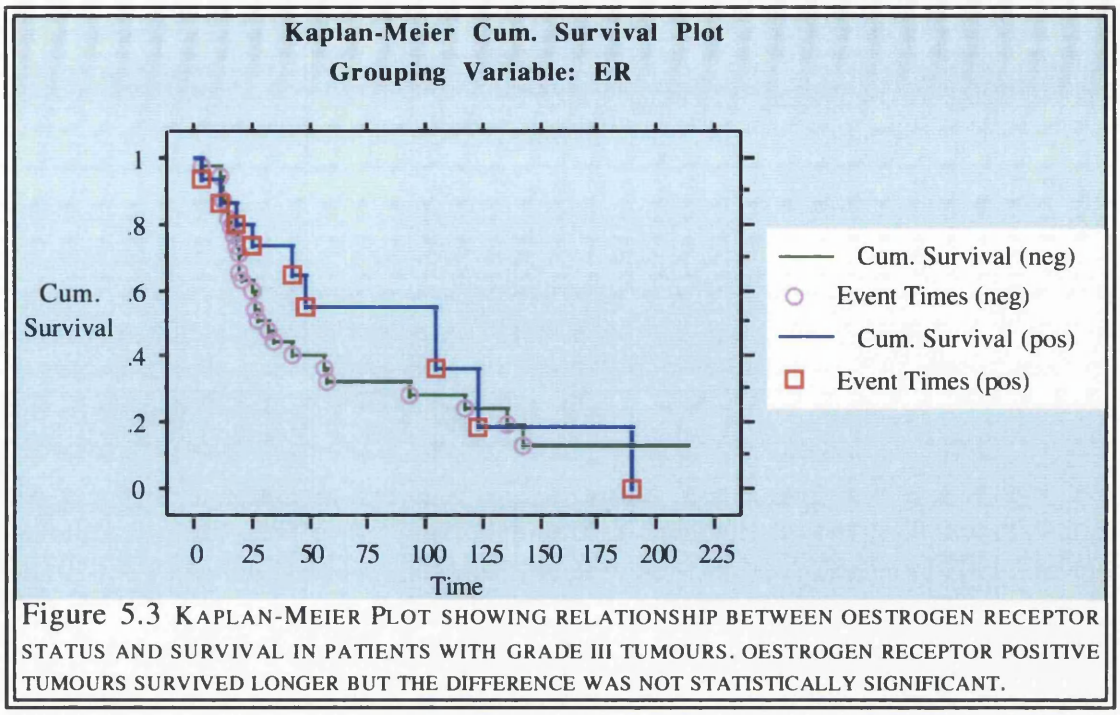

Figure 5.3 KAPLAN-MEIER PLOT SHOWING RELATIONSHIP BETWEEN OESTROGEN RECEPTOR STATUS AND SURVIVAL IN PATIENTS WITH GRADE III TUMOURS. OESTROGEN RECEPTOR POSITIVE TUMOURS SURVIVED LONGER BUT THE DIFFERENCE WAS NOT STATISTICALLY SIGNIFICANT.

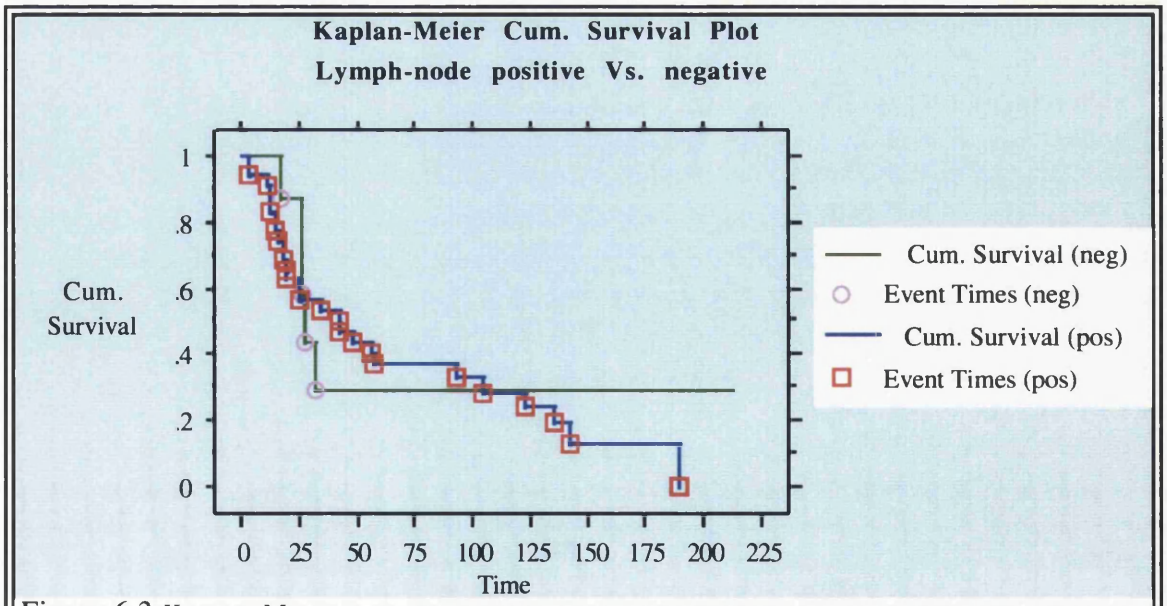

Figure 6.3 KAPLAN-MEIER PLOT SHOWING RELATIONSHIP BETWEEN LYMPH-NODE STATUS AND SURVIVAL IN GRADE III TUMOURS. THERE WAS NO DIFFERENCE IN SURVIVAL BETWEEN EITHER GROUP.

(29) were completely negative.

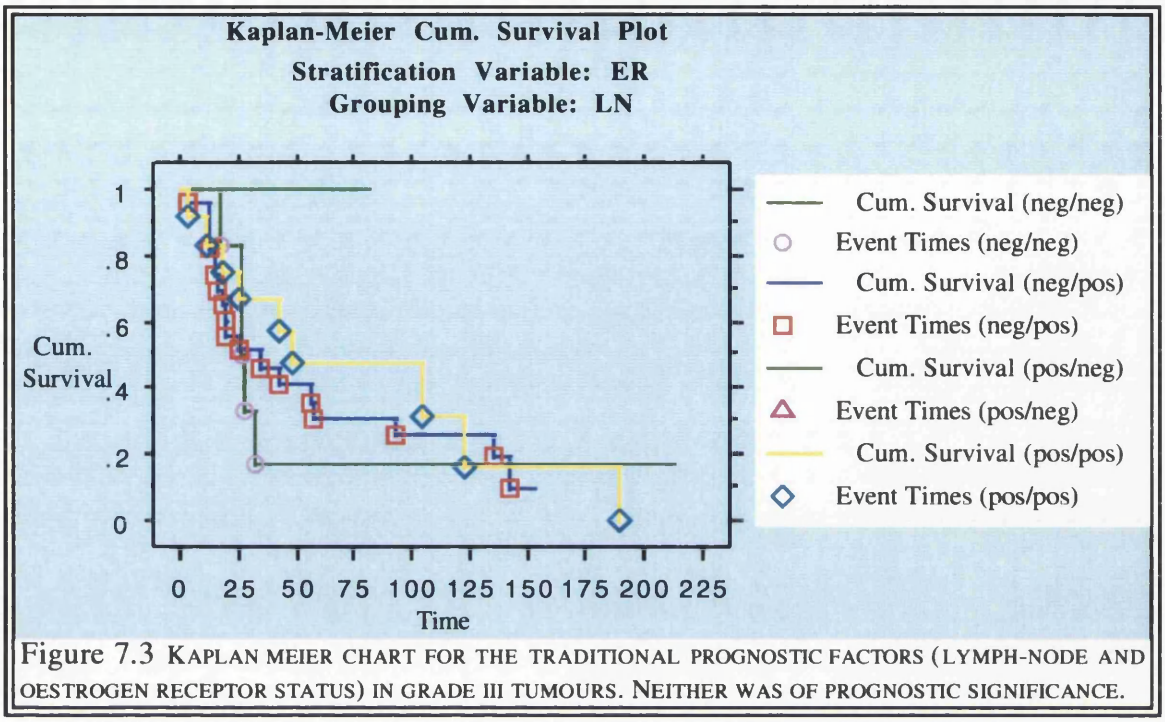

Figure 7.3 KAPLAN MEIER CHART FOR THE TRADITIONAL PROGNOSTIC FACTORS (LYMPH-NODE AND OESTROGEN RECEPTOR STATUS) IN GRADE III TUMOURS. NEITHER WAS OF PROGNOSTIC SIGNIFICANCE.

It is of interest that in some of these Bcl-2 negative tumours the lymphocytes stained positive and served as internal controls (Fig.s 1.3 & 2.3). Only 9 grade III tumours (16%) overexpressed the Bcl-2 protein in greater than 50 % of the tumour nuclei. Although the Bcl-2 and MIB-1 scores combined with the conventional mitotic count point towards uncontrolled proliferation as the method of tumour cell survival, this study does not clearly point towards the molecular mechanisms.

Fifty percent of patients in this group were dead within 24 months and Kaplan Meier plots showed that traditional prognostic factors were unable to predict prognosis with any reliability and although cyclin E positive patients after 24 months survived longer this was not significant (Fig.s 10.3 & 11.3). There was a weak correlation between cyclin E and the oestrogen receptor status ($p = .08$). Cyclin E expression corresponds to a shortened G1 phase of the cell cycle (Keyomarsi *et al.*, 1994, 1996) and its detection in breast tumours has been related to multiple mutated isoforms and poor prognostic factors (Qing-Ping Dou *et al.*, 1996). Less than one third (27 %) actually

scored three, this may be explained in as follows - cyclin E is undoubtedly a marker of proliferation but unlike MIB-1 is unlikely to be expressed in as many cells as its appearance marks the end of G1 phase and it appears probably only at this phase. In the low grade tumours only 6 % achieved a score of 3, confirming previous reports that cyclin E is expressed in high grade tumours and correlates with poor prognosis (Qing-Ping Dou *et al.,* 1996).

The interpretation of p53 overexpression in high grade tumours in the context of clinical outcome is complicated by the fact that a subset of tumours that are completely negative for the protein possess null mutations, which results in the production of no protein at all (Barnes *et al.,* 1992).

It is not uncommon for cells of developing tumours to undergo stress induced by anoxic conditions, or aneuploidy. This would explain why p53 mutations occur later in tumour progression, when stress would confer a selective advantage on cells with p53 mutations. This would explain why only one patient with pure atypical hyperplasia overexpressed the p53 protein, compared with 41 % with high grade breast carcinomas. Mutations would confer resistance to apoptosis - this would represent a net effect to the gain of function of Bcl-2. It has been suggested that in a subset of these tumours the functions of the p53 protein are hampered by sequestration and hence inactivation, by viruses. This has been proposed as a viable explanation for overexpression associated with cytoplasmic location (Riou *et al.,* 1993). This study failed to demonstrate a cytoplasmic location of the protein. This may be a result of differing patient populations, the heterogeneity of breast carcinomas, or differing clinical diagnostic criteria. The p53 protein was almost invariably located to the nucleus and therefore probably associated with mutation in the p53 tumour suppressor gene which encodes it. The strong association with the MIB-1 protein ($p < .0001$) a marker of proliferation, confirms the histologically obvious fact that these tumours are high grade and contain numerous abnormal mitoses. It is therefore not difficult to understand that the progressive increase of p53 positive tumours from 6 - 41 % implies a progressive

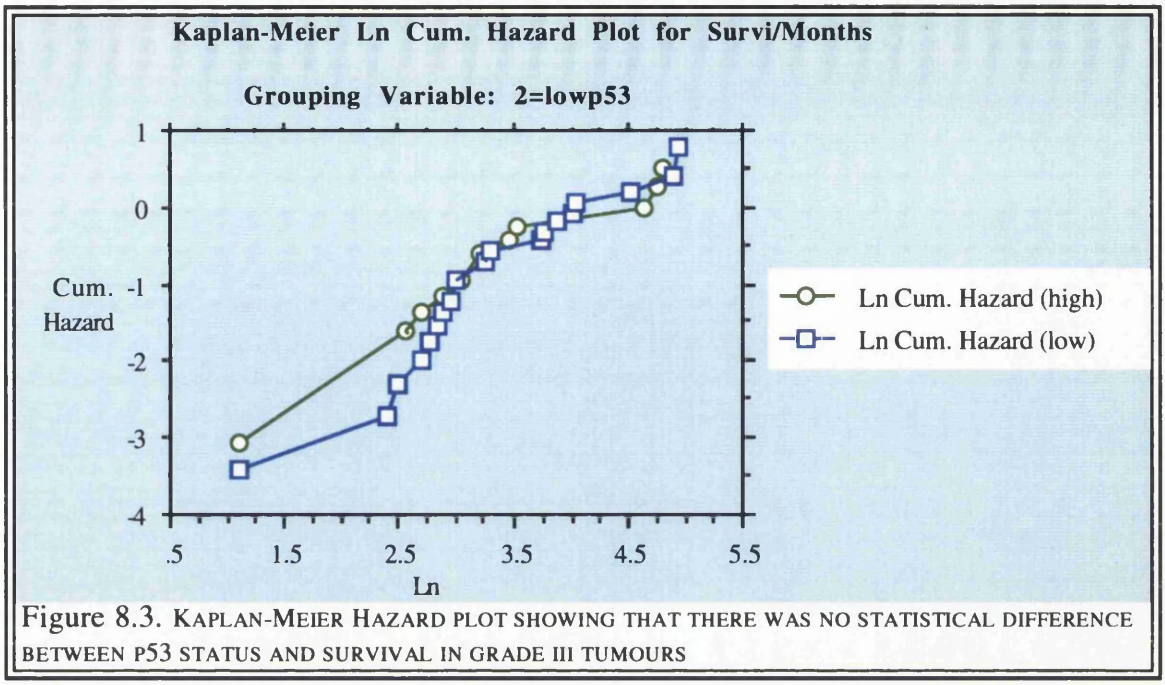

Figure 8.3. KAPLAN-MEIER HAZARD PLOT SHOWING THAT THERE WAS NO STATISTICAL DIFFERENCE BETWEEN P53 STATUS AND SURVIVAL IN GRADE III TUMOURS

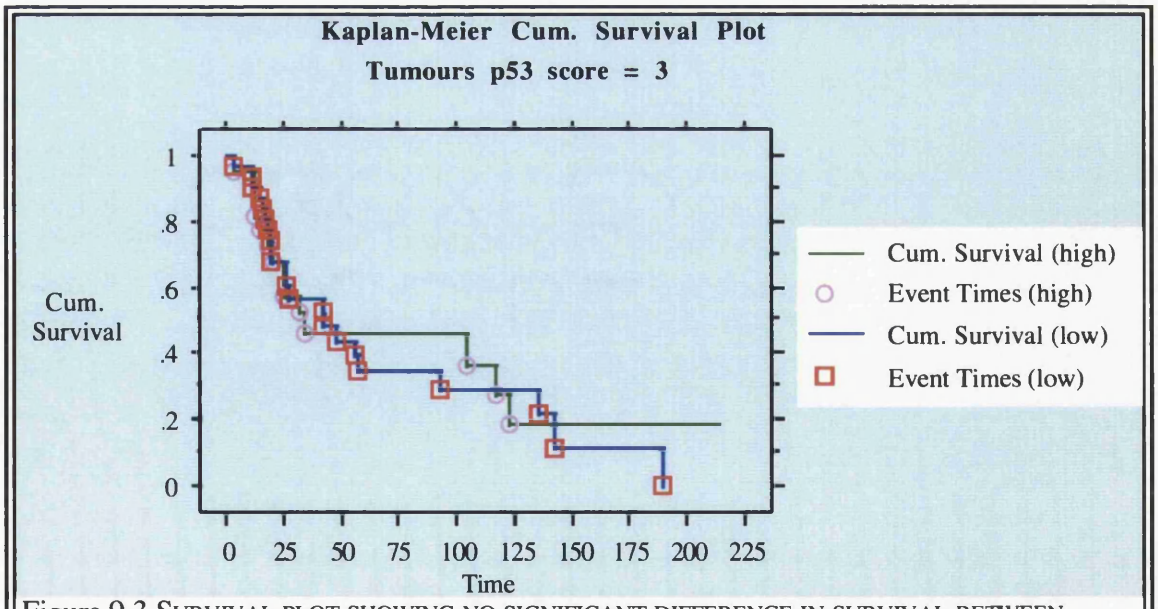

Figure 9.3 SURVIVAL PLOT SHOWING NO SIGNIFICANT DIFFERENCE IN SURVIVAL BETWEEN PATIENTS IRRESPECTIVE OF THEIR P53 STATUS.

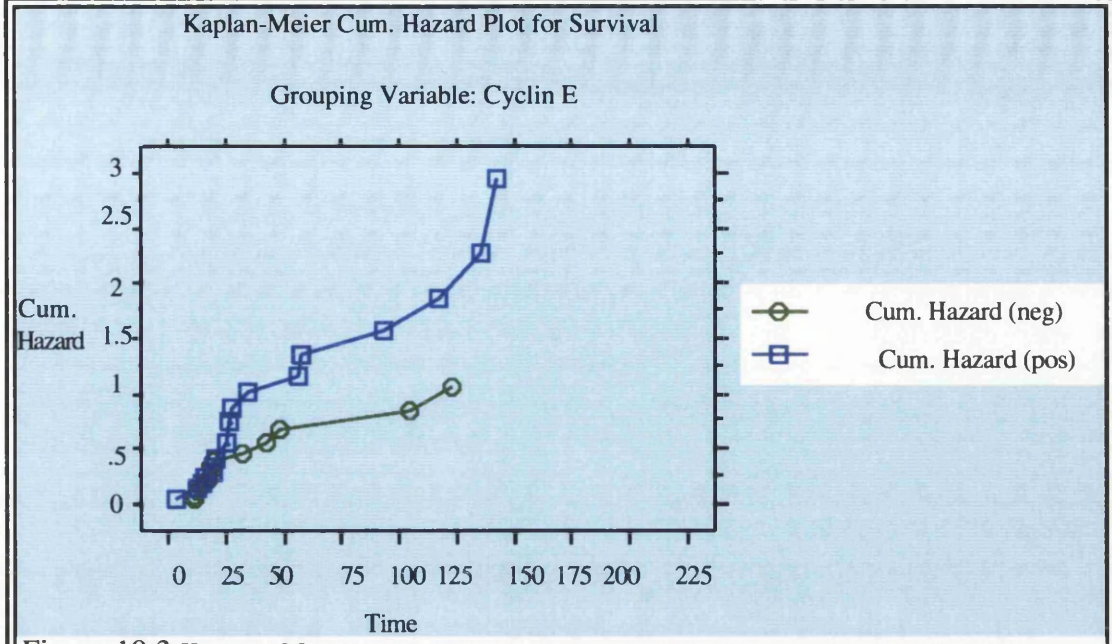

Figure 10.3 KAPLAN-MEIER PLOT SHOWING PATIENTS WITH CYCLIN E POSITIVE TUMOURS SURVIVED FOR SHORTER PERIODS THAN THOSE WITH TUMOURS THAT WERE NEGATIVE. THIS WAS NOT STATISTICALLY SIGNIFICANT P = .1

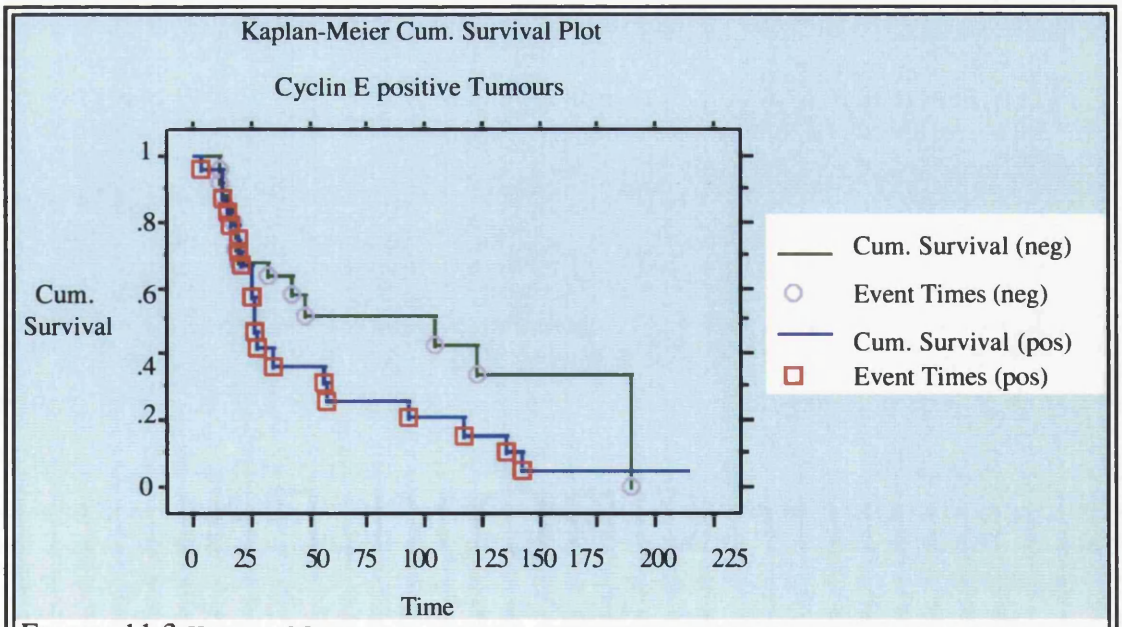

FIGURE 11.3 KAPLAN-MEIER PLOT SHOWING PATIENTS WITH CYCLIN E POSITIVE TUMOURS SURVIVED FOR SHORTER PERIODS THAN THOSE WITH TUMOURS THAT WERE NEGATIVE.

increase in resistance to chemotherapy and an increased risk of early recurrence. This is ironical, for although the grade III tumours had higher proliferation indices (high MIB-1 scores) which implies that they had more cells in cycle, they are less responsive to conventional chemotherapy and this must therefore be attributed to their p53 status. This study demonstrated a correlation between p53 expression and MIB-1 in grade III breast carcinomas ($p < .0001$) but not in T1N0 invasive ductal carcinomas. The subset of tumours that were Bcl-2 positive also tended to be oestrogen receptor positive, this is similar to the findings in less malignant tumours (Leek *et al.,* 1994, Bharghava *et al.,* 1994, Senna *et al.,* 1995).

p53 status did not affect survival, or early death (i.e. within 2 years), however, after 2 years cyclin E positive tumours survived longer ($p = .1$) but this was not statistically significant (Fig.s 12.2 & 13.2).

Further indirect evidence of the presence of a subset of tumours with an intact gene is the distribution of the overexpression of the MDM-2 gene protein product. The most common anomaly detected was overexpression of the MDM-2 protein which occurred in 65 % of tumours - this is lower than some studies the reported positivity varying between 7 - 97 % (McCann *et al.,* 1994, Buenos-Ramos *et al.,* 1996). It has been suggested that the interaction between the two proteins is via an auto regulatory loop (Oliner *et al.,* 1993) i.e. a rise in the level of one results in a fall of the other - this should therefore produce a negative correlation in patients with intact genes. However, the correlation between MDM-2 and both cyclin E ($p = .0008$) and MIB-1 ($p = .0019$) respectively, suggests that rather than an auto regulatory loop, a complete loss of control of the events of the cell cycle. Co-expression of p53 and MDM-2 has been associated with poor prognosis (Buenos-Ramos *et al.,* 1996). A weaker correlation between MDM-2 and the cyclin D which is of questionable statistical significance ($p = .077$) puts the extent of the genetic aberrations and loss of cell cycle control in the patients with the grade III tumours into context. It is significant that 25 % of these tumours expressed the cyclin E protein, which suggests that in these tumours the G1

phase is shortened and may reflect both the degree of proliferation and the disruption of the "R" point. A similar percentage (20 %) overexpressed cyclin D, but there was no correlation between either cyclin group. This may suggest that two separate pathways were followed in this group of tumours and may explain the treatment difficulties and the failure to respond to chemotherapy. Furthermore, both cyclins correlate with the marker of proliferation (MIB-1), which was expected as both cyclins are indirect markers of proliferation but are only present at specific times in the cell cycles. Thus the overall scores of the cyclins were not equal to the MIB-1 scores as only a percentage of cells would be cyclin positive. Furthermore, it suggests that following the disruption of the "R" point, either pathway is possible. One unexpected finding was the association of ER status with cyclin E expression and prolonged survival after 2 years. Although this was not statistically significant it warrant further evaluation and may suggest that when both are co-expressed patients may respond better to chemotherapy.

3.6 Breast Tumours in Patients with either DNS or Malignant Melanomas

There were twenty-two patients, nine with breast cancer and malignant melanomas and 13 with the dysplastic naevus syndrome (Fig. 2.4) and breast cancer respectively. Four patients had a family history of breast carcinoma in a first degree relative, only 2 patients developed carcinoma before the age of 25 years one also had a pancreatic tumour and a primary brain tumour in the same family.

One patient had a twin brother with hypernephroma and a father with lung carcinoma - this patient was nulliparous and had bone secondaries within one year. One patient had a sarcoma (Fig.s 2.2 & 4.2) and two patients had lobular carcinomas and lymph-node positive tumours respectively. Fourteen patients had invasive ductal carcinomas. There were 2 grade III tumours and the rest were grades I-II. The patient with the sarcoma also had a granulosa cell tumour of the vulva. Only 7 were ER positive. It is well recognised that a subset of patients with both breast cancer and malignant melanomas will harbour germline mutations in the p53 tumour suppressor gene. It is not clear however whether patients with the DNS, a putative precursor of malignant melanomas represent a specific subset, or are just one end of the spectrum. Sarcomas are one of the commonest tumours in the Li-Fraumeni syndrome, therefore the fact that one of the tumours was a sarcoma of the breast (Fig.s 2.2 - 4.2) is suggestive but not conclusive evidence that the combination of breast tumours and the dysplastic naevus syndrome may arise due to a defect in the p53 tumour suppressor gene.

The dysplastic naevus syndrome is a clear risk factor for the development of malignant melanomas. However, the association with breast cancer although reported (Lynch *et al.,* 1986) has not been associated with p53 aberrations. Although more than forty percent were positive for the protein there is no evidence that this occurred due to a germline mutation, but this will only be proved by sequencing the gene in these patients.

The correlation between p53 and MIB-1 persisted but the numbers were too small to

draw any conclusions from this.

3.7 Staining Patterns of the Oncoproteins in Malignant Melanomas

3.7.1 p53 in Malignant Melanomas

The protein was detected in three main areas, the dermo-epidermal junction, the melanocytes and the pilosebaceous units. Specimens positive in both tumour and dermo-epidermal junction tended to have more pilosebaceous units positive ($p = .07$) and ($p = .002$) respectively. It is of interest that the staining pattern observed in the melanoma cells was similar to that observed in a patient with Paget's disease of the nipple (Fig.s 8.2 & 9.2) which revealed positive cells in the dermo-epidermal junction as well as the pagetoid cells.

3.7.2 MIB-1 Localization in Malignant Melanoma

MIB-1 protein showed up in the dermo-epidermal junction - a known proliferative zone, in the individual melanocytes, the occasional pilosebaceous unit and in malignant melanoma cells (Fig. 8.3).

3.7.3 Bcl-2 Localization in Malignant Melanoma

In the melanomas the protein showed up at the dermo-epidermal junction as a faint "blush". In the individual melanoma cell it was visible in the cytoplasm - this was clearly shown in both the Alkaline phosphatase (Fig. 7.3) and the Streptavidin biotin methods (Fig. 9.3). The lymphocytic response found in the breast was mirrored in the melanomas and served as an internal control when the melanoma cells were negative (Fig. 9.3) but these were only clearly shown in the alkaline phosphatase method.

3.7.4 HMB-45 Localization in Malignant Melanoma

This stain was used for two reasons (a) to localize the melanoma cells on the histological section and (b) compare the positive cells with similar sections for other stains and confirm the histological diagnosis in cells which did not contain melanin (Fig. 2.2). Ninety-eight percent of tumours were positive and the melanocytes were the positive cells, lymphocytes were negative. This is in contrast to the Bcl-2 protein which stained both the melanocytes and the lymphocytes.

3.8 Malignant Melanomas

The seventy-four melanomas had a mean age of 51 years (range 1 - 87). Half of the sixty-six control melanomas (51 %) were located on a limb. Eleven and 13 % were located on the back and chest respectively (Fig. 8.2). A similar distribution was found in the nine patients with both malignant melanomas and breast cancer with 70 % having tumours on either the lower or upper limb. Twenty-two percent of the melanoma patients were aged between 41 and 50 years (Fig. 7.2) which is similar to the national average (OPCS, 1992). Forty percent were either level I or II melanomas, with 21 % and 33 % in level III or IV respectively. Only 6 % were level V (Fig. 10.2). Thirty-nine percent were relative good prognosis melanomas with a Breslow's thickness measurement less than 0.7 millimetre, but 36 % were over 1 millimetre thick (Fig. 9.2). The traditional, best known prognostic factors in melanomas are the Breslow's thickness measurement and the Clark's level of invasion, both are indirect measurements of the growth of the melanoma and statistically correlated with each other ($p < .0001$). It would therefore seem logical that estimation of a proliferative marker (MIB-1) would correlate with either measurement, but this was not the case. This can be explained in two ways - every bit of skin has a proliferative zone and the rate of proliferation has been reported to vary between 0.8 - 18.5 %. While an increased proliferative index of the individual skin may predispose to tumour formation, once this is induced, survival of the melanoma cell then occurs via other mechanisms apart from proliferation. This may indeed be related to the inhibition of apoptosis, for clinical observation clearly shows that even the neglected melanoma does not achieve considerable size, indeed Handley's initial description of a neglected melanoma recommended a five centimetre resection margin. However, in contrast to other cutaneous malignancies it tends to metastasize (Ball & Thomas, 1995).

It is interesting, but not altogether unexpected that tumours that overexpressed the p53 protein in the dermo-epidermal junction were more likely to harbour the protein in their nuclei.

Forty-six percent of melanomas achieved a score of 3, 22 % were completely negative and 18 % scored 1 (Table. 16.2, Figure 10.2). The two groups i.e scores zero and 1 probably consist of patients with intact p53 genes and the p53 detected represents normal p53 - indeed it has been shown that p53 is induced in response to sunlight and has been found in up to 28 % of skin biopsies in patients with other skin tumours (Barnes *et al.,* 1992).

Of the nine melanomas in patients with breast cancer two of the melanomas were melanomas *in-situ,* there was one each of the nodular, lentigo maligna melanoma and conjunctival melanoma (treated by enucleation) respectively. One patient had bilateral breast cancer, another had multiple malignancies and one died with melanoma secondaries in the scalp.

All the patients who had malignant breast tumours overexpressed the p53 protein. p53 expression in the melanoma cells correlated with over-expression, in the dermo-epidermal junction, of both p53 ($p < .0001$) and MIB-1 ($p = .02$), but not the MIB-1 score in the melanomas ($p = .21$). This reinforces the assumption that while the degree of proliferation in the skin may be critical in the initial development of the tumour, the melanoma cells do not then proliferate once transformation has occurred. Almost 40 % of tumours showed only the occasional cell positive for the MIB-1 protein, with just under 10 % having no cells in the tumour in cycle. This was mirrored by the expression of MIB-1 in the dermo-epidermal junction with 40 % having a score of 1 and only 9 % having all cells positive. Less than a quarter of specimens (23.7 %) showed evidence of significant overexpression of p53 at the dermo-epidermal junction and 42 % showed no activity at all. This lends weight to the suggestion by other workers that p53 expression in melanomas may only appear at a late phase of the development of these tumours (Albino *et al.,* 1992). Clark's level of invasion correlated with age ($p = .03$) and the MIB-1 score in the dermo-epidermal junction ($p = .05$) but not with any of the other oncogenic markers. Thirty-nine percent of melanomas scored 3 for Bcl-2 and only 16.9 % were completely negative. Furthermore, Bcl-2 expression in the melanomas

correlated with Breslow's thickness measurement (p = .08) and 46 % of tumours showed Bcl-2 positive reactive lymphocytes, reinforcing the suggestion that a significant proportion of melanomas survive by the inhibition of apoptosis.

Although the classic prognostic factors - Breslow's thickness and Clark's level suggest that proliferation is the key to understanding melanomas - this is by no means the case. The so called "thin melanoma" that metastasizes early decries this. In recent times angiogenetic factors and new prognostic indices have been introduced to get round these "odd cases".

Conclusion

This work does not establish a link between breast cancer, malignant melanoma and the dysplastic naevus syndrome. To prove a link would require extensive genealogical studies. Although all the tumours in patients with both breast cancer and malignant melanomas were "p53 positive"only sequencing the p53 gene would establish if they truly have the Li-Fraumeni syndrome and therefore germline mutations in the p53 gene. There were more p53 positive tumours in the patients with DNS, but this may be due to the higher grade of these tumours. Similar percentages in both tumour groups (breast cancer and malignant melanomas) were p53 positive, with higher grade tumours having more p53 positive cells.

However, the study establishes an argument for the evaluation of routine immunohisto-chemistry in the assessment of clinical outcome for breast and malignant melanomas. Molecular anomalies present in tumours excised over fifteen years ago may be examined and correlated with clinical outcome. The techniques are easily learnt, reproducible and cheap and armed with a working knowledge of the activities of these proteins may enhance our understanding of both groups of tumours and may modify our treatment modalities. However, larger studies exist which reveal that these factors may be of value only on univariate analysis (Pinder, *et al.,* 1998, Elston & Ellis, 1998).

The tumour numbers in each subset of breast tumours was small but establish that at least for low grade lymph-node negative tumours a prospectively validated study may be difficult to argue against. Bcl-2 has not shown any prognostic significance but the specific localisation in the endoluminal cells may act as a vehicle for the much touted gene therapy.

The cyclins may both be the "restriction point proteins" but are not of value in evaluation of prognosis in breast tumours. However, it must be stated that at the time of this study monoclonal antibodies were provided freeze-dried and it may well be that in the future more stable antibodies may be worth evaluating in relation to clinical

outcome.

MDM-2 has been associated with both good and poor prognosis tumours, but it does not appear to be of prognostic significance in breast tumours however, a significant number of high grade tumours will harbour an anomaly in its activity.

The monoclonal antibodies present for the evaluation of the oestrogen receptor have now become so reliable that it is no longer acceptable for any woman undergoing treatment for breast cancer to miss out on this estimation, although its prognostic significance on its own may be limited. It is valuable in predicting the hormone responsiveness of the individual patient (Pinder *et al.,* 1998).

The estimation of the mitotic count of the individual breast tumour will continue to be important in assessment of tumour grade - reliably done it is irreplaceable, but it is time consuming and anecdotal evidence suggests that there is great individual variation in its application (Hall & Coates, 1995). Furthermore, the mitotic count decreases by up to 50 % if the tumour is not processed within six hours (Pinder *et al.,* 1998) and it is well known that mitotic events proceed to completion after excision therefore, fewer mitoses are visible in the final section. This is as yet the only true argument for the routine introduction of assessment by the monoclonal antibody MIB-1. Provided there are internal and external controls it is cheap, reliable and easily reproducible and copies of the tumour stains amy be kept in the patient notes. A formal comparison between the mitotic count and apoptotic bodies and MIB-1 and Bcl-2 respectively in both sets of tumours may have revealed some interesting findings.

There is still no general agreement on the scoring methods in immunohistochemistry, with each group using differing methods of quantification, therefore, comparisons are difficult (Hall & Coates, 1995). In some cases rather than a single antibody, a cocktail of antibodies is used (Allred *et al.,* 1993), this is one reason why individual departments may need to validate each new prognostic factor.

Malignant melanomas fail to reach considerable size but afflict a young population before their prime. This study suggests that a subset of individuals with an active

dermo-epidermal junction acquire p53 anomalies which result in p53 positive melanomas. The correlation with Bcl-2 suggests a lesion in the apoptotic pathway may hold some as yet unanswered questions. The most significant result was the correlation of Bcl-2 with Breslow's thickness measurement but this in itself is not enough to suggest the routine evaluation of the protein. It however lays the ground for a larger study of patients with melanomas of varying thickness. Finally, it raises the questions of the possibility of "p53 inducers" in malignant melanomas and provides the basis for further work into the activities of the largest organ in the body - the skin.

Breast Tumours a Hypothesis

Based on the finding of this work and the present state of our knowledge a hypothesis on the natural history of breast tumours is proposed.

Almost all breast lesions result in an increased relative risk for the development of breast cancer rising from 1.5 for the pure fibrocystic disease to 5 in patients with atypical ductal hyperplasia. There is evidence that these benign proliferations arise from monoclonal aberrations and probably survive by the inhibition of apoptosis (Bcl-2 positive cells). As the disorder in the cell cycle increases the low-grade tumours appear, the cells continue to survive by the inhibition of apoptosis but rather than arising from a monoclonal population they arise from multiple cells proliferating at different rates - producing a differential score for the marker of proliferation (MIB-1) and the cyclins - which are also markers of proliferation. The lower grade tumours, exhibit less loss of cell cycle control (lower cyclin scores) are probably more responsive to chemotherapy and more likely to die by apoptosis (low p53 and high Bcl-2 scores).

Tumour recurrence may occur up to fifteen years after the initial excision and raises the possibility that nests of cells which may have been controlled by the primary tumour have remained in a state of "suspended animation" until such a time that a favourable environment reappears. Finally, the high grade tumours have high mitotic counts and MIB-1 scores respectively. These high-grade tumours consist of multiple groups of cells capable of varied individual propagation and possess multiple genetic aberrations

in the cell cycle proteins. The high grade tumours are unpredictable in their response to either chemotherapy, or radiotherapy and therefore traditional, pathological prognostic features are unreliable. It may be possible, or indeed necessary in the future to assess the individual tumour with a panel of antibodies to determine what genetic aberration is predominant in order to plan therapy. This is the promise of genetic engineering.

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Appendix I

Table of Results for Breast Cancer Patients with either Malignant Melanomas or DNS

Frequency Distribution for P53				
From (≥)	To (<)	Count	Rel. Freq.	Percent
0.000	1.000	7	.412	41.176
1.000	2.000	1	.059	5.882
2.000	3.000	1	.059	5.882
3.000	4.000	8	.471	47.059
	Total	17	1.000	100.000

Frequency Distribution for MIB-1				
From (≥)	To (<)	Count	Rel. Freq.	Percent
0.000	1.000	1	.059	5.882
1.000	2.000	5	.294	29.412
2.000	3.000	7	.412	41.176
3.000	4.000	4	.235	23.529
	Total	17	1.000	100.000

Frequency Distribution for Bcl2				
From (≥)	To (<)	Count	Rel. Freq.	Percent
0.000	1.000	4	.235	23.529
1.000	2.000	2	.118	11.765
2.000	3.000	1	.059	5.882
3.000	4.000	10	.588	58.824
	Total	17	1.000	100.000

Frequency Distribution for CD				
From (≥)	To (<)	Count	Rel. Freq.	Percent
0.000	1.000	5	.333	33.333
1.000	2.000	3	.200	20.000
2.000	3.000	5	.333	33.333
3.000	4.000	2	.133	13.333
	Total	15	1.000	100.000

Frequency Distribution for CE				
From (≥)	To (<)	Count	Rel. Freq.	Percent
0.000	1.000	2	.167	16.667
1.000	2.000	3	.250	25.000
2.000	3.000	4	.333	33.333
3.000	4.000	3	.250	25.000
	Total	12	1.000	100.000

Frequency Distribution for MDM				
From (≥)	To (<)	Count	Rel. Freq.	Percent
0.000	1.000	1	.071	7.143
1.000	2.000	1	.071	7.143
2.000	3.000	0	0.000	0.000
3.000	4.000	12	.857	85.714
	Total	14	1.000	100.000

Frequency Distribution for ER			
	Count	Rel. Freq.	Percent
neg	10	.588	58.824
pos	7	.412	41.176
Total	17	1.000	100.000

APPENDIX II
Clinical Details and Oncogene Scores for Grade III Tumours

path no.	Survi/Months	age	p53	MIB-1	Bcl-2	CD	CE	MDM	LN	ER
78.2513	213	51	3	2	0	1	2	2	neg	neg
78.2661267	142	56	0	1	0	3	3	2	pos	neg
78.6939	135	72	0	1	1	1	3		pos	neg
79.5923	123	44	3	2	3	3	1	3	pos	pos
79.6322	82	34	2	2	1			2	neg	pos
80.7137	189	57	0	2	0	3	0	3	pos	pos
80.7752	48	41	1	1	0	1	0	3	pos	pos
80.8827	43	51	0	1	0	0	0	2	pos	neg
81.1763	32	50	3	2	0	0	1	3	neg	neg
81.2581	18	43	3	3	3	2	1	3	pos	pos
81.5078	3	81	3	3	0	0	2	3	pos	neg
81.6347	26	33	2	2	0	2	3		neg	neg
81.6353	26	33	2	1	0	1	2	3	neg	neg
82.5944	42	29	0	3	0	0		3	pos	pos
83.4031	**3**	29	2	1	0	0		0	pos	pos
83.4804	153	83	3	3	0	3	1	3	pos	neg
84.0732	**13**	63	3	3	0	2	3	3	pos	neg
84.376	141	59	2	2	2	1	1	2	pos	neg
85.3395	129	63	0	1	2	3	0	1	pos	neg
85.3994	**17**	60	0	3	2	0	1	0	neg	neg
85.5695	37	78	2	2	1	1	1	3	pos	pos
86.5261	117	58	3	3	0	2	2	2		neg
86.5548	**19**	82	2	2	2	0	2	3	pos	neg
86.6341	25	86	0	2	3	3	3	3	neg	pos
87.8852	105	46	3	3	3	0	1	3	pos	pos
88.3173	**18**	27	3	3	0	0	1	3	pos	neg

APPENDIX II
Clinical Details and Oncogene Scores for Grade III Tumours

88.3647	93	52	2	2	2	2	3	3	pos	neg
88.3931	25	42	3	3	0	3	3	3	pos	pos
88.4314	**20**	72	0	1	0	3	2	3	pos	neg
88.5807	56	43	0	1	2	0	3	3	pos	neg
88.7874	15	47	2	1	0	2	0	2	pos	neg
89.4967	21	79	3	3	2	3	2	2		neg
89.7571	**15**	47	3	3	0	0	2	3	pos	neg
89.9383	**25**	30	3	3	3	0	3	3	pos	neg
89.9513		60	3						pos	
90.1301		75	2	2	0	0	2	3		pos
90.5737	57	59	3	0	1	1	1		pos	pos
90.7279	57	51	1	2	3	2	0	2	pos	pos
90.8156	**18**	38	2	3	0	2	1	3	pos	neg
91.0344	34	75	3	3	0	0	3	3	pos	neg
91.1391	**12**	50	1	2	3	1	1	3	pos	pos
91.2489	**13**	47	3	2	0	3	2	3	pos	neg
91.2689	13	60	3	3	3	3	1	3	pos	neg
91.6177	**2**	45	0	2	1	0	3	3	neg	neg
91.8022	57	64	0	2	0	0	3	3	pos	neg
91.8491	16	29	1	2	3	3	3	3	pos	neg
91.9108	27	65	1	2	0	3	3	3	neg	neg
92.174	20	43	0	3	0	0	2	3		neg
92.7673	45	38	3	3	1	2	2	3		neg
93.2297	**11**	40	0	0	1	0	0	3		neg
93.547	33	41	3	3	2	1	0	3	pos	neg
93.7855	33	44	2	2	2	1	1	3		neg
93.8783	26	48	0	1	0			2		pos

APPENDIX II
Clinical Details and Oncogene Scores for Grade III Tumours

93.9739	33	46	3	3	0	0	0	0		neg
94.4921	21	32	3	3	1	0	2	3		neg
95.1777	**21**	46	0	3	0	2	2	3		neg

APPENDIX III — Oncogene Scores for Patients with Grade I-II Tumours and Atypical Ductal Hyperpasia

DOB	p53	MIB-1	Bcl-2	CD	CE	Grade	ER
16844	1	1	2	2		1	pos
5031	0	3	1	2	0	2	pos
15885	P	3	3	2	1	2	neg
12867	0	1	1	1	0	1	pos
7939	1	3	3	1	2	2	pos
10789	1	3	3	0	2	2	pos
17478	3	1	3	3	1	1	neg
5959		3	3	2	1	1	neg
5856	2	1	3	1	1	1	pos
7501	0	1	0	2	0	1	pos
5523	1	3	3	2	1	2	pos
6715	0	0	3	1	1	1	pos
7912	0	2	2	3		2	pos
8929	0	3	0	2	2	1	pos
6539	0	2	2	3	0	2	pos
7896	0	1	0	3		1	pos
11654	0	3	2	3	1	1	pos
15608	2	3	3	0	1	2	neg
11142	0	2	3	0	2	2	pos
2979	2	3	3		2	2	pos
14954	1	3	3	3	2		pos
7406	1	3	3	3	2	2	pos
12866	2	3	3	3	3	2	neg
11229	1	3	3	2	0	1	pos
15907	1	3	3	3	1	2	neg
4563	1	3	3	1	2	2	neg
13787	3	2	3	0	1	2	pos
10182	1	3	2	3	3	2	pos
3930	2	1	2	1	0	1	neg
10685	2	1	0	1	1		pos
5840	0	1	0	0	0	3	neg
14769	0	2	0		0		neg
12367	3	1	2	0	0	2	pos
4146	2		3	1	1	2	pos
17279	3	1	3	3			neg
17027	0	1	3	0	1	3	pos
11340	3	1	3	0	1	2	neg

APPENDIX IV
Oncogene Scores for Malignant Melanomas

Age	Path no.	p53 Tumour	p53DEJ	p53SG	Bcl-2	Bcl-2LN
58	86.03799	3	3	pos	3	neg
77	87.04111	3	3	pos	3	pos
30	87.04467	1	1	pos	1	neg
55	87.05519	3	3	pos	3	neg
61	87.09292	3	3	pos	3	pos
27	88.01567	1	1	neg	1	pos
48	88.02884	3	3	neg	3	neg
72	88.6303				0	neg
45	88.7069	3	3	pos	3	neg
61	89.00322	0	0	neg	0	pos
67	89.01208	2	0	neg	2	pos
	89.02022	2	0	neg	2	neg
	89.02876	3	0	neg	3	neg
33	89.02877	3	3	pos	3	pos
52	89.05803	2	2	neg	2	neg
80	89.062	3	0	neg	3	neg
51	89.09626	0	0	neg	0	neg
23	89.09707	0	0	neg	0	neg
62	90.02114	3	3	neg	3	neg
81	90.02885	3	2	neg	3	pos
41	90.03339	0	0	neg	0	pos
36	90.03598	3	3	pos	3	pos
15	90.04614	2	1	pos	2	pos
39	90.07335	3	1	pos	3	neg
	90.09154			neg	-	neg
	90.4999	2	0	neg	2	pos
63	91.00327	3	2	pos	3	neg
79	91.01027	1	1	pos	1	pos
29	91.01728	3	1	pos	3	neg
27	91.03191	3	3	pos	3	pos
	91.03212					neg
12	91.05098	0	0	pos	0	neg
52	91.05663	1	0	pos	1	neg
47	91.08582	3	2	neg	3	pos
65	91.10773	3	0	neg	3	pos
26	92.0085					neg
29	92.01401	1	1	pos	2	pos
82	92.0277	1	0	pos	3	neg
73	92.04055	1	0	neg	2	neg
76	92.07223	3				neg
54	92.09501	3	0	pos	1	pos
48	93.00771				1	neg
44	93.0824002	3	0	neg	1	pos
24	93.08496	0	1	pos	2	neg
45	93.0851602	3	3	neg	1	pos
42	93.10664	1	1	neg	-	neg
43	93.11213	0	0	neg	1	neg
87	93.1203	0	0	neg	0	neg
71	94.00243	3	1	neg	3	neg

APPENDIX IV
Oncogene Scores for Malignant Melanomas

82	94.01484	2	0	neg	0	pos
71	94.01913	3	3	neg		neg
34	94.02355	1	1	neg	1	pos
87	94.03972	3	3	pos	3	pos
47	94.08469	0	0	pos	1	pos
1	94.08868	2	2	pos	1	pos
79	94.08987	0	0	neg	1	pos
59	94.09305	3	1	pos	0	neg
55	94.11988	3	3	pos	0	pos
50	94.1258	0	0	neg	3	pos
33	95.01008	2	1	neg	3	pos
49	95.01643	1	1	pos	1	neg
81	95.02181	1	0	neg	1	pos
56	95.04063	0	0	neg	1	neg
37	95.04204	3	1	pos	1	pos
49	95.04969	0	0	neg	2	neg