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by

Tomas Walsh BSc (Galway), MSc (Leicester)

Department of Pathology

University of Leicester

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ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

For Mary and Padraic

Abstract

Despite extensive research the pathways of breast cancer development remain largely unknown. The identification of key genetic alterations, particularly at the early stages of the disease, are central to elucidating the developmental pathways for this disease.

Pure populations of tumour cells were microdissected from well defined groups of breast lesions, comprising: ductal carcinoma in situ (DCIS) tubular carcinomas, and mammographically-detected, impalpable early stage, moderately to well differentiated invasive carcinomas, and analysed for alterations in polymorphic tandem repetitive sequences (microsatellites). This enabled analysis of microsatellite instability (MI), which has been demonstrated to be indicative of a mutator phenotype in colorectal cancer, and loss of heterozygosity (LOH), which may indicate the presence of a tumour suppressor gene.

MI was demonstrated to be a tumour specific alteration not present in benign proliferative disorders. It was present in 8 of 11 (73%) high grade lesions of DCIS, but only at a low frequency in low grade DCIS and invasive carcinomas and was absent from the tubular carcinomas. Two distinct types of alteration were observed: alterations to a single trinucleotide repeat (DM-1), and alterations of multiple microsatellite loci. Cases demonstrating this phenotype did not show alterations of candidate DNA repair genes (MSH2, MLH1 and PMS2), or in key cancer associated loci (TGFβRII, IGFIIR, Bax, and E2F-4), indicating that this phenotype is distinct to that described in colorectal tumours.

LOH studies were focused on chromosome 16q21-24.4, a site for which there is evidence of alteration in the early stages of the disease. A high frequency of LOH (greater than 40%) was observed in all carcinomas. A candidate tumour suppressor gene, E-cadherin, mapping to this region, was not found to be mutated in these cases demonstrating LOH. In vivo experiments suggested that this gene could be silenced by aberrant methylation.

In summary, MI was associated with high grade lesions, whereas LOH at 16q was observed at similar frequencies in all the carcinomas, possibly reflecting different roles in the development and progression of breast cancer.

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Abbreviations

ACF	Aberrant Cryptic Foci
ADH	Atypical Ductal Hyperplasia
Adr	Adriamycin Resistant
AH	Atypical Hyperplasia
ALH	Atypical Lobular Hyperplasia
APC	Adenomatous Polyposis Coli
AR	Androgen Receptor
AT	Ataxia Telangiectasia
ATM	Ataxia Telangiectasia *Mutated*
ATPγS	Adenosine-5'-O-(3-Thiotriphosphate)
bp	Base Pair
BSA	Bovine Serum Albumin
BW	Binding And Washing
CASH	Cancer And Steroid Hormone Study
cd	Codon Position
cdk	Cyclin Dependent Kinase
CGH	Comparative Genomic Hybridisation
cm	Centimeter
dd	Deionised
DAB	Diaminobenzidine
dATP	2'-Deoxyadenosine 5'-Triphosphate
DCC	Deleted In Colorectal Cancer
DCIS	Ductal Carcinoma In Situ
DMEM	Dulbecco's Modification Of Eagles Media
dNTP	2'-Deoxynucleotide 5'-Triphosphate
DM	Distant Metastasis
DNA	Deoxyribonucleic Acid
DRPLA	Denatorubral-Palliduysian Atrophy
ECL	Enhanced Chemilumesence
E. coli	*Escherichia coli*
EDTA	Disodium Ethylene Diamine Tetraacetate
ER	Oestrogen Receptor
EST	Expressed Sequence Tags
FAP	Familial Adenomatous Polyposis
FCS	Foetal Calf Serum
FISH	Fluorescence In Situ Hybridisation
FITC	Fluoroscine Isothiocyante
FA	Friedrich's Ataxia
g	Gravity
GTBP	G-T Binding Protein
h	Hour
HD	Huntingtons Disease
H&E	Haematoxylin And Eosin
HMEC	Human Mammary Epithelial Cells
HNPCC	Hereditary Non-Polyposis Colorectal Cancer
HRP	Horseradish Peroxidase
HRT	Hormone Replacement Therapy
IDC	Infiltrating Ductal Carcinoma
IGFII	Insulin-Like Growth Factor II
IGFIIR	Insulin-Like Growth Factor II Receptor

Inter	Intermediate
ILC	Infiltrating Lobular Carcinoma
IPTG	Isopropyl-1-Thio-β-D-Galactopyranoside
kb	Kilobases
kD	Kilodalton
l	Litre
LB	Luria Broth
LCIS	Lobular Carcinoma In Situ
LFL	Li-Fraumeni-Like
LFS	Li-Fraumeni Syndrome
LOH	Loss Of Heterozygosity
LNM	Lymph Node Metastasis
M	Molar
mA	Milliamps
Mamm	Mammographically
MBq	Megabecquel
MCMT	Microcell Mediated Transfer
MD	Myotonic Dystrophy
MDE	Mutation Detection Enhancement
5-meC	5-methyl-cytosine
mg	Milligram
µg	Microgram
MI	Microsatellite Instability
min	Minute
MIN	Microsatellite Instability (Thibodeau *et al.*, 1993)
MJD/SCA3	Machado-Joseph Disease
ml	Millilitre
µl	Microlitre
mm	Millimeter
mM	Millimolar
µm	Micrometer
MMAC1	Mutated In Multiple Advanced Cancers
MMR	Mismatch Repair
MS-PCR	Methylation Specific Polymerase Chain Reaction
NA	Not Applicable
ND	Not Described
ng	Nanogram
NIBH	N-iodacetyl-N'-biotinylhexylenediamine
NHSBSP	National Health Service Program National Coordinating Group For Breast Screening Pathology
nM	Nanomolar
nM	Nanometre
NP40	Nonyl Phenyl-Polyethylene Glycol 40
NST	Non Specific Type
OCP	Oral Contraceptive Pill
OD	Optical Density
ORF	Open Reading Frame
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PE	Predisposed Epithelium
PEG	Polyethylene Glycol

PgR	Progesterone Receptor
pmol	Picomoles
PMSF	Phenylmethylsulfonyl Fluoride
pRB	Phosphorylated Retinoblastoma Protein
PTEN	Phosphatase And Tensin Homologue Deleted On Chromosome 10
RER	Replication Error
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic Acid
rpm	Revolutions Per Minute
SBMA	Spinal And Bulbar Muscular Dystrophy
SCA	Spinocerebellar Ataxia
S. cerevisiae	*Saccharomyces cerevisiae*
SDS	Sodium Dodecyl Sulfate
Sec	Seconds
SRO	Smallest Region Of Overlap
SSCP	Single Stranded Conformational Polymorphism
StrepABC	Streptavidin Biotin Complex
SURF	Selective Ultraviolet Radiation Fractionation
Taq	*Thermus Aquaticus*
TAE	Tris Acetic Disodium Ethylene Diamine Tetraacetate
TBE	Tris Boric Disodium Ethylene Diamine Tetraacetate
TBS	Tris Buffered Saline
TBS-T	Tris Buffered Saline-Tween
TE	Tris Disodium Ethylene Diamine Tetraacetate
temp	Temperature
TGF-β	Transforming Growth Factor Beta
TGF-βIIR	Transforming Growth Factor Beta type II Receptor
USM	Ubiquitous Somatic Mutations
UTR	Untranslated Region
V	Volts
Vols	Volumes
VNTR	Variable Number Of Tandem Repeats
WHO	World Health Organisation
X-gal	(5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside)

Chapter 1

General Introduction to Breast Cancer

1.1 Introduction To Breast Cancer

Breast cancer is one of the most common cancers affecting women in the industrialised countries of the West (Boring *et al.*, 1993). The incidence of breast cancer in the UK is 25, 000 newly diagnosed cases each year with a mortality rate of 15, 000 cases each year. The UK has the poorest 5 year survival rate in Europe (Coleman *et al.*, 1993). The age specific incidence begins to rise after 35 years of age and it is a leading cause of morbidity and mortality in women over the age of 50.

The incidence of breast cancer has been rising steadily since the early eighties (Miller, 1992). Improvement in cancer registration is unlikely to account for more than 25 % of the increase that has occurred, and the introduction of screening mammography is thought to be responsible for the surge in increase.

Although mortality from breast cancer has generally been increasing world wide, there have been recent reports of declining mortality in the UK, USA, Norway, and Sweden (Blot *et al.*, 1987; Beral *et al.*, 1995; Hermon and Beral, 1996). Identification of environmental, biochemical and genetic factors that might contribute to the aetiology and progression of breast cancer are therefore essential in terms of improving prevention, diagnosis and therapy.

Epidemiological studies have evaluated the potential of several etiological factors and other variables associated with the development of breast cancer in women (detailed in Section 1.2). However, none of these factors alone or in combination can predict the occurrence or explain the variability of the disease. These studies generally state the relative risk to indicate the strength of a risk factor. However since these studies are purely observational, only an association, and no causation can be inferred.

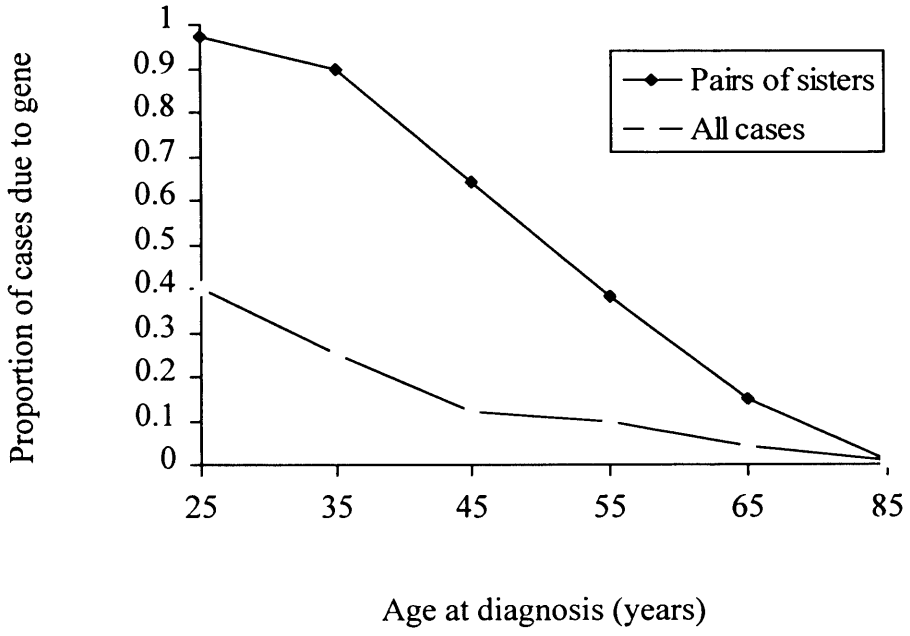
The epidemiological studies have highlighted a number of important areas for further investigation. By using molecular approaches to study these associations, it will be possible to determine the mechanisms underlying the natural history of breast cancer.

1.2 Risk Factors For The Development Of Breast Cancer

Family History Of Breast Cancer

Breast cancer has been recognised as having a familial component for many years (Brocca, 1886). Segregation analysis of data collected during the Cancer and Steroid Hormone Study (CASH), conducted by the Centres for Disease Control, has provided the largest estimate of the effect of family history (Claus *et al.*, 1991). This study found that familial clustering of breast cancer could be best explained by the inheritance of an autosomal dominant gene or genes. The model also proposed that if a single affected case is young at diagnosis, that individual has, at most a 36% chance of being a gene carrier if affected at age 20 to 29 years. However, if two first degree relatives are affected at age 30, the chance of a gene being present in the family, rises to 90%, as depicted in Figure 1.1.

Figure 1.1. The proportion of breast cancer cases due to autosomal dominant predisposition genes by age of the case (Eeles et al., 1994).



A familial component has also been recognised in ovarian cancer (Schildkraut *et al.,* 1988), and is sometimes associated with breast cancer (Schildkraut *et al.,* 1989). This suggests the existence of a gene predisposing to both familial breast and ovarian cancer (*BRCA1*), as described below.

Although the epidemiological data are consistent with a single major gene effect in both breast and ovarian cancer, it is clear that other predisposing genes are also involved. At least three other familial syndromes have been identified for which an increased risk of developing breast cancer is well established: Li-Fraumeni syndrome, Ataxia Telangiectasia, and Cowden disease, as detailed in the remainder of this section.

BRCA1 and BRCA2

In 1990, Hall *et al.,* found significant evidence of linkage between breast cancer and a marker on chromosome 17q21, in 23 families with multiple cases of early onset breast cancer. This locus was designated *BRCA1*. Linkage to 17q was confirmed in additional families with multiple cases of both breast and ovarian cancer (Narod *et al.,* 1991). Subsequent, positional cloning strategies led to the identification of *BRCA1* (Miki *et al.,* 1994).

Linkage analysis study for 214 families, has demonstrated that only about half of the families with breast cancer alone, can be attributed to *BRCA1*, although most of the families with breast and ovarian cancer were due to this gene (Easton *et al.,* 1993). Additional evidence, supporting the existence of a second susceptibility gene, came from a study of families with at least one case of male breast cancer (and generally several cases of female breast cancer and/or ovarian cancer) (Stratton *et al.,* 1994). The susceptibility gene, described as *BRCA2*, was again localised to chromosome 13q12-13, by linkage analysis (Wooster *et al.,* 1994a) and cloned a year later (Wooster *et al.,* 1995).

Like *BRCA1*, germline mutations in *BRCA2* are believed to account for up to half of the families with early onset breast cancer. In contrast to *BRCA1*, however, germ-line mutations in *BRCA2* predispose male carriers to an increased risk of breast cancer (Wooster *et al.,* 1995), and female carriers to an increase in ovarian cancer risk, which is less pronounced than that associated with germ line *BRCA1* mutations (Easton *et al.,* 1995).

Although, over 200 germline *BRCA1* and *BRCA2* mutations have now been described in affected familial cases, the presence of somatic mutations in women without a family history of breast cancer ('sporadic') has not been adequately demonstrated (Futreal *et al.,* 1994;

Hosking *et al.*, 1995; Merajver *et al.*, 1995; Takahahi *et al.*, 1995; Lancaster *et al.*, 1996; Miki *et al.*, 1996). There is recent evidence to suggest that somatic alteration may be due to another mechanism (detailed in Section 4.1).

Li-Fraumeni syndrome

Li-Fraumeni syndrome (LFS) is a rare dominant cancer syndrome involving childhood sarcomas, early onset breast cancer, brain tumours and a number of other cancers (Li, F. P *et al.*, 1988). The molecular basis underlying some LFS families was identified as a mutation in the tumour suppressor gene, *p53* (Malkin *et al.*, 1990, Srivastava *et al.*, 1990). Recent studies, have described *p53* mutations in 15 of 21 (71%) LFS families (Birch *et al.*, 1994; Varley *et al.*, 1997). Germline *p53* mutations have also been confirmed in families which do not fulfil the criteria of classical LFS, so called Li-Fraumeni-like (LFL). Of these families, 4 of 18 (21%) have *p53* mutations (Birch *et al.*, 1994; Varley *et al.*, 1997). In general, studies have shown that germline *p53* mutations account for less than 1% of all breast cancer cases (Borresen *et al.*, 1992; Sidransky *et al.*, 1992) and this appears to be mainly in the context of LFS or LFL.

The function of the *p53* gene and the role of somatic alterations in non-familial breast cancer is discussed in Section 1.5.

Ataxia Telangiectasia

Ataxia Telangiectasia (AT) is a rare autosomal recessive disease in which homozygotes develop a progressive cerebellar ataxia, hypersensitivity to ionising radiation and a predisposition to the development of cancer (Swift *et al.*, 1976). The gene responsible for AT (*ATM*) was recently identified on chromosome 11q22-23 (Sativsky *et al.*, 1995). Individuals homozygous for an *ATM* mutation develop cancer at a rate 100 times that of the general population and have an increased risk for breast cancer. It has been demonstrated that women heterozygous for an *ATM* mutation also have an increased risk of breast cancer (Swift *et al.*, 1976). However, recent studies have produced conflicting results concerning the risk associated. Athma *et al.*, (1997), found evidence of an approximately four-fold risk compared to non-carriers. However, another study (Fitzgerald *et al.*, 1997), has found no evidence of an increased risk.

Cowden disease

Cowden disease is characterised by benign tumours of multiple organs, including skin, intestine, thyroid and the breast (reviewed in Mallary, 1995). In addition to benign breast disease, breast cancer develops in approximately 30-50% of affected women (Starink *et al.*, 1986). Linkage analysis first identified a locus at chromosome 10q23 (Nelen *et al.*, 1996). A candidate gene mapping to this region was identified independently, by two different groups investigating sporadic tumours of the breast and brain, respectively. The gene has been designated *PTEN* (*phosphatase and tensin homologue deleted on chromosome 10*) (Li *et al.*, 1997), and *MMAC1* (*mutated in multiple advanced cancers*) (Steck *et al.*, 1997). Mutational analysis of *PTEN* in Cowden disease kindreds has recently identified germline mutations in four of five families (Liaw *et al.*, 1997).

Age

Breast cancer is a rare disease among young women, it has been suggested that the development of breast cancer before the age of 35 is an independent risk factor (Bonnier *et al.*, 1995). Above 35 years of age breast cancer rates double about every 10 years until after the menopause, when the rate of increase slows dramatically.

Age At Menarche

Women who begin to menstruate early in life have an increased risk of developing breast cancer. In addition, for a fixed age at menarche, women who establish regular menstrual cycles within 1 year of the first menstrual period have more than double the risk of breast cancer than women with a 5 year longer delay in onset of regular cycles (Henderson *et al.*, 1981). A delay in establishment of regular ovulatory cycles is thought to be protective (Apter and Vihko, 1983).

Age At Birth Of First Child And Parity

Nulliparity and late age at first birth both increase the lifetime incidence of breast cancer. The risk of breast cancer in women who have their first child after the age of 30 is about twice that of women who have their first child before the age of 20 (Anderson, 1974).

Age At Menopause

Women who experience a natural menopause before the age of 45 have half the breast cancer risk of those whose menopause occurs after the age of 55 (Trichopoulos *et al.*, 1972). Artificial menopause, by lateral oopherectomy or pelvic irradiation, also markedly reduces breast cancer risk.

Obesity

Obese women have a higher breast cancer mortality, thought to be due to delayed detection (Lew and Garfunkel, 1979). The relationship between weight and breast cancer risk is critically dependent on age. Subsequent to the menopause, the data suggests a modest increase in risk in women with higher relative weight only amongst older post-menopausal women (Choi *et al.*, 1978; Lubin *et al.*, 1985).

Smoking

Mutagens from cigarette smoke can come into direct contact with breast epithelial cells. Nipple fluid aspirated from smokers contains nicotine metabolites and is mutagenic. Aromatic amines found in tobacco smoke could be mutagenic and carcinogenic because they are metabolically activated and cause DNA damage in human breast epithelial cells, transform cultured mouse mammary epithelial cells, and induce mammary tumours in laboratory animals (Li *et al.*, 1996).

Most epidemiological studies have not found a clear association between smoking and breast cancer risk. Some have found elevated breast cancer risks (Morabia *et al.*, 1996), while others have reported a decreased risk (Field *et al.*, 1992). A recent study has considered genetic variability to cigarette smoke carcinogens (Ambrose *et al.*, 1996). Aromatic amines are detoxified and/or activated by metabolizing enzymes, including N-acetyltransferase. The level of activity of this enzyme determines the rates of aromatic amine detoxification and activation. Individuals can be classified as rapid or slow acetylators. Smoking appears to increase the risk for breast cancer 4-fold among postmenopausal women who are also slow acetylators (Ambrose *et al.*, 1996).

Oral Contraceptive Pill (OCP)

The relationship between oral contraceptive use and the risk of breast cancer continues to be a source of controversy (Pike *et al.*, 1983). The relative risk of breast cancer in women who

have taken the OCP has been estimated to be 1.15 (WHO study of neoplasia and steroid contraceptives 1990). The risk did not increase with duration of use and dropped when the pill was stopped. However, studies that have targeted specific groups for example, younger women have shown that the use of the pill for more than a few years is associated with increased risk of breast cancer irrespective of when they were used (UK national case control study group 1989; Miller *et al.*, 1989).

Hormone Replacement Therapy (HRT)

The association between HRT and the development of breast cancer is controversial. HRT is popular with post menopausal women as it has been shown to reduce the risk of cardiovascular disease, osteoporosis, stroke, Alzheimer's disease and increase the users overall quality of life (Lobo, 1995). Women who have taken HRT for 5 years or more have an increased risk of developing breast cancer (La Vecchia *et al.*, 1995; Colditz *et al.*, 1995). The risk among women of developing breast cancer using progestins in combination with oestrogens are similar to the risk of women using oestrogen alone (La Vecchia *et al.*, 1995).

Radiation

The carcinogenic effect of high doses of radiation on breast tissue has been well studied in atomic bomb survivors. From this data, it is known that risk is highest amongst women exposed before the age of 20, with definite evidence of a dose-response effect. The carcinogenic effect of radiation on the breast appears to diminish with increasing age and exposure, and is very small following exposure after the age of 40 (Tokunga *et al.*, 1984).

Geographical Variation

Age adjusted incidence and mortality for breast cancer varies greatly between countries, with Western countries generally having a higher incidence of breast cancer than Eastern. Studies have documented the gradual acquisition of the breast cancer rates of their adoptive countries by migrants from areas of lower incidence (Haenszel, 1961). For example, the incidence of breast cancer in Asia is low, yet studies of Japanese who have emigrated to America show that the second generation have an incidence of breast cancer approaching that of the native white population (Dunn, 1977). These studies suggest the importance of environmental factors in the development of breast cancer.

Benign Breast Disease

Benign breast diseases encompasses a heterogeneous group of lesions that clinically and radiographically span the entire spectrum of breast abnormalities. Dupont and Page (1985) categorised benign breast diseases into three main groups.

(i) Non proliferative lesions, e.g., cysts, papillary apocrine change, epithelial-related calcifications and fibroadenomas;
(ii) Proliferative lesions without atypia, e.g., intraductal hyperplasias, sclerosing adenosis and moderate or florid hyperplasia of usual type;
(iii) Proliferative lesion with atypia, such as atypical ductal or lobular hyperplasia.

All these groups have different relative risks for subsequent development of breast cancer. Studies by Dupont and Page (1985) and London *et al.*, (1992) have suggested a link between breast cancer and proliferative disease with atypia (Table 1.1).

Table 1.1. Relative risk for breast cancer with and without atypia.

	Dupont and Page (1985)		**London *et al.*, (1992)**	
Proliferative disease	Relative Risk	95% confidence Interval	Relative Risk	95% confidence Interval
Without atypia	1.9	1.2 - 2.9	1.6	1.0 - 2.5
With atypia	5.3	3.1 - 8.8	3.7	2.1 - 6.8

It is likely that more than one of the aforementioned factors may play a role in the pathogenesis of breast cancer in any given patient. However, 75% of all breast cancer patients do not have a recognisable risk factor (Strax, 1989).

1.3 Predicting The Behaviour Of Breast Cancer

The overall five year survival from breast cancer is approximately 62% (WHO, 1988). Prognosis in relation to breast cancer can be measured in terms of interval to recurrence after primary treatment, and length of survival. In general, these are closely related factors with a high frequency of recurrence correlating with reduced survival. However, treatment that delays recurrence but does not reduce its frequency may increase survival time, but not necessarily reduce the mortality from the disease.

Various factors are used to try and predict the behaviour of a breast cancer. These relate to clinical, pathological and biological features of the tumour.

Staging

Staging of a cancer is the grouping of patients according to the extent of spread of the disease. It is an established indicator for overall survival (Table 1.2). The two main systems used are the International Classification of Staging and the TNM system (Table 1.3).

The nodal status is best evaluated histologically as clinical evaluation has a high false positive and negative rate (Schottenfeld, 1976). The histologic involvement of axillary nodes, and the number of nodes involved correlates with prognosis (Valagussal *et al.*, 1978; Ferguson *et al.*, 1982).

Tumour size has also been cited as a prognostic indicator, although Fisher *et al.*, (1969) concluded that size alone was not as consequential to the patient's survival as lymph node involvement. A large study by the National Cancer Institute concluded that tumour size and node status were independent but additive prognostic indicators (Table 1.4). As tumour size increased, the prognosis was less favourable regardless of lymph node status and as lymph node involvement increased, survival status decreased regardless of tumour size (Carter *et al.*, 1989).

Table 1.2. Stage and survival at 5 years.

Stage	Survival at 5 years
I	84%
II	71%
III	48%
IV	18%

Table 1.3. The main staging systems used to assess the extent of spread of breast carcinomas.

International Classification	
I	Lump with slight tethering to skin but node negative
II	Lump with lymph node metastasis or skin tethering
III	Tumour which is extensively adherent to skin and / or underlying muscles, or ulcerating or lymph nodes are fixed
IV	Distant metastasis

TNM	
T_1	Tumour 20mm or less; no fixation or nipple retraction, including Paget's disease
T_2	Tumour 20-30mm, or less than 20mm but with tethering
T_3	Tumour greater than 50mm but less than 100mm; or less than 50mm but with infiltration, ulceration or fixation
T_4	Any tumour with ulceration or infiltration wide of it, or chest wall fixation, or greater than 100 mm in diameter
N_0	Node negative
N_1	Axillary nodes mobile
N_2	Axillary nodes fixed
N_3	Supraventricular nodes or oedema of arm
M_0	No distant metastasis
M_1	Distant metastases

Table 1.4. Five year breast cancer survival rates (%) by tumour size and lymph node status (Carter et al., 1989).

LN Status	Size (mm)				
	< 5	**5-10**	**10-19**	**20-50**	**>50**
Negative	99.2	94.9	90.6	89.4	82.2
1-3 Nodes	95.3	94	86.6	79.9	73
>4 Nodes	59	54.2	67.2	58.7	45.5
Total	96.2	94.9	90.6	79.8	62.7

Pathological Features

Histological Classification

The following outlines the differing histological types, adapted from the WHO classification of breast cancer (1981).

Non Invasive Carcinoma

These are lesions in which tumour cells are confined to the ductal or lobular units of the breast without evidence of invasion through the basement membrane into the surrounding stroma. They are characterised as either ductal or lobular, depending on the cytological features and growth patterns, although there may be overlap between the two lesions.

Ductal Carcinoma In Situ (DCIS)

DCIS is divided into high, intermediate and low grades (Bobrow *et al.*, 1995). In the past most women with a diagnosis of DCIS underwent mastectomy, so there is only limited data as to whether DCIS progresses to invasive carcinoma.

Lobular Carcinoma In Situ (LCIS)

These lesions originate from the terminal ductules or acini. It is multicentric, does not form a palpable tumour and has no characteristic radiographic signs. The true incidence of LCIS is consequently unknown. LCIS is a marker of increased risk of developing cancer.

Invasive Carcinoma

The incidence of different histological types of invasive carcinoma are given in Table 1.5.

Infiltrating Ductal Carcinoma (IDC, No Special Type or NST)

Invasive ductal carcinoma is the single largest group of malignant mammary tumours (Rosen *et al.*, 1979). To be defined as infiltrating ductal, over 90% of the tumour should not contain features of special tumour types and it is thought of as a classification of exclusion (WHO, 1981). There is considerable histological variation within this group with cell in trabeculae or solid groups, with the cells themselves ranging from regular to pleomorphic. In addition, there is variable or absent gland formation.

Infiltrating Lobular Carcinoma (ILC)

Typical clinical presentation is a palpable area of ill defined thickness in contrast to the prominent lump of ductal carcinoma and characterised by single cell infiltration, often around pre-existing breast structures. Infiltrating lobular carcinoma is far more frequently associated with bilateral disease than NST tumours and tends to be multicentric within the same breast.

Special Types

Medullary Carcinoma

These tumours are circumscribed, often large (up to 5-10cm) and are composed of syncytial cells with vesicular nuclei. Necrosis is common and the mitotic rate is often high. They have extensive lymphocytic infiltrate and scanty fibrous tissue.

Colloid or Mucinous Carcinoma

This is the most common tumour amongst older women, and are generally large due to late presentation rather than rapid growth rate. Histologically, the tumour is composed of nests, cords and even isolated cells lying in lakes of mucin which often accounts for more than half the volume of the tumour. It may be pure colloid or associated with other histological types. The presence of other histological types excludes it from the colloid category to that of mixed ductal-special type.

Tubular Carcinoma

These are highly differentiated infiltrating carcinomas composed of uniform cells arranged in well developed tubules. Diagnosis is made if tubule formation is greater than 90%, the exception being a combination of tubular and cribriform patterns where it is classified as tubular if the former component is greater than 50%. Generally, they are small lesions (1-2 cm). They are well differentiated and have an excellent prognosis. Ellis *et al.*, (1992) showed this type to be associated with a five year survival of approximately 90%.

Invasive Cribriform Carcinoma

Although similar to tubular carcinoma this type of tumour is classified as a special type due to its excellent prognosis. It is characterised by the presence of islands of small cells that are similar to cribriform DCIS. To be classified as cribriform carcinoma this pattern should be present in >90% of the section.

Histological typing provides useful prognostic information in patients with primary operable breast cancer (Ellis *et al.*, 1992). Mucinous, tubular, and cribriform carcinomas have a better prognosis than ductal carcinoma (Ellis *et al.*, 1992). These data are summarised in Table 1.6.

Table 1.5. Relative frequency of the different types of invasive breast tumours in a symptomatic population (Page and Anderson, 1987).

Type	Frequency (%)
Infiltrating ductal	73
Infiltrating lobular	10
Medullary	5
Mucinous	2
Tubular	3
Cribriform and Papillary	5
Mixed Tumours	2

Table 1.6. Ten year survival rate (%) for the different carcinoma types (adapted from Ellis et al., (1992). NA = Not applicable.

Type of Carcinoma	**Ten Year Survival**
Ductal Carcinoma In Situ	92
Lobular Carcinoma In Situ	NA
Infiltrating ductal	47
Infiltrating lobular	54
Special Types	
Medullary	51
Mucinous	80
Tubular-Cribriform	90

Histological Grade

The term grade refers to the level of differentiation within a tumour. The method of grading generally employed is a modification of the Bloom and Richardson method described in 1957 (Elston and Ellis 1991) and is based on evaluation of tubule formation, nuclear pleomorphism and mitotic count. Each of these variables is given a score of 1-3 for each feature that is assessed (Table 1.7). Scores are added together to obtain the overall tumour grade:

3-5 points - Grade 1 i.e. Well differentiated

6-7 points - Grade 2 i.e. Moderately differentiated

8-9 points - Grade 3 i.e. Poorly differentiated

The correlation between high histological grade and poor prognosis has been demonstrated in many studies (Fisher *et al.*, 1975; Contesso *et al.*, 1987; Elston and Ellis, 1991).

Table 1.7. Assessment scores for tubule formation, and nuclear pleomorphism.

Assessed Feature	**Score**
Tubule formation	
>75%	1
10-75%	2
<10%	3
Nuclear pleomorphism	
Small, regular, uniform cells	1
Moderate increase in size and variability	2
Marked variation	3
Mitotic counts	
Based on the number of mitosis per high power fields, which relates to the microscope	

Biological Features

Cell Kinetics

The relevance of cell kinetics as a tool to investigate growth pattern, biological heterogeneity and clinical progression of human tumours has been supported by a number of reports (Silvestrini *et al.*, 1989; Merkel and McGuire 1990). Consequently the measurement of the proliferative fraction of tumour cell populations (the ratio of cycling to non-cycling cells) has become increasingly important as a complement to the clinicopathologic findings in making clinical decisions.

The growth rate of a tumour can be defined as low, medium or high. A tumour with a lower rate of cell growth is generally associated with an improved prognosis. It is important to keep in mind that a high rate of cell differentiation may also be accompanied by a high rate of cell death and that aberrant cell division occurs such that one division fails to result in the doubling of the number of cells. Cell proliferation can be measured by counting of mitotic figures, [^{3}H]-thymidine uptake, flow cytometry and by use of monoclonal antibodies mainly directed against the Ki-67 antigen (Walker and Camplejohn, 1988; Bouzubar *et al.*, 1989).

Steroid Receptors

Many risk factors point towards the role of steroid hormones and their receptors in the progression of breast cancer. The steroids implicated are oestrogen and progesterone, with their hormonal action most likely via their cognate receptors, the oestrogen receptor (ER) and the progesterone receptor (PgR).

It is thought that two thirds of breast tumours are ER positive (Lippman and Allegra, 1980) with approximately half of these responding to anti-oestrogen therapy, while approximately 10% of ER negative tumours do respond (Anderson and Poulsen, 1989). Patients with ER negative tumours have shorter disease free intervals, earlier recurrence rates, and shorter survival times when compared to patients with ER positive tumours (Maynard *et al.*, 1978; Hartveit *et al.*, 1980). As PgR synthesis is regulated by ER (Lippman *et al.*, 1986), PgR is considered as an additional marker of response to endocrine therapy and overall prognosis (Table 1.8). PgR negative tumours are less likely to respond to endocrine therapy than PgR positive tumours (Pertschuk *et al.*, 1988). In addition, patients with PgR positive tumours have a longer disease free interval and longer overall survival (Fisher *et al.*, 1988; Chevellier

et al., 1978). In conclusion, evidence suggests that both the ER and PgR are prognostic indicators but neither is a strong predictor of behaviour.

Table 1.8 The prognostic significance of measuring oestrogen and progesterone receptors together (reviewed by Leong and Lee, 1995).

Combination of receptors	**Incidence**	**5 year disease free interval**	**Overall 5 year survival**
$ER^+ PgR^+$	50%	73%	91%
$ER^+ PgR^-$	20%	75%	93%
$ER^- PgR^+$	5%	68%	88%
$ER^- PgR^-$	25%	64%	77%

1.4 The Multistep Nature Of Carcinogenesis

Nowell (1976) was the first to propose that cancer can develop in a multistep manner and a substantial amount of evidence has been collected over the past decade which indicates that the genesis of malignancy requires the sequential accumulation of a number of genetic alterations (Fearon and Vogelstein, 1990). Colorectal carcinoma is an excellent model of the multistep nature of cancer in which distinct clinical stages of disease development are identifiable.

Epidemiological studies have suggested that approximately 15% of colorectal cancers occur in a dominantly inherited pattern (Houlston *et al.*, 1992). Two familial forms are now well defined, familial adenomatous polyposis (FAP) and hereditary non-polyposis colorectal cancer (HNPCC). FAP patients develop between hundreds and thousands of benign colorectal tumours (adenomas), some of which eventually progress to carcinomas. The disease is characterised at the molecular level by gross chromosomal alterations leading to losses of chromosome regions. This is identified at the molecular level as Loss of Heterozygosity (LOH), which is recognised as the hallmark of tumour suppressor gene inactivation (described in detail in Chapter 4). LOH is particularly prevalent at chromosomes: 5q, 17p and 18q, suggesting the involvement of tumour suppressor genes at these regions (Vogelstein *et al.*, 1988).

In the late 1980s, the adenomatous polyposis coli (*APC*) gene was identified on chromosome 5q and mutant *APC* alleles were shown to co-segregate with the affected FAP kindreds (Bodmer *et al.*, 1987; Leppert *et al.*, 1987). However, patients with germline mutations of *APC* do not necessarily develop colon cancer, but they are at an elevated risk of doing so over the general population. In order for such tumours to form, additional genetic alterations are required (Figure 1.2).

The other genetic alterations identified in the sequence of events include: the activation of the proto-oncogene K-*ras*, and the inactivation of the tumour suppressor gene *p53* (Fearon and Vogelstein, 1990). The identity of the putative tumour suppressor gene at the 18q locus remains controversial. The initial candidate was *DCC* (*deleted in colon cancer*) (Fearon *et al.*, 1990). However, recent evidence suggests that this gene may not have tumour suppressive functions (Fazeli *et al.*, 1997). Members of the *Mad* family, encoding proteins that transduce signals from the transforming growth factor β (TGFβ) family of cytokines, are possible candidates (Thiagalingam *et al.*, 1996) which are currently under investigation.

As the multistep progression for colorectal carcinogenesis has been refined over the years, it has emerged that the order in which the molecular aberrations occur is not important, except for in the initiation stage. Mutations in genes other than *APC* (e.g. *p53* or K-*ras*) do not efficiently initiate the neoplastic process. Therefore, it has been proposed that APC acts as cellular 'gatekeeper', that directly regulates colonic epithelial growth by inhibiting growth or promoting cellular death (apoptosis) (Morin *et al.,* 1996). The mechanisms by which APC normally control this switch are now emerging (Peifer *et al.,* 1997). In normal colonic epithelial cells, APC and a serine-threonine protein kinase (GSK) function together to maintain low levels of a key signalling molecule (β-catenin). However, in cells with inactive APC this control is abolished and the level of β-catenin increases. In turn, high levels of β-catenin lead to the formation of complexes with a group of transcription factors (Korinek *et al.,* 1997; Morin *et al.,* 1997), which may activate expression of genes involved in the stimulation of proliferation and inhibition of apoptosis.

In contrast to FAP, tumours of the HNPCC syndrome do not typically display loss of chromosomal regions. Instead these tumours are characterised by an elevated mutation rate which is manifest at the molecular level by replication errors or microsatellite instability (MI). HNPCC has been shown to be caused by germline mutations in four mismatch repair genes (Marra and Boland, 1995). Although the increase in genetic instability can be simply monitored by genome-wide changes at arbitrary loci, it is emerging that this defect may also target critical cancer-associated loci (Markowitz *et al.,* 1995) (described in detail in Chapter 3). The mismatch repair genes play a role in maintaining genome integrity, and have been described as 'caretaker' genes (Kinzler and Vogelstein, 1997). In HNPCC, it is believed that adenomas form at approximately the same rate as in the general population. However, an adenoma cell from a HNPCC patient will acquire mutations at an elevated rate, and the resultant accumulation of mutations in oncogenes and tumour suppressor genes may lead to a rapid progression to malignancy.

It has been assumed that the genetic instability, associated with MI, occurred in the later stages of the model, accelerating the rate of tumour progression (Kinzler and Vogelstein, 1996). However, a recent study has demonstrated that tumours with MI also have mutations of *APC* (Huang *et al.,* 1996). The specific type of APC mutations in the MI patients are distinctly different from those of the APC mutations in patients without MI, and are characteristic of tumours with the MI phenotype (described in detail in Chapter 3). This suggests that the genetic instability may precede *APC* mutations In these patients with MI

tumours, genetic instability may lead to tumourigenesis via *APC* inactivation and therefore is indirectly responsible for tumour initiation and progression.

A model of how 'gatekeeper' and 'caretaker' genes may co-operate in pathways of inherited susceptibility to cancer, and in particular the development of colon cancer, has recently been proposed (Kinzler and Vogelstein, 1997), and is illustrated in Figure 1.3. Inherited mutations of either a caretaker or gatekeeper gene can predispose an individual to neoplasia, however, additional genetic alterations are needed to convert a predisposed cell to a neoplastic cell. In the caretaker pathway, a mutation of one caretaker allele must be followed by three other mutations (the second caretaker allele and two gatekeeper alleles). In the gatekeeper pathway, mutation of one gatekeeper allele is followed by mutation of the other gatekeeper allele, in order to initiate neoplasia.

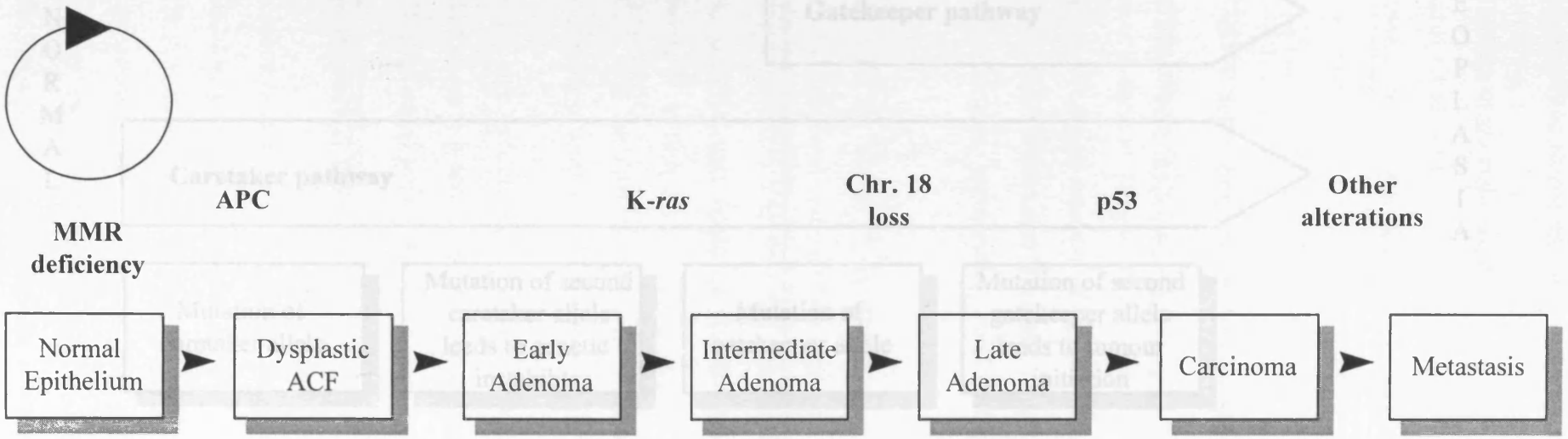


Figure 1.2. Genetic alterations associated with colorectal carcinogenesis (Adapted from Kinzler and Vogelstein, 1996)

Defects to the mismatch repair (MMR) proteins lead to mutations of the APC gene, as indicated by the 'rolling circle'. APC inactivation leads to the formation of dysplastic aberrant crypt foci (ACF), which are believed to be precursors of adenomas. These in turn progress to carcinoma as they acquire subsequent mutations (K-ras proto-oncogene activation, allelic loss of Chromosome 18, and finally p53 alterations).

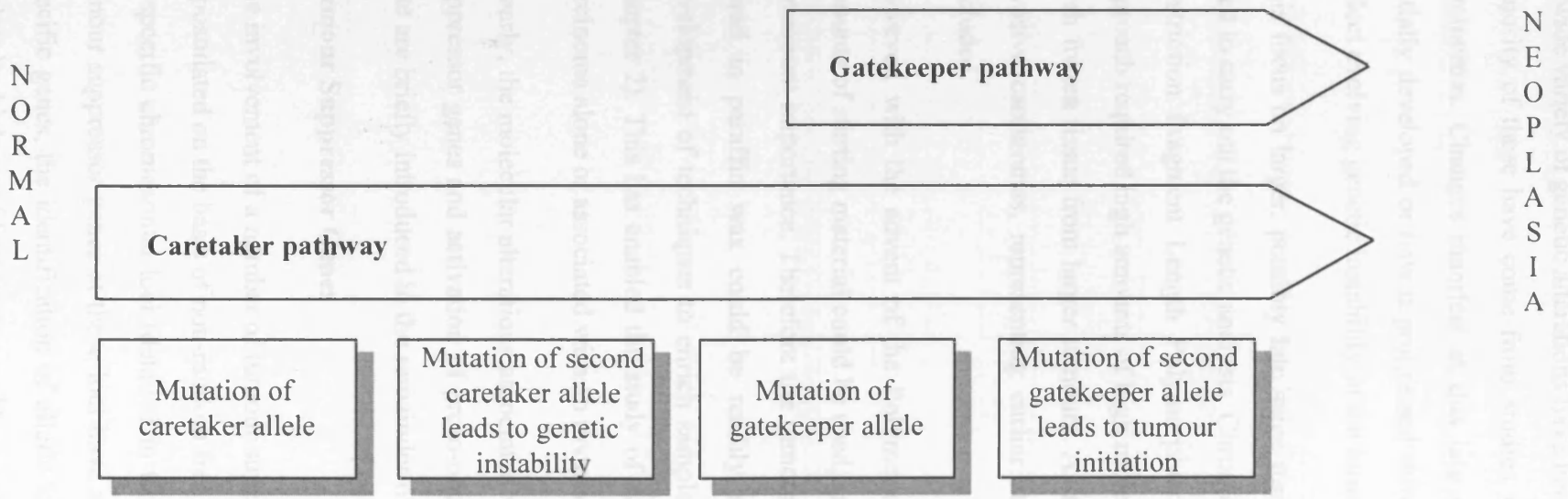


Figure 1.3. Caretaker and Gatekeeper pathways of inherited susceptibility to cancer (Kinzler and Vogelstein, 1997).

In the caretaker pathway, an inherited mutation is followed by three additional mutations, although the genetic instability that follows inactivation of the second caretaker allele accelerates the accumulation of the later mutations. In the gatekeeper pathway, one mutation coupled with the inherited mutation is needed to initiate neoplasia.

1.5 Genetic Alterations In Breast Cancer

A wide variety of genetic alterations have been described in breast cancer (outlined below) the majority of these have come from studies which analysed larger, possibly more advanced carcinomas. Changes manifest at this late stage do not necessarily reflect how a tumour initially developed or how it progressed through the early stages of disease and may merely reflect evolving genetic instability in the tumour.

This focus on larger, possibly late stage tumours reflected the limitations of the techniques used to carry out the genetic analysis. Chromosomal aberrations were detected primarily using Restriction Fragment Length Polymorphisms (RFLPs) and hybridisation protocols. This approach required high amounts of high molecular weight DNA, often necessitating the use of fresh frozen tissue from larger tumours. As a consequence the smaller tumours and the non-invasive carcinomas, representing earlier stages in the development of the disease, were excluded.

However, with the advent of the Polymerase Chain Reaction (Saiki *et al.*, 1985) smaller amounts of starting material could be used, and more critically the quality of DNA was not of paramount importance. Therefore the tumour samples which had been fixed in formalin and stored in paraffin wax could be readily studied. Another major advance has been the development of techniques to enrich samples for tumour cells (microdissection, detailed in Chapter 2). This has enabled the study of distinct tumour foci, for example areas of *in situ* carcinoma alone or associated with an invasive lesion (detailed in Section 1.6).

Clearly, the molecular alterations associated with breast cancer include inactivation of tumour suppressor genes and activation of proto-oncogenes. The key loci identified and analysed to date are briefly introduced in the remainder of this section.

Tumour Suppressor Genes

The involvement of a number of tumour suppressor genes in the development of breast cancer is postulated on the basis of non-random frequencies of allelic losses (Loss of Heterozygosity) at specific chromosomal loci (detailed in Chapter 4). However, the majority of the putative tumour suppressor genes at these loci have not yet been identified. In the absence of known specific genes, the identification of allelic losses is often used as the basis for studying the molecular biology of breast cancer. The most prevalent loci characterised to date are outlined

in Table 1.9. Two of these regions are located on chromosomes 13 and 17p, which are known to harbour the tumour suppressor genes: *RB1* and *p53*.

Table 1.9. Representative chromosome regions showing Loss of Heterozygosity in breast cancer. The majority of the studies described below utilised microsatellite markers and PCR.

Chromosomal region	LOH	Reference
1p31	28%	Nagai *et al.*, 1995
1q21-31	65%	Bieche *et al.*, 1995
3p14.2	38%	Ahmadian *et al.*, 1997
6q25-27	35%	Orphanos *et al.*, 1995
7q23	41%	Zenklusen *et al.*, 1994
8p12-21	50%	Kerangueven *et al.*, 1995
9p21-22	58%	Brenner and Aldaz, 1995
11p15.5	35%	Winqist *et al.*, 1995
11q23	42%	Koreth *et al.*, 1995
13q12-14	33%	Cleton-Jansen *et al.*, 1995a
16q22	67%	Iida *et al.*, 1997
17p13.3	48%	Ito *et al.*, 1995

The frequencies given for LOH are those from the cited references.

RB1

The retinoblastoma gene (*RB1*) has been mapped to 13q14 (Friend *et al.*, 1986) and encodes a 105kD protein which in its unphosphorylated form restricts cell cycle progression in G1, by interacting with E2F transcription factor (Chellappan *et al.*, 1991). Alterations to 13q (Lundberg *et al.*, 1987) and to the *RB1* gene itself (T'Ang *et al.*, 1988) have been found in breast carcinomas. Comparison of allele loss and protein expression have shown both loss of protein with loss of allele (Varley *et al.*, 1989) and high protein expression with allele loss (Borg *et al.*, 1992). Alterations were found either in advanced cases (Varley *et al.*, 1989) or in aneuploid high S phase cancer (Borg *et al.*, 1992) suggesting that *RB1* alterations were not an initiating event in breast cancer but as a consequence of an unstable genome.

p53

The *p53* gene is located on the short arm of chromosome 17. It was originally believed to be an oncogene (Linzer and Levine, 1979; Lane and Crawford, 1979). However, its ability to reverse the tumourigenic phenotype if transfected into tumour cell lines, the frequent finding that both alleles were inactivated in tumour cells and its association with hereditary cancers (Section 1.2) led to its reclassification as a tumour suppressor gene (Lane and Benchimol, 1980).

p53 functions as a cell cycle protein which exerts its function during the G1 phase of the cell cycle (Kastan *et al.*, 1991). The protein has been designated as 'guardian of the genome', since it has a major role in maintenance of cellular fidelity (Lane, 1992). In response to DNA damage, there is a rapid increase in the level of p53, which leads to arrest of the cell cycle. This enables the cell to initiate DNA repair pathways. However, if repair is not possible, p53 induces apoptosis (Haffner and Oren, 1995).

Most p53 mutational studies in sporadic breast tumours have focused on sequence changes in exons 5, 6, 7 and 8, within the highly conserved domains (Osbourne *et al.*, 1991; Varley *et al.*, 1991; Coles *et al.*, 1992; Mazars *et al.*, 1992; Andersen *et al.*, 1993; Merlo *et al.*, 1993; Caleffi *et al.*, 1994; Eyfjord *et al.*, 1995) although complete sequencing has also been undertaken (Bergh *et al.*, 1995). These studies have demonstrated an association between the presence of mutations and aggressive features within breast carcinomas, e.g. lack of ER expression (Mazars *et al.*, 1992; Andersen *et al.*, 1993; Caleffi *et al.*, 1994) and high S phase index (Merlo *et al.*, 1993). A significant association was found between p53 mutations and

disease-free and overall survival (Andersen *et al.*, 1993), and differences between location of mutation within p53, between node positive and node negative cases (Caleffi *et al.*, 1994). p53 mutations have also been documented in DCIS, suggesting that alteration is important early in the development of breast cancer (Munn *et al.*, 1996a).

Immunohistochemical staining of p53 is also used in combination with DNA sequencing to detect protein accumulation. Wild-type p53 has a short half-life and is not detected by immunohistochemical methods (Gronstajaski *et al.*, 1984). Prominent reactivity for p53 has been shown to be associated with the lack of ER expression, poor differentiation and high proliferation rates (reviewed in Walker *et al.*, 1997). Protein expression of p53 in DCIS is correlated with high grade (Rajan *et al.*, 1997).

Oncogenes

The most frequently observed mechanism of proto-oncogene activation in breast cancer is gene amplification (reviewed in Devilee *et al.*, 1994). The mechanism which leads to gene amplification often involves the surrounding chromosomal loci. The net result of this amplification is a 'chromosomal gain', detectable by molecular methods. Commonly amplified chromosomal regions in breast cancer are 17q12, 8q24, and 11q13 and encode c-*erb*-B2, c-*myc*, and cyclin D1, respectively.

c-erb-B2

The c-*erb*-B2 gene encodes a 185kD transmembrane glycoprotein, that has extensive homology to the epidermal growth factor receptor and is a putative growth factor receptor (Coussens *et al.*, 1985). Amplification of the c-*erb*-B2 gene has been found in 20-30% of invasive carcinomas (Slamon *et al.*, 1987; Varley *et al.*, 1987; Zhou *et al.*, 1987; Borresen *et al.*, 1990) with a correlation found between amplification and aggressive features and poor short term prognosis, although not in all studies (Clark and McGuire, 1991; Zhou *et al.*, 1989). Such c-*erb*-B2 gene amplifications have been shown to correlated with a corresponding over-expression of mRNA and protein levels (Walker *et al.*, 1989a; Borresen *et al.*, 1990; Venter *et al.*, 1987).

Many researchers have found c-*erb*-B2 overexpression to be an independent predictor of poorer disease free survival (Walker *et al.*, 1989b; Gullick *et al.*, 1991; Lovekin *et al.*, 1991; Winstanley *et al.*, 1991; Press *et al.*, 1993) and associated with more aggressive forms of the disease. Surprisingly, c-*erb*-B2 protein has been detected in ductal carcinoma *in situ* (van der

Vijver *et al.*, 1988), with more extensive studies demonstrating expression in 40-60% of cases but always associated with high grade DCIS (Bartkova, *et al.*, 1990; Lodata *et al.*, 1990; Ramachandra *et al.*, 1990).

c-*myc*

The c-*myc* gene encodes a nuclear phosphoprotein which acts as a transcriptional regulator controlling cell proliferation, differentiation and apoptosis (Evan *et al.*, 1992). Alterations to the c-*myc* gene, predominantly by amplification, have been found in approximately 25% of carcinomas (Bonilla *et al.*, 1988) Other studies have correlated c-*myc* alterations with aggressive features and/or poor prognosis (Escot *et al.*, 1986; Varley *et al.*, 1987; Berns *et al.*, 1992).

Cyclin D1

The cyclins are a family of cell cycle proteins. Cyclin D1 is a 36kD protein which is active in the G1 phase of the cell cycle. It forms a complex with cyclin dependant kinases 4 and 6 (cdk4 and 6) and following activation by cdk-activating kinases these complexes, along with cyclin E/cdk2, phosphorylate the retinoblastoma protein (pRB). Unphosphorylated RB is growth inhibitory and phosphorylation is required to remove this block in order to allow the cell to pass through the critical restriction point, thereby allowing the cell cycle to proceed to the S phase (Bates and Peters, 1995).

The *cyclin D1* gene is located at chromosome 11q13, and although amplification of this area is found in approximately 15-20% of breast cancers, overexpression of cyclin D1 mRNA and protein is seen in approximately 50% of cases (Gillet *et al.*, 1994). In this and another study (Gillet *et al.*, 1996) overexpression was associated with an overall longer disease free survival than with was found in women with low or non-expression of the protein. Other supporting studies have also correlated overexpression of cyclin D1 with factors which indicate a good prognosis (Scott and Walker, 1997).

H-*ras*-1

The H-*ras*-1 gene encodes a G protein involved in signal transduction. The role of activated *ras* genes in human cancer has not been fully elucidated. Mutations are rarely identified (Rochlitz *et al.*, 1989), although loss of one H-*ras*-1 allele has been correlated with aggressive features (Theillet *et al.*, 1986).

The H-*ras*-1 variable number tandem repeat (VNTR) polymorphism, located 1kb downstream of the H-*ras*-1 proto-oncogene (chromosome 11p15.5) is a possible genetic modifier of cancer penetrance. Individuals who have rare alleles of this VNTR have an increased risk of certain types of cancers, including breast cancer (Lidereau *et al.*, 1986; Garret *et al.*, 1993; Krontiris *et al.*, 1993).

Recent Approaches

Comparative genomic hybridisation (CGH) is a relatively new technique which compares two genomes for example, tumour and normal at the genomic level, and can identify all regions showing gain or loss in copy number (Kallioniemi *et al.*, 1992). Among the chromosomal loci which have been reported thus far to demonstrate consistent amplification in invasive breast tumours and lesions of DCIS are: 1q, 3q, 6p, 8q, 10q, 11q, 12q, and 20q (Isola *et al.*, 1994; Kallioniemi *et al.*, 1994; Muleris *et al.*, 1994; Reid *et al.*, 1995; James *et al.*, 1997; Kuukasjarvi *et al.*, 1997). Therefore, amplification of as yet unknown genes may often occur in breast cancer.

1.6 Natural History Of Breast Cancer

A model of breast tumourigenesis has been postulated in which a normal epithelium cell of the ducts or lobules gives rise to a focus of proliferating cells (hyperplastic without atypia and subsequently, with atypia) and then, through an accumulation of molecular abnormalities, evolves into a neoplasm, initially carcinoma *in situ*, followed by invasive disease and finally to metastatic disease (Russo and Russo, 1991).

This progression has been depicted by Devilee *et al.*, (1994), and is illustrated in Figure 1.4. As can be seen the proposed model is very complex, with the potential for breast cancer to develop through a variety of different pathways. For example, invasive carcinoma may develop from normal epithelium without progressing through a non-invasive step. Similarly it is possible that not all *in situ* disease progresses to invasive carcinoma. This model has been proposed mainly from histological observations and epidemiological data which suggest that certain histological subtypes confer an increased risk of developing breast cancer (Section 1.2).

Over the last few years, a major area of research as been in attempting to understand how the various precursor lesions behave at the molecular level. The studies described below provide some molecular evidence to underpin each of the pathways proposed by Devilee *et al.*, (1994).

The model proposed by Devilee *et al.*, (1994), only accounts for the development of infiltrating ductal carcinoma. Another model has recently been proposed which encompasses some of the other histological subtypes of breast cancer (Walker, 1997), illustrated in Figure 1.5. The early steps in this model are also supported by the molecular analysis of precursor lesions described below. However, this model also positions previously defined genetic alterations (Section 1.5) in the proposed pathways of breast cancer development, as well as the genes responsible for the familial component of the disease.

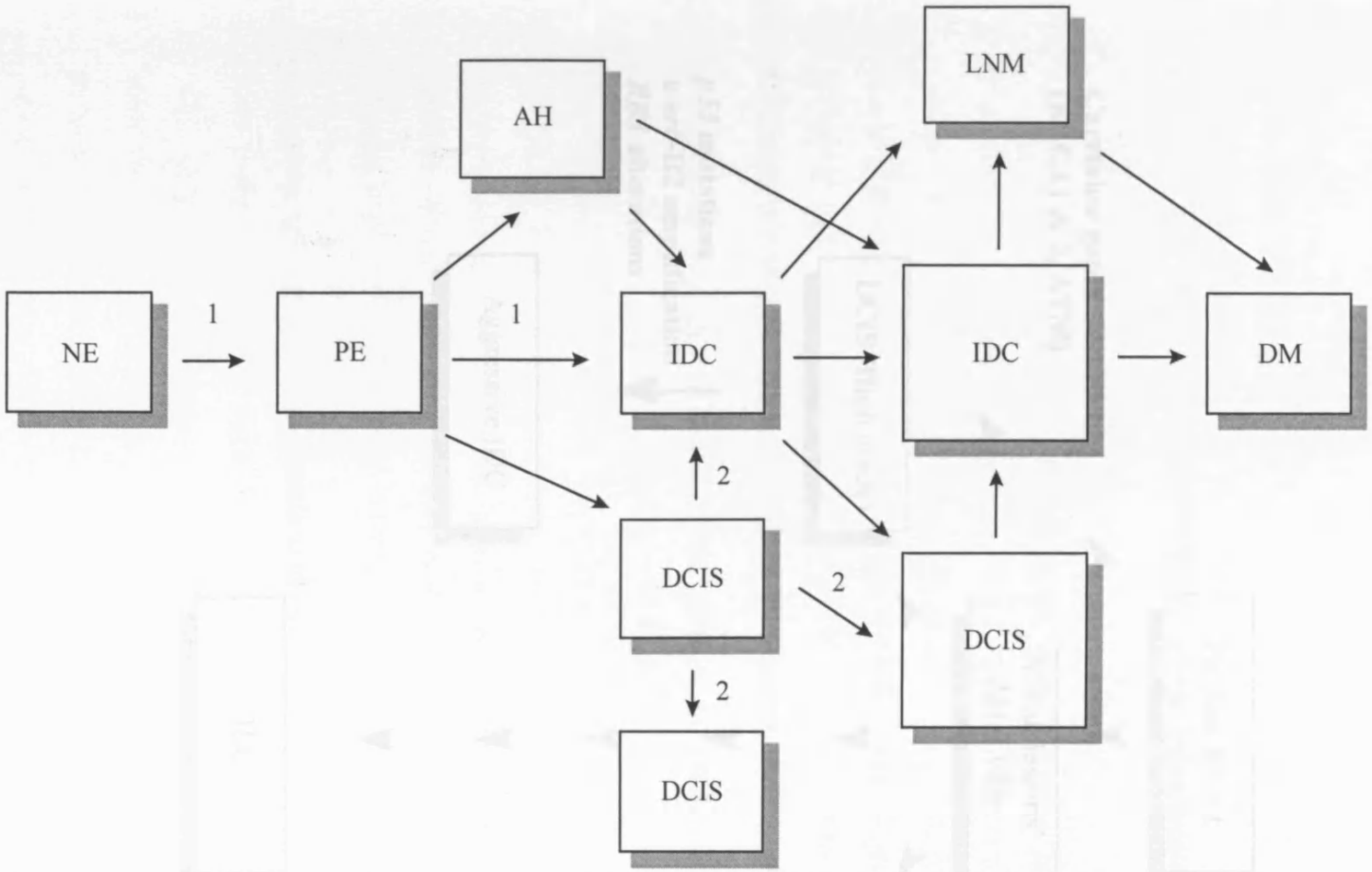
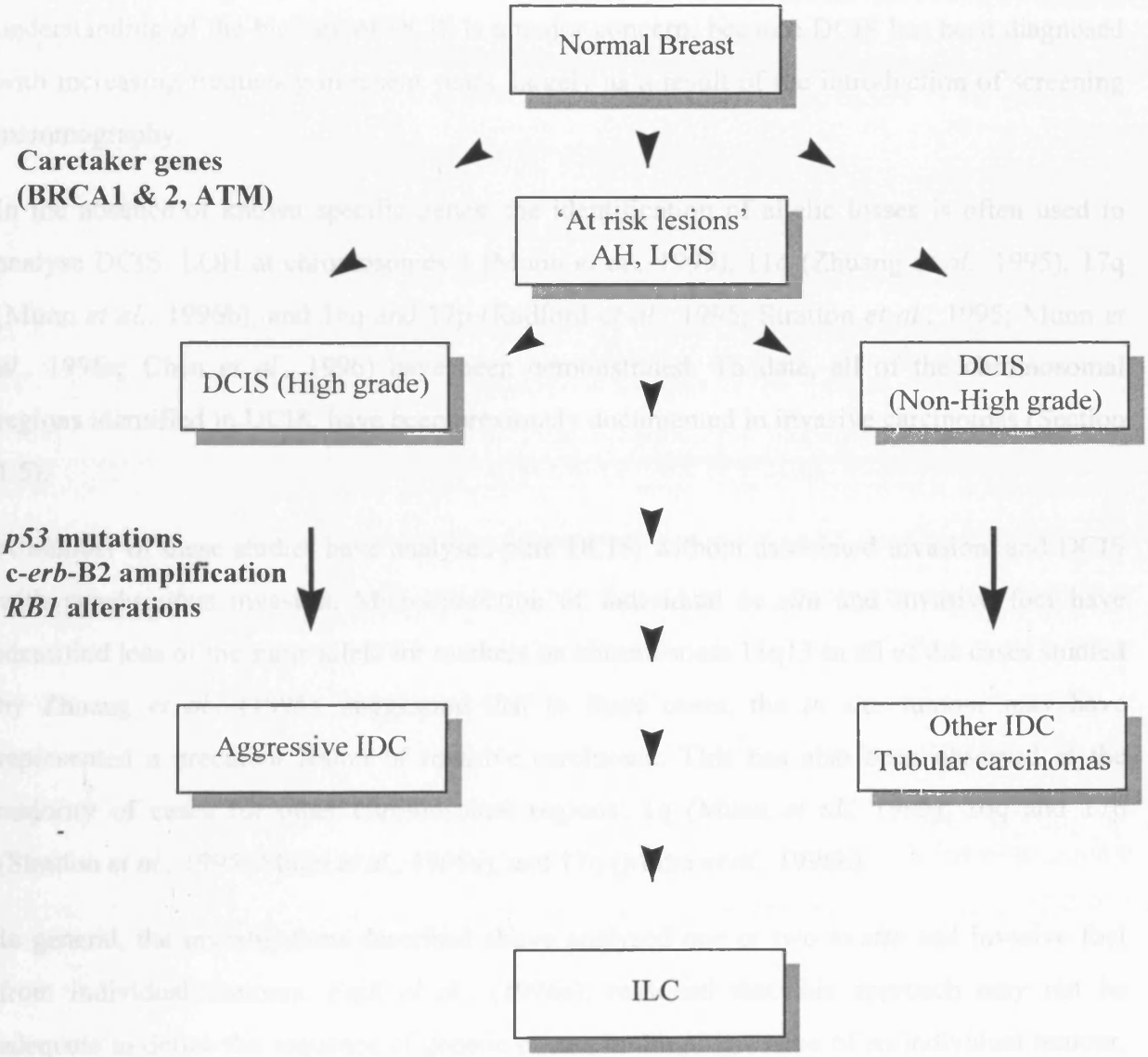


Figure 1.4. Hypothetical model for infiltrating ductal carcinoma (Adapted from Devilee et al., 1994)

(1) Normal (NE) or predisposed epithelium (PE) may develop into infiltrating ductal carcinoma (IDC), without proceeding through atypical ductal hyperplasia (AH) or ductal carcinoma in situ (DCIS), as proposed by Deng et al., 1996

(2) DCIS may progress to larger DCIS, with the accumulation of further genetic alterations, or develop into IDC, or remain as DCIS, as proposed by Fujii et al., 1996b. LMN indicates lymph node metastasis. DM is distant metastasis.

Figure 1.5. Model of breast cancer pathways (Walker, 1997).
The small arrows indicate possible pathways where the evidence is limited. The larger arrows indicate there is better evidence. AH indicates atypical hyperplasia, LCIS is lobular carcinoma in situ, DCIS is ductal carcinoma in situ, IDC is infiltrating ductal carcinoma, and ILC in infiltrating lobular carcinoma.



Ductal Carcinoma In Situ

Histological observations indicate that DCIS is a precursor to infiltrating ductal carcinoma of the breast because of finding it in association with invasive carcinoma (Page and Anderson, 1987). However, not all cases of DCIS appear to have the same potential of progressing to invasive cancer. This does depend on the nature of DCIS; low grade DCIS, misdiagnosed as benign, and incompletely excised can recur up to 24 years later (Page *et al.,* 1995). High grade DCIS recurs, with invasion, earlier and at a higher frequency (Lagios *et al.,* 1989). A greater understanding of the biology of DCIS is a major concern, because DCIS has been diagnosed with increasing frequency in recent years, largely as a result of the introduction of screening mammography.

In the absence of known specific genes, the identification of allelic losses is often used to analyse DCIS. LOH at chromosomes 1 (Munn *et al.,* 1995), 11q (Zhuang *et al.,* 1995), 17q (Munn *et al.,* 1996b), and 16q and 17p (Radford *et al.,* 1995; Stratton *et al.,* 1995; Munn *et al.,* 1996a; Chen *et al.,* 1996) have been demonstrated. To date, all of the chromosomal regions identified in DCIS, have been previously documented in invasive carcinomas (Section 1.5).

A number of these studies have analysed pure DCIS, without associated invasion, and DCIS with synchronous invasion. Microdissection of individual *in situ* and invasive foci have identified loss of the same allele for markers on chromosome 11q13 in all of the cases studied by Zhuang *et al.,* (1995), suggesting that in these cases, the *in situ* tumour may have represented a precursor lesion of invasive carcinoma. This has also been observed at the majority of cases for other chromosomal regions: 1q (Munn *et al.,* 1995), 16q and 17p (Stratton *et al.,* 1995; Munn *et al.,* 1996a), and 17q (Munn *et al.,* 1996b).

In general, the investigations described above analysed one or two *in situ* and invasive foci from individual tumours. Fujii *et al.,* (1996a), reasoned that this approach may not be adequate to define the sequence of genetic events in the progression of an individual tumour, since distinct foci at various stages of progression may be present within one tumour sample. In their study, multiple individual foci were microdissected and analysed at 7 chromosomal regions, in two groups of lesions: cases with DCIS and synchronous infiltrating breast cancer, and cases of pure DCIS. In 8 of 20 (40%) cases with synchronous DCIS and invasive cancer, loss of different alleles was observed in some of the *in situ* foci. This phenomenon has been described as 'allelic heterogeneity' (Chen *et al.,* 1992). In contrast the frequency of allelic

heterogeneity between ducts from the pure DCIS was only 8% (3 of 23). Further evidence for this difference between the pure DCIS, and DCIS with invasion, came from an extended study of pure DCIS, which also described low frequencies of allelic heterogeneity (Fujii *et al.*, 1996b).

Fujii *et al.*, (1996a, 1996b) proposed a model whereby the involved ducts in pure DCIS have evolved along a common pathway. As the tumour becomes invasive (DCIS with synchronous invasive cancer) several *in situ* tumour foci diverge along different pathways of genetic progression. In contrast allele heterogeneity was not observed between multiple foci of invasive carcinomas. Therefore it is possible that growth of invasive cancer is the result of a rapid expansion of one population from the divergent *in situ* cancer.

Clinically, DCIS is a heterogeneous group of pathological and biological subtypes, which may differ in behaviour. The lesions can be categorised by their nuclear morphology into high, intermediate and low nuclear grade (Bobrow *et al.*, 1995). The nuclear grade of a particular DCIS lesion can be predictive of the biological behaviour of the tumour. For example, high grade lesions may be at greater risk of local recurrence if incompletely excised and be associated with the development of invasion (Lagios *et al.*, 1982). Therefore it is important to characterise the genetic changes of the different categories of DCIS, and to determine the sequence of genetic changes in tumour evolution. The allelic loss studies described by Fujii *et al.*, (1996a) have suggested that the cumulative allelic loss for all loci and LOH at each of the chromosomal loci studied (with the exception of chromosome 16q) are significantly greater in intermediate or high-grade DCIS than in the low grade DCIS. This is consistent with the model proposed by Walker (1997), whereby high grade DCIS accumulates either more or different genetic alterations than non-high grade DCIS, and in turn may progress to a more aggressive form of invasive carcinoma than that of non-high grade DCIS.

Two of the groups of investigators who had investigated allele loss on chromosome 11q13 and chromosome 16q and 17p in DCIS, have extended their analyses to lesions of atypical hyperplasia (ADH) and lobular carcinoma *in situ* (LCIS), as described below.

Lobular Carcinoma In Situ

LCIS and atypical lobular hyperplasia (ALH) are generally regarded as being markers of an increased risk for breast cancer rather than direct precursors of invasive breast carcinoma (Page *et al.*, 1991). A study by Nayar *et al.*, (1996), noted 40% LOH at 11q13 in ILC and in

LCIS associated with ILC. In all cases in which synchronous LCIS and ILC from the same case showed LOH, the same allele was lost in both foci, similar to the previous study of DCIS (Zhuang *et al.,* 1995). This provides molecular evidence for the hypothesis that invasive lobular carcinoma develops from foci of LCIS. However, LCIS without associated invasive lobular carcinoma showed a very low frequency of LOH, comparable to a study of ALH (Chuqui *et al.,* 1997). The authors proposed that the observed differences in LOH frequency may indicate two sub-populations of ALH/LCIS, one that shows little genetic changes and indicates lesions that do not progress or possibly regress, and the second population, characterised by genetic alterations, which have the potential to progress to invasive carcinoma.

Cases of pure LCIS, and LCIS with associated DCIS or invasive cancer were examined for LOH at chromosomes 16q and 17p by Lakhani *et al.,* (1995a). In this study, multiple loci from seven cases were analysed. In one case, two distinct foci of pure LCIS showed allelic heterogeneity, suggesting the presence of independent genetic clones, as described for DCIS.

Atypical Ductal Hyperplasia

The study by Lakhani *et al.,* (1995b), demonstrated LOH for loci on chromosomes 16q and 17p in approximately 55% (5 of 9), and 20% (2 of 9) of ADH lesions, respectively. These findings suggest that ADH is a monoclonal, and therefore neoplastic proliferation which exhibits at least some of the genetic changes which characterise established *in situ* and invasive breast carcinoma. The incidence of LOH at chromosome 16q is similar to that reported in DCIS (Stratton *et al.,* 1995) and LCIS (Lakhani *et al.,* 1995a), suggesting that ADH, LCIS and DCIS may share a common path of evolution, as illustrated in Figure 1.5.

Morphologically Normal Breast Tissue

A recent investigation has demonstrated that some of the genetic aberrations found in invasive cancers are also present in morphologically normal breast epithelium from the same patient (Deng *et al.,* 1996). This study identified 8 of 30 cases with LOH, at three chromosome regions in morphologically normal ducts. In each of these cases the same allele was lost from the adjacent carcinoma cells. The highest frequency of LOH was observed at a marker positioned at 3p22-25. LOH was not found in normal ducts from surrounding tissue distant to the area of carcinoma, suggesting that the alteration was confined to normal ducts surrounding

the carcinoma. This has provided the first evidence that some invasive carcinomas may develop from morphologically normal epithelium (Figure 1.4).

Susceptibility Genes

A major incentive for studying the genetics of hereditary breast cancer was the belief that the genes responsible would also be somatically mutated or altered in a proportion of the non-familial or sporadic cases of the disease, similar to findings for colorectal cancer. To date, somatic mutations of *BRCA1* and *BRCA2* have not been identified outside of the familial cases. However, recent evidence suggests that these genes may fulfil the criteria of 'caretakers', as proposed by Kinzler and Vogelstein (1997). These have demonstrated that *BRCA1* and *BRCA2* bind to *Rad51*, a protein involved in maintaining genome integrity (Scully *et al.,* 1997; Sharan *et al.,* 1997). A model has been proposed whereby *Rad51*, *BRCA1* and *BRCA2* act as a complex together to repair damaged DNA (Brugarolso and Jacks, 1997). The *ATM* gene may also fulfil the criteria of a caretaker gene, since it has a central role in surveillance of DNA damage. It is part of a signal transduction pathway which activates cellular function in response to DNA damage (Meyn *et al.,* 1995).

Further insight in to the development and progression of breast cancer is likely to come with the identity of 'gatekeeper' genes.

1.7 Hypothesis and Aims

The hypothesis of this thesis was that genetic analysis of distinct and well defined groups of breast lesions would lead to the delineation of pathways of breast cancer development. It was reasoned that the starting point for genetic analysis must be a pure population of breast cancer cells. The genetic alterations chosen for this investigation (microsatellite instability and loss of heterozygosity) had previously been used to uncover distinct pathways ('mutator' and 'suppressor') in colorectal cancer development.

The specific aims associated with each type of genetic alteration are outlined below:

Loss of Heterozygosity

At the beginning of the project there was preliminary data to suggest that loss of regions on chromosome 16q maybe an 'early' event in the development of progression of breast cancer. The evidence came from cytogenetic analysis of breast tumours with little or no other genetic changes (Rodgers *et al.*, 1984; Dutrillaux *et al.*, 1990; Hainsworth *et al.*, 1991), and molecular analysis of invasive breast carcinomas (Larsson *et al.*, 1990; Sato *et al.*, 1990; Devilee *et al.*, 1991).

The aim of this thesis was to determine whether LOH at markers on chromosome 16q could be identified in well defined groups of breast lesions, each representing different stages in the hypothetical model of progression for the disease. The groups comprised: DCIS; tubular carcinomas, and mammographically detected early invasive carcinomas.

The approach involved preparation of tumour foci by microdissection. Each of these lesions could then be analysed by microsatellite PCR using highly polymorphic loci mapping to regions previously defined as undergoing LOH. Candidate tumour suppressor genes mapping to these regions could then by studied, in the cases which demonstrated LOH, using a combination of expression and mutation analyses.

The specific aims of this project were to:

- Determine if LOH on 16q occurs in early lesions;
- Investigate frequency of LOH on different regions of 16q;
- Analyse candidate tumour suppressor genes mapping to 16q in tumours which demonstrate LOH

Microsatellite instability (MI)

In 1994 there had been preliminary investigations of MI in breast cancer. The reported data were conflicting. Peltomaki *et al.*, (1993), and Han *et al.*, (1993) found no evidence of MI in breast tumours, whereas investigations by Yee *et al.*, (1994) and Wooster *et al.*, (1994b) did observe MI. It was clear that the presence or not and the significance of MI in breast cancer was a controversial issue.

MI was investigated in multiple microdissected foci of each of the breast lesions, using polymorphic microsatellites. The initial strategy of this project was to investigate the frequency of MI using different types of markers. Specific trinucleotide microsatellite markers were selected because they have been demonstrated to be intrinsically unstable in genetic diseases and therefore may represent sensitive markers of instability in cancer. A group of dinucleotides markers were studied because of concurrent projects with LOH mapping on chromosome 16q (and chromosome 6q by Steve Chappell). Finally, a number of mononucleotides were included in the analysis since it emerged that instability of this type may reflect an alternative pathway of mismatch repair.

The specific aims of this project were to:

- Determine if MI occurs in early lesions;
- Investigate the type and frequency of MI with different markers;
- Analyse candidate DNA repair genes;
- Analyse cancer-associated genes which may be targets for tumours with MI.

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Chapter 2

Materials and Methods

2.1 Introduction To Materials And Methods

The thesis has been concerned predominantly with the analysis of pre-invasive and mammographically-detected early invasive breast carcinomas. This does cause limitations since the lesions may not be diagnosed until after the tissue has been fixed and processed. There are advantages in that detailed analysis of different areas within individual cancers can be undertaken, and there are a variety of techniques now available which can be applied to fixed tissues.

Formalin Fixed Paraffin Embedded Tissues

Once removed from their normal physiological environment, morphological relationships between and within cells can only be preserved if their main structural components are stabilised and degradation prevented. Fixation by chemical methods relies on reagents that precipitate, denature or cross-link structural components within the tissue. Formaldehyde (formalin) works by reacting with basic amino acids to form cross-linking between proteins and DNA (Jackson and Chalkey, 1974).

Until the mid-1980s it was not known whether DNA could be prepared from formalin-fixed tissues. However, two studies (Goelz *et al.,* 1985; Dubeau *et al.,* 1986), demonstrated that DNA purified from fixed tissue remained partially intact, was double stranded, cleavable with restriction enzymes and therefore amenable to genetic analysis. The majority of DNA prepared from fixed tissues is in the range of 100 to 1500bp (Goelz *et al.,* 1985).

Tumour-specific alterations have traditionally been detected by Southern blot analysis. This technique generally requires microgram quantities of DNA, which are not available from many clinically obtained, small specimens. In addition this procedure requires DNA that can be digested by restriction enzymes. Warford *et al.,* (1988), demonstrated that although the extracted DNA was cleavable with some enzymes, not all could, and therefore there were still limitations in the use of fixed tissue.

Polymerase Chain Reaction

The polymerase chain reaction (PCR) is a technique which allows the amplification of a specific DNA region that lies between two regions of known DNA sequence (Saiki *et al.,* 1985). In the past few years, PCR has proven to be a fast and effective alternative for the analysis of small DNA regions that requires only minute amounts of DNA (Li H *et al.,* 1988).

In addition the quality of the DNA is not crucial because PCR allows amplification of DNA which has been partially degraded, in contrast to Southern blotting where the quality of DNA is important. The PCR has been used successfully on tissue samples from microscope slides (Meltzer *et al.*, 1991). Due to its exponential amplification, PCR can detect mutations in as little as one cell among a hundred thousand or more normal cells (Hardingham *et al.*, 1993). Therefore, when looking for tumour-specific alterations, the PCR is equally sensitive to the presence of non-tumour cells which can yield a PCR product and thus mask a tumour-specific alteration. Approaches to tumour enrichment are described later in this section.

The PCR applications utilised in this thesis are described below.

Microsatellites

Tandem dinucleotide repeats are one class of microsatellite sequences which are abundant interspersed repetitive DNA elements. Between 50 to 100,000 interspersed CA repeats are present in the human genome. The repeat sequences often exhibit length polymorphisms, which are inherited in a Mendelian manner, therefore representing an abundant source of genetic markers (Weber and May, 1989). The polymorphisms can then be analysed following PCR amplification using unique sequence primers that flank the microsatellite. The length variation of PCR amplification products can be revealed following fractionation by polyacrylamide gel electrophoresis. Microsatellite-PCR analysis is ideally suited to DNA prepared from fixed material because the amplification products are generally small (less than 200bp), and only nanogram quantities of DNA are required.

By comparing the microsatellite profiles of tumour and normal cells, from the same patient, it is possible to detect phenomena characteristic of some cancers, for example: genomic instability and loss of chromosomal regions (described in Chapters 3 and 4, respectively).

The majority of the microsatellites used in this thesis were dinucleotide repeats. In theory, heterozygous alleles can differ in size by a single repeat, i.e. 2bp. This type of resolution can not typically be achieved by agarose gel electrophoresis, therefore the microsatellite products are resolved on denaturing polyacrylamide gels. Following initial heat denaturation of the PCR products, they migrate through the polyacrylamide as single strands, in a size-dependent manner. Since polyacrylamide gels are larger than agarose gels, ethidium bromide staining is not practical. Therefore in order to visualise the PCR products a radioactive nucleotide (^{35}S dATP) is incorporated during the amplification procedure. This results in a radiolabelled PCR

product which can be visualised following exposure to autoradiographic film. DNA sequencing reactions (described below) which require 1bp resolution, were also analysed on denaturing polyacrylamide gels.

Mutation Detection

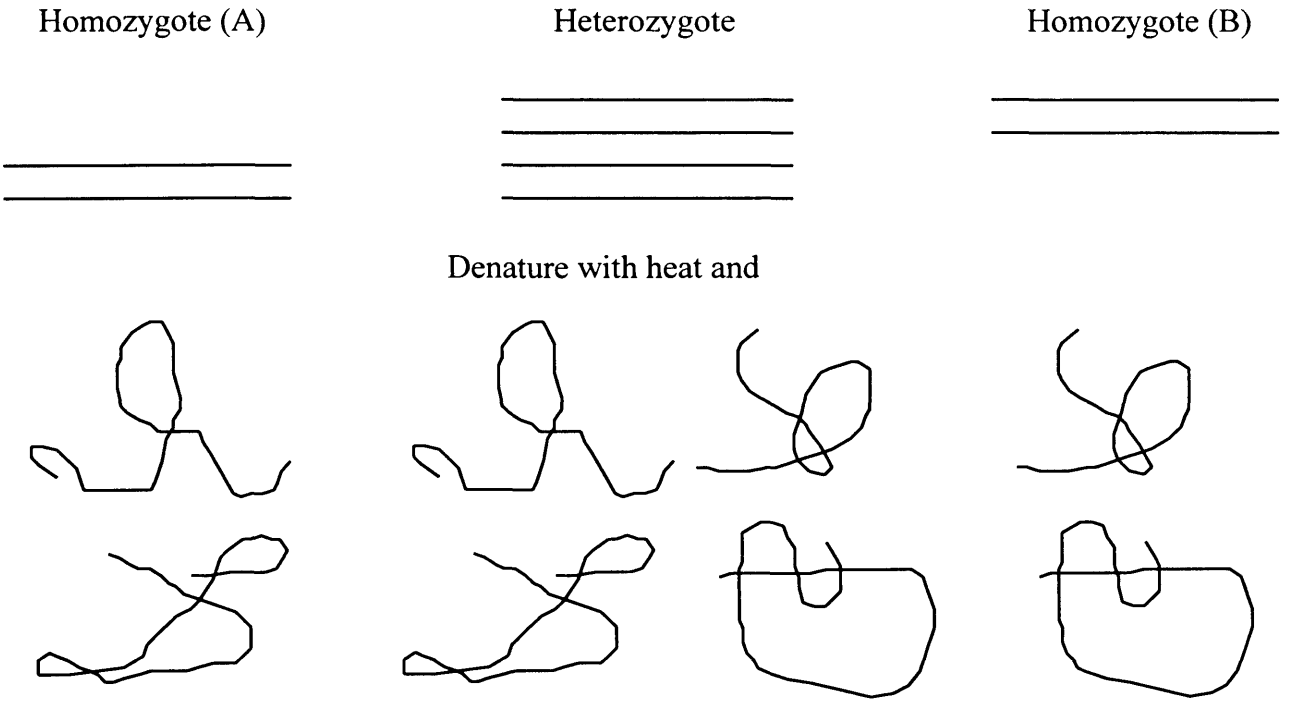
Single stranded conformation polymorphism analysis (SSCP) is a gel electrophoresis technique that allows the detection of mutations and polymorphisms in PCR products (Orita *et al.*, 1989a, 1989b). SSCP is based on the principle that the electrophoretic mobility of a molecule through a gel is dependent on size, charge, and shape of the molecule. Under non-denaturing conditions, single-stranded DNA has a folded secondary structure that is imposed by intramolecular interactions dictated by its nucleotide sequence. A single nucleotide difference between two similar sequences is sufficient to alter the secondary structure of one relative to the other. This results in a difference in mobility of the sequences. Incorporation of a radiolabel into the amplification reaction, allows the visualisation of these products following electrophoresis and autoradiography, as illustrated in Figure 2.1. Characterisation of mobility differences can then carried out by DNA sequencing.

DNA Sequencing

PCR products can also be sequenced directly without the need for subcloning. The approach used in this thesis was direct solid phase sequencing, as illustrated in Figure 2.2. One of the primers used for PCR is biotinylated at the 5′ end and following PCR amplification the product is captured with magnetic beads covalently coupled to streptavidin (Hultman *et al.*, 1989). The non-biotinylated strand is then eluted with alkali to yield single-stranded DNA immobilised at the 5′ end. The non-biotinylated primer can then be used as a sequencing primer in di-deoxy sequencing (Sanger *et al.*, 1977).

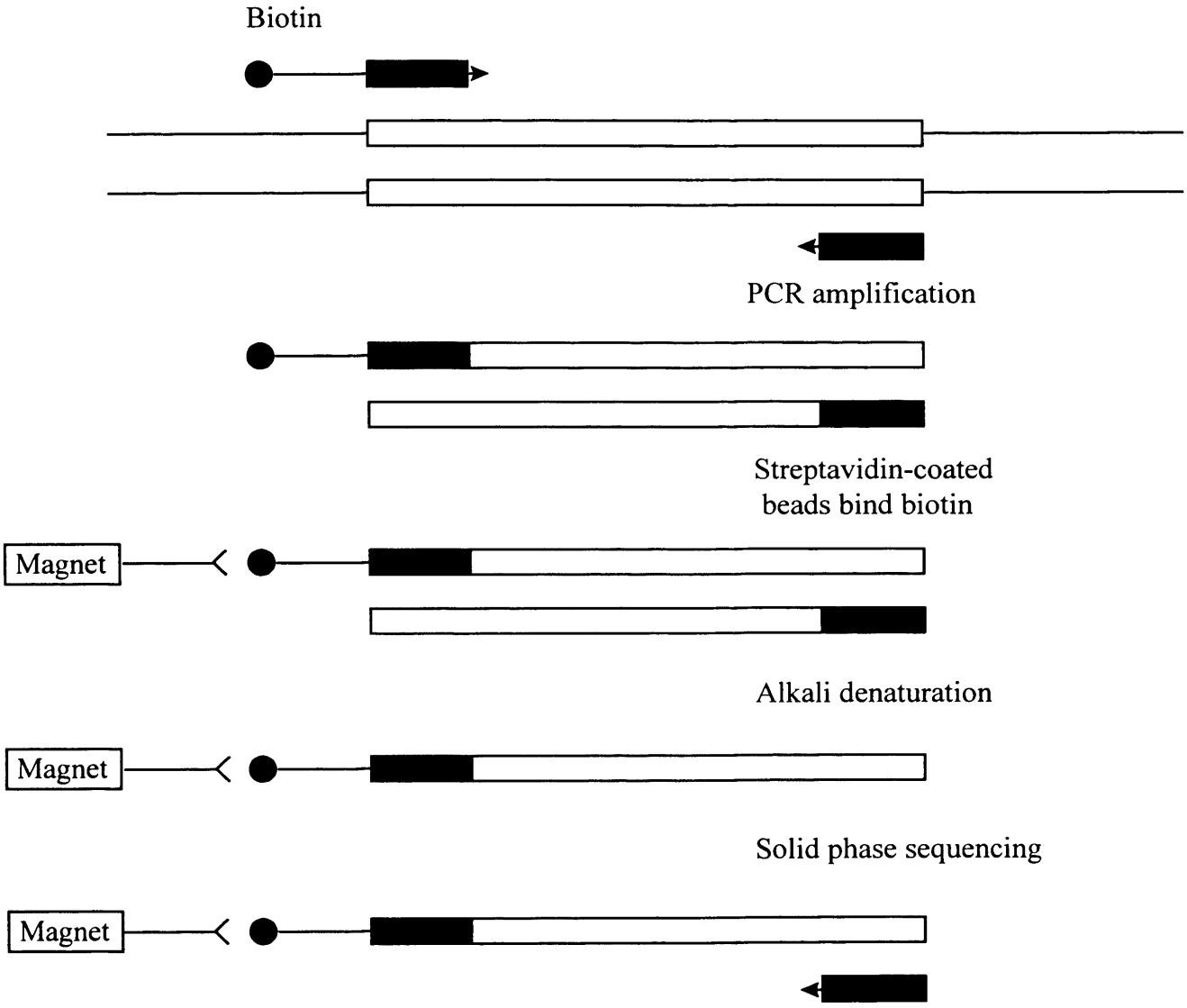
Figure 2.1. Diagrammatic representation of SSCP.

Following heat denaturation, double stranded character of PCR products is eliminated and folded single-stranded secondary structures emerge during cooling. The three dimensional structures are unique to the primary sequence and move at different rates through non-



denaturing polyacrylamide gels.

Figure 2.2. Direct solid-phase sequencing of PCR products (Newton and Graham, 1994). PCR amplification is carried out with a biotin labelled primer. Following amplification the products are captured by streptavidin coupled magnetic beads. The non-biotin strand is removed by alkali denaturation and the remaining strand is sequenced. Primers are indicated by black boxes.



Purification Of Tumour Cell Populations

Breast cancers are composed of a mixture of neoplastic and non-neoplastic cells. In tumour sections, neoplastic cells are surrounded by supporting stromal cells, leukocytes, necrotic cells and in some cases the pre-existing tissue of origin. DNA prepared from a single section of a paraffin embedded breast tumour is therefore likely to contain large amounts of non-tumour cell DNA, which may mask tumour specific alterations.

Individual breast tumour sections may also contain different histological components. For example, lesions of invasive carcinoma can contain areas of in situ carcinoma, each of which may harbour different genetic alterations. A tumour enrichment protocol must therefore be able to analyse multiple foci from a sample and correlate distinct alterations with each individual focus. The two main techniques which fulfil these criteria are: microdissection and selective ultraviolet radiation fractionation (SURF).

Microdissection

Tumour cells to be purified are identified and simply removed either manually by scraping cells from the tissue section with fine needles or capillary tubing (Going and Lamb, 1996), or by selectively adhering the cells to a transfer film with a pulse from a laser (Emmert-Buck *et al.*, 1996). DNA is then purified from procured cells, by standard extraction protocols, and amplified using PCR amplification.

SURF

This technique is essentially the reverse of microdissection. The tumour cells to be purified are protected by a covering of ink. The whole section is then irradiated with UV light, which leads to cross linking of DNA in all cells bar those protected by ink (Shibata *et al.*, 1992, 1993). DNA is then purified in the same was as described above.

A review of the current literature (Bohm and Wieland, 1997), shows that the microdissection approach is more widely used than SURF technique. The main disadvantage of SURF, is that it does not reliably inactivate short targets of DNA (less than 120bp). This may be a problem because many of the microsatellite markers used for PCR analysis generate PCR products less than 120bp.

Immunohistochemistry And Antigen-Retrieval

Although formalin fixation results in a high level of morphological preservation, the extensive cross-linking of proteins, may lead to a decrease in antigenic preservation. However, analysis of protein expression is possible. This is achieved by the technique of immunohistochemistry, whereby a specific antibody is targeted against the protein of interest and applied directly to the tissue section. Specific binding of antigen and antibody is then visualised microscopically. A variety of detection systems of increasing sensitivity have been developed, for example those based on avidin-biotin detection (Hsu *et al.*, 1981). There are also a number of 'antigen unmasking' or retrieval protocols which can improve protein detection (Shi *et al.*, 1991; Cattoretti *et al.*, 1993). Overall the method can qualitatively determine protein reactivity and also allow spatial distribution of protein expression.

In general, cryostat sections from fresh-frozen tissue give better antigenic preservation than formalin-fixed paraffin embedded tumours, but cytological and architectural detail can be poorer.

Confocal Laser Scanning Microscopy

An immunohistochemical approach using confocal laser scanning microscopy was adopted to gain more precise information about the localisation of cell adhesion molecules, and in particular to determine whether these proteins were expressed by breast myoepithelial cells.

In conventional microscopy, the illumination stimulates fluorescence throughout the whole depth of the specimen, thus contrast and resolution for objects within the focal plane can be compromised by background fluorescence from out of focus structures.

Confocal microscopy in contrast uses a combination of a focused illumination spot and a detection pinhole to restrict excitation and detection to within the focal plane. Consequently, out of focus structures do not contribute to background fluorescence as they receive little or no illumination. Image contrast and resolution are greatly improved in confocal microscopy and it is also possible to optically section specimens allowing three dimensional imaging of tissues (Ockleford, 1995).

Tissue Culture

A major limitation of fixed tissue is the inability to undertake functional assays. Cell lines have their limitations but can provide useful model systems and are of value for evaluating

new techniques, for example, methylation analysis. It is also possible to design experiments *in vitro* which may mimic what is occurring *in vivo*. For example, by adding anti-tumour drugs to the growth medium, it is possible to assess whether tolerance to these drugs is related to a decrease in the genetic stability of the cell lines or alterations in gene expression (Budworth *et al.*, 1997).

Over recent years tissue culture techniques have been developed to allow the maintenance of primary cultures, which have the advantage of allowing normal cells to be studied. For example, breast tissue from reduction mammoplasties can be maintained in culture. Individual cell populations, such as myoepithelial and epithelial cells can then by grown and studied independently, following cell separation protocols (Gomm *et al.*, 1995).

Methylation Analysis

It is now emerging that alterations in the DNA methylation machinery are among the most common changes associated with neoplasia and may have a causative role at an early stage in carcinogenesis (reviewed in Jones and Gonzalago, 1997). In higher order eukaryotes, DNA is methylated only at cytosine residues located 5′ to guanosine in the CG dinucleotide, giving rise to 5-methyl-cytosine (5-meC) (Jones, 1996). In humans, approximately 60% of genes contain CG-rich regions of DNA in their promoters. These 'CpG islands' are normally devoid of DNA methylation regardless of the expression status of the gene (reviewed in Jones, 1996). When present, DNA methylation of promoter-associated CpG islands leads to irreversible inhibition of gene expression, as demonstrated in X chromosome inactivation in women (Pfeiffer *et al.*, 1990), and in the transcriptionally silent copy of parentally imprinted genes (Sasaki *et al.*, 1992; Ferguson-Smith *et al.*, 1993; Stoger *et al.*, 1993). It is believed that methylation of these residues leads to the gene silencing through heterochromatisation (Bird, 1986). In recent years, inactivation of at least five tumour suppressor genes has been demonstrated to be due to de novo promoter methylation in sporadic cases (reviewed in Jones, 1996). Therefore, there is a need for a reliable technique which can detect aberrantly methylated gene sequences.

The detection of methylated regions of DNA has traditionally relied on Southern blotting approaches, based on the inability of methylation sensitive restriction enzymes (for example *Hpa*II, or *Cfo*I) to cleave sequences that contain one or more methylated CpG sites. This approach requires large amounts of high molecular weight DNA, and can detect methylation

only if present in greater than a few percent of alleles (Issa *et al.*, 1993). PCR-based methylation assays have been described which enable analysis of smaller amounts of template. These assays also use methylation specific enzymes to distinguish between methylated and unmethylated regions of DNA. Primers can be designed to amplify specific regions of interest following enzyme digestion (Singer-Sam *et al.*, 1993; Gonzalez-Zulueta *et al.*, 1995). However, due to the sensitive nature of PCR, a small percentage of undigested DNA can confuse the analysis.

A technique which reliably detects methylated DNA has recently been developed. It relies upon the ability of a chemical (sodium-bisulphite) to efficiently convert cytosine to uracil in single-stranded DNA, under conditions where methylated cytosines (5-MeC) are unreactive and remain as cytosine. The DNA sequence under investigation is then amplified by PCR with two sets of strand-specific primers to yield a pair of fragments, one derived from each strand, in which all uracil and thymine residues have been amplified as thymine and only 5-MeC residues have been amplified as cytosine (Frommer *et al.*, 1992). The PCR products can then be cloned and sequenced to provide methylation maps of single DNA molecules, or sequenced directly to determine the average methylation (Clark *et al.*, 1994).

More recently, a novel bisulphite-based PCR method, methylation-specific PCR (MS-PCR) has been developed which is specific and sensitive for methylation of CpG sites within CpG islands. The DNA sequence differences that result from the bisulphite reaction allow the design of PCR primers which distinguish methylated from unmethylated DNA. MS-PCR has recently been used for detecting aberrant methylation in four tumour suppressor genes in human cancer (Herman *et al.*, 1996).

2.2 Materials

Tissues

Impalpable carcinomas which had been detected by mammographic screening were fixed in 4% formaldehyde in saline for 18 h. After slicing, selected blocks 3-4 mm in thickness were processed through graded alcohols and xylene to paraffin wax.

All other tissues were received fresh within 30 min after surgery. Samples 8x5x3 mm were taken and frozen in liquid nitrogen, with subsequent storage in the vapour phase of a liquid nitrogen freezer. Parallel blocks of tissue 3-4 mm in thickness were taken and fixed for 18 h, then processed through graded alcohols and xylene to paraffin wax.

Following a review of haematoxylin and eosin-stained sections by Dr. R. A. Walker, a representative block was chosen for further study.

Histology

Ductal Carcinoma In Situ

DCIS from 23 patients was examined. 13 cases were identified by mammographic screening and 10 had presented clinically. Haematoxylin and eosin stained sections were examined and DCIS categorised for grade and architecture using the criteria in the NHS Breast Screening Programme National Co-ordinating Group for Breast Screening Pathology Guidelines, 1995 (NHSBSP), (Dr. R. A. Walker). These comprised 11 cases were of high nuclear grade, 3 of intermediate grade and 9 low grade, summarised in Table 2.1. For 2 cases lymph node tissue was available which was used as a source of normal DNA, whereas for 21 cases normal breast tissue, at least 3 cm away from the area of in situ carcinoma was the source of normal DNA. All tumour and normal samples had received the same fixation and processing.

Early Invasive Carcinomas

Thirty invasive breast carcinomas detected by screening mammography by the Leicestershire Breast Screening Service (between 1989 and 1992) were examined. All carcinomas were reported according to the NHSBSP guidelines (Dr. R. A. Walker). Infiltrating ductal carcinomas were graded using the modified Bloom and Richardson system (Elston and Ellis, 1991), summarised in Table 2.2. All had either axillary node sampling or axillary dissection. Lymph nodes, all of which negative for metastasis, were used as a source of normal DNA.

None of the tumours were from women with either a strong predisposition to the development of cancer or any known inherited predisposition to the development of tumours.

Tubular Carcinomas

Thirteen cases of tubular carcinoma were investigated. These were selected from the research files and were symptomatic cases. All tissues had initially been received fresh. They ranged in size from 10 mm to 35 mm, and four were lymph node positive.

Infiltrating Lobular Carcinomas

Twelve cases of infiltrating lobular carcinoma were investigated. These were selected from the research files and were symptomatic cases. All tissues had been received fresh. They ranged in size from 10 mm to 35 mm, and all were node positive.

Fibroadenomas

Eight cases of fibroadenoma and one benign phyllodes tumour (a neoplasm in which the stromal component predominates) were examined. These were selected from the research files. Normal surrounding breast tissue, at least 3 cm away from the lesion was the source of normal DNA.

Other Tissues

Extra-mammary tissues from selected patients were obtained from the Pathology files of the Leicester Royal Infirmary. All had been fixed and processed as described previously.

Table 2.1. Pathological features of DCIS.

DCIS Case	Nuclear Grade	Presentation	Source of normal DNA
1	High	Mamm. detected	Normal Breast
2	High	Mamm. detected	Normal Breast
3	Low	Mamm. detected	Normal Breast
4	Low	Mamm. detected	Normal Breast
5	High	Mamm. detected	Normal Breast
6	High	Mamm. detected	Normal Breast
7	High	Mamm. detected	Normal Breast
8	Low	Mamm. detected	Normal Breast
9	Intermediate	Mamm. detected	Normal Breast
10	Intermediate	Mamm. detected	Normal Breast
11	High	Clinically presenting	Normal Breast
12	High	Clinically presenting	Lymph Node
13	High	Clinically presenting	Normal Breast
14	High	Clinically presenting	Normal Breast
15	Intermediate	Clinically presenting	Normal Breast
16	High	Clinically presenting	Normal Breast
17	High	Clinically presenting	Normal Breast
18	Low	Mamm. detected	Normal Breast
19	Low	Mamm. detected	Normal Breast
20	Low	Clinically presenting	Normal Breast
21	Low	Clinically presenting	Lymph Node
22	Low	Mamm. detected	Normal Breast
23	Low	Clinically presenting	Normal Breast

Mamm. detected, indicates mammographically detected

Table 2.2. Pathological features of early invasive breast carcinomas.

Type	Grade	No. of Cases	Tumour size (mm)	No. of Cases
Tubular		4	<10	4
ILC/Tubular		1	10	10
IDC/ILC		1	11	2
ILC		1	12	4
IDC	I	10	13	2
IDC	II	11	14	0
IDC	III	2	15	8
Total		**30**		**30**

ILC, indicates Infiltrating Lobular Carcinoma, ICD, Infiltrating Ductal Carcinoma.

Chemical Reagents

All chemicals were 'analar' grade purchased from Sigma, with a few exceptions; Phenol, chloroform, isopropanol, acetone (Fisons), 2'-Deoxynucleoside 5'-Triphosphates (Pharmacia), ATPγS (Amersham USB), BSA (Boehringer Mannheim).

Bacteriological Reagents

Tryptone, Bacto-agar, and Yeast extract were purchased from Difco. Ampicillin, X-gal, and IPTG were from Sigma.

Tissue Culture Reagents

DMEM (without phenol red), FCS, L-glutamine and PBS were from Life Sciences. Trypsin and Dimethylsulfoxide were from Sigma. 75 cm^3 culture flasks were from Nunclon. Doxorubicin (2mg/ml) was kindly provided by Louise Reeve, Dept. Surgery.

Radioisotopes

α(^{35}S) dATP (0.4625 MBq/μl) was from either ICN Flow or Amersham USB.

DNAs

Plasmids containing trinucleotide repeats (CTG_5 and CTG_{20}) were kindly provided by Professor Keith Johnson, University of Glasgow

Genomic DNAs with previously characterised E-cadherin mutations (Berx *et al.*, 1996) were kindly provided by Dr Anne-Marie Cleton-Jansen, University of Leiden, as detailed in Table 2.3.

Table 2.3. Tumour samples with characterised E-cadherin gene mutations

Tumour Sample	Mutation Site	Exon	Nucleotide Change
BT1004	cd 254	6	Deletion of 17bp
BT1172	cd 321	7	Insertion of 5bp
BT823	cd 346	8	Substitution of 1bp
BT887	cd 368	8	Insertion of 1bp
BT995A	cd 408	9	Deletion of 1bp

cd indicates codon position (Berx et al., 1995a)

Antibodies and Sera

E-cadherin

Mouse monoclonal E-cadherin antibody (Clone 5H9) was purchased from EuroPath. The antibody reacts with 120 kD and 80 kD proteins in Western-blotting.

P-cadherin

Mouse monoclonal P-cadherin (NCC-CAD-299), available within the Department of. Pathology, was originally supplied by Dr. Setsuo Hirohashi, National Cancer Center Research Institute, Tokyo. The antibody reacts with a 72 kD protein in Western-blotting (Shimoyama *et al.*, 1989).

H-cadherin

Rabbit polyclonal H-cadherin was kindly provided by Dr. Sam Lee, Beth Israel Hospital, Harvard. The polyclonal was raised against a synthetic peptide spanning an extra-cellular domain of the H-cadherin molecule. The antibody detects proteins of 80 kD and approximately 110 kD by Western blotting (Lee, 1996).

MSH2

Mouse monoclonal MSH2 (NA27) was purchased from Oncogene Science. The antibody was raised against a carboxy-terminal fragment of human MSH2 protein, and reacts with a 100 kD protein in Western-blotting (Thibodeau *et al.*, 1996).

MLH1

Mouse monoclonal MLH1 (NA28) was purchased from Oncogene Science. The antibody reacts with a 88 kD protein in Western-blotting. The epitope from which the antibody was raised is unknown.

PMS2

Mouse monoclonal PMS2 (NA30), was purchased from Oncogene Science. The antibody reacts with a 96 kD protein on western-blotting. The epitope from which the antibody was raised is unknown.

Secondary antibodies

Biotinylated and FITC-labelled swine anti-rabbit immunoglobulin and rabbit anti mouse immunoglobulin antisera were obtained from Dako. StreptABC Complex HRP Kit was purchased from Dako.

Sera

Normal rabbit and normal swine sera were purchased from Life Sciences.

Cell lines

T-47-D - ER positive (Keydar *et al.*, 1979), MCF-7 - ER positive (Soule *et al.*, 1973), MDA-MB-231 - ER negative (Cailleau *et al.*,1974), cell lines were originally derived from malignant pleural effusions. HBL-100 - An ER positive cell line was derived from the milk of a lactating mother (Gaffney *et al.*, 1976). It is considered to be 'non-tumourigenic'. However, it was shown to be abnormal as early as passage 7 and has been demonstrated by some workers to be tumourigenic in nude mice (Ziche & Gullino, 1982). All cell lines were originally obtained from American Type Culture Collection.

MCF-7/Adr - A multiple drug-resistant derivative of MCF-7 was kindly provided by Dr. Gillian Hirst, Beatson Institute, Glasgow.

Pure populations of breast myoepithelial cells, prepared by cell separation of reduction mammoplasty samples, were kindly provided by Dr Louise Jones, Department of Pathology.

Enzymes

Commonly used restriction endonucleases were purchased from Life Sciences BRL and Boehringer Mannheim. *Taq* DNA polymerase was from Life Sciences, Promega and Bioline. Proteinase K and Protease (Type14) were from Boehringer Mannheim. RNase I and Trypsin were from Sigma. *Pfu* DNA polymerase was from Strategene.

Electrophoresis

Agarose was from ICN. Nusieve low melting agarose was from FMC. The λ/*Hind*III and ϕX174/*Hae*III DNA fragments used as size markers were obtained from Promega. Sequagel polyacrylamide was from National Diagnostics. Mutation Detection Enhancement (MDE) acrylamide was from AT Biochem. 3MM chromatography paper was from Whatmann.

Photography And Autoradiography

Photographs of gels were taken using a Sony Gel Documentation system 5000 and a UVP transilluminator, with Sony UPP-11-HD thermal paper. Biomax film was from Kodak. Exposed autoradiographs were developed automatically using a RP-X-OMAT Kodak processor.

Commonly Used Solutions

ddH_2O	Distilled and sterile throughout
PBS (20X)	2.6M NaCl, 60mM Na_2HPO_4, 140mM NaH_2PO_4, pH 7.4
TBS (20X)	0.05M Tris, 0.15M NaCl, 0.1% (w/v) BSA, pH 7.65
TE (1X)	10mM Tris-HCl (pH 8.0), 1mM EDTA (pH8.0)
TAE Buffer (50X)	2M Tris, 1M Glacial Acetic Acid, 0.005M EDTA, pH 8.0
TBE Buffer (10X)	108g Tris, 55g Boric Acid, 40ml 0.5M EDTA
Agarose loading buffer	0.025% Bromophenol blue, 0.025% Xylene cyanol, 2.5% Ficoll in water
PAGE loading buffer	95% formamide, 20mM EDTA, 0.05% Bromophenol blue 0.05% Xylene cyanol
SSCP loading buffer	As PAGE buffer with 50mM NaOH
Standard PCR buffer	45mM Tris-HCl (pH8.8), 11mM $(NH_4)_2SO_4$, 4.5mM $MgCl_2$, 200μM each dNTP, 113mg/ml BSA, 6.7mM β-mercaptoethanol, 4.4μM EDTA (pH 8.0)
^{35}S PCR buffer	As standard recipe except, dATP at 25μM
Binding Washing (BW) buffer (2X)	10mM Tris-HCl (pH 7.5), 1mM EDTA, 2.0 M NaCl.
Mayer's Haematoxylin Solution	0.1% Haematoxylin, 5% ammonium or potassium alum, 0.02% sodium iodide, 0.1% acid, 5% chloral hydrate
Eosin Solution	1% Aqueous water soluble eosin
Citric Acid Buffer	10mM Citric acid, pH 6.0

Homogenisation buffer	0.05M Tris-HCl, 2M urea, 1g/l NaCl, 1g/l EDTA, 1ml/l Brij 35, 0.1mM PMSF
SDS-PAGE sample buffer (2X)	0.05M Tris-HCl, 4% SDS, 20% glycerol, 0.2% bromophenol blue, 1.4M β-mercaptoethanol ,pH 6.7.
TBS-T	As TBS, without BSA, and with 0.1% Tween 20.
Blocking solution	TBS-T and 5% dried skimmed milk powder
LB/litre	10g bacto-tryptone, 5g yeast extract, 10g NaCl, 1g glucose, (15g agar/litre for plates).

2.3. Methods

Cell Culture

Maintenance Of Cell Lines

Established breast cancer cell lines were routinely maintained in $50cm^3$ culture flasks containing phenol red-free Dulbecco's modified Eagles medium (DMEM) supplemented with 10% FCS and 2mM L-Glutamine and incubated in a humidified atmosphere of 5% CO_2/95% air at 37^0C until 70-90% confluent.

Passage

Culture media was aspirated, cell monolayers were washed with PBS and cells removed by treatment with 2ml trypsin (0.5g/l). The cell suspension was then centrifuged at 3,000rpm for 5 min at 4^0C. Cell pellets were resuspended in 6ml preheated culture medium, and used to seed 3 flasks each containing 8ml of culture medium.

Freezing Down

A cell suspension was prepared as described above and resuspended in 0.5ml FCS and 0.5ml freezing solution (20% dimethylsulfoxide, 80% DMEM), placed at -70^0C overnight, then stored under liquid nitrogen.

Doxorubicin Experiments

An experiment was conducted to assess the tolerance of the MCF-7 cell line to increasing concentrations of the anti-tumour drug, doxorubicin. A flask of MCF-7 cells was grown to 70-90% confluence in the absence of doxorubicin. The cells were harvested and used to seed three flasks each containing medium with of 3nM doxorubicin. This was repeated for 30nM and 300nM doxorubicin. Cells were washed, trypsinised, and pelleted prior to DNA and protein extraction, as described later in this section.

DNA Extraction's

DNA template was prepared from tumour foci by means of microdissection. DNA was also prepared from both cell lines and fixed tissues, as described below.

Microdissection

A microdissection procedure was used to procure tumour cells from haematoxylin and eosin (H&E) stained sections of formalin-fixed, paraffin embedded tissues. DNA template was then prepared from the cells and used in subsequent genetic analysis.

Haematoxylin and Eosin (H&E) Staining Of Tissue Sections

Tissue sections (10μm) were cut onto glass microscope slides and placed in a 37°C incubator overnight. Dried sections were then dewaxed in xylene (1x 5min, 1x 2min) and rehydrated through a series of graded alcohols (2x 2min 99% alcohol; 1x 2min 95% alcohol) and rinsed in running tap water. Sections were then immersed in Mayer's haematoxylin solution for 1 min, rinsed in tap water, immersed in eosin for 15 sec and rinsed in running tap water. Stained section were then dehydrated, transferred to xylene and mounted in resinous mountant (XAM).

Microdissection Of Tissue Sections

The method used was based on the technique described by Koreth *et al.*, (1995). Two 10μm sections were cut and mounted on microscope slides, deparaffinised and stained with H&E, One H&E section was mounted and served as a reference section. Distinct foci were visualised and marked with pen by a Dr R. A. Walker. The corresponding foci were dissected from the unmounted section, with the aid of x40 magnification dissecting microscope (American Optical Corporation) using a sterile drawn-out capillary tube. The glass tip and procured cells was broken into a 0.5ml eppendorf containing 25μl digestion buffer (100mM Tris-HCl (pH8.8), 1mM EDTA (pH8.0), 200μg/ml Proteinase K). The samples were then incubated for 3h at 56°C, followed by heat inactivation of Proteinase K at 94°C for 10min, and then stored at 4°C. An aliquot (typically 5μl) was used as template for PCR amplification.

Fixed Tissues

Between two and six, 10μm sections were cut and placed in a sterile tube. Xylene (1ml) was added and they were allowed to stand at room temperature for 10 min. The samples were centrifuged at 13,000rpm for 5 min and the supernatant removed. The procedure was repeated with 1ml fresh xylene, then with 2x 1ml of absolute ethanol. The tissue pellets were air-dried and incubated for 3h at 56°C in 100μl digestion buffer (100mM Tris-HCl (pH8.8), 1mM EDTA (pH8.0), 200μg/ml Proteinase K). Samples were then extracted twice with equal volumes of phenol/chloroform, twice with chloroform and precipitated with three vols. of ethanol and 0.1 vol. of 3M sodium acetate (pH4.8). Following centrifugation (13,000 rpm for

10 min at 4^0C) the pellets were washed with 70% ethanol, air dried and resuspended in ddH_2O, prior to spectrophotometry.

Cell Lines

Cells were washed, trypsinised and pelleted as described previously. The cell pellet was then resuspended in 100µl extraction buffer (0.05M KCl, 0.01M Tris-HCl, pH8.3, 0.01M $MgCl_2$, 0.5% Tween 20, 0.5% NP40, 500µg/ml Proteinase K) incubated at 56^0C for 3h and phenol/chloroform extracted prior to ethanol precipitation, and resuspension in ddH_20. The concentration of the resulting DNA was determined by spectrophotometry.

Spectrophotometry

DNA concentrations were determined by spectroscopy. DNA samples diluted in ddH_20 were placed in silica cuvettes and the absorbency read at a wavelength of 260 and 280nm on a spectrophotometer (Pye Unicam, SP1700). DNA concentrations were calculated using the following approximation: OD_{260} of 1.0 is equivalent to 50µg/ml of double stranded DNA (Sambrook *et al.*, 1989a).

PCR Methodologies

To avoid contamination, all PCR reactions were prepared using PCR-dedicated reagents, tips and tubes. Reactions were prepared in a dedicated 'clean' area, in which no other experiments involving DNA, were carried out.

Primers

PCR primer information was obtained from cited publications (detailed in Table 2.4) the Genome Data Base (http://www.gdb.org), and the Chromosome 16 database at the Center for Human Genome Studies (http://www-ls.lanl.gov).

For a number of experiments, PCR primers were designed using the 'Prime' program of the Wisconsin Package (Genetics Computer Group). The default settings for the program were used on all occasions, except when designing primers to amplify GC-rich regions. For these primers, the percentage of CG content of the PCR primers and PCR products were increased to allow primer design. Primer combinations were then compared with EMBL/Genbank databases using the 'findpatterns' program to ensure unique primer binding sites. Primers were purchased from Life Sciences and Genosys, and supplied as desiccated oligonucleotide pellets.

Table2.4. (a) Primers used for microsatellite instability analysis.

Primer	Forward 5′-3′	Reverse 5′-3′	Tm (^{0}C)	Chr/Gene location	Size (bp)	Reference
DM	CTTCCCAGGCCTGCAGTTTGCCCATC	GAACGGGGCTCGAAGGGTCCTTGTAGC	67	3′ UTR	128-176	Brook *et al.*, 1992
X75b	GCCAGCCATTCAGTCATTTGAAG	CTGAAAGACACGTCACACTGGT	62	19q13	120-142	Jansen *et al.*, 1992
SBMA	GCCTGTTGAACTCTTCTGAGC	GCTGTGAAGGTTGCTGTTCCTC	65	Xq12	405-450	La Spada *et al.*, 1991
SCA-1	CAACATGGGCAGTCTGAG	AACTGGAAATGTGGACGTAC	57	6p22-23	210-	Orr *et al.*, 1993
TGFβRII	CTTTATTCTGGAAGATGCTGC	GAAGAAAGTCTCACCACG	63	n. 665-737	73	Myeroff *et al.*, 1995
IGFIIR	GCAGGTCTCCTGACTCAGAA	GAAGAAGATGGCTGTGGAGC	66	n.4029-4140	111	Souza *et al.*, 1996
Bax	ATCCAGGATCGAGCAGGGCG	ACTCGCTCAGCTTCTTGGTG	67	n. 91-185	94	Rampino *et al.*, 1997
E2F-4	CAACAACACTGGACACCCGGC	TCAAAGGAGGTAGAAGGGTTGG	67	[a]	130	Yoshitaka et al., 1996
BAT25	TCGCCTCCAAGAATGTAAGT	TCTCATTTTAACTATGGCTC	59	[b]	80	Liu *et al.*, 1995
BAT40	ATTAACTTCCTACACCACAAC	GTAGAGCAAGACCACCTTG	61	[c]	105	Liu *et al.*, 1995
cESR	GAC GCA TGA TAT ACT TCA CC	GCA GAA TCA AAT ATC CAG ATG	56	6q25.1	178-194	Orphanous *et al.*, 1995
D6186	TTA CCC ACT ACC TAC CCA GAG	GTC CCT TGG AAA ATT CTC CCT	56	6q26-27	235	Orphanous *et al.*, 1995
D6S193	AGA GCA GGC TCT GCA TGG TTA	CTG ACA AAA GAA CAT ATT GTT TCC C	56	6q26-27	190	Orphanous *et al.*, 1995

[a] *Reverse primer is intronic,* [b] *Intron of c-kit oncogene and* [c] *Intron of 3-β-hydroxysteroid dehydrogenase gene. n., is nucleotide position, UTR, is untranslated region.*

Table2.4. (b) Primers used for methylation analysis of E-cadherin promoter region.

Primer	Forward 5′-3′	Reverse 5′-3′	Tm (^{0}C)	Gene location	Size (bp)	Reference
EcadProm	ACCTAGACCCTAGCAACTCC	ACAGGTGCTTTGCAGTTC	63	See Appendix I	221	Not Applicable
EcadM	TTAGGTTAGAGGGTTATCGCGT	TAACTAAAAATTCACCTACCGAC	57	See Appendix I	116	Herman *et al.*, 1996
EcadU	TAATTTTAGGTTAGAGGGTTATTGT	CACAACCAATCAACAACACA	53	See Appendix I	97	Herman *et al.*, 1996

Table2.4. (c) Primers used for loss of heterozygosity and microsatellite instability analysis.

Primer	Forward 5′-3′	Reverse 5′-3′	Tm (^{0}C)	Chr. location	Size (bp)	Reference
D16S400	CTCATCCGACTTCTCACAGC	AATATGAACCCTCCATGCTG	60	16q21	192-202	Weissenbach *et al.*, 1992
D16S289	CACACATTATCATTTCTTCCAAGCTGTG	AGTTGGAGGAAGAGAGAAGCAG	62	16q23	156-172	Weissenbach *et al.*, 1992
D16S402	TTTTGTAACCATGTACCCCC	ATTTATAGGGCCATGACCAG	66	16q24.2	161-187	Weissenbach *et al.*, 1992
D16S3026	CTCCCTGAGCAACAAACACC	GGTCATTTATATGCGCCTGA	65	16q24.3	212-220	Dib *et al.*, 1996
D16S413	ACTCCAGCCCGAGTAA	GGTCACAGGTGGGTTC	65	16q24.4	131-149	Weissenbach *et al.*, 1992

Table2.4. (d) Primers used for SSCP and mutation analysis of E-cadherin gene. Primers are located in introns flanking each exon.

Primer	Forward 5′-3′	Reverse 5′-3′	Tm (^{0}C)	Gene location	Size (bp)	Reference
GEC14/24	ACCCAGTGTTGGGATCCTTC	GTTACCCCGGTGTCAACAAG	66	Exon 5	192	Kanai *et al.*, 1994
GEC12/22	TCCTCATCACAGCTCAAGTC	GGGTCCAAAGAACCTAAGAG	62	Exon 6	243	Oda *et al.*, 1994
GEC11/21	TGCCCAGTCCCAAAGTGCAG	TCCACACCCTCTGGATCCTC	65	Exon 7	253	Oda *et al.*, 1994
GEC13/23	CCTGACTTGGTTGTGTCGATC	GACCTTTCTTTGGAAACCCTC	65	Exon 8	178	Kanai *et al.*, 1994
Ecad9	GTACTTGTAATGACACATCTC	TGCCAGTTTCTGCATCTTGC	63	Exon 9	252	Berx *et al.*, 1995a

The oligonucleotide pellet was resuspended in 200µl ddH_20 and stored at -20^{0}C. An aliquot of this stock solution was then diluted to 10pmol/ml and used as the working stock solution. The sequence of each primer set is outlined in Table 2.4.

A number of PCR primers were 'biotinylated' to enable single-stranded DNA sequencing. This procedure is detailed the section describing DNA sequencing.

PCR Optimisation

PCR conditions for each primer set were optimised by evaluating different annealing temperatures. The initial annealing temperature was calculated using the following equation: 4(G+C) + 2(A+T). A number of reactions were then set up in parallel, and amplified at the estimated initial annealing temperature, and with temperatures increasing in increments of 2^{0}C. The optimal annealing temperature for each primer, was then judged as that which gave specific and consistent amplification. The optimised annealing temperatures for each of the primer sets is outlined in Table 2.4.

DNA Amplification

PCR amplification was carried out using the 'hot-start' procedure. This technique involves heating all of the reaction components, except for the PCR primers, to 94^{0}C. By adding the primers after the initial denaturation step, non-specific hybridisation can be avoided and in general this decreases amplification of spurious PCR products.

Typically, 25µl reactions were set up. DNA template (50-100ng or 5µl of microdissected material) were mixed with standard PCR reaction buffer (Section 2.2) and overlaid with a drop of mineral oil. This solution was then denatured for 5 min at 94^{0}C before primers (10pmoles each) and 1 Unit *Taq* DNA polymerase were added.

For radiolabelled PCR, amplification was carried out in the presence of 0.0925MBq ^{35}S dATP. The reaction components were similar to those described above, for non-radioactive PCR except that ^{35}S reaction buffer was used (Section 2.2). This contained dATP at a final concentration, eight times lower than that of the other dNTPs. The lower concentration of dATP facilitated the incorporation of the ^{35}S labelled dATP in the amplification products.

Reactions were amplified for between 30-35 cycles in a Perkin-Elmer Cetus (Version 2.2) or Hybaid Omnigene thermal cycler. Negative and positive controls were used throughout. A typical amplification protocol is outlined below.

Typical cycling conditions:

Initial denaturation	*94^{0}C - 5 min*
	94^{0}C - 1min
Cycling program (X35)	*60^{0}C - 1min (optimised annealing temperature)*
	72^{0}C - 1min
Final extension	*72^{0}C - 7min*

Electrophoresis

Restriction digests of genomic DNA and non-radioactive PCR products were resolved on agarose gels, and visualised by ethidium bromide staining of the DNA. DNA sequence products and microsatellite PCR products were resolved on denaturing polyacrylamide gels. Mutation analysis by SSCP, was carried out on non-denaturing polyacrylamide gels.

Agarose Gel Electrophoresis

PCR products were fractionated by electrophoresis through 1.0 - 2.0% Seakem agarose gels in 1X TAE buffer. Loading buffer was added to the samples to 1X concentration, prior to electrophoresis. Gels were stained in 0.5mg/ml ethidium bromide for 15 min to allow the DNA to be visualised on a UVP transilluminator. Electrophoresis was performed typically at 80 volts for 1-3h.

Denaturing Polyacrylamide Gel Electrophoresis

Radiolabelled PCR products and DNA sequencing reactions were diluted 2:1 in loading buffer. The samples (7μl) were denatured for 3min at 94^{0}C and loaded directly onto 6% polyacrylamide/8M urea gels. Electrophoresis was performed in 1X TBE at 60-90 Watts, 50^{0}C for 2-4 h. Gels were then fixed for 20min in 10% methanol and 10% acetic acid and then vacuum dried. Exposure to X-OMAT film (Kodak) took place at room temperature for 1 to 7 days.

Non-Denaturing Polyacrylamide Gel Electrophoresis (SSCP)

PCR products were diluted 2:1 in SSCP loading buffer, heat denatured, as above, and incubated on ice for 5 min, prior to loading samples on 0.5 - 0.8X MDE gels. Electrophoresis was performed in 0.6X TBE at 6-8 Watts for 12-18 h, at room temperature. Gels were dried and exposed to film as described above.

DNA Sequencing

DNA sequencing was carried out by the dideoxy chain-terminating method (Sanger *et al.*, 1977), using the Sequenase Version 2.0 Kit (Amersham). DNA sequencing was carried out on subcloned PEG-purified plasmid and biotinylated-PCR products. The preparation of each of these templates is described below.

Preparation Of Plasmid

DNA regions to be sequenced were PCR-amplified and subcloned into restricted plasmid. The subcloning procedure, transformation, and subsequent plasmid preparation of recombinant clones is detailed below.

Subcloning

Direct subcloning of PCR products was carried out with the pCR-Script SK(+) Cloning Kit (Stratagene), according to the manufacturers directions. *Pfu* DNA polymerase was used in the initial amplification reaction to facilitate blunt ended ligation of vector to insert.

Transformation

Following subcloning, 10ng of the ligation reaction was transformed into supercompetent *E. coli* XL-1 Blue cells, by 'heat-pulse', as per the manufacturers guidelines. The transformation reaction was plated onto selective agar plates, and recombinant colonies were differentiated from transformants by colour selection, with X-gal and IPTG. Recombinant colonies were then restreaked onto new plates and stored in a glycerol suspension at -70^{0}C (Sambrook *et al.*, 1989b).

Plasmid Preparation (Birnboim and Doly, 1979)

A single colony was inoculated into 10ml of LB/Ampicillin (50μg/ml) and grown overnight at 37^{0}C with shaking (200 rpm). A 10ml overnight culture of the clone of interest was then pelleted by centrifugation at 13,000rpm for 1min, and resuspended in 400μl of chilled buffer P1 (100μg RNase A, 50mM Tris-HCl pH 8.0, 10mM EDTA pH 8.0) and split into two eppendorfs. 200μl of freshly prepared buffer P2 (200mM NaOH, 1% SDS) was added, mixed gently by inversion, and incubated at room temperature for 5 min. 200μl chilled buffer P3 (3M Potassium Acetate, pH 5.5) was added, mixed gently by inversion, and incubated on ice for 15 min. Supernatants were transferred to fresh tubes following centrifugation at 13,000 rpm for 5 min, followed by phenol/chloroform extraction and ethanol precipitation.

The plasmid was resuspended in 32µl of ddH_2O and polyethylene glycol (PEG) precipitated by the addition of 8µl 4M NaCl and 30µl 13% PEG. Following incubation on ice, the plasmid was centrifuged as before, washed in 70% ethanol, and finally resuspended in ddH_2O. Sequencing was carried out with 3-5µg RNA-free PEG purified, alkali denatured plasmid, using the conditions described by the manufacturers.

Single Stranded Sequencing

Single stranded DNA template was prepared by streptavidin-biotin selection of PCR products. Streptavidin coated Dynabeads (Dynal) were used to capture biotinylated PCR products. The primer biotinylation, and Dynabead purification is detailed below.

Primer Biotinylation

Primers were biotinylated essentially as described in the Oligonucleotide Labelling Kit (Amersham). Briefly, 500pmoles of primer was incubated with 1X T4 polynucleotide kinase buffer, 5mM ATPγS and 30 units of T4 polynucleotide kinase. At 37^0C for 1h, followed by inactivation at 70^0C for 10min. The primer was then ethanol precipitated and resuspended in 100mM Potassium Phosphate (pH 8.0), NIBH, and incubated at 50^0C for 1h. The reaction was made up to 100µl with ddH_2O and purified on pre-equilibrated Sephadex G25 spun columns by centrifugation at 1200g for 5 min.

Spun-Column Chromatography

Spun columns were prepared by plugging 1ml disposable syringes with polyallomer wool and then adding a slurry of pre-swollen Sephadex G25/TE. Columns were spun at 1200g for 5min and topped up with slurry until the column was packed. The column was then equilibrated by adding 100µl TE and centrifuged as before. After each centrifugation the volume of eluent was measured and the procedure was repeated until the eluent was 100µl.

Dynabead Separation

Following PCR amplification with one biotinylated primer (typically the reverse primer) and a non-biotinylated primer (forward), the PCR products were captured using Dynabeads coated with Streptavidin, essentially as described in the Dynabead M-280 kit (Dynal). 10µl Dynabeads (1mg/ml) were washed in an equal volume of 1X Binding and Washing (BW) buffer, by placing in a magnet for 30 sec, and resuspended in 40µl of 2X BW. Washed beads were added to an equal volume of biotin-labelled PCR products and incubated with constant pipetting for 15 min at room temperature The pellet was washed in 40µl of 1x BW, and then incubated in 8µl 0.1M freshly prepared NaOH with constant mixing for 10 min. This was

followed by washes in: 50µl 0.1M NaOH, 40µl 1X BW, 50µl 1X TE, and finally resuspended in ddH_2O.

Methylation Analysis

Methylation analysis of the E-cadherin promoter region was carried out on DNA prepared from the MCF-7 and MDA-MB-231 cell lines using a PCR based methylation assay, and the Methylation-Specific PCR protocol (Herman *et al.,* 1996). Appendix I, illustrates the E-cadherin promoter region.

PCR-based Methylation Assay

1µg cell line DNA was digested for 3 h at 37^0C with 25 Units *Hpa*II, in a total volume of 25µl. Samples were then inactivated by heating at 100^0C for 10min. 50ng of digested DNA was then used in PCR amplification.

Bisulphite Treatment

The bisulphite reaction was carried out on linear fragments of DNA (Herman *et al.,* 1996). This was achieved by digestion with a restriction enzyme which did not cut within the target sequence. DNA (typically 1µg) was denatured by the addition of freshly prepared NaOH, to a final concentration of 0.3M and incubated for 15 min at 37^oC. 3.1M Sodium bisulphite (pH 5.0) and 0.5mM hydroquinone were added to the denatured DNA. The sample was gently mixed, overlayed with mineral oil and incubated at 50^oC for 16 h. The DNA was recovered by snap freezing the reaction and removing the unfrozen mineral oil.

Removal of free bisulphite was achieved by using a desalting column (Wizard Columns, Promega), according to the manufacturer's instructions, and eluted in 50µl of ddH_20. The sample was then denatured with NaOH, as before, and neutralised by the addition of NH_4OAc, pH 7.0, to a final concentration of 3M and the DNA was ethanol precipitated, dried, and resuspended in ddH_2O. Amplifications with the MS-PCR primers were performed on 1-5µl of bisulphite-treated genomic DNA.

Immunohistochemistry - Immunoperoxidase

Immunohistochemistry was performed to detect protein expression/antigen reactivity of cryostat tissue sections and formalin-fixed, paraffin-embedded tissue samples.

Cryostat Tissue Sections

Frozen sections (7μm)were cut on a cryostat (Bright) at -20^{0}C. Sections were stored at -70^{0}C overnight and air dried, prior to fixation in acetone for 10 min at 4^{0}C. Following air drying, the sections were equilibrated in PBS and immunostained as outlined below.

Antigen Retrieval/Presentation Conditions For Formalin-Fixed Paraffin Embedded Tissues

Sections (4μm) were cut onto glass slides coated with 2% 3-aminopropyltriethoxysilane and placed in a 37^{0}C incubator overnight. Sections were then dewaxed and rehydrated, as previously described (DNA extractions).

Pressure Cooking

The dewaxed sections were immersed in a Prestige pressure cooker (Model Number 6869) containing boiling 10mM citric acid buffer, pH6.0. The sections were incubated for 1 min, once the pressure gauge had risen completely. After completion slides were immediately transferred to PBS prior to immunostaining.

Protease Digestion

The dewaxed sections were immersed in a solution of 0.05% protease type-14, prepared in 0.01% $CaCl_2$/PBS (preheated to 37^{0}C) for 20 min. Slides were then rinsed in PBS prior to immunostaining.

Proteinase K Digestion

The dewaxed sections were immersed in a solution of 2-5mg/ml proteinase K in PBS (preheated to 37^{0}C) for 20 min. Slides were then rinsed in PBS prior to immunostaining.

Antibody Dilutions

The working dilutions of the primary and secondary antibodies are summarised in Table 2.5 - 2.7.

Avidin-Biotin Complex Peroxidase (ABC) Technique

Hydrated sections were equilibrated in PBS for 5 min. Excess buffer was wiped from around the sections and placed in a humidity chamber on a rocker platform and incubated with 100μl of 20% normal serum, diluted in PBS, for 10 min at room temperature. Excess serum was shaken and wiped off, and 100μl of the diluted primary antibody was added for 12-16 h at 4^{0}C. After washing in PBS, secondary antibody was added for 30 min. The ABC solution was then prepared (1μl Streptavidin and 1μl Biotinylated Horseradish Peroxide in 1ml PBS) and

added to the rinsed sections for 30 min. Diaminobenzidine (DAB) solution (500µl DAB and 9·5ml deionised water filtered through a Whatman No. 1 filter paper and, immediately before use, the addition of 100µl of 3% hydrogen peroxide) was then added for 5 min. The slides were then rinsed briefly in PBS, washed in running tap water and counterstained with Mayers haematoxylin. Following dehydration through the series of graded alcohols, sections were mounted in XAM using appropriately sized coverslips.

For each case a negative control section was tested whereby the primary antibody step was omitted and the normal serum left on. In addition, with each batch of slides processed a known positive control was included to ensure consistency between batches.

Table 2.5. Antibodies used for staining formalin-fixed paraffin embedded tissue sections.

1^0 Antibody	Dilution	Pretreatment	2^0 Antibody (biotinylated)	Dilution
E-cadherin	1:50	Protease	Rabbit anti mouse immunoglobulins	1:400
MSH2	1:40	Pressure Cooking	Rabbit anti mouse immunoglobulins	1:400
MLH1	1:10	Pressure Cooking	Rabbit anti mouse immunoglobulins	1:400
PMS2	1:40	Pressure Cooking	Rabbit anti mouse immunoglobulins	1:400

Table 2.6. Antibodies used for staining cryostat tissue sections.

1^0 Antibody	Dilution	Pretreatment	2^0 Antibody (biotinylated)	Dilution
H-cadherin	1:10	Not Applicable	Swine anti rabbit immunoglobulins	1:600
P-cadherin	Neat	Not Applicable	Rabbit anti mouse immunoglobulins	1:400

Table 2.7. Antibodies used for immunofluorescent studies.

1^0 Antibody	Dilution	Pretreatment	2^0 Antibody (FITC)	Dilution
E-cadherin	1:50	Not Applicable	Rabbit anti mouse immunoglobulins	1:50
H-cadherin	1:10	Not Applicable	Swine anti rabbit immunoglobulins	1:50
P-cadherin	Neat	Not Applicable	Rabbit anti mouse immunoglobulins	1:50
Cytokeratin14	1:10	Not Applicable	Rabbit anti mouse immunoglobulins	1:50

Immunohistochemistry - Immunofluorescence

Immunofluorescent microscopy was performed to detect and localise protein expression in pure populations of breast myoepithelial cells. Cells were grown on glass coverslips by Dr. Louise Jones.

Cell Fixation

Cells adhered to coverslips were fixed in acetone for 10 mins at 4^0C and washed in PBS prior to immunsostaining.

Immunofluorescent Detection

Excess buffer was tipped off the coverslips and incubated with 100µl of 20% normal serum, diluted in PBS, for 20 min at room temperature. Excess serum was washed off with PBS, and 100µl of the diluted primary antibody (Table 2.7) was added for 2 h. After washing in PBS, secondary antibody (Table 2.7) was added for 1 h. Following a PBS wash, the coverslips were mounted in aqueous mountant and stored in the dark prior to immunofluorescent microscopy.

For each batch a negative control section was tested whereby the primary antibody step was omitted and the normal serum left on. In addition, a known positive control was included.

Confocal Microscopy

Sections were examined using a BioRad MRC 600 laser confocal microscope, and images recorded.

Western Blotting

Western blotting was carried out to determine qualitative protein expression in the breast cancer cell lines.

Protein Extraction

Cell suspensions were resuspended in 300µl homogenisation buffer and homogenised for 1 min using a Pellet Pestle Motor (Kontes). The suspension was centrifuged at 13,000rpm for 1min at 4^0C, and the supernatant was stored at -80^0C until required.

Protein Estimation

The protein concentration of samples was determined using the Bio-Rad protein assay, based on the method of Bradford *et al.,* (1976). A standard curve of absorbance (595nm) versus protein concentration was constructed using a series of protein standards (0.1µg/ml to

200μg/ml of BSA). Protein sample to be analysed (50μl) was mixed with 50μl PBS, then added to 900μl 1X Protein assay reagent (BioRad), and the absorbance read at 595nm, and the concentration read from the standard curve.

SDS-PAGE

Electrophoresis of proteins was carried out using a mini-gel unit (Hoeffer), as adapted by Towbin *et al.*, (1979). Protein samples (20μg) were heated at 95^0C in an equal volume of sample buffer for 3min and immediately loaded onto 3.75% stacking gels and 7.5% resolving polyacrylamide gels and run for 50V, through the stacking gel, and then at 75V for 2-3h, in running buffer (0.025M Tris, 0.192M glycine; pH 8.3). Molecular weight protein markers (BioRad) were run along side the samples to allow size determination of the resulting protein bands.

Electroblotting

Following electrophoresis the protein gel was transferred to Hybond C^+ nitrocellulose membrane (Amersham) by electroblotting at 300mA for 5 h in transfer buffer (0.025M Tris, 0.192M glycine, 20% methanol; pH 8.3).

ECL Immunodetection

Protein detection was determined by a chemiluminescent technique, using an ECL kit (Amersham). Following electroblotting the membrane was rinsed of residual polyacrylamide with TBS and incubated for 1h in blocking solution at room temperature, with shaking. The membrane was then rinsed in TBS-T, twice, followed by washes of 1 x 15min, and 2 x 5min, prior to the addition of primary antibody against either MSH2, PMS2, or MLH1 all diluted in blocking solution to 1:100 for 1h. The membrane was then washed as before, and incubated with secondary antibody diluted in TBS-T to 1:2000 for 1h. After washing the membrane was developed by incubating with the ECL solutions for 1min, followed by exposure to film (X-OMAT, Kodak) for 30secs to 1min.

Chapter 3

Microsatellite Instability

3.1 Genetic Instability And Human Disease

Trinucleotide Repeat Expansion

The expansion of trinucleotide repeat sequences has been shown to be the underlying cause of 11 human genetic diseases (Mitas, 1997). In general, expansion of these triplet repeats is characterised clinically by 'anticipation'. As the disease is transmitted from one generation to the next, the symptoms progress in severity and/or there is an earlier age of onset of the disease.

The disorders fall into two broad categories, summarised in Table 3.1 and 3.2. Type I disorders are categorised by progressive neuronal loss. Members of this group includes Huntingdon's disease (HD) (The Huntington's Disease Collaborative Research Group, 1993), spinal and bulbar muscular dystrophy (SBMA) (La Spada *et al.*, 1991), dentatorubral-pallidluysian atrophy (DRPLA) (Koide *et al.*, 1994), Machado-Joseph disease (MJD/SCA3) (Kawaguchi *et al.*, 1994), and the spinocerebellar ataxias (SCAs) (Orr *et al.*, 1993; Imbert *et al.*, 1996; Pulst *et al.*, 1994; Sanpei *et al.*, 1996; Zhuchenko *et al.*, 1997).

The second group of disorders includes myotonic dystrophy (DM) (Brook *et al.*, 1992), fragile X syndrome (Kremer *et al.*, 1991; Verkeck *et al.*, 1991; Gu *et al.*, 1996), and Friedrich's ataxia (FA) (Campuzano *et al.*, 1996). These diseases are characterised by a more multisystem pathology (Table 3.2). DM is characterised by muscle weakness and wasting. Fragile X syndrome is characterised by mental retardation. Friedrich's ataxia is characterised by spinocerebellar degeneration.

Type I disorders, are associated with shorter repeat expansion lengths compared with type II disorders. In general, type I disorders have repeat lengths in the range of 40 to 70 CAGs, in contrast to lengths ranging from 50 into the thousands for type II disorders. In all type I disorders the expanded repeat encodes a polyglutamine segment within the open reading frame of an expressed gene. In contrast, repeat sequences of the type II diseases are not translated, as depicted in Figure 3.1.

Table 3.1. Type I trinucleotide repeat diseases (Adapted from Reddy and Housman, 1997).

Disease	Repeat	Repeat Number Normal	Repeat Number Disease	Location of the Repeat	Gene product	Gene function	Change in gene function
HD	CAG	6-34	36-121	ORF	Huntingtin	Unknown	Gain
DRPLA	CAG	7-25	49-88	ORF	Atrophin	Unknown	Gain
SBMA	CAG	11-34	40-62	ORF	Androgen receptor	Transcription factor	Gain
SCA1	CAG	6-39	41-81	ORF	Ataxin-1	Unknown	Gain
SCA2	CAG	15-29	35-59	ORF	Ataxin-2	Unknown	Gain
MJD/SCA3	CAG	13-36	68-79	ORF	Ataxin-3	Unknown	Gain
SCA6	CAG	4-16	21-27	ORF	Ataxin-6	Calcium channel	Gain

ORF indicates open reading frame

Table 3.2. Type II trinucleotide repeat diseases (Adapted from Reddy and Housman, 1997).

Disease	Repeat	Repeat No. Normal	Repeat No. Premutation	Repeat No. Disease	Location of the Repeat	Gene product	Gene function	Change in gene function
DM	CTG	5-7	50-180	200->2000	3'UTR	DMPK	Serine/threonine protein kinase	?
FRAXA	CGG	6-52	60-200	200->2000	5'UTR	FMR-1	RNA-binding protein	Loss
FRAXE	GCC	6-25	43-200	>200	?	?	?	?
FA	GAA	7-22	-	200->900	Intron	Frataxin	Unknown	Loss

UTR indicates untranslated region, ? is unknown.

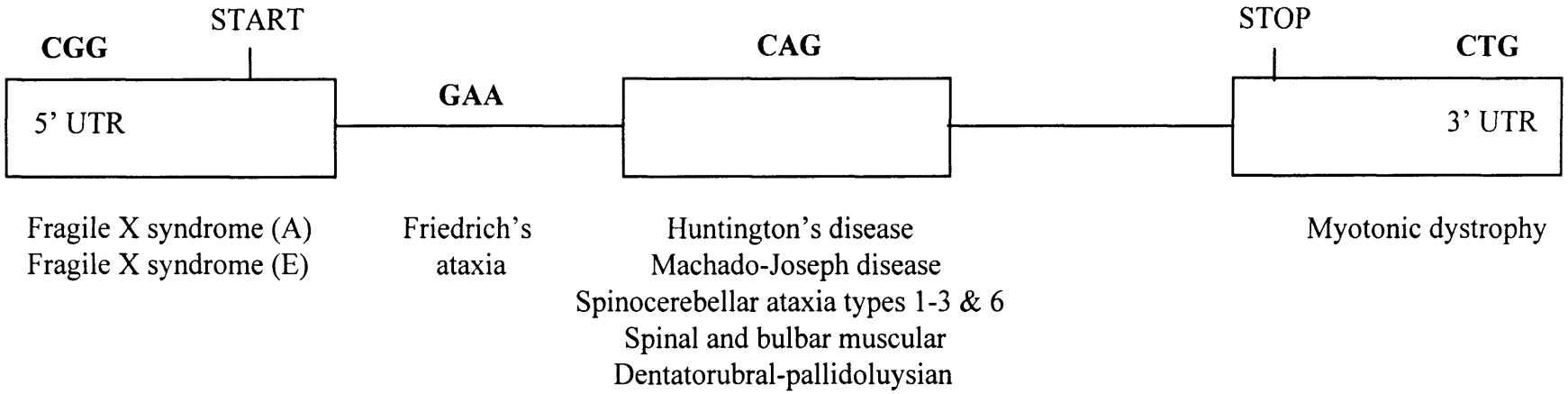


Figure 3.1 Triplet repeat expansions with respect to gene organisation (Adapted from Mitas, 1997)

Blocks indicate exons and untranslated regions (UTRs), lines indicate introns.

3.2. Microsatellite Instability And Cancer

Hereditary non-polyposis colorectal cancer (HNPCC) is a common cancer predisposition, which accounts for approximately 2-4% of the total colorectal cancers in the Western world (Lynch *et al.*, 1996). HNPCC patients develop colorectal cancer at an average age of 42, more than two decades earlier than the general population. Although colorectal carcinoma is the major cancer in HNPCC families, about 35-40% of the people in these families have other types of tumours, of which endometrial and ovarian tumours are the most common. In addition, approximately 35% of people in HNPCC families develop more than one tumour. The detection of 'microsatellite instability' in colorectal tumours from HNPCC patients led to the identification of a new class of gene causing cancer predisposition.

In 1993, three articles reported that an unusual form of somatic mutation occurred in 12-15% of all colorectal tumours and was found in virtually all the colorectal tumours of HNPCC. These mutations consisted of changes in the length of DNA tandem repeat sequences that are normally interspersed throughout the genome and were termed 'ubiquitous somatic mutations' (USMs) in simple repeated sequences by Ionov *et al.*, (1993) 'replicative errors' (RERs) by Aaltonen *et al.*, (1993) or 'microsatellite instability' (MIN) by Thibodeau *et al.* (1993). All three investigating groups recognised that errors of insertion or deletion of dinucleotide repeats or other short repeated sequences occurred during the replication of DNA by neoplastic cells in HNPCC tumours. It was recognised that RERs could possibly represent a new mechanism contributing to tumour development. (The microsatellite instability phenotype is referred to as MI^+ in the remainder of this thesis).

In bacteria this mutational fingerprint is characteristic of a failure of the mismatch repair (MMR) enzymatic complex. The best known MMR pathway is the *E. coli* MutHLS system that promotes a 'long-patch' methyl-directed repair of single base-pair mismatches and short mismatched loops in newly synthesised DNA, thus increasing the fidelity of DNA replication (reviewed in Modrich, 1991). The bacterial MutS protein binds to DNA mismatches, and other proteins of the MutHLS system distinguish the newly synthesised unmethylated strand, excise the incorrectly synthesised nucleotides, and resynthesise double-stranded DNA. Failure of the bacterial MutHLS repair system results in genetic instability. The MMR pathway in *Saccharomyces cerevisiae* requires MSH (a homologue of bacterial MutS) and two MLH proteins (homologues of the bacterial MutL proteins). Mutation of MMR genes in *S.*

cerevisiae results in an increase in the rate of expansion and contraction of dinucleotide repeat sequences.

Mismatch Repair Genes

The human homologue of the MutS, *hMSH2* (or human MutS Homologue), was subsequently cloned and disease-causing mutations were characterised in HNPCC kindreds (Fishel *et al.*, 1993; Leach *et al.*, 1993). Several additional human mismatch repair genes (*hMLH1* and *hPMS1* through *hPMS8*) have been cloned by virtue of homology to bacterial and yeast MutL homologues (Nicolaides *et al.*, 1995). Of these *hMLH1* (Bronner *et al.*, 1994) has been confirmed as another major locus in HNPCC (Table 3.3). Although HNPCC kindreds with *hPMS1* and *hPMS2* have also been identified, mutations at these loci are less prevalent than mutations in either *hMSH2* and *hMLH1* in patients tested to date (Nicolaides *et al.*, 1994; Kinzler and Vogelstein, 1996). Another MutS homologue, *hMSH6* or *GTBP* (G-T binding protein) has been identified through biochemical characterisation (Drummond *et al.*, 1995; Palambo *et al.*, 1995). The yeast MSH3 (DUG) gene is another MutS gene, but it is not yet clear whether it plays any role in human mismatch repair (Fujii and Shimada, 1989). The role of this gene in mismatch repair is currently under investigation.

Table 3.3. HNPCC germline mutations (Adapted from Rhyu, 1996, Kinzler and Vogelstein, 1996, Eshleman and Markowitz, 1996).

Gene	*E. coli* homologue	Mutated in HNPCC (%)	Chromosome location
hMSH2	MutS	45	2p16
hMSH6	MutS	-	2p16
hMSH3	MutS	-	-
hMLH1	MutL	49	3p21
hPMS1	MutL	*	2q31-33
hPMS2	MutL	6	7p22

** Indicates that a hPMS1 mutation was identified in a single kindred (Liu et al., 1996), Dash signifies not yet reported.*

Mechanism Of Mismatch Repair

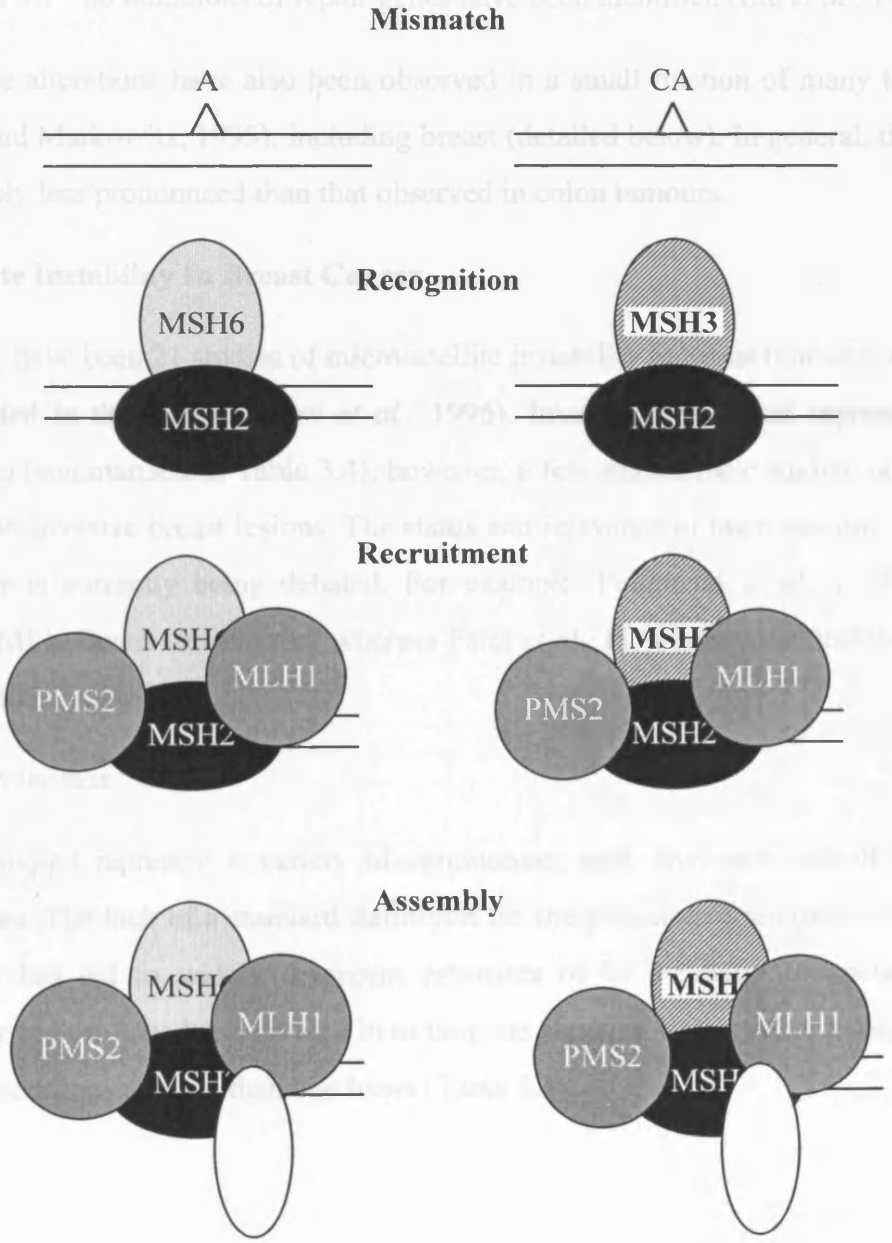
In human cells, mismatches are recognised by a heterodimer, hMutSα, comprising hMSH2 and hMSH6 (Figure 3.2). Biochemical and genetic data indicate that inactivation of each partner has a different effect on genome stability (Karran, 1995). Mutations in *hMSH6* are associated with a selective inability to repair single base mismatches and single nucleotide loops. Dinucleotide loops are repaired efficiently. In contrast, cells with *hMSH2* mutations are unable to repair single base mismatches, single base loops or dinucleotide loops. It has now been proposed that hMSH2 participates, in combination with hMSH3, in a second mismatch repair function that stabilises dinucleotide or larger repeats (Johnson *et al.*, 1996).

After mismatch recognition, binding of MutL occurs. The MutL component is also a heterodimer (hMutLα) of hMLH1 and hPMS2. The fact that these MutL homologues form functional heterodimers, combined with evidence that a mutation in hPMS1 is also associated with HNPCC, suggests that hPMS1 may form a heterodimer with a different MutL homologue. This putative partner may be among the family of six MutL-like genes (hPMS3-hPMS8) on chromosome 7.

The later steps in mismatch repair are less clear, but the interaction of Mut S and MutL heterodimers is likely to lead to the recruitment of polymerases, nucleases and other proteins required for the repair processes. Recently, the hMSH2 gene has been postulated to be a novel p53-regulated target gene (Scherer *et al.*, 1996). This may indicate a direct involvement of p53 in repair mechanisms via DNA binding of a mismatch repair gene.

Figure 3.2. Steps in mismatch repair (Karran, 1995, 1996)

Displaced single bases (e.g. -A-) are recognised by the mismatch binding heterodimer MSH2:MSH6 which bind to the mispaired region. Dinucleotide loops (e.g. -CA-) are bound by an alternative complex comprising MSH2:MSH3. The MLH1:PMS2 heterodimer is then recruited to the bound complexes and this facilitates further assembly of other proteins



leading to excision and resynthesis of a stretch of DNA containing the mismatched region.

Microsatellite Instability In Sporadic Cancers

Sporadic tumours with microsatellite instability account for 13% of all colorectal cancers, but the molecular mechanisms responsible for the instability has not been fully elucidated. Approximately 65% of these cases have somatic mutations of the same MMR genes causing HNPCC (Kinzler and Vogelstein, 1996), while others have mutations of *hMSH6* or *hMSH3*, which are rarely mutated in HNPCC (Papadopoulos *et al.*, 1995; Malkohysan *et al.*, 1996) or in the proof-reading domain of polymerase δ (da Costa *et al.*, 1995). In other colorectal tumours with MI^+, no mutations of repair genes have been identified (Liu *et al.*, 1995).

Microsatellite alterations have also been observed in a small fraction of many tumour types (Eshleman and Markowitz, 1995), including breast (detailed below). In general, the instability is considerably less pronounced than that observed in colon tumours.

Microsatellite Instability In Breast Cancer

To date there have been 21 studies of microsatellite instability in breast tumours, including the work presented in this thesis (Shaw *et al.*, 1996). Invasive carcinomas represent the most studied group (summarised in Table 3.4), however, a few studies have studied non-malignant as well as non-invasive breast lesions. The status and relevance of microsatellite instability in breast cancer is currently being debated. For example, Peltomaki *et al.*, (1993) found no evidence of MI in breast carcinomas, whereas Patel *et al.*, (1994) detected instability in all of the tumours they studied.

Invasive Carcinomas

The cases studied represent a variety of carcinomas, each analysed with different set of microsatellites. The lack of a standard definition for the presence of microsatellite instability in a tumour has led to widely divergent estimates of its incidence in sporadic cancers. Therefore the results have been divided in to tumours showing instability at a single locus and those with alterations at more than one locus (Table 3.4).

Table. 3.4. Microsatellite instability in invasive breast carcinomas.

	No. of tumours studied	No. of tumours with MI	MI at single locus	MI more than one locus
Peltomaki *et al.*, 1993	84	0 (0%)	-	-
Han *et al.*, 1993	26	1 (4%)	1	0
Wooster *et al.*, 1994b	104	11 (10%)	10	1
Yee *et al.*, 1994	20	4 (20%)	1	3
Glebov *et al.*, 1994	14	6 (3%)	6	0
Patel *et al.*, 1994	13	13 (100%)	1	12
Jonsson *et al.*, 1995	30	2 (7%)	ND	ND
Contegiacomo *et al.*,1995	28	6 (21%)	1	5
Aldaz *et al.*, 1995	52	12 (23%)	ND	ND
Karnick *et al.*, 1995	69	20 (29%)	19	1
Shaw et al., 1996	30	4 (8%)	2	2
Huang *et al.*, 1995	29	9 (31%)	8	1
Paulson *et al.*, 1996	37	25 (67%)	14	11
Toyama *et al.*, 1996a	100	8 (8%)	6	2
DeMarchis *et al.*,1997	81	27 (33%)	7	20
Sourvinos *et al.*, 1997	42	11 (26%)	9	2
Rush *et al.*, 1997	46	13 (28%)	8	5
Yang *et al.*, 1997	21	10 (48%)	10	NA
Dillon *et al.*, 1997	100	13 (13%)	12	1

NA indicates non-applicable since only a single microsatellite was investigated, ND indicates that type of MI has not been described in the cited publications.

Non-Invasive Carcinomas

The most comprehensive study to date of DCIS has been described by Aldaz *et al.*, (1995). They investigated MSI in 23 cases of pure DCIS. Three (13%) tumours with MI were identified. Dillon *et al.*, (1997) also investigated MI in 33 cases of DCIS. MI was detected in one (3%) case. Toyama *et al.*, (1996b), studied two foci of DCIS from two invasive tumours which had previously been demonstrated to show microsatellite instability (Toyama *et al.*, 1996a). Both areas demonstrated the same pattern of instability to that seen in the invasive components.

Non-Malignant Lesions.

The study described by De Marchis *et al.*, (1997) included seven cases of fibroadenoma. Eight microsatellites were analysed, but there was no evidence of instability in these cases. Rosenberg *et al.*, (1997) examined 12 separate atypical hyperplastic lesions, from 6 breast cancer patients, for microsatellite instability. Alterations were detected in 5 of the 12 ADH lesions, from three patients. Kasami *et al.*, (1997) demonstrated MI in 2 of 8 breast biopsies with hyperplastic lesions. Dillon *et al.*, (1997) also investigated 18 specimens of epithelial hyperplasia (without atypia), one (6%) of which demonstrated MI.

Mismatch Repair Genes In Breast Cancer

To date mutational analysis or expression studies of candidate MMR genes have not been reported in breast tumours displaying MI. However, a recent study has identified a breast tumour (as an integral tumour within the HNPCC syndrome) which displayed MI and a had a frameshift mutation in *hMLH1* (Risinger *et al.*, 1996). This was the first evidence that breast cancer, albeit within the context of HNPCC, may result from the inheritance of a mutant mismatch repair gene.

3.3 Mismatch Repair Mechanisms And Cancer

Exactly how such mismatch-repair defective mutations lead to the development of cancer is unclear at present. One possibility is that cells which have a mismatch repair defect have a significantly higher mutation rate. If this causes mutations to accumulate in tumour suppressor genes and/or oncogenes, then the mutant cells will probably progress to become tumour cells. This has been termed the mutator hypothesis of tumourigenesis (Loeb 1991, 1994). The targeted genes are now beginning to emerge.

TGFβRII

TGFβ is one of the most potent inhibitors of normal cell growth. However, many malignancies of epithelial origin are resistant to TGFβ, suggesting that developing resistance to growth inhibitory cytokines plays an important role in tumourigenesis (reviewed in Polyak, 1996). Tumours can become resistant to TGFβ in many different ways. However, mutation of one of the TGFβ signalling receptors (type II) has recently been associated with microsatellite instability in colon cancer (Markowitz *et al.*, 1995). The TGFβ type II receptor (*TGFβRII*) contains a region of 10 consecutive adenines which is a target for up to 90% of colon tumours with the MI^+ phenotype. Insertions or deletions of adenines within this repeat produce frameshift mutations, resulting in truncated type II receptors, and hence resistance to TGFβ. However, this does not seem to be a universal target for all MI^+ tumour types. Endometrial tumours with microsatellite instability do not show mutations at the *TGFβRII* poly A tract (Myeroff *et al.*, 1995).

IGFIIR

The insulin-like growth factor type II receptor (*IGFIIR*) or mannose-6-phosphate receptor plays roles in a number of cellular functions, the most critical of which is the activation of the potent growth inhibitor TGFβ (Dennis and Rifkin, 1991). This receptor also inhibits cellular proliferation mediated by IGFII, which is a potent growth stimulant, by internalising and degrading this protein (Kornfield, 1992). Recent studies have suggested that *IGFIIR* functions as a tumour suppressor gene, in a number of tissues, including breast (Hankins *et al.*, 1996). The *IGFIIR* gene harbours a tract of 8 guanines in its coding region. Recent studies have characterised mutations of this sequence in MI^+ tumours of the gastrointestinal tract and endometrium (Souza *et al.*, 1996, Ouyang *et al.*, 1997).

Bax

The development and growth of tumours are controlled by a combination of cellular replication and cell death (Williams, 1991). The most common mechanism of cell death is programmed cell death, a precisely regulated process that culminates in a set of characteristic and morphological and structural alterations referred to as apoptosis (Wyllie *et al.*, 1980). Many apoptotic stimuli induce cell death through a pathway that is regulated by members of the Bcl-2 family. These promoters function to either promote or inhibit apoptosis. Bcl-2 is a suppressor of apoptosis and can heterodimerise with Bax, a pro-apoptotic member of this family of proteins (Oltavi *et al.*, 1993).

The ability of Bax to promote cell death suggests that it may function as a tumour suppressor gene, with reductions in Bax giving cells a survival advantage and therefore contributing to their expansion. The *Bax* gene contains a tract of 8 consecutive guanines in exon 3 (Oltvai *et al.*, 1993). One nucleotide insertions and deletions within the poly G tract were identified in 51% of colon tumours which were also MI^+, but not in any tumours characterised as MI^- (Rampino *et al.*, 1997). The frameshift mutations resulted in truncated proteins which result in loss of BAX function. Cells lacking Bax protein are likely to have a diminished capacity to trigger apoptosis after receiving a death signal, thereby facilitating tumourigenesis.

E2F-4

The E2F family of transcription factors are involved in the regulation of the cell cycle and apoptosis (La Thangue, 1994). Some of the genes were found to be potential oncogenes (Xu *et al.*, 1995). E2F-4 is a member of this family, and is involved in the transition from G_0 to G_1 phase of the cell cycle, as well as in early G_1, facilitating the transactivation of genes necessary for cellular proliferation (Sardat *et al.*, 1995). This gene contains a long spacer region encoded by 13 consecutive serine (CAG) residues, which is likely to function as a transactivation domain. This repeat is polymorphic in the normal human population and therefore a functional effect of mutation in this region can not be assumed. However, modification of the transactivation domain may alter the expression of E2F-4 responsive genes. Yoshitaka *et al.*, (1996) demonstrated that two (10%) of patients with sporadic colorectal carcinoma had tumour specific alterations at the 13 consecutive trinucleotide repeats. Of the twenty tumours studied only the two with *E2F-4* alterations were classified as MI^+.

In an extended study Souza *et al.*, (1997), examined 86 neoplastic lesions, including 46 sporadic colorectal carcinomas (31 of which were MI^+ and 15 MI^-) gastric adenocarcinomas, endometrial cancers and prostate carcinomas, all of which were classified as MI^+. Of the MI^+ gastrointestinal tumours, 37% demonstrated tumour specific alterations of the CAG repeats. None of the MI^- gastrointestinal tumours or the MI^+ endometrial or prostate tumours showed alterations, suggesting that this gene is a frequent target of microsatellite instability in gastrointestinal tumourigenesis.

APC

Huang *et al.*, (1996) have recently demonstrated a relationship in colorectal cancer between the *APC* mutational spectrum and the presence of MI^+ (Section 1.4). The prevalence of APC mutations was similar when MI^- and MI^+ tumours were compared. However, in a total of 101 sequenced mutations, there was a substantial excess of *APC* frameshift mutations in the MI^+ cases, particularly at mononucleotide repeats. This suggests that the *APC* gene is also a mutational target for MI^+ colorectal tumours.

MSH3 and MSH6

Interestingly some of the mismatch repair genes themselves may be targets for slippage induced frameshift at repetitive coding sequences. Malkhosyan *et al.*, (1996), demonstrated that approximately 30% and 40% of MI^+ colorectal tumours contained frameshifts in the *MSH6* and *MSH3* genes, respectively. These frameshift mutations were at tracts of 8 adenines in the coding region of MSH3 and in a run of 8 cytidines in the *MSH6* gene, suggesting that they were the consequence of a previous defect in mismatch repair.

Other Targets

At present, the four genes described above represent the only targets which have definitively been correlated with the presence of microsatellite instability in a large number of tumours. However, there are a wealth of candidate genes which possess similar repeat motifs in their coding regions. For example, the *BRCA2* gene contains a number of poly A tracts in it coding region. Insertion of an adenine at one of these regions has been described in an ovarian tumour which also showed microsatellite instability (Takahashi *et al.*, 1996). A recent review of *BRCA1* mutations also found that mutations at sites of homonucleotide and short repeats was common (Rodenhiser *et al.*, 1996).

3.4 Mismatch Repair Deficiency And Drug Resistance

It is emerging that the mismatch repair proteins may function in other aspects of genome integrity. One topic under investigation at present is the role mismatch repair proteins play in recognition of DNA damage induced by anti-tumour drugs. It has been proposed that alterations to the mismatch repair genes may enable tumours to become resistant to treatment with anti-cancer drugs (described below).

A number of widely used chemotherapeutic agents act by forming DNA adducts. The exact mechanism by which these adducts cause cell death is not known. However, it is likely that a failure to repair the damaged DNA causes the formation of double-strand breaks or single-strand gaps in the DNA, which lead to chromosome aberrations and breakage during mitosis (reviewed in Chaney and Sancar, 1996). Ultimately, the DNA adducts or the damage induced by the adducts triggers apoptosis. Therefore it has been proposed that repair of these adducts would cause resistance to some anticancer drugs.

Cisplatin induces cytotoxic DNA damage predominantly in the form of intrastrand cross-links at adjacent guanine bases (reviewed in Sanderson *et al.*, 1996). Doxorubicin (Adriamycin) another effective anticancer drug intercalates into DNA and is believed to be cytotoxic due to the induction of DNA double-strand breaks (Cummings *et al.*, 1991).

The observation that mismatch repair proteins recognise insertion/deletion mispairs and single-base mismatches, suggested that they may also recognise the drug-induced structural distortions in DNA. Mello *et al.*, (1996) and Yamada *et al.*, (1997) demonstrated that purified MSH2 protein recognises and binds specifically to the DNA adducts induced by cisplatin treatment. The mechanism by which this binding leads to cell death is unclear. However, it is possible that the initial recognition of these adducts could lead to the induction of an apoptotic signal, or that the mismatch repair proteins shield the adduct from DNA repair enzymes thereby allowing incorporation of the adduct into the genome.

It was predicted from the model that inactivation of the mismatch repair pathway would lead to tumours that were resistant to the action of cisplatin. This has now been demonstrated in a series of studies. Anthoney *et al.*, (1996) first demonstrated that an MI^+ phenotype appears to be selected for during the acquisition of anticancer drug resistance. This study revealed that microsatellite instability was a common phenotype in ovarian and breast cancer cell lines, selected for resistance to cisplatin and adriamycin. The drug resistant ovarian lines were later

shown to be mismatch-repair deficient owing to defective expression of hMLH1 (Drummond *et al.*, 1996).

Aims Of This Chapter

The objectives of this part of the thesis were to determine if MI occurs in cases of DCIS, mammographically detected impalpable early invasive carcinomas and tubular carcinomas by analysing microdissected tumour foci for alterations in a panel of microsatellites (mono-, di-, and trinucleotide repeats). Cases which did demonstrate instability, could then be screened for alterations in candidate DNA repair loci (MSH2, MLH1, and PMS2) and frameshift mutations in a number of cancer-associated genes (TGFβRII, IGFIIR, Bax, and E2F-4).

The relationship between MI and the development to drug resistance was also investigated by using breast cancer cell lines and the anti tumour drug, doxorubicin.

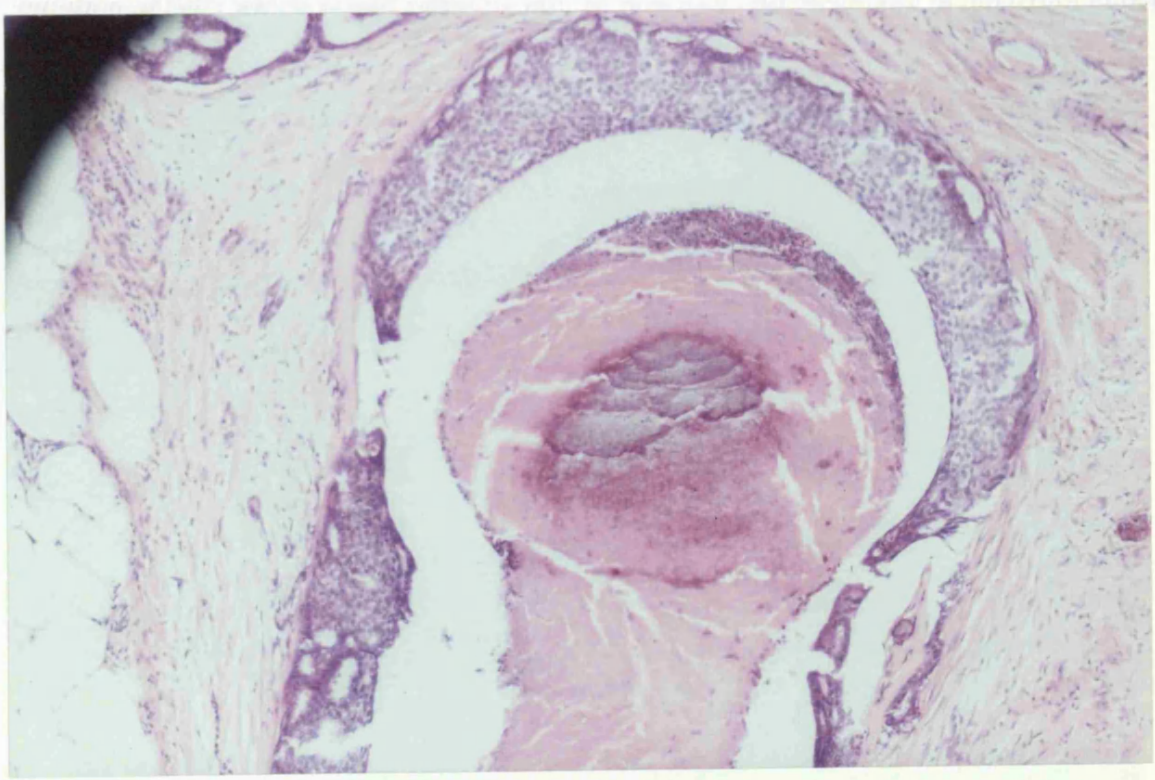
3.5 Microdissection

Tumour cells were initially removed from the tissue sections with sterile needles (21 gauge). However, this did not prove reliable since the cells did not always become detached when placed in buffer. Sterile drawn-out capillary tubes were then used. This method was more reliable as it was possible to break the tip of the tubing, containing cells, directly into the digestion buffer.

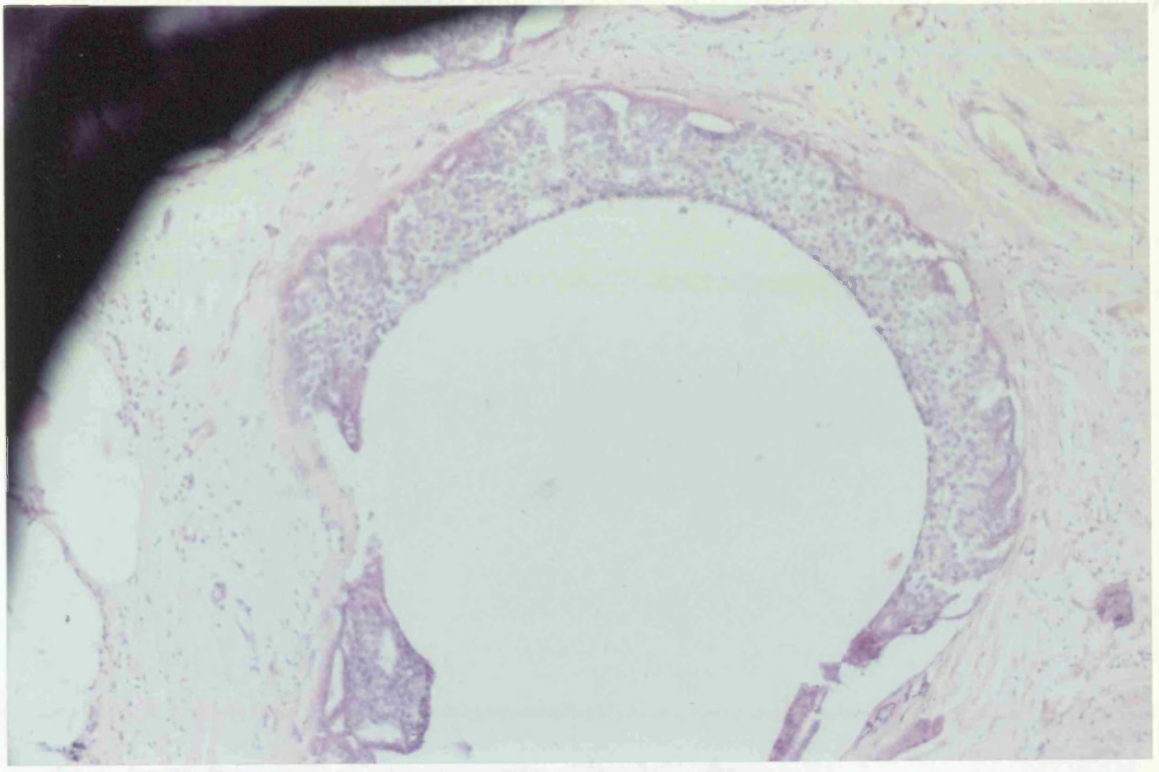
In order to reduce the presence of non-tumour cells in the microdissection preparations it was often beneficial to first remove surrounding non-malignant cells and areas of necrosis. This was particularly useful when procuring cells from cases of DCIS (as illustrated in Figure 3.3). For other cases, tumour foci were generally removed straight from the section (as illustrated in Figure 3.4).

Figure 3.3. Preparation for microdissection.

(a) Haematoxylin and Eosin (H&E) stained section of DCIS showing duct (Case No. 10). Black is edge of area that has been marked.



(b) As in (a), demonstrating removal of necrosis.



(c) As above, demonstrating removal of surrounding tissue around duct, leaving pure population of cells which would subsequently be removed and incubated in digestion buffer.

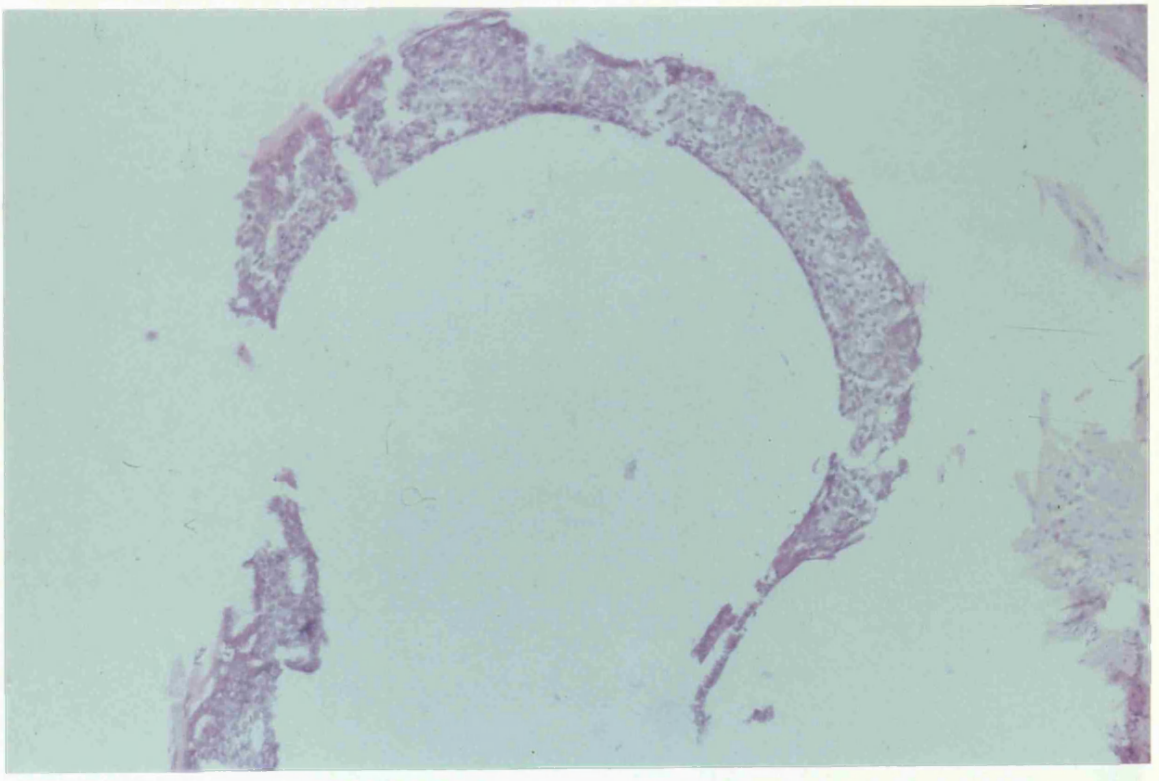
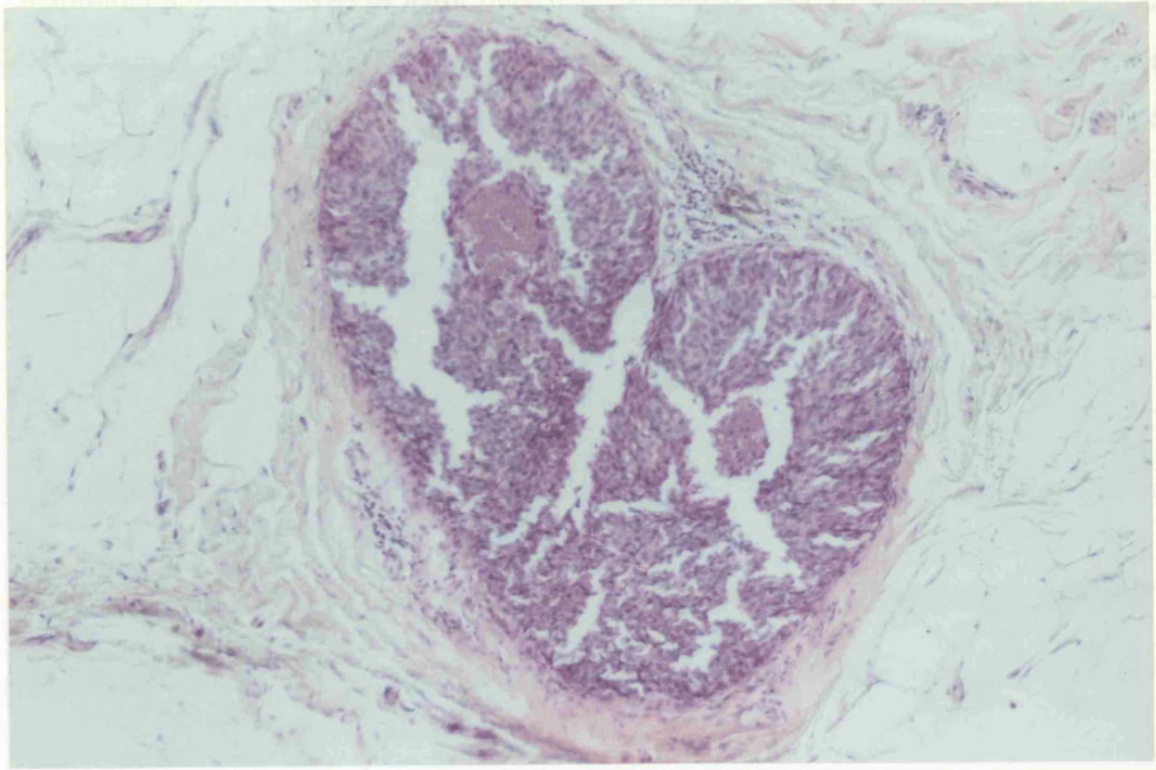
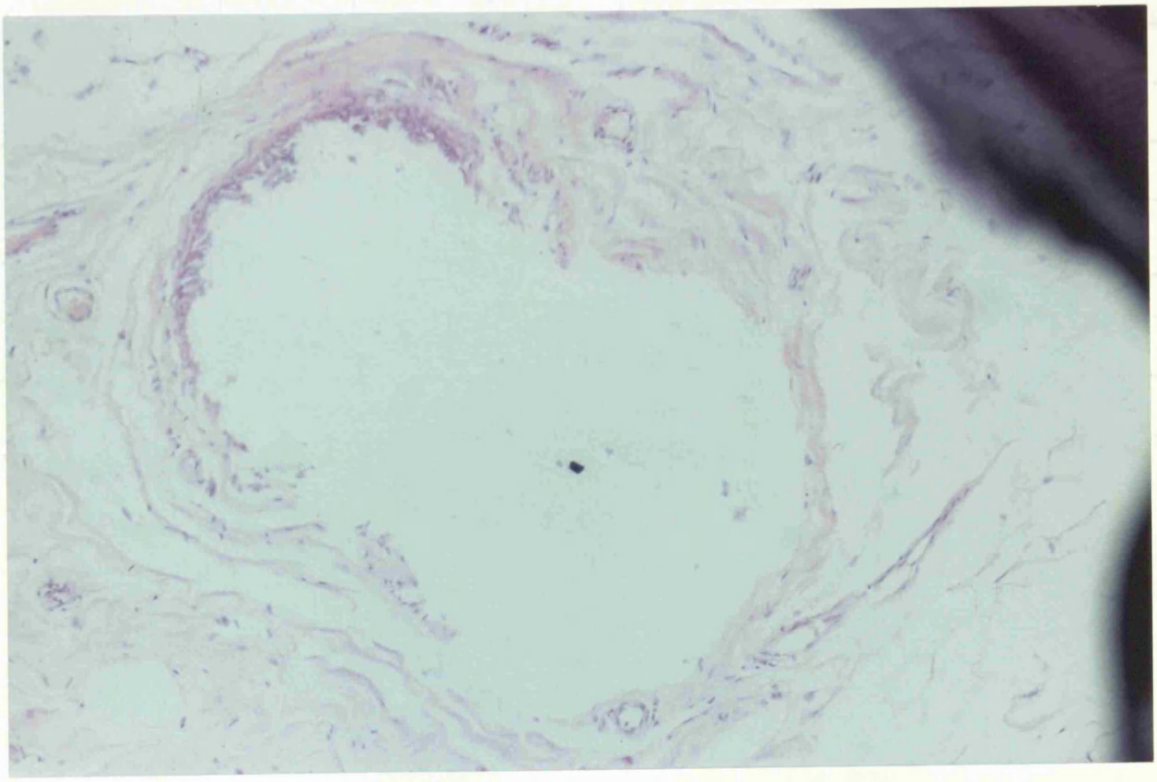


Figure 3.4. Direct microdissection of DCIS

(a) Haematoxylin and Eosin (H&E) stained section of DCIS (Case No.15) showing duct filled with malignant cells and area of necrosis.



(b) Parallel H&E section after removal of cells by microdissection.



3.6 Microsatellite Instability Analysis

Four types of breast lesion were investigated for evidence of microsatellite instability (MI). These comprised 8 fibroadenomas and one phyllodes, 23 cases of DCIS, 30 mammographically detected early invasive carcinomas, and 13 tubular carcinomas. MI was investigated by analysing each of the lesions for tumour-specific alterations in a series of microsatellites. The microsatellite markers used to investigate the occurrence and frequency of MI are detailed in Table 3.5.

Table 3.5. Microsatellite markers used to investigate MI.

Microsatellite	Repeat	Chromosome region	Associated gene (where known)
BAT25	A	4q11-13	c-*kit* oncogene (intronic)
BAT40	A		3β hydroxysteroid dehydrogenase (intronic)
D6S193	CA	6q26-27	
D6S186	CA	6q26-27	
ESR	TA	6q25.1	Oestrogen receptor (1kb upstream)
D16S400	CA	16q22	
D16S413	CA	16q24.3	
D16S289	CA	16q24.3	
D16S402	CA	16q24.3	
X75b	CA	19q13	
DM-1	CTG	19q13.3	DM protein kinase (3'UTR)
SCA-1	CAG	6p21	Spinocerebellar ataxia type I
SBMA	CAG	Xq12	Androgen receptor (exon 1)
TGFβRII	A_{10}	3p22	TGFβ type II receptor
IGFIIR	G_8	6q26-27	Insulin-like growth factor II receptor
BAX	G_8		Bax
E2F-4	CAG	16q24.3	E2F-4

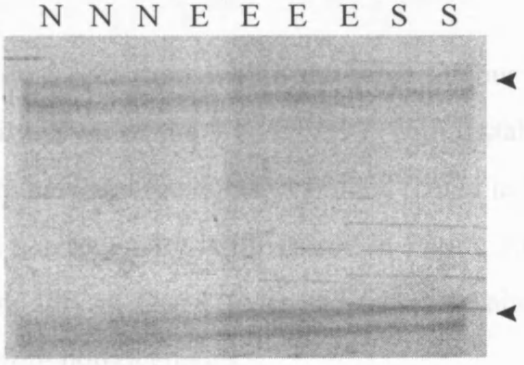
Chromosome 6q analysis was performed by Steve Chappell

Fibroadenomas

In fibroadenomas, hyperproliferation affects both the connective tissue component and the epithelial cells, which together grow to form well-circumscribed benign breast tumours. Little is known about the implication of genetic alterations in the development of fibroadenomas. In order to determine whether microsatellite instability might be a feature of proliferation, 8 cases of fibroadenoma and one benign phyllodes tumour (a neoplasm in which the stromal component predominates) were investigated for MI.

At least three distinct epithelial and three stromal components from each of the fibroadenomas and the phyllodes tumour were separately microdissected and compared with normal ducts from adjacent surrounding tissue. For two of these cases, areas of ductal hyperplasia in the surrounding tissue were also examined. Microdissected foci were analysed with two microsatellites. Instability of the DM-1 trinucleotide was investigated, since during the course of this thesis it emerged as a sensitive marker of MI (see Chapter 3 - Discussion). The second marker analysed was the D16S400 dinucleotide. Analysis of this marker allowed concomitant assessment of LOH at chromosome 16q (as described in Chapter 4). However, MI was not observed for any of the samples (Figure 3.5).

Figure 3.5. Representative example of microsatellite instability analysis in fibroadenomas. Analysis of normal ducts (N), epithelial (E), and stromal components (S) by PCR, at the DM-1 marker, followed by electrophoresis on 6% denaturing gels and autoradiography. Arrows indicate alleles.



DCIS

MI was initially investigated, in this study group, using DNA prepared from whole tumour sections. However, using this approach, microsatellite alterations were not observed (data not shown). This was attributed to the masking of tumour specific alterations by the vast quantities of non-tumour cells present in the DCIS tissue sections (see Chapter 3 - Discussion).

Therefore, the analysis was repeated using DCIS samples which had been prepared by microdissection. In general, three individual non-contiguous foci from each case were compared with non-involved lymph node or surrounding breast tissue, using a series of microsatellite markers (summarised in Table 3.6). MI was characterised by the appearance of alleles of altered length in tumour samples, indicating an alteration in microsatellite size. A tumour was classified as MI^+ when alterations in microsatellite size at two or more independent genomic sites were observed, as defined by Aaltonen *et al.*, (1993).

MI was documented in 12 cases. However, different patterns of instability were observed. Five of 23 (22%) DCIS cases showed instability at 2 or more loci (MI^+), and a further 7 (30%) cases showed alterations only at the DM-1 marker. Representative examples of cases with MI^+ and those with alterations of DM-1 are illustrated in Figure 3.6. (a) to (d). The pathological characteristics of the tumours displaying MI are summarised in Table 3.7. Eleven cases were of high nuclear grade, three of intermediate grade and 9 low grade (as determined by Dr. R. A. Walker). MI^+ and instability at DM-1 was found in high grade (8/11) and intermediate grade (2/3) at a higher frequency than in the low grade (2/9), but this was not statistically significant ($p=0.06$).

Microdissection of individual foci from within the same tumour section allowed analysis of intratumoural heterogeneity. Two of the 7 cases (9%) with instability only at DM-1 showed heterogeneity in instability between the ducts examined (listed in Table 3.7). A representative example of intratumoural heterogeneity is illustrated in Figure 3.6 (e). All other cases which were MI^+ or showed instability at DM-1 (MI) had the same alteration in each of the ducts examined, and were therefore homogeneous.

Analysis of morphologically normal breast ducts adjacent to areas of in situ carcinoma was also attempted. New slides for each of the MI^+ tumours and those with alterations to DM-1 were prepared and re-evaluated by Dr. R. A. Walker. Four of the cases with alterations

contained DCIS and adjacent normal ducts. These were marked and microdissected and analysed using the DM-1 marker. However, only Case No.14 yielded PCR products, as illustrated in Figure 3.6 (f). Two of the areas of DCIS showed altered alleles, as previously demonstrated Figure 3.6 (a). Whereas each of the adjacent normal ducts and a hyperplastic region showed the same alleles as those present in the surrounding normal breast tissue distant from the DCIS. The third area of DCIS did not demonstrate instability indicating heterogeneity between the ducts.

Table 3.6. Microsatellite markers analysed for instability in DCIS cases.

Mononucleotides	BAT25, BAT40
Dinucleotides	DI6S400, D16S413, X75b, D6S186, D6S193, ESR
Trinucleotides	DM-1, SCA-1

Table 3.7. Pathological and microsatellite instability data for DCIS cases.

Case No.	**Grade**	**Microsatellite Instability**
2	High	DM-1, D6S193
5	High	DM-1
7	High	D6S193, D6S186
12	High	DM-1
13	High	DM-1*
14	High	DM-1
16	High	DM-1
17	High	DM-1, D6S193, D16S400, D16S413
10	Inter.	DM-1, D6S193
15	Inter.	DM-1*
3	Low	DM-1
20	Low	BAT25, BAT40

Inter. Indicates intermediate, and * indicates cases with intratumoural heterogeneity

Figure 3.6. Representative examples of microsatellite instability in DCIS.

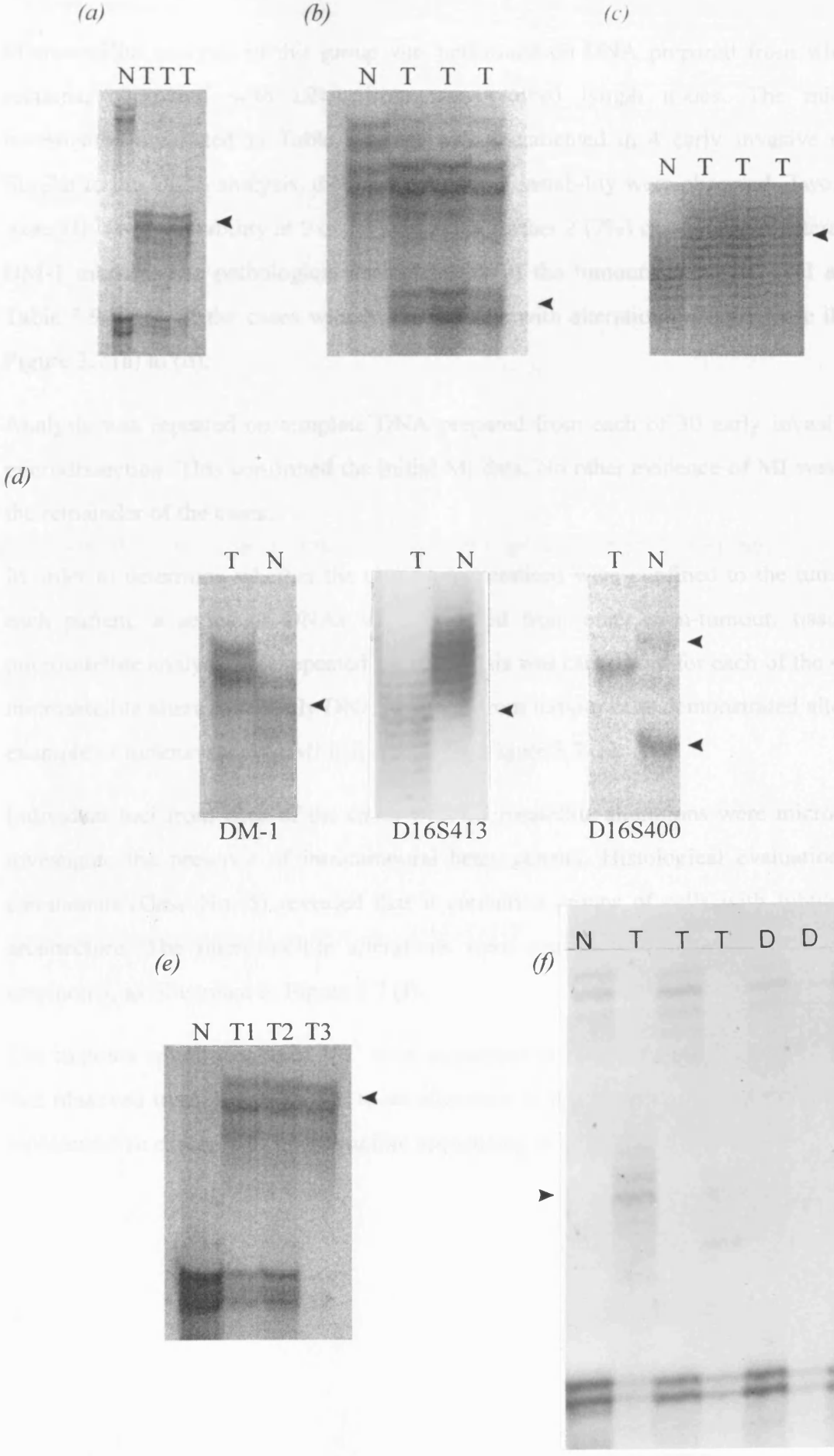
Analysis of normal (N) and microdissected DCIS (T) by PCR, followed by electrophoresis on 6% denaturing gels and autoradiography. (a) and (b) Case Nos. 14 and 16 showing alterations to the DM-1 locus, (c) Case No. 20 with instability of the BAT40 mononucleotide, and (d) Case No. 17 with instability at multiple loci. Arrows indicate alleles showing MI.

(e) Representative example of intratumoural heterogeneity in DCIS.

Analysis of normal (N) and microdissected ducts (T1, T2, T3), from Case No. 13 by PCR, at the DM-1 marker. The foci labelled T1 and T2 each contain two alleles, one of which is present in the normal sample, whereas the T3 duct contains only one allele (indicated by the arrow) which is distinct from that seen in the normal DNA sample.

(f) Analysis of DCIS and adjacent morphologically normal ducts.

Analysis of normal surrounding breast tissue (N), microdissected ducts (T), adjacent morphologically normal ducts (D) and hyperplastic region (H), from Case No.14 by PCR at the DM-1 marker. Two of the involved ducts show alterations of one allele, whereas the remaining involved duct, the adjacent normal ducts and the hyperplastic duct, each contain the same alleles that are present in the surrounding breast tissue.


(a)
N T T T
(b)
N T T T
(c)
N T T T
(d)
T N
T N
T N
DM-1
D16S413
D16S400
(e)
N T1 T2 T3
(f)
N T T T D D H

Early Invasive Carcinomas

Microsatellite analysis in this group was performed on DNA prepared from whole tumour sections, compared with DNA from non-involved lymph nodes. The microsatellites investigated are listed in Table 3.8. MI was documented in 4 early invasive carcinomas. Similar to the DCIS analysis, different patterns of instability were observed. Two of 30 (7%) were MI^+ (with instability at 9 of 10 loci) and a further 2 (7%) cases showed alterations at the DM-1 marker. The pathological characteristics of the tumours displaying MI are listed in Table 3.9. Each of the cases with MI^+ and those with alterations of DM-1 are illustrated in Figure 3.7 (a) to (d).

Analysis was repeated on template DNA prepared from each of 30 early invasive cases by microdissection. This confirmed the initial MI data. No other evidence of MI was detected in the remainder of the cases.

In order to determine whether the observed alterations were confined to the tumour cells of each patient, a series of DNAs were prepared from other, non-tumour, tissues and the microsatellite analysis was repeated. This analysis was carried out for each of the 4 cases with microsatellite alterations. Only DNA prepared from tumour cells demonstrated alterations. An example of tumour-specific MI is illustrated in Figure 3.7 (e).

Individual foci from each of the cases with microsatellite alterations were microdissected to investigate the presence of intratumoural heterogeneity. Histological evaluation of one of carcinomas (Case No. 5) revealed that it contained groups of cells with tubular and solid architecture. The microsatellite alterations were confined to the solid component of the carcinoma, as illustrated in Figure 3.7 (f).

The tumours which displayed MI^+ were sequenced at a number of different loci, to confirm that observed instability was due to an alteration in the number of microsatellite repeats. A representative example of microsatellite sequencing is illustrated in Figure 3.7 (g).

Table 3.8. Microsatellite markers analysed for instability in early invasive carcinomas

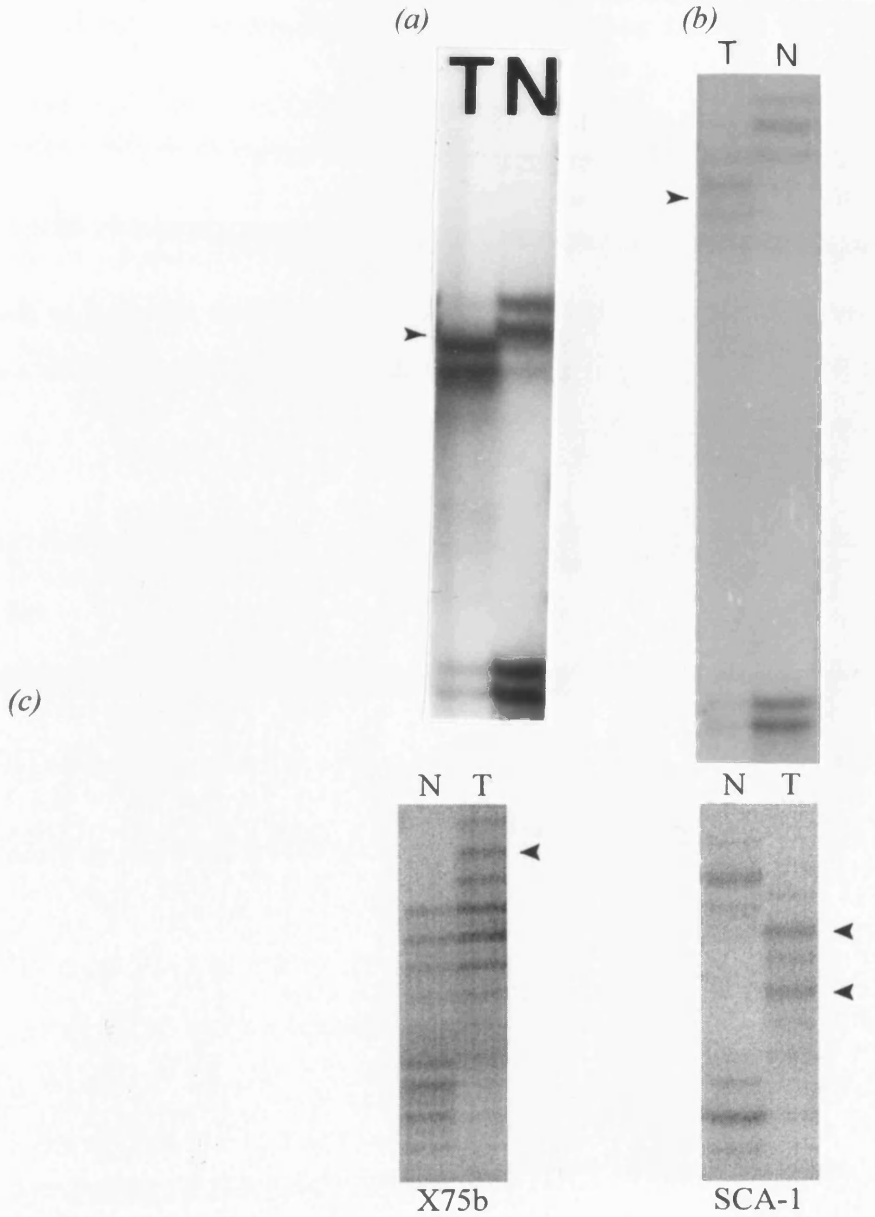
Dinucleotides	DI6S400, D16S413, D16S289, D16S402, X75b, D6S193, ESR
Trinucleotides	DM-1, SBMA, SCA-1

Table. 3.9. Pathological and microsatellite instability data for invasive carcinomas.

Tumour No.	**Type**	**Grade**	**Microsatellite Instability**
5	IDC/ILC	II	DM-1, SCA-1, SBMA, X75b, ESR, D6S193, D16S400, D16S402, D16S413
55	IDC	II	DM-1, SCA-1, SBMA, X75b, ERTA, D6S193, D16S400, D16S402, D16S413
59	IDC	II	DM-1
61	IDC	I	DM-1

IDC indicates Infiltrating ductal carcinoma, ILC is Infiltrating lobular carcinoma.

Figure 3.7. Representative examples of microsatellite instability in invasive carcinomas. Analysis of normal (N) and tumour DNA (T) by PCR, followed by electrophoresis on 6% denaturing gels and autoradiography. (a) and (b) Case Nos 37 and 59 with instability at DM-1, respectively. (c) Case No.5 with MI at X75b and SCA-1, (d) Case No.55 with MI at D16S413 and at D16S400. Arrows indicate alleles showing tumour-specific alterations.

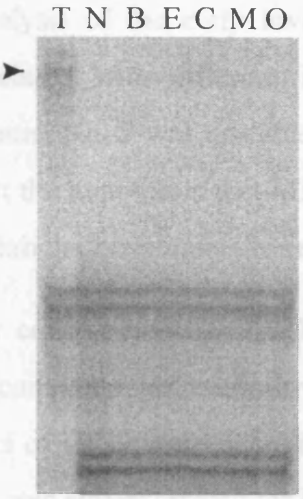

(a)
TN
(b)
T N
(c)
N T
X75b
N T
SCA-1
(d)
N T
D16S400
TN
D16S413

(e) Analysis of non-tumourous tissues for evidence of microsatellite instability.
Analysis of DNAs, prepared from tumour (T), lymph node (N), histologically normal breast (B), endometrium (E), cervix (C), myometrium (M), and ovary (O), from Case No.55, by PCR at the DM-1.

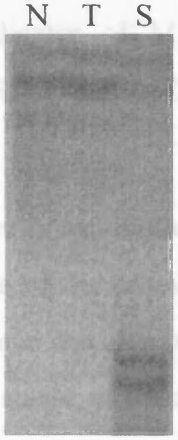
(f) Example of intratumoural heterogeneity in invasive carcinoma
Analysis of lymph node (N) and microdissected solid (S) and tubular foci (T) from Case No. 5 by PCR, at the DM-1 marker. The tubular area contains one allele, which is identical to that seen in the normal sample, whereas the area of solid tumour contains an additional allele not present in the normal or tubular areas (indicated by the arrow).

(g) Case No.55 with MI^+ atD16S400 and sequence analysis of the D16S400 PCR products.

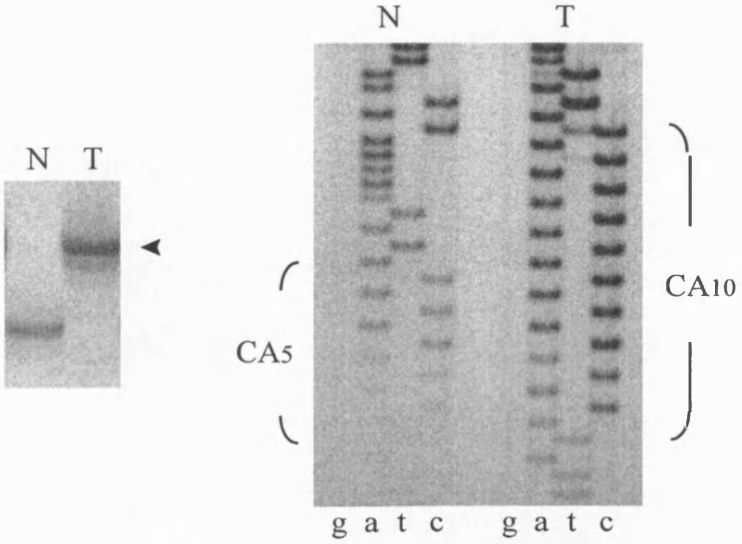
(d)



(e)



(f)



(g)

Tubular Carcinomas

The data obtained from the analysis of the early invasive carcinomas suggested that the presence of MI may be associated with different histological components. The solid component from invasive carcinoma No. 5 was unstable compared to the tubular component of this carcinoma. In order to test the hypothesis that MI may represent different pathways of tumour development, a series of tubular carcinomas were examined for MI.

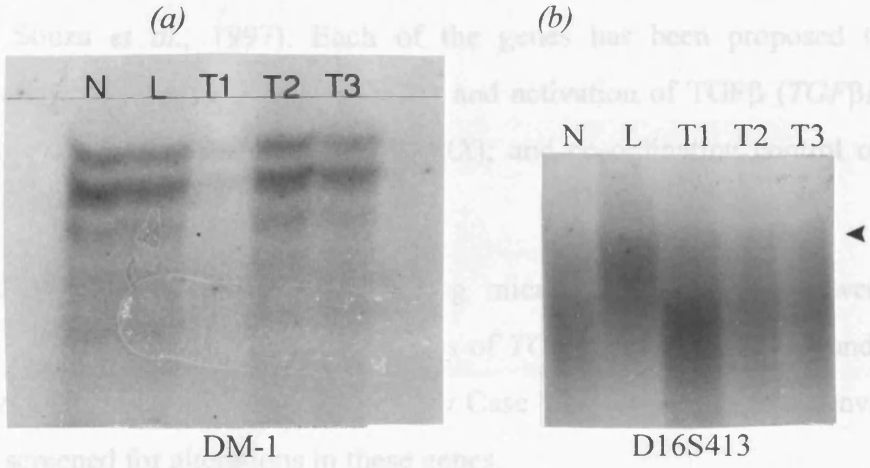
In general, three distinct tubular components from each of the 13 tubular carcinomas were separately microdissected and compared with non-involved lymph node from the same patient. For two of the cases, foci of DCIS were microdissected and examined. Another case contained an area of LCIS which was also examined for MI.

Analysis was performed with trinucleotide and dinucleotide microsatellites. The strategy was similar to that adopted for the analysis of the fibroadenomas cases. Three markers were analysed: DM-1 (as a sensitive indicator of MI), and two dinucleotides mapping to chromosome 16q (D16S289 and D16S413) so that LOH could also be assessed (as described in Chapter 4)

MI was not observed in any of the tubular carcinomas. However, instability of D16S413 was observed in LCIS associated with one of the cases. This pattern of instability was distinct from that of the previous findings, since the alteration was present in a single dinucleotide repeat and the DM-1 marker remained stable, as illustrated in Figure 3.8 (a).

Figure 3.8. Microsatellite instability in tubular carcinomas.

Analysis of normal (N), microdissected tubular foci (T1, T2, T3), and an area of LCIS (L) from Case No. 13 by PCR, followed by electrophoresis on 6% denaturing gels and autoradiography. (a) Analysis of the DM-1 marker, no evidence of instability. (b) Analysis of the D16S413 marker, with instability present in the LCIS component. Arrow indicates allele showing MI.



3.7 Analysis Of Cancer-Associated Genes

The existence of a mutator phenotype, characterised by MI^+, may be responsible for aberrations to critical cancer-associated loci (Loeb *et al.*, 1991, 1994). Among the targets identified to date are repetitive sequences in the coding regions of the TGFβ type II receptor (*TGFβRII*) (Myeroff *et al.*, 1995), the insulin-like growth factor II receptor (*IGFIIR*) (Souza *et al.*, 1996, Ouyang *et al.*, 1997), *Bax* (Rampino *et al.*, 1997) and the *E2F-4* gene (Yoshitaka *et al.*, 1996, Souza *et al.*, 1997). Each of the genes has been proposed to have tumour suppressive functions relating to: resistance and activation of TGFβ (*TGFβRII* and *IGFIIR*, respectively); maintenance of apoptosis (*BAX*); and co-ordinating control of the cell cycle (*E2F-4*).

Each of the 12 DCIS cases demonstrating microsatellite alterations were screened for frameshift mutations within repetitive motifs of *TGFβRII*, *IGFIIR*, *Bax*, and *E2F-4*. Due to the paucity of tissue available for study, only Case No. 55 from the early invasive carcinoma group was screened for alterations in these genes.

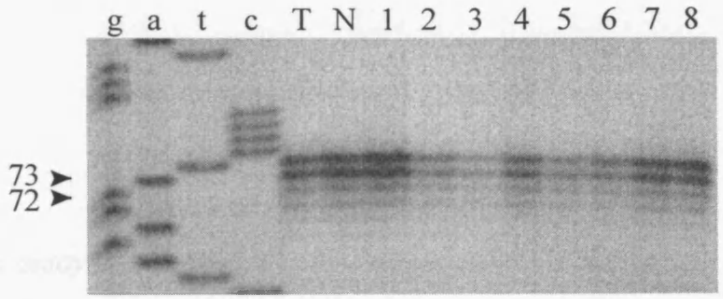
TGFβRII

The wild-type *TGFβRII* gene contains a tract of 10 consecutive adenines. Amplification across this region using published primers (Myeroff *et al.*, 1995), results in a single band of 73bp with a 'stutter' band migrating at 72bp (-1 *Taq* stutter band). Prior to testing the tumour samples a series of normal DNAs were tested to establish the wild-type banding pattern (personal communication, Lois Myeroff), as illustrated in Figure 3.9 (a).

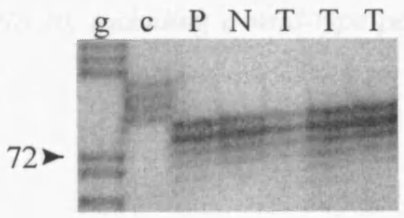
Frameshift alterations at this region were not observed in either the early invasive case or the DCIS cases, as illustrated in Figures 3.9 (a) and (b), respectively. A number of PCR products from the assay described above were sequenced directly, to confirm that the 73bp product contained 10 adenines only. A representative example of *TGFβRII* sequencing is illustrated in Figure 3.9 (c).

Figure 3.9 TGFβRII assay.

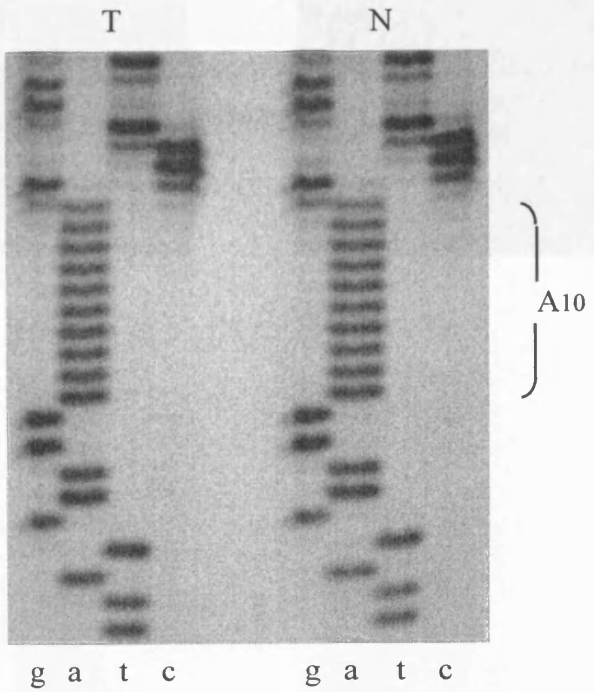
Analysis of tumour (T) and corresponding normal DNA (N), by PCR at the TGFβRII gene, followed by electrophoresis on 6% denaturing gels and autoradiography. (a) Early invasive carcinoma Case No. 55 and normal DNAs (1-8), including an M13 DNA sequence ladder with sizes in base pairs (b) DCIS Case No.20, including a wild-type positive control (P) from Figure 3.11 (a), and (c) Sequence analysis of the TGFβRII PCR products in tumour and normal tissues from early invasive carcinoma Case No. 55.



(a)



(b)



(c)

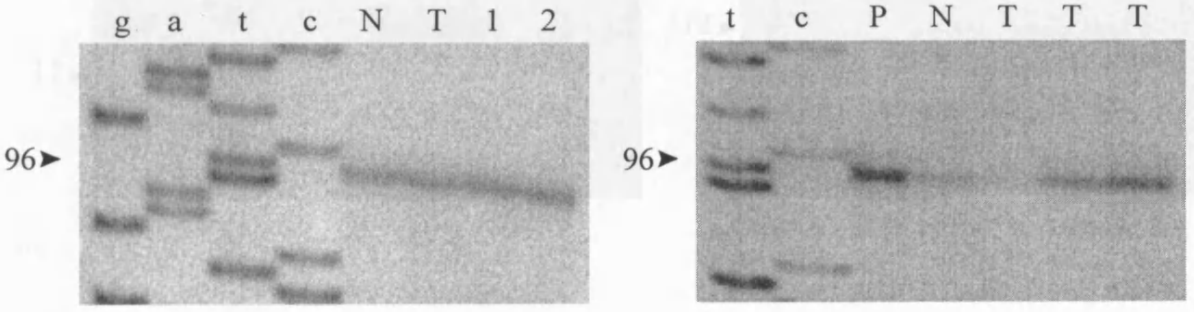
Bax

Amplification of the region spanning the G_8 tract of the *Bax* gene results in a PCR product of 93bp. It was not necessary to establish the wild-type banding pattern for this PCR product, since there are no '*Taq* stutter' bands generated during PCR amplification.

Frameshift alterations of the *Bax* G_8 region were not detected in the early invasive carcinoma or the DCIS cases, as illustrated in Figures 3.10 (a) and (b) respectively.

Figure 3.10. Bax assay

Analysis of tumour (T) and corresponding normal DNA (N), by PCR at the Bax gene, followed by electrophoresis on 6% denaturing gels and autoradiography. (a) Early invasive carcinoma Case No. 55 and normal DNAs (1and 2), including an M13 DNA sequence ladder with sizes in base pairs (b) DCIS Case No.20, including a wild-type positive control (P) from Fig 2.5 (a).



(a) **(b)**

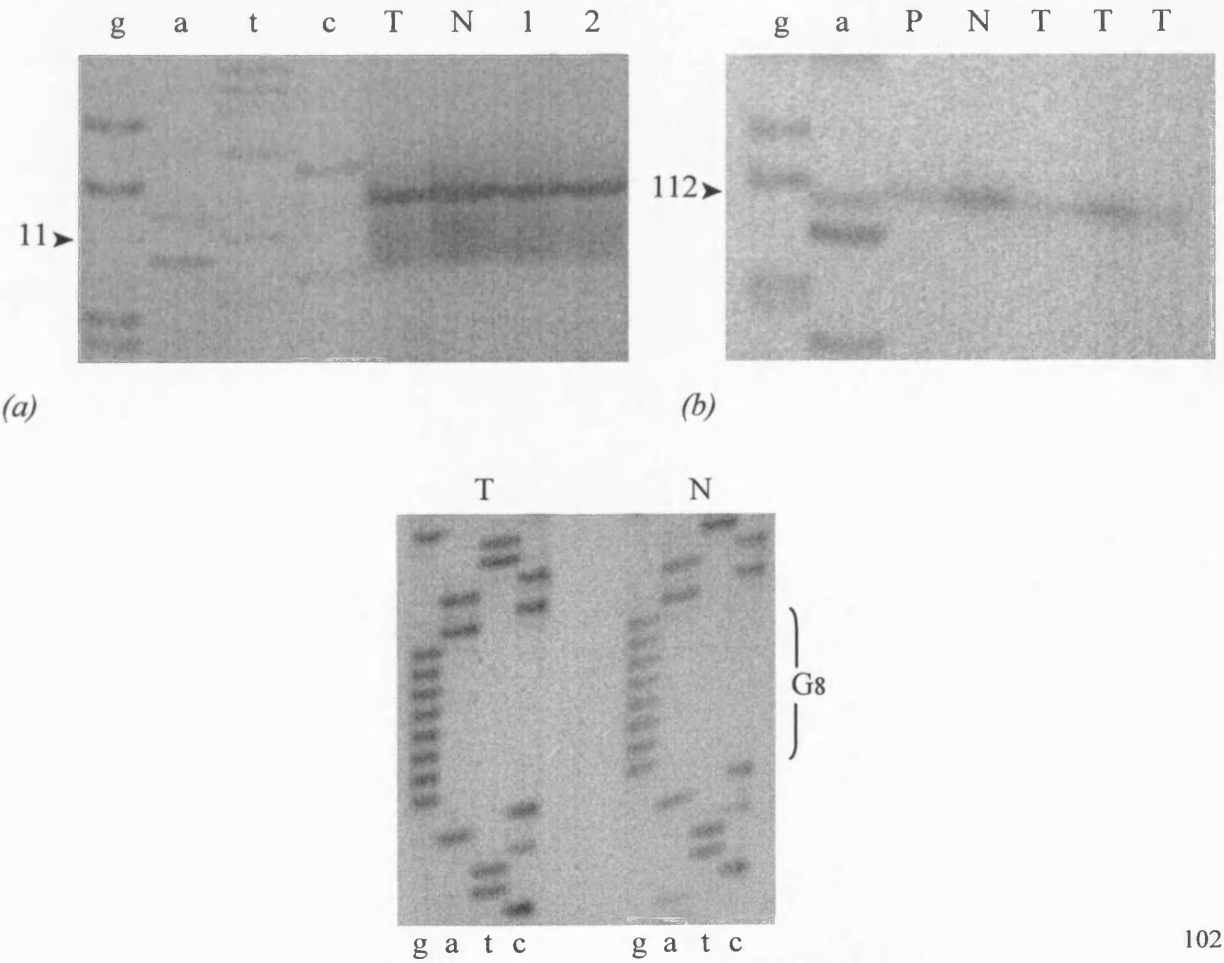
IGFIIR

Amplification of the region spanning the G_8 tract of the *IGFIIR* gene results in a PCR product of 111bp. Similar to the *Bax* gene, it was not necessary to establish the wild-type banding pattern for this PCR product, since there are no '*Taq* stutter' bands generated during PCR amplification.

Frameshift alterations of the *IGFIIR* G_8 region were not detected in the early invasive carcinoma or the DCIS cases, as illustrated in Figures 3.11 (a) and (b) respectively. Sequence analysis of the *IGFIIR* PCR products confirmed that the 111bp product contained 8 consecutive guanine residues only, as illustrated in Figure 3.11 (c).

Figure 3.11. IGFIIR assay

Analysis of tumour (T) and corresponding normal DNA (N), by PCR at the IGFIIR gene, followed by electrophoresis on 6% denaturing gels and autoradiography. (a) Early invasive carcinoma Case No. 55 and normal DNAs (1 and 2), including an M13 DNA sequence ladder with sizes in base pairs (b) DCIS Case No.20, including a wild-type positive control (P) from Fig 3.11 (a), and (c) Sequence analysis of the IGFIIR PCR products in tumour and normal tissues from early invasive carcinoma Case No. 55.



E2F-4

This gene contains 13 consecutive (CAG) repeats. However, this region is polymorphic, with variation of the number of repeats occurring throughout the population. Amplification of the region was initially attempted with a primer set which amplified a 330bp product. However, a number of the cases did not yield PCR products. Therefore the analysis was repeated with a primer set which amplified a smaller 135bp product.

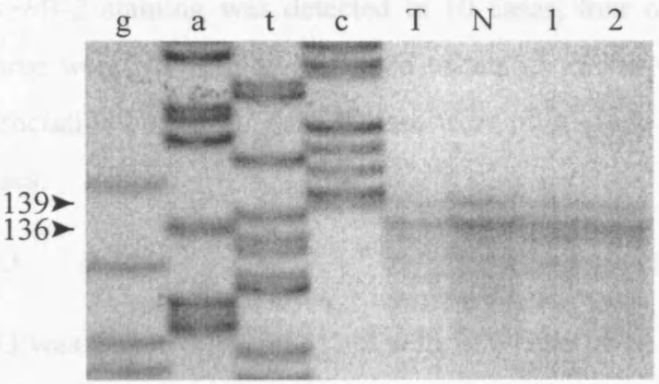
Alterations of the CAG repeat were not observed in the early invasive carcinoma. Analysis of the DCIS cases identified one case (No. 13) that contained an extra allele corresponding to 14 copies of the CAG repeat. However, the same number of repeats was also present in the corresponding normal tissue for this case, and therefore this was not a tumour-specific alteration, as illustrated in Figures 3.12 (a) and (b) respectively.

Sequence analysis of the *E2F-4* PCR products confirmed that the early invasive case No. 55 contained $(CAG)_{13}$ as illustrated in Figure 3.12 (c).

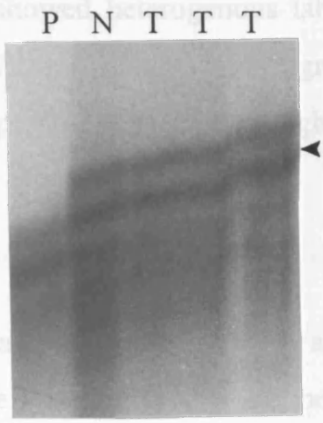
Figure. 3.12. E2F-4 assay

Analysis of tumour (T) and corresponding normal DNA (N), by PCR at the E2F-4 gene, followed by electrophoresis on 6% denaturing gels and autoradiography. (a) Early invasive carcinoma Case No. 55 and normal DNAs (1 and 2), including an M13 DNA sequence ladder with sizes in base pairs (b) DCIS Case No.13, including a CAG_{13} control (P) from Fig 3.12 (a), and (c) Sequence analysis of the E2F-4 PCR products from (c) early invasive carcinoma Case No. 55.

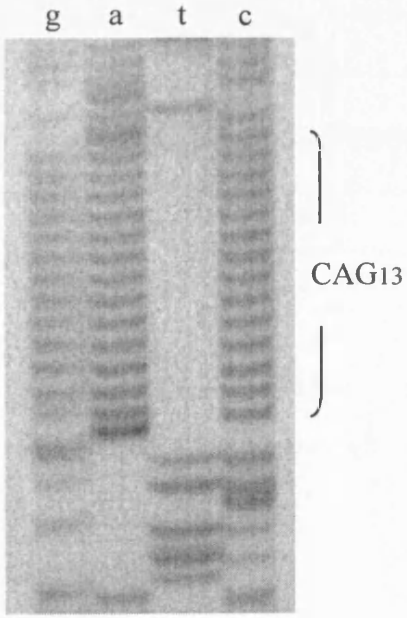
(a)



(b)



(c)



c-*erb*B-2 and p53

Each of the DCIS cases had been screened for expression of p53 and c-*erb*B-2 by immunohistochemistry and data was provided by Dr. RA Walker. Comparison of data was undertaken to determine if the presence or not of microsatellite alterations in the DCIS cases, related to alterations to p53 and c-*erb*B-2. The immunohistochemical and MI data are summarised in Table 3.10.

c-erbB-2

c-*erb*B-2 staining was detected in 10 cases, four of which showed heterogenous labelling. Three were MI^+ and five showed instability exclusively at DM-1, and this was a significant association ($p=0.02$). All of these were high grade and comprised 10 of the 11 high grade cases.

p53

p53 was detected in 20-85% of cells in 6 cases. One was MI^+ and four had instability at DM-1. Five were high grade and one low grade. However, there was no relationship between immunoreactive p53 and microsatellite instability.

Table 3.10. Pathological and microsatellite instability data for DCIS cases.

Case No.	Grade	Microsatellite Instability	p53	c-*erb*B-2
1	High		-	-
2	High	DM-1, D6S193	-	Het. +
3	Low	DM-1	-	-
4	Low		-	-
5	High	DM-1	85%	+
6	High		-	Het. +
7	High	D6S193, D6S186	-	Het. +
8	Low		-	-
9	Inter.		-	-
10	Inter.	DM-1, D6S193	-	-
11	High		-	+
12	High	DM-1	50%	+
13	High	DM-1	20%	Het. +
14	High	DM-1	80%	+
15	Inter.	DM-1	-	-
16	High	DM-1	-	+
17	High	DM-1, D6S193, D16S400, D16S413	45%	+
18	Low		-	-
19	Low		-	-
20	Low	BAT25, BAT40	-	-
21	Low		30%	-
22	Low		-	-
23	Low		-	-

p53 reactivity was determined by calculating the percentage of positive cells present in 10 ducts. Inter. designates Intermediate; Het. is Heterogenous; - is Negative; + is Positive

3.8 Analysis Of Mismatch Repair Proteins

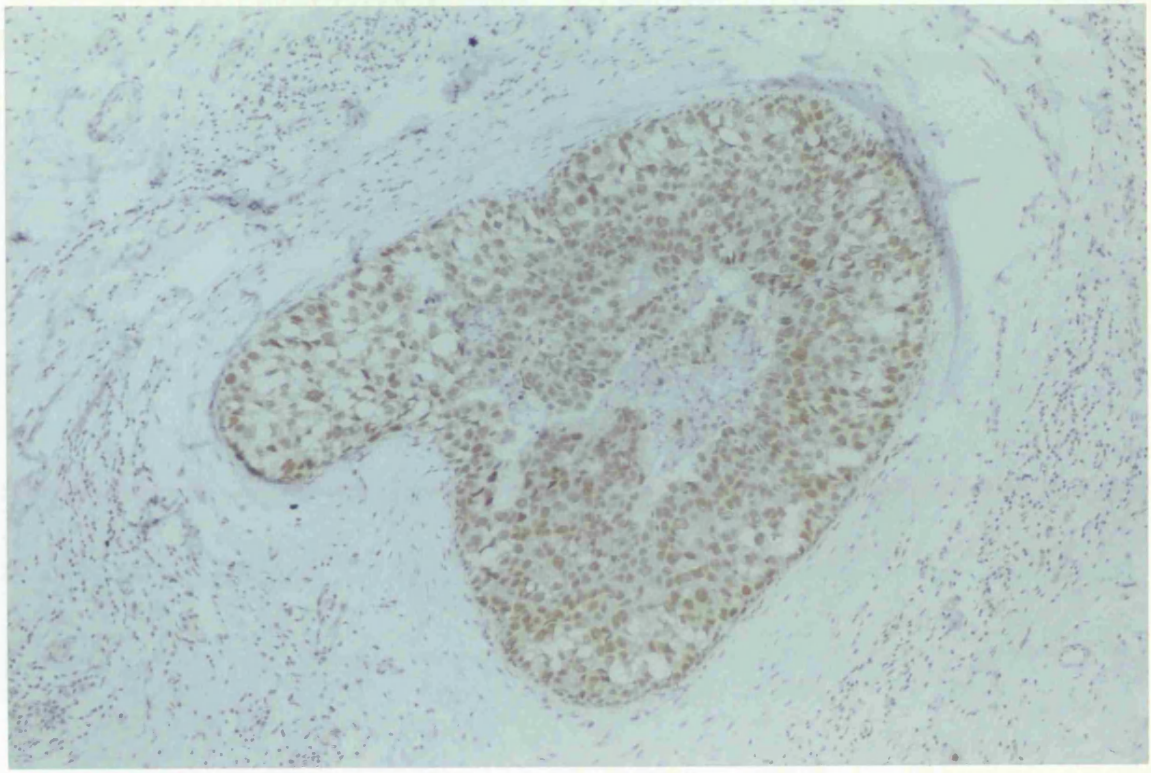
Mismatch repair protein expression was examined by immunohistochemistry. The first MMR protein to be examined was PMS2 with the Clone 9 antibody. This antibody had not been previously tested on formalin-fixed paraffin-embedded tissues. It was initially tested on untreated breast tissue sections with primary antibody dilutions ranging from, 1:10 to 1:50. No reactivity was observed. The sections were then pre-treated by protease digestion or pressure cooking and tested with the same dilution range. Pressure cooking gave consistent and reproducible nuclear reactivity.

The other MMR proteins investigated were MLH1 (Clone 14) and MSH2 (FE11). These antibodies had previously been demonstrated to stain paraffin sections, pre-treated with 10mM sodium citrate buffer for 10 mins at 95^0C, with and without proteinase K digestion (personal communication, Oncogene Science). This protocol was compared directly with the pressure cooking procedure. Pressure cooking led to antigen unmasking and consistent nuclear reactivity, whereas reactivity was not observed using the 'boiling sodium citrate' protocol. Therefore, pressure cooking was favoured for all subsequent immunohistochemical staining with these antibodies.

Evaluation of MMR protein staining was confirmed by Dr. R. A. Walker. DCIS reactivity was compared to that seen in normal breast tissue in the same section, in relation to presence and intensity. Two cases showed stronger PMS2 staining in the DCIS as compared to normal (Case Nos. 3 and 22). Case No. 3 had an alteration at the DM-1 repeat, whereas case No. 22 showed no alterations. The staining for MSH2 was stronger and generally there was clear nuclear reactivity in normal breast and DCIS. Overall there was no difference in the reactivity for PMS2, MLH1 and MSH2 between those cases showing MI and those not. Immunohistochemical staining of PMS2, MSH2, and MLH1 is illustrated in Figures 3.13, 3.14, and 3.15, respectively. The immunohistochemical staining for each of the mismatch repair proteins is summarised in Table 3.11.

Figure 3.13 PMS2 Immunohistochemistry.

(a) Pure DCIS of intermediate grade (Case No. 10) showing staining of majority of nuclei. A few cells have no labelling, which is also seen in the corresponding normal breast, demonstrating variability. This case did not demonstrate microsatellite instability.



(b) Micropapillary DCIS (Case No.16) with clear nuclear staining. This case did not demonstrate microsatellite instability.

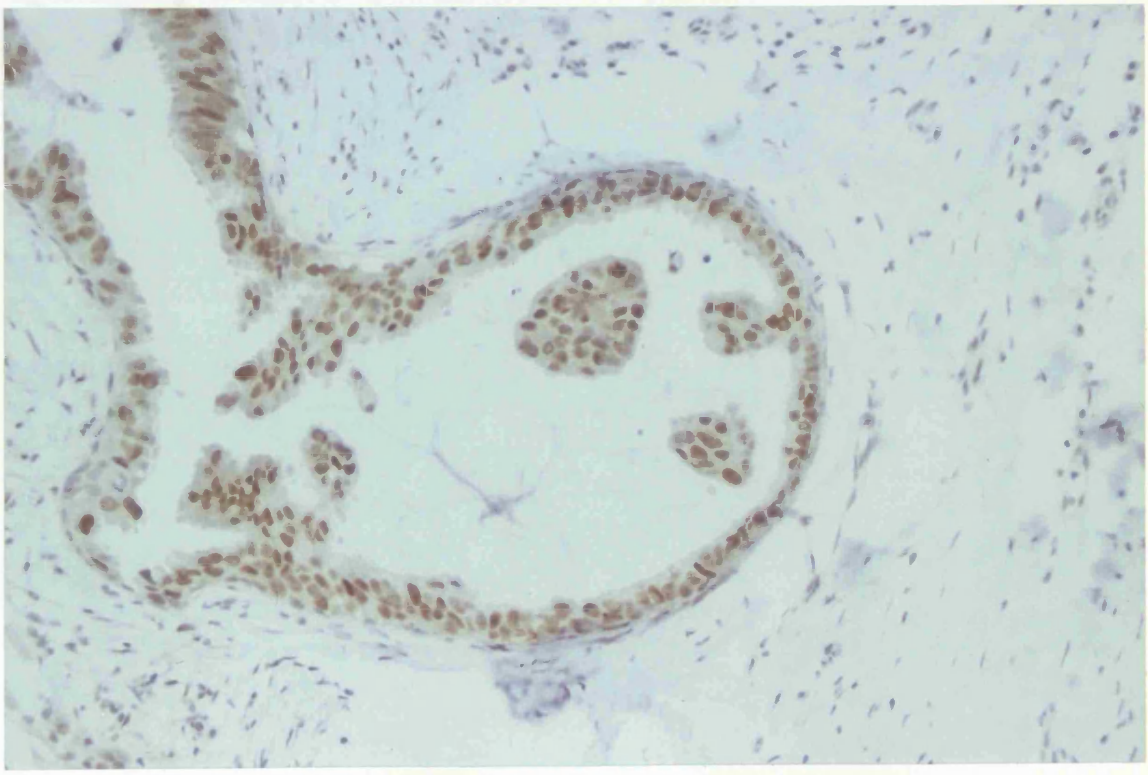
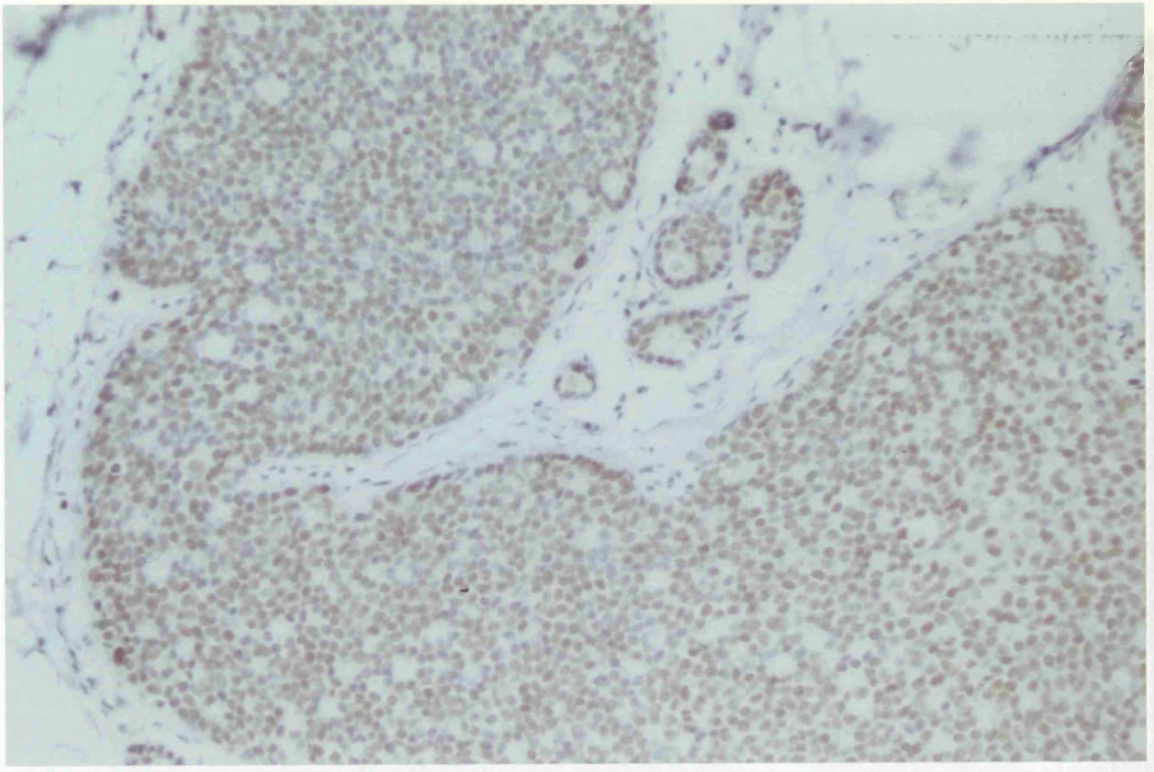


Figure 3.14. MSH2 Immunohistochemistry.

(a) Area of DCIS of low grade (Case No. 19) showing staining of majority of nuclei. This case did not demonstrate microsatellite instability.



(b)Adjacent hyperplastic breast with strong nuclear staining.

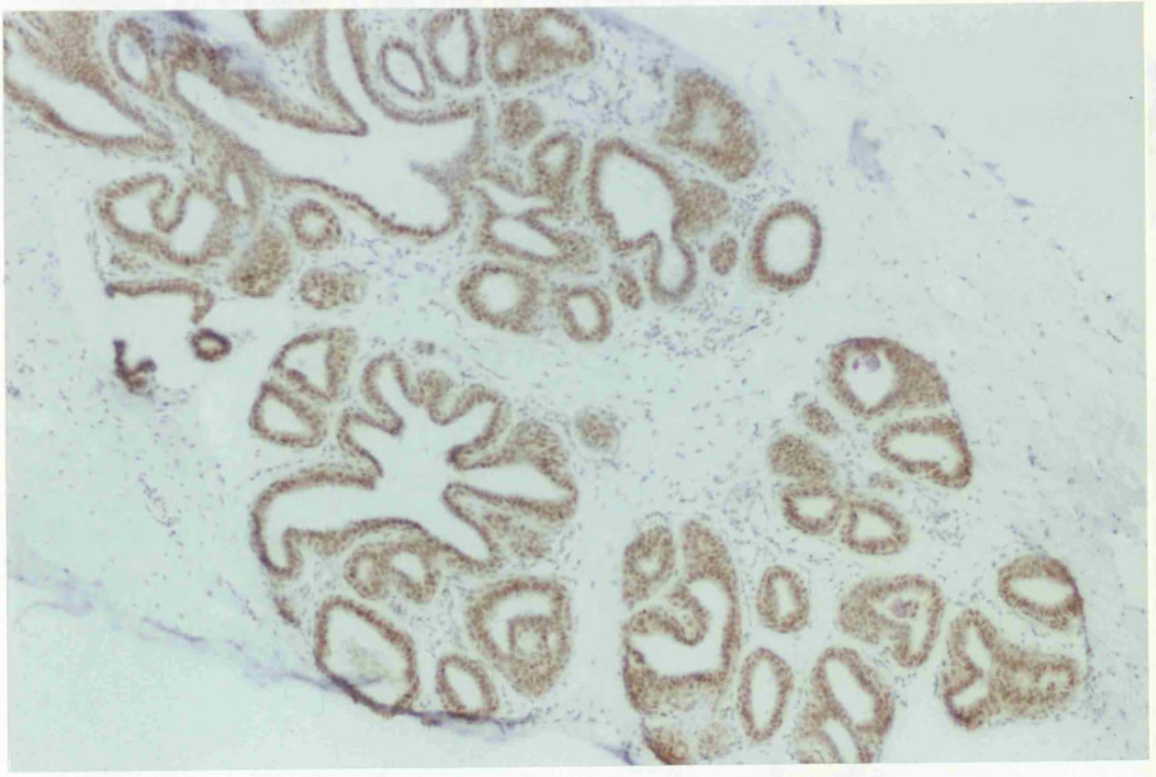
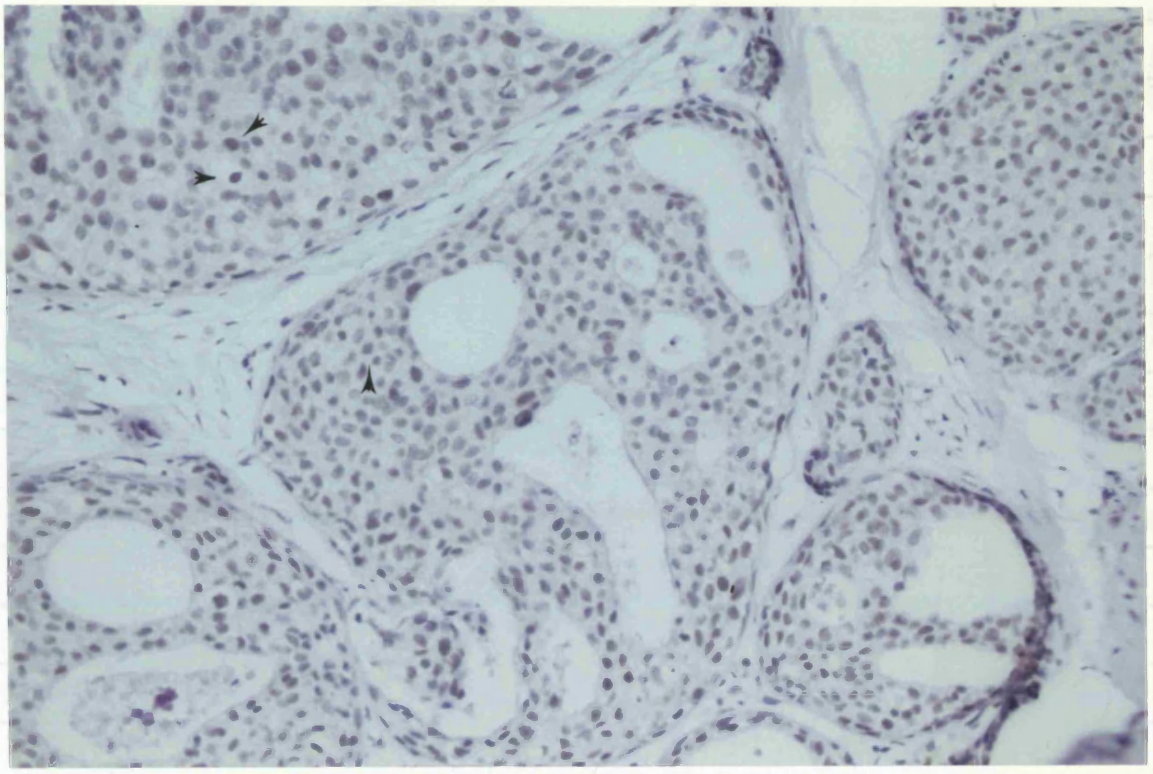


Figure 3.15. MLH1 Immunohistochemistry.

(a) Same case as stained for MSH2. Variable nuclear staining in DCIS. Arrows indicate positive cells.



(b) Adjacent hyperplastic breast with variable nuclear staining.

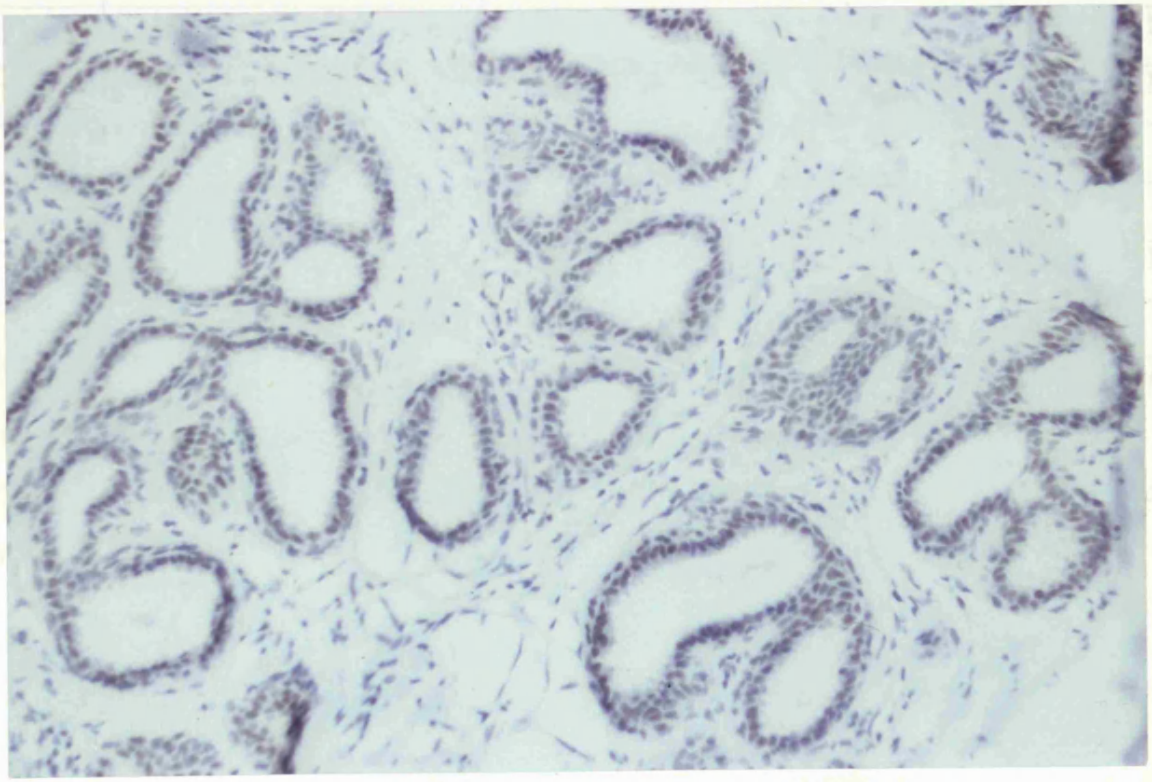


Table 3. 11. Mismatch repair protein reactivity in DCIS.

Case No.	PMS2	MSH2	MLH1
1	ND	Staining in N*	ND
2	Staining in DCIS and N	Staining in DCIS and N	ND
3	Staining stronger in DCIS	Staining in DCIS and N	Staining in DCIS and N
4	No staining	Staining in DCIS and N	ND
5	Weak staining	Staining in N*	Staining in DCIS
6	Weak staining	Variable staining	Staining in DCIS and N
7	Weak staining		Staining in DCIS and N
8	ND	Weaker in papillary areas	No staining
9	No staining	Staining in DCIS and N	ND
10	Staining in DCIS	Staining in DCIS and N	ND
11	Weak staining	Staining in DCIS and N	Staining in DCIS and N
12	Weak staining	Staining in DCIS and N	Staining in N*
13	Staining in DCIS and N	Staining in DCIS	Staining in DCIS
14	Staining in DCIS and N	Staining in N*	Staining in DCIS and N
15	ND	Staining in DCIS and N	ND
16	Staining of DCIS	Variable staining	Staining in DCIS and N
17	Weak staining	Staining in N*	Staining in N*
18	Weak staining	Staining in DCIS and N	Staining in DCIS and N
19	Weak staining	Staining in DCIS and N	Staining in DCIS and N
20	No staining	Staining in DCIS and N	Variable staining
21	Staining of DCIS and N	ND	Variable staining
22	Staining stronger in DCIS	Staining in DCIS and N	Staining in DCIS and N
23	Weak staining	Variable staining	No staining

Staining refers to DCIS, unless normal ducts (N) are present within the same section, where staining is then described in relation to that seen in DCIS.

** Indicates that there is no DCIS in the section, ND is not determined due to insufficient tissue.*

3.9 Microsatellite Instability And Development Of Drug Resistance

This project was developed to determine whether there was an association between the presence of microsatellite instability and the development of drug resistance in culture. This was investigated by using the multidrug resistant derivative of the MCF-7 cell line, called MCF-7/Adr (kindly provided by Gillian Hirst) and the anti-tumour drug, doxorubicin (adriamycin). The studies were designed to answer two main questions:

1. Is the presence of MI^+, in the MCF-7/Adr cell line, characterised by:
(i) a decreased expression of the mismatch repair proteins (MSH2, MLH1, and PMS2), and
(ii) the presence of frameshift mutations of the cancer-associated genes (TGFβRII, IGFIIR, Bax, and E2F-4).
2. Can microsatellite instability be 'induced' in MCF-7 cells by treatment with increasing concentrations of doxorubicin.

MCF-7/Adr Cell Line

The MCF-7/Adr cell line had previously been shown to demonstrate MI^+ (Anthoney *et al.*, 1996). In this study the cell line was shown to be unstable at 6 of 13 loci tested. These were predominantly dinucleotide markers mapping to the long arm of chromosome 17.

Prior to analysing the cancer-associated genes in the MCF-7/Adr cell line, the microsatellite profiles of an extended panel of markers were compared in the MCF-7 and MCF-7/Adr cell lines. The markers chosen were from each of the three repeat types available: the BAT25 mononucleotide, D16S400 dinucleotide, and DM-1 trinucleotide. The analysis revealed that for each marker tested, the MCF-7/Adr cell line showed alterations to both alleles compared to the alleles form the MCF-7 cell line (Figure 3.16a). Each of the repeat regions from the cancer associated genes (*TGFβRII*, *IGFIIR*, *Bax*, and *E2F-4*) were then investigated in both cell lines, however the there were no alterations from the wild-type alleles (Figure 3.16b).

Alterations of multiple microsatellite loci may suggest an aberration to one of the mismatch repair genes in the MCF-7/Adr cell line. Therefore, the expression levels of PMS2, MLH1 and MSH2 from both cell lines were compared by Western blot analysis. Each of the blots detected a single band, within the correct size range, for the MCF-7 and MCF-7/Adr samples. For each of the mismatch repair proteins there was no difference in band intensity between MCF-7 and MCF-7/Adr cell lines (Figure 3.17) suggesting equivalent levels of expression of

these proteins in both cell lines. However, determination of equivalent protein loading was not determined.

Figure 3.16. Microsatellite Instability analysis of MCF-7 and MCF-7/Adr cell line.
Microsatellite analysis of breast cancer cell lines (MCF-7, and MCF-7/Adr) by PCR, followed by electrophoresis on denaturing gels and autoradiography. (a) DM-1 marker, (b) Bax G_8 region. Arrow indicates altered alleles in MCF-7/Adr in comparison to MCF-7.

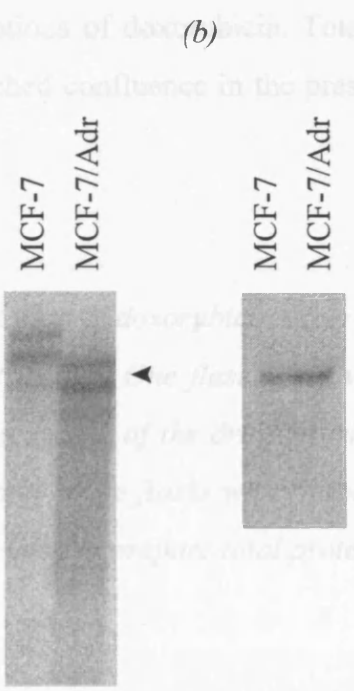
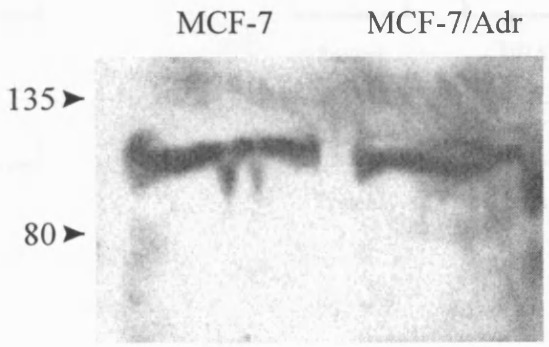


Figure 3.17. Mismatch protein analysis of MCF-7 and MCF-7/Adr cell line.
Protein extracts (20μg) from MCF-7 and MCF-7/ADR cell lines were electrophoriesed on 10% SDS-PAGE, blotted onto Hybond C^+ and probed with MSH2 antibody. Sizes are in kD.

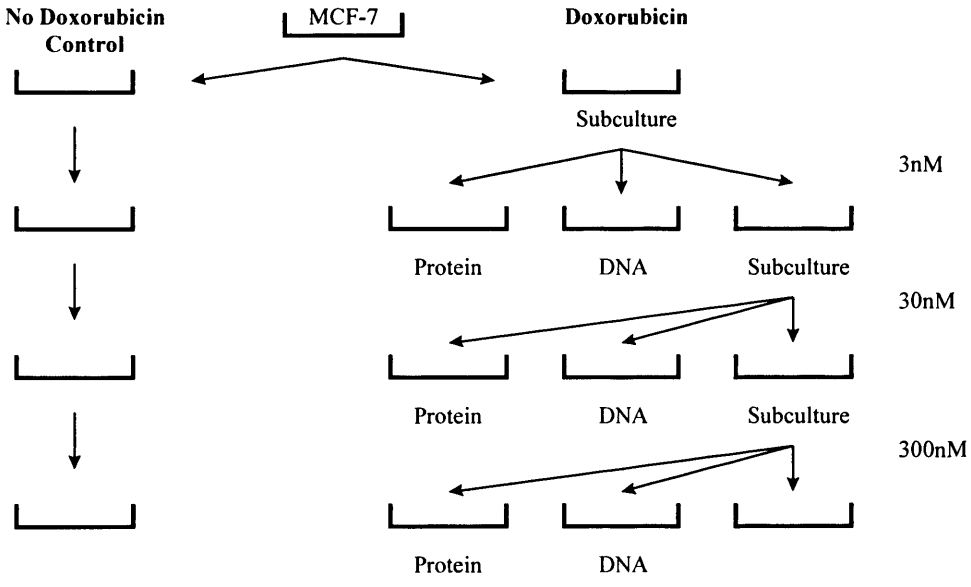


Doxorubicin Treatment Of MCF-7 Cell Line

The second part of this study was to determine whether increasing concentrations of doxorubicin could lead to the development of microsatellite instability. The initial concentration of doxorubicin used in these experiments (3nM) was based upon a recent publication (Ogretman and Safa, 1997) which conducted cell survival assays and calculated the concentration of doxorubicin that inhibited MCF-7 cell survival by 50% (IC_{50}).

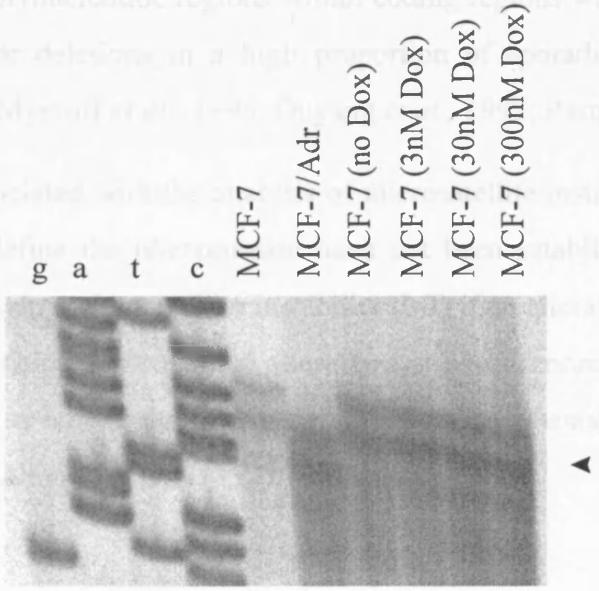
The schematic (Figure 3.18) illustrates the experimental design. MCF-7 cells were grown in triplicate in increasing concentrations of doxorubicin. Total DNA and protein extracts were prepared when the cells had reached confluence in the presence of 3nM, 30nM, and 300nM, respectively.

Figure 3.18. Schematic representation of doxorubicin experiment.
A flask of MCF-7 cells are split in two. One flask is grown in the absence of doxorubicin, while the other is grown in the presence of the drug, in triplicate. When cells are confluent, one flask is used to subculture three more flasks with (increasing doxorubicin concentration) and the two remaining flasks are used to prepare total protein and DNA extracts respectively.



Due to time constraints, the expression levels of each of the mismatch repair proteins in the doxorubicin-treated cells were not determined. The DNA from each of the subcultures and the untreated control were analysed with respect to stability of the DM-1 marker. This marker was selected because it was judged to be a sensitive marker of microsatellite instability. There was no evidence of microsatellite instability in any of the four doxorubicin-treated MCF-7 cell derivatives (Figure 3.19).

Figure 3.19. Doxorubicin treatment of MCF-7 cell line.
Microsatellite analysis of DNA prepared from MCF-7, MCF-7/Adr cell lines, and MCF-7 cells, cultured in the presence of increasing concentrations of doxorubicin (Dox), at the DM-1 marker by PCR, followed by electrophoresis, along side an M13 DNA sequencing ladder. Arrow indicates the altered alleles of the MCF-7/Adr cell line.



3.10 Microsatellite Instability

The accumulation of widespread mutations (insertions and deletions of one or a few nucleotides) in simple repeated sequences of a tumour cell genome is the diagnostic feature of cancer of the microsatellite instability phenotype (MI^+) (Ionov *et al.,* 1993; Perucho *et al.,* 1994). MI^+ tumour represent a well characterised and supportive example of the cancer as a mutator phenotype hypothesis (Loeb *et al.,* 1991, 1994) and of the mechanisms responsible for cancer susceptibility (Knudson *et al.,* 1971, 1985). Mutations in these unstable repeat sequences (reviewed in Eshelman and Markowitz, 1995) accumulate because of the failure by the DNA mismatch repair machinery (MSH2, MLH1, PMS2, PMS1, and MSH6) to correct the errors of replication due to slippage by strand misalignment (Streisinger *et al.,* 1966). Mutated cancer associated genes in MI^+ tumours are generally different from those found in tumours of the classical suppressor pathway (Fearon and Vogelstein, 1990) because they contain targets for slippage-induced frameshift mutations. For example, the *TGFβRII, IGFIIR,* and *Bax* genes contain polynucleotide regions within coding regions which are subject to one or two base insertions or deletions in a high proportion of sporadic gastrointestinal and urogenital MI^+ tumours (Myeroff *et al.,* 1995; Ouyang *et al.,* 1997; Rampino *et al.,* 1997).

One of the problems associated with the analysis of microsatellite instability in breast cancer is that clear criteria to define the phenomenon have not been established. In this thesis, a tumour was defined as having microsatellite instability (MI) if an alteration of a single marker was observed. Tumours which demonstrated alterations at two or more independent genomic locations were described as having the microsatellite instability *phenotype* and designated as MI^+, as first defined by Aaltonen *et al.,* (1993).

Technical Problems

The DNA template procured from microdissected tumour foci produced consistent amplification of the microsatellites used in this study. In general, microdissection of a single involved duct or group of carcinoma cells produced sufficient template for analysis with multiple markers. However, analysis of morphologically normal ducts adjacent to DCIS unfortunately produced a high (3 of 4) failure rate of amplification, suggesting that there was insufficient template in these ducts. The study by Deng *et al.,* (1996) overcame this problem by PCR-amplifying their markers for 45 cycles. Amplification of the microsatellites used in this study for 45 cycles did result in PCR products but with additional non-specific products which made interpretation of the results difficult. The efficiency of DNA preparation from

very small numbers of cells would be greatly improved by using the recently described technique of laser captured microdissection (Emmet-Buck *et al.*, 1996).

Fibroadenomas

Although it was subsequently demonstrated that MI was a tumour specific alteration, it was possible that observed instability was simply a consequence of the increased proliferation rate of the tumours. This was tested by examining a group of hyperproliferative breast lesions (fibroadenomas) for MI. Analysis with DM-1 and a dinucleotide did not reveal MI in any of the cases, demonstrating that this alteration was not solely due to an increased rate of proliferation. Interestingly, a proportion of the cases did show allelic loss for the chromosome 16q marker (described in Chapter 4), suggesting that genetic alterations could be detected in this type of lesion.

Invasive Carcinomas

One problem associated with MI analysis of breast tumours is that, to date, each investigation has used a different panel of microsatellite markers, therefore making comparisons between different groups of tumours and different tumour types very difficult. Other problems for the analysis of invasive carcinomas relate to the investigation of unselected groups, for example early and advanced groups combined. In order to establish the role of MI in early stages of invasive tumours, small (less than 15mm) node negative tumours were investigated. The analysis in this group was initially performed on DNA prepared from whole tumour sections, similar to the majority of other studies performed on invasive carcinomas (summarised in Table 3.4). The analysis identified four tumours that showed two types of instability.

Two (7%) of the cases had alterations exclusively to the DM-1 marker. Both of these tumours showed contraction of the larger DM-1 allele by a single repeat unit (three base pairs). It is noteworthy that the only other study to use the DM-1 marker to assess MI, also characterised a tumour with a 3bp contraction of the larger allele (Wooster *et al.*, 1994b). As the analyses progressed it emerged that the DM-1 marker may be a sensitive marker of MI^+ in breast cancer. It was observed that when a tumour had alterations to both DM-1 alleles, this was always accompanied by alterations to several other microsatellite markers. In contrast, alteration to just one DM-1 allele was typically not accompanied by further genetic changes. This is unlikely to be a reflection of the repeat length at DM-1 because other microsatellites used (for example, AR) generally had longer repeats than DM-1. It is possible that this

instability has implications for the myotonic dystrophy protein kinase (DMPK) gene in which the repeat is located (as discussed later in this section).

The second type of instability was more pronounced. Two (7%) cases displayed instability at 9 of 10 loci tested (including both dinucleotide and trinucleotide markers) and were designated MI^+. In the majority of instances both alleles were altered for each of the markers analysed, including DM-1. Breast carcinomas with similar widespread alterations have also been reported by other investigators. For example, Contegiacomo *et al.,* (1995), characterised a tumour with alterations to 6 of 8 markers, and Toyama *et al.,* (1996a), demonstrated alterations to 8 of 12 loci in one tumour in their study. These cases have instability similar to that reported in the HNPCC kindreds, however, the pattern of instability is different. In tumours from HNPCC patients there is generally a ladder of unstable alleles which results in 'smearing' of the PCR products. The tumours described here generally had two new alleles not present in the corresponding normal sample. The possibility of a sample mix up was discounted by repeating the analysis with alternative tumour blocks from the same patient, since heterozygous stable alleles were observed for at least one other locus.

The group of invasive carcinomas studied were predominantly infiltrating ductal, with a small number of tubular carcinomas or lobular-tubular carcinomas. MI and MI^+ was only identified in the infiltrating ductal carcinomas. Other studies have included infiltrating lobular carcinomas and have correlated the presence of MI with this phenotype (Aldaz *et al.,* 1995; Contegiacomo *et al.,* 1995).

Microdissection of individual foci from each of the carcinomas confirmed the initial MI results obtained from DNA prepared from an entire tumour tissue section, and did not lead to the identification of any additional cases with alterations. This suggests that microdissection may not be necessary for the analysis of infiltrating carcinomas where the majority of cells in the tissue section are malignant, in contrast to the non-invasive carcinomas where there are high proportions of non-malignant cells surrounding malignant foci (described later in this section). However, the microdissection analysis was of value in tumours with solid and tubular components. Microsatellite analysis of DNA prepared by microdissection on each of these components demonstrated that only the solid groups of cells were altered with respect to the normal tissue. This provided preliminary evidence that MI may be associated with dedifferentiation. Within individual tumours clones of MI could occur, and may be the focal points for progression.

To further investigate the role of MI in breast cancer pathways, a group of tubular carcinomas were investigated. The analysis was conducted with the DM-1 marker and two dinucleotides (D16S413 and D16S289) mapping to chromosome 16q. There was no evidence of MI in any of the tubular carcinomas. However, instability of the D16S413 marker was observed in LCIS associated with one of the cases. This type of instability, of a single dinucleotide, was not observed in any of the other cases examined in this thesis but it has been reported by a number of investigators (Table 3.4) and is best exemplified by the data of Karnick *et al.*, (1995). In their study, the authors examined a series of dinucleotide repeats mapping to chromosome 11p, but demonstrated instability of only one in 19 of 69 (28%) cases. A number of other investigators have studied the D16S413 marker with respect to Loss of Heterozygosity of chromosome 16q in microdissected breast lesions and found no evidence of instability with this marker (Aldaz *et al.*, 1995; Lakhani *et al.*, 1995a; Lakhani *et al.*, 1995b; Stratton *et al.*, 1995), suggesting that the instability of D16S413 described above, does not merely reflect that this marker is inherently unstable.

DCIS

The benefits of microdissection analysis were particularly evident when studying MI in the group of DCIS. Microsatellite analysis in these lesions using DNA template prepared from whole tissue sections revealed no alterations, as would be expected since tumour cells may often represent a low percentage of the total cells from cases of this type. Microdissection enabled enrichment for tumour cells and the analysis of multiple ducts from the same tumour section. The types of MI were similar to that seen in the early invasive carcinomas, although the frequencies were higher, with 7 (30%) cases showing alterations to one allele of the DM-1 marker, and 5 (22%) characterised as MI^+. However, there were different patterns of MI^+. Three cases showed alterations at DM-1 and different dinucleotides, one showed alterations to two dinucleotides, and the remaining tumour was altered at the BAT25 and BAT40 mononucleotide repeats. This last case is particularly interesting because alterations restricted to mononucleotide repeats may prove to be indicative of alterations to a separate mismatch repair pathway. This is supported by the study of Yee *et al.*, (1994), who investigated MI in 20 invasive carcinomas with mono-, di-, tri- and tetranucleotide repeats. One tumour showed an alteration exclusively at a mononucleotide repeat, a (T)n repeat within an Alu sequence.

Aldaz *et al.*, (1995) also studied MI in 23 cases of pure DCIS, and found a MI^+ frequency of 13% (3 of 23). Unfortunately, the markers showing instability in these cases were not

described. The nuclear grade of the cases was also not reported. However, the data recently presented by Dillon *et al.,* (1997) seems to contradict these findings. They examined 33 cases of DCIS with a panel of microsatellites, and found only one case to be altered. However, it is not clear from this paper whether microdissected tumour foci or DNA from whole sections were used. Considering the evidence presented in this thesis it seems likely that the vast quantities of non-malignant cells present in cases of pure DCIS would lead to 'masking' of tumour specific alterations.

Microsatellite instability was detected at a higher frequency in the high and intermediate grade cases, compared to the low grade cases. It is not possible to demonstrate correlation between MI^+ and grade from the other DCIS studies of Aldaz *et al.,* (1995) and Dillon *et al.,* (1997). However, MI^+ in invasive breast carcinomas has been correlated with indicators commonly associated with poor disease prognosis, such as involvement of lymph node (de Marchis *et al.,* 1997), and reduced overall survival (Paulson *et al.,* 1996).

Heterogeneity between ducts was observed in two cases, as demonstrated by different instability of the DM-1 alleles. This low frequency (9%) of heterogeneity is similar to that described by Fujii *et al.,* (1996b), who demonstrated 3 of 23 (13%) cases with heterogeneity between ducts, as determined by allelic loss. From their studies on DCIS, Fujii *et al.,* (1996b) proposed a model of how DCIS may progress to invasive carcinoma. The involved ducts evolve along a common pathway, until a point where several foci diverge along pathways of genetic progression, which is associated with synchronous invasion. The growth of invasive cancer is then hypothesised to result from expansion of one population of the divergent in situ carcinoma. This is supported by the identification of allelic heterogeneity which is present in a low frequency in the pure DCIS, increased in DCIS with synchronous invasion and not present in invasive carcinomas. Although analysis of DCIS with synchronous invasion is central to this model, the allelic heterogeneity data presented here (with respect to instability of the DM-1 marker) supports this model. There was a low level of genetic heterogeneity in the cases of pure DCIS, and no heterogeneity in the invasive foci.

Overall Findings

The overall findings indicate that, in the DCIS and early invasive breast lesions, MI was a tumour specific alteration. There was no evidence of MI in fibroadenomas, ruling out MI occurring as a sole consequence of increased proliferation. The data from the DCIS cases together with evidence from other investigations suggests that in cases of DCIS and invasive breast carcinomas instability is associated with high nuclear grade and an aggressive phenotype. This is also supported by the findings of low frequency of MI in well to moderately differentiated early invasive carcinomas and absence of microsatellite alterations in the tubular carcinomas.

3.11 Mismatch Repair Proteins And Cancer-Associated Genes

Despite a large number of MI studies in breast cancer, there have been no reports correlating this phenotype with alterations in the mismatch repair proteins or any of the cancer-associated loci containing targets for slippage-induced frameshift mutations. The only data reported thus far is a frameshift mutation in MLH1 in a MI^+ breast tumour from a patient with HNPCC (Risinger *et al.,* 1996). This was the first evidence that breast cancer, albeit as an integral tumour within the HNPCC syndrome, may result from the inheritance of a mutant MMR gene.

Following the identification and characterisation of MI in various breast malignancies, the project then questioned the 'cause' and 'effect' of the observed instability. Subsequent analysis of the mismatch repair genes and cancer-associated loci was confined to the cases of DCIS, where 5 cases were categorised as MI^+, and a further seven showed alterations at the DM-1 marker.

Is MI^+ 'caused' by inactivation of key mismatch repair genes (MSH2, MLH1, and PMS2) in breast cancer ?

HNPCC is caused by germ-line mutations in at least four mismatch repair genes (MSH2, MLH1, PMS1, and PMS2) (Marra and Bolon, 1995). At-risk individuals have much to gain from genetic screening because frequent colonoscopic examination allows early detection and curative removal of pre-malignant polyps (Lynch *et al.,* 1993). Somatic mutation analysis of these genes and the other mismatch repair genes is also required to address the origin of the MI^+ phenotype in sporadic cancers. Even limiting the mutation analysis to the MSH2 and MLH1 genes still entails scanning 35 exons over 150kb of genomic DNA (Kolodner *et al.,*

1995). Approximately 70% of MSH2 mutations result in inactivation by premature stop codons, frameshifts, or alternative splicing. This type of alteration can be readily detected by the protein truncation test, which involves in vitro transcription and translation and subsequent analysis of protein products (Powell *et al.*, 1993). However, this technique cannot readily be applied to paraffin embedded tissues, which was the majority of material available in this study.

An alternative strategy for the identification of altered mismatch repair expression has been described which directly examines tumour specimens for the presence or absence of mismatch repair protein expression by immunohistochemistry (Thibodeau *et al.*, 1996). This investigation examined the expression pattern of MSH2 and MLH1 in a large series of patients with colorectal cancer (both HNPCC and sporadic colorectal cases). The results demonstrated that an absence of protein expression for both proteins was associated with the presence of MI^+. The technique therefore has the potential to be a rapid and convenient screen for identifying defects in mismatch repair genes.

In order to gain further insight into the nature of microsatellite instability in breast cancer, each of the DCIS cases was screened for expression of three MMR (MSH2. MLH1, and PMS2) proteins by immunohistochemistry. The immunohistochemical analysis of the DCIS cases was for MSH2 was performed with the same antibody clone used by Thibodeau *et al.*, (1996) to identify cases with MSH2 mutations. The immunohistochemical analysis described in the cases of DCIS was not carried out in the presence of a known mutant control. Fixation processes generally vary from institute to institute, and reactivity of particular epitopes, on tissue sections, is known to be affected by the type of fixation process used. Thus in order for a mutant control to be used effectively along side the DCIS cases, it would have to be processed in the same manner as the breast tissues. The antigen retrieval protocol used for this epitope has been shown previously to be effective for the study of nuclear antigens (Scott and Walker, in press), and is similar to the protocol used by Thibodeau *et al.*, (1996). The staining for MSH2 was present in nuclei of normal ducts and DCIS. Immunohistochemical analyses with the MLH1 and PMS2 antibody clones has not been described, and in general the staining was weaker for these antibodies when compared to MSH2. This may reflect that these antibodies are less well characterised and have been raised against unknown epitopes.

However, despite these shortcomings, all the MI^+ tumours in this study demonstrated expression of MSH2, MLH1 and PMS2, and there were no significant differences in staining

for MMR protein between cases showing MI^+ and those without alterations to microsatellites. It is important to note that the immunohistochemical detection of these proteins does not necessarily imply normal DNA mismatch repair function. However, it is also possible that aberrations to the other MMR genes are responsible for the observed MI^+.

The cases in this study that showed less widespread instability, that is alterations only at the DM-1 repeat, are unlikely to reflect aberrations to the MSH2 and MLH1 genes since HNPCC tumours with MMR gene mutations show widespread instability at all types of loci examined. Moreover, colorectal cell lines that contain mutations in MSH2 and MLH1, show very similar or identical DM-1 repeat lengths to that of mismatch proficient cell lines (Kramer *et al.,* 1996) suggesting that instability at this locus is unusual in HNPCC. Other candidate mutator loci need to be investigated in these breast cancers. Based on the enzymology of mismatch repair in yeast, inactivating mutations in the human homologue of MSH3 (hMSH3) might be predicted, since mutations in MSH3 do not cause the extreme microsatellite instability and spontaneous mutability observed in MSH2 mutants (Johnson *et al.,* 1996).

Does MI^+ 'effect' key cancer-associated loci (TGFβRII, IGFIIR, Bax, and E2F-4) in breast cancer ?

To date, mutations of the mononucleotide coding regions within the *TGFβRII*, *IGFIIR*, and *Bax* genes have been identified predominantly in MI^+ tumours of the gastrointestinal and urogenital tracts (Myeroff *et al.,* 1995; Ouyang *et al.,* 1997; Rampino *et al.,* 1997). In each gene a single nucleotide or in some cases two nucleotides are deleted or inserted at these mononucleotide regions, resulting in frameshifts, leading to introduction of premature stop codons, and consequently truncated proteins with impaired function. These types of alteration can be detected by PCR amplification and standard polyacrylamide gel electrophoresis. Each of the investigations, to date, has also limited the size of the PCR products so that material from paraffin embedded blocks can be readily analysed. Despite that fact that these alterations have been predominantly identified in MI^+ gastrointestinal and urogenital tumours, the genes also play important roles in breast tissue (as described below) and are therefore worthy of analysis.

TGFβRII

Loss of a growth inhibitory response to transforming growth factor β has been proposed to contribute to breast cancer progression, and TGFβ responsiveness often correlates with

TGFβRII expression in breast cancer (reviewed in Koli and Arteaga, 1997). There are several lines of evidence to suggest the TGFβRII encodes a tumour suppressor gene involved in breast cancer development. For example, transfection of a TGFβRII construct into MCF-7 breast cancer cell lines produces reversion of malignancy, as determined by soft agar assay and tumourigenicity in mice (Sun *et al.,* 1994). This suggests that *TGFβRII* could be a target for MI^+ breast tumours.

A tract of 10 adenines in exon 3 of the gene is mutated in up to 90% of MI^+ colon cancers and 70% of MI^+ gastrointestinal tumours. This can be readily analysed by PCR amplification of a 73bp region (Myeroff *et al.,* 1995). In the assay it was important to establish the wild-type banding pattern of this locus because the assay was optimised without a mutant positive control (for example DNA with 9 or 11 adenines at this region). Amplification across the region produced a 73bp product and a *Taq.* 'stutter' band migrating at 72bp. This is due to slippage by *Taq.* polymerase and is often associated with the amplification of longer repetitive sequences (Huage and Litt, 1993). Direct DNA sequence analysis of the products confirmed the presence of 10 consecutive adenines only. Although there was no deviation from this allelic pattern in any of the tumour samples analysed, it is possible that a homozygous or heterozygous alteration, leading to the loss of a single adenine, may have been 'masked' by the stutter band. An alternative strategy may have been to electrophorese the products on non-denaturing gels. An alteration of one base pair could produce a 'band shift' more easily identifiable than the extra stutter band that was seen on the denaturing polyacrylamide gel. However, resolution of base differences from PCR products less than 200bp in size by SSCP is sub-optimal (Orita *et al.,* 1989a, 1989b).

Vincent *et al.,* (1996) also failed to demonstrate mutations in 10 cases of breast cancer. This study used a different set of primers which amplified a 140bp product and did not co-amplify 'stutter bands'. However, the breast tumours investigated in this study were not characterised for MI. Studies have also failed to demonstrate alterations in MI^+ tumours of the lung, pancreas and endometrium, suggesting that this alteration may in fact be confined to tumours from the stomach and colorectum (Abe *et al.,* 1996).

IGFIIR

Chromosome 6q has been shown to be a frequent site for loss of heterozygosity in breast cancer (Devilee *et al.,* 1991; Chappell *et al.,* 1997). The *IGFIIR* maps to chromosome 6q26-27, and functions in the activation of TGFβ. LOH of the *IGFIIR* locus with accompanying

mutation of the remaining allele has been reported in liver tumours (De Souza *et al.*, 1995) and recently in two cases of high grade DCIS, suggesting that *IGFIIR* may be tumour suppressor gene important in the development of some breast cancers (Hankins *et al.*, 1996).

Frameshift mutations within a tract of eight guanines of this gene have been described in a high proportion of MI^+ gastrointestinal tumours (Souza *et al.*, 1996; Ouyang *et al.*, 1997) However, there was no evidence of alteration in the MI^+ cases identified. The assay was also conducted in the absence of mutant controls, however, there were no stutter products associated with amplification of this region, making detection of altered alleles more recognisable than in the *TGFβRII* assay.

Bax

In recent years, it has become clear that defective apoptosis contributes significantly to the origins and progression of breast cancer (Reed, 1996). Two groups have reported marked reductions in the expression of Bax in breast cancers. Bargou *et al.*, (1995) showed that Bax mRNA levels were reduced compared to normal breast tissue in 10 of 10 breast tumour specimens. In an extended analysis, the same investigators demonstrated reduced Bax mRNA levels in 35 of 36 breast carcinomas and in 4 of 4 in situ carcinomas (Bargou *et al.*, 1996). Krajewski *et al.*, (1995) have correlated reduced Bax expression (as determined by immunohistochemistry) with patients who did not respond to therapy and with shorter overall survival. The molecular mechanisms that result in reduced expression in breast cancer are not clear. Tumour suppressive qualities for this gene have also been suggested by a study that showed the growth of breast cancer cell lines, in immuno-compromised mice, can be suppressed by gene transfer-mediated overexpression of Bax (Bargou *et al.*, 1996).

The analysis at the guanine tract (G_8) in the *Bax* gene was similar to the *IGFIIR* analysis. The PCR product amplified a single product without any stutter bands, thereby facilitating simple identification of any alteration at this region. However, again there was no evidence to suggest that this region was altered in any of the MI^+ cases.

E2F-4

There is little direct evidence to suggest that this gene is involved in the development of breast cancer. However, to date it remains the only gene, with a trinucleotide repeat, which is associated with alterations in MI^+ tumours. Considering the high frequency of alterations in the DM-1 trinucleotide repeat this gene was considered to be a candidate worthy for analysis.

The gene contains a region of 13 consecutive CAGs encoding a poly-serine region involved in the transactivation activities associated with the gene. The region is known to be polymorphic in the general population, and therefore alterations at this region cannot be inferred directly. Also, because this repeated region is a trinucleotide, alterations in the number of repeats, will not result in the introduction of a premature stop codon into the gene. An alteration of 3bp was detected at this region in one case. However, the extra repeat was also present in the normal tissue of that patient suggesting that it was merely a polymorphism.

Overall the data suggested that each of the four previously defined targets for frameshift mutations do not appear to be selected for in the MI^+ breast tumours identified in this study. Wild-type alleles were observed in each of the mononucleotide regions, even for the case with alterations to the long mononucleotide markers. Similarly, *E2F-4* (trinucleotide) alterations were not present in the cases with changes in the DM-1 trinucleotide.

It is possible that there are other cancer-associated genes targeted in MI^+ breast cancers. A recent study has conducted an extensive search for other targets which may be altered in MI^+ gastrointestinal tumours (Simms *et al.,* 1997). The putative targets included coding and non-coding repeats of mono-, di-, and tri-, tetra-, and pentanucleotide repeats from other cancer associated loci involved in tumour suppression (*p53*), cell adhesion (β-*catenin*), and apoptosis (*Bcl-2*). However, each of the loci were stable in MI^+ gastrointestinal tumours. Among the other targets which remained stable was a combined trinucleotide and mononucleotide repeat within exon 1 of the oestrogen receptor gene. Considering the central role of the oestrogen receptor in the development and progression of breast cancer, this region may represent a particularly good candidate for future mutation analyses in MI^+ breast tumours.

Other genes

In order to gain further insight into the nature of microsatellite instability in breast cancer, each of the DCIS cases was screened for expression of the oncogene c-*erb*B-2 and the tumour suppressor gene p53. Since MI was associated with high nuclear grade, as is c-*erb*B-2 overexpression (Bobrow *et al.,* 1995) this may account for the association. A proportion of the tumours did display strong nuclear reactivity for p53, four of which showed alterations to DM-1. Immunohistochemically reactive p53 represents stabilised p53 which, in those case with greater reactivity is more likely to be due to a mutation (Gretasdottir *et al.,* 1996). This may indicate that DM-1 alterations lead to alteration of repetitive sequences within the p53

gene. An extensive review of p53 mutations by Greenblatt *et al.*, (1996), suggest that there are numerous targets for slippage-induced mutations within the gene.

DM-1 and DMPK ?

It is possible that the DM-1 specific instability, observed in cases of DCIS and early invasive carcinoma, indicates a role for the gene in which the repeat is located. The DM-1 repeat is located in a 3' region of the *DMPK* gene which is transcribed but not translated (Brook *et al.*, 1992). Expression studies for this gene have not been described in breast tissues. Alterations in the number of CTGn repeats may alter mRNA stability or the translational controls that normally function to regulate *DMPK*. This may be worthy of further investigation particularly as *DMPK* has been identified as an orthologue of the Drosophila *warts/lats* tumour suppressor gene (reviewed in Watson, 1995), and an association between defects in human mismatch repair genes and those of transcription coupled excision repair has recently been described (Mellon *et al.*, 1996).

3.12 Microsatellite Instability And Drug Resistance

The development of drug resistance is a major clinical problem. Doxorubicin is widely used as an anti cancer drug in breast cancer (Cummings *et al.*, 1991) but its clinical effectiveness is often limited by the emergence of drug-resistant tumour cell populations. The experiments described in this chapter provided a preliminary investigation as to the role of MI^+ in the resistance of breast cancer cell lines to doxorubicin.

The ovarian cancer cell line A2780/AD was isolated by in vitro selection for doxorubicin resistance of its parental A2780 cell line (Anthoney *et al.*, 1996). The doxorubicin resistant derived cell line has been shown to be MI^+ in contrast to the MI^- doxorubicin sensitive cell line (Anthoney *et al.*, 1996). The basis for the observed MI^+ was subsequently shown to be due an absence of the mismatch repair protein, MLH1 (Drummond *et al.*, 1996; Brown *et al.*, 1997).

The doxorubicin resistant breast cancer cell line (MCF-7/Adr) which is dervied from the MCF-7 cell line, was also demonstrated to be MI^+, as determined by 6 of 13 alterations at dinucleotide repeats (Anthoney *et al.*, 1996). However, the molecular basis for the microsatellite instability has not yet been described. The microsatellite profile of the MCF-7 and MCF-7/Adr cells confirmed that the doxorubicin derivative was MI^+ and that it was not limited to dinucleotide repeats, since alterations were observed at the BAT25 mononucleotide

and DM-1 trinucleotide repeats. However, the subsequent investigation of mismatch proteins (MLH1, MSH2 and PMS2) demonstrated no significant differences in expression between the MCF-7 and MCF-7/Adr cell lines by Western blotting. This may have been attributable to the culturing conditions. Prior to arrival in this laboratory the cells had been routinely maintained in the presence of doxorubicin (Gillian Hirst, personal communication). Subsequent passaging was performed in the absence of the drug. A recent study has suggested that in order to maintain a high level of resistance, MCF-7/Adr cells have to be cultured in the continuing presence of doxorubicin (Budworth *et al.,* 1997). It is therefore possible that the cultured cells contained a mixed population of cells, and that there were insufficient numbers of resistant clones to enable detection of microsatellite intability. In contrast, the presence of non-resistant cells prevented accurate determination of mismatch repair protein expression. An alternative strategy would be to establish independent subclones from single doxorubicin resistant cells.

Treating MCF-7 cells with increasing concentrations of doxorubicin (up to 300nM) did not result in any microsatellite alterations, as determined by analysis with the DM-1 marker. A recent investigation has performed cell survival analysis of MCF-7 and MCF-7/Adr cells and demonstrated that the doxorubicin derivative is 6,000-fold more resistant to doxorubicin than the MCF-7 cells (Ogretmen and Safa, 1997). This would suggest that the selection with increasing concentrations of drug may need to be over much longer periods of time (and with increasing concentrations of the drug) to determine whether emerging resistance is associated with the development of MI^{+} and concomitant decrease in MMR protein expression. Similar to the experimetns with the MCF-7/Adr cell line, an alternative strategy would be to repeat the analysis on cell populations initially derived from a single cell.

Chapter 4

Loss of Heterozygosity

4.1 The Tumour Suppressor Gene Concept

Somatic Cell Genetics

In 1969, Harris *et al.,* first proposed the existence of genetic elements which were capable of suppressing tumour proliferation. In their experiments, a cancerous cell, capable of forming tumours in animals, was fused with a non-tumourigenic cell. The hybrid cell line, that resulted from the fusion, grew in culture and maintained many of the properties of cancer cells, however, it was no longer capable of producing tumours in animals. During the course of these studies and others (reviewed by Stanbridge *et al.,* 1992), it was observed that an occasional hybrid cell line would produce a tumour in an animal. Analysis of these rare clones, demonstrated that they had lost one or more chromosomes that originated from the normal parent cells. This suggested that the normal cells had contributed one or more genes whose product suppressed the ability to form tumours in animals. The chromosomes that contained these putative tumour suppressor genes were identified using microcells, which carry only a single chromosome, derived from the normal parent and then fused with a tumourigenic cell.

The main conclusion to be drawn from these studies is that tumour suppressor genes loose their functions in cancer cells, which can be restored by a single dominant wild-type allele, and so both alleles of a tumour suppressor gene must be lost by mutation in cancerous cells. Karyotypic analysis of tumours has supported this notion. Different types of cancerous cells have been shown to contain loss of complete chromosomes or deletions of specific portions of chromosomes (reviewed in Mitelman *et al.,* 1997).

Retinoblastoma And The 'Two-Hit Hypothesis'

Epidemiological studies (Knudson, 1971) of the childhood cancer, retinoblastoma, provided further evidence for the role of tumour suppressor genes in cancer. Knudson noted that 40% of the cases of retinoblastoma occurred in young infants with a mean age of 14 months, and these tumours were almost always bilateral with an average of three independent tumours per patient. In some of these cases there was a family history of tumours, suggesting an inherited predisposition for these cancers. By contrast, Knudson noted, about 60% of the retinoblastomas did not fit this pattern. In these cases, there was no familial component. The tumours in these patients were some what different than the first group. They were detected

at a later age, and originated only in one eye, with only one cancer per patient. This class of retinoblastoma was also quite rare, occurring in about one in 30,000 people.

Knudson developed a hypothesis (the so-called 'two-hit' hypothesis) to explain the origins of both categories of retinoblastoma. He suggested that some children inherited a mutant allele in a gene (subsequently designated *RB1*) and that a second somatic mutation in a retinoblast cell would then give rise to this cancer. He also hypothesised that the children who developed retinoblastoma at a later age do not inherit a mutant *RB1* allele. Instead, two independent mutations in a single retinoblast must occur to give rise to late-onset tumours, which are rare. The first group have one inherited mutant allele and one somatic mutation, while the second group has two somatic mutations, which, because they are the same genetic loci as in the first group, cause the same disease.

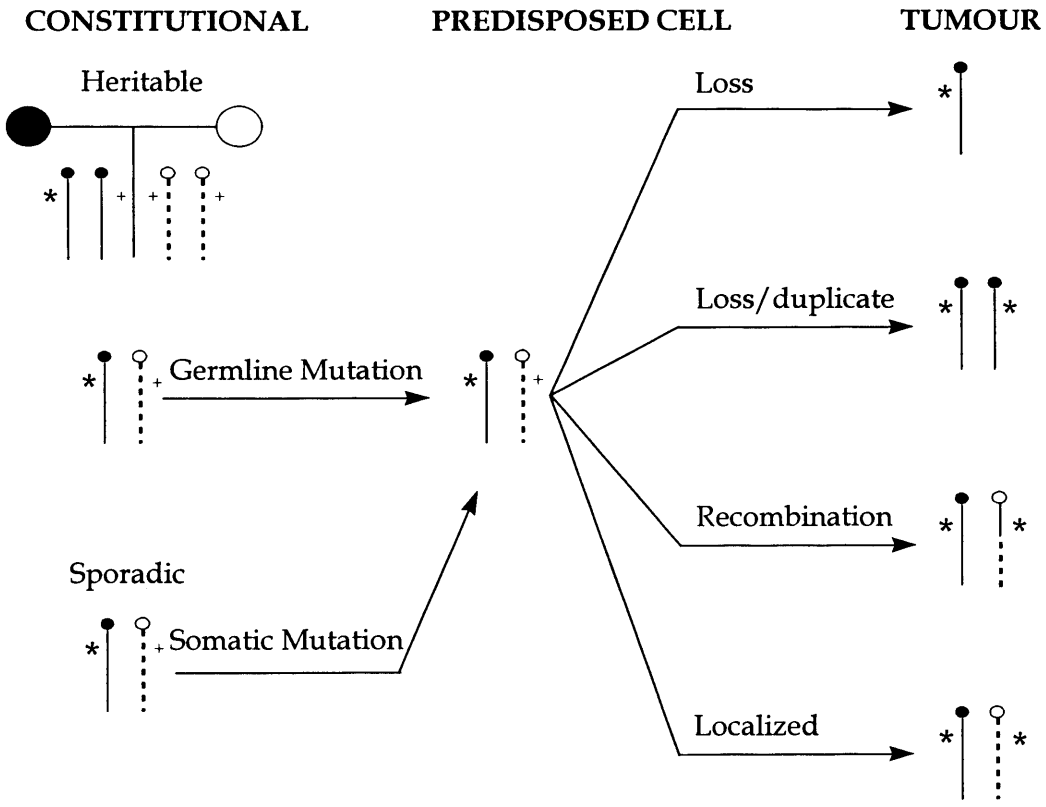
Some of the familial retinoblastoma were reproducibly shown to contain small deletions in chromosome 13q14 (Yunis and Ramsey, 1978), suggesting a locus for the *RB1* gene. Several DNA probes spanning the chromosome 13q14 region were shown to detect natural polymorphisms of human DNA, restriction fragment polymorphisms (RFLPs). Interestingly, some of these probes showed a reduction to homozygosity in the cancerous tissue, that is while the normal tissue contained two alleles the tumour tissue only contained one of these alleles (Cavenee *et al.,* 1983). This could arise from a loss of a chromosome, a deletion of the genetic locus, or a gene conversion where one allele is copied (via replication or recombination) and inserted into the other allele (Figure 4.1).

The reduction to homozygosity or loss of heterozygosity (LOH) at 13q14 was observed in both the inherited and 'sporadic' forms of retinoblastoma. The *RB1* gene, mapped to chromosome 13q14, was then isolated (Friend *et al.,* 1986), therefore permitting a test of Knudson's hypothesis. It was subsequently demonstrated that *RB1* gene from retinoblastoma carried mutations in both alleles, or in one allele with the second lost by LOH (Weinberg, 1992).

The genetic mechanisms for *RB1* were subsequently demonstrated in other tumour suppressor genes, e.g. *APC* (colorectal cancer), *NF2* (Neurofibromatosis type 2), *VHL* (Von Hippel-Lindau disease) and *p53* (breast cancer). Therefore, the finding of consistent LOH, at a chromosomal region, in any malignancy could signify the presence of a nearby tumour suppressor locus. This has led to investigators comparing the constitutional genotype of cancer patients with that of their tumours, using a series of polymorphic markers, to

determine allelic losses in tumour DNA as compared with normal tissue from the same patient (Lasko *et al.*, 1991). LOH analysis for deletion is therefore analogous to cytogenetic analysis, with the advantages of enhanced resolution.

Figure 4.1. Mechanism of loss of heterozygosity (Lasko et al., 1991).
*A general model for mechanism eliciting loss of heterozygosity. Upper left: In heritable disease, a recessive defect (labelled *) at the tumour locus is inherited, giving a genotype */+ in all cells. The predisposition is unmasked by elimination of the wild-type alleles, usually by one or the chromosomal mechanisms described in the text, followed by other alterations during malignant progression. Lower left: In sporadic disease, somatic mutation at the tumour locus results in formation of a predisposed precursor cell. Predisposition is unmasked in mechanistically similar ways as in heritable disease.*



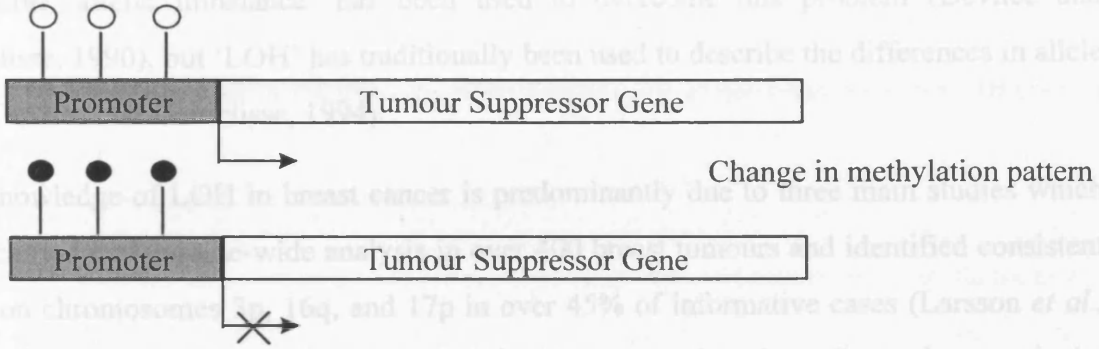
De novo Methylation

Alternative mechanisms of tumour suppressor gene inactivation exist. The most common mechanism is aberrant methylation of promoter regions of tumour suppressor genes. It is now emerging that alterations in the DNA methylation machinery are among the most common changes associated with neoplasia and may have a causative role at an early stage in carcinogenesis (Jones and Gonzalgo, 1997). In higher order eukaryotes, DNA is methylated only at cytosine residues located 5' to guanosine in the CG dinucleotide, giving rise to 5-methyl-cytosine (Bird, 1986). In humans, approximately 60% of genes contain CG-rich regions of DNA in their promoters, which function in gene regulation by binding specific transcription factors. These 'CpG islands' are normally devoid of DNA methylation, regardless of the expression status of the gene (reviewed in Jones, 1996). When present, DNA methylation of promoter-associated CpG islands leads to irreversible inhibition of gene expression, as demonstrated in X chromosome inactivation in women (Pfeiffer *et al.,* 1990), and in the transcriptionally silent copy of parentally imprinted genes (Sasaki *et al.,* 1992; Ferguson-Smith *et al.,* 1993; Stoger *et al.,* 1993). It is believed that methylation of these residues leads to gene silencing through heterochromatisation (Jones, 1996).

In recent years, inactivation of at least five tumour suppressor genes has been demonstrated to be due to de novo promoter methylation in both familial and sporadic cases of cancer. These include *RB1* in 15% of sporadic Retinoblastoma, (Sakai *et al.,* 1991), *VHL* in 20% of sporadic renal tumours (Herman *et al.,* 1994), and most recently p16 in a variety of sporadic human tumours (Merlo *et al.,* 1995, Herman *et al.,* 1995, Gonzalez-Zulueta, *et al.,* 1995). These studies have also shown that inactivation of these genes can occur through LOH of one allele coupled with methylation of the remaining allele. Cell culture studies have also demonstrated that E-cadherin and the oestrogen receptor genes are also transcriptionally silenced by *de novo* methylation in breast cancer cell lines (reviewed in Jones, 1996). There is also recent evidence demonstrating that methylation of the *BRCA1* promoter region occurs in sporadic breast cancer (Dobrovic and Simpfendofer, 1997).

Hypermethylation of a gene promoter region is depicted in Figure 4.2. As can be seen this *de novo* methylation results in gene silencing.

Figure 4.2. Abnormal silencing of tumour suppressor genes (Jones and Gonzalgo, 1997). Circles represent CpG dinucleotides; open circles represent unmethylated cytosines; solid circles represent methylated cytosines.



4.2 LOH In Breast Cancer - The Role Of Chromosome 16q

The polymorphic DNA markers used to analyse tumour and normal pairs can reveal any imbalance in parental alleles, that is they can detect loss of an allele as well as a gain in allele copy number. This suggests that not all differences in allele intensity are due to LOH. The term 'allelic imbalance' has been used to overcome this problem (Devilee and Cornelisse, 1990), but 'LOH' has traditionally been used to describe the differences in allele loss (Devilee and Cornelisse, 1994).

Our knowledge of LOH in breast cancer is predominantly due to three main studies which have carried out genome-wide analysis in over 400 breast tumours and identified consistent LOH on chromosomes 3p, 16q, and 17p in over 45% of informative cases (Larsson *et al.*, 1990; Sato *et al.*, 1990; Devilee *et al.*, 1991). Other researchers have focused on particular areas of specific chromosomes in which key regions of LOH have been identified (summarised in Table 1.9). The cumulative evidence highlights the complexity of chromosome involvement in breast cancer, given that half the cases studied showed LOH at more than 2 different chromosomes (Devilee *et al.*, 1994).

LOH on the long arm of chromosome 16 has been postulated to play an early role in the development of breast cancer. Evidence for this comes from two main types of study, firstly cytogenetic data of tumours with few chromosomal aberrations, and secondly molecular analysis of 'early' breast cancer lesions, for example non-malignant lesions and in situ carcinomas.

Cytogenetic Evidence

In 1984, Rodgers *et al.*, described the first detailed cytogenetic study on direct preparations from primary breast carcinomas. The chromosome analysis was made from cell suspensions of the tumour material without any preceding cell culture, thereby excluding the secondary chromosome changes associated with prolonged cell culture. Cytogenetic banding techniques revealed extremely complex chromosome abnormalities in a high proportion of cases. The only chromosome feature common to all of the breast tumours was the presence of extra chromosomal material from 1q. However, monosomy or partial monosomy of chromosome 16 was observed in 6 of the 9 cases, including two cases with the simplest alterations. This was the first evidence that loss of regions of chromosome 16 could have a role in breast cancer.

In an attempt to differentiate primary chromosome abnormalities from secondary changes Hainsworth *et al.*, (1991) focused on tumours with diploid range karyotypes (32-57 chromosomes per cell). Of 4 such tumours, 3 displayed translocations with a 16q22 breakpoint. This was the first study to suggest that the 16q 21-24 segment might be of relevance for the initiation of malignant transformation in breast cancer. Among the non-diploid tumours in this study (which represent a later stage of tumour evolution) only one had a non-random rearrangement of chromosome 16 suggesting that the 16q21-24, important in early stages of breast carcinogenesis, may become submerged among the other abnormalities associated with tumour progression.

Analysis of another 20 cases of breast cancer with near-diploid karyotypes (Dutrillaux *et al.*, 1990) also demonstrated that rearrangements of chromosome 16, which principally led to loss of 16q, were frequent.

Molecular Studies

In the near diploid tumours described above, almost all deletions and losses should lead to the loss of one of the two parental chromosomes or chromosome segments and therefore to loss of heterozygosity. In the late 1980s, molecular approaches were initiated to investigate the areas of loss uncovered by cytogenetic analysis. The polymorphic sites used to study LOH were RFLPs. As previously described in Chapter 1 (Section 1.5), this technique required large amounts of good quality DNA, thereby confining investigations to the larger invasive carcinomas. The advent of the Polymerase Chain Reaction (PCR) enabled smaller quantities of starting material to be used. Moreover, DNA prepared from archival specimens of fixed tissue proved to be suitable template for the PCR. The molecular studies of chromosome 16q losses are described below.

Analysis Of 'Early' Lesions'

Microdissection techniques designed to enrich samples for tumour cells, have recently been adapted to analyse lesions of non malignant and preinvasive disease. LOH on chromosome 16q has now been investigated in cases of DCIS, LCIS and atypical ductal hyperplasia. Stratton *et al.*, (1995) demonstrated LOH at a marker (D16S413) located at 16q24 in 40% of microdissected DCIS samples. Using the same microsatellite marker, Lakhani *et al.*, (1995a) identified 20% of 10 cases of pure LCIS cases with LOH. The same group demonstrated

LOH at D16S413 in 5 of 9 microdissected samples of atypical ductal hyperplasia (Lakhani *et al.*, 1995b).

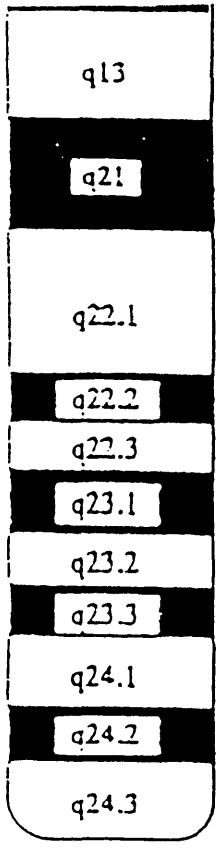
These studies complemented the existing cytogenetic evidence, and also lend further support to the theory that the putative tumour suppressor gene(s) residing on chromosome 16q are important in the early stages of breast carcinogenesis.

Distinct Regions Of LOH On Chromosome 16q

In an effort to clone the putative tumour suppressor gene(s) on chromosome 16q, a number of groups have tried to localise, more accurately, the region commonly deleted on this chromosome arm (Figure 4.3). This was facilitated by the refinement of physical and genetic chromosome maps using a large number of novel polymorphic markers (Kozman *et al.*, 1993).

Tsuda *et al.*, (1994), detected a high frequency of LOH (49%) on 16q in predominantly invasive carcinomas. Of the tumours which showed LOH, 98% demonstrated loss in the 16q24.2 - qter region. Cleton-Jansen *et al.*, (1994) studied 79 invasive tumours using 20 polymorphic markers mapping to 16q. Only samples which contained greater than 50% tumours cells where analysed. Two regions involved in allelic loss on 16q were observed. One region at q24.3 and a second at q22.1. Dorion-Bonnet *et al.*, (1995), analysed 45 breast tumours with 11 microsatellites, and also found a high frequency of LOH at q22.1 region. The most recent study of LOH on 16q (analysing 210 breast carcinomas) has reiterated the presence of the q24.3 deleted region (Iida *et al.*, 1997). However, the q22 region of deletion identified by Iida *et al.*, (1997), does not correspond to the site defined by Cleton-Jansen *et al.*, (1994).

The only study to date to focus entirely on 16q in DCIS (Chen *et al.*, 1996) confirmed the presence of the two commonly deleted regions, similar to the invasive carcinoma studies. However, the highest frequency of LOH (77%) was present in a region between q23.1 and q24.1, a locus not previously reported.



Marker		Cleton-Jansen *et al.*, 1994	Skirnisdottir *et al.*, 1995	Chen *et al.*, 1996
D16S400				64% (7/11)
	P-cadherin			
	E-cadherin			
D16S398		29% (16/56)		46% (13/28)
D16S421			58% (35/60)	55% (12/22)
D16S260				50% (8/16)
D16S289		37% (21/57)		
D16S422				48% (10/21)
D16S402			45% (45/97)	74% (14/19)
D16S413			46% (53/116)	37% (10/27)
	H-cadherin			

Figure 4.3. Chromosome 16q and LOH in breast cancer.

Representative studies which have analysed LOH in breast carcinomas with the markers used in this thesis (emboldened). Each marker is positioned beside its corresponding cytogenetic location. Percentages refer to the LOH at each particular marker for informative cases in brackets. Candidate tumour suppressor genes investigated in this thesis are also positioned on the map.

4.3 Candidate Tumour Suppressor Genes On Chromosome 16q

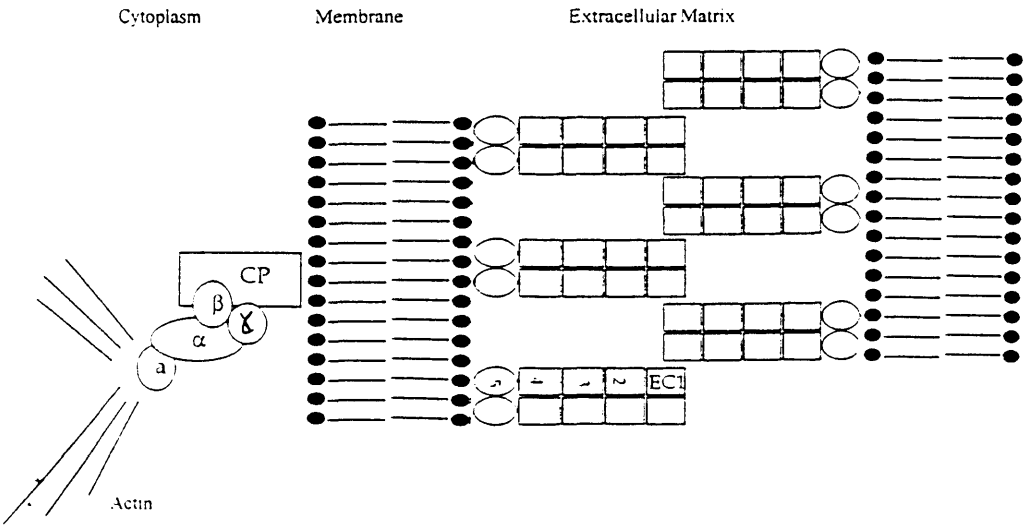
Chromosome 16q22.1

E-Cadherin

Cadherins are calcium-dependent transmembrane glycoproteins which mediate cell-cell adhesion by binding to cadherin molecules of the same type on adjacent cells. E-cadherin is a 120kD transmembrane glycoprotein that mediates the adhesion of epithelial cells. The cytoplasmic portion of the molecule is linked via the catenins to the actin cytoskeleton (Cowin *et al.*, 1994), whereas the extracellular domain is involved in a molecular zipper mediating cell-cell adhesion (Shapiro *et al.*, 1995), illustrated in Figure 4.4.

A role for E-cadherin as a potential tumour suppressor protein was proposed following experiments which demonstrated that inhibition of E-cadherin function by specific antibodies or antisense mRNA, resulted in changes to cellular growth pattern with the acquisition of invasive potential in vitro (Behrens *et al.*, 1989). In contrast, the invasive potential was suppressed when cell lines were transfected with E-cadherin cDNA (Nagafuchi *et al.*, 1987). An in vitro model has shown that a cell without E-cadherin, or with dysfunctioning E-cadherin, is easily washed off a main tumour mass by lymphatic or venous shear forces (Byers *et al.*, 1995).

Figure 4.4. Interaction of E-cadherin molecules (Adapted from Jiang, 1996)
Extracellular domains (EC1-5) involved in homophilic binding of E-cadherin, and cytoplasmic portion (CP) interacting with α, β, and γ catenins and actin filaments (a).



Expression Of E-cadherin In Breast Cancer

Immunohistochemical studies have demonstrated that the expression of E-cadherin is partially or totally lost in various human epithelial tumours (Birchmeier and Behrens, 1994). In breast carcinoma, reduced expression of E-cadherin is associated with invasiveness and loss of differentiation characteristics. Immunohistological studies have shown that approximately 50% of infiltrating ductal carcinomas show reduced E-cadherin expression, whereas reduced or negative expression is a uniform finding in infiltrating lobular carcinomas (Moll *et al.,* 1993; Oka *et al.,* 1993; Gamallo *et al.,* 1993; Palacios *et al.,* 1995; Siitonen *et al.*, 1996; Jones *et al.*, 1996).

Gupta, S.K., *et al.,* (1997) examined E-cadherin expression semi-quantitatively in 96 cases of pure DCIS, using an intensity distribution method. They demonstrated that well differentiated DCIS had significantly higher expression of E-cadherin than poorly differentiated tumours. This study was the first to suggest that progressive loss of E-cadherin may occur at an early stage of breast carcinoma development.

Mutation Analysis Of E-cadherin.

The first investigation of E-cadherin mutation in breast tumours was conducted by Kanai *et al.,* (1994). Focusing on the cadherin domain encoded by exons 5-8, they identified two infiltrating lobular carcinomas (10%) with an identical missense mutation, which was likely to abolish the adhesive function of the molecule. A similar study of 25 infiltrating ductal carcinomas failed to identify mutations in exons 6 to 9 (Kashiwaba *et al.,* 1995). Berx *et al.,* (1995a) performed an extensive mutation screen, combined with LOH analysis and immunohistochemistry, in 49 breast tumours (predominantly infiltrating ductal carcinomas). They identified four truncation mutations in infiltrating lobular carcinomas out of seven. Each of the tumours demonstrated LOH at the 16q22.1 chromosomal band and lacked E-cadherin expression. None of the infiltrating ductal carcinomas in this study demonstrated mutations. The same group then reported mutation analysis of a larger series of infiltrating lobular carcinomas (Berx *et al.,* 1996). A further 23 (56%) of the infiltrating lobular carcinomas had mutations (predominantly nonsense mutations and frameshifts), the majority in combination with LOH of the wild-type locus. Consistent with these findings, Candidus *et al.,* (1996) also described truncation mutations in infiltrating lobular carcinomas. However, this study also identified a point mutation in a infiltrating ductal carcinoma.

De novo Methylation Of The E-cadherin Promoter

Accumulating evidence supports the hypothesis that *de novo* methylation (or hypermethylation) of CpG islands located in the 5' regulatory and proximal promoter regions causes transcriptional inactivation of tumour suppressor genes in human carcinomas (reviewed in Jones, 1996). GC-rich regions have recently been identified in the promoter region (Bussemakers *et al.*, 1994) and spanning intron 1 of the E-cadherin gene (Berx *et al.*, 1995a)

CpG methylation of the E-cadherin promoter has been demonstrated in many cancer cell lines (Yoshiura *et al.*, 1995), including breast cancer cell lines (Henning *et al.*, 1995; Graff *et al.*, 1995; Herman *et al.*, 1996). The study by Graff *et al.*, (1995) also examined primary tissue from breast tumours and normal breast samples and concluded that *de novo* methylation of the E-cadherin CpG island was tumour specific and not limited to cultured cancer cell lines. This study also demonstrated the direct involvement of *de novo* methylation in the suppression of E-cadherin gene expression. Treatment of E-cadherin negative cell lines with de-methylating reagents led to the re-activation of E-cadherin gene expression.

P-Cadherin

A cadherin first described in placental tissue (P-cadherin) has been shown to be present in the myoepithelium of normal breast (Shimoyama *et al.*, 1989). The P-cadherin gene has been localised 32kb upstream of the E-cadherin gene, on 16q22.1 (Bussemakers *et al.*, 1994), but the genomic organisation of this gene has not been described. Two studies have analysed the possible role of P-cadherin in breast carcinogenesis, using immunohistochemistry. Rasbridge *et al.*, (1993) studied expression in a series of benign biopsies, DCIS, and cases of infiltrating ductal and lobular carcinoma. Staining was not observed in normal luminal or hyperplastic breast epithelium, but there was strong reactivity of myoepithelial cells. Only one tumour (an infiltrating lobular carcinoma) was P-cadherin positive. The remaining carcinomas did not express P-cadherin, although staining was observed in myoepithelial cells surrounding foci of carcinoma in situ.

Palacios *et al.*, (1995) investigated a larger group of infiltrating carcinomas. In most of the carcinomas analysed, staining for P-cadherin was observed in myoepithelial cells around areas of carcinoma in situ, or normal breast adjacent to the carcinoma, similar to the report

of Rasbridge *et al.*, (1993). However, 20% of the infiltrating ductal carcinomas also expressed P-cadherin. These tumours tended to be of high nuclear grade and negative for hormone receptors, suggesting that P-cadherin expression may identify a subset of infiltrating ductal carcinoma with a particularly poor prognosis.

Chromosome 16q24.3-qter

H-Cadherin

A novel member of the cadherin family was recently identified by subtractive hybridisation using normal mammary epithelial cells and mammary tumour cell lines (Lee, 1996). H-cadherin has been mapped to chromosome 16q24 by FISH analysis. The gene contains many of the motifs associated with cadherins, including an extracellular region containing several cadherin domains and a transmembrane domain. The absence of a cytoplasmic domain suggests that H-cadherin has a different regulatory pathway from E-cadherin. Introduction of H-cadherin cDNA into established breast cancer cell lines resulted in markedly decreased cell growth, and loss of anchorage independent growth in soft agar assays. In vitro invasion assays suggested that loss of H-cadherin may lead to an invasive phenotype in cancer cells. Immunohistochemistry of a small number of paraffin embedded tissues revealed H-cadherin expression is restricted to normal mammary epithelial cells and non-cancerous benign breast tissue. It was not detected in situ or invasive breast carcinomas. To date, mutation analysis of H-cadherin has not been reported.

Aims Of This Chapter

The objectives of this part of the thesis were to determine if LOH of chromosome 16q occurs in cases of DCIS, mammographically detected impalpable early invasive carcinomas and tubular carcinomas by analysing microdissected tumour foci for allele loss in a panel of microsatellites: D16S400 (16q21), D16S289 (16q23.3), D16S402 (16q24.2), D16S3026 (16q24.3), and D16S413 (16q24.4). Cases which did demonstrate allele loss, could then be screened for alterations in candidate tumour suppressor genes by mutation analysis (E-cadherin) and by immunohistochemistry (E-cadherin, P-cadherin and H-cadherin).

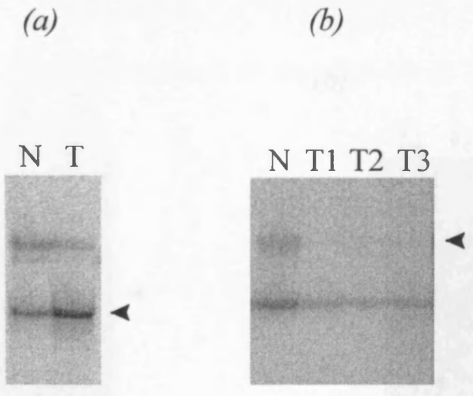
The relationship between promoter methylation and silencing of E-cadherin gene expression was also investigated by using breast cancer cell line assays to detect aberrant methylation.

4. 4 Microdissection

The 'allelic imbalance' data presented in this chapter was predominantly loss rather than gain. The microdissection technique, used in Chapter 3, confirmed the reduction in allelic intensity in tumour compared to normal DNA.

For example, Figure 4.5 demonstrates how microdissection helped distinguish allele loss from gain. DNA was prepared from a complete tissue section for early invasive Case No.57, and analysed in parallel with corresponding normal DNA, at the D16S400 marker on chromosome 16q21. (Figure 4.5a). As can be seen there is a slight reduction in allele intensity when the larger allele in the tumour DNA is compared with the same allele in the normal DNA. However, when comparing the smaller alleles, the tumour DNA shows an increase in allele intensity compared to that of the normal. This might suggest that this tumour is undergoing allelic gain rather than allelic loss (LOH). However, microdissection of individual tumour foci from this case, and subsequent reanalysis at the same marker (Figure 4.5b), revealed that the larger allele was completely lost, confirming LOH.

Figure 4.5. Analysis of allelic imbalance by microdissection.
Analysis of normal DNA (N), tumour DNA (T) and DNA template from three microdissected tumour foci (T1, T2, T3), from early invasive Case No.57, by PCR at the D16S400 marker, followed by electrophoresis on 6% denaturing gels and autoradiography.
(a) Gain in intensity of the smaller allele in tumour DNA (T), relative to the same allele in the normal DNA (N), as indicated by the arrow.
(b) Complete loss of the larger allele in the microdissected tumour foci (T1, T2, T3) compared with the same allele in the normal (N), as indicated by the arrow.



4.5 Loss of Heterozygosity Analysis

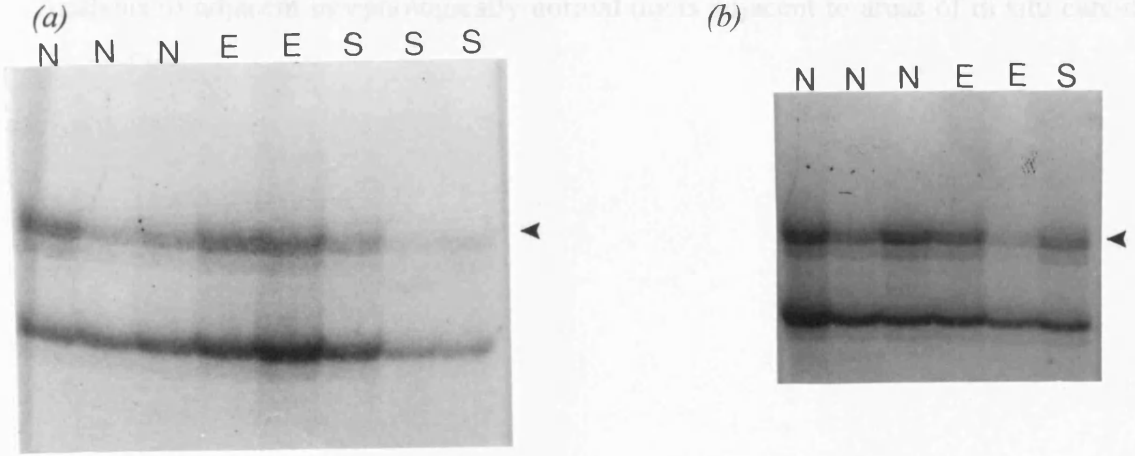
Fibroadenomas

In order to determine whether LOH on chromosome 16q was a genetic alteration associated with development of fibroadenomas, microdissected foci from 8 cases of fibroadenoma and one benign phyllodes tumour were analysed with the D16S400 marker. This marker was selected because it was highly informative, did not produce stutter bands which may mask allele loss, and it is located at q21 within a region of chromosome 16q which is know to undergo allelic loss in breast carcinomas.

In general, three distinct epithelial and three stromal components from each of the fibroadenomas and phyllodes tumour were separately microdissected and compared with normal ducts from adjacent surrounding tissue. For two of these cases, areas of ductal hyperplasia in the surrounding tissue were also examined.

Seven of the nine cases (78%) were heterozygous for the D16S400 marker. LOH was observed in 1 of 6 (17%) informative fibroadenomas, and the phyllodes tumour. However, LOH was not observed at all of the components. In Case No. 5 (phyllodes), LOH was observed in 2 of 3 stromal components but not in the epithelial components of the tumour (Figure 4.6a). In contrast, Case No.9 displayed LOH in 1 of 3 epithelial components but not in the stroma of this lesion (Figure 4.6b).

Figure 4.6. Loss of Heterozygosity analysis in fibroadenomas and a phyllodes tumour. Analysis of normal ducts (N), epithelial (E), and stromal components (S) by PCR, at the D16S400 marker, followed by electrophoresis on 6% denaturing gels and autoradiography. Arrows indicate alleles showing LOH. Case No. 5 (phyllodes) with LOH at S2 and S3, (b) Case No. 9, with LOH at E2.



DCIS

LOH analysis was conducted on 20 of the 23 DCIS cases which had previously been analysed for MI. LOH analysis was initially investigated using DNA prepared from whole tumour sections. However, using this approach, microsatellite alterations were not observed (data not shown). This was attributed to the masking of tumour specific alterations by the vast quantities of non-tumour cells present in the DCIS cases, as previously described for MI analysis (Chapter 3).

The analysis was repeated using DCIS samples which had been prepared by microdissection. In general, three individual non-contiguous foci from each case were compared with non-involved lymph node or surrounding breast tissue, using the D16S413 and D16S400 markers. The D16S400 marker was selected for the same reasons it was used in analysis of the fibroadenomas. The D16S413 marker was selected because it maps to 16q24.4, the second site of frequent deletion (as defined by Cleton-Jansen *et al.,* 1994).

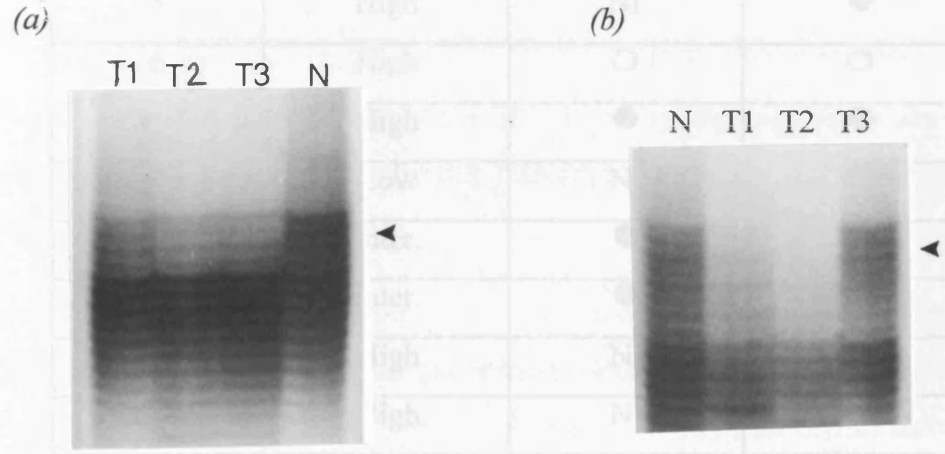
LOH at one of the markers was documented in 10 of 20 DCIS cases. Five cases showed LOH at both loci. The D16S400 marker showed LOH in 8 of 12 (67%) informative cases. Seven of 14 (50%) cases showed LOH at the D16S413 marker. Representative examples of cases with LOH are illustrated in Figure 4.7 (a) and (b).The pathological characteristics of the DCIS cases displaying LOH are summarised in Table 4.1. Four cases were of high nuclear grade, three of intermediate grade, and three of low grade. There was no association between presence of LOH and grade of DCIS.

Intratumoural heterogeneity was observed in one case (5%). This was categorised by the loss of different alleles (of the D16S400 marker) in distinct ducts, and is illustrated in Figure 4.7 (c).

Analysis of adjacent morphologically normal ducts adjacent to areas of in situ carcinoma was not performed.

Figure 4.7. Representative examples of LOH in DCIS.

Analysis of normal (N) and microdissected ducts (T1, T2, T3) by PCR at D16S413, followed by electrophoresis on 6% denaturing gels and autoradiography. (a) Case No. 16 showing LOH in all ducts, and (b) Case No. 5 with LOH in ducts T1 and T2, but with retention of heterozygosity in T3. Arrows indicate alleles showing LOH.



(c) Intratumoural heterogeneity in DCIS.

Analysis of normal (N) and microdissected ducts (T1, T2, T3), from Case No. 19 by PCR, at the D16S400 marker. The T1 shows reduction in intensity of the upper allele, whereas T3 shows reduction in intensity of lower allele, compared with the normal sample. Arrows indicate the heterozygous alleles in the normal sample.

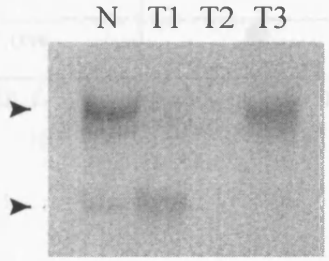


Table 4.1. Pathological and Loss of Heterozygosity data for DCIS cases

Case No.	Grade	D16S400	D16S413
1	High	❍	NI
2	High	●	●
3	Low	●	●
4	Low	NI	NI
5	High	NI	●
6	High	❍	❍
7	High	●	●
8	Low	NI	NI
9	Inter.	●	NI
10	Inter.	●	●
11	High	NI	❍
12	High	NI	NI
13	High	NI	❍
14	High	NI	NI
15	Inter.	●	●
16	High	NI	●
17	High	MI	MI
18	Low	❍	❍
19	Low	●	❍
20	Low	●	❍

❍ indicates allele retention, ● is LOH, and NI is non informative (homozygous), MI is microsatellite instability

Early Invasive Carcinomas

Loss of heterozygosity analysis in this group was initially performed on DNA prepared from whole tumour sections. The D16S400 (q21), D16S289 (q23.3), D16S402 (q24.2), D16S413 (q24.4) microsatellites were analysed in each of the 30 carcinomas and corresponding normal tissues from non-involved lymph nodes. Due to paucity of tissue the D16S3026 (q24.3) marker was only analysed in 10 of the cases.

This analysis identified 13 (43%) cases with allelic imbalance for at least one marker, two of which were interpreted as allelic gain rather than loss. For the cases which demonstrated loss, the reduction in intensity was never greater than approximately 50%, as determined by visual inspection.

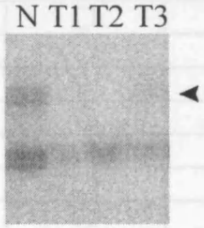
Individual foci from each of the cases were microdissected to confirm the initial result and to investigate the presence of intratumoural heterogeneity. All of the cases which demonstrated partial reduction in allele intensity were confirmed as allelic loss (LOH) using the template DNA prepared by microdissection. The cases with possible allelic gain were also were subsequently demonstrated to show LOH (as demonstrated in Section 4.5).

Alleic heterogeneity was not observed. In a small number of cases a weak normal allele was sometimes seen, for example, Figure 4.8a. This may reflect the presence of non-malignant cells within the microdissected invasive foci.

The highest frequency of LOH (50%) was observed at the D16S3026 marker at q24.3, however there were only 4 informative cases, two of which demonstrated LOH. The D16S400 marker at q22.1 showed 40% LOH. The frequency of LOH at the other q24 markers was approximately 35%, as summarised in Table 4.2. The LOH frequencies are summarised in Table 4.2, and the cumulative LOH analysis for each case is depicted in Table 4.3.

Figure 4.8. Loss of Heterozygosity analysis in invasive carcinomas.
Analysis of normal DNA (N), DNA template from three microdissected tumour foci (T1, T2, T3) by PCR followed by electrophoresis on 6% denaturing gels and autoradiography. Arrows indicate alleles showing LOH. (a) Case No. 21 at the D16S400 marker, (b) Case No.45 at the D16S413 marker.

(a)



(b)

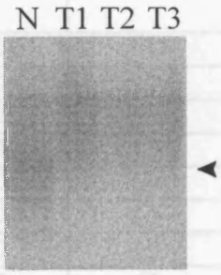


Table 4.2 Summary of Loss of Heterozygosity data for invasive carcinomas

Marker	Cases Analysed	Informative Cases	LOH Cases
D16S400	30	20/30 (67%)	8/20 (40%)
D16S289	27	23/27 (85%)	4/25 (16%)
D16S402	20	15/20 (75%)	7/20 (35%)
D16S3026	10	4/10 (40%)	2/4 (50%)
D16S413	29	25/29 (86%)	9/25 (36%)

Table 4.3. Loss of Heterozygosity data in early invasive carcinomas

Case No.	D16S400	D16S289	D16S402	D16S3026	D16S413
1	❍	❍	ND	ND	ND
3	❍	❍	❍	ND	❍
5	MI	MI	MI	ND	MI
7	❍	❍	❍	ND	❍
9	❍	❍	❍	ND	❍
11	❍	❍	❍	ND	❍
13	❍	❍	❍	ND	❍
15	❍	❍	❍	ND	❍
17	❍	NI	●	ND	❍
19	●	❍	ND	NI	●
21	●	●	NI	NI	●
23	NI	❍	●	NI	●
25	❍	ND	ND	ND	❍
27	NI	❍	NI	ND	❍
29	❍	❍	ND	ND	❍
31	❍	❍	❍	ND	❍
33	●	❍	●	●	❍
35	NI	ND	❍	ND	❍
37	NI	❍	ND	NI	●
39	●	❍	ND	NI	●
41	NI	❍	●	ND	❍
43	●	❍	❍	ND	●
45	●	●	●	❍	❍
47	NI	❍	ND	ND	●
49	●	NI	●	❍	●
51	❍	❍	ND	ND	NI
53	NI	●	●	●	●
55	MI	NI	MI	ND	MI
57	●	●	ND	ND	●
59	●	ND	ND	❍	●

❍ indicates retention, ● is loss of heterozygosity, NI is non-informative, ND is not determined, MI is microsatellite instability,

Tubular Carcinomas

In general, three distinct tubular components from each of the 13 tubular carcinomas were separately microdissected and compared with non-involved lymph node from the same patient. For two of the cases, foci of DCIS were microdissected and examined. Another case contained an area of LCIS which was also examined for LOH. The D16S413 and D16S289 markers were analysed in each of the foci.

The D16S289 marker showed LOH in 4 of 10 (40%) informative cases. Five of 9 (55%) cases showed LOH at the D16S413 marker (Table 4.4). LOH was not observed in any of the in situ foci associated with tubular carcinomas, and all invasive foci showed loss of the same allele, thereby ruling out allelic heterogeneity (Figure 4.9).

Table 4.4. Loss of Heterozygosity in tubular carcinomas and associated areas of in situ carcinoma.

Case No.	D16S289	D16S413
1	●	●
2	❍	❍
2 (DCIS)	❍	❍
3	●	●
4	●	●
5	NI	❍
5 (DCIS)	NI	❍
6	❍	NI
7	●	●
8	NI	❍
9	❍	●
10	❍	❍
11	❍	NI
12	NI	NI
13	❍	NI
13 (LCIS)	❍	MI

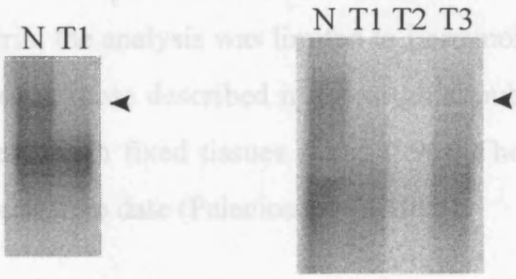
Figure 4.9. Loss of Heterozygosity in tubular carcinomas.

Analysis of normal (N), and microdissected tubular foci (T1, T2, T3), by PCR, followed by electrophoresis on 6% denaturing gels and autoradiography. Arrow indicates allele showing LOH

(a) Analysis of the D16S289 marker, in Case No.1 with LOH at T1

(b) Analysis of the D16S413 marker, in Case No. 9 with LOH in the foci T1 and T3 foci.

(a) (b)



4.6 Analysis Of Candidate Tumour Suppressor Genes

The experimental strategy adopted was to analyse each of the candidate genes, by a combination of approaches, in the tumours which demonstrated LOH at one or more loci on chromosome 16q. The analysis was confined to the early invasive carcinomas and the DCIS cases.

The approaches adopted depended on how well the gene had been characterised. For E-cadherin, the genomic structure of the intron-exon borders was available, thereby facilitating mutation screening and DNA sequencing of exons encoding key regions of the gene.

For H-cadherin and P-cadherin, the analysis was limited to immunohistochemistry. At present the only antibodies available are those described in the original publications. The H-cadherin clone has been shown to react with fixed tissues (Lee, 1996). The P-cadherin antibody has only been tested on frozen section to date (Palacios *et al.*, 1995).

For the majority of cases studied the paraffin embedded tissue blocks represented the only source of material available for analysis. '

E-cadherin Mutation Analysis

LOH analysis identified 13 early invasive carcinomas and 9 DCIS cases with LOH at one or more markers on chromosome 16q. Microdissected foci from 9 of the 13 invasive carcinomas, and 8 of 9 of the DCIS cases, were further analysed for mutations in exons 5 - 9 of the E-cadherin gene. For the remaining cases there was insufficient tissue to perform SSCP analysis, and these cases were sequenced directly (as described later in this section). The optimum SSCP conditions for each exon were identified by electrophoresing PCR products from mutant controls (kindly provided by Dr. Anne-Marie Cleton-Jansen and detailed in Table 2.3) on different concentrations of non-denaturing (MDE) gels.

Analysis of the early invasive and DCIS cases did not reveal any mobility shifts in the presence of known controls. Representative examples of SSCP analysis and subsequent DNA sequence analysis are illustrated in Figure 4.10.

DNA sequence analysis was performed on the cases which were excluded from SSCP analysis because of paucity of tissue. Case No.57 from the early invasive group showed extra bands at exon 7, the strongest of which is an adenine at amino acid position 857 of the gene. No other tissue was available to repeat the analysis in order to confirm this alteration. Analysis of the

corresponding normal DNA for this case, showed wild-type sequence suggesting that the putative alteration was a tumour-specific change. A number of the controls used in the SSCP analysis were also sequenced to confirm the observed mobility shifts. Representative examples of DNA sequencing are illustrated in Figure 4.11.

In light of the publications describing a high incidence of E-cadherin mutations in infiltrating lobular carcinomas, 10 infiltrating lobular carcinomas were selected from the files and analysed at two exons (7 and 8). Each of these cases had previously been shown to be negative for E-cadherin protein expression, as determined by immunohistochemistry (Jones J. L *et al.,* 1996). However, no alterations were observed at the exons studied during SSCP analysis (data not shown).

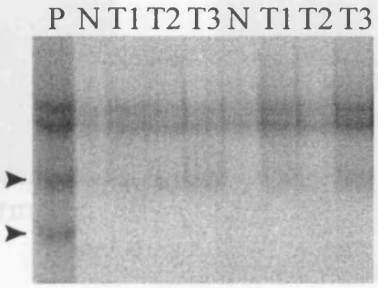
Figure 4.10. SSCP analysis in early invasive carcinomas and DCIS cases.

Analysis of normal (N), microdissected tumour foci (T1, T2, T3), and mutant controls (P), by PCR, followed by electrophoresis on 0.6xMDE non-denaturing gels and autoradiography. Arrows indicate altered mobility shifts.

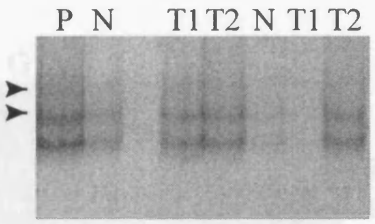
(a) Exon 6 analysis of early invasive carcinoma Case Nos. 21 and 33, and positive control (BT1004 deletion of 17bp).

(b) Exon 7 analysis of DCIS Case Nos. 7 and 10, and positive control (BT1172 insertion of 5bp).

(c) Exon 9 analysis of early invasive carcinoma Case Nos. 49 and 53, and positive control (BT995A deletion of 1bp).



(a)



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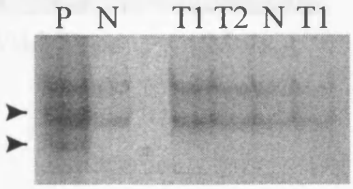


(c)

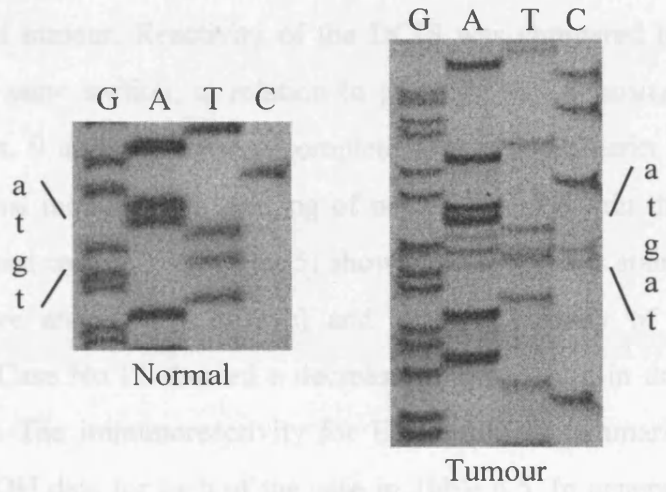
Figure 4.11. DNA sequence analysis of E-cadherin gene.

Sequence analysis of PCR products, followed by electrophoresis on 6% denaturing gels and autoradiography.

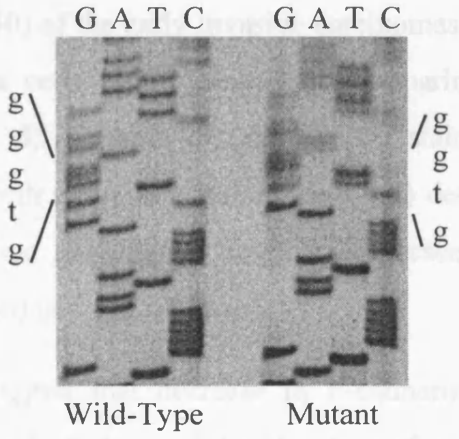
(a) Exon 7 sequence analysis of early invasive Case No. 57 with possible 1bp insertion restricted to tumour DNA.

(b) Exon 9 sequence analysis of BT995A with 1bp deletion, and wild-type sequence from early invasive Case No. 21.

(a)



(b)



E-cadherin Immunohistochemistry

E-cadherin protein expression was examined by immunohistochemistry. The pre-treatment protocol for E-cadherin immunohistochemical analysis had previously been optimised by Sheila Dearing. All of the early invasive carcinomas (carried out by Sheila Dearing) and cases of DCIS were examined for E-cadherin expression.

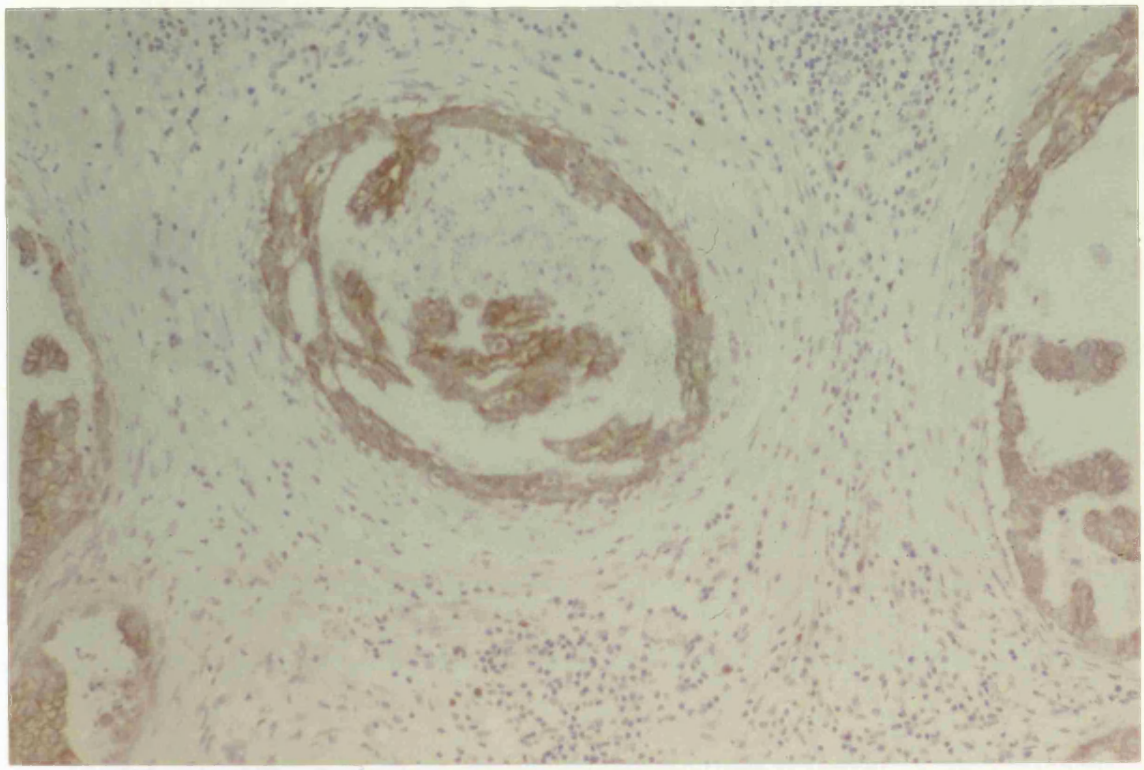
Evaluation of protein expression was confirmed by Dr. R. A. Walker. No reactivity was observed in 5 of 23 DCIS cases, and maybe associated with fixation problems, since this was for both normal and tumour. Reactivity of the DCIS was compared to that seen in normal breast tissue in the same section, in relation to presence and intensity (Figure 4.12a). Two cases of DCIS (Nos. 9 and 13) showed complete loss of E-cadherin reactivity in involved ducts, but had normal membranous staining of normal ducts within the same tissue section (Figure 4.12b). A third case of DCIS (No. 5) showed heterogenous staining of involved ducts (some ducts positive and some negative) and strong reactivity of adjacent normal and hyperplastic ducts. Case No.14 showed a decrease of E-cadherin in ducts, in approximately 50% of the section. The immunoreactivity for E-caderhin is summarised in relation to the chromosome 16q LOH data for each of the case in Table 4.5. In general, LOH does correlate with a decrease or absence of E-cadherin protein reactivity. Case Nos. 9 and 14 were non-informative for both of the markers. However, Case Nos. 13 and 5 both demonstrated LOH at the D16S400 and D16S413 markers.

A higher proportion (11 of 30) of the early invasive carcinomas failed to show any reactivity. Reactivity of the carcinoma cells was evaluated by comparing foci throughout the tissue section. Three cases (Nos. 3, 27, and 29) showed no staining in the carcinoma cells. Comparison of LOH data with expression data (Table 4.6) demonstrated that each of these cases retained all chromosome 16q markers tested. A representative example of E-cadherin immunoreactivity is illustrated in Figure 4.12 (c).

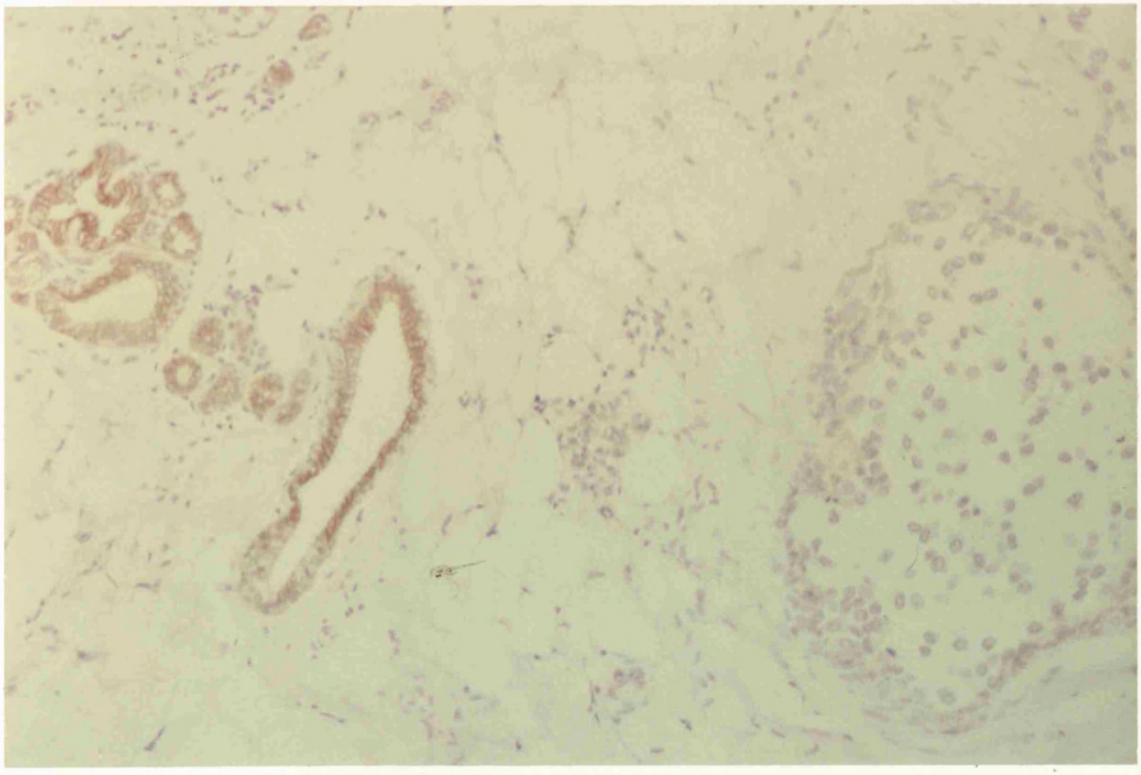
Taken together the data suggest that decrease in E-cadherin protein expression may be occurring via a mechanism which does not involve loss of regions of chromosome 16q, as detailed in Section 4.8.

Figure 4.12. E-cadherin immunohistochemistry.

(a) DCIS case No. 3. Normal breast, hyperplastic and DCIS showing E-cadherin membrane reactivity. This case retained heterozygosity at the D16S400 and D16S413 markers.



(b) DCIS case No.5. Normal breast showing E-cadherin membrane staining, whereas adjacent DCIS does not show labelling. This case demonstrated LOH at D16S413 marker, but was non-informative for the D16S400 marker.



(c) Early invasive Case No. 33. Normal breast (lower left hand side) showing labelling for E-cadherin. Adjacent tumour cells also show labelling but more variable. This case demonstrated LOH at the D16S400 and D16S402 markers.

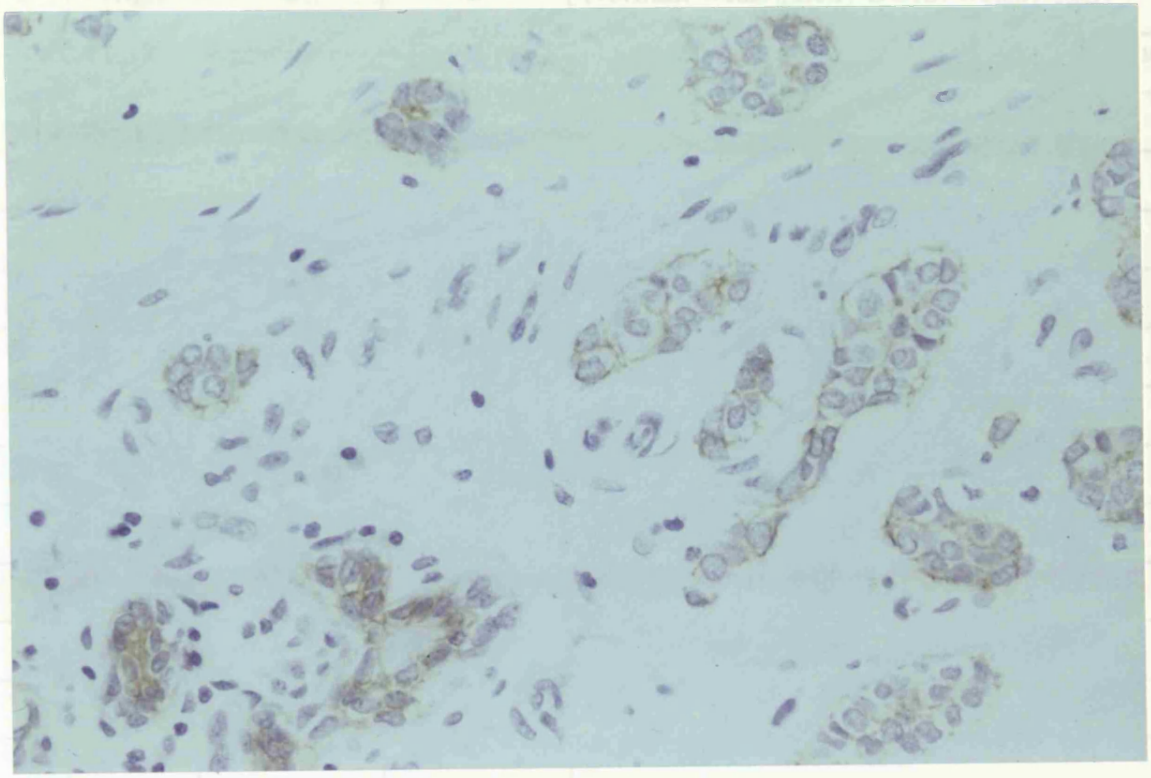


Table 4.5. LOH and E-cadherin immunohistochemical data in DCIS cases.

No.	Grade	D16S400	D16S413	E-cadherin immunohistochemical data
1	High	❍	NI	Normal: pos. DCIS: pos (cyto and memb)
2	High	●	●	Normal: weak/patchy DCIS: weak/patchy
3	Low	●	●	Normal/Hyperplasia: strong memb. DCIS: het+/-
4	Low	NI	NI	Normal: weak, DCIS: weak
5	High	NI	●	Normal: pos, DCIS: neg
6	High	❍	❍	DCIS: clear memb
7	High	●	●	Normal: pos, DCIS: neg
8	Low	NI	NI	Normal: pos, DCIS: pos
9	Inter.	●	NI	Excluded
10	Inter.	●	●	Normal: variable, DCIS: variable
11	High	NI	❍	Normal: strong, DCIS: weak
12	High	NI	NI	Het. memb staining
13	High	NI	❍	DCIS: weak (but no loss)
14	High	NI	NI	Normal: strong, DCIS: strong
15	Inter.	●	●	DCIS: weak, clear memb
16	High	NI	●	DCIS: clear memb
17	High	MI	MI	No staining in section
18	Low	❍	❍	Normal: strong, DCIS: strong except some ducts
19	Low	●	❍	DCIS: weak (cyto and memb)
20	Low	●	❍	Memb staining of all ducts
21	Low	ND	ND	No staining in section
22	Low	ND	ND	No staining in section
23	Low	ND	ND	No staining in section

❍ indicates retention, ● is loss of heterozygosity, NI is non-informative, ND is not determined, MI is microsatellite instability, Inter., is intermediate grade.

Het. indicates heterogeneous staining, memb, is membranous staining, cyto, is cytoplasmic staining, pos, is positive, and neg, is negative

Table 4.6. LOH and E-cadherin immunohistochemical data in invasive carcinomas

No.	D16S400	D16S289	D16S402	D16S3026	D16S413	E-cadherin immunohistochemical data
1	❍	❍	ND	ND	ND	No staining in section
3	❍	❍	❍	ND	❍	No staining in tumour
5	MI	MI	MI	ND	MI	Cytoplasmic and membrane staining
7	❍	❍	❍	ND	❍	No staining in section
9	❍	❍	❍	ND	❍	Het. cytoplasmic staining >50% reactivity
11	❍	❍	❍	ND	❍	No staining in section
13	❍	❍	❍	ND	❍	Het. membrane staining (>50% reactivity)
15	❍	❍	❍	ND	❍	Membrane staining
17	❍	NI	●	ND	❍	No staining in section
19	●	❍	ND	NI	●	Het. normal staining (Weak cyto. <50%)
21	●	●	NI	NI	●	Het. membrane staining
23	NI	❍	●	NI	●	Het. membrane staining (>50% reactivity)
25	❍	ND	ND	ND	❍	Het. membrane staining (<50% reactivity)
27	NI	❍	NI	ND	❍	No staining in tumour
29	❍	❍	ND	ND	❍	No staining in tumour
31	❍	❍	❍	ND	❍	Membrane staining
33	●	❍	●	●	❍	Cytoplasmic and membrane staining
35	NI	ND	❍	ND	❍	No staining in section
37	NI	❍	ND	NI	●	No staining in section
39	●	❍	ND	NI	●	No staining in section
41	NI	❍	●	ND	❍	No staining in section
43	●	❍	❍	ND	●	No staining in section
45	●	●	●	❍	❍	Membrane staining
47	NI	❍	ND	ND	●	No staining in section
49	●	NI	●	❍	●	Membrane staining
51	❍	❍	ND	ND	NI	No staining in section
53	NI	●	●	●	●	No staining in section
55	MI	NI	MI	ND	MI	Het. cytoplasmic staining of tumour (<50%)
57	●	●	ND	ND	●	Homogen. cytoplasmic staining of tumour
59	●	ND	ND	❍	●	No tumour cells remaining

❍ indicates retention, ● is loss of heterozygosity, NI is non-informative, ND is not determined, MI is microsatellite instability.

Het. indicates heterogeneous staining, Homog. is homogeneous staining.

H-cadherin

The strategy for H-cadherin analysis was to characterise reactivity of this protein in the cases previously analysed for LOH with chromosome 16q markers, so that correlations between expression levels and LOH could be attempted.

Immunohistochemistry was attempted with polyclonal H-cadherin antibodies (kindly provided by Dr Sam Lee). This antibody had previously been tested on sections of paraffin embedded breast tissues and shown to be present in the membrane of normal and benign ducts but absent from in situ and infiltrating carcinomas (Lee, 1996).

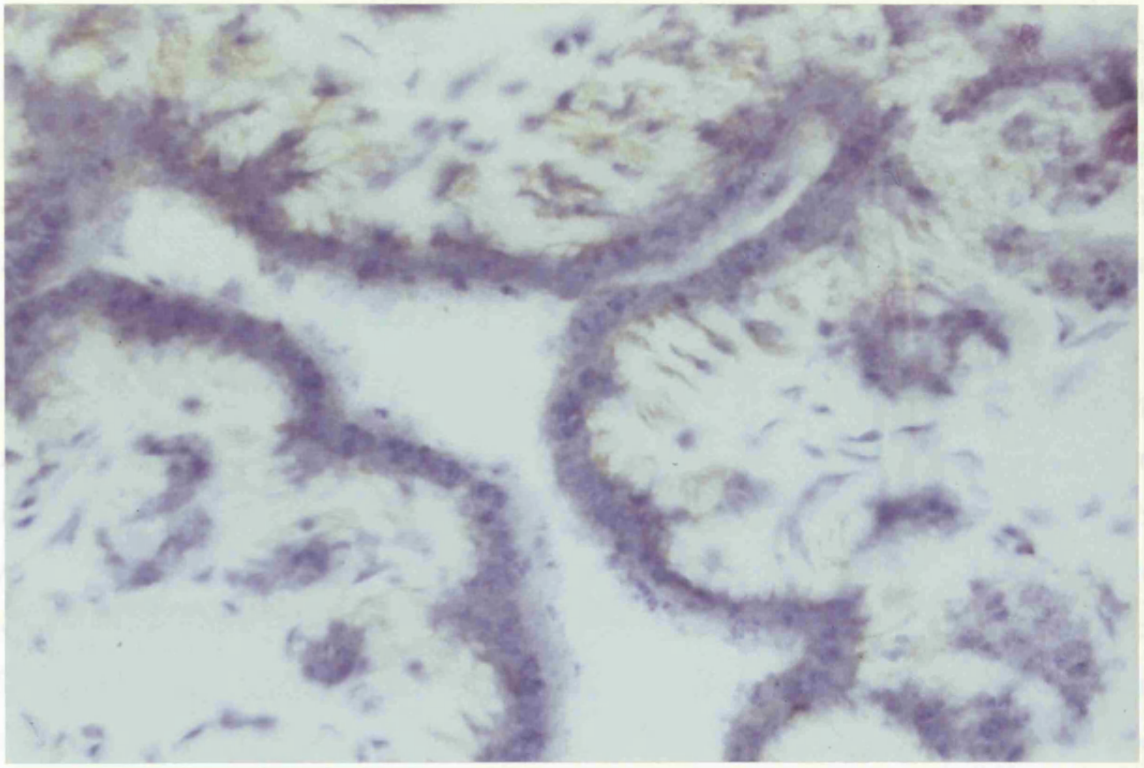
Tissue sections of DCIS containing adjacent normal breast tissue were tested with dilutions ranging from neat to 1:100. However reactivity was not observed. The same dilution range was applied to tissue sections which had been pre-treated by pressure cooking and protease digestion, but this also failed to produce reactivity. Frozen sections of normal breast tissue were then tested and reactivity was observed. Using a 1:10 dilution of the H-cadherin antibody, reactivity was only observed in the breast myoepithelial cells surrounding medium sized ducts, as determined by Dr. R.A Walker, and illustrated in Figure 4.13 (a).

In order to further characterise the putative myoepithelial expression of H-cadherin, immunofluorescence microscopy was performed on purified populations of breast myoepithelial cells (kindly provided by Dr. Louise Jones) stained with a range of antibody dilutions. Peripheral reactivity was observed (Figure 4.13b). The immunofluorescence experiments were carried out in the presence of antibodies raised against specific myoepithelial-specific proteins (Cytokeratin 14 and P-cadherin, the latter demonstrated below).

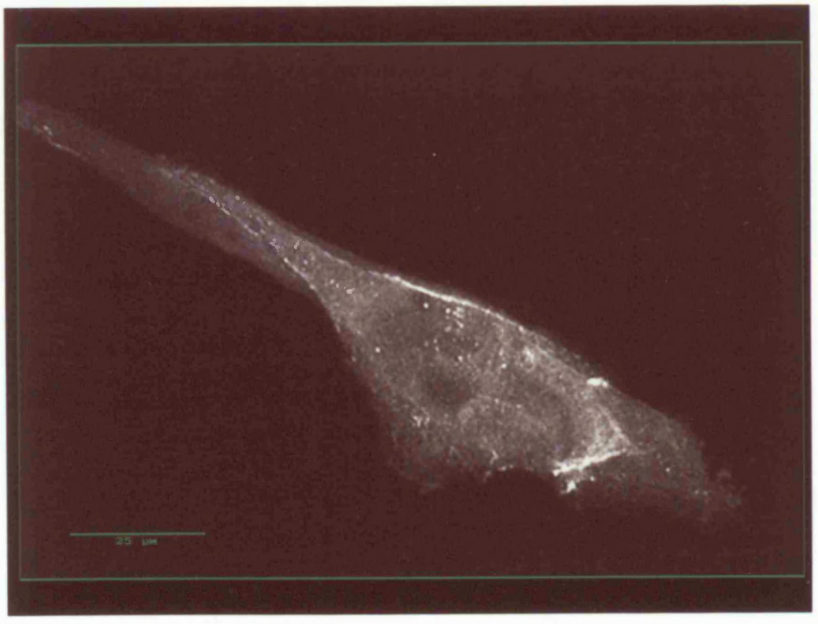
There was insufficient antibody to perform Western blot analysis.

Figure 4.13. H-cadherin immunohistochemistry.

(a) High power view of normal breast duct (frozen section) showing staining of elongated cells around periphery of duct in position of myoepithelial cells.



(b) Confocal microscopic analysis of H-cadherin in isolated myoepithelial cells showing membrane localisation.



P-cadherin

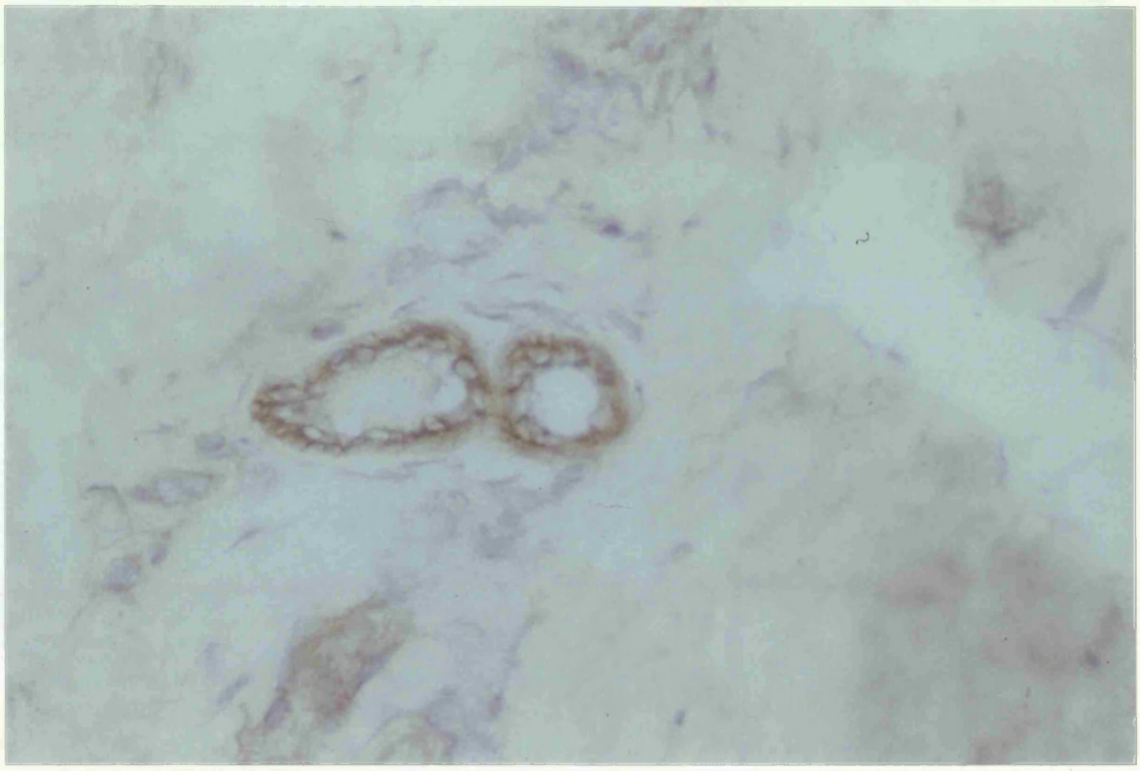
The strategy for analysing P-cadherin was similar to that for H-cadherin. The P-cadherin antibody (NCC-CAD-299) although demonstrated to be reactive on frozen tissue in a number of studies (Shimoyama *et al.,* 1989; Rasbridge *et al.,* 1993; Palacios *et al.,* 1995), had not previously been tested on paraffin embedded tissue sections. The antibody has been demonstrated to react with breast myoepithelial cells (Shimoyama *et al.,* 1989; Rasbridge *et al.,* 1993) and some infiltrating ductal carcinoma cells (Palacios *et al.,* 1995). Basal keratinocytes and sweat glands of the skin are a positive control for P-cadherin expression (Shimoyama *et al.,* 1989), as illustrated in Figure 4.14 (a).

Reactivity of breast myoepithelial cells were observed by immunohistochemistry using an undiluted stock of this antibody on frozen sections of DCIS and surrounding normal breast tissue, as illustrated in Figure 4.14 (b) and (c). In order to determine whether this antibody would be reactive on formalin fixed paraffin embedded tissue, a section of skin from a reduction mammoplasty was fixed and processed as described in Section 2.2. Tissue sections from the embedded block of skin were then pre-treated by protease digestion and pressure cooking prior to immunohistochemistry with an undiluted aliquot of P-cadherin antibody. However, no reactivity was observed.

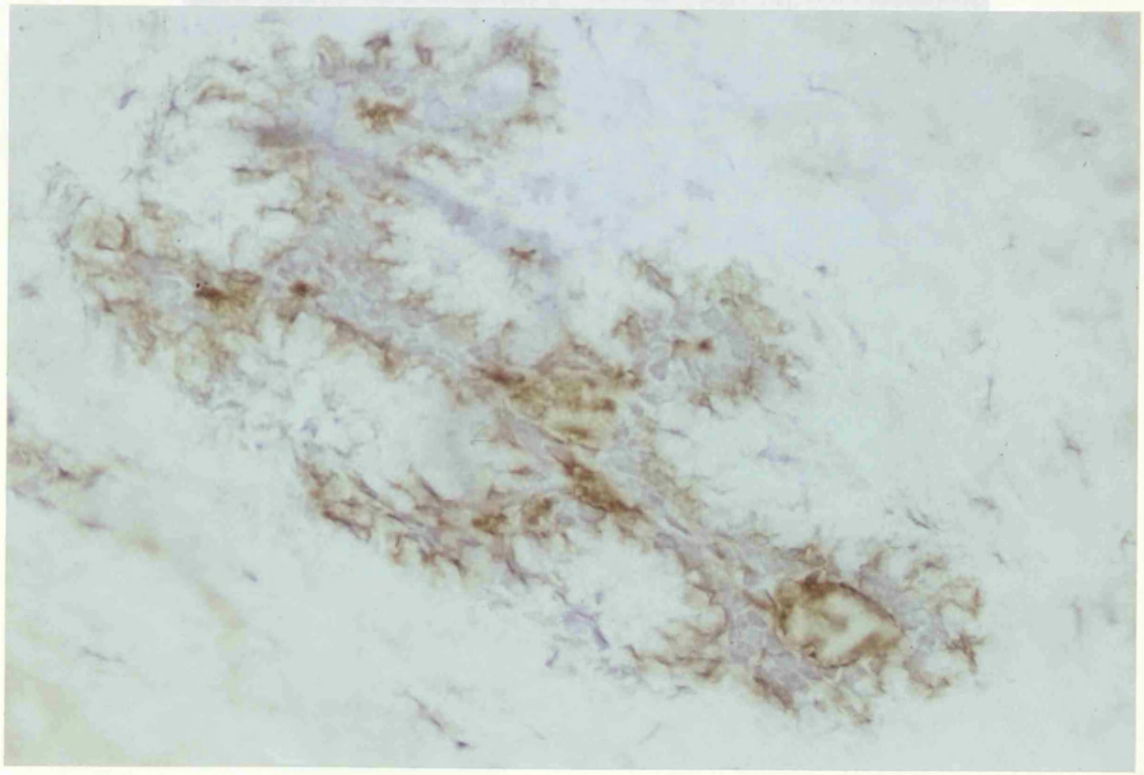
Immunofluorescence studies on purified myoepithelial cells with this antibody confirmed the myoepithelial expression observed in the sections of frozen tissue, as illustrated in Figure 4.14 (d).

Figure 4.14. P-cadherin immunohistochemistry.

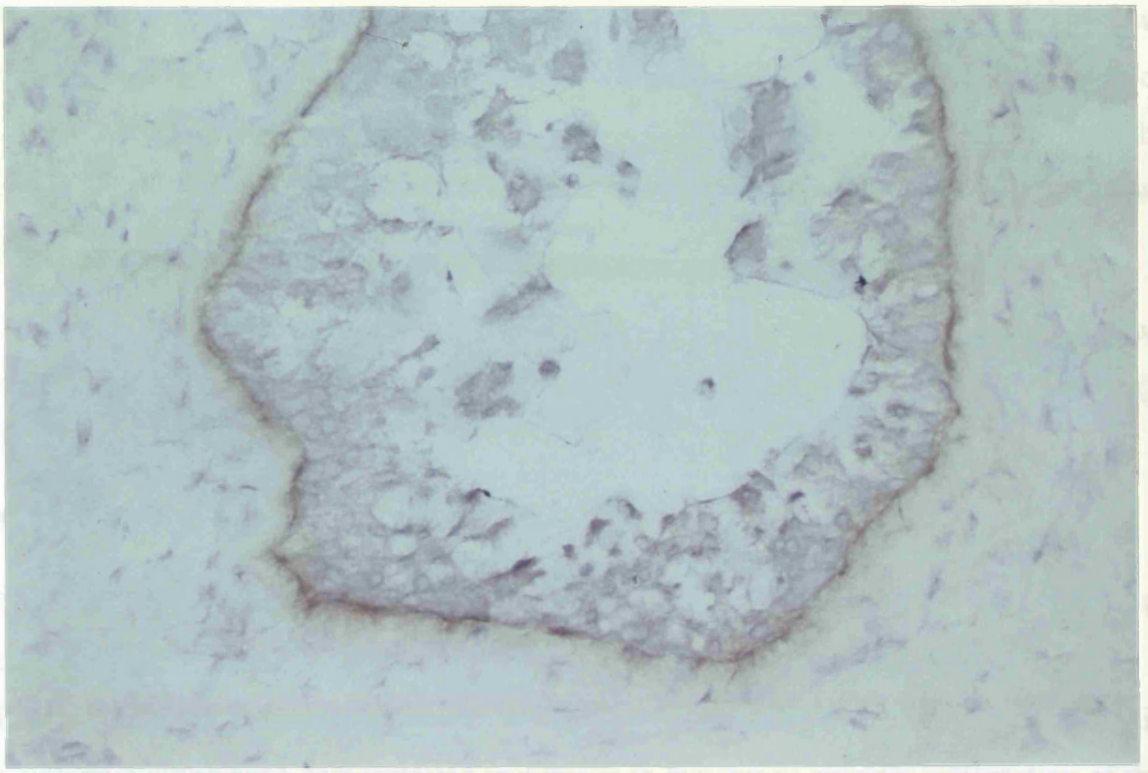
(a) Skin. Two eccrine glands in frozen section of skin showing strong staining predominantly of outer layer (myoepithelium)



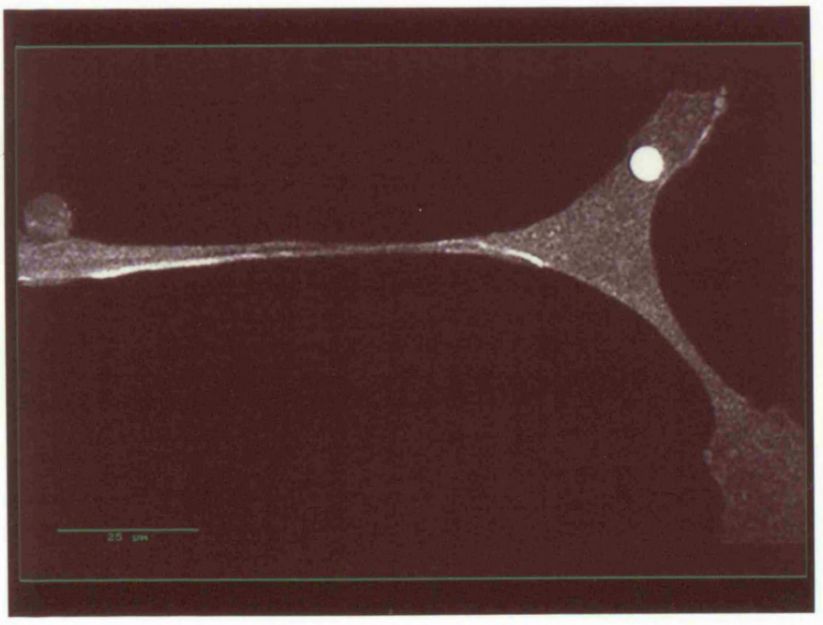
(b) Normal breast. Duct from frozen section showing labelling of peripheral cells with elongation - myoepithelia.



(c) DCIS. Labelling of cells around periphery of DCIS - myoepithelia.



(d) Confocal microscopic analysis of purified myoepithelial cells showing membrane localisation for P-cadherin. Density is residual bead from purification procedure.



4.7 Methylation Analysis

Recent evidence has suggested that de novo methylation of promoter regions may lead to transcriptional silencing of tumour suppressor genes, including the E-cadherin genes (Section 2.1). This was investigated in breast cancer cell lines with well established E-cadherin expression profiles. The long term aim of this project was to develop a technique that would be applicable to fixed tissues.

The experimental strategy involved two approaches, the development of a PCR-based methylation assay, and the recently described method of methylation-specific PCR (MS-PCR).

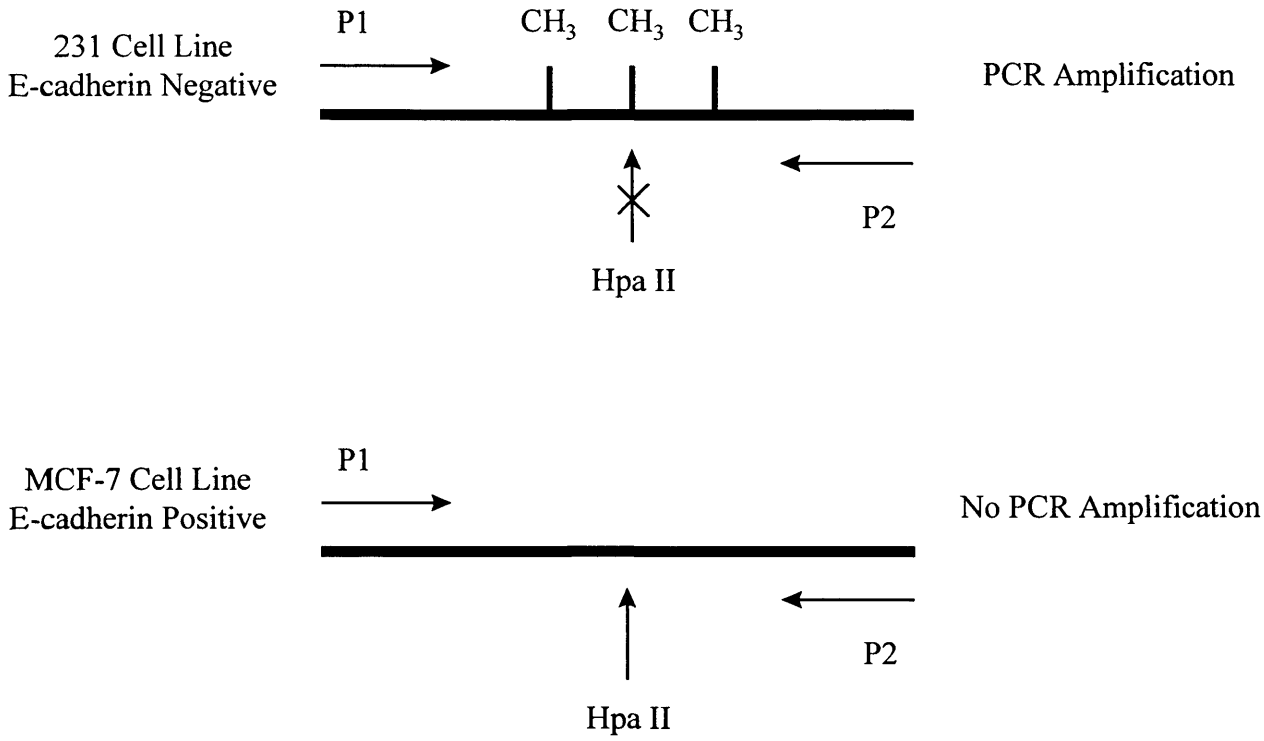
PCR-Based Methylation Assay

The MCF-7 and MDA-MB-231 (231) breast cancer cell lines had previously been demonstrated to be positive and negative for E-cadherin expression, by immunohistochemistry, respectively (personal communication, Louise Jones). Studies using Southern blotting have demonstrated that this is in fact due to aberrant methylation of the E-cadherin promoter region (Graff *et al.*, 1995). The 231 cell line is methylated at regions within the E-cadherin promoter region, whereas the MCF-7 cell line is unmethylated

This PCR-based methylation assay relied on the inability of particular methylation sensitive restriction enzymes (for example *Hpa*II) to digest DNA specifically at methylated bases in the promoter region of the E-cadherin gene. Following digestion, the DNA is PCR amplified with primers flanking the restriction site, as illustrated in Figure 4.15 and Appendix I.

Figure 4.15. Schematic Of PCR-based Methylation Assay.

DNA from 231 (E-cadherin negative) and MCF-7 (E-cadherin positive) is digested with the methylation-sensitive HpaII enzyme. The enzyme does not restrict methylated DNA (231) but does restrict unmethylated DNA (MCF-7). Following PCR amplification with primers flanking the restriction sites, only the DNA from the 231 cell line produces a product.



The presence of methylated regions is detected by the lack of an amplifiable PCR product. Therefore it essential to include a positive control for PCR amplification. The exon 9 primer was selected as a positive control for amplification because it does not contain *Hpa*II sites, and was sufficiently different in size to allow resolution of both products (promoter region and exon 9) on agarose gels.

Genomic DNA prepared from 231 cell line digested (with *Hpa*II) and undigested was amplified with both sets of primers in a multiplex PCR. This led to amplification of promoter and exon 9 products from *Hpa*II-digested and undigested DNA, as illustrated in Figure 4.16 (a). This was an expected result for DNA with methylated bases within the promoter region.

The experiments with DNA prepared from the MCF-7 cell line were conducted in the presence of radio-isotope to improve the sensitivity of detection. In order to determine the optimum number of cycles for the assay, a cycle titration curve was set up. This involved removing aliquots from the amplification reaction at 26, 29, 32, and 35 cycles and electrophoresis on polyacrylamide gels (Figure 4.16b).

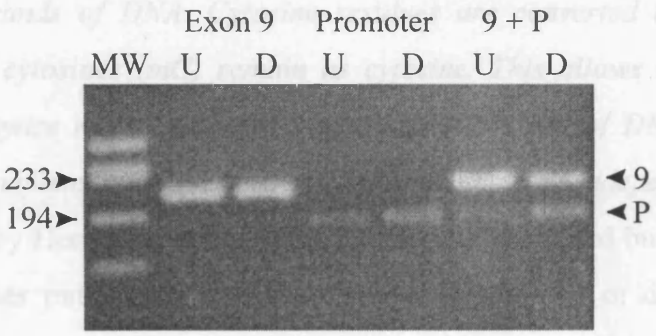
Digestion of MCF-7 DNA with *Hpa*II and subsequent amplification with the promoter and exon 9 primers led to the amplification of the exon 9 product only. However, the titre experiment detected amplification products above 32 cycles in *Hpa*II digested DNA, suggesting that at this cycle number incompletely digested DNA could be lead to the amplification of a PCR product, as illustrated in Figure 4.16 (b).

Experiments were then planned to determine the effect of the fixation process on the digestibility of DNA with the *Hpa*II enzymes. However, at this time, another technique became available which offered more scope for application to fixed tissues, as described below.

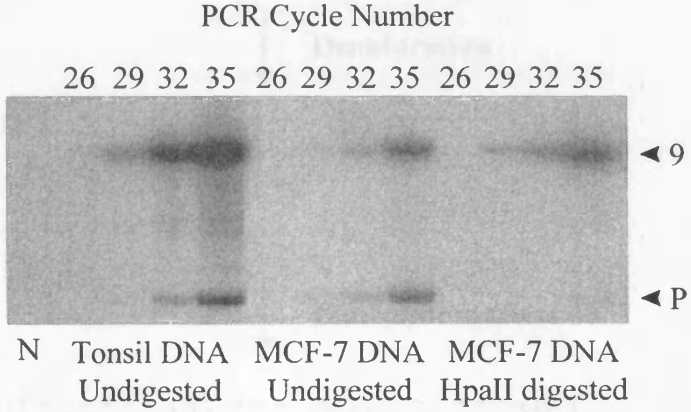
Figure 4.16. PCR-based Methylation Assay

(a) Undigested (U) and HpaII digested DNA samples (D) from the 231 cell line were amplified with exon 9 primers (9), promoter-specific primers (P) and a combination of both sets (9+P). PCR products were electrophoresed on 4% agarose gels alongside ϕX174/HaeII molecular weight markers. Sizes are indicated in base pairs.

(b) Titration experiment with undigested and HpaII digested DNA from MCF-7 cell line, and undigested tonsil DNA, amplified with exon 9, promoter-specific, and both sets of primers. Aliquots were removed at the cycle number indicated and electrophoresed on 6% denaturing polyacrylamide gels followed by autoradiography. Negative control (N) without DNA was amplified for 35 cycles.



(a)



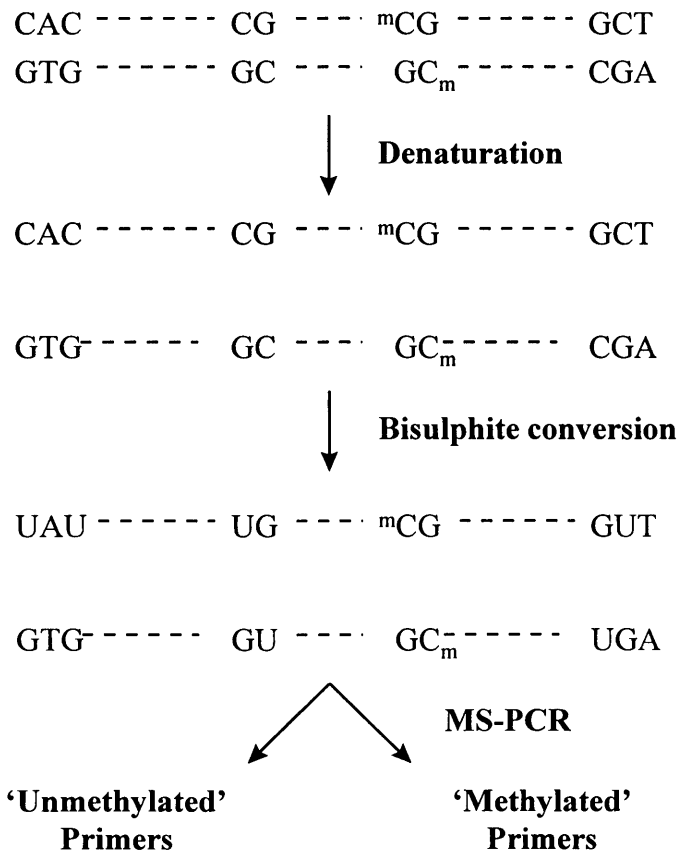
(b)

Methylation Specific-PCR

This recently described technique relies on the ability of sodium bisulphite to convert cytosine to uracil. Methylated cytosines are not converted to uracil and remain as cytosine as described in Section 2.1. and depicted in Figure 4.17. The DNA sequence changes that result from the bisulphite conversion allow the design of PCR primers which distinguish methylated from unmethylated DNA, thereby producing a positive display for methylated regions. This technique has recently been applied to the analysis of methylation in the E-cadherin gene (Herman *et al.,* 1996). They confirmed, using MS-PCR, that DNA from MCF-7 cells showed no evidence of methylation, and that DNA from 231 cells displayed prominent methylation in the E-cadherin promoter region.

Figure 4.17. Bisulphite conversion and MS-PCR (Adapted from Clark et al., 1994).
Two complementary strands of DNA. Cytosine residues are converted to uracil residues whereas as methylated cytosines (mC) remain as cytosine. This allows design of primers which specifically recognise methylated and unmethylated regions of DNA. The two DNA strands are no longer complementary and therefore can be amplified independently.

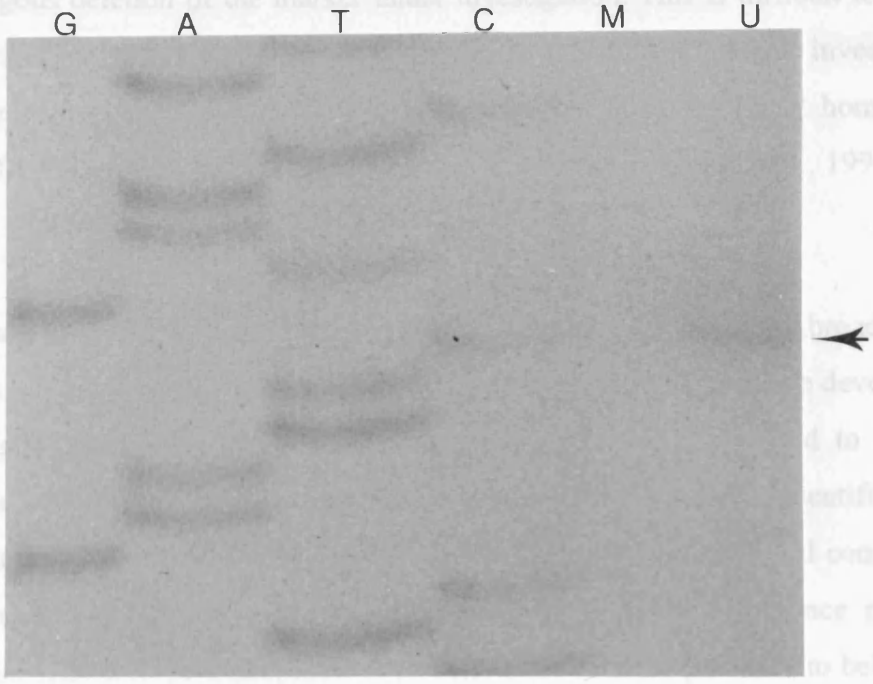
The protocol described by Herman *et al.,* (1996) was initially attempted but failed to produce PCR products. An earlier publication suggested shearing the DNA or digesting it with a



The protocol described by Herman *et al.*, (1996) was initially attempted but failed to produce PCR products. An earlier publication suggested shearing the DNA or digesting it with a restriction enzyme prior to bisulphite conversion, improved the efficiency of conversion (Clark *et al.*, 1994). The DNA was restricted with *Eco*RI which does not cleave within the E-cadherin promoter region. This led to the amplification of MS-PCR products. The 231 cell lines only amplified a product with primers designed to detected methylated regions, whereas the MCF-7 cells produced a product with primers designed for unmethylated DNA, as illustrated in Figure 4.18.

Figure 4.18. Methylation-Specific PCR (MS-PCR)

Bisulphite treated DNA from the MCF-7 cell line was PCR amplified with primers which were designed to amplify unmethylated (U) and methylated DNA (M), followed by electrophoresis along side an M13 DNA sequence ladder. The arrow indicated the 93bp product obtained with the unmethylated specific primers.



4.8 Loss of Heterozygosity

Technical Difficulties

Characterisation of LOH and MI in the same cases can be difficult to interpret. MI is characterised by alteration to the size of alleles, whereas LOH is defined by loss of an allele. It is possible that a tumour with MI may have an allele alteration to the position of the other allele which could be interpreted as LOH. The data from Chapter 3 suggested that MI^+ cases had alterations of both DM-1 alleles and subsequently other alleles. Alterations of the 16q microsatellite markers in all MI^- cases and those with alterations to a single DM-1 allele were described as LOH.

A proportion of the microdissected tumour foci from each of the carcinoma study groups failed to produce amplification products at some loci but not at others. Each locus was analysed separately, so it is possible that sampling errors may have occurred, for example, the omission of template in some reactions. It is also possible that absence of PCR products is a result of homozygous deletion of the marker under investigation. This is difficult to analyse because it results in a 'negative' result. However, this may be worthy of further investigation, and could be conducted in the presence of a positive control with a defined homozygous mutation at 16q, for example the SK-BR-3 breast cancer cell line (Pierceall *et al.,* 1995).

Fibroadenomas

The low frequency of LOH (17%) at chromosome 16q21 observed in the fibroadenomas, suggests that loss of this region may not be an important genetic alteration in the development of fibroadenomas. A frequency of LOH between 10 and 15% is considered to reflect a 'background' level, of random genetic alteration (Orphanos *et al.,* 1995). The identification of LOH in 2 of 3 stromal components, and retention of the locus in both epithelial components, in the single phyllodes tumour may be worthy of further investigation since phyllodes tumours have been shown to be a neoplasm of stromal cells with the potential to behave in a malignant fashion. Analysis of 50 fibroadenomas and 8 phyllodes tumours (Lizard-Nacol *et al.,* 1995) failed to demonstrate any genetic alterations, either LOH (at loci on chromosomes 1p, 3p, 7q, 11p, 17p, 17q, and 18q), or gene amplification (with probes for c-*myc*, cyclin D1 and c-*erb*B-2). However, these investigators analysed DNA from complete fibroadenoma and phyllodes specimens, and compared polymorphisms with DNA prepared from leukocytes of the same patients, which might not be sufficiently sensitive to detect alterations.

DCIS

LOH at the chromosome 16q markers was observed in a high proportion of the cases. The frequencies of LOH were 67% for the D16S400 marker and 50% for the D16S413 marker. These markers have been used in a number of other studies that have investigated LOH in cases of pure DCIS. In general the frequency of LOH at the D16S413 marker is higher than observed in previous studies (21% (4/19), Radford *et al.*, (1995), 28% (11/39) Stratton *et al.*, (1995), 25% (5/20) Aldaz *et al.*, (1995) and, 37% (14/19) Chen *et al.*, (1996)). The reasons for the observed higher frequency are unlikely to reflect differences in preparation of tumour material, because all of the studies have used similar microdissection protocols. It may simply be due to differences in sample size. It is possible that the cases in this thesis are displaying different patterns of LOH than those in the other published studies. This is supported by the data presented by Chen *et al.*, (1996), who observed different patterns of LOH. Approximately one third of their cases displayed large terminal deletions or recombinations involving most or all of the whole chromosome 16q arm, whereas the remaining two thirds of LOH cases appeared to be mostly the result of interstitial deletions or more complex recombination events. Whether this is the case in tumours presented in this thesis could only be proved by examining an extended panel of flanking markers in these cases.

Chen *et al.*, (1996) also analysed the D16S400 marker and demonstrated 64% LOH (7/11), which is comparable to the observed frequency of 67% (8/12) in this study and confirms that this locus is commonly deleted in cases of DCIS.

In contrast to MI (Chapter 3), there was no association between LOH at chromosome 16q and histological grade of DCIS. The frequency in low grade cases was not significantly different from the frequency in high grade DCIS. This has also been observed by Chen *et al.*, (1996). Fujii *et al.*, (1995b) examined LOH at 10 chromosomal regions and demonstrated that for each of the regions, with the exception of 16q, there was a significantly greater frequency of LOH in high and intermediate grade than in the low grade DCIS.

Intratumoural heterogeneity, with respect to LOH, was observed in one case. This has not been observed previously (Radford *et al.*, (1995), Stratton *et al.*, (1995), Aldaz *et al.*, (1995) or Chen *et al.*, (1996)). However, in each of the aforementioned investigations it was not clear how many individual ducts from each case were examined. It is possible that the occurrence of heterogeneity could not be investigated because only a single duct was examined. In

contrast, Fujii *et al.*, (1996b) analysed up to eight individual involved ducts and observed heterogeneity of a chromosome 16q marker in 1 of 19 cases showing LOH.

Early Invasive Carcinomas

LOH in the early invasive group was conducted with five markers. The frequencies of LOH detected ranged from 40% at q22.1 (D16S400), 16% at q23.2 (D16S289) to 35-50% at the q24 markers (D16S402, D16S3026, D16S413). Comparison of LOH frequencies between different studies is not feasible because of the variety of carcinomas studied. It is however possible to compare patterns of LOH between studies. Approximately half of the early invasive cases showed LOH confined to the q24 markers, whereas the remainder showed complex patterns of LOH, which might suggest interstitial deletions and recombination events. A recent investigation has proposed that LOH of 16q in vivo is primarily due to mitotic recombination (Gupta, P.W., *et al.*, 1997).

The highest frequency of LOH detected in this study was at the q24 and the q22.1 regions. However, because only one marker, mapping between these regions, was studied it is not possible to confirm that these regions are two independent sites of LOH as proposed by Cleton-Jansen *et al.*, (1994).

The microdissection analyses of distinct tumour foci proved beneficial. Distinguishing between allelic gain and loss was difficult without this. The subsequent demonstration of LOH in these cases is consistent with previous cytogenetic data (Hainsworth *et al.*, 1991) and the recent data provided by comparative genomic hybridisation analysis (James *et al.*, 1997) which has not demonstrated gains on the long arm of chromosome 16.

Intratumoural heterogeneity was not observed in any of the early invasive carcinomas cases. However, analysis of multiple foci did reveal foci with incomplete allele loss. This may have been due to the presence of non-neoplastic stromal cells in the microdissected invasive cancer, In contrast this was generally not observed in the DCIS cases were there were fewer non-neoplastic cells and where it was easier to identify individual ducts. Stratton *et al.*, (1995), have calculated that the presence of more than 20 percent of non-neoplastic cells in invasive breast cancer makes LOH difficult to detect by PCR of microsatellite repeats.

Tubular Carcinomas

The frequency of LOH in this group was higher than in the early invasive group, with 40% LOH at the D16S289 marker and 55% LOH at the D16S413 marker. A recent investigation of LOH in tubular carcinomas (Mau *et al.,* 1996) has demonstrated 40% LOH at a marker mapping to 16q24.2 (D16S402).

Overall

The overall findings indicate that LOH of chromosome 16q was common in all of the malignant lesions, but was not identified at a significant frequency in benign fibroadenomas, which will not progress to malignancy.

The frequency of LOH did vary between the groups of carcinomas, with the highest frequency observed in the DCIS cases. However, this is difficult to interpret because of differences in sample size. In contrast to MI, there was no association with grade of DCIS. It has been proposed that high and low grade DCIS proceed along different pathways of breast cancer development (Walker, 1997). The finding of relative equal frequency of 16q LOH in high and non-high grade DCIS suggest that ducts with this alteration are unlikely to diverge along any one particular pathway, which is different to that observed by MI analysis.

4. 9 Analysis Of Candidate Genes

The loss of heterozygosity data provided the cases which would be investigated for alterations in candidate tumour suppressor genes mapping to chromosome 16q.

E-cadherin

The SSCP assay used has been demonstrated to be sensitive for the detection of a single base pair mutation. However, no evidence of alterations was found. This suggests that each of the early invasive and DCIS cases demonstrating LOH at markers on chromosome 16q did not contain mutations in the exons 5 to 9 of the E-cadherin gene. DCIS cases have not previously been investigated for mutations of E-cadherin. However, the data from the early invasive carcinomas is consistent with previous studies which have demonstrated low frequencies of mutations in infiltrating ductal carcinomas (Kanai *et al.,* 1994; Berx *et al.,* 1995b; Berx *et al.,* 1996; Candidus *et al.,* 1996). These same studies have demonstrated that approximately half of all infiltrating lobular carcinomas, examined to date, contain mutations in this gene. Data from the literature suggests that the mutations are not confined to the functionally important

domains of the gene, but are scattered over all 16 exons. It is therefore possible that the DCIS cases and invasive carcinomas are mutated in exons other than those investigated. The recently described multiplex-SSCP technique (Berx *et al.,* 1997) would greatly improve the efficiency of detection of alterations.

Direct sequence analysis did produce a putative tumour-specific alteration in one infiltrating ductal carcinoma at exon 7. However, this could not be confirmed due to paucity of tissue available for study. The additional bands did not produce an 'overlap' between wild-type and mutant sequence as would be expected from a true insertion of one base pair. This alteration is therefore likely to have resulted from a DNA sequencing artefact, for example 'pausing' of the polymerase. Interestingly, this case was the only one to demonstrate homogeneous cytoplasmic reactivity of E-cadherin as demonstrated by immunohistochemistry. The significance of the change from membraneous to cytoplasmic reactivity is not clear at present but may be due to altered cadherin-catenin interactions (Jones *et al.,* 1996).

The immunohistochemical analysis of all the DCIS and invasive carcinomas provided an estimate of qualitative E-cadherin protein expression. This uncovered a number of cases which seem to have lost expression of the protein whilst retaining chromosome 16 markers flanking the E-cadherin locus. This provided preliminary evidence for an alternative mechanism of inactivation for this gene, for example aberrant methylation of the E-cadherin promoter regions.

The association between methylation and E-cadherin gene inactivation had been previously demonstrated in culture (Graff *et al.,* 1995) and was investigated during this thesis using two different approaches. In the first approach, a PCR-based methylation assay was modified to enable application to microdissected tumour foci. However, the difficulties encountered with the technique made it apparent that the technique would only produce reliable results with intact DNA, because it depends upon complete digestion of DNA. The bisulphite approach developed by Herman *et al.,* (1996) has much more potential for application to fixed tissue. Establishing the methylation status of the breast cancer cell lines was important since it will provide controls for further investigations. In particular it will be important to determine whether the fixation process will effect the bisulphite conversion process. This can be addressed by fixing cells from the MCF-7 and 231 cell lines in formalin and processing through to wax, and repeating the MS-PCR procedure along side freshly prepared DNA from these lines.

H-cadherin and P-cadherin

H-cadherin was proposed as a candidate tumour suppressor gene in breast cancer primarily on the evidence that expression of the protein, while present in normal and benign tissues was not present in DCIS and invasive carcinomas, as determined by immunohistochemistry with an anti-H-cadherin antibody (Lee, 1996). The immunofluorescence and immunohistological data presented in this thesis, using the same antibody clone, would suggest that H-cadherin protein may be expressed in breast myoepithelial cells. This would in part explain the immunohistological observations of Lee (1996), since myoepithelial cells are not present in invasive carcinomas. However, this does not explain the absence of expression in DCIS, where they can be around ducts, that was observed by Lee (1996).

There are a number of lines of evidence to suggest that myoepithelial specific genes may play tumour suppressive roles in cancer. In particular, these cells have been proposed to act as a host defence mechanism against cancer development (Sternlicht and Barsky, 1997). Removal of myoepithelial cells from surrounding epithelial cells is unlikely to be achieved even with laser capture techniques and therefore it will be difficult to analyse P-cadherin and H-cadherin with respect to allele loss and mutation analysis. Further investigations should focus on how the expression of H-cadherin and P-cadherin contribute to the host-defence mechanism proposed for the myoepithelial cells.

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compared polymorphisms with DNA prepared from leukocytes of the same patients, which might not be sufficiently sensitive to detect alterations.

DCIS

LOH at the chromosome 16q markers was observed in a high proportion of the cases. The frequencies of LOH were 67% for the D16S400 marker and 50% for the D16S413 marker. These markers have been used in a number of other studies that have investigated LOH in cases of pure DCIS. In general the frequency of LOH at the D16S413 marker is higher than observed in previous studies (21% (4/19), Radford *et al.,* (1995), 28% (11/39) Stratton *et al.,* (1995), 25% (5/20) Aldaz *et al.,* (1995) and, 37% (14/19) Chen *et al.,* (1996)). The reasons for the observed higher frequency are unlikely to reflect differences in preparation of tumour material, because all of the studies have used similar microdissection protocols. It may simply be due to differences in sample size. It is possible that the cases in this thesis are displaying different patterns of LOH than those in the other published studies. This is supported by the data presented by Chen *et al.,* (1996), who observed different patterns of LOH. Approximately one third of their cases displayed large terminal deletions or recombinations involving most or all of the whole chromosome 16q arm, whereas the remaining two thirds of LOH cases appeared to be mostly the result of interstitial deletions or more complex recombination events. Whether this is the case in tumours presented in this thesis could only be proved by examining an extended panel of flanking markers in these cases.

Chen *et al.,* (1996) also analysed the D16S400 marker and demonstrated 64% LOH (7/11), which is comparable to the observed frequency of 67% (8/12) in this study and confirms that this locus is commonly deleted in cases of DCIS.

In contrast to MI (Chapter 3), there was no association between LOH at chromosome 16q and histological grade of DCIS. The frequency in low grade cases was not significantly different from the frequency in high grade DCIS. This has also been observed by Chen *et al.,* (1996). Fujii *et al.,* (1995b) examined LOH at 10 chromosomal regions and demonstrated that for each of the regions, with the exception of 16q, there was a significantly greater frequency of LOH in high and intermediate grade than in the low grade DCIS.

Intratumoural heterogeneity, with respect to LOH, was observed in one case. This has not been observed previously (Radford *et al.,* (1995), Stratton *et al.,* (1995), Aldaz *et al.,* (1995) or Chen *et al.,* (1996)). However, in each of the aforementioned investigations it was not clear

how many individual ducts from each case were examined. It is possible that the occurrence of heterogeneity could not be investigated because only a single duct was examined. In contrast, Fujii *et al.,* (1996b) analysed up to eight individual involved ducts and observed heterogeneity of a chromosome 16q marker in 1 of 19 cases showing LOH.

Early Invasive Carcinomas

LOH in the early invasive group was conducted with five markers. The frequencies of LOH detected ranged from 40% at q22.1 (D16S400), 16% at q23.2 (D16S289) to 35-50% at the q24 markers (D16S402, D16S3026, D16S413). Comparison of LOH frequencies between different studies is not feasible because of the variety of carcinomas studied. It is however possible to compare patterns of LOH between studies. Approximately half of the early invasive cases showed LOH confined to the q24 markers, whereas the remainder showed complex patterns of LOH, which might suggest interstitial deletions and recombination events. A recent investigation has proposed that LOH of 16q in vivo is primarily due to mitotic recombination (Gupta, P.W., *et al.,* 1997).

The highest frequency of LOH detected in this study was at the q24 and the q22.1 regions. However, because only one marker, mapping between these regions, was studied it is not possible to confirm that these regions are two independent sites of LOH as proposed by Cleton-Jansen *et al.,* (1994).

The microdissection analyses of distinct tumour foci proved beneficial. Distinguishing between allelic gain and loss was difficult without this. The subsequent demonstration of LOH in these cases is consistent with previous cytogenetic data (Hainsworth *et al.,* 1991) and the recent data provided by comparative genomic hybridisation analysis (James *et al.,* 1997) which has not demonstrated gains on the long arm of chromosome 16.

Intratumoural heterogeneity was not observed in any of the early invasive carcinomas cases. However, analysis of multiple foci did reveal foci with incomplete allele loss. This may have been due to the presence of non-neoplastic stromal cells in the microdissected invasive cancer, In contrast this was generally not observed in the DCIS cases were there were fewer non-neoplastic cells and where it was easier to identify individual ducts. Stratton *et al.,* (1995), have calculated that the presence of more than 20 percent of non-neoplastic cells in invasive breast cancer makes LOH difficult to detect by PCR of microsatellite repeats.

Tubular Carcinomas

The frequency of LOH in this group was higher than in the early invasive group, with 40% LOH at the D16S289 marker and 55% LOH at the D16S413 marker. A recent investigation of LOH in tubular carcinomas (Man *et al.,* 1996) has demonstrated 40% LOH at a marker mapping to 16q24.2 (D16S402).

Overall

The overall findings indicate that LOH of chromosome 16q was common in all of the malignant lesions, but was not identified at a significant frequency in benign fibroadenomas, which will not progress to malignancy.

The frequency of LOH did vary between the groups of carcinomas, with the highest frequency observed in the DCIS cases. However, this is difficult to interpret because of differences in sample size. In contrast to MI, there was no association with grade of DCIS. It has been proposed that high and low grade DCIS proceed along different pathways of breast cancer development (Walker, 1997). The finding of relative equal frequency of 16q LOH in high and non-high grade DCIS suggest that ducts with this alteration are unlikely to diverge along any one particular pathway, which is different to that observed by MI analysis.

4. 9 Analysis Of Candidate Genes

The loss of heterozygosity data provided the cases which would be investigated for alterations in candidate tumour suppressor genes mapping to chromosome 16q.

E-cadherin

The SSCP assay used has been demonstrated to be sensitive for the detection of a single base pair mutation. However, no evidence of alterations was found. This suggests that each of the early invasive and DCIS cases demonstrating LOH at markers on chromosome 16q did not contain mutations in the exons 5 to 9 of the E-cadherin gene. DCIS cases have not previously been investigated for mutations of E-cadherin. However, the data from the early invasive carcinomas is consistent with previous studies which have demonstrated low frequencies of mutations in infiltrating ductal carcinomas (Kanai *et al.,* 1994; Berx *et al.,* 1995; Berx *et al.,* 1996; Candidus *et al.,* 1996). These same studies have demonstrated that approximately half of all infiltrating lobular carcinomas, examined to date, contain mutations in this gene. Data from the literature suggests that the mutations are not confined to the functionally important

domains of the gene, but are scattered over all 16 exons. It is therefore possible that the DCIS cases and invasive carcinomas are mutated in exons other than those investigated. The recently described multiplex-SSCP technique (Berx *et al.,* 1997) would greatly improve the efficiency of detection of alterations.

Direct sequence analysis did produce a putative tumour-specific alteration in one infiltrating ductal carcinoma at exon 7. However, this could not be confirmed due to paucity of tissue available for study. It may have resulted from a DNA sequencing artefact, for example 'pausing' of the polymerase. However, a number of samples set up in parallel on tissue of the same type, and analysed under the same electrophoretic conditions, did not display this alteration.

The putative change introduced by the 1bp insertion would lead to a premature stop codon and hence a truncated transcript. This could not be confirmed by RT-PCR because of the inability to obtain reliable yields of mRNA from fixed tissue. However, it is possible that the truncation would affect the cellular localisation of this product. Interestingly, this case was the only one to demonstrate homogeneous cytoplasmic reactivity of E-cadherin as demonstrated by immunohistochemistry. The significance of the change from membraneous to cytoplasmic reactivity is not clear at present but may be due to altered cadherin-catenin interactions (Jones *et al.,* 1996).

The immunohistochemical analysis of all the DCIS and invasive carcinomas provided an estimate of qualitative E-cadherin protein expression. This uncovered a number of cases which seem to have lost expression of the protein whilst retaining chromosome 16 markers flanking the E-cadherin locus. This provided preliminary evidence for an alternative mechanism of inactivation for this gene, for example aberrant methylation of the E-cadherin promoter regions.

The association between methylation and E-cadherin gene inactivation had been previously demonstrated in culture (Graff *et al.,* 1995) and was investigated during this thesis using two different approaches. In the first approach, a PCR-based methylation assay was modified to enable application to microdissected tumour foci. However, the difficulties encountered with the technique made it apparent that the technique would only produce reliable results with intact DNA, because it depends upon complete digestion of DNA. The bisulphite approach developed by Herman *et al.,* (1996) has much more potential for application to fixed tissue. Establishing the methylation status of the breast cancer cell lines was important since it will

provide controls for further investigations. In particular it will be important to determine whether the fixation process will effect the bisulphite conversion process. This can be addressed by fixing cells from the MCF-7 and 231 cell lines in formalin and processing through to wax, and repeating the MS-PCR procedure along side freshly prepared DNA from these lines.

H-cadherin and P-cadherin

H-cadherin was proposed as a candidate tumour suppressor gene in breast cancer primarily on the evidence that expression of the protein, while present in normal and benign tissues was not present in DCIS and invasive carcinomas, as determined by immunohistochemistry with an anti-H-cadherin antibody (Lee, 1996). The immunofluorescence and immunohistological data presented in this thesis, using the same antibody clone, would suggest that H-cadherin protein may be expressed in breast myoepithelial cells. This would in part explain the immunohistological observations of Lee (1996), since myoepithelial cells are not present in invasive carcinomas. However, this does not explain the absence of expression in DCIS, where they can be around ducts, that was observed by Lee (1996).

There are a number of lines of evidence to suggest that myoepithelial specific genes may play tumour suppressive roles in cancer. In particular, these cells have been proposed to act as a host defence mechanism against cancer development (Sternlicht and Barsky, 1997). Removal of myoepithelial cells from surrounding epithelial cells is unlikely to be achieved even with laser capture techniques and therefore it will be difficult to analyse P-cadherin and H-cadherin with respect to allele loss and mutation analysis. Further investigations should focus on how the expression of H-cadherin and P-cadherin contribute to the host-defence mechanism proposed for the myoepithelial cells.

Chapter 5

Conclusions and Future Studies

5.1 Conclusions

Microdissection

Breast carcinomas have long been recognised as heterogeneous in their biological and clinical behaviour. The development of microdissection of different tissue components within breast cancers has enabled the analysis of heterogeneity at the molecular level. This has been particularly important in the study of DCIS. By analysing multiple involved ducts it was possible to compare genetic differences between ducts from the same case. The differences observed between ducts, for certain genetic alterations, may suggest that DCIS is composed of distinct populations of genetically divergent clones. The technique has also allowed comparison of DCIS and invasive components of the same carcinoma, and similarly different types of differentiation. The significance of this will be discussed later.

The microdissection technique was initially adopted to enable enrichment of tumour cells and thus identify tumour-specific alterations which may be masked by analysis of samples composed of large amounts of non-malignant tissue. Whilst this was critical for the analysis of DCIS, it seems less so for invasive carcinomas. However, it has been empirically determined that even a small proportion of non-neoplastic cells (20%) can lead to misinterpretation of LOH (Stratton *et al.,* 1995). Therefore it is still important to enrich samples for tumour cells by microdissection. This does have some associated advantages.

Analysing DNA template from whole tumour sections can be associated with problems of LOH interpretation. For example a difference in allele intensity between a tumour and normal sample can be interpreted as allelic gain as well as loss. As demonstrated in this thesis, the analysis of template from microdissected tumour foci can greatly clarify this interpretation.

Microsatellite Instability

The identification of MI in breast cancer remains a controversial issue. The data presented here suggests that MI^+ breast tumours do not have targeting of the same cancer-associated loci (*TGFβRII*, *IGFIIR*, *Bax* and *E2F-4*) that have been characterised in colorectal tumours with this phenotype. The preliminary immunohistochemical data suggests that altered expression of three of the mismatch repair proteins (MSH2, MLH1, and PMS2) is also not associated with MI^+ in breast cancer. This questions the significance of the instability in breast cancer. Analysis of other cancer-associated genes that contain repeat motifs, targets

for slippage-induced mutations (for example the oestrogen receptor gene), and other candidate DNA repair loci (for example MSH3) is needed to clarify the role of MI^+ in breast cancer.

Further evidence for different features of MI in breast comes with the association with high nuclear grade in DCIS. This is in contrast to colorectal carcinomas where MI^+ is associated with less aggressive features and overall better survival rates than MI^- patients.

A greater understanding of the significance of MI in breast cancer is likely to come when a set of criteria for analysis have been established. Only then can inter-study comparisons be made. The panel of microsatellites investigated should include at least one of the long mononucleotide markers, in particular BAT26. Alterations, specifically contractions of this repeat, have been indicative of the MI^+ phenotype in over 90% of colorectal cancers (Hoang *et al.,* 1997). A range of dinucleotides will also be important, particularly those mapping to regions not associated with allele loss in breast cancers. Finally, in light of the finding of high incidence of alterations at the DM-1 locus which have been presented in this thesis, it will be important to establish whether this is also a feature of symptomatic and more advanced breast carcinomas.

The instability exclusively at the DM-1 marker is distinct from the widespread instability associated with MI^+. This has also been demonstrated by others (Wooster *et al.,* 1994) and may have implications for the role of DMPK in breast cancer development. Immunohistochemistry with an anti-DMPK antibody on a panel of breast carcinomas and normal breast tissues would give an insight into the expression levels and cellular localisation of this protein. The relationship between trinucleotide repeat length and expression levels of this gene, can be addressed using RT-PCR of DMPK transcripts in breast cancer cell lines which have different length DM-1 alleles, for example MCF-7 and MDA-MB-231 (data not shown).

LOH

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However, in light of the high frequency of LOH at the DCIS stage it would suggest that this alteration is indeed an early event in the progression of the disease.

The identity of the putative tumour suppressor gene(s) mapping to chromosome 16q remains unknown. It seems likely that the E-cadherin gene is not the proposed tumour suppressor gene, at least in infiltrating ductal carcinoma. This contrasts with the infiltrating lobular carcinomas, where inactivating mutations have been described in 56% of carcinomas studied (Berx *et al.,* 1996). Moreover, 90% of infiltrating lobular carcinomas with LOH at markers on 16q22.1 have been demonstrated to have E-cadherin mutations. It is conceivable that a high proportion of the lobular carcinomas, not demonstrating LOH within the vicinity of the E-cadherin genomic locus, will have reduced or absent E-cadherin expression as a consequence of promoter methylation.

The developmental experiments presented in this thesis suggest that the MS-PCR protocol represents a more suitable approach to studying promoter methylation in fixed tissues, than techniques which rely upon the inability of methylation specific to cleave specific methylated sites. Herman *et al.,* (1996) demonstrated that MS-PCR could be applied to fixed tissues, so it is likely that tumour foci showing absence of E-cadherin reactivity, as determined by immunohistochemistry in this thesis, could be analysed with respect to methylation status of the E-cadherin gene.

The immunological data presented for the H-cadherin protein have added to the understanding of how this novel protein may relate to the development of breast cancer. The initial report describing the cloning of this gene (Lee, 1996) proposed a tumour suppressor role for H-cadherin, primarily on the basis that there was a lack of expression in DCIS and invasive carcinoma whilst normal/benign tissues expressed the protein. If H-cadherin expression is genuinely confined to myoepithelial cells then this would explain the lack of expression in invasive carcinomas, since this cell type is not present in invasive carcinomas. An extended analysis with DCIS cases would clarify the myoepithelial expression of H-cadherin.

Key tumour suppressor candidate genes will emerge following collaborative LOH mapping, refinement of the smallest region of overlap (SRO) and large scale genomic sequencing. It is also possible that the location of the proposed gene(s) will be further refined by linkage analysis in breast cancer families which show genetic linkage to this chromosomal region (Hall *et al.,* 1990).

Breast Cancer Development

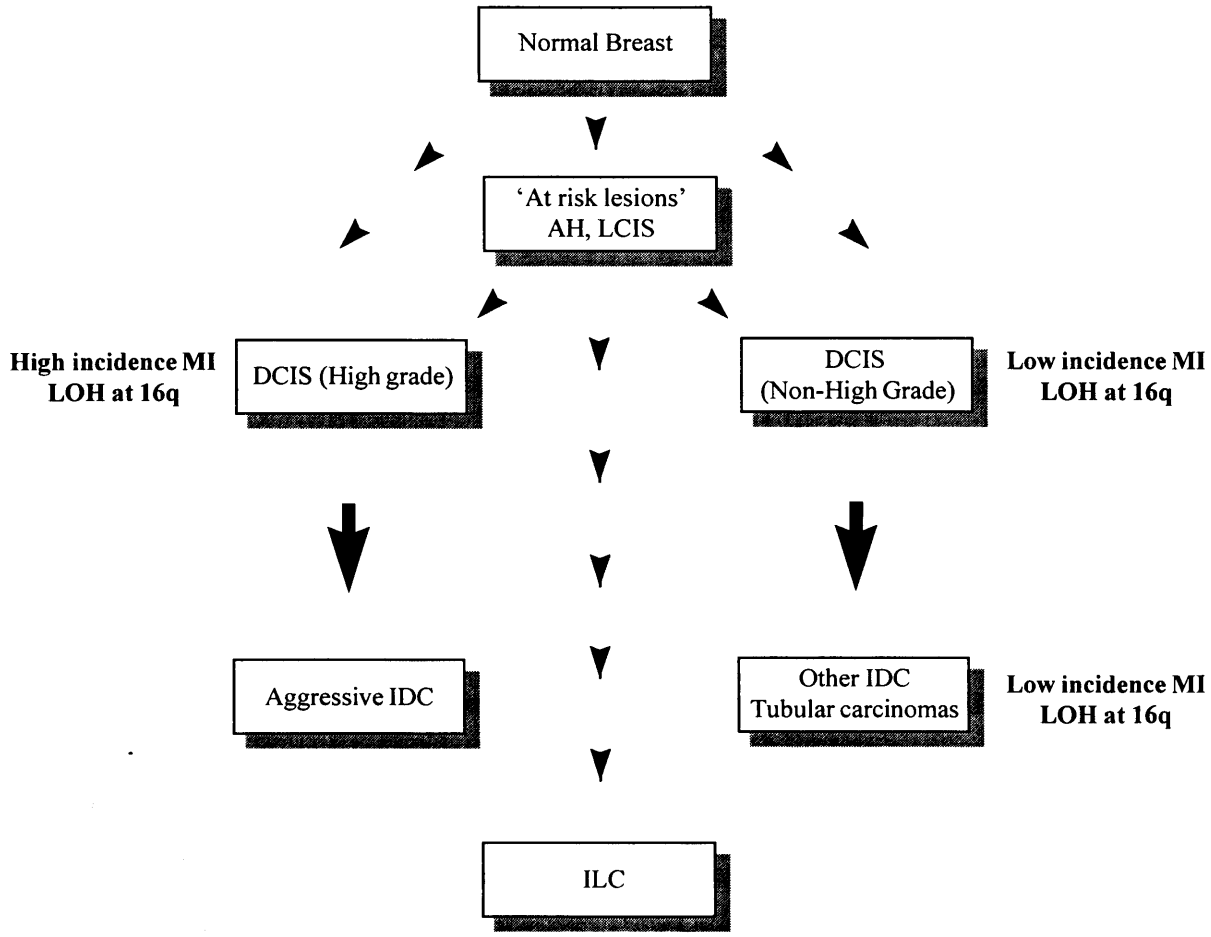
The main aim of this thesis was to study key genetic alteration in specific tumour types with the view to defining possible pathways of breast cancer development. The results from the 16q LOH analysis suggest that this type of alteration is common to all the tumour lesions analysed, and interestingly is not associated with grade of DCIS. In contrast the MI analysis has provided circumstantial evidence that low grade DCIS may progress to well to moderately differentiated invasive carcinomas. Of interest is the lack of instability in tubular carcinomas and tubular components within invasive carcinomas, suggesting that an inverse relationship with good structural differentiation.

The finding of a high frequency of MI in high grade DCIS, and the existing data regarding association between MI and aggressive features in invasive carcinomas (Paulson *et al.*, 1996; De Marchis *et al.*, 1997) suggests that high grade DCIS may progress to poorly differentiated, high grade, invasive carcinomas. Whether this is independent of other genetic alterations for example c-erbB-2 amplification remains to be classified. Additional evidence for this proposed pathway of breast cancer development could be provided by analysing an extended group of high grade DCIS and high grade poorly associated invasive carcinomas. The hypothetical pathways described here are represented in the scheme proposed by Walker (1997), Figure 5.1.

It would have been of interest, and relevance, to analyse 'at risk' lesions whose role in breast cancer development is still uncertain, for example the atypical ductal hyperplasias and lobular carcinoma in situ. The one example of the latter studied did show instability of D16S413. Obtaining tissue showing these alterations without associated malignancy is difficult but investigation of this would be of value.

Figure 5.1. Possible pathways of breast cancer progression with respect to microsatellite instability and loss of heterozygosity at 16q.

A high frequency of MI in high grade DCIS may suggest progression to high grade invasive carcinoma, whereas, a low frequency of MI in low grade DCIS and well to moderately differentiated invasive carcinomas may suggest a common pathway. LOH at 16q is common to each of the divergent pathways



5.2 Future Approaches

DM-1 and DMPK In Breast Cancer

Although there is a wealth of clinicopathological information available from archival tissues, the techniques which can be applied to these tissue types are limited to PCR-based approaches and immunohistochemistry. However, those cases in which a diagnosis is made preoperatively the tumour can be bisected, and one half is fixed and processed to wax, whereas the other half is snap-frozen in liquid nitrogen. This enables histological evaluation in sections prepared from paraffin-embedded tissue. These can then be microdissected and analysed with markers, for example DM-1. Any alterations that are documented between different tumour foci can then be further analysed by microdissection of the corresponding region in the frozen tissue, thereby allowing analysis of mRNA. It may therefore be possible to determine whether alterations in the length of the DM-1 repeat are correlated with an alteration in expression levels of DMPK mRNA, using a quantitative RT-PCR protocol which has been optimised for use with small amounts of starting material (Bicknell *et al.*, 1996).

An important question to address is the function, if any, of DMPK in breast cancer development. Sequence analysis has revealed homology between DMPK and members of a subfamily of serine/threonine protein kinases, which are closely related to cyclic AMP-dependant protein kinases. Members of this family are present in a diverse variety of organisms. The greatest amino acid sequence similarities exist within the catalytic domains of the WARTS (*wts*) gene in *Drosophila melanogaster* (Justice *et al.*, 1995)

Studies of *wts* indicates a role in cell differentiation and proliferation. Homozygous *wts*$^-$ clones develop prominent outgrowths on the adult cell body, resulting from increased proliferation of the *wts*$^-$ cells (Justice *et al.*, 1995). This indicates that *wts* has tumour suppressive functions. In myotonic dystrophy (DM) the presence of neural-crest derived tumours and small bowel carcinomas in some patients may reflect DMPKs role as a tumour suppressor gene.

Testing the tumour suppressive role of DMPK in breast cancer could be performed in culture using antisense techniques in cell culture. The human mammary epithelial cells (HMECs) represent a well studied immortalised 'non-tumourigenic' cell line. By transfecting an antisense DMPK construct into HMECs and breast cancer cell lines it will be

possible to assess the effects of transiently 'knocking-out' this gene. Assays which detect differences in cell doubling time, contact inhibition, and anchorage dependence can then be performed to determine whether decrease in DMPK expression leads to an increased growth and invasive capacity, as a prelude to the acquisition of a tumourigenic phenotype.

LOH And Tumour Suppressor Genes

Although LOH is considered as the hallmark of tumour suppressor gene inactivation, there is no functional evidence to suggest that regions of chromosome 16q encode a tumour suppressor gene important in the development of breast cancer. Introduction of individual normal chromosomes in cancer cells by the process of microcell fusion-mediated transfer (MCMT) represents an alternative approach for the identification of chromosomes which contain tumour suppressor genes (Trent *et al.,* 1990). If chromosome 16q does contain a tumour suppressor gene (other than E-cadherin) then introduction of chromosome 16q into tumourigenic breast cancer cell lines should result in the generation of sublines with a suppressed phenotype. Structural analysis of the 16q segments retained in these hybrid sublines and non-suppressed sublines after fusion by MCMT may refine the location of the putative tumour suppressor gene(s).

Breast Cancer Development

The development of the laser capture microdissection technique (Emmet-Buck *et al.,* 1996) has greatly improved the accuracy and efficiency of procuring DNA template from small foci of tumour cells. The technique has the added advantage of being able to yield high quality RNA from frozen specimens. This allows the construction of short stretches of cDNA or expressed sequence tags (ESTs) for each area analysed. The Cancer Genome Anatomy Project has amalgamated these two techniques and has begun to comprehensively characterise different lesions in a number of cancers.

Analysis of the various histological subtypes including normal breast, atypical hyperplasia, pure DCIS, DCIS with associated invasion, and invasive carcinoma, using these approaches will provide a wealth of information regarding the pattern of gene expression at each stage of the hypothetical progression of the disease. This will hopefully lead to the delineation of individual pathways of development with the identification of key genetic alterations for each stage in the natural history of the disease.

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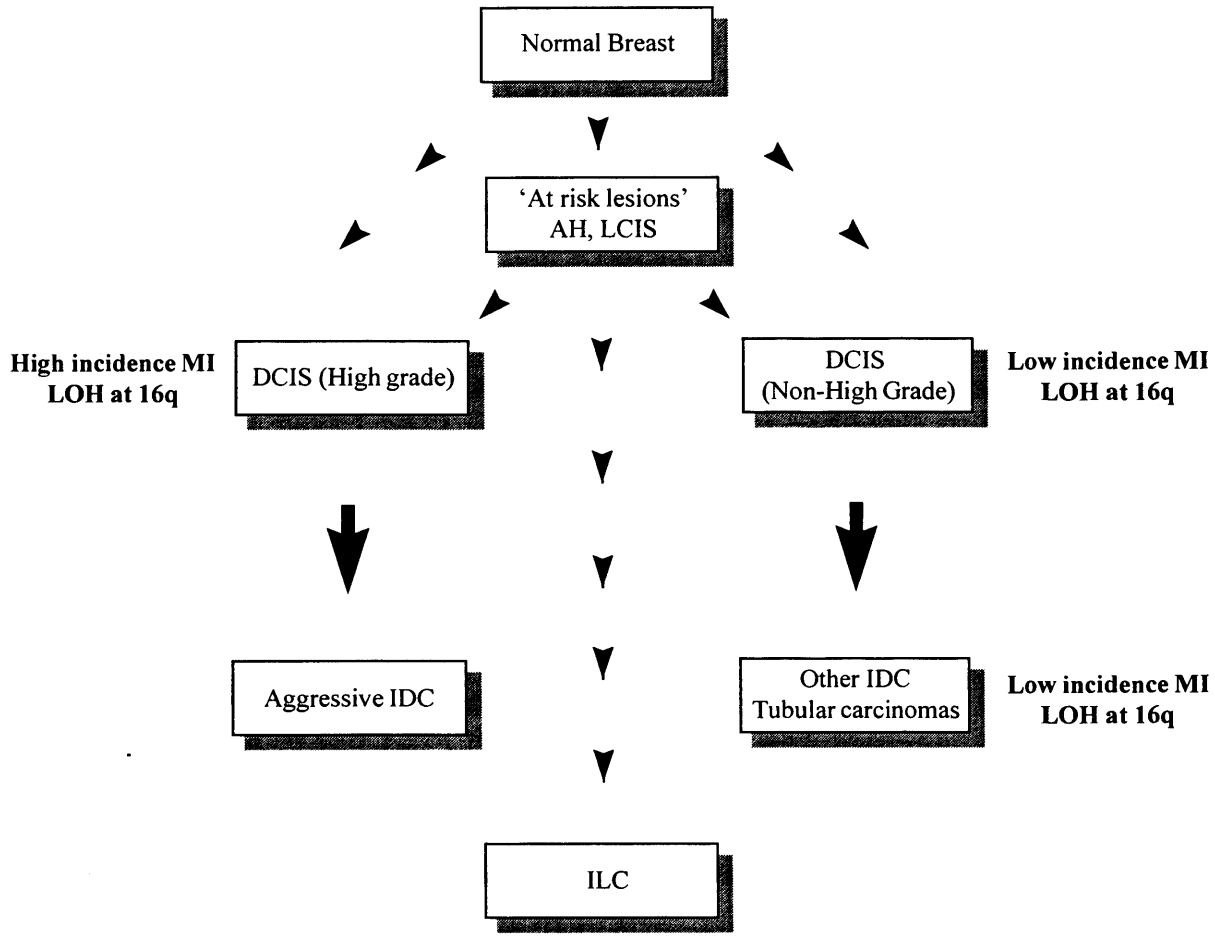

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Testing the tumour suppressive role of DMPK in breast cancer could be performed in culture using antisense techniques in cell culture. The human mammary epithelial cells (HMECs) represent a well studied immortalised 'non-tumourigenic' cell line. By transfecting an antisense DMPK construct into HMECs and breast cancer cell lines it will be

possible to assess the effects of transiently 'knocking-out' this gene. Assays which detect differences in cell doubling time, contact inhibition, and anchorage dependence can then be performed to determine whether decrease in DMPK expression leads to an increased growth and invasive capacity, as a prelude to the acquisition of a tumourigenic phenotype.

LOH And Tumour Suppressor Genes

Although LOH is considered as the hallmark of tumour suppressor gene in activation, there is no functional evidence to suggest that regions of chromosome 16q encode a tumour suppressor gene important in the development of breast cancer. Introduction of individual normal chromosomes in cancer cells by the process of microcell fusion-mediated transfer (MCMT) represents an alternative approach for the identification of chromosomes which contain tumour suppressor genes (Trent *et al.,* 1990). If chromosome 16q does contain a tumour suppressor gene (other than E-cadherin) then introduction of chromosome 16q into tumourigenic breast cancer cell lines should result in the generation of sublines with a suppressed phenotype. Structural analysis of the 16q segments retained in these hybrid sublines and non-suppressed sublines after fusion by MCMT may refine the location of the putative tumour suppressor gene(s).

Breast Cancer Development

The development of the laser capture microdissection technique (Emmet-Buck *et al.,* 1996) has greatly improved the accuracy and efficiency of procuring DNA template from small foci of tumour cells. The technique has the added advantage of being able to yield high quality RNA from frozen specimens. This allows the construction of short stretches of cDNA or expressed sequence tags (ESTs) for each area analysed. The Cancer Genome Anatomy Project has amalgamated these two techniques and has begun to comprehensively characterise different lesions in a number of cancers.

Analysis of the various histological subtypes including normal breast, atypical hyperplasia, pure DCIS, DCIS with associated invasion, and invasive carcinoma, using these approaches will provide a wealth of information regarding the pattern of gene expression at each stage of the hypothetical progression of the disease. This will hopefully lead to the delineation of individual pathways of development with the identification of key genetic alterations for each stage in the natural history of the disease.

a) Region 800-1050 of the E-cadherin (acc no. L34545) containing 3 HpaII sites. Primers designated EcadProm R/F flank these sites.

b) E-cadherin promoter region 840-990 and 840-940 for primer sets EcadM F/R and EcadU F/R respectively. Sequence differences between modified primers and unmodified DNA are emboldened and differences between methylated/bisulfide modified and unmethylated/bisulfide modified are underlined.

a)PCR Based E-cadherin Promoter Methylation Analysis

EcadProm F+R

```
                                          acc tagaccctag caactcc→
800 atcagaaccg tgcaggtccc ataacccacc tagaccctag caactccagg

                                        HpaII
850  ctagagggtc accgcgtcta tgcgaggccg ggtgggcggg ccgtcagctc

                                                    HpaII
900  cgccctgggg aggggtccgc gctgctgatt ggctgtggcc ggcaggtgaa

                                                HpaII
950  ccctcagcca atcagcggta cgggggggcgg tgctccgggg ctcacctggc


1000 gcagccacgc acccccctctc agtggcgtcg gaactgcaaa gcacctgtga
                                       ←cttgacgttt cgtggaca
```

b) MS-PCR E-cadherin Promoter Methylation Assay

EcadM F/R

```
         ttagg ttagagggtt atcgcgt→
840 caactccagg ctagagggtc accgcgtcta tgcgaggccg ggtgggcggg


890 ccgtcagctc cgccctgggg aggggtccgc gctgctgatt ggctgtggcc
                                                      ←cag

940 ggcaggtgaa ccctcagcca atcagcggta cgggggggcgg tgctccgggg
    ccatccactt aaaaatcaat
```

EcadU F/R

```
    taattttagg ttagagggtt attgt→
840 caactccagg ctagagggtc accgcgtcta tgcgaggccg ggtgggcggg

                                    ←ac acaacaataa ccaacac
890 ccgtcagctc cgccctgggg aggggtccgc gctgctgatt ggctgtggcc
```

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PUBLICATIONS AND ABSTRACTS

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Microsatellite Instability In Early Invasive Sporadic Breast Cancer
JA Shaw, T Walsh, SA Chappell, N Carey, K Johnson, RA Walker
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Microsatellite Instability In Ductal Carcinoma In Situ
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Loss of Heterozygosity At Chromosome 6q In Early Invasive And Preinvasive Breast Carcinomas
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Molecular Pathology Of Breast Cancer And Its Application To Clinical Management
RA Walker, JL Jones, S Chappell, T Walsh, JA Shaw
Cancer and Metastasis Reviews 16: 5-27 (1997)

Loss of heterozygosity at the mannose 6-phosphate/insulin-like growth factor 2 receptor gene correlates with poor differentiation in early breast carcinomas.
SA Chappell, T Walsh, JA Shaw, RA Walker
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ABSTRACTS

Microsatellite Instability In Ductal Carcinoma In Situ
T Walsh, SA Chappell, RA Walker, JA Shaw
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Microdissection-Microsatellite Analysis Of Early Invasive Breast Cancers - Lack Of Instability In Tubular Carcinomas
T Walsh, SA Chappell, RA Walker, JA Shaw
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Identification Of A Mutator Phenotype In Non-Invasive Breast Cancer
T Walsh, SA Chappell, RA Walker, JA Shaw
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Loss Of Heterozygosity At Chromosome 6q In Early Invasive Breast Cancer
SA Chappell, T Walsh, JA Shaw, RA Walker
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Loss Of Heterozygosity At Chromosome 6q In Non-Invasive Breast Cancer
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Microsatellite instability in early sporadic breast cancer

JA Shaw[1], T Walsh[1], SA Chappell[1], N Carey[2], K Johnson[3] and RA Walker[1]

[1]*Breast Cancer Research Unit, Department of Pathology, University of Leicester, Clinical Sciences, Glenfield General Hospital, Groby Road, Leicester LE3 9QP, UK;* [2]*Departments of Biochemistry and Surgery, Charing Cross and Westminster Medical School, Fulham Palace Road, London W6 8RF, UK;* [3]*Laboratory of Genetics, University of Glasgow, Pontecorvo Building, Anderson College, 56 Dumbarton Road, Glasgow G11 6NU, UK.*

Summary We have studied the incidence of microsatellite instability at three trinucleotide repeats and seven dinucleotide repeats from five chromosomal regions, in a group of 30 mammographically detected 'early' invasive breast cancers and correlated its occurrence with clinicopathological parameters. The myotonic dystrophy (DM-1) trinucleotide repeat was analysed in 48 additional cases. In 4 out of 78 (5%) paired tumour-normal DNA samples we found evidence of somatic microsatellite instability at DM-1: a novel allele of a different size was seen in the tumour DNA which was not present in the normal DNA sample. All four tumours that showed evidence of instability were from the core group of 30 cases (13%) and were well or moderately differentiated, oestrogen receptor-positive, infiltrating ductal carcinomas. Two of these tumours were unstable at nine of ten loci studied, both trinucleotide and dinucleotide repeats. DNA prepared from different normal tissues showed no evidence of instability, for all four instability cases. These data indicate that microsatellite instability is specific to the tumour DNA and is an early event in the genesis of some sporadic breast cancers.

Keywords: breast carcinoma; mammography; microsatellite instability

Breast cancer is a heterogenous disease, both clinically and with regard to the genetic alterations involved in tumorigenesis. Hence, multiple somatic and inherited genetic changes that lead to loss of growth control may contribute to the development of breast cancer. Despite notable recent advances, with the cloning of *BRCA1* (Miki *et al.*, 1994; Futreal *et al.*, 1994) and mapping of *BRCA2* (Wooster *et al.*, 1994*a*), there is no clear understanding of the natural history of the disease. This contrasts with colorectal carcinoma where extensive studies have identified a benign to malignant progression with recognisable molecular changes, frequently point mutations that involve proto-oncogene and tumour-suppressor gene loci (Fearon and Vogelstein, 1990).

Recently, a novel alteration based on DNA repeat misalignment mutagenesis has been described (Aaltonen *et al.*, 1993; Thibodeau *et al.*, 1993; Ionov *et al.*, 1993). This type of mutagenesis occurs in microsatellite DNA sequences in which one- to six-nucleotide motifs are tandemly repeated and are often highly polymorphic (Weber and May, 1989). Mono-, di- and trinucleotide repeats are unstable in hereditary non-polyposis colorectal cancer (HNPCC) as well as sporadic colorectal cancer cells. Germline mutations in four DNA mismatch repair genes, including *hMSH2* on chromosome 2p21-22, have been implicated as the cause of the hereditary non-polyposis syndrome and the associated microsatellite instability (Fishel *et al.*, 1993; Leach *et al.*, 1993). Microsatellite instability may reflect defective function of DNA mismatch repair genes and be manifested when both copies of a mismatch repair gene are inactivated (Parsons *et al.*, 1993).

If similar mismatch repair defects are involved in the relatively early stages of breast cancer, then microsatellite instability should be found in 'early' carcinomas and 'at risk' lesions. In a preliminary study we have detected somatic microsatellite instability at the myotonic dystrophy (DM-1) associated-CTG repeat in 'early' mammographically detected breast cancers (Shaw *et al.*, 1995). We now report our findings from analysis of ten polymorphic markers, three trinucleotide repeats and seven dinucleotide repeats in a group of 30 'early' sporadic breast cancers together with the analysis of DM-1 in a total of 78 cases. The markers analysed map to five chromosomal regions: 6p (SCA-1), 6q (ERTA and D6S193), 16q (D16S289, D16S400, D16S402, D16S413), 19q (DM-1 and X75b) and Xq (AR). The oestrogen receptor maps to 6q25 (Menasce *et al.*, 1993) and D6S193 maps to 6q27 (Saito *et al.*, 1992). The chromosome 16q markers span over 50 cM (Weissenbach *et al.*, 1992) and DM-1 and X75b reside within 90 kb of each other (Jansen *et al.*, 1992). We have analysed the frequency and type of microsatellite instability and correlated these data with clinicopathological findings. Since the markers studied are highly polymorphic concurrent assessment of allelic loss was also possible.

Materials and methods

Patients

A total of 78 invasive breast carcinomas which were impalpable and detected by mammography were studied. All were from the prevalent round of screening and were detected by the Leicestershire Breast Screening Service. Cases 15 mm or less in maximum diameter were examined. All had either axillary node sampling or axillary dissection. None of the tumours were from women with a strong family history of breast cancer.

Tissues

All tissues were fixed in 4% formaldehyde in saline for 18-36 h. After slicing, selected blocks were processed through graded alcohols and xylene to paraffin wax. Following review of haematoxylin and eosin stained sections representative blocks were chosen for further study. Additional normal tissue from hysterectomy specimens were retrieved from the pathology files of the Leicester Royal Infirmary. These were sampled and processed at different time periods to the original tumour material.

Histology

The carcinomas were reported according to the Royal College of Pathologists working party guidelines (1990). Infiltrating ductal carcinomas were graded using the modified Bloom and Richardson system (Elston and Ellis, 1991). All

Correspondence: JA Shaw, Department of Pathology, University of Leicester, Robert Kilpatrick Clinical Sciences Building, PO Box 65, Leicester Royal Infirmary, Leicester LE2 7LX, UK.
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histology was undertaken by RAW. The clinicopathological features are shown in Table I.

Oestrogen receptor

Oestrogen receptor was determined immunohistochemically using antigen retrieval and 1D5 monoclonal antibody (Dako) (Rajakariar and Walker, 1995).

DNA extraction

Formalin-fixed, paraffin-embedded tissue from breast tumour samples and non-involved lymph nodes served as the sources of tumour and normal DNA respectively. For each sample, DNA was extracted from 7 μm paraffin-embedded tissue sections or material prepared by microdissection. Briefly, sections were dewaxed and rehydrated by sequential addition, mixing and removal of 2 × 1 ml xylene, 2 × 1 ml 99% ethanol and 2 × 1 ml 95% ethanol. Air-dried pellets were resuspended in 250 μl proteinase K solution (1 mg ml^{-1} in 50 mM Tris HCl, pH 8.0, 1% sodium dodecyl sulphate), and incubated overnight at 37°C. Samples were then extracted twice with phenol–chloroform, precipitated with ethanol and resuspended in distilled water.

Table I Clinicopathological features of 78 'early' sporadic breast cancers

Type	*Grade*	*Number of cases*	*Tumour size (mm)*	*Number of cases*
Tub		13	<10	15
Lob/Tub		2	10	17
Idc/Ilc		3	11	4
Ilc		3	12	8
Idc	I	22 (2)	13	6
Idc	II	31 (1)	14	3
Idc	III	4	15	25
Total		78		78

Tub, tubular carcinoma; Lob/Tub, lobular and tubular carcinoma; Idc/Ilc, infiltrating ductal with infiltrating lobular carcinoma; Ilc, infiltrating lobular carcinoma; Idc, infiltrating ductal carcinoma; numbers in brackets, node-positive cases.

PCR analysis

Microsatellite repeats were analysed by polymerase chain reaction (PCR). Primer pairs and amplification conditions were as described in previous reports. Trinucleotide repeats comprised: DM-1 (Brook *et al*., 1992), SCA-1 (Orr *et al*., 1993) and AR (La Spada *et al*., 1991). Dinucleotide repeats were: X75b (Jansen *et al*., 1992), a (TA)n repeat in the upstream region of the human oestrogen receptor gene (ERTA) (Del Senno *et al*., 1992), D6S193 (Saito *et al*., 1992), D16S289 (Shen *et al*., 1992), D16S400, D16S402 and D16S413 (Weissenbach *et al*., 1992). The PCR products were labelled by the addition of 3 μCi of [α-^{35}S]dATP to the reaction. The labelled PCR products were electrophoresed through denaturing 6% polyacrylamide gels at 70 W for 1–3 h depending on the fragment size. Gels were dried and exposed to radiographic film for 1–4 days. Comparison of the migration of alleles from paired normal and tumour DNA samples that showed the appearance of alleles of altered length in tumour DNA served to indicate microsatellite instability. Where instability was detected, the analyses were repeated using freshly prepared DNA using adjacent sections prepared from the paraffin blocks. Allele sizes were estimated by comparison with a M13mp 18 DNA sequence ladder.

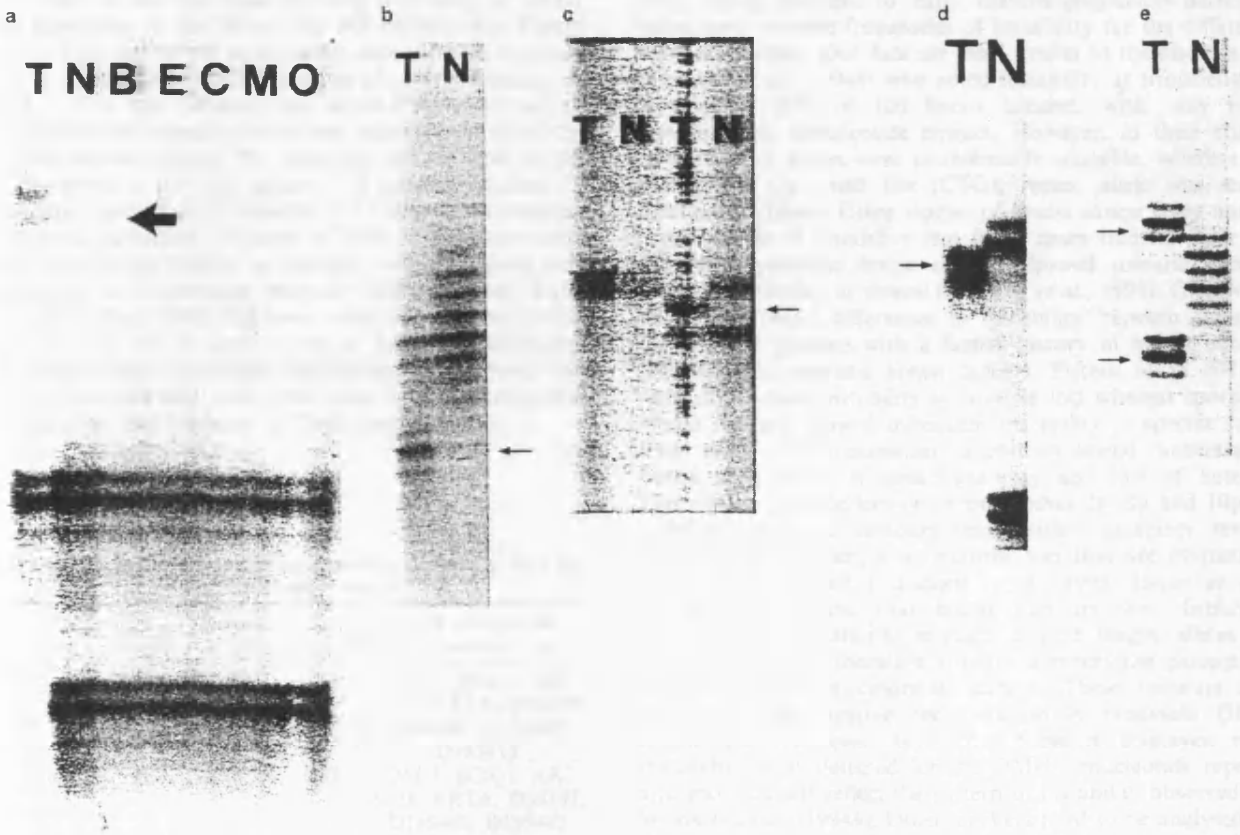

Figure 1 Microsatellite instability in early breast cancer. Genomic DNA samples from paired normal lymph node (N) and microdissected tumour (T) samples were compared by PCR amplification, electrophoresis on 6% sequencing gels and autoradiography. (a) Expansion at DM-1 in microdissected tumour from case 2 with analysis of six normal tissues: lymph node (N), normal breast (B), endometrium (E), cervix (C), myoendometrium (M) and ovary (O). (b) Contraction at X75b in case 1. (c) Contraction at AR in case 2. (d) Contraction at DM-1 in case 4. (e) Contraction at SCA-1 in case 1. Case numbers refer to Table II. Arrows indicate altered length alleles in tumour compared with normal tissue DNA indicating somatic microsatellite instability.

Results

Ten polymorphic microsatellite markers, three trinucleotide repeats and seven dinucleotide repeats, from five chromosomal regions were amplified from 30 tumour–normal DNA pairs using the PCR. The DM-1 (CTG) repeat was analysed through 48 additional tumour–normal DNA pairs.

The appearance of alleles of altered length in tumour DNA indicated an alteration in microsatellite size (Figure 1 and Table II). Microsatellite instability was maximally detected at the DM-1 trinucleotide repeat in four of 78 (5%) tumours. Two of these four tumours showed instability at nine loci, both trinucleotide and dinucleotide repeats. These data were replicated firstly with freshly prepared DNA samples from adjacent sections from the paraffin blocks and secondly with DNA samples prepared by microdissection of small areas of tumour within a section.

In order to verify whether these DNA changes are restricted to the tumour DNA, we next analysed DNA prepared from other normal tissues for these instability cases. DNA prepared from uninvolved breast, endometrium and cervix showed no evidence of microsatellite instability for all four cases. Figure 1a shows microsatellite instability at DM-1 in tumour 2. None of six normal tissues analysed (lymph node, histologically normal breast, endometrium, cervix, myoendometrium and ovary) showed any evidence of microsatellite instability, suggesting that instability is indeed specific to the tumour cell population.

For the DM-1 repeat, all of the novel alleles seen in tumour DNA lie within the normal population range, although the new allele sizes differed by up to 16 repeat units from the alleles seen in normal DNA. These data have been confirmed for two of the cases by cloning and sequencing of the altered length alleles (data not shown).

The incidence of microsatellite instability in tumour DNA was less frequent at the two other trinucleotide repeats studied. Two of the four cases showing instability at DM-1 showed instability at the SCA-1 and AR repeats (e.g. Figure 1c and e). The size of the novel alleles seen in these tumours lies within the normal population range of polymorphisms, as for DM-1. The two tumours that showed instability at all three trinucleotide repeats also showed instability at six of the seven dinucleotide repeats. No instability was detected at the D16S289 repeat in the core group of 30 tumours studied.

The clinicopathological features of the four cases showing microsatellite instability are listed in Table II. All were node-negative infiltrating ductal carcinomas, well or moderately differentiated and oestrogen receptor (ER) positive (Rajakariar and Walker, 1995). However, when we analysed DM-1 through a total of 78 cases, none of 53 other infiltrating ductal carcinomas, 13 tubular carcinomas, two tubular and lobular carcinomas and three infiltrating lobular carcinomas studied showed any evidence of DNA instability.

Table II Clinicopathological and microsatellite instability data for early sporadic breast cancers

Case	*Type*	*Grade*	*Tumour size (mm)*	*ER H-score*	*MSI detected at markers*
1	Idc IIc	II	15	215	DM-1, SCA-1, AR, X75b, ERTA, D6S193, D16S400, D16S402, D16S413
2	Idc	II	15	183	DM-1, SCA-1, AR, X75b, ERTA, D6S193, D16S400, D16S402 D16S413
3	Idc	II	11	194	DM-1
4	Idc	I	14	231	DM-1

Idc IIc, infiltrating ductal with infiltrating lobular carcinoma; Idc, infiltrating ductal carcinoma; ER, oestrogen receptor; MSI, microsatellite instability. All cases node negative.

Discussion

Expansion of specific trinucleotide repeats was first noted in several heritable neuromuscular diseases including fragile X syndrome, myotonic dystrophy and Huntington's disease (Miwa, 1994). All these repeats are polymorphic in normal populations as a result of variation in the number of trinucleotide repeat units. Although instability of these repeats is a feature of expanded disease-specific alleles, 'smearing' of the signal from a single allele of trinucleotide repeat genes has not been reported in normal individuals, indicating that somatic microsatellite instability is uncommon in the normal population.

We have detected somatic microsatellite instability at the DM-1 (CTG)n repeat in four of 78 (5%) 'early' sporadic breast cancers. Two tumours showed instability at multiple loci: the DM-1, SCA1 and AR trinucleotide repeats and six of seven dinucleotide repeats. It seems unlikely that the instability seen in breast tumours represents a random background instability for this reason. Analysis of DNA samples prepared from different normal tissues (uninvolved breast, cervix and uterus) showed no evidence of microsatellite instability for the four instability cases. These data provide firm evidence that the instability seen was specific to the breast tumour DNA. Parsons *et al.* (1995) reported recently that rare cells in a normal tissue population from HNPCC patients may harbour microsatellite alterations. Our data from analysis of different normal tissues do not conflict with this finding. The analysis of total DNA prepared from a 7 μm normal tissue section would not be sufficiently sensitive to detect a rare variant normal cell and microsatellite instability in these breast cancers may have arisen by a different mechanism to that seen in HNPCC.

Our data show a lower level of microsatellite instability (5%) than other published reports. This may reflect differences between the groups of tumours studied, with our study being restricted to 'early' mammographically detected cases, and variable frequencies of instability for the different markers studied. Our data are most similar to the findings of Wooster *et al.* (1994*b*) who noted instability at trinucleotide repeats in 10% of 100 breast cancers, with only rare instability at dinucleotide repeats. However, in their study larger DM-1 alleles were preferentially unstable, whereas in our group the small five $(CTG)_n$ repeat allele was most frequently altered. Other studies of breast cancer have noted higher levels of instability, but fewer cases studied. Four of 20 (20%) sporadic breast cancers showed somatic microsatellite instability at several loci (Yee *et al.*, 1994). Glebov *et al.* (1994) noted differences in instability between tumour DNA from patients with a family history of breast cancer (FHBC) and sporadic breast cancers. Fifteen of 18 FHBC tumours showed instability at multiple loci whereas sporadic breast cancers showed infrequent instability at specific loci. Patel *et al.* (1994) examined 13 primary breast cancers and noted high levels of both instability and loss of heterozygosity for specific loci on chromosomes 2p, 8p and 10p.

Most colorectal tumours that display instability reveal alleles of altered length at multiple loci that are frequently dinucleotide repeats (Aaltonen *et al.*, 1993; Ionov *et al.*, 1993). Two of the four breast tumours that displayed microsatellite instability, revealed altered length alleles at multiple loci and therefore appear to reflect the pattern of instability seen in colorectal cancer. These tumours are worthy of investigation for mutation in candidate DNA repair loci. However, two other tumours displayed rare instability, only detected for the DM-1 trinucleotide repeat, and more closely reflect the pattern of instability observed by Wooster *et al.* (1994*b*). Other markers need to be analysed in these cases to confirm whether the instability is indeed restricted to specific trinucleotide repeat loci.

The variation in frequency of instability seen for the ten repeats studied imply that some loci may be more unstable than others. For our trinucleotide repeat data, DM-1 appears to be a more 'sensitive' locus than either AR or SCA-1 for

studying microsatellite instability. This appears not to be a function of repeat length, as the number of repeats at the AR locus for example tends to be longer than at DM-1. Six of seven dinucleotide repeats studied showed evidence of instability in one tumour and again the variation in frequency appears not to be a function of repeat length. These data suggest that some chromosomal regions are more unstable than others. Both chromosome 6q (Devilee *et al.*, 1991) and 16q (Sato *et al.*, 1991) have been shown previously to harbour areas of loss of heterozygosity in breast cancer. Our data provide other evidence for genomic instability in these chromosomal regions and for specific trinucleotide repeats on 6p, 19q and Xq in breast cancer. Any structural perturbation of these chromosomal regions may alter the function of gene(s) harboured on the specific chromosomes.

The DNA instability observed in the four breast cancers could be a manifestation of errors in DNA repair as has been found for HNPCC (Fishel *et al.*, 1993; Leach *et al.*, 1993). The relaxed genome stability, observed as microsatellite instability, could be initiated by alteration of genes involved in either DNA replication or repair and would be an early event in carcinogenesis (Loeb, 1994). Such unstable cancer cell genomes could promote a cascade of mutations some of which enable the cancer cells to bypass the host regulatory process. Similarly the allele instability observed in our series of 'early' breast cancers may be a sensitive indicator of genomic hypermutation in these tumours. Although the DM-1 and AR microsatellites are expressed, it is unlikely that these loci themselves contribute to the development of breast cancer since all of the unstable alleles lie well within the normal population range and their sizes are common in the normal population. However, different length repeat alleles, even within the normal range, may have subtle influences on cellular metabolism, which may manifest in breast cancer.

The clinicopathological features of the four breast tumours that display instability were examined for possible correlation with this phenotype. All were infiltrating ductal carcinomas, well or moderately differentiated, and node-negative. Oestrogen receptor was detected in all at moderate to high levels (Rajakariar and Walker, 1995). No evidence of instability at DM-1 was detected for 13 tubular, two mixed lobular and tubular cases and three infiltrating lobular carcinomas from a total of 78 tumours that were screened. Linell *et al.* (1980) have suggested that tubular carcinomas may progress to less differentiated carcinomas if left untreated, and the tubular mixed carcinomas described by Ellis *et al.* (1992) may lend support to this. If this is the case, our findings would suggest either that instability occurs at a particular stage of development and progression or only with certain pathways of development and progression.

In summary, we have detected somatic microsatellite instability in 5% of 78 'early' sporadic breast cancers. These data for 'early' breast cancers support the suggestion that microsatellite instability may be an early event in the genesis of some sporadic breast cancers (Yee *et al.*, 1994). Moreover, our data demonstrate that instability is not found between different normal tissues from the same individual, but appears to be specific to DNA prepared from within a tumour. An extended study on a larger range of lesions including additional tubular and lobular carcinomas, cases of ductal carcinoma *in situ* and 'at risk' lesions (e.g. florid and atypical hyperplasia) will be important to verify these observations and to determine the role of these DNA changes in the natural history of breast cancer.

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Loss of heterozygosity at chromosome 6q in preinvasive and early invasive breast carcinomas

SA Chappell, T Walsh, RA Walker and JA Shaw

Breast Cancer Research Unit, Department of Pathology, University of Leicester, Clinical Sciences Building, Glenfield General Hospital, Groby Road, Leicester LE3 9QP, UK

Summary We have used polymerase chain reaction (PCR) analysis to study the incidence of allelic imbalance at four polymorphic microsatellite markers on chromosome 6q25.1–27, three dinucleotide repeats and one trinucleotide repeat, for microdissected tumour foci from a group of 75 'early' breast carcinomas. The tumours comprised 16 preinvasive cases of ductal carcinoma in situ (DCIS) and 59 mammographically detected early invasive carcinomas. Loss of heterozygosity (LOH) was detected at all four loci and in all types and grade of disease. The frequency of LOH ranged from 23% to 50% depending on the marker studied. The highest frequency of LOH was observed at the D6S186 locus for the cases of DCIS and at the oestrogen receptor locus for the invasive carcinomas. These data suggest that the inactivation of tumour-suppressor genes within this region on chromosome 6q is important for the development of these early lesions.

Keywords: breast carcinoma; mammography; loss of heterozygosity; tumour-suppressor genes

According to the multistep model of carcinogenesis, tumours may develop and progress as a result of alterations in oncogene and tumour-suppressor gene loci. In colon cancer, a benign to malignant progression with recognizable molecular changes has been described (Fearon and Volgelstein, 1990). However, the situation for breast cancer is less clear, since there is no clear understanding of the natural history of the disease.

Cytogenetic analyses of primary breast tumours have identified frequent alterations to a number of chromosomes, notably deletions, suggesting the potential localization of tumour-suppressor genes (for a review, see Devilee and Cornelisse, 1994). These studies demonstrated that deletion of chromosome 6q was one of the most frequent chromosomal changes (Dutrillaux et al, 1990; Mars and Saunders, 1990). A subsequent study, using Southern analysis of restriction fragment length polymorphisms to compare constitutional and tumour DNAs, identified chromosome 6q as the second most frequent site for allelic loss (loss of heterozygosity) after 17p in breast cancer (Deville et al, 1991).

Other evidence for the presence of putative tumour-suppressor genes on chromosome 6q comes from chromosome-mediated transfer experiments of normal chromosome 6 into melanoma cell lines (Trent et al, 1990), uterine endometrial cell lines (Yamada et al, 1990) and the breast cancer cell lines, MDA-MD231 and MCF-7 (Negrini et al, 1994), all resulting in the suppression of tumorigenesis.

The advent of polymerase chain reaction (PCR) analysis of microsatellite polymorphisms (Weber and May, 1989) has confirmed the cytogenetic evidence for chromosomal deletion at 6q and has enabled construction of a more detailed deletion map. Allelic loss at 6q24–27 has been observed in different tumour types, including breast carcinoma (Orphanos et al, 1995), ovarian carcinoma (Saito et al, 1992; Rodabaugh et al, 1995), hepatic carcinoma (De Souza et al, 1995), small-cell lung carcinoma (Merlo et al, 1994), renal cell carcinoma (Morita et al, 1991), malignant melanoma (Millikin et al, 1991; Walker et al, 1994) and non-Hodgkin's lymphoma (Menasce et al, 1994). The reported frequencies of allelic loss range from 30% to 60% depending on the tumour types and markers studied. This shared region of allelic loss may harbour putative tumour-suppressor genes that are pleiotropic for these tumour types and reflect a common mechanism of tumorigenesis.

Recent detailed analyses of microsatellite markers on chromosome 6q in breast cancers have highlighted two key regions showing high levels of LOH at 6q13 and 6q26–27, indicating the presence of at least two tumour-suppressor genes (Devilee et al, 1991; Orphanos et al, 1995). Since these studies were concerned with symptomatic, well-established breast carcinomas, it is not clear whether allelic loss on chromosome 6q is an early event in the development of breast cancers. Small, mammographically detected breast cancers form a useful group for study of the involvement of tumour-suppressor genes in tumour development and earlier stages of progression. In this report, we examined LOH at the more distal region, chromosome 6q25–q27, using four polymorphic microsatellite markers, in a group of 75 'early' lesions comprising 59 mammographically detected invasive carcinomas and 16 preinvasive lesions of ductal carcinoma in situ (DCIS).

The markers span a chromosomal region of approximately 15 Mb. Two of the markers (D6S186 and D6S193) were analysed previously in well-established carcinomas (Orphanos et al, 1995). The two other markers studied comprise repeats at or within coding sequences that might be candidates for 'early' mutations in breast cancer: a $(TA)_n$ repeat positioned 1 kb upstream of the oestrogen receptor gene (ESR) (Del Senno et al, 1992) and a $(CAG)_n$ repeat within the human TATA box-binding protein (TBP) (Polymeropoulos et al, 1991). We have analysed the frequency of LOH in the two groups of cases and correlated these data with oestrogen receptor (ER) and progesterone receptor (PR) status and other clinicopathological findings.

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Correspondence to: JA Shaw

Table 1 Clinicopathological features of 59 mammographically detected early invasive breast carcinomas

Type	Grade	Number of cases	Tumour size (mm)	Number of cases
Tub	I	6	< 10	10
Lob/tub	I	1	10	14
IDC/ILC	II	1	11	4
ILC	II	1	12	5
IDC	I	17 (1)	13	3
IDC	II	29 (2)	14	2
IDC	III	4	15	21
Total		59		59

Tub, tubular carcinoma; Lob/tub, lobular and tubular carcinoma; IDC/ILC, infiltrating ductal with infiltrating lobular carcinoma; ILC, infiltrating lobular carcinoma; IDC, infiltrating ductal carcinoma. Numbers in brackets, node-positive cases.

MATERIALS AND METHODS

Patients

A total of 59 invasive breast carcinomas that were impalpable and detected by mammography were studied. All were from the prevalent round of screening and were detected by the Leicestershire Breast Screening Service. Cases of 15 mm or less in maximum diameter were examined. All had either axillary node sampling or axillary dissection. None of the tumours were from women with either a strong family history of breast cancer or any known inherited predisposition to the development of tumours. Some 56 cases were node negative.

A total of 16 cases of pure ductal carcinoma in situ (DCIS) were studied. These comprised three low, three intermediate and ten high nuclear grade cases. Ten of these were mammographically detected and six were clinically presenting.

Tissues

All tissues were fixed in 4% formaldehyde in saline for 18–36 h. After slicing, selected blocks were processed through graded alcohols and xylene to paraffin wax. Following review of haematoxylin and eosin-stained sections, representative blocks were chosen for further study. Tissue from histologically normal lymph node served as the source of normal DNA.

Histology

All carcinomas were reported according to the NHS Breast Screening Programme National Coordinating Group for Breast Screening Pathology Guidelines (1995). Infiltrating ductal carcinomas were graded using the modified Bloom and Richardson system (Elston and Ellis, 1991). Cases of DCIS were graded as low, intermediate or high nuclear grade. All histology was undertaken by RA Walker. The clinicopathological features of the invasive carcinomas are shown in Table 1.

Oestrogen receptor and progesterone receptor immunohistochemistry

Avidin–biotin complex peroxidase immunohistochemistry was carried out for the 59 early invasive carcinomas as described

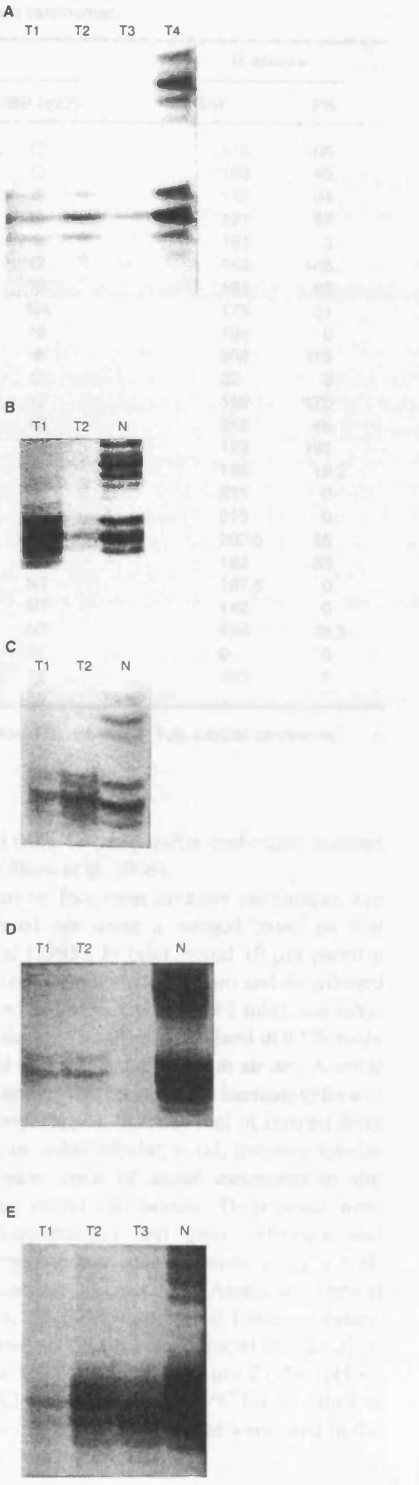

Figure 1 Loss of heterozygosity in early breast cancer. Genomic DNA samples from paired normal lymph node (N) and microdissected tumour (T) samples were compared by PCR amplification, electrophoresis on 6% sequencing gels and autoradiography. (**A**) LOH at D6S193 in case 37: (T1–T3) solid component of infiltrating ductal carcinoma. (**B**) LOH at D6S186 in case 21: (T1 and T2) solid component of infiltrating ductal carcinoma. (**C**) LOH at D6S193 in case 102: (T1 and T2) solid component of infiltrating ductal carcinoma. (**D**) LOH at D6S186 in DCIS case D5: (T1 and T2) individual ducts. (**E**) LOH at ERTA in case 57: (T1) area of DCIS, (T2) tubular component, (T3) tubular component

Table 2 Pattern of loss of heterozygosity observed using four microsatellite markers from 59 early invasive breast carcinomas

Case no.	Type	Grade	Loss of heterozygosity at markers				H scores	
			ESR (q25.1)	D6S186 (q26)	D6S193 (q27)	TBP (q27)	ER	PR
3	ILC	II	●	○	NI	○	175	106
7	IDC	I	●	NI	○	○	182	49
13	Tub	I	○	○	NI	●	142	94
15	IDC	I	●	NI	NI	○	181	97
17	Tub	I	○	○	NI	●	195	3
19	IDC	II	○	NI	●	○	162	168
21	IDC	II	●	●	●	NI	151	92
23	IDC	I	●	●	NI	NA	175	31
29	Lob/tub	I	●	NI	○	NI	196	0
31	IDC	I	MSI	NI	NI	●	202	115
37	IDC	II	NI	○	●	○	22	3
41	IDC	II	NI	○	●	○	192	170
49	IDC	II	NI	●	NI	NI	215	46
57	IDC	I	●	NI	○	NT	173	192
59	IDC	I	●	●	●	NI	196	19.2
70	IDC	I	●	NI	NA	NI	231	0
76	IDC	I	●	NI	NA	NI	219	0
78	IDC	I	NI	●	○	NT	202.5	85
80	Tub	I	NI	NI	●	NI	162	83
98	IDC	II	●	●	●	NT	187.5	0
102	IDC	II	●	●	●	NT	142	0
106	IDC	II	○	NI	●	NT	195	33.5
108	IDC	II	●	NI	●	NI	0	0
122	IDC	II	NI	NI	●	NI	160	0

●, loss of heterozygosity; ○, heterozygosity; MSI, microsatellite instability; NI, not informative; NA, no amplification; NT, not tested, Tub, tubular carcinoma; Lob/tub, lobular and tubular carcinoma; ILC, infiltrating lobular carcinoma; IDC, infiltrating ductal carcinoma.

Table 3 Pattern of loss of heterozygosity observed using three microsatellite markers from 16 preinvasive lesions of DCIS

Case no.	Grade	Chromosome 6q markers			
		ESR (q25.1)	D6S186 (q26)	D6S193 (q27)	TBP (q27)
D2 (M)	High	NA	●	NI	NT
D3 (M)	Low	●	NI	○	NT
D4 (M)	Low	NI	●	●	NT
D5 (M)	High	●	●	●	NT
D8 (M)	Low	NI	NI	●	NT
D12 (C)	High	NI	●	●	NT
D13 (C)	High	●	NI	○	NT
D14 (C)	High	NI	NI	●	NT

●, loss of heterozygosity; ○, heterozygosity; NI, not informative; NA, no amplification; NT, not tested; (M), mammographically detected; (C), clinically presenting.

previously (Rajakariar and Walker, 1995) with minor modifications. For antigen retrieval pretreatment, sections were exposed to two cycles of microwaving for oestrogen receptor [mouse monoclonal ID5 (Dako)] and progesterone receptor [mouse monoclonal NCL-PgR (NovaCastra)].

DNA extraction and microdissection from paraffin embedded sections

Formalin-fixed, paraffin-embedded tissue from breast tumour samples and non-involved lymph nodes served as the source of tumour and normal DNA respectively. For each tumour–normal pair, DNA was extracted from 10-μm paraffin-embedded sections as described previously (Shaw et al, 1996).

Microdissection of tumour foci from invasive carcinomas and areas of DCIS was carried out using a method based on that described by Koreth et al (1995). In brief, serial 10-μm paraffin sections were deparaffinized in xylene (2 × 5 min) and dehydrated in 99% ethanol (2 × 2 min) and 95% ethanol (1 × 2 min), and rehydrated in water before staining. Tissues were stained in 0.5% eosin solution for 20 s, washed in water and allowed to air dry. A serial reference slide for each tumour was stained with haematoxylin and eosin, dehydrated and coverslipped. Tumour foci of interest from the invasive carcinomas included tubular, solid, invasive lobular components and preinvasive areas of ductal carcinoma in situ (DCIS) within infiltrating ductal carcinomas. These areas were visualized using the haematoxylin and eosin reference and microdissected from corresponding eosin sections using a × 40 magnification microdissection microscope (American Optical Corporation) using sterile, 20-μl drawn-out glass Pasteur pipettes. Tumour foci (approximately 100 cells) were placed into 25 μl of digestion buffer [100 mM Tris-HCl (pH 7.6), 1 mM EDTA (pH 8), 200 μg ml^{-1} proteinase K] and incubated at 55°C for 3 h, then at 94°C for 10 min. Volumes (2 μl) of this mixture were used in the PCR analysis.

PCR analysis at 6q25.1–27

A total of 75 'early' breast carcinomas were studied for LOH at four polymorphic markers from chromosome 6q25.1–27: the oestrogen receptor (ESR) at 6q25.1 (Del Senno et al, 1992), D6S186 (6q26) and D6S193 (6q27) (Saito et al, 1992; Orphanos et al, 1995) and the TATA box-binding protein (TBP) gene at 6q27

(Polymeropoulos et al, 1991; Saito et al, 1994). PCR reaction conditions were as follows: 45 mM Tris-HCl, pH 8.8, 11 mM ammonium sulphate, 4.5 mM magnesium chloride, 200 μM dTTP, dCTP and dGTP, 25 μM dATP (Pharmacia, UK), 0.3 μl [α-^{35}S]deoxyadenosine-5'-triphosphate (600 Ci mmol^{-1}, 10 mCi ml^{-1}; ICN Pharmaceuticals, UK), 113 μg ml^{-1} bovine serum albumin (Boehringher Mannheim), 6.7 mM β-mercaptoethanol, 4.4 μM EDTA, pH 8.0, 10 pmol of forward and reverse primers, 2 μl of microdissected DNA and 1 unit *Taq* DNA polymerase (Gibco BRL, UK) in a total volume of 25 μl. Hot-start PCR was carried out using the following cycles: 5 min denaturation at 94°C followed by 30 (40 for microdissections) cycles of 1 min denaturation at 94°C, 1 min annealing and 1 min extension at 72°C with a final extension of 7 min at 72°C on a DNA Thermal Cycler (Perkin Elmer Cetus, UK). Analysis of PCR products was as described previously (Shaw et al, 1996).

Detection of LOH

Autoradiographs were scored independently by two individuals (SC and JS) and the results compared. All examples of LOH were confirmed by microdissection analysis to prepare multiple tumour foci and then by repeating the PCR analysis where possible.

RESULTS

A total of 59 early invasive breast tumours and 16 preinvasive lesions of DCIS were screened for LOH with four polymorphic microsatellite markers, mapping to chromosome 6q25.1–q27. LOH was considered to be present when the constitutive tissue DNA was heterozygous (informative) for the locus under investigation, and where there was complete or > 50% loss of one allele in the corresponding tumour DNA as estimated by visual inspection. The complex heterogeneity of the disease and the presence of non-tumour cells can mask LOH, therefore all analyses were confirmed using DNA prepared by microdissection from different histological tumour foci within the same tumour section (Figure 1). The use of microdissected tumour material produced almost complete allelic loss (Figure 1), such that densitometric analysis of the data was not considered necessary.

For example, Figure 1 B, C and D shows two invasive carcinomas and one case of DCIS that all exhibit complete LOH for two separate microdissected foci at the markers studied. The tumours analysed in Figure 1A and E show some evidence of heterogeneity with variation between different microdissected foci. For example Figure 1E is an infiltrating ductal carcinoma grade I that exhibits LOH at ERTA. Analysis of three distinct microdissected foci shows an area of in situ carcinoma with LOH (T1), a tubular component with heterogeneity of LOH (T2) and a second tubular component with complete LOH (T3). This discrepancy could be attributable to the presence of contaminating non-neoplastic stromal cells in the tubular component, even when it was dissected away from normal tissue. Although microdissection analysis revealed occasional heterogeneity of distinct structural components, e.g. in situ, solid or tubular lesions within a tumour section, with some foci showing clear LOH and others showing no evidence of LOH, no clear correlation was seen between specific structural components and LOH at any particular locus.

Table 2 and 3 summarize the observed patterns of LOH at 6q25.1–q27 for the invasive and preinvasive study groups respectively. LOH was seen for all types and grades of disease studied. Altogether, 24 of 59 invasive carcinomas (48%) showed evidence of LOH. Of these, 17 exhibited LOH only at a single locus (Table 2) and one tumour (case 31) also showed clear microsatellite instability at ESR. The situation was similar for the cases of DCIS with eight of 16 cases (50%) showing evidence of LOH and five of these only exhibiting LOH at a single locus (Table 3). The frequency of LOH at individual markers ranged from 23% to 40.6% for the early invasive cases and from 33.3% to 50% for the DCIS group (Table 4). The highest frequency of LOH was observed at the ESR locus for the invasive carcinomas and at the D6S186 locus for the cases of DCIS. LOH was observed in both high- and low-grade DCIS. The cases of DCIS were not studied for LOH at the TBP marker owing to the paucity of available material for study.

In addition, the 59 early invasive carcinomas were studied for oestrogen receptor and progesterone receptor status by immunohistochemistry (Table 2). In all, 53 (90%) were oestrogen receptor positive and 27 (46%) were progesterone receptor positive. Thirteen of the early invasive carcinomas showed LOH at the ESR locus. Of these, 12 were ER positive (92%) and four were PR positive (31%) by immunohistochemistry (Table 2). Therefore, LOH at ESR is not necessarily reflected in negative values for ER and/or PR. There was no significant relationship between LOH at ESR and either ER or PR status.

DISCUSSION

Using microdissection of distinct structural components from within a tumour tissue section and PCR amplification of microsatellite repeats, we have demonstrated loss of heterozygosity (LOH) at chromosome 6q25.1–27 in foci of both DCIS and 'early' invasive carcinomas. Comparing the proportion of in situ lesions with the proportion of invasive lesions exhibiting LOH at each locus revealed similar frequencies. Moreover, there was a general spread of LOH detected for all types and grades of disease studied. These data for the 'early' carcinomas suggest that the majority of allele losses previously reported at these loci in symptomatic

Table 4 Summary of chromosome 6q LOH data in early invasive tumours and preinvasive cases of DCIS

Locus	Chromosomal location	No. of cases tested		No. of informative cases (%)		No. of cases with LOH (%)	
		Invasive	DCIS	Invasive	DCIS	Invasive	DCIS
ESR	6q25.1	57	15	32 (56)	9 (60)	13 (40.6)	3 (33.3)
D6S186	6q26	54	16	23 (42.6)	8 (50)	7 (30.4)	4 (50)
D6S193	6q27	57	16	35 (61.4)	12 (75)	11 (31.4)	5 (41.7)
TBP	6q27	28	–	13 (46)	–	3 (23)	–

invasive cancers (Orphanos et al, 1995; Iwase et al, 1995) can be found in preinvasive carcinomas. This suggestion is supported by evidence from a small number of the infiltrating ductal carcinomas (e.g. Figure 1) in which it was possible to analyse an invasive and in situ component from the same tumour section. In each case, LOH was detected in both lesions. In combination, these data suggest that loss of alleles on chromosome 6q is an early event in the progression of malignancy in the breast.

Although the highest frequency of LOH was observed at the ESR locus (40.6%) for the invasive carcinomas and at the D6S186 locus (50%) for the cases of DCIS, these differences may merely reflect the different groups of cases studied and the difference in sample size between the two groups. In addition, the slightly higher frequency of LOH noted for the cases of DCIS may reflect the fact that most were of high nuclear grade, and therefore a more aggressive disease type. It is interesting to note that LOH was found for all three markers studied in both high- and low-grade DCIS, suggesting the early involvement of loci on 6q in the development of these lesions. In a study of chromosome 1, differences were found between chromosomal regions for the different subtypes of DCIS with no alteration at two regions in low-grade DCIS (Munn et al, 1995).

The prevalent detection of LOH at a single locus rather than multiple loci in both the 'early' invasive carcinomas and cases of DCIS argues against random losses resulting from general chromosomal instability and gross chromosomal alterations. Some invasive carcinomas and cases of DCIS showed LOH at more than one locus. Since the markers studied map from 6q25–27, a distance of several megabases, it is not possible to say whether a contiguous region harbouring the relevant loci has been lost, or whether there are distinct areas of LOH within this region of chromosome 6q.

Knowledge of the ER status of a carcinoma is of value in aiding prediction of hormone responsiveness and can provide some prognostic information. Tumours lacking ER and PR generally grow faster than those containing both ER and PR (McGuire and Clark, 1989). Overall, 46.1% of informative cases for the invasive carcinomas exhibited LOH at the oestrogen receptor locus (ESR), and 33% of the informative cases of DCIS showed LOH at ESR. These frequencies are higher than the 19% LOH at ESR observed by Iwase et al (1995) and may reflect different groups of cases, or might be due to the more informative analysis of material prepared by microdissection in this study. The high incidence of LOH at the ESR locus in the invasive carcinomas was not reflected by loss of ER as detected by immunohistochemistry. Indeed, the majority of the group of early invasive lesions were ER positive. This would be expected, since the group studied was predominantly well or moderately differentiated. Evolving tumour cells may later acquire new proliferative pathways as a consequence of multiple genetic alterations, enabling the tumour cells to bypass oestrogen-dependent proliferation (Liu et al, 1988).

Other studies have found no relationship between LOH on chromosome 6q and ER status (Devilee et al, 1991; Iwase et al, 1995), suggesting that allele loss may not play an important role in the lack of ER function in breast cancer tissues. However, our results have identified nine of 13 tumours exhibiting LOH at ESR that were ER positive and PR negative. This might indicate inactivation of the remaining ESR allele leading to production of an inactive but detectable ER protein, and hence loss of PR. These cases are candidates for screening for either mutations or spliced variants of the oestrogen receptor gene. The identification of spliced variants would seem most likely, since ER-positive/PR-negative phenotype breast tumours were shown by Fuqua et al (1993) to contain a variant ER (missing exon 7) that was unable to function as a transcriptional inducer of PR expression.

Only three invasive carcinomas showed evidence of LOH at the TBP locus (23%), at 6q27. This frequency of LOH is only marginally raised above expected levels for random background loss. These data suggest that inactivation of this region of the chromosome is of lesser importance than of that harbouring the ESR, D6S186 and D6S193 loci in these early lesions.

The mannose 6-phosphate/insulin-like growth factor 2 receptor (M6P/IGFr) functions in the intracellular trafficking of lysosomal enzymes, the degradation of IGF2, a mitogen often overproduced in tumours (Kornfeld, 1992), and the activation of the potent growth inhibitor, transforming growth factor β (Dennis and Rifkin, 1991). Some 70% of human hepatocellular carcinomas show LOH at this locus, which maps to chromosome 6q26–27 (Laureys et al, 1988), and 25% of these show point mutations in the remaining allele (De Souza et al, 1995). Clearly, M6P/IGFr might be inactivated in breast cancers also. Recently, Hankins et al (1996) reported point mutations in two comedo-type (high-grade) DCIS cases, suggesting that this is a candidate tumour-suppressor gene in some breast cancers. Our preliminary analyses of this locus have shown no evidence of LOH in the well to moderately differentiated invasive carcinomas suggesting that inactivation of M6P/IGFr is not common in these tumours (manuscript in preparation). In combination, these data suggest that inactivation of M6P/IGFr may occur only within certain more aggressive subgroups (poorly differentiated cases) of breast cancers. The frequent LOH that we have detected at 6q25.1–q27 might, therefore, be caused by inactivation of other tumour-suppressor genes on chromosome 6q as well as M6P/IGFr.

In summary, we have detected frequent LOH at three polymorphic loci from chromosome 6q25.1–27 in cases of both high- and low-grade DCIS and all types and grades of early invasive carcinomas. In combination, these data confirm distal chromosome 6q as a major site for genetic change in the early stages of development of some sporadic breast cancers, and form the starting point to identify the corresponding genes.

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Molecular pathology of breast cancer and its application to clinical management

Rosemary A. Walker, J. Louise Jones, Stephen Chappell, Tom Walsh and Jacqueline A. Shaw
Department of Pathology, University of Leicester, Breast Cancer Research Unit, Clinical Sciences, Glenfield Hospital NHS Trust, Groby Road, Leicester LE3 9QP, UK

Key words: breast cancer, genetic analysis, steroid receptor, epidermal growth factor receptor, cell adhesion, proteases

Abstract

Breast cancer is a major cause of morbidity and mortality in women in many parts of the world. Breast carcinomas are heterogenous in their biological and clinical behaviour and a greater understanding of how they develop and progress could lead to more directed forms of screening and therapy. It is important to determine the molecular mechanisms underlying the natural history of breast cancer.

Developments in the techniques for molecular analysis have meant that they can now be applied to a large range of clinical material such as cytological preparations and fixed, embedded material, so increasing the potential for relating any molecular alterations to clinical behaviour and response to therapy.

In this review we consider recent developments in three areas of importance to breast cancer: genetic analysis – oncogenes, tumour suppressor genes, loss of heterozygosity, microsatellite instability, familial breast cancer; steroid receptors, oestrogen regulated proteins, epidermal growth factor receptor, growth factors particularly transforming growth factor beta; and cell adhesion, invasion and metastasis – E-cadherin, integrins, proteases. These are discussed in relation to potential for screening, prognosis and treatment.

Introduction

Breast carcinoma is the most common malignancy in women in North America and Western Europe and is a major cause of morbidity and mortality. Although there have been advances in treatment only modest improvements in survival have been achieved. One of the important points to appreciate about breast cancers is that they are heterogeneous in the way they behave both biologically and clinically. It is, therefore, essential that the nature of individual tumours is characterised and used to plan the most appropriate therapy. A greater understanding of how breast cancers develop and how they progress could lead to more directed forms of screening and therapy. This is why it is necessary to determine the molecular mechanisms underlying the natural history of breast cancer.

The techniques which have been available for analysis have changed considerably so that many can now be applied to routine, diagnostic histopathological material and to aspirates/small biopsies. The various approaches which can be used will be referred to in the different sections where relevant. A summary of the possible ways of analysing breast cancers is given in Table 1.

It is not possible to cover every aspect of breast cancer and we will concentrate on three areas. However, for each of these the concern is to consider the following:

- can alterations be identified which represent early events and have potential for screening

- do any alterations/abnormalities provide information about how individual cancers will behave
- can the molecular alterations be used for the selection of the most appropriate form of treatment or be targets for new forms of therapy.

Genetic alterations

Oncogenes

Alterations to a number of proto-oncogenes appear to be of importance in a proportion of breast cancers.

c-*myc*

This encodes a nuclear phosphoprotein which acts as a transcriptional regulator, controlling cell proliferation, differentiation and apoptosis [1]. Whilst studies of c-*myc* in mouse systems suggest that alterations to the gene may be an important early event in the development of tumours [2], the evidence from human breast carcinomas is less conclusive. Alterations to the c-*myc* gene, predominantly amplification, have been found in approximately 25% of carcinomas but whilst Bonilla et al. [3] considered this to be associated with the development of breast cancer, other studies have found c-*myc* alterations to correlate with aggressive features and/or poor prognosis [4–6]. When c-*myc* mRNA and protein is determined in carcinomas by Northern analysis, *in-situ* hybridisation, immunohistochemistry and flow cytometry it is evident that there is a poor correlation between levels and extent of expression and amplification of the gene [7–8] and hence these approaches are of no value in determining prognosis.

ras

Although activation of the *ras* proto-oncogene family is important in rodent mammary tumours the role of *ras* genes in human breast cancer is less clear.

Table I. The various techniques which can be applied to breast cancer samples of different types

	Method	Detect	Application
DNA	Southern blotting	Amplification, deletion, allelic loss, imbalance	Fresh, frozen tissue.
	PCR amplification	Amplification, deletion allelic loss, imbalance, microsatellite instability.	Fresh, frozen tissue. Fixed, paraffin embedded tissue. Fine needle aspiration.
	Flow cytometry	Ploidy status.	Fresh, frozen tissue, fixed, paraffin embedded tissue, Aspirates (if sufficient cells).
	Fluorescent and non-radioactive *in-situ* hybridisation	Deletions, allelic loss, aneusomy.	Fixed, paraffin embedded tissue. Fine needle aspirates.
RNA	Northern blotting	Over expression, mutation	Fresh, frozen tissue.
	RT-PCR	Expression (can be quantified)	Fresh, frozen tissue. Fine needle aspirates.
	RT-PCR with SSCP[a]/CDGE[b]	Variants. Mutations.	Fresh, frozen tissue. Fine needle aspirates.
	In-situ hybridisation	Expression with localisation.	Fresh, frozen tissue. Fixed, embedded tissue.
Protein	Western blotting	Molecular weights of proteins, plus relative levels.	
	Immunohistochemistry, with or without antigen retrieval	Localisation of protein. Extent of reactivity e.g. receptors.	Fresh, frozen tissue. Fixed, embedded tissue. Fine needle aspirates.

Abbreviations: [a] SSCP – Single strand conformational polymorphism; [b] CDGE – Constant denaturing gel electrophoresis.

Mutations have rarely been identified [9], although loss of one H-*ras*-1 allele has been correlated with aggressive features [10].

Immunohistochemical studies give conflicting results regarding *ras* protein expression, some of which may result from the lack of specificity of the antibody used by several groups. Although some studies found greater expression in carcinomas [11–12], we did not [13]. Going et al. [14] found an increase in staining from normal to *in-situ* carcinomas, but surprisingly found greater staining in myoepithelial cells.

Of greater significance from a screening point of view comes from the finding of the tight linkage of H-*ras*-1 to a minisatellite locus which consists of four common alleles and several rare alleles. There is a significant association of the rare alleles with cancer and as many as 1 in 11 breast cancers might be attributed to this [15].

c-*erb*B-2

This proto-oncogene has proved to be of particular interest in human breast cancer. Also called *neu* or HER2, it encodes a 185 kD transmembrane glycoprotein, that has extensive homology with epidermal growth factor receptor and is a putative growth factor receptor [16].

Amplification of the gene is found in 20–30% in invasive carcinomas [5, 17–19] and a correlation has been found between amplification and aggressive features and poor short term prognosis, although not by all [20–21]. The vast majority of these studies have used Southern blotting techniques but more recent work has employed differential polymerase chain reaction since it can be applied to fixed, embedded tissue. Liu et al. [22] identified amplification in 21% of breast cancers but Hubbard et al. [23] found it at a much higher incidence.

Importantly, there is a good concordance between c-*erb*B-2 gene amplification and over-expression of mRNA and protein as detected by Northern analysis, *in situ* hybridisation and immunohistochemistry [7, 19, 24]. C-*erb*B-2 can be readily detected in fixed, paraffin embedded tissue using immunohistochemistry. It is our experience that antigen retrieval should not be used, since this can result in a greater incidence of low level staining and loss of the correlation with amplification. Although some studies failed to find a relationship between over-expression and prognosis [21], or only for node positive cases [25], many have found c-*erb*B-2 over-expression to be an independent predictor of poorer disease free interval and survival [26–30] and we continue to do so. There is now a wealth of antibodies available and a detailed analysis of them found their ability to detect over-expression to be variable, which may account for some of the differences concerning the relationship between c-*erb*B-2 and prognosis [31].

Amplification and over-expression of c-*erb*B-2 does appear to be associated with an aggressive form of breast cancer, so it was surprising that in immunohistochemical studies the protein could be detected in ductal carcinoma *in situ* [32]. More extensive studies found c-*erb*B-2 expression in 40–60% of cases but always associated with the high grade (comedo) type [33–35]. Allred et al. [36] proposed that either over-expression of c-*erb*B-2 decreases as carcinomas evolve from *in-situ* to invasive or that many invasive carcinomas arise *denovo* by mechanisms not involving c-*erb*B-2. We would propose that there are several molecular pathways by which breast carcinomas arise and alteration to c-*erb*B-2 is just one of them.

Knowledge of c-*erb*B-2 status can be of value in determining therapy since there is clear evidence that c-*erb*B-2 positive tumours show a poor response to endocrine therapy [37–39].

CCND1

Interest in genes on chromosome 11q13 came originally from retroviral studies in mice, where the oncogenic effect of mouse mammary tumour virus is due to the transcriptional activation of the cellular proto-oncogenes *int*-1 and *int*-2 (now *Wnt*-1 and *FgF*-3). Initially, the *int*-2/FGF3 gene, which is at 11q13, was studied and shown to be amplified in 10–20% of breast cancers. An association was found with the presence of oestrogen receptor [40] although others found that amplification was associated with poor prognosis [41, 42]. It was subsequently shown that FGF3 is not expressed in human breast cancers and so the amplification was just a

useful indicator of amplification of another gene in that region of the chromosome.

CCND1 is at 11q13 and encodes cyclin D1 [43]. This, when complexed with its associated cyclin-dependent kinase, controls cell cycle progression in G1 by phosphorylating retinoblastoma protein [44]. Over-expression of cyclin D1 has been found in breast cancer cell lines in both the presence and absence of amplification and dysregulation of expression of cyclin D1 may be a potential factor in the pathogenesis of breast cancer [45].

Antibodies have been generated against recombinant human cyclin D1. Over-expression has been identified in breast cancers, both with and without amplification [46, 47]. In one study patients whose carcinomas co-expressed cyclin D1 with epidermal growth factor receptor were found to have a poorer prognosis [48]. Barnes et al. [49] found a good correlation between immunohistochemically detectable cyclin D1 and oestrogen receptor and noted that cyclin D1 was a very good marker of likely response to endocrine therapy. Using a different monoclonal antibody and pressure-cooker antigen retrieval methods, we have found a similar correlation between cyclin D1 and oestrogen receptor ($p < 0.001$). It could clearly form an alternative to progesterone receptor or pS2 as a marker of hormone responsiveness.

Tumour suppressor genes

Retinoblastoma gene

Retinoblastoma gene (RB1) is the classical example of a tumour suppressor gene. It has been located on 13q14 [50] and encodes for a Mr105000 protein which in its unphosphorylated form restricts cell cycle progression in G1, by interacting with E2F transcription factor [51].

It would potentially appear to be a candidate gene to be altered in the early stages of the disease. Alterations to chromosome 13q have been found at several loci in breast carcinomas [52] as have structural alterations in the RB1 gene [53]. Two studies have compared allele loss and protein expression, either by immunohistochemistry or immunoblotting, with one finding loss of protein with allele loss [54] and the other high expression with allele loss [55]. Of significance was that alterations were found either in advanced cases [54] or in aneuploid, high S phase cancers [55], suggesting that alterations to RB are not an initiating event in breast cancer but is an event occurring in an unstable genome.

p53

There is substantial evidence that alterations to p53 can provide information about many aspects of breast cancer.

Germline p53 mutations have been found in families with the Li-Fraumeni Syndrome [56], a rare syndrome in which there is young onset sarcoma associated with breast cancer, primary brain tumour or leukaemia in a first degree relative under the age of 45 years. The mutations were initially found in exon 7, but have subsequently been found in other areas in the conserved region. However, only about half the Li-Fraumeni families have p53 mutations [57] and germline p53 mutations are rarely found in cases of early-onset breast cancer and those with a strong family history [58, 59].

Although allelic loss on 17p and in the region of the p53 gene has been reported by many groups, it would appear that the presence and nature of mutations provide more information that is of clinical value.

Several approaches have been made for identifying p53 mutations. These have included generating cDNA from RNA or starting from DNA; subsequent single-stranded conformation polymorphism analysis or constant denaturing gel electrophoresis with sequencing to confirm potential mutations, or cDNA sequencing. Most studies have focused on sequence changes in exons 5, 6, 7 and 8 with highly conserved domains [60–67] although complete sequencing has been undertaken [68]. The other method used which was initially considered to detect mutations was immunohistochemistry. It is now evident that an increase in p53 expression can occur in response to DNA damage and can be detected by immunohistochemistry [69]. The incidence of mutations and immunoreactive protein in the same group of carcinomas does differ [60, 70]. The main advantage of immunohistochemistry is that many antibodies can be applied to fixed, embedded tissue

and the use of antigen retrieval (microwave, pressure cooker) improves results, but care has to be taken in interpretation and it is best to consider staining to represent stabilised, possibly mutant, protein.

There is an association between the presence of mutations and aggressive features within breast carcinomas e.g. lack of oestrogen receptor [62, 65, 66], high S phase index [64]. Andersen et al. [65] found a significant association between p53 mutations and disease-free and overall survival. Bergh et al. [68] who screened the whole coding sequence found differences in the sites of mutations between node positive and node negative cases. Mutations in conserved regions II and V were associated with a significantly worse prognosis. Although immunohistochemistry may not always identify mutations the results also relate to tumour characteristics. It is important that there are clearly defined cut-off points for defining positive and negative cases [71]. Prominent reactivity for p53 is associated with lack of oestrogen receptor, poor differentiation, high proliferation rates, and presence of epidermal growth factor receptor [70, 72–74]. It has also been shown to be an independent marker of prognosis [72, 75, 76], although sequencing has been found to give better prognostic information than the monoclonal antibody Pab1801 [77].

Besides being of value for the prediction of prognosis, p53 can aid in the selection of therapy. Bergh et al. [68] found adjuvant tamoxifen therapy to be of less value in p53 mutation lymph node positive cases. Response to chemotherapy and radiotherapy can be affected by altered p53 function, due to its role in regulating DNA damage response [78].

Loss of heterozygosity

The frequent loss of heterozygosity (LOH) at a certain chromosomal locus in tumours indicates that this could be the sight of a tumour suppressor gene. There have been many studies which have examined a variety of chromosomes for LOH in series of breast cancers. Specific allelic losses have been reported for many chromosomes (reviewed by Devilee and Cornelisse [79]). Certain allelic losses or imbalances appear to be late events, whereas changes affecting 7p, 16q, 17p and 17q appear to be early abnormalities since they have been found in 25–30% of ductal carcinoma *in situ* cases [80]. Clearly identification of such changes at very early stages of the disease could be of value as use as markers in women who are at increased risk.

We have been studying ductal carcinoma *in situ* and small, impalpable lymph node negative invasive carcinomas detected by mammographic screening, concentrating on chromosome 6q since it is the second most frequent site for allelic loss. The 6q 25-27 region has been analysed using PCR analysis of four polymorphic microsatellite markers. Tumour foci and individual ducts from ductal carcinoma *in situ* were microdissected from formalin-fixed, paraffin embedded sections and DNA was extracted from fixed, embedded corresponding normal tissue for comparison.

The microdissection ensures that there is no contamination from non-tumour cells and can also demonstrate heterogeneity within individual tumours. We have found LOH in 48% of invasive carcinomas and 50% of ductal carcinoma *in situ*, with loss at single and multiple loci. Changes were observed in all types of invasive and *in situ* carcinomas which suggests that inactivation of tumour suppressor genes in this region on chromosome 6q could occur relatively early and would be an area worthy of study in 'at risk' lesions.

Microsatellite instability

The simple random repeat DNA sequences of mono-, di- and trinucleotides represent a very common and highly polymorphic class of genetic elements, which are used in gene mapping and linkage analysis. Microsatellite instability, demonstrated by expansion or contraction of repeat elements was first reported in neoplasms in colorectal tumours [81]. In hereditary nonpolyposis colorectal carcinoma (HNPCC) this instability has been shown to be caused by inherited and somatic mutations in DNA mismatch repair genes.

Microsatellite instability has been found in breast carcinomas. Wooster et al. [82] concluded that in-

stability occurred almost exclusively at higher order tri- and tetranucleotide repeats and that instability at more than one locus was rare. Other studies [83, 84] have identified tumours showing instability at more than one locus, including dinucleotide repeats, which is reminiscent of that seen in HNPCC kindreds. Yee et al. [83] suggested that microsatellite instability is an early event in mammary tumourigenesis, whilst LOH may occur at a later stage.

We have undertaken an analysis of microsatellite instability in early, mammographically-detected, invasive carcinomas [85] and *in situ* carcinomas, using multiple markers. A small number of invasive carcinomas showed instability with 7% showing instability at multiple loci. There was no instability in tubular carcinomas, including cases which presented clinically. Microsatellite instability was detected in *in situ* carcinoma but only when microdissection was performed. The incidence was greater in the high grade type. Differences between ducts from the same case was found in 3 of 23 cases. The data suggest that microsatellite instability is an early event in the genesis of some sporadic breast cancers and that it could have potential as a screening tool.

Familial breast cancer

The majority of breast cancers are due to acquired mutations, with only 5 to 8% of breast cancer patients having a strong family history, indicative of inheritance of mutations.

Inherited early onset breast cancer has been linked to two genes, BRCA1 [86] and BRCA2 [87]. The genomic structure of BRCA1 is complex. Currently the protein truncation test promises to become a valuable technique for detecting mutations. Hogervorst et al. [88] used it to screen for mutations in exon 11 which encodes 61% of BRCA1. However, there is no hard evidence that these genes are important in sporadic breast cancers. Beckmann et al. [89] investigated LOH of BRCA1 and BRCA2 in sporadic cancers using PCR-based fluorescent DNA technology and found that it was not of the same prognostic value as for familial cancer. No BRCA1 mutations have been found in sporadic cases [90].

Steroid and growth factor receptors, oestrogen related regulated proteins and growth factors

Oestrogen receptor

Determination

The evaluation of the oestrogen receptor status of a breast cancer has been used for over 20 years to help determine the likely response to hormonal therapy. Oestrogen receptor (ER) can be detected in 60–70% of breast cancers, of whom about a half (i.e. a third overall) will respond to hormonal manipulation [91]. The predominant assay method has been ligand binding in nature, in which radiolabelled steroid is added to homogenised breast tumour cytosol and binding determined after removal of free steroid by dextran-coated charcoal. It has been replaced in some laboratories by an enzyme immunoassay, utilising an antibody generated against the receptor. Both these approaches can be quantified, but they do require fresh tissue which can be a limiting factor.

The introduction of antibodies to ER has meant that immunohistochemistry can be used. The initial antibodies were used predominantly on frozen sections [92], although with various enzyme pretreatments, reactivity could be achieved in fixed material [93]. Further antibodies have been generated and are available commercially which work on formalin-fixed material. These require antigen retrieval, either by microwaving or pressure cooking in citrate buffer, but give results which are comparable with quantitative enzyme immunoassay [94]. They clearly have the advantage in that they can be applied to routine pathology material and also to fine needle aspirates or small biopsies. However, evaluation of reactivity and defining cut-off points which will provide information about response to hormone therapy, can cause problems. It can only be semiquantitative, there are problems in reproducibility [95] and there can be problems in achieving consistency in staining intensity. With care both the more complex H score and simpler scoring systems can provide useful clinical information [96].

Significance of ER

Whilst the main interest in oestrogen receptor has

been its role in the clinical management of breast cancer, there are several important questions surrounding the significance of ER in breast cancer:

- do receptors in normal breast epithelial cells and malignant cells have the same structure and function?
- why are a proportion of carcinomas ER negative?
- why do a proportion of ER positive cancers fail to respond to endocrine therapy?
- why do a small number of ER negative tumours response to hormone therapy?
- what is the role of ER in breast cancer development and progression?

The cloning of ER DNA [97] has made possible structural and functional analyses of ER which could contribute to our understanding of the above problems.

ER has 6 conserved domains, A–F [98] with different functions: transcriptional activation and repression, nuclear localisation, DNA binding and hormone binding. The DNA binding domain is responsible for the recognition of specific enhancer sequences found in hormone-responsive genes, e.g. progesterone receptor. Discrimination between different hormone response elements is determined by three amino acids at the base of the first zinc finger of the DNA binding region [99] so mutations could have important functional consequences. The E region, which contains both the hormone binding domain and the region required for stable dimerization, is very complex and mutations within this region could have profound consequences on receptor function.

Alterations to ER

There are some alterations which have been identified which can contribute to explaining some of the questions outlined above.

Analysis of DNA has shown no good evidence for amplification of the gene in breast cancers [100]. Using Southern blotting techniques Watts et al. [101] failed to find any gross rearrangements.

Several reports have suggested that a polymorphism associated with the restriction endonuclease *Pvu*II was linked to ER expression in human breast cancer [102] with absence of one allele being related to failure to express ER. However Yaich et al. [103] failed to find any such association. Andersen et al. [104] analysed *Bst* VI and *Pvu*II polymorphisms and found alleles with the *Pvu*II restriction site were more frequent in patients with progesterone receptor negative tumours, although there was no association between any polymorphisms and ER status.

One mechanisms that could block transcription of the ER gene in ER negative tumours without structural alterations in the gene is methylation of cytosine-rich areas, CpG islands, in the 5′ regulatory region of the gene. Hypermethylation has been found in ER negative tumours [105, 106] and cell lines [107]. Supporting the concept that methylation can affect expression is the finding that demethylation of the ER gene in ER negative breast cancer cells can reactivate expression [108].

However, the major focus of research into the genetic analysis of ER has been concerned with the identification of mutations or variants which may play a role in receptor dysfunction. The basis for some of these studies is because Sluyser [109] proposed that mutated or truncated forms of the steroid receptor family have oncogenic potential, with aberrant forms completing with normal receptor for binding to hormone response elements, so interfering with normal transcription mechanisms.

One of the first studies [110] used RNase protection assays to screen primary breast carcinomas and identified modifications in the B region of ER which correlated with low levels of oestrogen binding. However, this was subsequently found at a similar frequency in both ER positive and ER negative tumours [111], suggesting it may be of no functional significance.

Alterations to the DNA binding domain could be important in determining receptor function. Scott et al. [112] used gel-retardation assays to measure ER-DNA binding capacity and reported that two thirds of cancers with high ER levels retain DNA binding ability while most tumours with low or intermediate levels have lost this activity. Also, they found an association between ER-DNA binding and progesterone receptor. Both DNA binding and non DNA binding receptors could be found in the same tumour demonstrating the heterogeneity of

breast cancers. Fuqua et al. [113] identified a variant which resulted from a deletion of exon 3 of the DNA-binding domain such that the second zinc finger was missing. Although this would appear to be important as a dominant negative receptor, it may be a natural alternative spliced mistake which is functionally insignificant.

Murphy and Dotzlaw [114] identified variant ER mRNAs in breast cancer biopsies, and the smaller variants appeared to be mising some or all of the E and F domains of the receptor. Since these would have altered hormone binding some of them were cloned and sequenced [115]. One, Clone 4, which was present in multiple tumours, had sequences identical to wild type exons 1 and 2 but then diverged. To determine whether this variant is of importance, 106 carcinomas which were ER+ PgR+, ER+ PgR- and ER- PgR- were assayed using an RNase protection assay. Significantly higher levels of clone 4 variant mRNA were found in PgR negative tumours and in tumours with markers of poor prognosis [116]. This particular variant could be important in the progression of breast cancer from hormone dependence to independence.

Fuqua et al. [117] developed a sensitive polymerase chain reaction (PCR) assay to detect ER mRNA using small amounts of RNA, so as to semiquantitate ER expression and to identify rare transcripts. Using this approach they identified a variant lacking exon 5 of the hormone binding domain, which was the predominant ER RNA expressed in ER- PgR+ tumours [118] and could account for this particular phenotype. They also studied tumours of the ER+ PgR- phenotype and identified a truncated ER lacking Exon 7, which was unable to induce transcription and which prevented normal ER function.

There has been interest about the role of ER variants/mutations in tamoxifen resistance. Karnik et al. [120] screened tamoxifen resistant and tamoxifen sensitive tumours for mutations and identified a base-pair replacement and a base pair deletion in exon 6 in 2 of 20 tamoxifen resistant tumours. Since these were at a low frequency they suggested that mutations did not account for tamoxifen resistance. Daffada et al. [121] investigated the exon 5 deletion splice variant in a similar group of tumours using RT-PCR. Higher levels of the variant were found in the ER- PgR+ or pS2+ group and there was significantly greater variant exon 5 mRNA expression in the ER+ pS2+ tamoxifen-resistant group. While the variant is unlikely to be responsible for tamoxifen resistance in most breast cancers, it may be important in some.

Roodi et al. [122] screened DNA from ER positive and negative tumours for deletions/insertions or point mutations, amplifying exons 1 through to 8. No deletions/insertions were identified and only 2 mutations were found in the same ER negative tumour. A polymorphism in codon 325 showed a strong association with family history and this warrants further study.

The studies to date go towards explaining some of the questions posed e.g. the ER- PgR+ pheno-

Table 2. Summary of the alterations identified in the ER gene

Alteration	Significance	Reference No
Polymorphism	Absence of one allele, failure to	102
Pvu II	express ER.	
	Absence of one allele, no effect.	103
	Association with PgR negative tumours.	104
Codon 325	Associated with family history.	122
Modification to B region	No functional significance.	111
Deletion of exon 3	No functional significance.	113
Wild type exon 1 and 2 then divergence (clone 4)	Higher levels in PgR negative tumour, tumours with markers of poor prognosis.	116
Exon 5 variants	Associated with ER- PgR+ phenotype	118
	Associated with tamoxifen resistance in ER+ PS2+ tumours	121

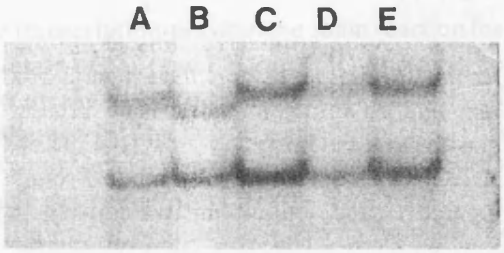

Figure 1. Four carcinomas (lanes A, B, D, E) and ER cDNA (lane C). The DNA binding domain has been analysed by RT-PCR and single stranded conformational polymorphism analysis. There is a band shift in carcinoma B (to be sequenced).

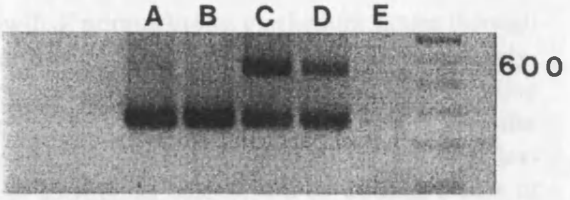

Figure 2. Four carcinomas (lanes A to D) analysed for the second half of the hormone binding domain by RT-PCR. The negative control (E) and 100 bp ladder is shown. Carcinoma A has a weak (upper) wild type band and a stronger lower variant band, carcinoma B only has the variant, and carcinomas C and D have both wild type (600 bp) and variant.

type, but there is still a lot to understand about ER in normal cells, and ER function. The published data regarding the ER gene is summarised in Table 2.

We have investigated a group of cancers different from those in published series. These have been mammographically-detected small invasive carcinomas which have a much higher incidence of ER positivity [123]. RNA has been extracted from frozen sections using a Dynabead method, reverse-transcribed and regions of the DNA binding and hormone binding domains amplified by PCR. These have been analysed by single stranded conformational polymorphism analysis. Using primers to amplify a region covering the DNA binding domain including the zinc fingers, band shifts have been identified in seven of 45 carcinomas (Figure 1). One carcinoma showed a band shift when exon 5 was amplified. Interestingly, 11 of the 45 cases showed splice variants in the second half of the hormone binding domain when a region spanning exons 6 to 8 was amplified (Figure 2). We are in the process of sequencing all abnormal patterns and relating the abnormalities to characteristics of the tumours such as PgR and proliferation status. It would be of value also to screen non-invasive carcinomas and 'at-risk' lesions such as atypical ductal hyperplasia to determine whether similar alterations are found in these even earlier changes.

Progesterone receptor

Progesterone receptor (PgR) is regulated by oestrogen acting through ER and is itself a gene regulator. It is a useful predictor of response to endocrine therapy [91].

PgR can be detected by binding assays, similar to those used for ER providing a synthetic progestogen is used. Monoclonal antibodies have been developed and these can be used reliably on formalin fixed material [123], and on aspirates.

Absence of PgR in breast cancers may be due to defects in ER function, as described above, or to molecular alterations in PgR. Fuqua et al. [124] did not find any major gene rearrangements to PgR in a study of 132 tumours and concluded that this mechanism could not explain the lack of PgR expression for the majority of breast tumours. Although LOH has been detected within the PgR gene it did not correlate with loss of immunoreactive PgR [125]. Loss of PgR more likely relates to altered ER function.

Oestrogen regulated proteins

Various oestrogen regulated proteins have been identified from the MCF-7 breast cancer cell line, either by analysis of culture medium after oestrogen stimulation or by differential screening of cDNA from hormone treated and untreated cells. The pS2 gene was identified by the latter approach [126] and subsequently identified by other groups and called pNR2 [127] and Md2 [128]. Expression of pS2 mRNA and protein is related to oestrogen receptor within breast carcinomas [129–131] and is a useful marker of potential hormone responsiveness [132]. Westley's group has developed a competitive

reverse transcription-polymerase chain reaction for quantitation of pS2 (and ER and PgR) which can be used to measure expression in small numbers of cells obtained by fine needle aspiration [133]. This type of approach is of particular value for tumours in elderly patients.

pS2 is a trefoil peptide [134] and its function remains unclear. It can be found in tissues which are oestrogen unresponsive e.g. gastric mucosa and here it may be involved in healing. The gene is also responsive to epidermal growth factor, TPA, c-H-*ras* and c-*jun* [135]. There may be factors other than oestrogen regulating its expression in breast cancers which may account for the complex staining pattern seen with immunohistochemistry and the finding of pS2 in ER negative tumours [136].

Other proteins which were originally identified as being oestrogen regulated appear to be markers of aggressive behaviour, which contrasts with ER. Cathepsin D, a major lysosomal protease, was initially identified in the medium of MCF-7 cells cultured in the presence of oestrogen [137]. However, it is constitutively overexpressed in ER negative breast cancer cell lines and several studies have failed to find a relationship between cathepsin D and ER in primary breast carcinomas [131, 138–140]. Transfection of cathepsin D resulted in transformed cells increasing their metastatic capacity [141]. Clinical studies, using immunoassays, have suggested that cathepsin D is a marker of poor prognosis [138–140] but immunohistochemical studies have shown that the cathepsin D may be in the stromal component, rather than the tumour cells and that this may be a reflection of macrophage infiltrate [142].

Another oestrogen regulated protein, heat shock stress response protein (Srp-27) has also been associated with tumours being aggressive, despite a correlation with ER [143]. Manning et al. [144] have identified an oestrogen-regulated gene, pLIV1, which is associated with lymph node involvement and may represent a candidate gene for metastatic spread in ER+ cancers.

Epidermal growth factor receptor

Epidermal growth factor (EGF) is necessary for the growth of normal breast epithelium, acting through its receptor (EGFR) which is a transmembrane glycoprotein. This has an extra cellular ligand binding domain and an intracellular tyrosine kinase domain, with close similarity to v-*erb*B [145]. High levels of EGFR, as determined by binding assays of homogenates and immunohistochemistry, have been shown to correlate with a poor prognosis and failure to respond to hormone therapy [146–149]. In most tumours there is an inverse relationship with ER. EGFR can provide useful clinical information.

The increased expression is not due to gene amplification. Increased levels of EGFR mRNA are found [150]. We have undertaken *in-situ* hybridisation for EGFR mRNA in both benign and malignant breast tissue using a digoxigenin-labelled oligonucleotide probe. In normal/benign tissue mRNA is detectable to a greater extent than protein as demonstrated by immunohistochemistry, but this difference is more striking in the carcinomas. Two thirds of the cancers assessed had demonstrable mRNA (Figure 3), whereas only one third had detectable protein. Those cases with both were all ER and PgR negative, but the group with EGFR mRNA but no protein comprised a mixture of ER+ PgR+, ER+ PgR– cases, and ER-PgR. It has been suggested [151] that regulation of the EGFR gene involves a number of interactions between positive regulatory factors and repressors (some of which may be oestrogen regulated) with binding sites in both the promoter and first intron and that there is a progression from an ER positive with low levels of EGFR, considered negative, to an ER negative, EGFR overexpressing tumour. We would suggest that there could be other reasons and that it is important to consider EGFR expression in the different cell types present in normal breast e.g. could there have been development from an ER negative EGFR positive myoepithelial cell.

Growth factors

Peptide growth factors act in an autocrine/paracrine manner. Primary breast carcinomas consist of epithelium, stroma, vascular and other elements and interactions between these are important for

tumour growth. Altered cellular expression and/or response to different growth factors by epithelia and/or stromal cells could clearly be important in the development and progression of breast cancers.

Various growth factors have been studied e.g. insulin growth factor 1 and 2, transforming growth factor alpha, fibroblast growth factors. Our own studies have been concerned with transforming growth factor beta, and this will be considered in more detail.

Transforming growth factor beta

Transforming growth factor beta (TGF-β) comprises a group of multifunctional regulatory proteins which have effects on many processes which could be of importance in the overall behaviour breast cancer [152]. These include proliferation, differentiation, stimulation of extracellular matrix formation, cell migration, angiogenesis and immune function.

There are some conflicting findings regarding the role(s) of TGF-β in breast carcinomas. TGF-β has been reported to inhibit [153] and stimulate [154] proliferation of breast cancer cells. Tamoxifen has been shown to modify the expression of TGF-β_1 [155] and it has been proposed that this may be a secondary mechanism for the mediation of its antitumour effects. In this *in vivo* study [155] stromal fibroblasts were demonstrated as the source of extracellular TGF-β, which would then act in a paracrine fashion on tumour cells. Subsequent *in vitro* studies [156] suggest that synthesis of TGF-β by fibroblasts is increased by tamoxifen, but not necessarily the secretion which is needed for the paracrine effect. MacCallum et al. [157] examined mRNA for the three mammalian TGF-β isoforms in carcinomas before and after tamoxifen treatment and found that TGF-β_2 rather than TGF-β_1 expression tended to increase. The role of TGF-β in the regulation of proliferation and its relationship to tamoxifen is obviously complex and requires further study.

There is evidence from several studies, including our own, that TGF-β expression by breast carcinomas is associated with poorer prognostic features [158–161]. We identified a difference in TGFβ_1 protein, as detected by immunohistochemistry, between *in-situ* and invasive carcinomas, with fewer *in-situ* cases having reactivity. We also demonstrated that TGF-β_1 but not TGF-β_2 expression was associated with lymph node metastasis [161]. It was also associated with increased reactivity for the stromal components fibronectin and tenascin and altered macrophage and T lymphocyte infiltration, all of which may be important in enhancing invasion and metastasis. In an *in-situ* hybridisation study, using digoxigenin-labelled riboprobes, we demonstrated TGF-β_1 mRNA in the epithelial tumour cells and not stromal cells [162] which is contrary to some immunohistochemical studies.

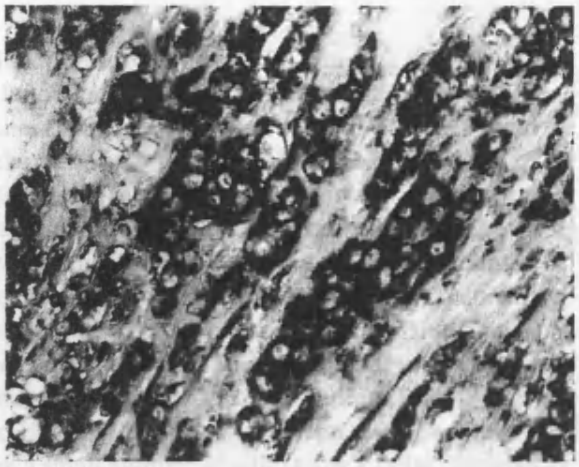

Figure 3. Breast carcinoma hybridised with digoxigenin-labelled oligonucleotide probe to EGFR, showing strong labelling of tumour cells. EGFR protein was detected immunohistochemically.

Due to the complex, multifunctional properties of TGF-β further studies are required to elucidate its value in clinical management.

Cell adhesion, invasion, metastasis

This is a large topic which includes cell-cell interactions, cell-stromal interactions, proteases, angiogenesis, growth factors, anti-metastasis genes such as nm23, and interaction of tumour cells with specific organ environments. Since it is not possible to cover all of these aspects comprehensively, we will concentrate on areas of particular interest to our laboratory.

E-cadherin

E-cadherin plays a critical role in initiating and maintaining cell-cell contacts and is a member of the growing family of calcium-dependent cadherin adhesion molecules [163]. E-cadherin molecules are located within adherans junctions and are transmembrane structures; the cytoplasmic region interacts with the catenins, which are in turn connected to the actin microfilament network [164]. Evidence that E-cadherin is important in suppressing invasion comes from studies in which this is observed in cells transfected with the cDNA [165].

Studies of primary breast carcinomas have given inconsistent results. The predominant method of analysis has been immunohistochemistry; some studies have used frozen material and some have used fixed tissue. The interpretation and definition of positive/negative has also varied and some of this may account for the discrepancies.

Reduced E-cadherin has [166, 167] and has not [168, 169] been related to poorer differentiation of infiltrating ductal carcinomas. We [169] and other [167] have found a relationship between reduced membrane staining and lymph node metastasis. We also demonstrated a highly significant association between cytoplasmic reactivity for E-cadherin in tumour cells and nodal metastasis. This altered E-cadherin localisation may be the result of abnormal catenin expression, or be due to local environmental influences. We also demonstrated a correlation between the presence of EGFR and cytoplasmic reactivity. There is evidence that phosphorylation of EGFR leads to dissociation of E-cadherin/catenin complexes from the cytoskeleton [164] and so may modify function.

One of the interesting, and potentially useful diagnostically, observations that we and other have made is that infiltrating lobular carcinomas and lobular carcinoma *in situ* do not express E-cadherin [166, 168–170]. This may be of relevance in explaining the infiltrative nature of this type of invasive carcinoma.

The E-cadherin gene maps to chromosome 16q; deletions of this region occur frequently and have been associated with distant metastasis [171]. We have used microsatellite markers covering 16q21 to 16q24.3 and identified LOH ranging from 25–44%, depending on the marker. One third of the informative cases showed LOH for D16S400, the closest to the region of the E-cadherin gene but we failed to find any relationship between LOH and loss of E-cadherin staining. Those cases showing LOH were then screened for mutations in exons 6, 7 and 9 of the E-cadherin gene. One had a 1 bp insertion in exon 7 but no other mutations were identified. This was a well differentiated infiltrating ductal carcinoma with homogenous cytoplasmic reactivity for E-cadherin. Mutations have been identified by others in a small number of infiltrating lobular carcinomas [172, 173], but not in infiltrating ductal carcinomas [174].

The control of E-cadherin in breast carcinomas is complex and alterations in the link between membrane signalling pathways and E-cadherin expression may be the key to its role in invasion.

Integrins

Integrins are cell adhesion molecules which are involved in cell-stromal interactions, and possibly cell-cell interactions. They are heterodimers composed of non-covalently liked α and β subunits which provide a transmembrane link between the cytoskeleton and specific extracellular matrix proteins [175]. The integrins are classified according to their β subunit. They include receptor for collagen, laminin and fibronectin.

Because they play an important role in cell adhesion and migration, alterations in expression and/or function could be important in altered growth and invasion in neoplasms. There have been several studies of integrin expression in primary breast carcinomas, using immunohistochemistry although Zutter et al. [176] have examined mRNA expression of α_2, α_5 and β_1 with radioactive-labelled riboprobe *in-situ* hybridisation. All studies have shown reduced expression at the protein [177–180] and mRNA [176] levels for $\alpha_2\beta_1$, $\alpha_6\beta_1$ and $\alpha_6\beta_4$. We [180] found no relationship to grade or node status, although others have found loss of $\alpha_2\beta_1$ to be greater in poorly differentiated carcinomas [177, 178]. As with E-cadherin, we found that reactivity could be

cytoplasmic rather than membrane [180] and this loss of polarization has been reported by Pignatelli et al. [181]. Overall alterations in integrin expression have not been of value in providing prognostic information.

We observed an alteration in the staining for $\alpha_6\beta_4$ in non-involved tissue from cancer-containing breasts, in that two thirds of the cases showed loss of reactivity, identical to that of the corresponding tumour [180]. This was not seen for other integrins and suggests that altered $\alpha_6\beta_4$ integrin staining may be an early event in the neoplastic process, and as such, may be of use as a marker of pre-malignant change.

Proteases

Proteolytic enzymes that could degrade extracellular matrix may play an important role in invasion and metastasis. Proteases fall into three main groups: aspartyl, which includes cathepsin D, which has already been discussed; serine, such as plasminogen activators; and metal dependent, which includes metalloproteinases and stromelysins.

Plasminogen activators

There are two main forms of plasminogen activators, uPA and tPA, which activate plasmin; uPA also participates in tissue remodelling under normal and pathological conditions [182]. The uPA pathway is regulated by two inhibitors PAI-1 and PAI-2 and by a cell surface receptor for uPA (uPAR) which binds both pro-uPA and active uPA. Thus the cell surface may be the physiological site of uPA-initiated fibrinolysis.

There has been interest as to whether the plasminogen activators could provide prognostic information and there have been several reports that carcinomas with high levels of uPA and PAI-1 have a high risk of relapse [183–186]. These studies have used ELISA assays of tissue homogenates, which has the advantage of quantification but does require fresh tissue. Certainly uPA and PAI-1 appear to be useful prognostic markers.

These approaches though do not provide information about cellular location of the different components and their inter-relationships. Using immunohistochemistry uPA can be detected in tumour cells, with a greater intensity than normal/benign epithelium and also weakly in stromal cells [187]. A similar pattern of staining is seen for PAI-1 [187]. Results for the localization of uPAR have been conflicting. Pyke et al. [188] and Bianchi et al. [189] did not detect uPAR in normal and benign breast, but the former only found the receptor on tumour associated macrophages, while the latter found it on tumour cells as well in a proportion of cases. In contrast Constantini et al. [187] detected uPAR on normal and tumoural epithelial cells and stromal cells, but to a greater extent in tumours. One reason for some of the differences could be due to differences in the sensitivities of the antibodies used, since the glycosylated variants of uPAR may have been detected to different degrees.

The finding of reactivity in tumour and stromal cells suggests the existance of complex paracrine interactions. However, staining may reflect bound enzyme and does not necessarily indicate the site of synthesis. Also, it is not known whether the expression observed in carcinomas is a primary alteration or is simply a marker of the effects of cytokines and growth factors on tumour cells. A more detailed understanding of this would be of value so as to identify the most appropriate targets, for the design of new therapeutic agents.

Matrix metalloproteinases

The matrix metalloproteinases (MMP) are a family of zinc dependent enzymes capable of degrading different components of the extracellular matrix [190]. At least 11 members are now described [191] which can be broadly categorized into collagenases, gelatinases and stromelysins acording to their specificity. The collagenases include MMP-1 (interstitial collagenase), MMP-8 (neutrophil collagenase) and MMP-13 (collagenase 3), and degrade fibrillar interstitial collage. The gelatinases, also known as type IV collagenases, are particularly potent against denatured collagen and include MMP-2 (gelatinase A, 72 kd gelatinase) and MMP-9 (gelatinase B, 92 kd gelatinase). Stromelysins 1, 2 and 3 (MMP-3, MMP-10 and MMP-11 respectively) have

a broader substrate specificity and degrade glycoproteins such as laminin and fibronectin.

MMP activity can be controlled at multiple levels which include transcriptional activation, conversion of latent precursor to active enzyme and inhibition via interaction with the specific tissue inhibitors of the MMPs, TIMP-1, 2 and 3 [192, 193].

Both immunohistochemistry and zymography have demonstrated increased reactivity for MMP-2 in breast carcinomas in comparison to normal and benign breast (194, 195], with some studies showing a relationship to lymph node involvement [196]. Our own studies have confirmed the reactivity but not found any relationship with node status (manuscript in preparation). MMP-9 immunoreactivity has also been shown to correlate with nodal metastasis [197]. MMP-11 or stromelysin 3 [198] has been detected in most invasive and high grade *in situ* carcinomas but is rarely detected in benign breast disease [199, 200]. Levels of stromelysin 3 mRNA may be of prognostic value in that one study found recurrence more common in patients with tumours having high as compared to low levels [201].

Because of the interactions between MMPs, their activators and inhibitors, assessment of one alone or even a panel may be of limited value with regards to prognosis. This is illustrated by a study using quantitative RT-PCR to compare MMP-2 and TIMP-2 mRNA in node negative and positive cases [202]. When levels of mRNA were analysed separately no relationship was found, but when the ratios of MMP-2 mRNA to TIMP-2 mRNA were examined, ratios of 2–4:1 were associated with node metastasis and < 1:1 with negative nodes. Analysis by Northern blotting did not find any relationship between MMP-2 mRNA levels and malignancy, only higher levels of MMP-11 mRNA in carcinomas [203].

Whilst immunohistochemical studies show staining of MMPs in tumour and stromal cells, *in-situ* hybridization studies have localised MMP transcripts predominantly to the stromal cells [204, 205]. This apparent discrepancy can be explained by the presence of receptors for MMPs on tumour cells [206]. Also a new form of MMP has recently been described, the membrane type MMP (Mt-MMP) [207–209]. The three molecules, MT1-MMP, MT2-MMP and MT3-MMP, have in common a transmembrane domain and are localised at the cell membrane. Both MT1-MMP and MT2-MMP are potent activators of progelatinase A [207, 210] and so expression of these receptors on tumour cells would localise proteolytic activity to the site of invasion, mirroring the strategy displayed by the uPA receptor [187]. Elevated levels of MT1-MMP has been demonstrated in various tumour tissues [211, 212]. Work in our laboratory has revealed consistently high reactivity for MT1-MMP in breast carcinomas and suggests that combined expression of MT1-MMP and MMP-2 show a better correlation with prognosis than MMP-2 alone (manuscript in preparation).

There has been considerable interest in the development of agents which could modify MMP function, that would have therapeutic applications for many diseases, including neoplasia. Application of these in breast cancer would clearly be of interest.

Conclusions

It is not possible to cover every aspect of the molecular pathology of breast cancer which could be of clinical importance e.g. multi-drug resistance genes. However, many aspects have been covered which show that our greater understanding of molecular mechanisms and the increasing ability to investigate these in primary breast carcinomas should aid the clinical management of breast cancer. We highlighted three important areas in the development and progression of breast cancer and have identified alterations which could be of relevance to three aspects of clinical management:

Early changes/screening potential

BRCA1 and BRCA2.

Loss of key chromosmal regions e.g. 6q.

Microsatellite instability as a marker of DNA repair defects.

P53 mutations in certain families.

Certain ER variants and polymorphisms.

Altered $\alpha 6\beta 4$ integrin expression in non involved cancerous breast.

Behaviour/prognosis
C-erb2 over-expression and prognosis.
P53 mutations and prognosis.
EGFR and prognosis.
Cathepsin D and prognosis.
TGF-β1 and lymph node metastasis.
E-Cadherin and lymph node metastasis.
uPA and PAI-1 and prognosis.
MMP-2 and MT1-MMP and prognosis.

Treatment strategy/new therapies
C-erbB-2 and poor response to endocrine therapy. Potential target site for new drug.
P53 mutation and poor response to endocrine therapy; development of therapeutic agents against mutants.
ER and response to endocrine therapy, possibility of variants explaining tamoxifen resistance.
PgR, PS2 and Cyclin D1 response to endocrine therapy.
EGFR and poor response to endocrine therapy.
Plasminogen activators and inhibitors (uPA and PAI-1) and metalloproteinases as targets for design of new therapeutic agents.

The only way forward is to ensure that both biological and clinical features of breast carcinoma are considered together since we need to have an understanding of the former if we are to improve the clinical outcome of breast cancer. There is still much work to be done.

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Address for offprint: R.A. Walker, Breast Cancer Research Unit, Clinical Sciences, Glenfield Hospital, Groby Road, Leicester, LE3 9QP, UK

SPECIAL NOTE

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