The Identification of Genetic Alterations in Pre-Invasive Breast Cancer

Christopher Reid Wilson

This thesis is submitted to the University of Glasgow in part fulfilment of the degree of Doctor of Medicine

University Department of Medical Oncology

&

University Department of Surgery, Western Infirmary

July 1997

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

CONTENTS

List of Figures

List of Tables

Chapter 5 : Interphase Cytogenetic Analysis of the ERBB2 Gene

Chapter 6 : Identification of Chromosome 18 Imbalances in DCIS

Abbreviations

ADH	Atypical ductal hyperplasia
BRCA-1	breast cancer 1 gene
BRCA-2	breast cancer 2 gene
CGH	comparitive genomic hybridisation
DAPI	4',6-diamino-2-phenylindole
DCIS	ductal carcinoma in-situ
DIG	digoxigenin
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside triphosphate
EDTA	ethylene diamine tetra-acetic acid
EGF	epidermal growth factor
FISH	fluorescence in-situ hybridisation
FITC	fluoroscein isothiocyanate
IgG	immunoglobulin G
LOH	loss of heterozygosity
NaCl	sodium chloride
NaOH	sodium hydroxide
PBS	phosphate buffered saline

PCR	polymerase chain reaction
PDWA	proliferative disease without atypia
RNA	ribonucleic acid
SSC	sodium chloride/sodium citrate (buffer)
TAE	Tris/acetate buffer
Taq	*Thermus aquaticus* DNA polymerase
TBE	Tris/borate buffer
TE	Tris / EDTA buffer
TEMED	N,N,N',N'-tetramethyl-ethylenediamine

Abstract

In recent years much interest has focused on the early detection of breast carcinoma and the potential for prevention strategies for women at high risk, and this has been fuelled by the implementation of breast screening programmes which have increased the diagnosis of all forms of pre-invasive disease. The management of women found to have proliferative breast disease is unsatisfactory with mammographic surveillance being the only realistic option for these patients. It is therefore particularly important that the genetic profile of these lesions is comprehensively studied in order to detect potential biomarkers for progression and hence future avenues for therapeutic intervention and prevention.

The aim of the study is to reconstruct the molecular evolution of breast cancer progression with particular emphasis on the critical pre-invasive steps. It is hypothesised that a normal breast epithelium can evolve to breast cancer through the intermediary stages of proliferative disease without atypia [PDWA], atypical ductal hyperplasia [ADH] and ductal carcinoma *in situ* [DCIS].

The characterisation of genetic alterations shared by DCIS and invasive breast cancer has reinforced evidence that DCIS is a true non-obligate precursor of breast cancer, however there is little genetic evidence to substantiate the hypothesis that PDWA is a precursor of invasive breast cancer.

Our group had previously shown DCIS to have chromosomal imbalances involving chromosomes 1, 3, 10, 17 & 18, therefore 31 cases of PDWA were analysed by fluorescence in-situ hybridisation [FISH] using chromosome specific centromeric probes for chromosomes 1,3,10,17 &18.

Chromosomal imbalances affecting those chromosomes were detected in PDWA which mirror those found in DCIS but at a reduced frequency

suggesting that a subpopulation of PDWA resembles DCIS at a molecular level and are at a greater risk of progression.

A huge increase in the frequency of chromosomal imbalances affecting chromosomes 3 & 17 occurs between PDWA and DCIS thus key genetic events involved in the progression of PDWA to DCIS may be present on those chromosomes.

In contrast chromosome imbalances affecting chromosome 18 were present at a high frequency in both PDWA and DCIS suggesting that this chromosome harbours genes responsible for the earliest stages of breast cancer progression. There is therefore a requirement to define the regions of change in chromosome 18 in more detail to identify candidate genes.

Multiple foci of DCIS from sections known to have a loss of chromosome 18 sequences were therefore microdissected and three polymorphic microsatellite markers for chromosome 18 were PCR amplified in order to detect regional alterations to chromosome 18 and to investigate the phenomenon of intratumour genetic heterogeneity.

A high frequency of allelic imbalance was found for all the markers with the highest frequency incorporating the region of 18q23, suggesting that this region harbours genes crucial to the early progression of breast cancer.

Individual foci of DCIS exhibited a high degree of genetic heterogeneity for chromosome 18 which suggests that several subclones of DCIS may coexist within an individual.

These results set the agenda for the future investigation of genetic alterations in pre-invasive breast disease.

Acknowledgements

I would like to take this opportunity to acknowledge the many people who have made this project possible. In particular I would like to give special mention to the following.

I am extemely grateful to my supervisor Dr. Nicol Keith for giving me the opportunity to join his laboratory group and for providing me with invaluable advice and guidance.

Many thanks go to Dr Liz Mallon and Dr James Going for their pathological assistance.

Iwould also like to thank Professor Douglas McGregor for his encouragement and assistance during this work.

The members of the ' FISH LAB' require a special mention for their assistance. Without Sharon, Stacey, Anja and latterly Karen, life for the duration of this project would have been considerably less entertaining !!

Last but not least I would like to thank my wife Esther and my family for their help and enthusiasm during the past two years.

Overview.

This thesis postulates a genetic basis for a hypothetical model of breast cancer progression whereby a normal breast epithelium can evolve through a number of morphological stages culminating in the invasive phenotype.

Recently developed techniques have been applied in order to identify and compare common genetic changes in benign and malignant tumours of the breast . The work has succeeded in showing that the same changes are present in the DNA of both types of lesions. This provides evidence for a common path, and also points to the early changes which may be responsible for the disease.

Chapter 1 examines why investigating the genetic basis of breast cancer is crucial and reviews the clinical evidence which is compatible with the hypothetical model of breast cancer progression. The chapter moves on to review the present knowledge of genetic alterations in both invasive breast cancer and pre-invasive disease.

Chapter 2 describes the molecular techniques employed to analyse pre-invasive breast disease by the author.

Chapter 3 marks the beginning of the results section and describes, with examples, how the experimental data was analysed and quantified.

Chapter 4 presents and discusses the results of the genetic analysis of proliferative breast disease without atypia [PDWA], which reveals genetic alterations similar to that of DCIS and invasive cancer.

Chapter 5 presents data and discussion on the involvement of the ERBB2 oncogene in PDWA and DCIS.

Chapter 6 analyses and identifies regional changes on chromosome 18 in DCIS. Chromosome 18 was chosen for more detailed analysis due to the high frequency of genetic alterations found in both PDWA and DCIS which suggests that chromosome 18 harbours genes responsible for the earliest stages of breast cancer progression.

Chapter 7 provides a general summary of the thesis bringing together concepts from each of the chapters.

INTRODUCTION

Chapter 1.

Introduction.

1. Breast Cancer

1.1. Incidence and mortality

Breast cancer is the most common malignancy affecting women in Europe (180 000 cases per year) and the USA (130 000 cases per year). In European countries breast cancer constitutes 20% of all malignancies and represents a major public health problem (Willett et al. 1992). Steady progress continues to be made in the clinical management of breast cancer. However, advances in our understanding of the biology of breast cancer and pre-invasive breast cancer, particularly the genetic basis of inherited susceptibility to breast cancer are occurring at a more rapid pace. As these biologic processes are elucidated, intensive efforts are being made to translate this fundamental science into progress in the prevention, diagnosis and therapy of this common cancer.

Current estimates indicate that the cumulative lifetime risk of developing breast cancer is ~10% and the lifetime risk of dying of breast cancer is ~3.6%. Half of a woman's risk occurs over the age of 65, with the majority of cancers occurring in women over 55. The risk for younger women, in the absence of other major risk factors other than age, is low and the chance of developing breast cancer between the ages of 35 and 55 is 2.5% (Hankey et al. 1994; Gelb et al. 1985; Willett et al. 1992).

The incidence of breast cancer has been increasing in most Western countries since 1950 and continues to do so at a mean rate of 1 - 2% annually. The rate of increase is more pronounced in lower risk populations and thus the difference in incidence between women in low risk populations such as the Far East, and women living in the West will decrease (Willett et al. 1992). The most recent surge in breast cancer incidence rates in the 1980's may well be attributable to the implementation of breast cancer screening programmes,

however early mammographic detection cannot explain the underlying, long-term annual increase in incidence of this disease,(Figure 1).

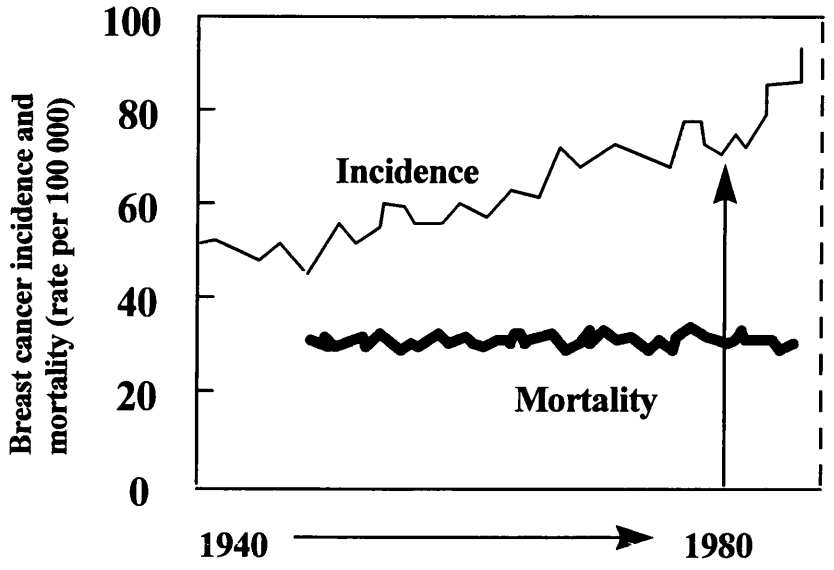


Figure 1.: **Age standardised incidence of breast cancer and mortality (The data are summerised from ICRF, Epidemiology Unit)**

Although the incidence of breast cancer continues to increase, age - standardised mortality rates for breast cancer, world-wide, are tending to remain constant, (Figure 1) (Beral and Hermon, 1996). Several factors could be responsible for the decrease in mortality rates from breast cancer in the face of an increasing incidence: changes in death certification practises; changes in incidence rates due to changes in risk factors; increases in a more benign form of the disease; earlier detection; and improvements in treatment. Indications are that the earlier detection and possible improvements in treatment have improved survival to the extent that the increases in incidence are being offset (Willett et al. 1992). However, as the incidence of breast cancer increases it is crucial that multidisciplinary research into the aetiology of breast cancer continues, to decrease both the incidence and mortality of this disease.

1.2. Therapy.

Currently therapy for primary operable breast cancer is based on the co-operative expertise of a surgeon, oncologist and radiotherapist, who aim to manage the primary tumour, the axillary lymph nodes and prevent locoregional and distant recurrence by using adjuvant hormonal or chemotherapeutic agents.

The surgical management of breast cancer involves treatment of the primary tumour by either modified radical mastectomy or conservative breast surgery and either the sampling, clearing or monitoring of the ipsilateral axillary lymph nodes.

Many randomised trials have now established that in appropriately selected cases, a combination of wide local excision of the tumour together with radiotherapy is as effective as mastectomy, thus offering women a less mutilating and psychological scarring alternative (Early Breast Cancer Trialists' Collaborative Group, 1995; Friedman, A. et al. 1995). Many studies are now questioning whether radiotherapy is necessary for all tumours and in particular tumours less than 2cm.

The management of the axilla is more controversial. Preoperative clinical or radiological assessment of lymph node involvement is inaccurate and only pathological examination of excised nodes can provide accurate information. Those who support axillary clearance contend that it yields prognostic information on which oncologists can base decisions about adjuvant chemotherapy. However, others contend that decisions on adjuvant chemotherapy have already been made based on the pathological examination of the primary tumour and hence the 50% of women with breast cancer who have node negative disease would be spared the morbidity of an axillary procedure (Osteen and Harris, 1985; Feldman and Gardner, 1993; Hellman, 1994). Current practise in Scotland is to clear the axilla. A possible answer to this dilemma is the ''sentinel node biopsy'' (the node most likely to drain the primary tumour) which can be found by a vital dye or radioactive tracer. The

main studies of this confirm near perfect correlation with the nodal status of the dissected axilla. Thus, a simple local anaesthetic technique may well be able to predict the status of the axilla (Ku et al. 1996; Krag and Alex, 1996; Krasne et al. 1995).

However, despite advances in techniques, the surgical management of breast cancer has not made an impact on survival. Over half of women with operable breast cancer who undergo locoregional surgical management alone will die from metastatic disease and hence the only way to improve the chance of survival of these women is to use effective systemic treatment.

Randomised clinical trials have shown that chemotherapy, tamoxifen and ovarian ablation can reduce the frequency of relapses and prolong survival among patients with operable breast cancer and ipsilateral lymph-node metastases (Early Breast Cancer Trialists' Collaborative Group, 1992). As a result almost all patients receive adjuvant systemic therapy. Multiagent chemotherapy is the agent of choice for pre-menopausal women with node positive disease (Veronesi et al. 1977); for post-menopausal women, endocrine therapy, alone or in combination with chemotherapy is advantageous (Legault-Poisson et al. 1990; Ludwig Breast Cancer Study Group, 1984). If you summarise the effects of adjuvant therapy, the use of tamoxifen in women greater than 50 will reduce the annual odds of recurrence by 30% and the annual odds of death by 19% which equates with 8 extra women alive at 10 years for every 100 women treated with stage I & II cancer. The use of adjuvant chemotherapy in women less than 50 reduces the annual odds of recurrence by 37% and the annual odds of death by 30%, which equates with an extra 14 women alive at 10 years for every 100 women treated with stage I & II cancer (Perren, 1995). Further follow up is required to determine whether these women are cured or that their ultimate death has just been delayed. Unfortunately, it is therefore apparent that cytotoxic and endocrine therapies have significant limitations and present survival estimates remain depressing, (Table 1).

Survival of patients with breast cancer according to lymph node status		Survival of patients according to tumour stage	
	survival at 10 years	stage	survival at 5 years
All patients	45.9%	I	84%
Node -ve	64.9%	II	71%
Node +ve	24.9%	III	48%
1 - 3 + nodes	37.5%	IV	18%
>4 + nodes	13.4%		

Table 1. **Patient Survival According to Lymph Node Status and Stage of Breast Cancer. (Data from " The ABC of Breast Disease," BMJ, Publications.)**

In attempt to improve survival, intensive efforts have been made in the areas of genetic, clinical and population research to identify risk factors in women which can predict their susceptibility to breast cancer development in the hope that the natural history of developing breast cancers could be altered or prevented by early diagnosis and therapeutic intervention.

1.3. Risk factors for the development of breast cancer.

The aetiology of breast cancer appears to be multifactorial with a number of endogenous and exogenous factors known to increase the risk of breast cancer. Age, family history, benign breast disease and reproductive history are all endogenous factors which contribute to risk. Ionising radiation, diet, alcohol and pesticides are all exogenous factors which have been suggested to increase breast cancer risk, however other than the recognition of these factors, there is limited understanding of the scientific basis of the risk or the interactions between risk factors. The strength of a risk factor is indicated by its relative risk, however an elevated risk does not necessarily imply causation, but it does provide a framework for further research, (Figure 2).

RISK	RISK CATEGORY	RELATIVE RISK
Family History	mother affected before 60 yrs	2.0
	mother affected after 60 yrs	1.4
	two 1st degree relatives	4 - 6
Age at menarche	11 to 14yrs	1.5
	15 yrs	1.3
Age of child at 1st birth	20 - 24 yrs	1.3
	25 - 29 yrs	1.6
	> 30 yrs	1.9
	nulliparous	1.9
Age at menopause	after 45yrs	1.5
	before 45 yrs	0.7
	oophorectomy <35 yrs	0.4
Benign Breast Disease	any benign disease	1.5
	PDWA	2.0
	ADH	4.0
Radiation	atomic aomb	3.0
	repeat x-rays	1.5 - 2.0
Oral Contraceptive	current use	1.5
	past use	1.0
Hormone replacement	current use all ages	1.4
	ever use	1.0
	past use	1.0
	use >10yrs	1.5
Alcohol	1 drink/day	1.4
	2 drinks/day	1.7
	3 drinks/day	2.0

Figure 2. **Established and Probable Risk Factors for Breast Cancer. (Modified from NEJM, Vol 327, No.7)**

1.31. Age

The incidence of breast cancer increases with age, slowing after the menopause.

1.32. Family history

Family history is the most widely recognised breast cancer risk factor. There appear to be two different levels of risk in women with a positive family history. **(i)** The first is due to a genetically inherited predisposition to breast cancer which accounts for only 4 - 5% of breast cancer cases. **(ii)** The second level of risk into which most women with a family history fall is associated with an increased familial incidence of breast cancer where familial clustering may be a result of shared genes, shared environmental exposures or both. The level of risk varies with the closeness of the affected relative and the age at which the relative developed breast cancer, (Figure 2). In contrast to the huge increase in risk group one affords, those women in group two have a risk of developing breast cancer which rarely exceeds 30% (Anderson, 1992; Howell et al. 1994).

At present, mutations in three genes, p53, BRCA-1 and BRCA-2 have been associated with a genetic predisposition to breast cancer, (group one). The familial characteristics suggestive of a genetic predisposition to breast cancer are : multiple relatives with breast cancer; history of breast and ovarian cancer; early onset breast cancer; and bilateral breast cancer (Howell et al. 1994).

BRCA-1 . Mutations of the breast cancer predisposition gene BRCA-1, located on chromosome 17q21, are associated with a substantial risk of both breast and ovarian cancer. For women with these mutations, the estimated risk of developing breast cancer before the age of 50 is 50%, rising to 85% by the age of 65. The risk of a second breast cancer is 65% by the age of 70.
The risk of ovarian cancer is less well quantified and ranges from 20% to 50%.

In addition mutations of BRCA-1 are also associated with an increased risk of prostate cancer and colon cancer. Over 100 mutations of this gene have now been identified, but the risk attributable to each mutation is as yet unknown which also illustrates the complexity of breast cancer genetics (Collins, 1996).

BRCA-2 . Mutations of BRCA-2 located on chromosome 13q12-13 carry the same level of risk for breast cancer as BRCA-1, however there is only a small increase in the risk of ovarian cancer. In addition BRCA-2 mutations are also associated with an increased risk of male breast cancer (Page et al. 1996).

p53. The Li Fraumeni syndrome, in which breast cancer occurs at an early age associated with soft tissue sarcoma, osteosarcoma, adrenal tumours, gliomas and other childhood tumours may be caused by mutations of the p53 tumour suppresser gene. Analysis has shown that approximately half of the families with this syndrome have a p53 mutation (Page et al. 1996).

1.33. Benign breast disease.

The strongest risk factors associated with the development of breast cancer have been shown to be a genetic predisposition and secondly the histological evidence of benign breast disease (Rosen, 1993). The term benign breast disease encompasses a group of pathological entities which have to be specifically defined as they have varying degrees of risk. Hence, benign breast lesions are now defined as being non-proliferative, proliferative and / or atypical (Dupont and Page, 1985).

Non-proliferative breast lesions are not associated with an increased risk of breast cancer, while proliferative breast disease is associated with a relative risk of (1.5 - 2.0) of subsequent breast cancer. Atypical hyperplasia (ductal or lobular) carries the greatest risk with a relative risk of (4 - 5). The interaction of risk factors is illustrated in those women who demonstrate both a family history and a diagnosis of atypical ductal hyperplasia. Their absolute risk of breast cancer increases to 20% at 15 years which equates with a relative risk of 10, (Figure 3) (Page and Dupont, 1993; Palli et al. 1991; London et al. 1992;

Dupont and Page, 1985; Page et al. 1984; Page and Dupont, 1990). Additionally, no further increase in the risk of breast cancer was demonstrated in those women who used exogenous oestrogen after their biopsy (Dupont et al. 1993).

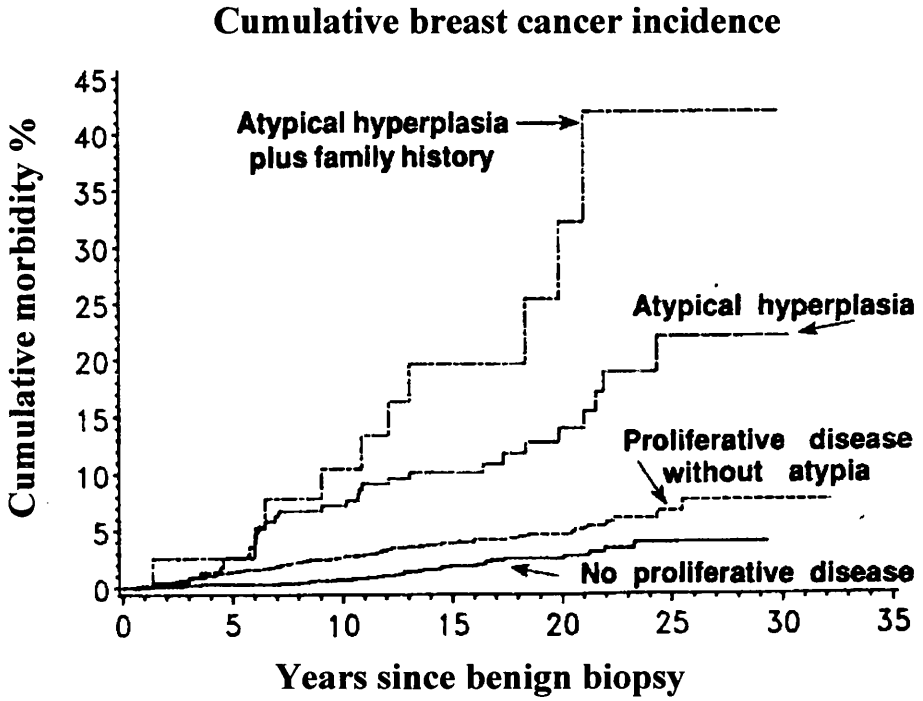


Figure 3. **The absolute risk of subsequent breast cancer following a biopsy showing ADH. (Modified from Page & Dupont New England Journal of Medicine, 1985.)**

1.34. Reproductive history and hormonal factors.

Breast cancer is certainly related to hormonal factors with many studies linking breast cancer incidence to the age of menarche, menopause and first pregnancy with the greatest risk associated with a long period of uninterrupted menstrual cycles. Some authors estimate a 20% reduction in breast cancer risk for each year that menarche is delayed (Lobo et al. 1987), however, others are more conservative in their estimates. Other factors that delay regular menstruation such as physical exercise may also decrease risk (Brown et al. 1973). The age at menopause is also influential with women whose natural menopause occurs before 45 having half the relative risk of breast cancer than those women with a menopause after 55 (Cole et al. 1972).

Nulliparous women are at greater risk than those who are parous with a relative risk of about 1.4. The effect of term pregnancy on risk varies with age at first birth with some authors proposing that women whose first pregnancy occurs after 30 have a 2 - 5 fold increase in risk compared to women whose first pregnancy is before 18 (Bzsuch et al. 1989). Again this risk varies between different investigators, (Figure 2). The effects of an incomplete pregnancy are as yet inconclusive (Bzsuch et al. 1989; Cole et al. 1983).

The effect of the oral contraceptive pill and hormone replacement therapy have been and are being extensively studied, but no clear evidence on risk is obvious. Overall, their is no convincing evidence of an increased risk of breast cancer in women who have ever used oral contraceptives (Romieu et al 1990). Analyses of hormone replacement therapy have demonstrated more variable results, but two meta-analyses suggest small increases in risk associated with duration of usage (Rodigues-Canteras et al. 1992; Weiss et al. 1993).

1.35. Dietary factors

The evidence for diet affecting breast cancer risk is limited, however the observation that national per capita fat consumption is correlated with breast cancer incidence and mortality suggests a possible link. Epidemiological studies have been inconclusive with a number of prospective trials failing to establish a correlation (Colditz et al. 1987).

Evidence does however exist which supports a relationship between a higher alcohol intake and breast cancer risk (Orza et al. 1988).

1.36. Environmental factors

Exposure to ionising radiation, either secondary to nuclear explosion or medical diagnostic and therapeutic procedures increases breast cancer risk, however, less than 1% of breast cancer is estimated to result from common diagnostic radiology (Sherman et al. 1989). Recently, a marked increase risk of breast cancer has been seen in women receiving irradiation for Hodgkin's disease (Hoppe et al. 1993).

Other environmental influences such as exposure to electromagnetic fields, occupational exposures and organochlorine pesticides have been suggested as potential risks, but evidence is still lacking.

1.4. Breast screening programmes

Despite intensive research into various risk factors, the pathogenesis of breast cancer remains unresolved , with the prospect of primary prevention based on the identification and understanding of risk factors a distant reality.

However, one potentially important strategy in reducing mortality from breast cancer is earlier detection, based on the hypothesis that detecting the tumour before it has developed metastatic ability will prevent deaths. To date the only proven method of screening is mammography (Shapiro, 1989). Critics of screening suggested that simply by detecting tumours earlier will not

necessarily decrease mortality. Screening may simply detect lethal cancers sooner (lead time bias), cancers that are growing slowly are less likely to be lethal (length time bias), or tumours with questionable malignant potential (overdiagnosis bias). In addition, women who opt to participate in screening programme may induce a selection bias.

Several randomised trials were set up in attempt to eliminate these criticisms and overall indicate that screening a population of women over 50 appears to decrease breast cancer mortality by approximately 25% (Anderson, J. et al. 1990; U.K.Trial of Early Detection of Breast Cancer Group, 1988; Gad et al. 1992; Janzon et al. 1988; Shapiro, 1989). Breast cancer screening was introduced in the U.K. in 1989.

As a result of screening, a new population of breast cancers are being seen with an increase in the diagnosis of small node negative invasive tumours, ductal carcinoma *in-situ*, tumours of a special type (with a better prognosis) and proliferative breast lesions (Dixon and Blamey, 1994).

Thus, we are now faced with the clinical dilemma of treating a population of tumours and potential pre-invasive lesions whose natural history and biological nature are undetermined. However, this explosion in the diagnosis of proliferative breast lesions and ductal carcinoma in-situ has fuelled intensive scientific interest in these lesions and their potential for subsequent progression to breast cancer.

1.5. Breast cancer progression.

This thesis postulates a genetic basis for a hypothetical model of breast cancer progression. This section reviews the clinical evidence which is compatible with such a model.

The present morphological model of breast cancer progression is based on Vogelstein's observations that a normal colonic epithelium can evolve through

a series of well defined genetic events, closely associated with morphological tumour progression to a colonic carcinoma (adenoma - carcinoma sequence) (Fearon and Vogelstein 1990). This delineation of the genetic events involved in the evolution of colonic carcinoma has paved the way for the investigation of other solid tumours, such as melanoma (Hayward et al. 1995) and bladder cancer (Cordon-Cardo et al. 1993).

In the model proposed for breast cancer, a normal breast epithelium is hypothesised to evolve to invasive cancer via the pre-invasive stages of proliferative disease without atypia (PDWA), atypical ductal ductal hyperplasia (ADH) and ductal carcinoma in-situ (DCIS) (Allred et al. 1993; O'Connell et al. 1994). Each lesion within the model is envisioned as a non-obligate precursor of the next, in the sense that most will pursue a stable natural history while others will accumulate a critical number of genetic alterations and progress to the next stage. While the majority of potential precursors never progress, they are morphologically similar to those that may have already undergone initiating or transforming genetic events, (Figure 4).

The genetic evidence for this hypothesis is limited and it is based primarily on epidemiological evidence and animal studies. For instance, the model is consistent with post-mortem studies which demonstrated that PDWA, ADH and DCIS are found progressively less frequently in the breasts of women dying from causes other than breast cancer (Bartow et al. 1987). Perhaps more compelling evidence is afforded by those studies which show an increased risk of later developing breast cancer after excision of either PDWA [Relative risk =(1.2 - 2)], ADH [Relative risk = (4 -6)] and DCIS [Relative risk = (10 - 12)] (Palli et al. 1991; London et al. 1992; Dupont and Page, 1985; Dupont et al. 1993; Page et al. 1984; Page and Dupont, 1990). Additionally, lesions such as PDWA, ADH and DCIS are observed concurrently in over 50% of breasts containing invasive breast cancer (Bartow et al. 1987). Data from various sources suggest the risk for carcinoma is equally divided between both breasts following a diagnosis of PDWA or ADH (Alpers and Wellings, 1985). This bilateral risk is in striking contrast to the strong tendency for subsequent

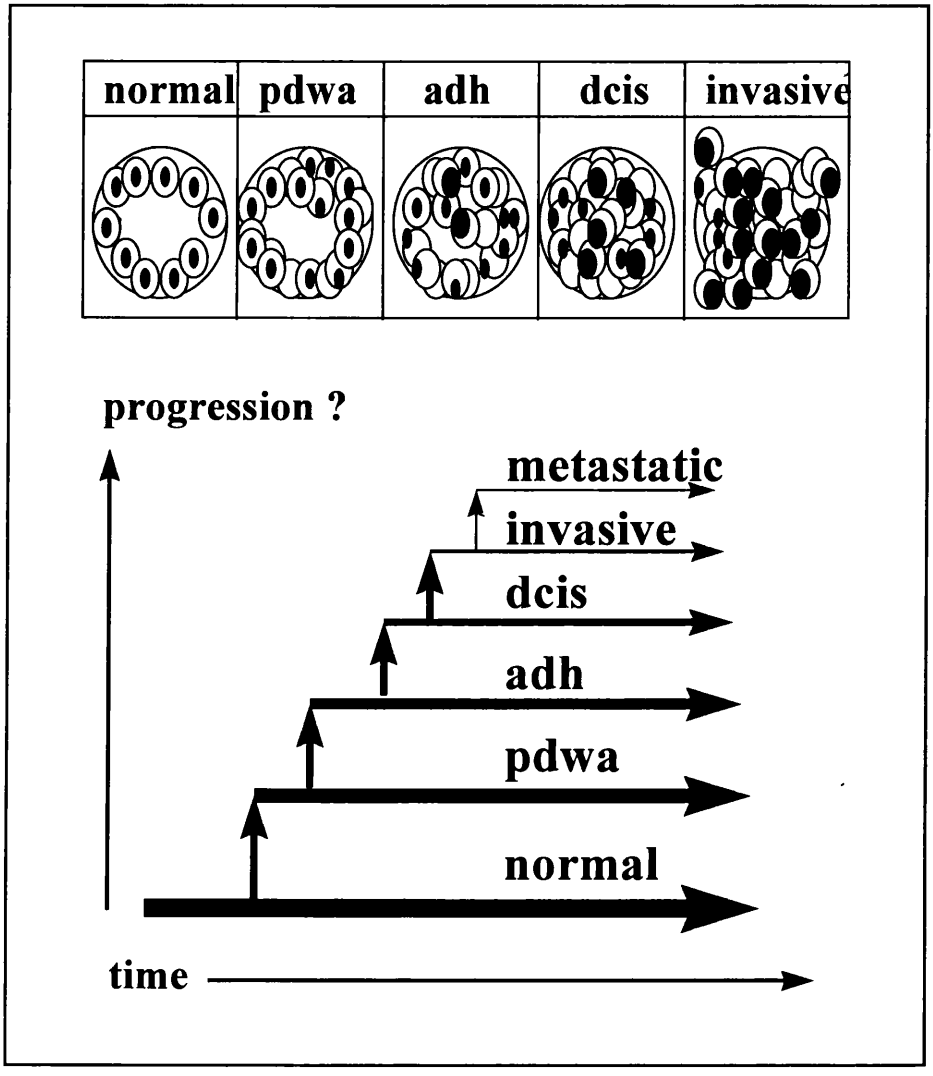


Figure 4. **Morphological model of breast cancer evolution. Ductal breast cancer is hypothesised as evolving from a normal epithelium through a series of increasingly abnormal , but non-obligatory, cellular changes from hyperplasia to invasive carcinoma. (Modified from Allred et al 1993)**

invasive carcinoma to arise in the ipsilateral breast following diagnosis of DCIS.

The hypothesis that breast cancers can evolve from precursor lesions in a stepwise fashion is further supported by experiments on mice in which breast epithelial cells in these animals undergo nodular proliferation to form hyperplastic alveolar nodules. When those lesions are transplanted into mammary fat pads they develop into tumours more frequently than normal breast tissue (Lakhani et al. 1996).

This model provides a framework for the investigation and identification of the morphological, cellular and molecular events involved in the progression to malignant breast disease. Adding to our present knowledge of pre-invasive breast disease by delineating specific genetic changes for each proposed stage of progression and their biological consequences should lead to a better understanding of the natural history of breast cancer and pre-invasive breast disease allowing the development of more rational means of treatment and prevention.

2. Pre-invasive breast disease.

2.1. Ductal carcinoma *in-situ* .

2.11. Epidemiology.

Until recently DCIS was regarded as a clinical oddity accounting for <5% of patients with symptomatic breast malignancies. DCIS frequently contains calcification and hence, with the widespread use of mammographic screening DCIS now compromises 15% - 30% of newly diagnosed carcinomas. This previous pathological oddity now imparts a significant impact on society, emphasising the importance of understanding its biology (Frykberg and Bland, 1994).

2.12. Pathology and natural history of DCIS.

The essential histologic feature of DCIS is ''confinement of malignant epithelial cells within their natural basement membrane boundaries'' (Broders, 1932). Previously DCIS was classified according to growth patterns within the duct system, (papillary, cribriform, solid and mixed) however this has proven to be unsatisfactory as there is much observer variation and DCIS specimens tend to have a mixture of patterns, (Figure 5a). Consequently, the classification has little relationship to clinical outcome. Evidence is now emerging that DCIS is a heterogeneous entity both morphologically and biologically, hence there is a need to develop a reproducible morphological classification which can aid the clinician their planning of appropriate therapy.

Until recently most cases of DCIS were treated with mastectomy, however with the advent of conservative breast surgery this practise is being re-evaluated. Follow up studies show that following a biopsy of DCIS (not complete excision), 14 -30% of women will subsequently develop invasive breast cancer, therefore mastectomy may be a radical overtreatment for many women (Frykberg and Bland, 1994). In trials with complete excision of DCIS there appears to be a recurrence rate of between 15% and 25% of which half are invasive, which is reduced with the addition of radiotherapy by 50% (Lagios and Page, 1993). Consequently, wide local excision with or without radiotherapy may well be an appropriate treatment for DCIS, therefore it is important to identify which types of DCIS are more likely to recur and which are amenable to excision alone. There are a number of new proposed classifications which combine pathological findings and clinical outcome, however the Van Nuys DCIS classification seems to be gaining preliminary acceptance as it divides DCIS into three easily distinguishable groups (non-high grade, without comedo necrosis; non-high grade, with comedo necrosis; and high grade) each of which has a different likelihood of recurrence if treated with breast conservation therapy (Bellamy et al. 1993; Lennington et al. 1994; Holland et al. 1994; Silverstein et al. 1995). Randomised clinical trials, however, are still required to evaluate this and other proposed classifications of DCIS.

H & E Photomicrographs

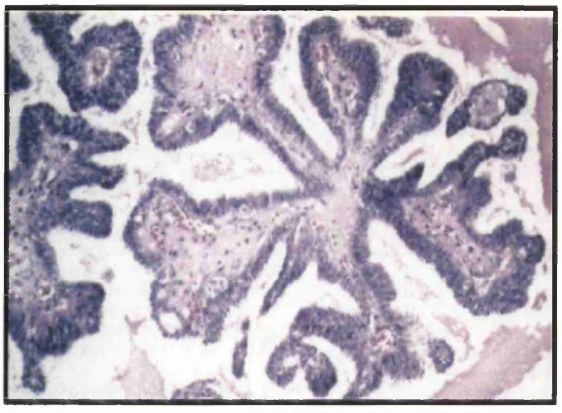


Fig.5a.

Ductal carcinoma in-situ with a papillary growth pattern.

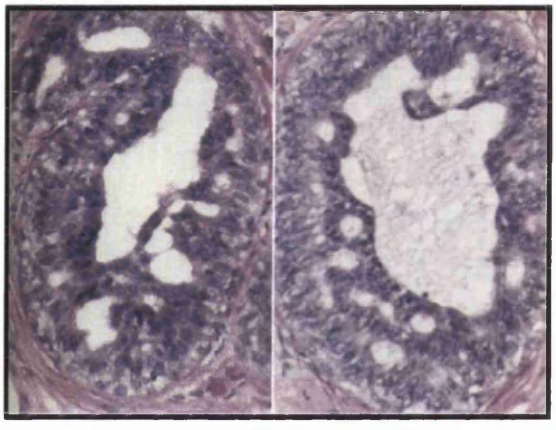


Fig.5b.

Two ducts demonstrating the features of atypical ductal hyperplasia

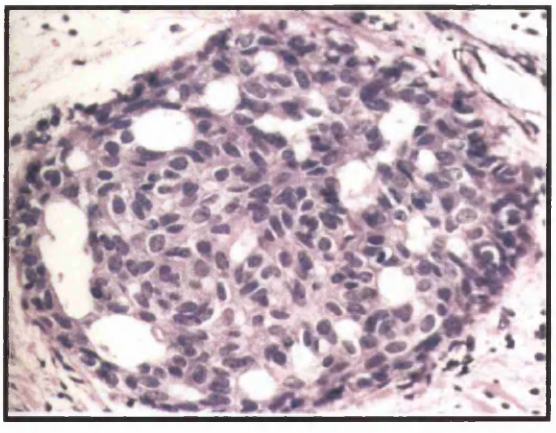


Fig.5c.

Florid proliferative disease without atypia

2.2. Proliferative breast disease.

There is a wide range in the degree of epithelial proliferation in benign breast disease and various attempts have been made to quantify both the degree of change and the magnitude of risk for breast cancer, however the studies of Dupont and Page have provided the gold standard, which has led to the American College of Pathologists recommending that patients with benign breast disease be grouped into Dupont and Page's three categories of risk (Page and Dupont, 1993).

Risk associated with proliferative breast disease:

I	No or mild hyperplasia:	No risk
II	Moderate or florid hyperplasia:	1.5 - 2 times the risk
III	Atypical ductal hyperplasia	5 times the risk

2.21. Proliferative disease without atypia.

This area of breast pathology has been fraught with different terminology (papillomatosis, epitheliosis) leading to confusion, however most pathologists are now adopting the above classification where mild PDWA is made up of 3 to 4 epithelial cells in thickness and moderate to florid PDWA when the appearances are more pronounced, (Figure 5c). A number of specific features identifying the benign nature of these lesions are also described. As yet there are no morphological indications of a particular subpopulation at a greater risk of progression.

2.22. Atypical ductal hyperplasia.

Post-mortem studies confirm that ADH has a prevalence of approximately 2.5% - 4% in women in their forties (Bartow et al. 1987). ADH confers a relative risk = (5) for the development of breast carcinoma and a relative risk =(12) if associated with a positive family history (Dupont and Page, 1985). Further evidence to suggest the pre-cancerous nature of these lesions is the

observation that invasive carcinoma and DCIS can arise in foci of ADH (Cardiff and Morris, 1987; Blair et al. 1959).

2.23. Pathology and Natural history

Atypical ductal hyperplasia is defined as ''hyperplasia of the ductal epithelium with some cytological and / or architectural criteria of DCIS, but is incompletely developed and not uniformly present throughout two complete duct spaces'' (Figure 5b) (Page et al. 1984). The full histopathological criteria for the recognition of ADH are well recognised, however pathologists still fail to concur frequently, which is well illustrated in a recent study where six experienced breast pathologists examined cases of PDWA, ADH and DCIS. Strict criteria were laid down for diagnosing ADH, but the pathologists agreed in only 58% of cases (Gelman, R. et al) . Given the similarity of ADH to low grade non-comedo DCIS there is a school of thought that ADH represents the lower end of a continuum of DCIS. A grey area therefore exists at that end of the spectrum of DCIS. This may account for the low incidence of reported ADH because a diagnosis of DCIS is the 'safer option' as these women will undergo formal treatment, whereas women with ADH will merely undergo clinical and mammographic follow-up.

3. Somatic genetic alterations in breast cancer.

3.1. Introduction.

Invasive breast cancer has been the subject of intensive molecular genetic investigation in recent years with the intention of identifying common genetic alterations which will enhance the present understanding of breast carcinogenesis and hopefully lead to the development of improved therapies and novel treatments.

The contemporary view of carcinogenesis, whereby a tumour arises from cellular transformation, loss of contact inhibition and acquisition of invasive capacity as a result of successive mutations, is in keeping with the present model of breast cancer progression (Fearon and Vogelstein, 1989; Nowell, 1976; Fearon and Vogelstein, 1990; Devilee et al. 1994a). According to this

hypothesis, a cell that acquires specific genetic alterations may develop a proliferative advantage and the clonal expansion of this cell driven by successive mutations, can lead to tumour progression. A substantial amount of evidence is available which indicates that the process of carcinogenesis requires this sequential accumulation of a number of genetic alterations. These genetic alterations are thought to be effected through activating and inactivating mechanisms involving two classes of gene (protooncogenes and tumour suppresser genes) (Bieche, and Lidereau, 1995; Fearon and Vogelstein, 1990).

An elegant example of how these type of genes may interact to cause disregulated cell growth and tumour promotion is the recent model of tumour - promoting events within the G1 control pathway (Figure 6). This has resulted from the recent discoveries in the fields of cell-cycle regulation and tumourigenesis merging to reveal the molecular basis of the ''R'' point or ''restriction point.'' This is a kind of molecular switch, within the cell-cycle, between the alternate routes of cell division, temporary cell-cycle arrest, quiescence, differentiation and cell death. If defective, loss of restriction point control has been found to be associated with malignant transformation (Bartek et al. 1995).

Recent discoveries have confirmed earlier predictions that R-point regulation can be ascribed to proteins encoded by known genes. Implicated are the D-type cyclins (cyclin D1), the retinoblastoma protein (pRB) as the major negative regulator and the cyclin-dependent kinases and their inhibitors (p16). Thus amplification of the protooncogene cyclin D1 or loss of the tumour suppresser genes p16 and pRB can independently or co-operatively disregulate the equilibrium of cell cycle control and facilitate progression into the S-phase and possible malignant transformation. (Bartek et al. 1995).

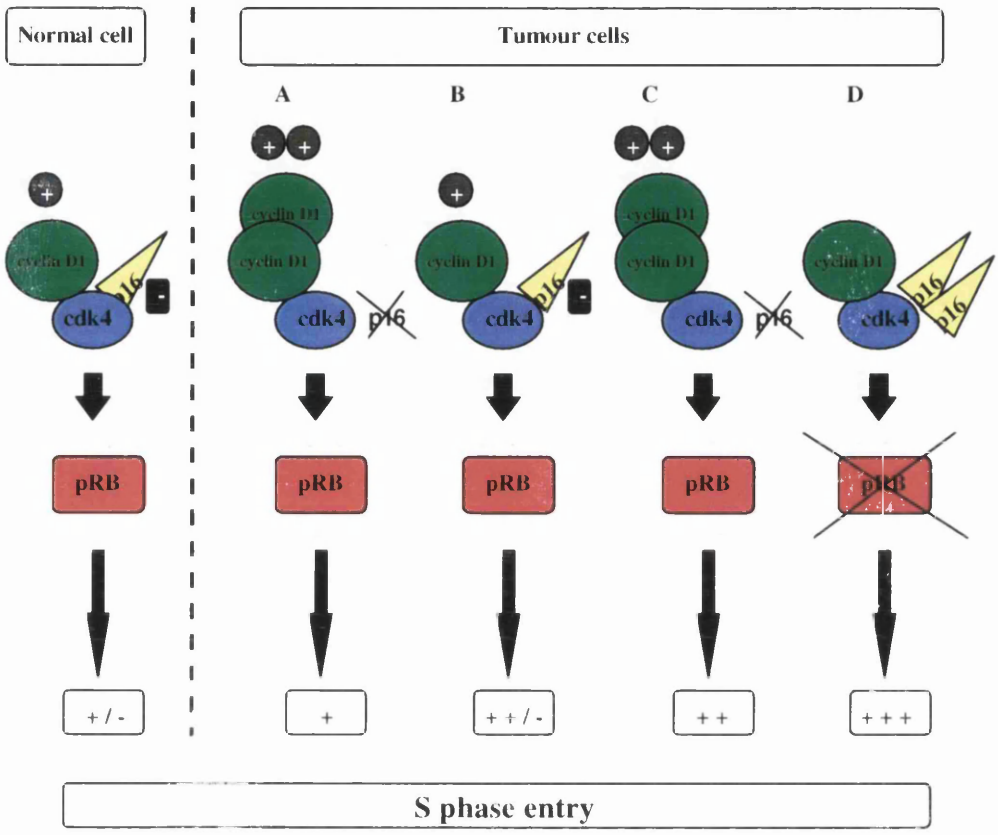


Figure 6. **Model of tumour producing events within the G1 control pathway; Progression into S-phase is facilitated by either loss of p16 [A]. or overexpression of cyclin D1 [B], or loss of the pRB target normally restraining premature S-phase entry[D]. (Modified from Bartek et al 1995)**

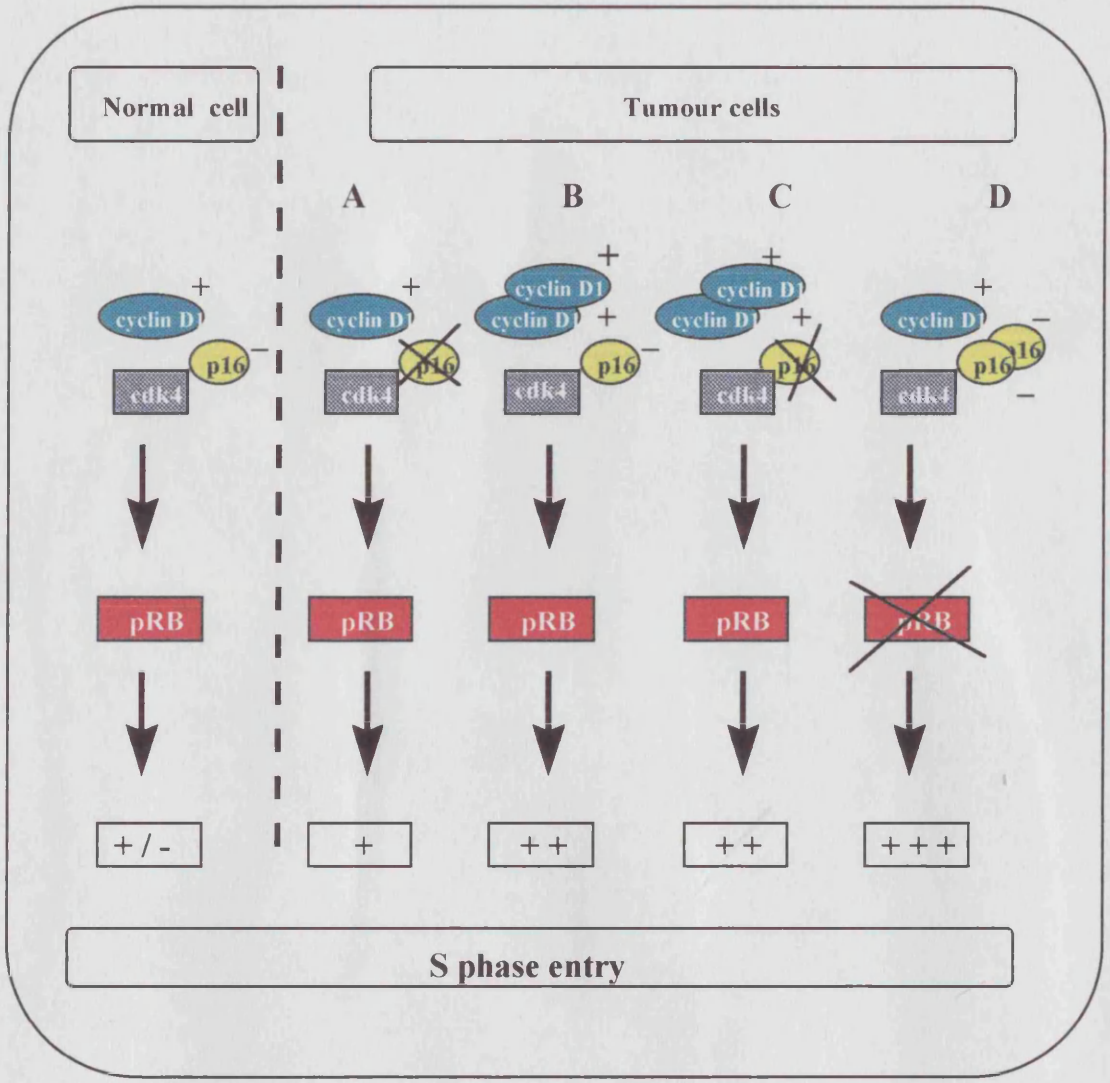


Figure 6. **Model of tumour producing events within the G1 control pathway.**

Progression into S - phase is facilitated by either :-

A. Loss of the p16 tumour suppresser gene.
B. Amplification of the protooncogene cyclin D1.
C. Loss of p16 and amplification of cyclin D1.
D. Loss of the pRB target ormally restraining premature S - phase entry.

(Modified from Bartek,J., Lukas,J. and Strauss,M. Nature Medicine (1995) 1245 - 1248)

Somatic genetic alterations have been studied in breast cancer using a number of techniques: **Flow cytometry; cytogenetics, in-situ hybridisation and molecular analysis.**

3.2. Flow cytometry.

Breast cancer has been extensively studied by flow cytometry demonstrating that 60% -80% of invasive carcinomas are aneuploid and that 10% - 15% contain multiple aneuploid stemlines, providing evidence of tumour heterogeneity. Disappointingly, ploidy has subsequently been found to be of little prognostic value (Devilee and Cornelisse, 1990).

3.3. Cytogenetic analysis.

Prior to the advent of advanced cytogenetic and molecular techniques, classical cytogenetic analysis involved the karyotyping of breast tumours. These studies revealed that numerical changes affecting whole chromosomes were the most prevalent change, involving all chromosomes. (Trisomy of chromosomes 7 and 18 and monosomies of chromosomes 6,8,11,13,16,17,22 & X). A small number of studies have also identified a single clonal abnormality which may have been crucial to tumourigenesis, [i(1q) & del(3)(p12-p14)] (Heim et al. 1995; Zafrani and Dutrillaux, 1990). Due to the technical difficulties of karyotyping solid tumours relatively few breast tumours have been analysed in this way, however the abnormalities are consistent with those found and further refined by molecular techniques.

3.4. In-situ hybridisation.

Fluorescence in-situ hybridisation (FISH) and comparative genomic hybridisation (CGH) are new developments in cytogenetic analysis which increases its resolution and avoids the need for in-vitro cell culture.

CGH has the ability to detect, in particular, regions of amplification in a genome. The technique involves in-situ hybridisation of DNA from normal

and tumour DNA to metaphase chromosomes. Each DNA is labelled with a different modified nucleotide which is revealed separately by specific antibodies labelled with fluorochromes. Increases or decreases in fluorochrome ratios along the chromosome indicate regions of increased or decreased copy number in the tumour. CGH in combination with chromosome microdissection has revealed some 20 amplified regions in breast cancers (Waldman and Kallioniemi, 1994; Trent et al. 1994; Muleris et al. 1995, James et al. 1997).

The most frequently amplified regions are shown in (Table 5). Many whole arm changes have been reported before, however most of the regional copy-number changes have not. 17q22-24 and 20q13 have emerged as major regions of amplification by several authors, suggesting that these loci may harbour previously unknown genes important in breast cancer progression (Waldman and Kallioniemi, 1994; James et al 1997).

FISH detects, locates and quantifies specific nucleotide sequences (DNA or RNA) in tissue sections, cells or chromosomal preparations. It has been used to study several genes (ERBB2, RB1) and is also useful for studying chromosomal mechanisms leading to loss of heterozygosity (LOH) as detected by molecular techniques.

3.5. Molecular analysis.

The advent of recombinant DNA technology has revealed that DNA amplification (mainly protooncogenes encoding growth factors and their receptors) and mutations and LOH that could inactivate tumour suppresser genes are the most frequently observed genetic anomalies in breast cancer.

3.51. Loss of heterozygosity / allelic imbalance

The first tumour suppresser gene to be isolated was the gene for retinoblastoma (RB1) and is thought to contribute to carcinogenesis by its functional inactivation of both the alleles of the gene. This has also been demonstrated in other genes. In familial tumours, the first inactivating

mutation is recessive and thus can be passed through the germline. The second mutation occurs at the somatic level resulting in the absence of the gene product. In non-familial tumours the ''two hits'' both occur at the somatic level. (Knudson, 1993).

In the case of RB1 the second mutation leads to loss of the wild type allele which can be demonstrated via molecular techniques which exploit natural polymorphisms in human DNA which distinguish the parental and maternal alleles. Thus in a tumour heterozygous for a certain polymorphism the loss of one of the alleles can be demonstrated. The tumour is now said to display loss of heterozygosity (LOH) as it is hemizygous for the mutated allele. These mechanisms proposed for RB1 have been verified in other tumour suppresser genes.

Hence, the finding of frequent LOH at a particular chromosomal locus highlights the presence of a tumour suppresser gene or genes at that locus. In practise polymorphic DNA markers will reveal gain and loss of an allele therefore it can be difficult to interpret allelic signals, hence the adoption of the term allelic imbalance to describe this concept.

A vast number of publications have demonstrated that chromosome involvement in breast cancer is complex with 16 chromosome arms showing an average LOH frequency of over 20% (Barnes et al. 1990; Smith et al. 1989; Saunders et al. 1989; Evans et al. 1988; Smith et al. 1992; Nakamori et al. 1990; Callahan et al. 1990; Wiseman et al. 1992; Nakamura et al. 1994; Devilee et al. 1994; Callahan et al. 1987; Callahan et al. 1989; Carter et al. 1993; Sellberg et al. 1992; Nakamori et al. 1919; Lidereau and Bieche, I, 1993; Wenngren et al. 1992; Liscia et al. 1992; Cornelisse et al. 1991a; Cornelisse et al. 1991b; Cornelisse and Devilee, 1990; Fearon and Vogelstein, 1989). Despite this huge amount of data uncertainty still exists on how to interpret this data, however there is cytogenetic and molecular concurrence on the involvement of chromosomes 1, 3, 11p, 16q, 17p, 17q, 18q and 22q in

breast cancer progression. Table 2, summarises LOH data from a number of those studies of invasive breast cancer.

Most of the altered regions are also subject to LOH in other solid tumours which implies that in those regions, a single suppresser gene may be implicated in the progression of different tumours, perhaps at different stages or alternatively there may be several genes in each region, each of which have a role in one type of cancer.

Certainly a number of deleted regions in breast cancer involve known tumour suppresser loci, (Table 3). (Bieche, and Lidereau, 1995) Hence, there are a number of candidate genes which could be involved in breast carcinogenesis, (Table 6). It should be emphasised that many of the deleted regions observed in breast cancer will not lead to the discovery of tumour suppresser genes as certain deletions may deregulate the expression of neighbouring genes by a gene dosage effect.

3.52. Gene amplification.

Amplification of a gene is the result of overreplication that confers a growth advantage through increased levels of the protein encoded by the amplified gene. Usually a genomic region of considerable size is co-ampified, thus making it difficult to determine which of the genes in an amplified region provides a growth advantage. To date several amplified genes and regions have been detected in breast cancer, (Table 4 and Table 5), (Waldman and Kallioniemi, 1994; Trent et al. 1994; Muleris et al. 1995, James et al. 1997, Bieche, and Lidereau, 1995). Three regions harbouring protooncogenes that encode growth factors or their receptors are the most commonly amplified regions in breast cancer: ERBB2, MYC and 11q13, (Table 4). Gene amplifications are usually correlated with a high level of mRNA and protein but not universally as overexpression without amplification may occur due alterations of the regulatory sequences of the gene.

Chromosome arms	Average LOH (%)	Range (%)	No. of studies
1p	23	3 - 52	12
1q	32	12 - 34	9
3p	19	10 - 47	5
6q	20	7 - 48	9
7q	22	0 - 40	3
8p	24	18 - 33	3
9q	25	9 - 36	12
11p	16	10 - 41	9
11q	27	0 - 43	12
13q	21	15 - 45	3
14q	23	13 - 28	3
15q	29	15 - 37	7
16q	49	28 - 63	21
17p	39	34 - 75	17
17q	49	22 - 79	8
18q	23	3 - 34	6
22q	26	0 - 38	14

Table 2. **Loss of heterozygosity in breast cancer.**

Gene	Malignancy	Locus
BRCA-1	Breast & ovarian	**17q21**
BRCA-2	Breast	**13q12 - 13**
p53	Breast (Li Fraumeni)	**17p13.1**
MSH2	Colon (HNPCC)	**2p15 - 16**
MLH1	Colon (NNPCC)	**3p**
APC	Colon (polyposis coli)	**5q21**
MCC	Colon	**5q21**
DCC	Colon	**18q21**
RET	Thyroid (MEN 2A/B)	**10q11 - 12**
WTI	Wilms' tumour	**11p13**
RB1	Retinoblastoma	**13q14**
NF1	Neurofibromatosis I	**17q11.2**
NF2	Neurofibromatosis II	**22q12**
VHL	Von Hippel-Lindau	**3p25 - 26**

Table 3. **Genes associated with tumour suppression or increased cancer susceptability**

Gene	Locus	%
FGFR1	1q21	6
EGFR	7p12-13	3
MYC	8q24	6-23
FGFR2	10q26	6
FGF3	11q13	4-23
IGF1R	15q25	2
ERBB2	17q12	15-30

Table 4. **Genes Known to be Amplified in Breast Cancer and Frequency of amplification**

Ampified loci by CGH
1q21
8q22-24
9p13
11p13
16p11
17q21.1
20q12-13.2

Table 5. **Loci Found to be amplified in Breast Cancer by CGH**

ERBB2.

The ERBB2 protooncogene belongs to the ERBB family (ERBB1, ERBB2, ERBB3 & ERBB4) which encode transmembrane receptors with tyrosine kinase activity. ERBB1 encodes the epidermal growth factor receptor (EGF) . ERBB2 encodes for a 185-kDa transmembrane receptor whose extracellular region is very similar to that of the epidermal growth factor receptor (Bieche, and Lidereau, 1995). ERBB2 is the most extensively studied gene in breast cancer and is amplified and overexpressed in 15% - 25% of invasive breast cancers and with variable frequency in other solid tumours, (Table 4) (Bieche, and Lidereau, 1995; Chen et al. 1995; Allred et al. 1992). Association of ERBB2 amplification and overexpression with rapid proliferation, low oestrogen receptor content and high grade DCIS suggest that this oncogene plays an important role in breast cancer progression (Allred et al. 1992).

In addition to genetic studies ERBB2 has and is being extensively investigated in clinical trials as a molecular marker of potential prognostic significance in breast cancer. In the U.K. about 50% of breast cancer sufferers present without axillary node involvement and appear to be cured, but unfortunately some 25% to 30% of these women will die of disseminated disease. At the moment there is no molecular markers which can help to prospectively identify women

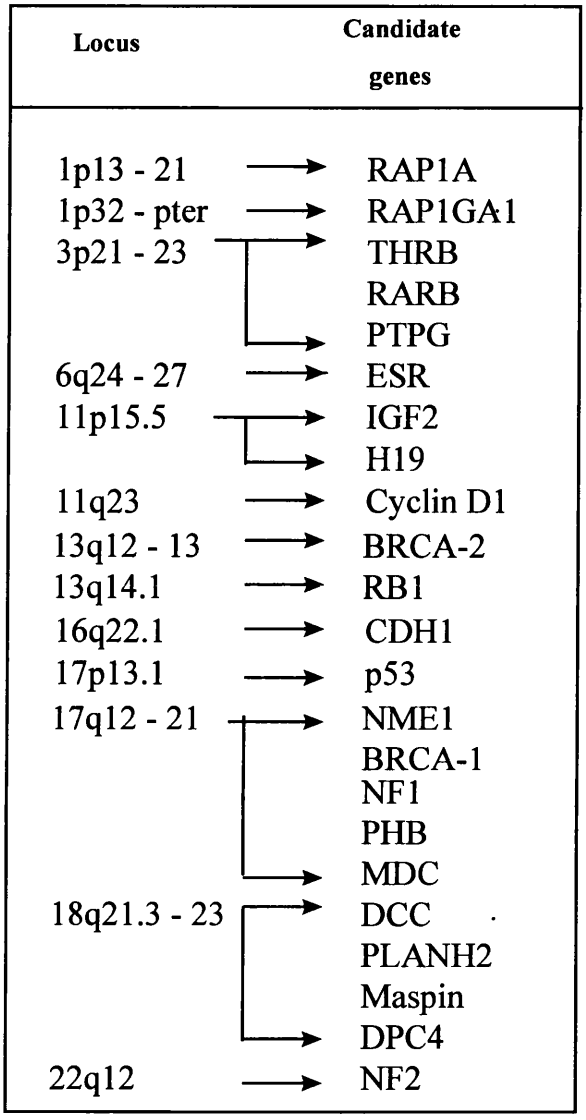


Table 6. **Candidate Genes Involved in Breast Cancer**

at greater risk of recurrence who would benefit from systemic treatment. Most node negative pre-menopausal women receive chemotherapy, which results in 70% of women suffering the morbidity of chemotherapy unnecessarily. Studies to date have shown ERBB2 amplification and/or overexpression to be associated with a poor prognosis only in node positive disease with results from node negative disease remaining inconclusive (Miller,WR. 1994).

MYC.

Myc is a member of a family of genes including MYCL1 and MYCN, however only MYC is amplified in breast cancer. The frequency of reported amplification varies from 4% to 41%. The MYC protooncogene encodes for a 59 - 62 kDa nuclear protein involved in the control of normal growth, differentiation and apoptosis. It has been proposed to play an important role in embryogenesis, cell growth and the tissue repair process. Overexpression is reported without amplification, therefore MYC may be activated by means other than gene amplification. Clinical trials have failed to show MYC of any prognostic significance to date (Riou et al. 1988).

Amplification of 11q13.

The 11q13 band is amplified in 15% - 20% of invasive breast cancers (Escot et al. 1988). Results demonstrate that there are at least four independently amplified regions and different genetic rearrangements. The amplicon contains a number of genes potentially involved in breast tumourigenesis: FGF3, FGF4, GSTP1, EMS1 & CCND1 (Bieche, I and Lidereau, 1995).

FGF3 and FGF4 are frequently co-amplified in breast cancer and due to their involvement in mouse tumourigenesis were thought to be good candidate genes for a role in human cancer, however their expression is undetectable which implies that the amplicon contains a gene responsible for a selective advantage (Bieche, and Lidereau, 1995).

GSTP1, EMS1 and CCND1 amplification is associated with overexpression of their products. GSTP1 is amplified in 5% - 10% of breast cancers and has been muted as a gene responsible for amplicon selection due to its proximal

site on the amplicon. CCND1 encodes a protein of the cyclin family, coding for cyclin D1. Cyclin D1 seems to regulate the G1 - S restriction point in association with CDK4 kinase. The role of the cyclins in breast cancer progression remains to be seen, although overexpression of cyclin D1 is common in breast cancer (Weinstat-Saslow, D., et al 1995).

3.6. Tumour heterogeneity.

Flow cytometry has demonstrated breast carcinomas with multiple aneuploid clones which provides good evidence of intratumour genetic heterogeneity. In studies the degree of heterogeneity found is proportional to the number of areas sampled with up to 60% of tumours showing greater than one aneuploid cell line (Cornelisse and Devilee. 1990). This hypothesis of intratumour heterogeneity is supported by karyotypic and in-situ hybridisation studies (Fearon and Vogelstein, 1990).

Ductal carcinoma in-situ constitutes a diverse and complex group of lesions with extensive intertumour heterogeneity. Recent molecular analyses, where multiple tumour foci were analysed for LOH, have suggested that DCIS may also exhibit extensive intratumour heterogeneity (Tsuda et al. 1995). This observation suggests the existence of multiple genetically diverse subclones within a section of DCIS and has implications for the future analysis and interpretation of LOH studies. For instance if only one tumour focus is investigated the frequency of genetic alterations may be underestimated or missed. More importantly, if multiple subclones of DCIS exist, there is the potential to detect genetic alterations in different clones and perhaps identify alterations that are associated with transformation to the malignant phenotype.

4. Somatic Genetic Alterations in Pre-invasive Breast Disease.

The role of chromosome abnormalities in invasive breast cancer has been extensively studied. However, due to the complexity of the alterations no clear genetic model of the critical events involved in breast cancer progression has emerged. However, the methodology employed and the data obtained from invasive cancer has set the agenda for the investigation of pre-invasive breast disease. During the process of clonal evolution and malignant transformation, critical genetic changes could occur at an early stage, hence it is important to understand the emerging order of genetic alterations in breast cancer progression and their relation to histopathological stage.

By gaining a better understanding of the molecular characteristics of pre-invasive breast disease, the critical issue of the likelihood that PDWA, ADH and most importantly DCIS will progress to breast cancer may be answered and the model of breast cancer progression strengthened.

4.1. Genetic alterations in ductal carcinoma in-situ.

The characterisation of genetic alterations shared by DCİS and invasive breast cancer has reinforced evidence that DCIS is a true precursor of breast cancer, however, relatively little is known about early genetic changes associated with the transformation of normal breast epithelium to the malignant phenotype.

Progress in defining the molecular characteristics of DCIS indicates that DCIS is a non - obligate precursor of invasive breast cancer, but markers for identifying DCIS with a high potential for invasion have not yet been found. Evidence for this is primarily based on LOH studies of DCIS and comparative LOH and CGH studies of DCIS and invasive carcinoma from the same specimen (Waldman and Kallioniemi, 1994; Trent et al. 1994; Fearon and Vogelstein, 1990; Muleris et al. 1995; James et al. 1997).

The allelotyping of DCIS has demonstrated a high frequency of allelic imbalance on chromosomes 1p,1q,6q,8p,11q,13q,16q,17p,17q and 18q which mirrors alterations found in invasive cancer, (Table 7), (James et al 1997; Stratton et al. 1995; Aldaz et al. 1995; Murphy et al. 1995; Fujii et al. 1996; Allred et al. 1993; Harrison et al. 1995; Radford et al. 1995). The relation of LOH% to grade of DCIS suggests that alterations affecting 16q and 17p occur at a high frequency in low grade DCIS suggesting that those chromosomes may be involved in an early stage of progression. When invasive disease co-existed the majority of allelic imbalances were present in both in-situ and invasive components.

Preliminary studies using comparative genomic hybridisaton reveal that chromosome alterations in pure DCIS and DCIS in the presence of invasive cancer, are of a similar pattern. Gains involving 1q, 8q, and 17q were frequent as were losses of 8p, 16q and 17p. Paired samples of DCIS and invasive cancer from the same patients showed almost identical changes, suggestive of close clonal relationships (Waldman and Kallioniemi, 1994; Trent et al. 1994; Muleris et al. 1995).

The amplification of the ERBB2 oncogene in DCIS has been reported as high as 66% (Liu et al. 1992; Hubbard et al. 1994; Allred et al. 1992), however, those analyses relied on differential PCR techniques and, in the absence of control loci from chromosome 17, polysomy of chromosome 17 may be misinterpreted as ERBB2 amplification. It is therefore possible that those studies overestimated the true ERBB2 gene frequency in DCIS because of the high frequency of polysomy of chromosome 17. Indeed, subsequent studies using FISH which are controlled for chromosome 17 polysomy, demonstrate frequency of amplification in keeping with that of invasive cancer (Murphy et al. 1995).

Preliminary analysis also reveals the overexpression of BRCA-1 in DCIS which may indicate a role as a tumour suppresser during the evolution of DCIS to invasive disease (Munn et al. 1996).

Locus	DCIS - LOH frequency (%)				
1p	30				
1q	28				
2p-ter					
6q	32				
8p			18.7		
9p	30				
11p	44				
11q	33				
13q	39		18		
16q	51	29	28.6	25	33
17p	68	41	37.5	29	
17q	60	11	15.9	29	22
18q		16	10.7		
Authors	Fujji	Tsuda	Radford	Aldaz	O'Connell

Table 7. **Loss of Heterozygosity in Ductal Carcinoma *in-situ* (Summary of the most Recent Data)**

4.2. Genetic alterations in proliferative breast disease.

Substantial evidence exists to support the view that a fundamental feature of breast cancer evolution is the accumulation of multiple genetic alterations, however evidence of such alterations in ADH and PDWA is sparse and inconclusive. For instance flow cytometry has demonstrated aeuploidy in approximately 36% of atypical ductal hyperplasia. (Micale et al. 1994). LOH analysis of PDWA and ADH has been very limited with a small number of studies showing low frequencies of allelic imbalance on 16q and 17p, although a wide array of markers were not used (Lakhani et al. 1995). Levels of Expression of Cylcin D1 have been found to be markedly lower in ADH than DCIS, which was interpreted to differentiate between low grade DCIS and ADH (Weinstat-Saslow et al. 1995). Other than this a comprehensive genetic analysis of proliferative disease is not yet available.

5. Identification of Genetic Alterations in Pre-invasive Breast Disease.

In recent years much interest has focused on the early detection of breast carcinoma and the potential for prevention strategies for women at high risk, and this has been fuelled by the implementation of breast screening programmes which have increased the diagnosis of all forms of pre-invasive disease. The management of women found to have proliferative breast disease is unsatisfactory with mammographic surveillance being the only realistic option for these patients. It is therefore particularly important that the genetic profile of these lesions is comprehensively studied in order to detect potential biomarkers for progression and hence future avenues for therapeutic intervention and prevention.

Traditional molecular and cytogenetic analyses have been hampered by the small and heterogeneous nature of DCIS and proliferative lesions. Our group and other investigators have used cytogenetic techniques to study

chromosomal alterations in DCIS, as interphase cytogenetics allows the histological distribution of genetic alterations to be identified. Our group have previously found DCIS to have extensive chromosomal imbalances affecting chromosomes 1, 3, 10, 17, and 18 (Murphy et al. 1995). The aim of the study was to firstly establish a genetic link between PDWA and DCIS using FISH and thereafter use the more sophisticated recombinant DNA technology to pusue in more detail the genetic alterations which could link PDWA and DCIS.

6. Aims

The purpose of the following study was two-fold : -

(i) **To analyse sections of isolated PDWA using interphase cytogenetics (fluorescence in-situ hybridisation) to identify gene alterations and chromosomal imbalances in PDWA, the earliest proposed morphological stage of breast cancer progression.**

(ii) **To analyse multiple, microdissected tumour foci from sections of DCIS, previously found to demonstrate a loss of chromosome 18 sequences by FISH, in order to investigate the phenomenon of intra tumour heterogeneity and identify regional alterations on chromosome 18**

MATERIALS and METHODS

Chapter 2.

Materials and Methods

2.1. Materials.

2.11. Chemicals.

All chemicals were obtained from the following manufacturers unless otherwise stated: BDH Chemicals Ltd., GIBCO BRL, and Pharmacia LKB Biotechnology.

Alpha Laboratories, Eastleigh, Hants.

Streck Tissue Fixative

Amersham International PLC, Amersham, Bucks.

α^{32}P dCTP

Beatson Institute Central Services

PBS

Boehringer Mannheim UK Ltd., Lewes, East Sussex.

proteinase K

DAPI

Taq polymerase

PCR buffer / Mg^{2+}

Block

National Diagnostics, Atlanta Georgia

Sequagel Sequencing System

Sequagel Concentrate

Sequagel Diluent & Buffer

Promega Ltd., Milton Keynes, Bucks.

dNTPs

Taq polymerase

Research Genetics, Huntsville, AL.

Human MapPairs™

Sigma Chemical Co., Ltd., Poole,Dorset

Tween 20

TEMED

bromophenol blue

xylene cyanol

proteinase K

Pepsin

Propidium iodide

Vector Laboratories Inc., Burlington, USA

Vectashield antifade mounting medium.

2.12. General Solutions.

20 x SSC

3 M NaCl

0.3M Tri-sodium citrate

TBE (x1)

89mM Tris Borate

89mM Boric Acid

2.5mM EDTA

TAE (x1)

40mM Tris base

20mM EDTA

20mM NaCl

20mM Na Acetate

PBS

20 PBS (Dulbecco 'A') formulated tablets (OXOID, Unipath LTD.)

PER 2 Litres of distilled water

2.13. Gel running Solutions.

8% Polyacrylamide Gel

15mL Sequagel Concentrate

39mL Sequagel Diluent

6mL Sequagel Buffer

150 μL APS

75μL TEMED

DNA Loading Dye

0.25% bromophenol blue

0.25% xylene cyanol FF

30% glycerol

2.14. FISH solutions.

70% Hybridisation Solution for Centromeric Probes

70% formamide

2 x SSC

5% Dextran sulphate

500ug/mL salmon sperm DNA

50% Hybridisation Solution for Single-Copy Probes

50% formamide

2 x SSC

10% Dextran sulphate

500ug/mL salmon sperm DNA

Denaturation Solution

70% formamide in 2 x SSC at 70 - 80°C

10% Block Solution Stock

10% Boehringer Mannheim blocking reagent in maleic buffer.

Microwave until solution becomes turbid and autoclave.

Used at a concentration of 0.5% in maleic acid buffer/Tween

4 x SSC-T or PN-T buffer

4 x SSC Wash Solution

4 x SSC

0.05% Tween 20

PN buffer

Mix of 0.1M NaH2PO4 and Na2HPO4

PN-T

PN buffer

0.05% Tween

2.15. Antibodies.

Boehringer Mannheim UK Ltd., Lewes, East Sussex

anti-digoxigenin (affinity purified sheep IgG)

Stratech, Luton, Bedfordshire

Fluorescein (FITC) conjugated AffinitiPure Donkey Anti-Sheep IgG

2.16. FISH probes.

Appligene Oncor, Durham

Chromosome-specific repetitive sequence probes

Vysis Inc., Downers Grove, Il

Locus Specific Identifier probe (LSI®) Her 2/neu Spectrum Orange™ : CEP® 17 Spectrum Green

2.17. Equipment and Plasticware

Eastmann Kodak Co., Rochester, New York

X - ray film

Fujji Photo Co., Ltd., Japan

X - ray film

Gibco Europe, Life Technologies Ltd., Paisley

Gel running apparatus

Greiner Laboratechnik Ltd., Dursley

Eppindorfs

aerosol resistant pipette tips

Hybaid Ltd., Teddington, Middlesex

Omnislide in-situ system

PCR thermal cycler

Whatman International Ltd., Maidstone

3MMM paper

2.2. Fluorescence In-Situ Hybridisation.

2.21. Study Population.

A group of 37 formalin-fixed, paraffin embedded blocks of tissue from 31 patients with isolated proliferative disease without atypia were studied. The tissue blocks were collected from 1986 through 1992. Each block was given a number from P1 to P31 with two blocks from the same patient designated 'a' and 'b' . However, it is difficult in this material to assess the true relationship between blocks and these blocks may represent independent sites of proliferative disease or contiguous disease.

Histological characterisation of PDWA, with particular reference to exclusion of atypical ductal hyperplasia, was carried out independently by two experienced breast pathologists (Dr. E.E.A. Mallon & Dr. J.J.Going) and showed absolute concurrence.

Histology on sections from the 37 blocks showed 22 to be predominantly moderate PDWA, 11 to be florid PDWA and 4 to be mild.

2.22.Tissue Section Preparation.

Sections, 5 μm thick, from formalin-fixed paraffin-embedded tissue were placed on aminopropyltriethoxysilane-treated slides, to avoid the sections lifting from the slide during analysis. Before use the slides were baked at 65°C for 24 hours. The slides were then dewaxed with xylene (3 washes of 10 min each) followed by methanol (2 washes 5 min each). Slides were then digested with pepsin (0.4% pepsin in 0.2N hydrochloric acid) for 45 min at 37°C. After washing 5 times with distilled water and 5 times in PBS, the slides were post-fixed in 1% formaldehyde in PBS, 50mM $MgCl_2$ for 10 min at room temperature (Streck Laboratories, Inc.,Omaha, NE). Finally the sections were dehydrated in ethanol.

2.23. DNA Probes and Probe Labelling.

Chromosome-specific repetitive sequence probes for the following loci were purchased from Oncor, Inc. (Gaithersburg, MD):

D1Z5 (Chromosome 1)	D10Z1 (Chromosome 10)
D3Z1 (Chromosome 3)	D17Z1 (Chromosome 17)
D6Z1 (Chromosome 6)	D18Z1 (Chromosome 18)

Chromosome specific repeat sequence centromeric probes were diluted in a hybridisation mix consisting of 70% formamide, two times the standard concentration of standard saline citrate (SSC) (1XSSC IS 0.15*M* NaCl and 0.015*M* sodium citrate [pH 7], 500μg / mL salmon sperm DNA, and 10% dextran sulphate. These commercial probes were labelled with digoxigenin.

2.24. In-Situ Hybridisation

The chromosome specific digoxigenin labelled probe was added to the tissue section, a coverslip applied and sealed with cowgum. For the detection of chromosome-specific repetitive sequences, the probe in the hybridisation mix and the DNA in a tissue section were denatured together using the Omnislide modular system (Hybaid Ltd, London, U.K.) at 80 °C for 5 minutes.

The probe was then allowed to hybridise to the tissue DNA for 24 hours at 37°C in a humidity chamber to prevent the slides from drying out.

After hybridisation, the coverslips were removed from the slides in 2 x SSC and washed twice in 50% formamide and 1 x SSC at 42°C for 10 minutes and twice in 2 x SSC at 42°C for 10 minutes each time.

Prior to immunocytochemical detection, slides were blocked for 30 minutes in 4 x SSC-TB (4 x SSC, 0.05% Tween 20, and 0.5% Boehringer blocking agent (Boehringer Mannheim Corp., Indianapolis, IN.)

2.25. Immunocytochemical Detection.

The **first detection layer** consisted of sheep anti-digoxigenin (sheep anti-digoxigenin at 1:200 dilution in 4 x SSC-TB for one hour at room temperature) followed by a wash in 4 X SSC-T for 10 min at roon temperature.

The **second detection layer** consisted of donkey anti-sheep fluorescein isothiocyanate (FITC) (at 1:300 dilution 4 x SSC-TB for one hour at room temperature) followed by a wash of 20 min in 4 x SSC-T at room temperature.

The slides were then dehydrated in ethanol and counterstained with propidium iodide (0.4μg/mL) and mounted in antifade medium (Vectashield; Vector Laboratories, Inc.).

Fluorescence was analysed on a Bio-Rad (Richmond, CA) MRC-600 laser scanning confocal microscope equipped with a krypton argon laser. Original unedited images were stored as separate files on optical disks. Images were processed using edge enhancement algorithms (Comos Software, Bio-Rad) to aid definition of nuclear boundaries and hybridisation sites. Images were merged using image Comos and Nexus software (Bio-Rad). Optimal colour balance of the pseudocolour images was achieved using image processing software, (Photomagic, Micrografx, Arapaho Richardson, Texas, USA).

2.26. ERBB2 / Chromosome 17 Double Hybridisation's.

2.261.Tissue section preparation was as described before.

2.262.Probe preparation .

ERBB2 and chromosome 17 sequences were detected using the Locus Specific Identifier probe (LSI®) Her 2/neu Spectrum Orange^{TM} : CEP® 17

Spectrum Green ™ (Vysis Inc., Downers Grove, Il.). The probe was diluted in distilled water and LSI® hybridisation buffer. The probe was then denatured separately from the tissue section at 74°C for 5 min.

2.263. In-situ hybridisation.

The tissue sections were denatured in a 70% formamide solution at 80°C for 5min as previously described, then dehydrated in ice-cold ethanol. The denatured probe was then added to the tissue sections, sealed with a coverslip and hybridised in a humidity chamber for 24 hours at 37°C. After hybridisation, the coverslips were removed from the slides in 2 x SSC and washed twice in 50% formamide and 1 x SSC at 42°C for 10 minutes and twice in 2 x SSC at 42°C for 10 minutes each time.

As the probe was directly labelled with two fluorochromes no immunocytochemical detection was necessary. The slides were therefore dehydrated in ethanol and counterstained using DAPI. Fluorescence was analysed and images captured using a CCD camera. Original unedited images were stored on optical disks

2.3. Microsatellite Analysis.

2.31. Tumour Samples.

A group of 11 formalin-fixed, paraffin embedded blocks of ductal carcinoma in-situ, previously found by interphase cytogenetics to demonstrate chromosome 18 loss, was studied. Histology demonstrated the sections to be of the comedo, high grade end of the spectrum. Several ducts within each section were identified, by an experienced breast pathologist (Dr.J.J.Going) together with a normal terminal duct lobular unit, to act as a normal control.

2.32. Tissue Microdissection.

Sections (5μm) of formalin-fixed, paraffin embedded tissue were cut and mounted on untreated glass slides (to aid microdissection), dewaxed in xylene and rehydrated. The hydrated sections were stained in 0.1% toluidine blue for 30 seconds. Each identified duct with DCIS was individually microdissected, an approach which ensured a minimum of contamination of normal tissue. Tissue microdissection was carried out with the aid of a dissecting microscope (model SZ11; Olympus Optical Co., London, U.K.) equipped with a Leitz mechanical micromanipulator (type M; Leica Inc., Buffalo,NY.).

2.33. Preparation of Samples and DNA Extraction.

Microdissection was carried out under a drop of proteinase K buffer (10mM Tris and 1 mM EDTA [pH 8.3]), and tissue fragments consisting of individual ducts were transferred to a mictocentrifuge tube in 12.5μL or 25μL of the same buffer. An equal volume of proteinase K buffer that contained proteinase K (1mg/mL) and 1% Tween was then added. After overnight digestion at 37°C, proteinase K was heat inactivated at 95°C for 10 minutes. Four microlitres of this crude DNA lysate was then used for each PCR reaction.

2.34. PCR Amplification using Chromosome 18 Microsatellites

50μL reactions were performed with 4uL of crude DNA lysate in a reaction mixture with 200uM of each dNTP, 2 oligonucleotide primers at 200nmol each, 0.2 units of *Taq* polymerase (Boehringer Mannheim, U.K.) supplied with Buffer (50mM KCl, 20mM Tris-HCl pH 8.3, 5mM $MgCl_2$) and 1μCi [^{32}P] deoxycytidine triphosphate.

All reaction were conducted in a Hybaid Omnigene PCR thermal cycler using a touchdown PCR protocol as follows :

95°C	4 minutes	1 cycle
95°C	1 minute	
52 - 62°C	2 minutes	3 cycles
72°C	30 seconds	
95°C	1 minute	
52 - 62°C	2 minutes	3 cycles
72°C	30 seconds	
95°C	40 seconds	
52 - 62°C	30 seconds	30 cycles
72°C	30 seconds	
72°C	4 minutes	1 cycle

The annealing temperatures were varied to give the best amplification at highest stringency for each of the amplimers used. The amplimers were obtained from Research Genetics, Huntsville, Alabama.

2.35. Separation of PCR Amplified Products.

PCR products were resolved on 6% acrylamide denaturing gels formed using 20 x 38cm glass plates (Model S2 gel apparatus, Life Technologies, Gibco, BRL.) coated / polished with ''Mr Sheen'' (Beckit & Cowan Products Ltd,Hull.). PCR products were loaded with 50% glycerol, 1 x TBE, 0.25% bromophenol blue, 0.25% xylene cyanole and electrophoresis was conducted at 40^0C for 4 hours at 60 Watts. Allelic imbalance was assigned when only the complete loss of an allele was observed.

RESULTS

Chapter 3.

Quantification of FISH.

3.1 Introduction.

The technique of fluorescence in-situ hybridisation is based on the same principle as Southern blot analysis, ie, the ability of single-stranded DNA to anneal to complementary DNA. As in Southern blot analysis, the target DNA is attached to a substrate; in the case of FISH , the target DNA is the nuclear DNA of interphase cells. The test probe is labelled, most commonly by enzymatic incorporation of biotin or digoxigenin-labelled nucleotides. The cellular DNA and the labelled probe are denatured to form single stranded DNA. A solution containing the probe DNA is then added to the slide, covered and allowed to hybridise. Thereafter, the unbound probe is removed by washing steps. The labelled probes are then detected with fluorochromes (Fig.1.)

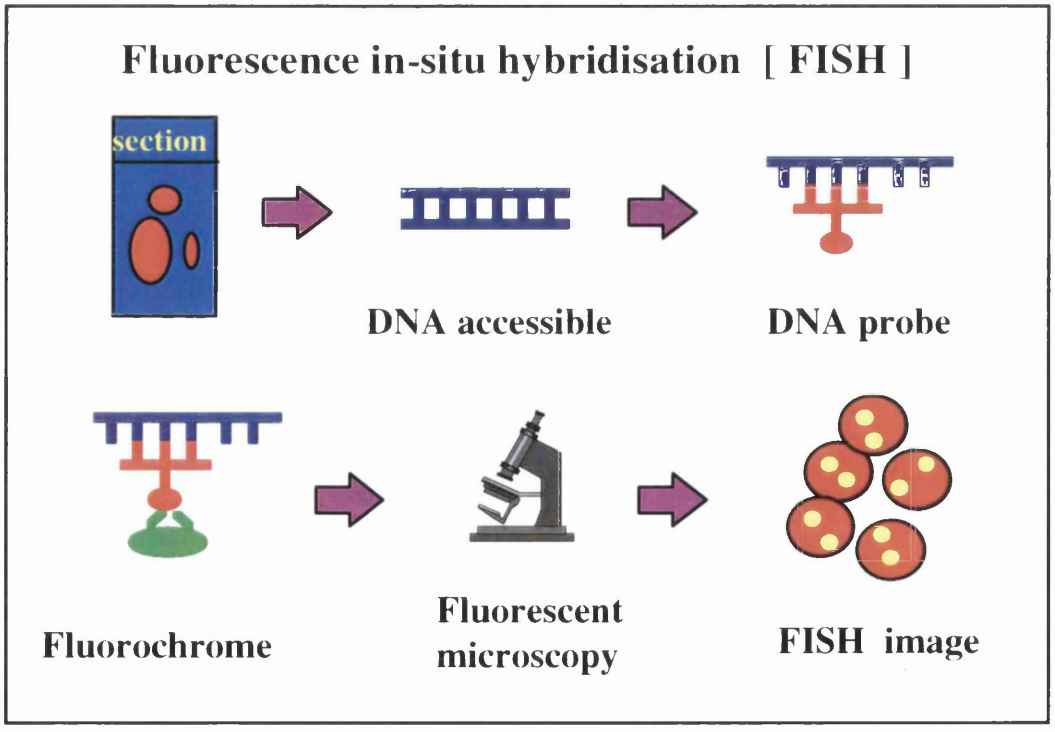


Figure.1. **Schematic Representation of FISH.**

3.2. Quantification of Hybridisation Signals.

Chromosome -specific centromeric probes were hybridised to sections of normal breast tissue and sections of PDWA. The evaluation and interpretation of FISH signals were carried out as described by Murphy et al., (Murphy et al. 1995) in which overlapping nuclei were not analysed, minor hybridisation signals identifiable by their low intensity and smaller spot area compared with optimal hybridisation's were not counted and fluorescent patches inconsistent in shape with hybridisation signals were not counted. Poor quality hybridisation's were not analysed. For each section of PDWA, 200 nuclei from an area of PDWA, 200 nuclei from a normal lobule within the section and 200 nuclei from a normal breast section, included in each experiment as an external control, were counted.

This information was recorded in frequency-distribution table, and examples are shown in Table 1, which shows examples of the frequency of hybridisation sites for chromosome 1 on four sections of normal breast tissue. Table 1, shows the analysis of the number of hybridisation's per nucleus for chromosomes 1 in normal breast tissue, while (Table 2), shows the basic statistical analysis of the hybridisation data presented in Table 1 . The data documented in Table 1 are represented in (Figure 2), which show the frequency distribution of the number of hybridisation sites per nucleus. As can be seen from (Figure 2), 60% to 70% of the nuclei in normal tissue sections show two hybridisation signals. The majority of other nuclei have either one or no hybridisation signals. This observation is due to nuclear truncation during sectioning, (Figure 3).

Sample	Number of hybridisations / nucleus				
	0	1	2	3	4
normal breast 1	28	48	144	4	0
normal breast 2	21	36	151	6	0
normal breast 3	30	56	132	4	0
normal breast 4	18	38	141	3	0

Table 1. **Chromosome 1 Copy Number in Normal Breast Tissue**

Sample	N	mean	median	SDEV	SE	Range
normal breast 1	224	1.55	2	0.73	0.05	0 - 3
normal breast 2	214	1.66	2	0.69	0.05	0 - 3
normal breast 3	222	1.49	2	0.75	0.05	0 - 3
normal breast 4	200	1.64	2	0.66	0.05	0 - 3

Table 2. **Statistical Analysis of Chromosome 1 Content**

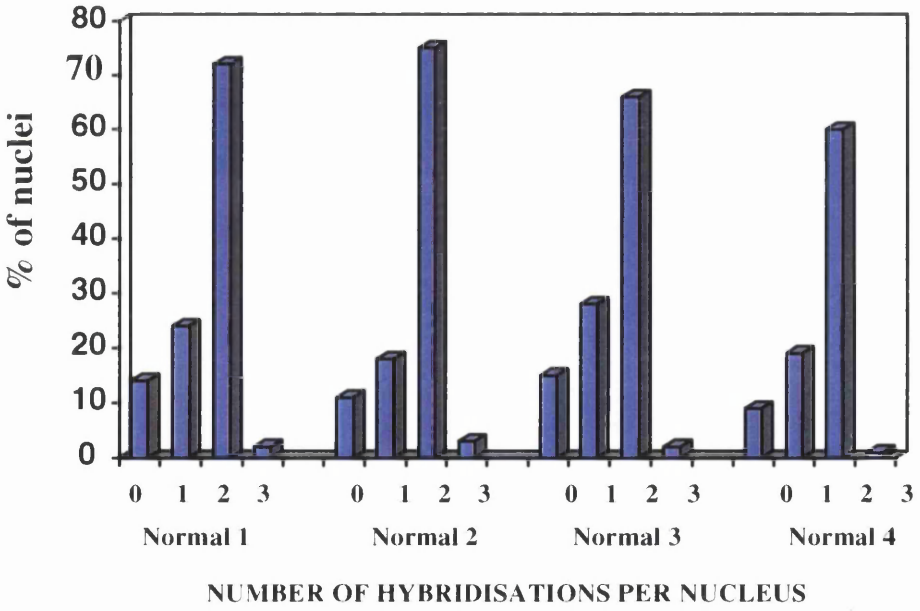


Figure.2. **Frequency distribution of chromosome 1 hybridisation signals in sections of 4 normal breast sections.**

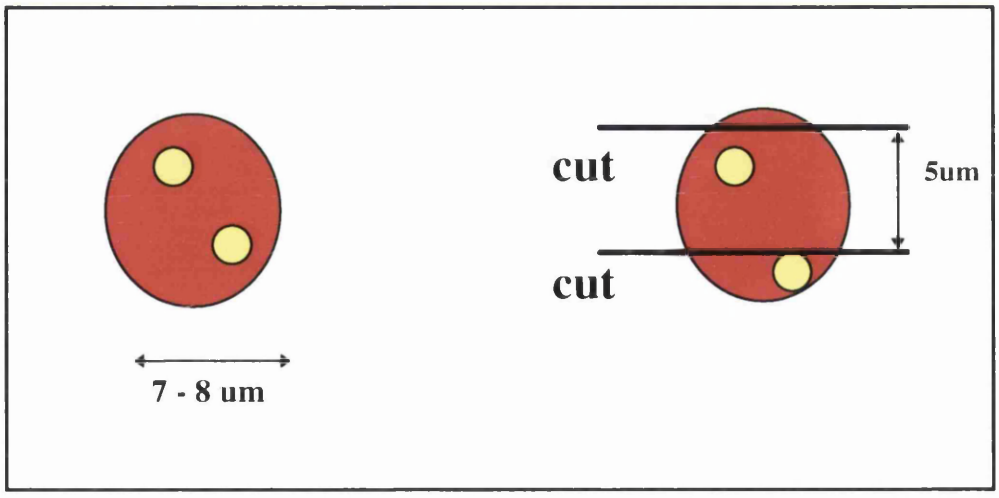


Figure 3. **Schematic representation of how 5μm sectioning of paraffin sections can lead to nuclear truncation and loss of a predictable percentage of hybridisation signals from normal breast tissue.**

The hybridisation data were analysed in two ways to assess the degree of chromosome imbalance for each section and each chromosome: The chromosome index and the signal distribution.

3.21. Chromosome Index. [CI]

The chromosome index is calculated by dividing the total number of hybidisation signals by the total number of nuclei counted. The chromosome index gives an average chromosome copy number and is therefore better suited to describe clonal changes within a tumour.

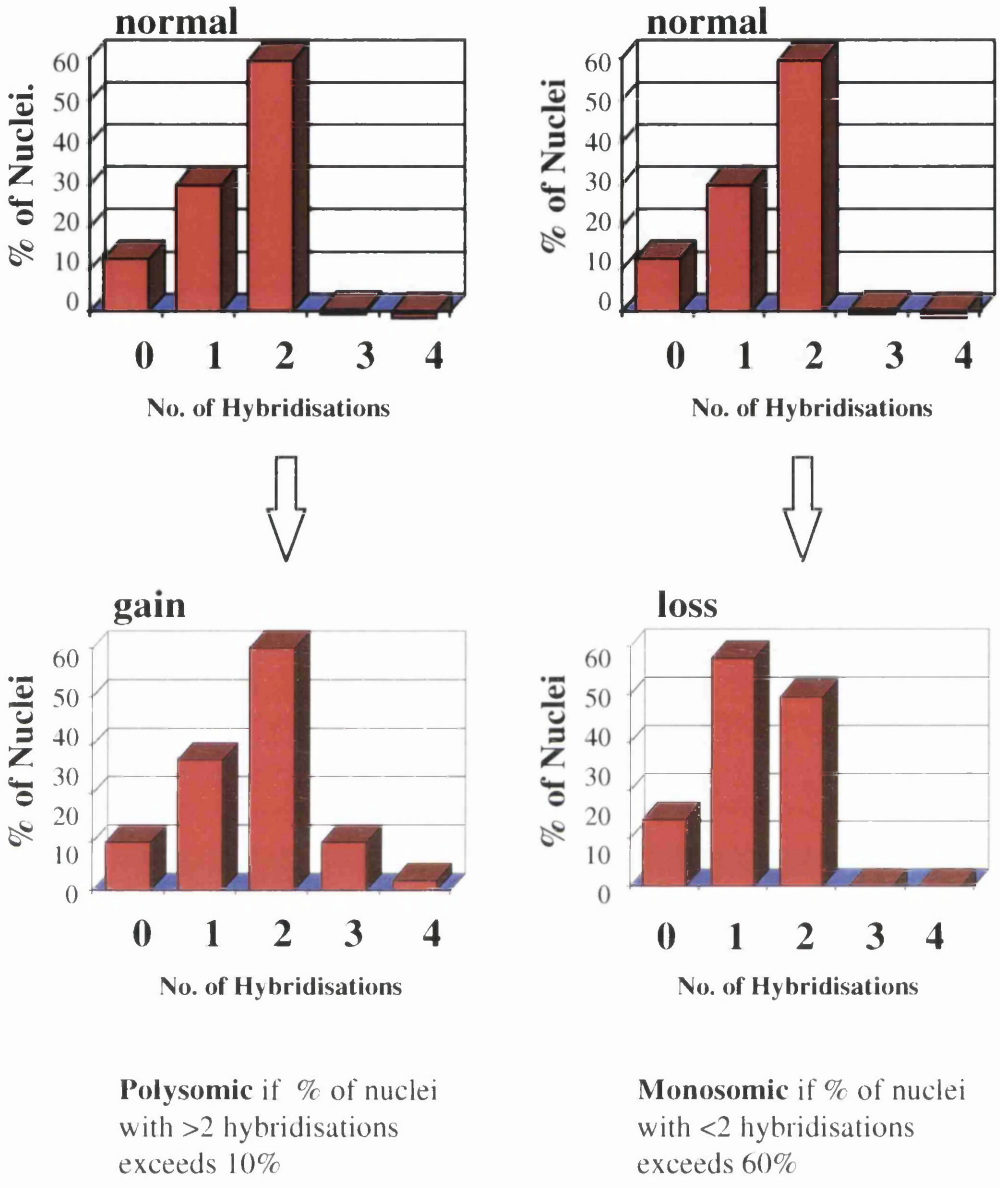
50 hybridisations were carried out on normal breast sections, using centromeric probes for chromosomes 1, 3, 6, 10, 17 and 18. The chromosome indices were calculated and the mean chromosome index for normal breast tissue was 1.55 and three standard deviations from the mean gave values of 1.33 and 1.69.

When using the chromosome index as a measure for chromosome imbalances any section with a CI >1.69 was defined as polysomic and a CI<1.33 was defined as monosomic.

3.22. Signal Distribution.

The second method that can be used to define chromosome polysomy or monosomy is the signal distribution. A section was defined as polysomic for a given chromosome if the percentage of nuclei with >2 hybridisation signals was >10% of the nuclei counted. A section was defined as monosomic if the percentage of nuclei with fewer than two hybridisation signals was >60% of the nuclei counted. The analysis of signal distribution can potentially detect relatively small populations of cells with numerical chromosome imbalances, (Figure 4).

These criteria for both signal distribution and chromosome index are based on published estimates and the considerable experience of our group and takes into account nuclear truncation.



Polysomic if % of nuclei with >2 hybridisations exceeds 10%

Monosomic if % of nuclei with <2 hybridisations exceeds 60%

Figure 4. **Graphic Representation of Polysomy and Monosomy as Defined by the Signal Distribution of a Section.**

Chapter 4.

Chromosomal Imbalances in Proliferative Disease Without Atypia [PDWA]

4.1. Introduction.

Substantial evidence exists to support the view that a fundamental feature of breast cancer evolution is the accumulation of multiple genetic events, which have also been demonstrated in DCIS. However, evidence of such alterations in PDWA or ADH is lacking. If the evolution from a premalignant lesion to the malignant phenotype is accompanied by molecular alterations, characterisation of such genetic changes should reinforce the recognition of proliferative disease as a premalignant stage.

4.2. Characterisation of Genetic Alterations in PDWA.

Previously our group have shown alterations to chromosomes 1, 3, 10, 17 and 18 in sections of ductal carcinoma *in-situ* (Murphy et al. 1995), therefore chromosome-specific repetitive sequence probes for those chromosomes were hybridised to sections of PDWA together with a chromosome 6 probe which demonstrated little deviation from normal in DCIS. Figure.1, shows an example of a section of PDWA hybridised with a chromosome 18 probe and shows both a high and low power image of a small PDWA lesion and a normal lobule which acts as the internal control. Figure 1, demonstrates the retention of spacial relationships that FISH affords, which allows the cytogenetic images and data to be directly compared to the original histology.
Sections hybridised to centromeric probes specific for chromosomes 1, 3, 6, 10, 17 and 18 were analysed as previously described [see *Chapter 3*] and the chromosome index and signal distribution for each PDWA lesion and its internal and external control determined. The data for both chromosome index and signal distribution are presented in (Table 1a & b). This table presents the chromosome index of the sections and the percentage of nuclei with <2

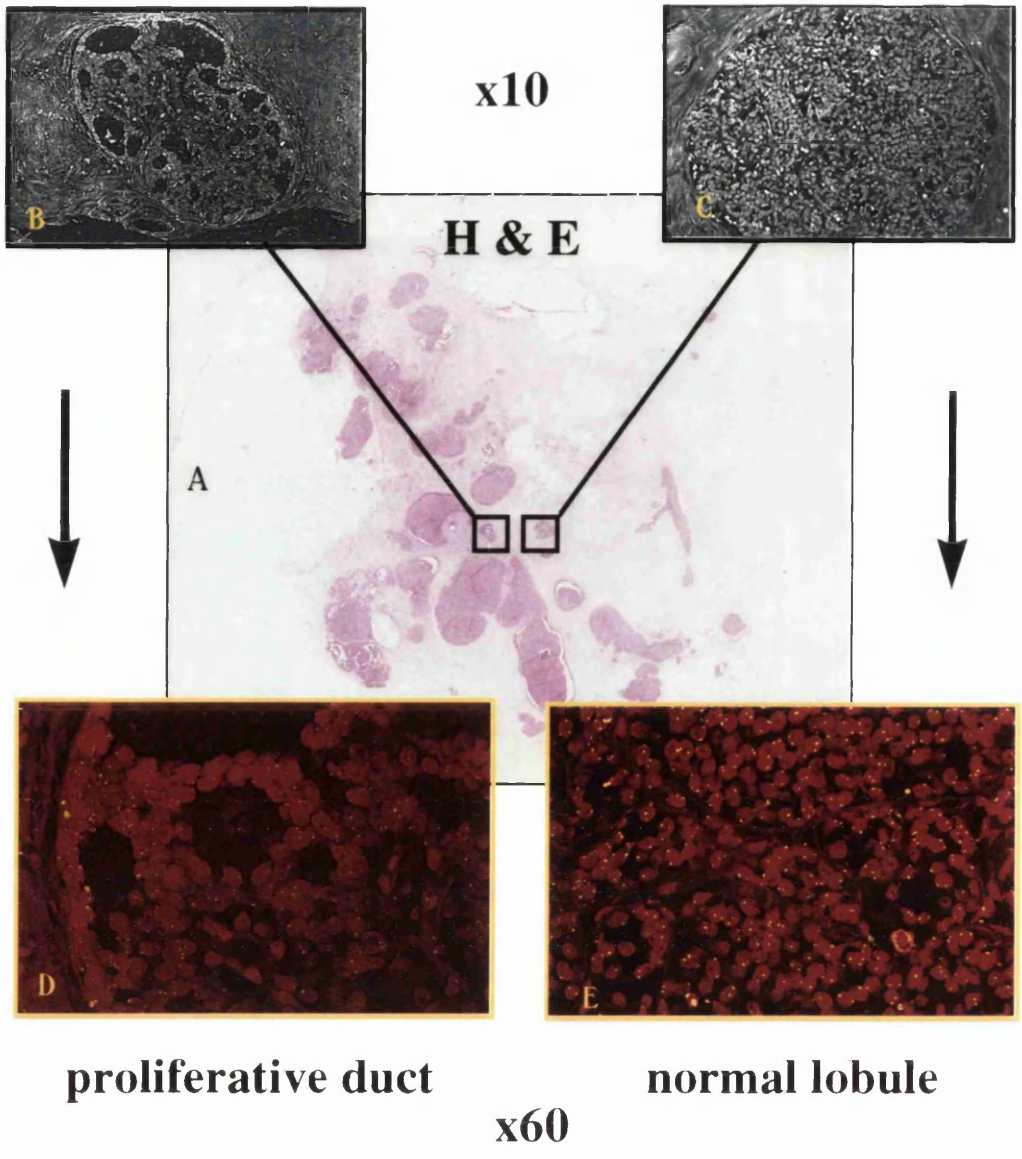


Figure 1. **Analysis of PDWA by FISH.**

[A] Low power image of a section stained with H&E. A serial section was then used for FISH. [B] & [C] Low power image of a section that has undergone FISH using a chromosome 18-specific probe. Image shows propidium iodide staining of the nuclei. Area [B] is a region of PDWA and a high power image of the hybridisation is shown in [D]. [C] is a normal lobule as confirmed by examination of the H&E and a high power image of the hybridisation is shown in [E]. Analysis of the PDWA lesion showed it to have a loss of chromosome 18 sequences in comparison to the normal lobule.

No.	section	Chromosome 18			Chromosome 17			Chromosome 10		
		CI	<2	>2	CI	<2	>2	CI	<2	>2
1	9137	**1.29**	**63**	1	1.57	37	2	1.47	45	0
2	4700eb	1.51	44	0	1.48	41	0	1.56	33	0
3	5344a	1.48	42	0	1.64	37	7	1.58	34	0
4a	7303e	1.41	52	1	**1.71**	33	**11**	1.43	51	2
4b	7303f	1.52	38	1	1.52	38	2	1.49	43	2
5	6828	1.52	38	1	1.52	38	2	1.49	43	2
6	5390	1.52	38	1	1.52	38	2	1.49	43	2
7	7302b	1.47	46	3	1.53	44	6	1.44	45	0
8	0813c	1.6	34	1	**1.75**	32	**11**	1.61	35	3
9	7667a3	1.48	39	1	1.46	47	2	1.64	31	2
10a	9041k	1.59	33	0	1.55	41	0	1.48	39	0
10b	9041h	1.6	36	2	**1.8**	29	**13**	1.35	51	0
11a	4655b	1.68	30	4	1.63	33	3	1.62	36	0
11b	4655a	1.58	36	0	1.61	36	1	**1.21**	**65**	1
12	0881	1.5	41	1	1.64	36	3	1.58	34	1
13	6642	1.34	52	0	1.46	45	1	1.44	44	1
14	5520	1.54	41	1	1.49	38	1	1.52	36	0
15	2641a	1.59	34	1	**1.8**	32	**15**	1.49	42	2
16	11158	1.55	38	0	1.55	40	1	1.41	47	2
17	8429	1.5	40	1	1.34	51	0	1.59	36	1
18	7882	1.47	42	1	1.58	41	8	1.42	44	0
19	3593a	1.44	48	2	1.34	54	0	1.51	38	0
20	10605	1.44	46	0	1.63	31	2	1.35	51	0
21	5060ae	**1.04**	**76**	0	1.61	35	2	1.49	43	0
22	8062	**1.27**	59	0	1.51	40	1	1.51	37	0
23a	6913e	1.26	57	0	1.49	39	0	1.34	57	0
23b	6913f	**1.18**	**67**	0	1.52	39	0	**1.23**	59	0
24	4093b	**0.97**	**81**	0	**1.86**	25	**21**	1.57	39	1
25a	6190a5	1.52	38	1	1.59	33	0	**1.28**	**64**	0
25b	6190a9	1.54	38	0	1.58	33	2	1.58	48	1
26	5644	1.30	38	0	1.58	33	2	1.58	48	1
27	6834	1.47	44	0	1.6	34	2	1.52	38	0
28	8430	**1.29**	56	0	1.55	36	2	1.42	45	1
29	3804	**1.22**	**65**	0	1.6	36	0	1.51	42	1
30	11403	1.59	34	3	1.39	45	0	**1.32**	52	0
31a	3893g	1.45	44	0	1.44	49	0	**1.30**	52	0
31b	3893h	**1.32**	52	0	1.36	44	0	1.49	50	2

Table 1 [a]. **Chromosome Index and Signal Distribution Data for Sections of PDWA.(Sections with a Gain or Loss are Highlighted in yellow, while sections showing a tendency to a Gain or loss are highlighted in pink.)**

No	sections	Chromosome 6			Chromosome 3			Chromosome 1		
		CI	<2	>2	CI	<2	>2	CI	<2	>2
1	9137	1.41	48	4	1.43	45	1	1.6	40	1
2	4700eb	1.35	50	0	1.42	44	0	1.56	35	1
3	5344a	1.44	45	0	1.42	45	1	**1.32**	54	0
4a	7303e	1.64	33	2	1.49	39	1	**1.32**	52	0
4b	7303f	1.52	37	1	1.51	41	0	1.33	57	0
5	6828	1.52	37	1	1.51	41	0	1.33	57	0
6	5390	1.52	37	1	1.51	41	0	1.33	57	0
7	7302b	1.4	48	0	1.48	40	0	1.52	40	0
8	0813c	1.52	41	0	**1.3**	57	0	1.61	35	5
9	7667a3	1.33	54	1	1.62	31	2	1.38	48	0
10a	9041k	1.64	30	1	1.63	36	1	1.47	45	1
10b	9041h	1.52	44	1	**1.74**	33	**18**	1.42	45	0
11a	4655b	1.55	38	3	1.45	44	2	1.41	49	1
11b	4655a	1.46	47	0	1.33	56	1	1.43	45	2
12	0881	1.63	30	1	1.47	43	1	1.6	33	1
13	6642	1.51	42	0	1.68	31	7	1.59	33	0
14	5520	1.43	44	0	1.39	47	0	1.58	35	3
15	2641a	1.52	37	1	**1.32**	52	0	**1.3**	56	1
16	11158	1.38	48	0	**1.3**	56	0	1.46	45	0
17	8429	1.62	31	0	1.52	39	1	**1.3**	55	0
18	7882	1.48	44	0	1.62	38	8	1.43	45	0
19	3593a	1.59	33	0	1.66	33	9	1.4	47	2
20	10605	1.47	41	0	1.5	39	0	1.41	48	1
21	5060aeb	1.35	52	0	1.38	51	0	1.59	35	2
22	8062	1.43	44	1	1.41	44	0	**1.3**	55	0
23a	6913e	1.57	38	3	1.53	38	1	1.51	38	2
23b	6913f	1.42	46	0	1.42	46	0	1.53	37	0
24	4093b	1.63	37	5	1.49	38	0	1.37	53	2
25a	6190a5	1.59	37	0	1.58	36	2	**1.24**	**61**	0
25b	6190a9	1.45	46	0	1.46	45	2	**1.29**	59	0
26	5644	1.28	46	0	1.46	45	2	**1.29**	59	0
27	6834	1.42	47	0	1.27	38	3	1.4	46	0
28	8430	1.42	48	0	1.55	37	2	1.58	33	1
29	3804	1.59	35	0	**1.79**	29	**12**	1.35	49	1
30	11403	1.45	43	0	1.47	43	3	1.37	47	0
31a	3893g	**1.29**	53	0	1.47	41	2	**1.28**	55	0
31b	3893h	1.46	45	1	1.37	49	0	1.39	46	1

Table 1 [b]. **Chromosome Index and Signal Distribution Data for Sections of PDWA. (Sections with a Gain or Loss are Highlighted in yellow, while sections showing a tendency to a Loss or Gain are highlighted in pink).**

hybridisation signals and the percentage of nuclei with > 2 hybridisation signals.

The **chromosome index** is a direct method of analysing chromosomal imbalances and allows a comparison of the average chromosome copy number for each PDWA lesion to be made. The chromosome index data for each centromeric probe are presented in Figure. 2 (i - vi) which include the CI's of 50 normal breast sections from which the mean chromosome index was calculated; the CI's for each section of PDWA and the CI's for the normal controls of each PDWA showing a deviation from normal. When the chromosome index data are summarised together, (Figure.3), it is apparent that the majority of PDWA sections are within the normal range, [see *Chapter 3*]. However, a pattern begins to emerge of a subpopulation of PDWA which are showing a low frequency of chromosomal imbalances. For example, from (Figure 3), a subset of of PDWA sections show gains in the frequency of centromeric markers for chromosomes 3 and 17, when compared to normal tissue; whereas loss of centromeric markers for chromosomes 1, 10 and 18 are found in a small population of PDWA. Chromosome 6 showed little deviation from the internal and external controls.

In order to quantify chromosome imbalances in more detail the **signal distribution** of each section was analysed, which is more sensitive at identifying small populations of cells, within a section, with imbalances. These chromosome imbalances may be missed by the chromosome index, which is a measure of the average chromosome copy number of all cells within a duct. Hence, chromosomal loss or gain was assigned only if both criteria (chromosome index and signal distribution) indicated an imbalance. If only one criterion was met, the section was recorded as showing a tendency to a loss or gain. Using this criteria, the frequency of chromosomal imbalances was calculated and is demonstrated in (Table 2). The data shown in (Table 2) resembles the low frequency pattern of chromosomal imbalances presented in (Figure 3). Of the 37 sections analysed, it can be seen from (Table 1) and (Table 2) that chromosome 18 shows the highest frequency of loss in PDWA

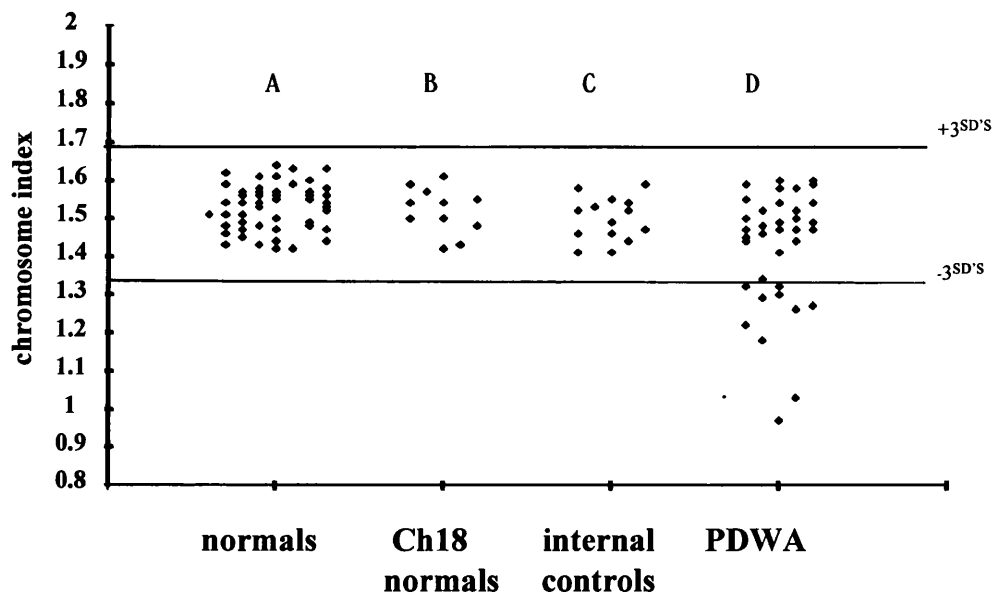


Figure 2 (i).

Group A

The chromosome indices of 50 normal breast sections analysed by all centromeric probes and used to determine the normal range, indicated by +/- $3^{sd's}$.

Group B : External controls.

The chromosome indices of normal breast sections analysed using a chromosome 18 centomeric probe.

Group C : Internal controls.

The chromosome indices of normal breast lobules within the sections of PDWA.

Group D :

10 sections of PDWA from 31 patients demonstrate a loss of chromosome 18 sequences .

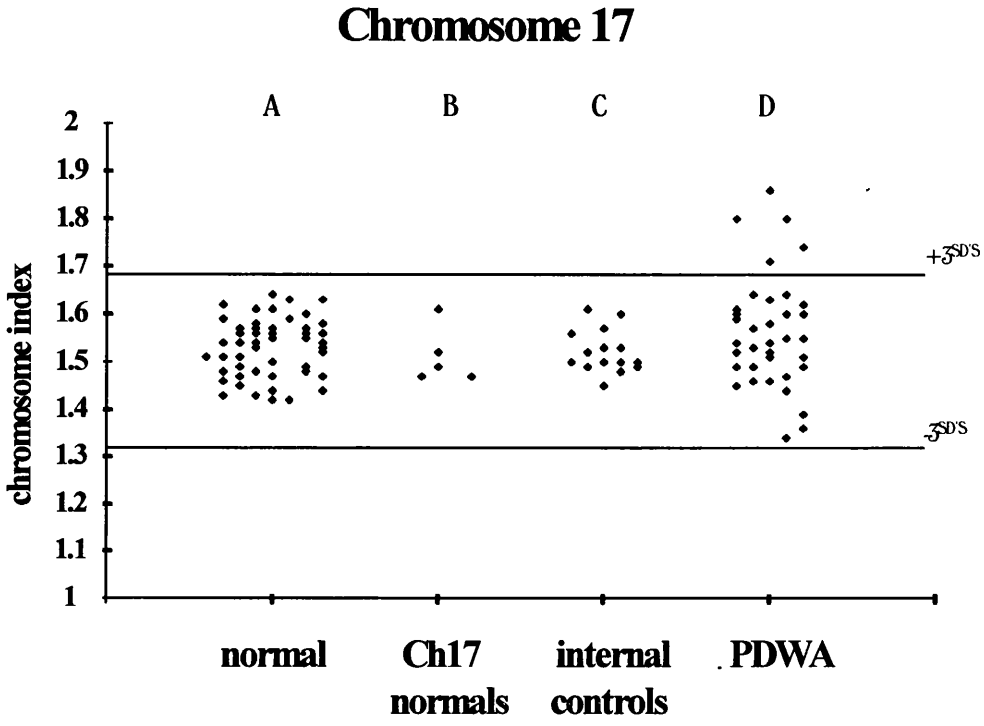


Figure 2 (ii).

Group A

The chromosome indices of 50 normal breast sections analysed by all centromeric probes and used to determine the normal range, indicated by +/- $3^{sd's}$.

Group B : External controls.

The chromosome indices of normal breast sections analysed using a chromosome 17 centomeric probe.

Group C : Internal controls.

The chromosome indices of normal breast lobules within the sections of PDWA.

Group D :

5 sections of PDWA from 31 patients are demonstrating a gain of chromosome 17 centromeric sequences.

Chromosome 10

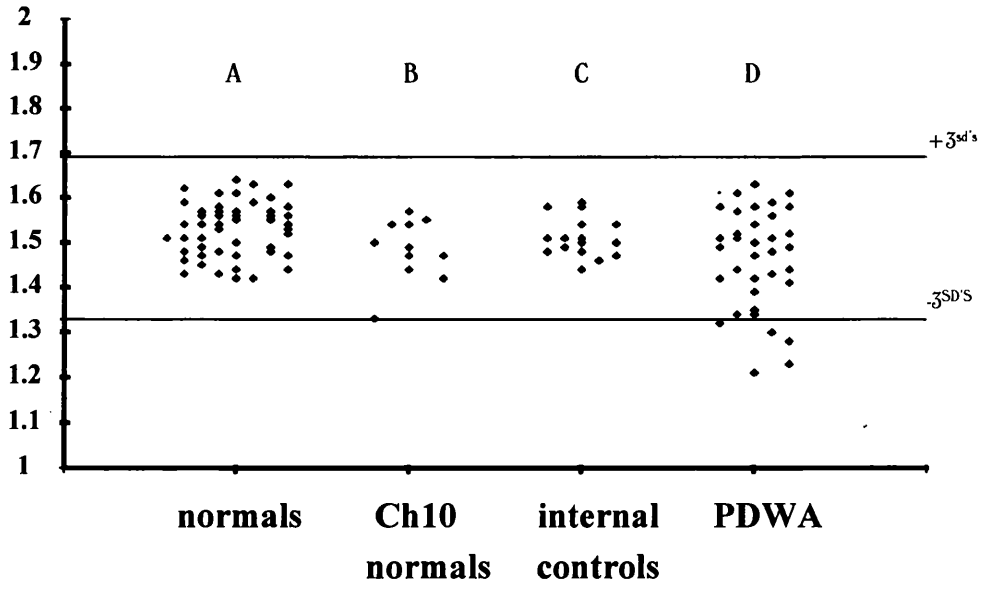


Figure 2 (iii)

Group A

The chromosome indices of 50 normal breast sections analysed by all centromeric probes and used to determine the normal range, indicated by +/- $3^{sd's}$.

Group B : External controls.

The chromosome indices of normal breast sections analysed using a chromosome 17 centomeric probe.

Group C : Internal controls.

The chromosome indices of normal breast lobules within the sections of PDWA.

Group D :

5 sections of PDWA from 31 patients are showing a loss of chromosome 10 sequences.

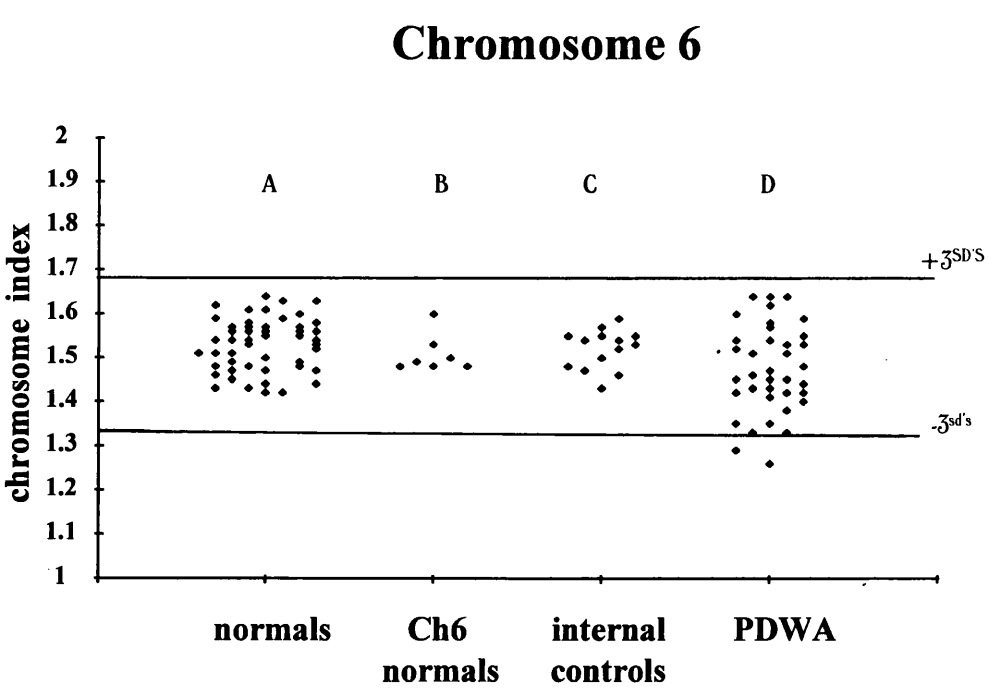


Figure 2 (iv)

Group A

The chromosome indices of 50 normal breast sections analysed by all centromeric probes and used to determine the normal range, indicated by +/- $3^{sd's}$.

Group B : External controls.

The chromosome indices of normal breast sections analysed using a chromosome 17 centomeric probe.

Group C : Internal controls.

The chromosome indices of normal breast lobules within the sections of PDWA.

Group D :

2 sections of PDWA from 31 patients are showing a loss of chromosome 6 sequences.

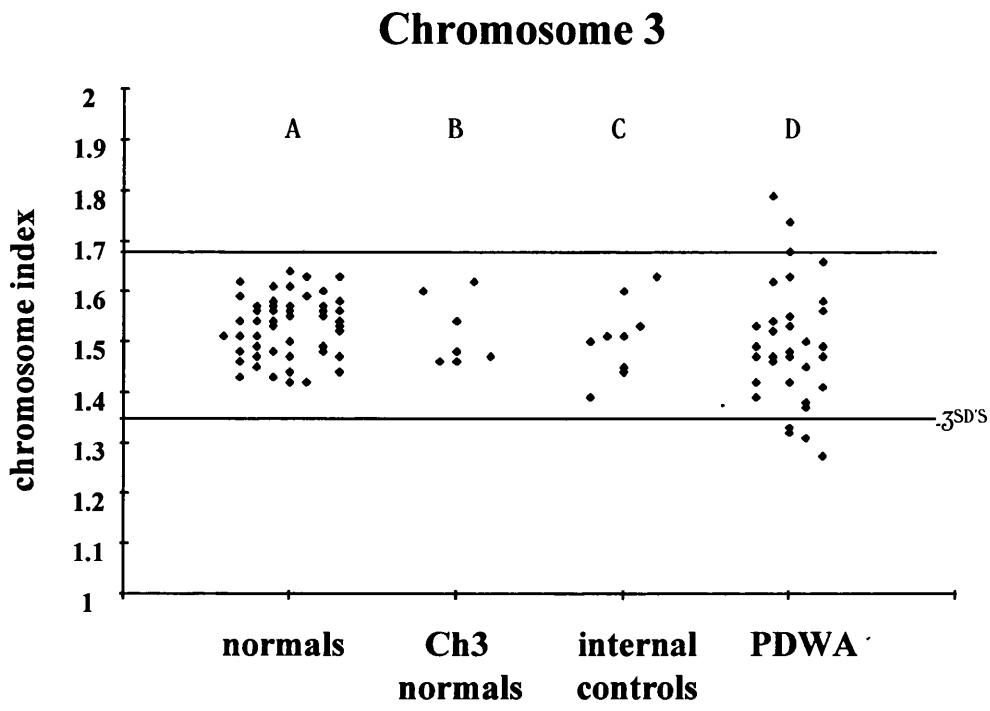


Figure 2 (v)

Group A

The chromosome indices of 50 normal breast sections analysed by all centromeric probes and used to determine the normal range, indicated by +/- $3^{sd's}$.

Group B : External controls.

The chromosome indices of normal breast sections analysed using a chromosome 17 centomeric probe.

Group C : Internal controls.

The chromosome indices of normal breast lobules within the sections of PDWA.

Group D :

2 sections of PDWA are showing a gain of chromosome 3 sequences while 4 sections are showing a loss of chromosome 3 sequences.

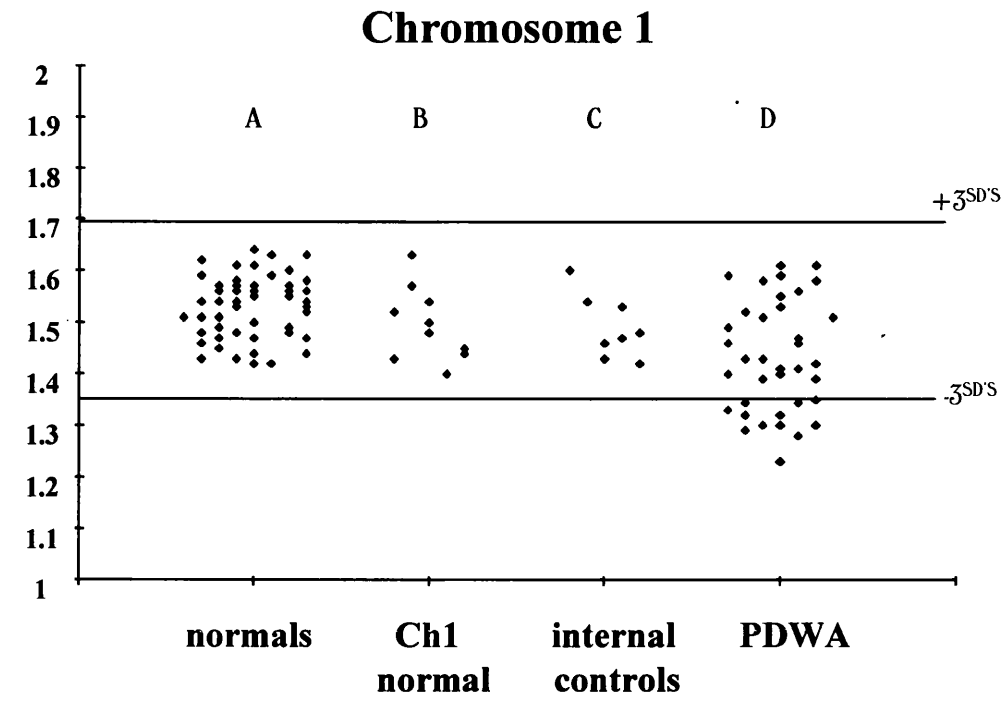


Figure 2 (vi)

Group A

The chromosome indices of 50 normal breast sections analysed by all centromeric probes and used to determine the normal range, indicated by +/- $3^{sd's}$.

Group B : External controls.

The chromosome indices of normal breast sections analysed using a chromosome 17 centomeric probe.

Group C : Internal controls.

The chromosome indices of normal breast lobules within the sections of PDWA.

Group D :

9 sections of PDWA from 31 patients are showing a loss of chromosome 1 sequences

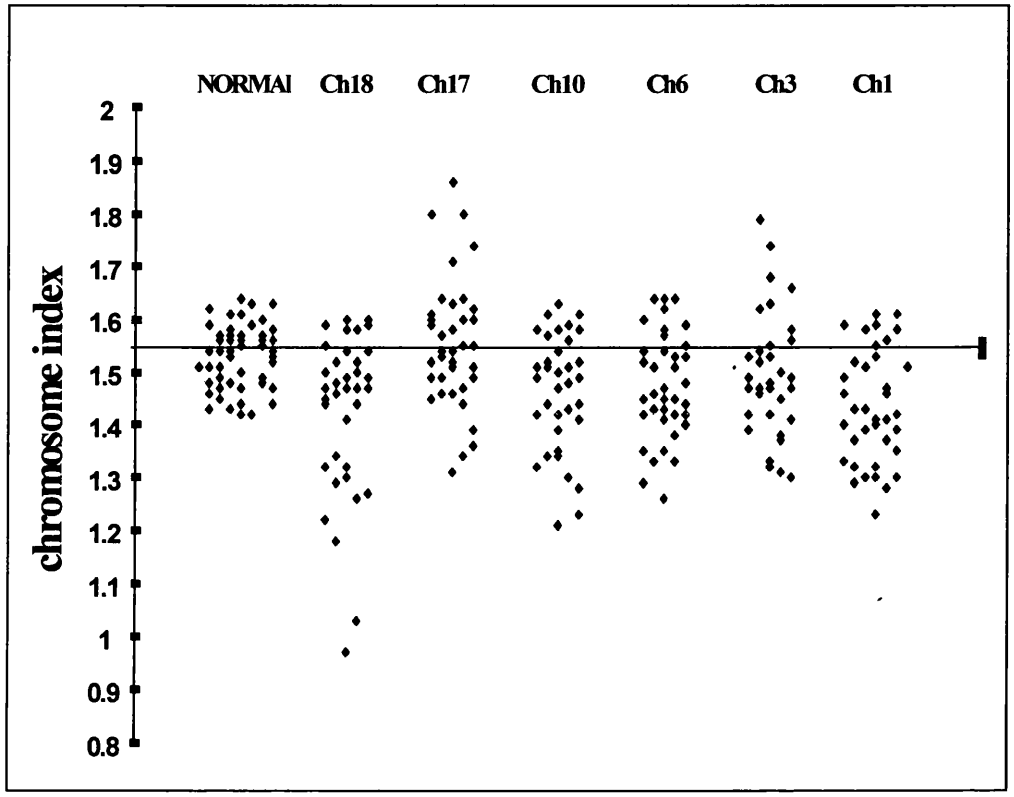


Figure 3. **Summary of chromosome index data from normal breast sections and PDWA. The vast majority of proliferative sections lie within the normal range, however a small sub-population are demonstrating gains of chromosome 17 and 3 centromeric sequences while chromosomes 1, 10 and 18 are showing losses of centromeric sequences.**

Chromosome No.	Loss	Balanced	Gain	Total
18	5(5)	32	0	37
17	0	32	5	37
10	2(3)	35	0	37
6	0(2)	37	0	37
3	0(4)	35	2	37
1	1(8)	36	0	37

Table 2. **Chromosomal Imbalances in Proliferative Disease Without Atypia. A sample had to show imbalance by both chromosome index and signal distribution to be counted. Imbalances determined by either chromosome index or signal distribution alone are shown in brackets. [See Chapter 3 for details].**

The number of alterations in individual lesions ranged from 0 to 3, with 11 sections showing 2 or more chromosome imbalances, (Table 1 and Table 4).

with 5 sections showing a loss of chromosome 18-specific sequences and a further 5 sections showing a tendency to a loss, (Figure 1). Chromosomes 1 and 10 also show a loss. Chromosome 17 shows the highest frequency of gain in PDWA with 5 sections demonstrating a gain of chromosome 17-specific sequences. (Figure 4) shows a low and high power image of a PDWA section with a gain of chromosome 17 sequences. Chromosome 3 also shows a gain of chromosome 3 sequences in 2 sections but also the tendency to a loss in a further 4 sections.

The number of alterations in individual lesions ranged from 0 to 3, with 11 sections showing 2 or more chromosome imbalances, (Table 1and Table 4).

4.3. Analysis of Intratumour Heterogeneity using FISH.

Sections of PDWA clearly show individual regions of disease. Thus, there is potential for intratumour heterogeneity. In an attempt to ascertain whether heterogeneity exists within sections and whether FISH is sensitive enough to detect it, five sections of PDWA were analysed using FISH. In this instance the chromosome index and signal distribution of multiple areas of PDWA within each section were individually calculated. There was no difference in chromosome index or signal distribution between PDWA lesions of the same section for the panel of centromeric probes. Data for section 5 are shown in (Table3). FISH did not provide any evidence of genetic heterogeneity in this instance and indeed this data probably analyses the question of intraobserver variation rather than intratumour heterogeneity, hence the adoption of microsatellite analysis in Chapter 6 to address the question of intratumour heterogeneity in sections of DCIS.

4.4. Florid versus Non-Florid PDWA.

The identification of specific molecular alterations within specimens of PDWA prompted us to examine the relationship between histology and chromosomal abnormalities in more detail. The PDWA group was therefore split into biopsies with florid hyperplasia versus mild to moderate hyperplasia,

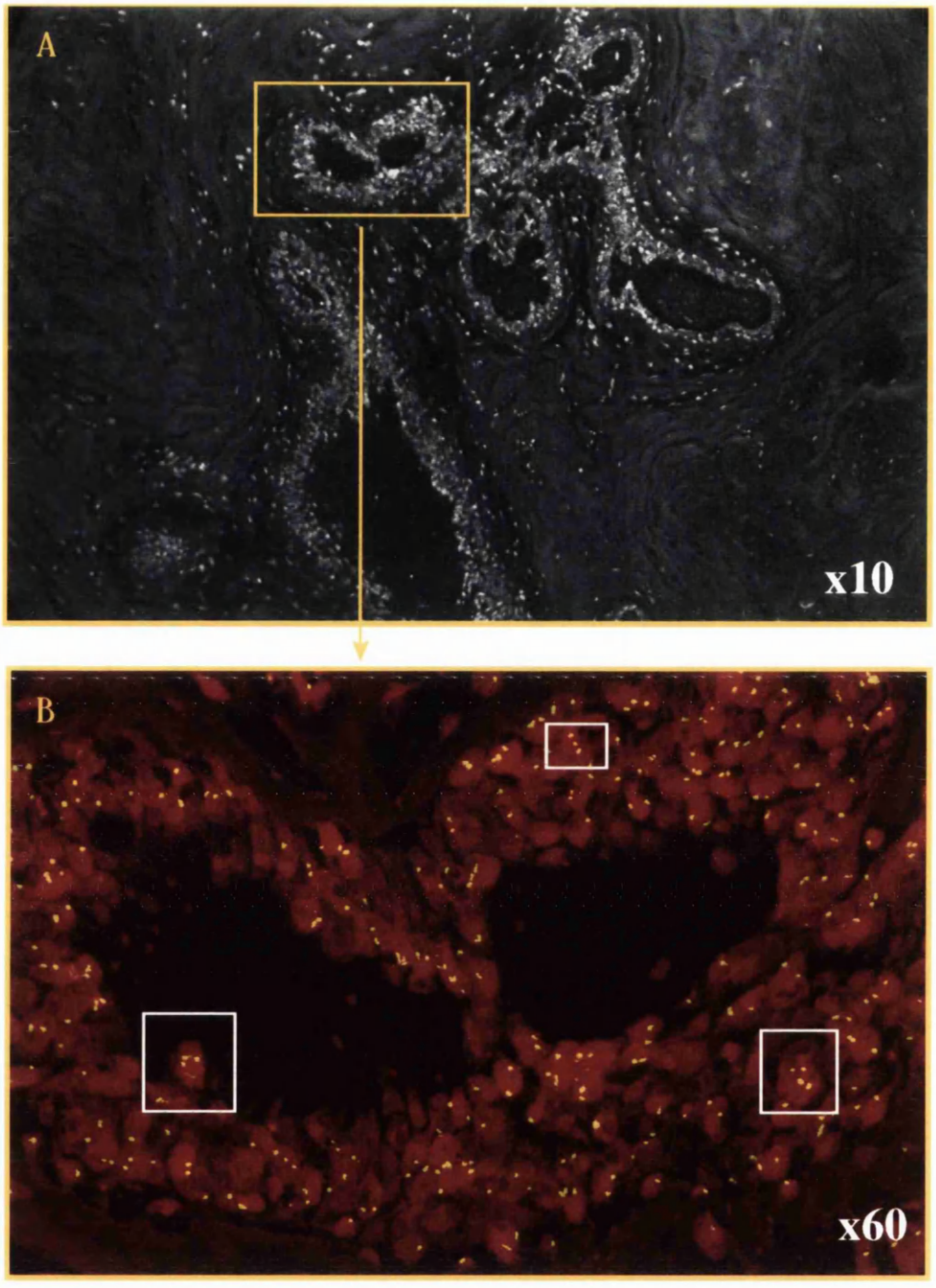


Figure 4. **Section of PDWA demonstrating a gain of chromosome 17 sequences.**

[A] Low power image of a section of PDWA that has undergone FISH using a chromosome 17-specific probe . Image shows propidium iodide staining of the nuclei.
[B] Is a high power image of the region of PDWA shown above. The boxed nuclei are examples of nuclei with >2 hybridisation signals.

Section 5	Number of hybridisations					CI
	0	1	2	3	Total	
A	22	35	141	2	200	1.45
B	18	53	129	0	200	1.41
C	28	34	135	3	200	1.49
D	24	45	131	0	200	1.40
E	29	44	125	2	200	1.37

Table 3. **Chromosome 18 Copy Number and Chromosome Index in multiple areas of PDWA, Within Section 5. (A -E represent data from different foci of PDWA)**

Sections	Pathology	Chromosome Imbalances
P1	mild pdwa	Ch18↓
P2	mild pdwa	
P3	mild pdwa	(Ch1↓)
P4a	mild pdwa	Ch17↑ (Ch1↓)
P4b	moderate pdwa	(Ch1↓)
P5	moderate pdwa	
P6	moderate pdwa	
P7	moderate pdwa	
P8	moderate pdwa	Ch17↑ (Ch3↓)
P9	moderate pdwa	
P10a	moderate pdwa	
P10b	moderate pdwa	Ch17↑ Ch3↑
P11a	moderate pdwa	
P11b	moderate pdwa	Ch10↓
P12	moderate pdwa	
P13	moderate pdwa	
P14	moderate pdwa	
P15	moderate pdwa	Ch17↑ (Ch3↓ Ch1↓)
P16	moderate pdwa	(Ch3↓)
P17	moderate pdwa	(Ch1↓)
P18	moderate pdwa	
P19	moderate pdwa	
P20	moderate pdwa	
P21	moderate pdwa	Ch18↓
P22	moderate pdwa	(Ch18↓ Ch1↓)
P23a	moderate pdwa	(Ch18↓)
P23b	florid pdwa	Ch18↓ (Ch10↓)
P24	florid pdwa	Ch18↓ Ch17↑
P25a	florid pdwa	Ch10↓ Ch1↓
P25b	florid pdwa	(Ch1↓)
P26	florid pdwa	(Ch18↓ Ch6↓ Ch1↓)
P27	florid pdwa	(Ch3↓)
P28	florid pdwa	(Ch18↓)
P29	florid pdwa	Ch18↓ Ch3↑
P30	florid pdwa	(Ch10↓)
P31a	florid pdwa	(Ch10↓ Ch6↓ Ch1↓)
P31b	florid pdwa	(Ch18↓)

Table 4. **Summary of Chromosome Imbalances in Proliferative Disease without Atypia. Chromosomal gains or losses are in red type. Chromosomes. (Ch). showing a trend to loss or gain are shown in brackets.**

(Table 4 and Table5). A total of 19 chromosome imbalances (chromosomal loss or gain plus a tendency to a loss or gain) were shown in the 11 cases of florid PDWA and 19 chromosomal imbalances were shown in the 26 cases of non-florid PDWA, (Table 4 and Table 5). Applying a two-sided Mann-Whitney test to compare the number of genetic changes between the florid and non-florid PDWA demonstrated a significant difference between the two groups, (p=0.006), therefore florid PDWA is associated with a higher frequency of chromosomal imbalances than non-florid within our study group.

		Non-florid PDWA	Florid PDWA
Number of chromosome imbalances per section	**0**	**13** (50%)	**0** (0%)
	1	**8** (31%)	**5** (45.5%)
	2	**4** (15%)	**5** (45.5%)
	3	**1** (4%)	**1** (9%)
		No. = 26	**No. = 11**

Table 5. **Comparison of Genetic Alterations Between Florid and Non-Florid PDWA.**

4.5. Discussion.

We have used interphase cytogenetics to analyse chromosomal imbalances in PDWA. Using this approach our group previously identified a high frequency of imbalances in DCIS involving particularly chromosomes 1, 3, 10, 17 and 18. The pattern of chromosomal imbalances identified in PDWA mirrored that found in DCIS but at a reduced frequency, (Figure 5). Loss of chromosome 1,

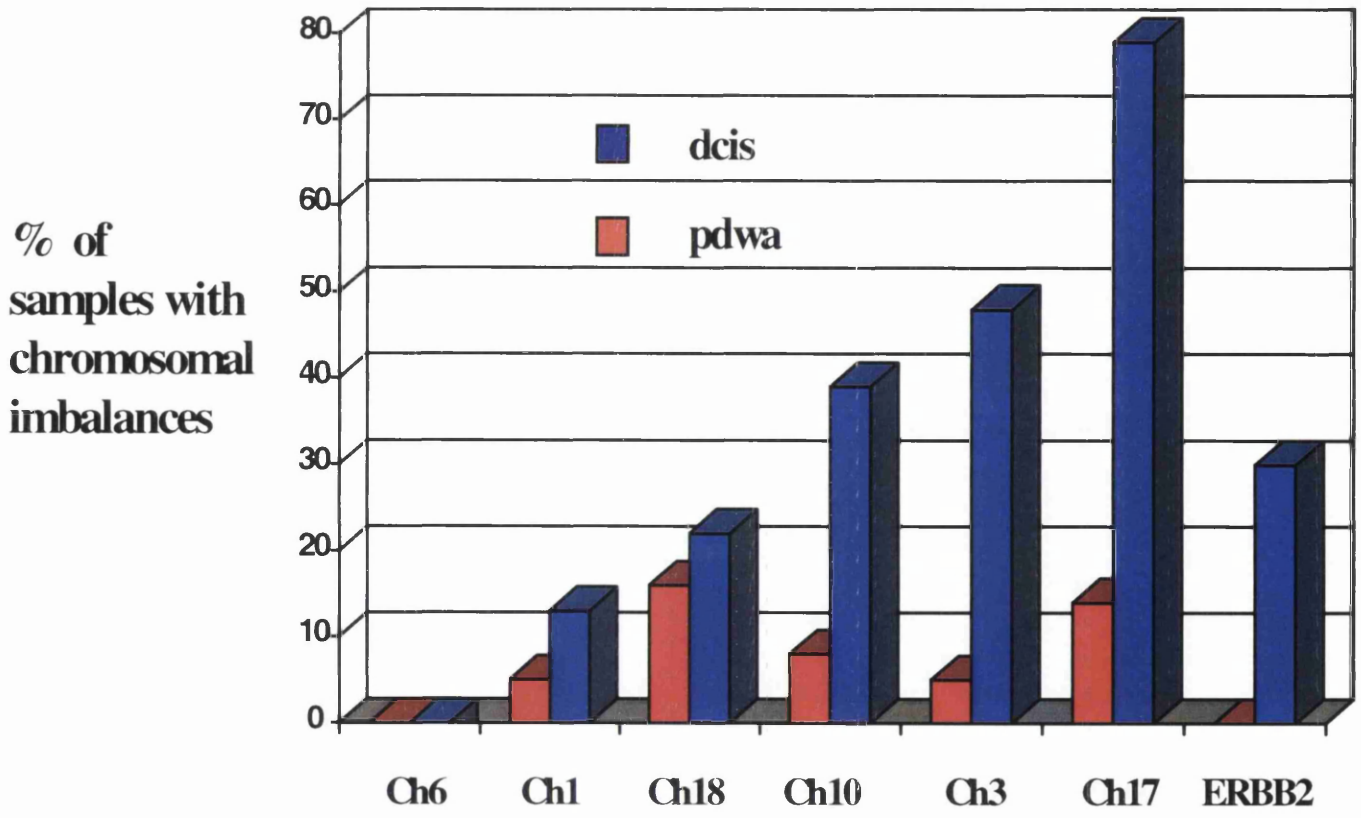


Figure 5. **A comparison of the frequency of chromosomal imbalances and ERBB2 amplification in PDWA and DCIS.**

10 and 18 centromeric sequences were common to both PDWA and DCIS as was gain of chromosome 3 and 17 centromeric sequences. Chromosome 6 showed little deviation from normal in either group. Thus we may be identifying genetic alterations in a sub-population of PDWA which confers a greater propensity toward progression, (see Figure 5). The histological examination of benign breast biopsies and subsequent clinical follow-up has identified a number of risk categories for the subsequent development of invasive carcinoma. Thus, PDWA is associated with a slight risk while DCIS is associated with a high risk. However, although this may be useful in the clinical setting for prognostication, the histological stratification is largely descriptive rather than mechanistic. Within this stratification of risk, wide confidence limits are assigned to each group suggesting the magnitude of risk for each individual within a group can vary, and indeed some individuals may in fact have a higher risk than predicted by histology alone.

Interestingly, within the PDWA specimens, the molecular alterations associated with florid PDWA rather than non-florid PDWA, ($p=0.006$), Table 5. These data identify the first molecular alterations distinguishing florid from non-florid PDWA, and suggest the transition to DCIS is associated with these same alterations, (Figure 5). Thus, the molecular events may identify individuals with PDWA with a high risk for invasive cancer than can be attributed by histological examination alone. The identification of chromosomal imbalances common to both PDWA and DCIS sets an agenda for the further molecular investigation into early breast cancer progression.

When the frequency of chromosomal imbalances and ERBB2 amplification [see *Chapter 5*] are compared between PDWA and DCIS (Figure 5), it is evident that there is a large increase in the frequency of chromosomal imbalances affecting chromosome 3 and chromosome 17 between PDWA and DCIS suggesting loci responsible for the progression of PDWA to DCIS may be present on those chromosomes. This pattern is also present for chromosome 10 but to a lesser extent. In contrast the frequency of chromosomal imbalances affecting chromosome 18 remain relatively constant, which may imply loci

involved in the earliest stage of breast cancer progression are present on chromosome 18. Hence, the necessity to identify regional alterations on chromosome 18 [see *Chapter 6*].

Particularly striking however is the large increase in the frequency of chromosome alterations to chromosome 3 and chromosome 17 between PDWA and DCIS suggesting that those chromosomes may harbour loci responsible for the progression of PDWA to DCIS.

Our group have recently mapped the human telomerase RNA gene; (hTR), to chromosome 3q26.6 and have demonstrated the hTR gene to be amplified in a number of solid tumours and a putative telomerase repressor has been localised to 3p. (Soder et al. 1997) While there is no evidence to suggest that telomerase is essential for normal cellular proliferation, the reactivation of telomerase activity may be a fundamental feature of progression to the malignant phenotype. (Parkinson, 1996) A study combining FISH analysis and microsatellite analysis of microdissected DCIS and PDWA should define whether this locus is subject to amplification or regional deletions in pre-invasive disease.

Chromosome 17 and loci on chromosome 17 are frequently altered in invasive breast cancer and DCIS. This data suggests that chromosome 17 alterations may already be present in PDWA and that progression to DCIS involves an increase in chromosome 17 alterations and the development of ERBB2 amplification, (Figure 5) [see *Chapter 5*].

In summary, interphase cytogenetics has identified chromosomal alterations in PDWA which parallel those found in DCIS, providing strong evidence that PDWA is a true precursor of DCIS and invasive cancer. An interesting extension to this work will be to analyse sections of ADH to determine whether or not they share the same chromosomal imbalances as PDWA and DCIS and may provide valuable molecular data which could help validate or

repudiate the distinction usually drawn between ADH and low grade DCIS, which is an area of pathological and clinical difficulty.

Chapter 5.

Interphase Cytogenetic Analysis of the ERBB2Gene.

5.1. Introduction.

ERBB2 amplification has been described to occur at a high frequency in DCIS and indeed our group have previously shown by interphase cytogenetics that ERBB2 amplification occurs in 27% of DCIS which is a slightly lower figure than those quoted by other authors.(Liu et al. 1992; Hubbard et al. 1994; Murphy et al. 1995; Allred et al. 1992) However, benign breast disease in general has not been associated with amplification of this oncogene (Allred et al. 1993). The gene is localised to 17q21.

5.2. ERBB2 / Chromosome 17 Analysis of PDWA.

To determine whether PDWA is associated with amplification of ERBB2, 37 sections of PDWA (previously analysed by centromeric probes, Chapter 4) were analysed using double hybridisation's to simultaneously detect ERBB2 sequences and chromosome 17 centromeric sequences. Hence, chromosome 17 polysomy masquerading as ERBB2 amplification can be detected and provides a more accurate measure of amplification.

No ERBB2 amplification was observed in our PDWA sections.

Due to the high quality of hybridisation of the ERBB2 / Ch17 probe, single copies of ERBB2 were easily identified, therefore it was possible to analyse regional alterations to chromosome 17 at the ERBB2 locus in 17 sections of PDWA. Included in these 17 sections were two shown to have gain of chromosome 17-specific centromeric sequences, (*Chapter 4,* Table 1 and Table 2, P4a & P8). The sections P4a & P8 have been renamed sections 5 & 15 respectively for the purposes of this analysis. The remaining 15 sections did

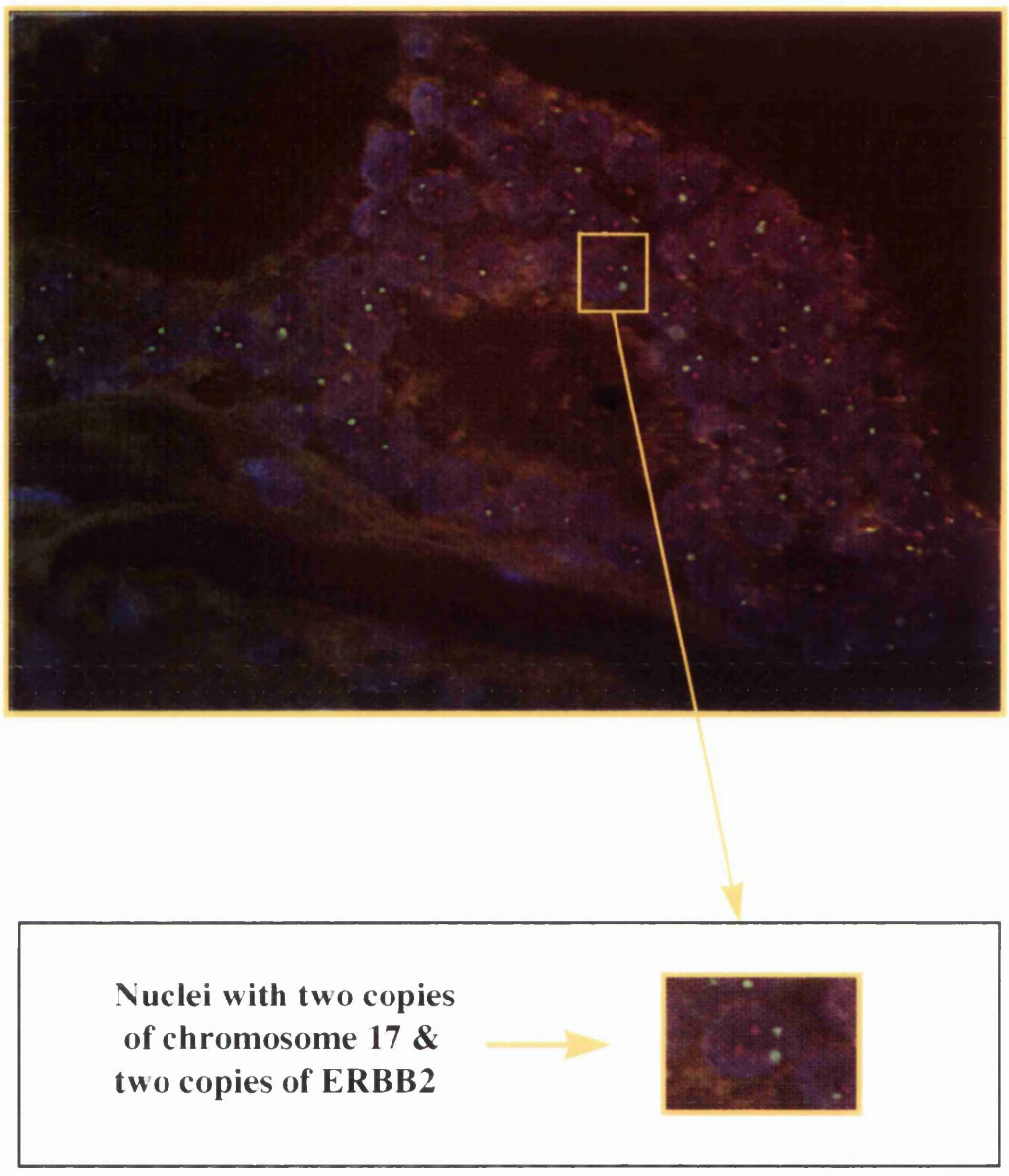


Figure 1. **ERBB2 / Chromosome 17 double hybridisation of a section of PDWA .**

The nuclei of the section of PDWA are pseudocoloured blue; the ERBB2 hybridisation signals are red; and the chromosome 17 hybridisation signals are green. Analysis showed that the section had a normal copy number of ERBB2 and Chromosome 17 sequences.

not demonstrate chromosome 17 imbalances. (Figure 1) provides an illustration of a ERBB2 / Ch17 double hybridisation for a section of PDWA.
The chromosome index and the signal distribution were determined as previously described, for each section of PDWA and an area of normal tissue within each section. The number of ERBB2 hybridisation signals and the number of the chromosome 17 hybridisation signals were counted in the same 200 nuclei per section. The data for the chromosome indices and signal distributions are presented in (Tables 1 to 4) and the chromosome index for each PDWA section and it's normal control are graphically displayed in (Figure 2). The data confirmed that no increased copy number of ERBB2 was found and this was confirmed using the Mann-Whitney test to compare the ERBB2 hybridisations of normal tissue to those of PDWA ($p=0.45$).

This comparison of the two probes is possible because the efficiency of each probe is similar as is demonstrated in (Figure 2), (Mann Whitney, $p = 0.33$), where the chromosome index for normal and PDWA sections all lie within, previously defined normal limits [see *Chapter 3*]. The experiment also reproduced the gain of chromosome 17 sequences in sections 5 and 15, (Table 2), however, this was not accompanied by an increased copy number of ERBB2, (Figure 3) .

5.3. ERBB2 / Chromosome 17 Analysis of Recurrent DCIS.

To determine whether recurrent DCIS is associated with a higher frequency of ERBB2 amplification, a small group of patients were identified in whom initial and recurrent DCIS tissue blocks were available.
Ten patients were identified who had undergone initial wide local excisions for DCIS + /- radiotherapy. All patients had a recurrence of DCIS within 5 years and four recurrences had an invasive component. All patients underwent a modified radical mastectomy and axillary clearance in whom an invasive component had been identified and a simple mastectomy if only DCIS was present. Thankfully this is not a large population to study as they probably represent treatment failure.

Chromosome 17 normals

No	section	CI	<2	>2
1	5344	1.42	46	0
2	7822	1.47	40	1
3	6832	1.38	48	0
4	3593	1.39	50	0
5	3804	1.45	40	0
6	842	1.52	37	1
7	8062	1.49	41	0
8	8430	1.49	41	1
9	0881	1.51	39	1
10	9137	1.52	39	1
11	5520	1.54	39	0
12	7302	1.51	40	0
13	3893	1.37	50	0
14	2641	1.41	47	0
15	0813	1.46	44	0
16	6828	1.49	46	0
17	5390	1.43	49	0

Table 1.

Chromosome 17 PDWA

No	section	CI	<2	>2
1	5344	1.37	50	0
2	7822	1.42	49	1
3	6832	1.38	46	1
4	3593	1.43	47	0
5	3804	1.61	37	12
6	842	1.43	44	0
7	8062	1.36	49	1
8	8430	1.43	44	1
9	0881	1.38	47	0
10	9137	1.39	50	0
11	5520	1.43	45	0
12	7302	1.47	44	1
13	3893	1.40	48	0
14	2641	1.51	39	0
15	0813	1.61	35	14
16	6828	1.48	48	0
17	5390	1.49	49	0

Table 2.

ERBB2 normals

No	section	CI	<2	>2
1	5344	1.55	37	0
2	7822	1.41	48	1
3	6832	1.5	43	1
4	3593	1.48	40	0
5	3804	1.49	41	1
6	842	1.45	43	0
7	8062	1.55	37	0
8	8430	1.40	48	0
9	0881	1.48	41	1
10	9137	1.47	43	0
11	5520	1.47	42	0
12	7302	1.45	43	1
13	3893	1.45	40	0
14	2641	1.47	43	1
15	0813	1.49	44	1
16	6828	1.57	36	0
17	5390	1.50	41	0

Table 3.

ERBB2 PDWA

No	section	CI	<2	>2
1	5344	1.48	43	0
2	7822	1.44	45	0
3	6832	1.52	41	0
4	3593	1.46	42	2
5	3804	1.55	40	1
6	842	1.5	35	0
7	8062	1.40	48	1
8	8430	1.54	38	1
9	0881	1.39	49	0
10	9137	1.54	39	2
11	5520	1.49	44	0
12	7302	1.45	41	1
13	3893	1.48	34	1
14	2641	1.5	44	0
15	0813	1.53	42	0
16	6828	1.44	41	0
17	5390	1.43	38	0

Table 4.

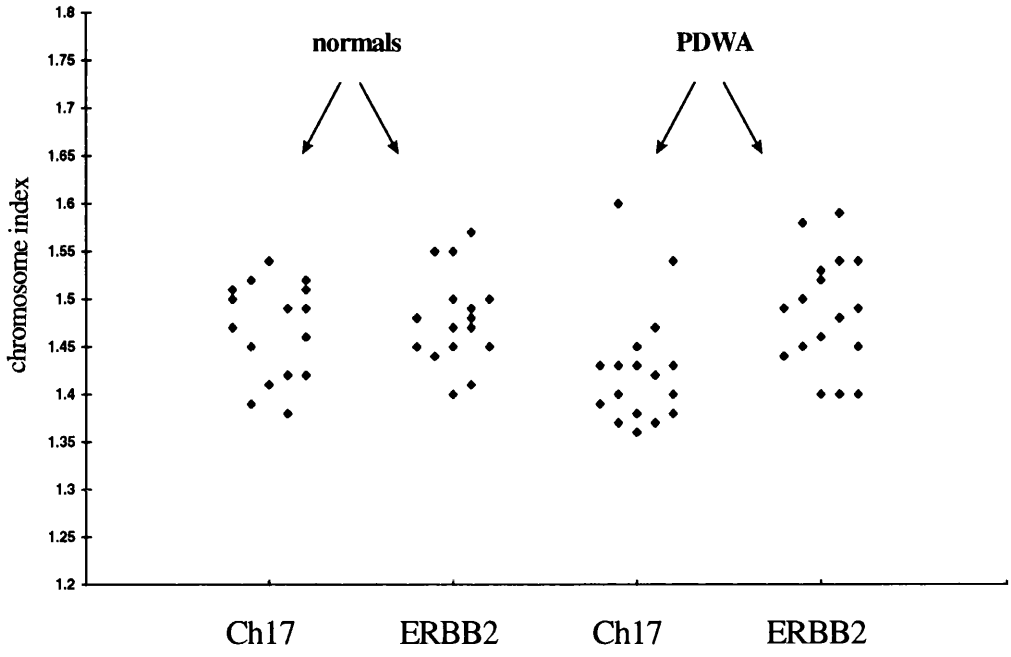


Figure 2. **Graphic representation of the chromosome indices of ERBB2 Chromosome 17 hybridisations of 17 PDWA sections and their normal controls. This demonstrates the efficiency of each probe is similar and thus the results are comparable.**

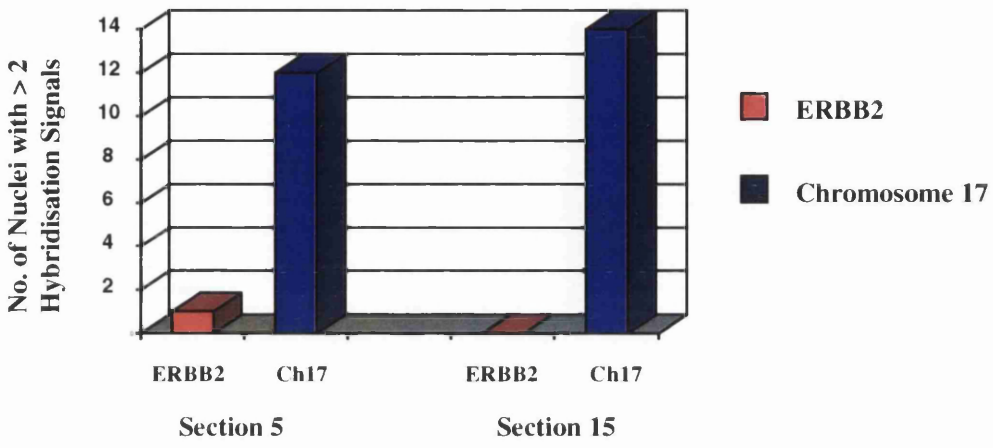


Figure 3. **PDWA Sections 5 and 15 Demonstrating Polysomy of Chromosome 17 without Increased Copy Number of ERBB2.**

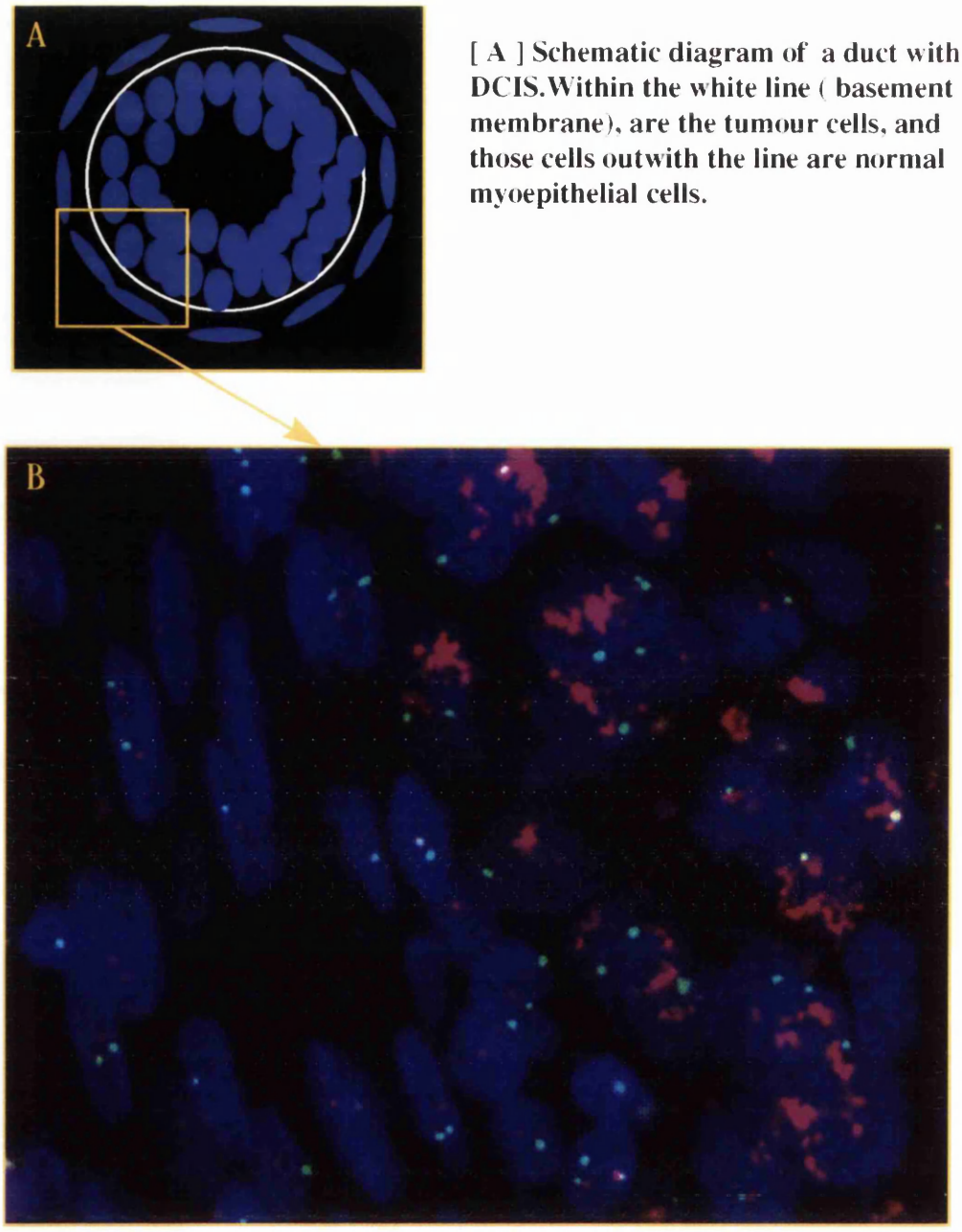
Paraffin-embedded, formalin-fixed sections were prepared and analysed as previously described [see *Materials and Methods*].An example of a ERBB2 / Chromosome 17 hybridisation is presented in (Figure 4), which demonstrates ERBB2 amplification in the tumour cells within the duct, but only single copy of ERBB2 and chromosome 17 in the normal myoepithelial cells surrounding the duct.

The results of the ERBB2 / chromosome 17 hybridisation's are presented in (Table 5) . Two of the ten initial samples of DCIS showed amplification of ERBB2. The recurrent biopsies of those patients whose initial biopsy demonstrated ERBB2 amplification also showed amplification of the ERBB2 oncogene. There was no other evidence of amplification in the remainder of the recurrent biopsies, thus our small group of recurrent DCIS had the same frequency of ERBB2 amplification as the group of initial biopsies. Of the two recurrences with amplification one had an invasive component and one did not. ERBB2 amplification was demonstrated in both the DCIS and invasive component of the recurrence.

5.4. Discussion.

Analysis of ERBB2 has established that PDWA is not associated with amplification of this oncogene, however our data suggests that chromosome 17 alterations are already present in PDWA and that progression to DCIS involves an increase in the frequency of chromosome 17 alterations and the development of ERBB2 amplification.

The analysis of recurrent DCIS implies that ERBB2 amplification is not associated with the development of recurrent disease and that it is the persistence of an established subclone of DCIS, with existing genetic alterations, that results in recurrences, probably due to inadequate excision. In addition, ERBB2 does not seem to be crucial in the development of invasive cancer in the recurrences, as three DCIS recurrences with invasion did not show amplification of the ERBB2 oncogene.



[A] Schematic diagram of a duct with DCIS. Within the white line (basement membrane), are the tumour cells, and those cells outwith the line are normal myoepithelial cells.

Figure 4. **The FISH image is an example of an ERBB2 / Chromosome 17 double hybridisation.**

The nuclei are pseudocoloured blue; the ERBB2 hybridisations are red and the chromosome 17 hybridisations are green. The tumour cells are demonstrating amplification of ERBB2 while the normal peripheral myoepithelial cells are demonstrating normal copy number for ERBB2 and chromosome 17.

Section	Initial Biopsy Pathology	ERBB2 amplification	Recurrent Biopsy Pathology	ERBB2 amplification
1	DCIS	-	DCIS / INVASIVE	-
2	DCIS	-	DCIS	-
3	DCIS	-	DCIS	-
4	DCIS	-	DCIS / INVASIVE	-
5	DCIS	AMP	DCIS / INVASIVE	AMP
6	DCIS	-	DCIS	-
7	DCIS	-	DCIS	-
8	DCIS	AMP	DCIS	AMP
9	DCIS	-	DCIS	-
10	DCIS	-	DCIS / INVASIVE	-

Table 5. **Results of ERBB2 amplification analysis on initial and recurrent DCIS.**

The finding of polysomy of chromosome 17 for a section of PDWA associated with a normal ERBB2 copy number is particularly interesting because it infers that a regional deletion encompassing the ERBB2 locus on chromosome 17, with subsequent reduplication of chromosome 17 is present in these PDWA lesions, (Figure 5), a situation previously observed in DCIS using LOH analysis (Murphy et al. 1995).

This interpretation of the FISH data is in keeping with the model of the genetic evolution of breast cancer by Dutrillaux and Devillee, (Figure 6). This model envisages two phases, the first of which suggests that genetic instability is initiated by mutations to a crucial gene or genes. In this phase, structural chromosomal rearrangements are thought to be crucial and would be associated with loss of heterozygosity and chromosome losses, as well as leading to DNA amplification. This hypodiploid clone would then enter phase two which involves endoreduplication which is thought to confer a growth advantage to the resultant clone because of a gene dosage phenomenon. A single aneuploid clone may arise or more likely several subclones including the origonal hypodiploid clone would persist. The identification of genetic changes in PDWA consistent with the presence of a hypotetraploid clone suggests that the process of endoreduplication may occur earlier in breast cancer progression than was proposed in Figure 6.

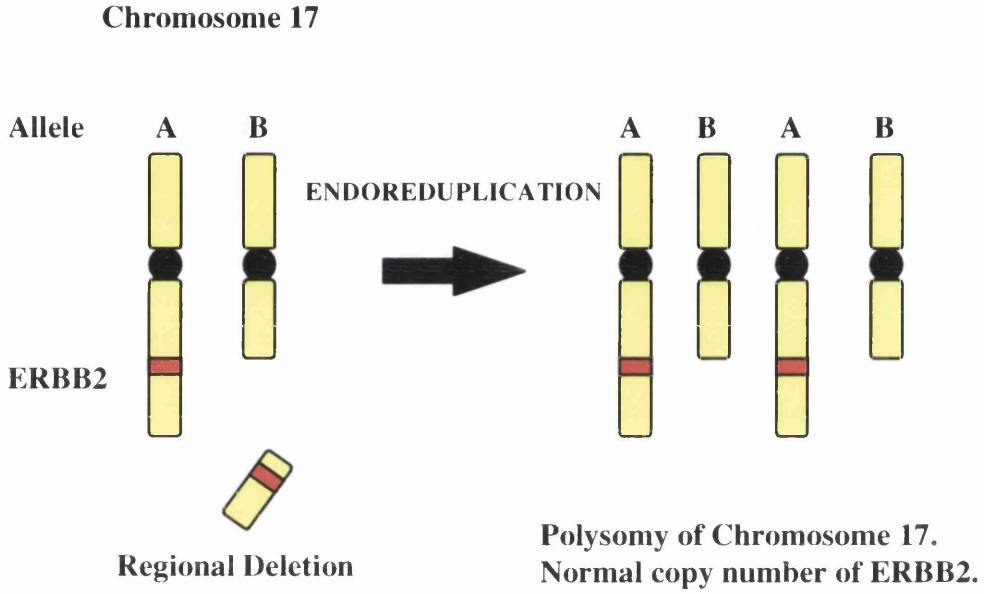


Figure 5. **Schematic representation of the mechanism by which a PDWA lesion can have increased copy number of chromosome 17 but a normal copy number of a regional marker such as ERBB2.**

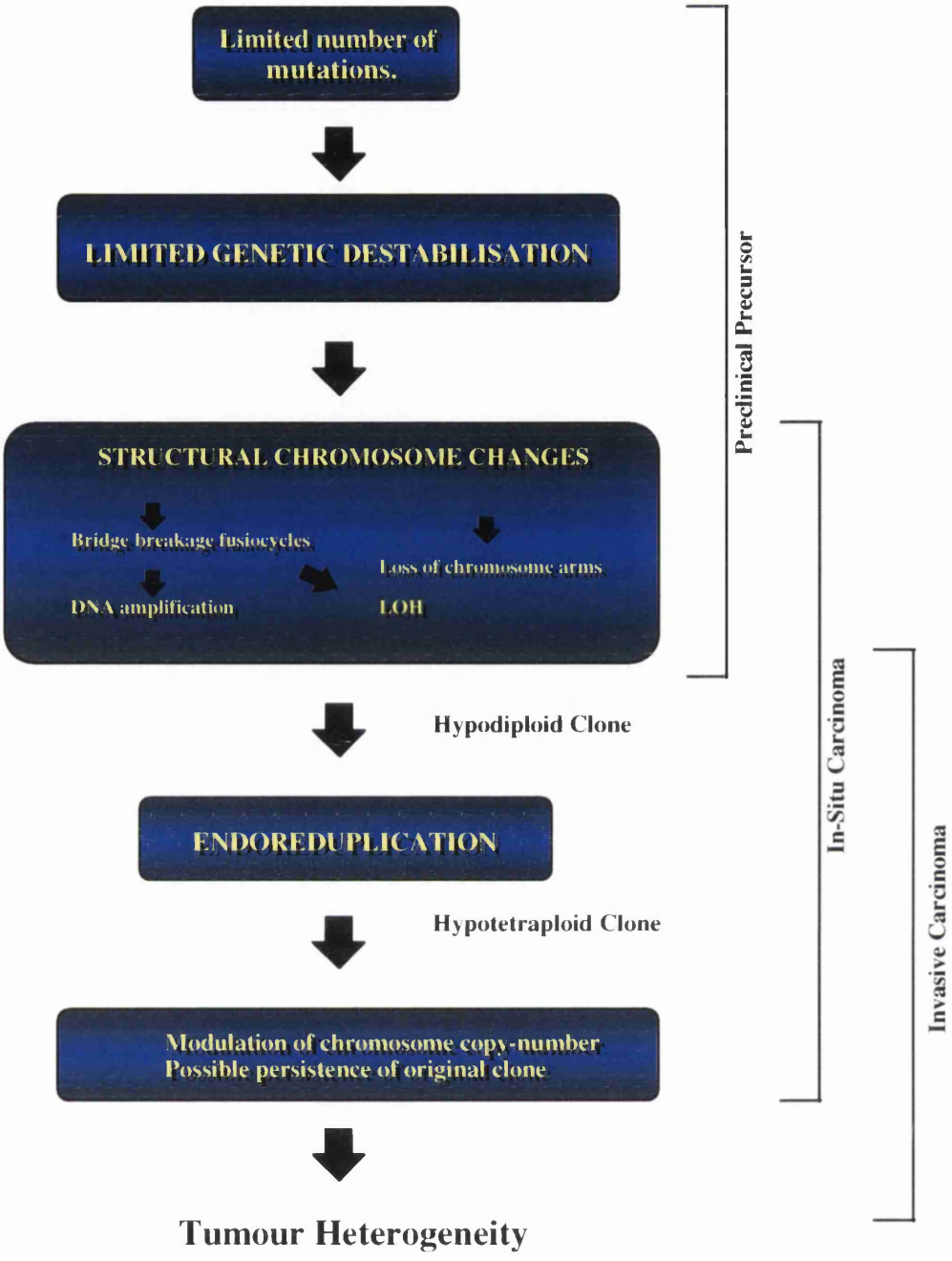


Figure 6. **Genetic Model of Breast Cancer Evolution.**
(Modified from Cornelisse & Devilee 1990)

Chapter 6.

Identification of Chromosome 18 Imbalances in Ductal Carcinoma *in-situ.*

6.1. Introduction.

Molecular studies have demonstrated a number of chromosomes which may harbour key genes involved in the progression of breast cancer. It is hypothesised that genetic alterations important for tumour progression are produced early in development and that progression related genetic events or randomly acquired alterations are probably generated later. Hence, the molecular analysis of pre-invasive breast lesions should lead to the discovery of genetic alterations crucial to the early stages of breast cancer initiation and progression.

A striking feature of the interphase cytogenetic analysis of these pre-invasive lesions is the high frequency of chromosome 18 loss in both DCIS and PDWA. LOH studies of invasive breast cancer and DCIS have also demonstrated a high frequency of LOH in chromosome 18 (James et al 1997; Stratton et al. 1995; Aldaz et al. 1995; Murphy et al. 1995; Fujii et al. 1996; Allred et al. 1993; Harrison et al. 1995; Radford et al. 1995). Thus, chromosome 18 may harbour loci involved in the earliest stages of breast cancer progression or that a subset of lesions may progress via this route. Hence, there is a need to study chromosome 18 in more detail, using microsatellite analysis, in order to identify regional changes on chromosome 18.

Three polymorphic DNA markers were chosen to analyse chromosome 18. Two on the p-arm (D18S59 & D18S71), chosen due to the paucity of information about this arm and one from the q-arm (D18S70), chosen because a high frequency of LOH that has been found at this locus (18q23) in invasive breast and other cancers,(Huang et al.1995, Devilee et al. 1991), (Figure1).

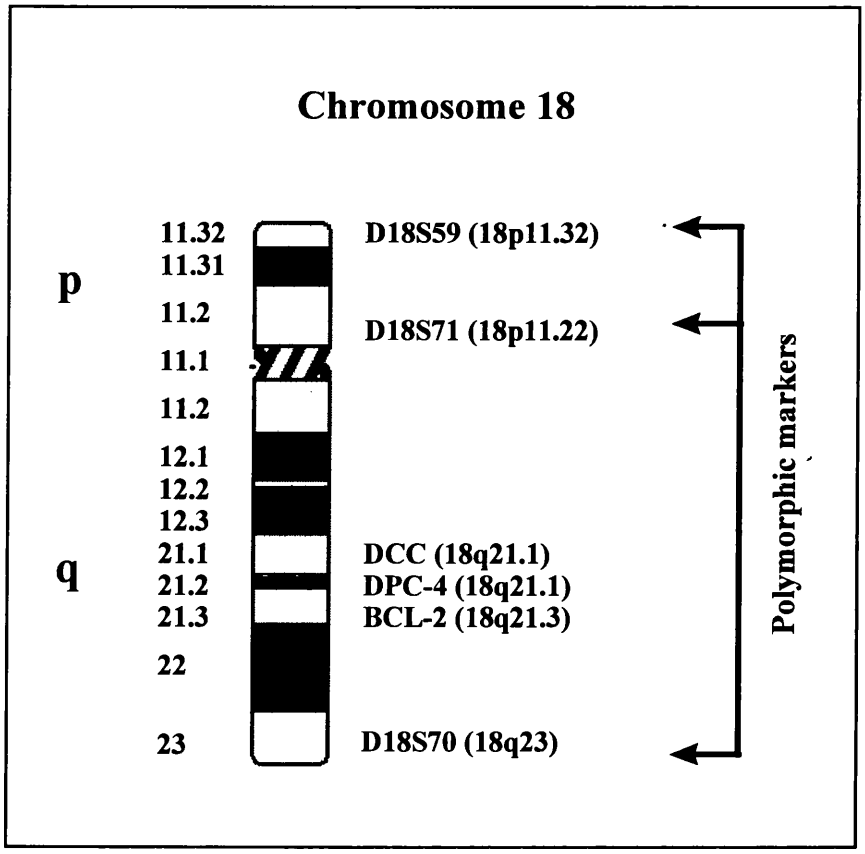


Figure 1. **Chromosome 18 ideogram illustrating the position of the polymorphic DNA markers. Genes proposed to be involved in breast carcinogenesis are also included, (DCC, DPC4).**

A number of molecular studies now indicate that DCIS may exhibit intratumour genetic heterogeneity whereby individual foci of DCIS, from the same patient, show differing patterns of allelic imbalances (Fujii et al. 1996).

Thus, in order to identify regional alterations of chromosome 18 and to address the phenomenon of intratumour heterogeneity of DCIS microsatellite analysis was performed, using three polymorphic markers for chromosome 18, on 62 individually microdissected foci of DCIS from 11 patients previously found to have chromosome 18 loss by interphase cytogenetics, (Figure 2).

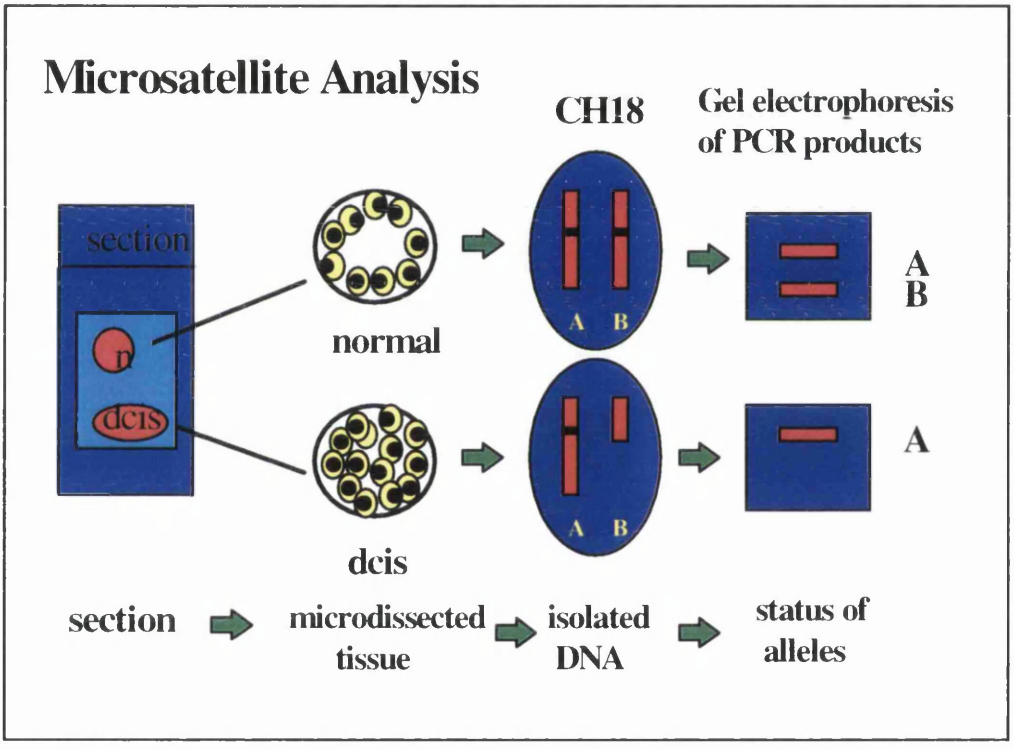


Figure.2. **Strategy for Determining the Allelic Status of Chromosome 18.**

Foci of ductal carcinoma in-situ and normal tissue were separated by microdissection of routine histological sections from resected breast specimens. Total genomic DNA was isolated from each sample. The polymerase chain reaction (PCR) was used to amplify polymorphic microsatellite markers capable of distinguishing maternal and paternal copies of chromosome 18. Allelic loss was observed as the absolute loss of the PCR product corresponding to the lost **chromosomal arm.**

6.2. Frequency of Allelic Imbalance on Chromosome 18 in Ductal Carcinoma *in-situ.*

Eleven patients with isolated DCIS were analysed with three polymorphic markers for chromosome 18 and the results of the analysis are presented in (Table 1).

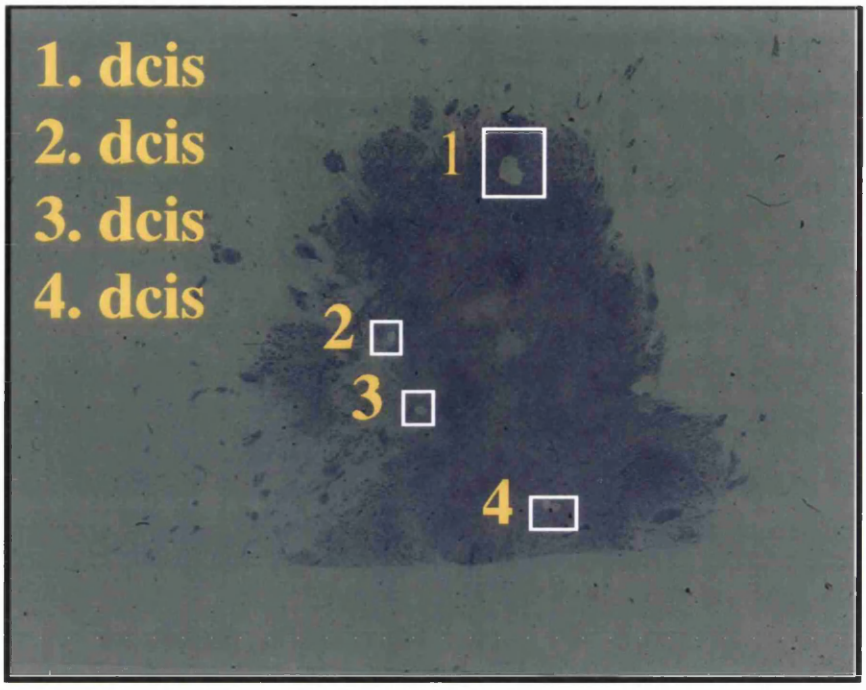
Chromosome Region	No. of cases with LOH
18p11.32	**8 / 11** (72%)
18p11.22	**6 / 11** (55%)
18q23	**9 / 11** (82%)

Table 1. **Frequency of Allelic Imbalances in DCIS for Chromosome 18 markers, D18S59 (18p11.32), D18S71 (18p11.22) D18S70 (18q23).**

The polymorphic markers for the region 18p11.32 showed allelic imbalance in 8 / 11 patients, (72%); while the marker for 18p11.22 showed allelic imbalance in 6 / 11 patients, (55%); the highest frequency of allelic imbalance was demonstrated for the region 18q23 which showed allelic imbalance in 9 / 11 patients, (82%). This data complements the interphase cytogenetic findings for each section which demonstrated a loss of chromosome 18-centromeric sequences.

6.3. Intratumour Heterogeneity for Allelic Imbalances.

Each section of DCIS had up to nine separate ducts containing DCIS microdissected from toluidine blue stained paraffin sections, the DNA extracted and the subsequent PCR products separated on acrylamide gels to detect allelic imbalances,(Figure 3).



LOH Analysis

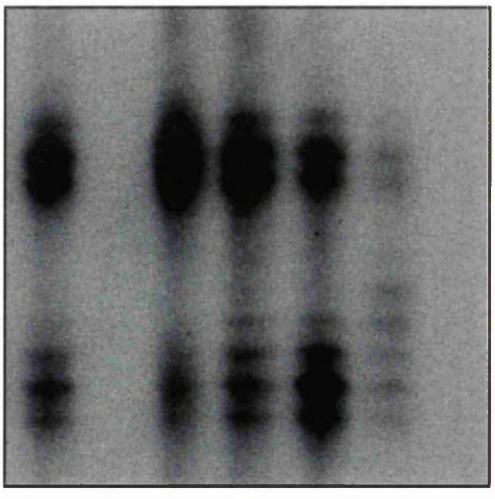


Figure 3. **Example of PCR products from a microdissected section separated on an acrylamide gel.**

Of the 11 patients analysed 10 patients had tumour foci informative for all three polymorphic markers and it was only those 10 sections that were included in the analysis, (Table 2).

Of the 62 individual ducts microdissected from 11 patients, 31 of these ducts from 10 patients were informative for all three markers, (Table 2). D18S59 showed allelic imbalance in 8 / 10 patients and in 14 / 31 ducts (LOH frequency of 45%) while D18S71 showed allelic imbalance in 6 / 10 patients and 12 / 31 ducts (LOH frequency of 39%). The highest frequency of allelic imbalance for an chromosomal region was for the marker D18S70, with 9 / 10 patients showing allelic imbalances and 20 / 31 ducts (LOH frequency of 65%). This estimation of the frequency of allelic imbalance may be a more accurate reflection of the rate of LOH for DCIS as it takes into account the number of DCIS foci examined in each section, whereas the percentage of patients with LOH, does not, (Table 1).

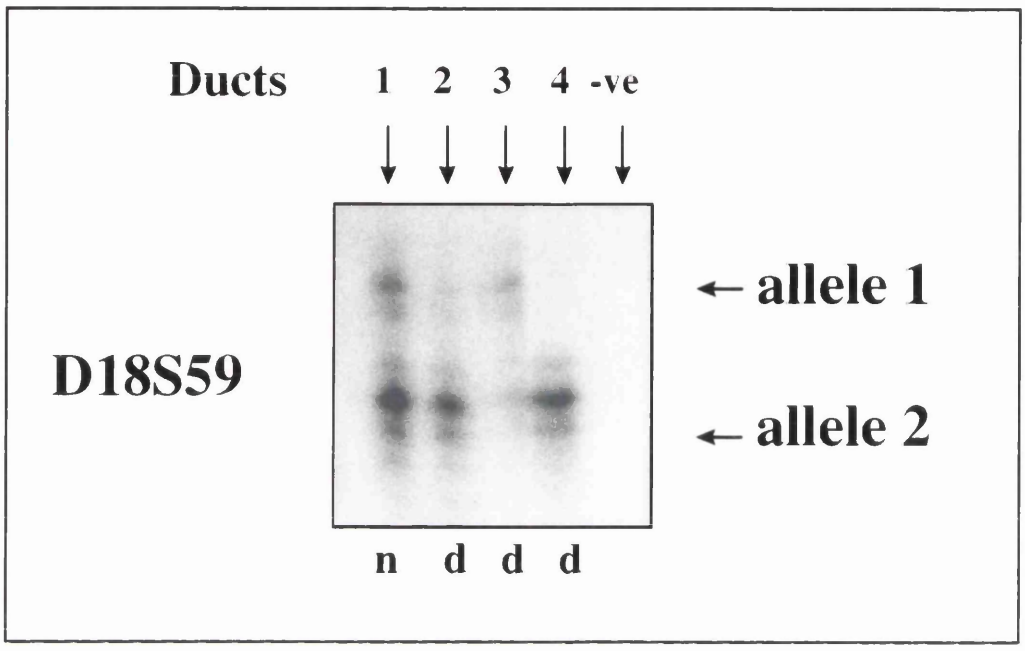
The striking feature of (Table 2), is the extensive heterogeneity of allelic imbalances within sections of DCIS. The data from patient 3 illustrates this phenomenon, (Figure 4 & Figure 5). Patient 3 had three separate foci of DCIS microdissected together with a normal lobule. The normal lobule retained both alleles for all three markers. Duct 1 retained both alleles for the p-arm markers but lost the upper allele for the q-arm marker. Duct 2 again retained both the alleles of the p-arm markers however lost the opposite lower allele for the q-arm marker. Duct 3 lost the upper allele for D18S59 but retained both alleles for the other markers.

This genetic disparity between foci of DCIS was present in all the sections analysed and is schematically represented in (Figure 5).

In some sections, such as section 3, (Figure 4), genetic alterations on chromosome 18 were similar in different foci of DCIS and similar to the invasive component of the section. However, in other sections, such as section 6, the invasive component demonstrated different alterations.

Patient	1	2	3	4	5	6	7	8	9	10	11	Total
No. of ducts informative for all 3 markers	6	3	3	4	2	2	2	0	3	4	2	31
Locus	⌜ Ducts demonstrating allelic imbalance ⌝											LOH %
D18S59	1	1	1	0	0	2	2	0	3	3	1	14/31 (45%)
D18S71	1	0	0	3	0	2	0	0	2	3	1	12/31 (39%)
D18S70	4	1	2	4	0	1	1	0	3	2	2	20/31 (65%)

Table 2. **Results of PCR amplification of 3 polymorphic markers for multiple foci of DCIS. (eg: Patient one had 6 DCIS foci informative for all three markers. 1 / 6 ducts showed allelic imbalance for the marker D18S59 and D18S71 while 4 / 6 ducts showed allelic imbalance for the marker D18S70. The remaining ducts showed retention of both alleles.)**



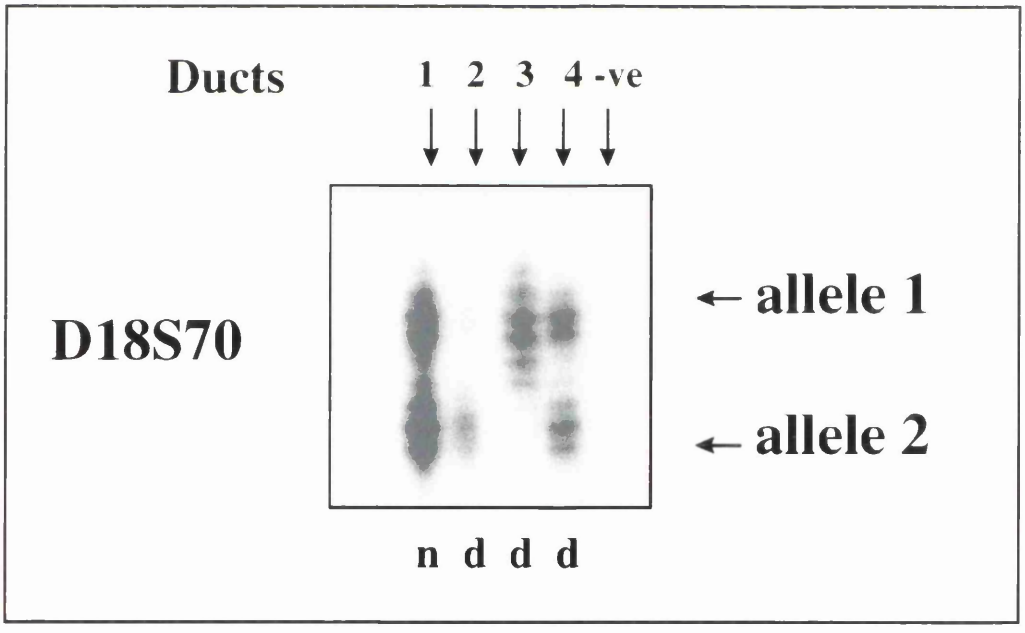
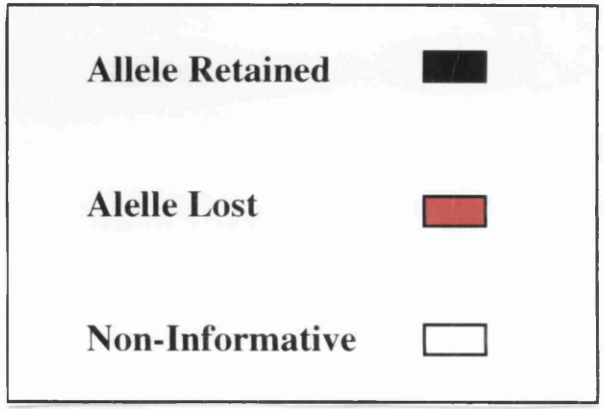
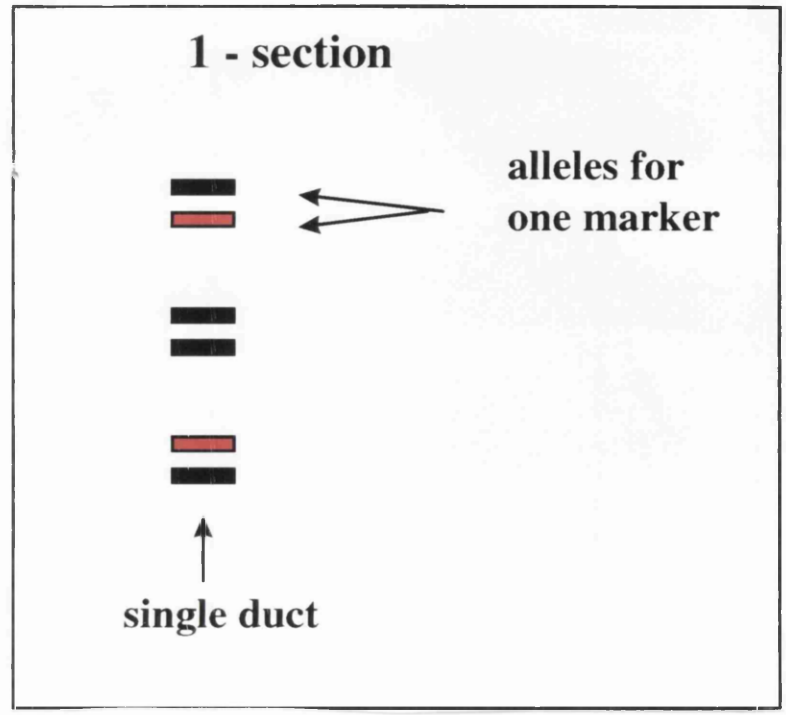


Figure 4. **Microsatellite analysis of section 3.**

(n - normal; d - DCIS)


Allele Retained
Alelle Lost
Non-Informative


1 - section
alleles for
one marker
single duct

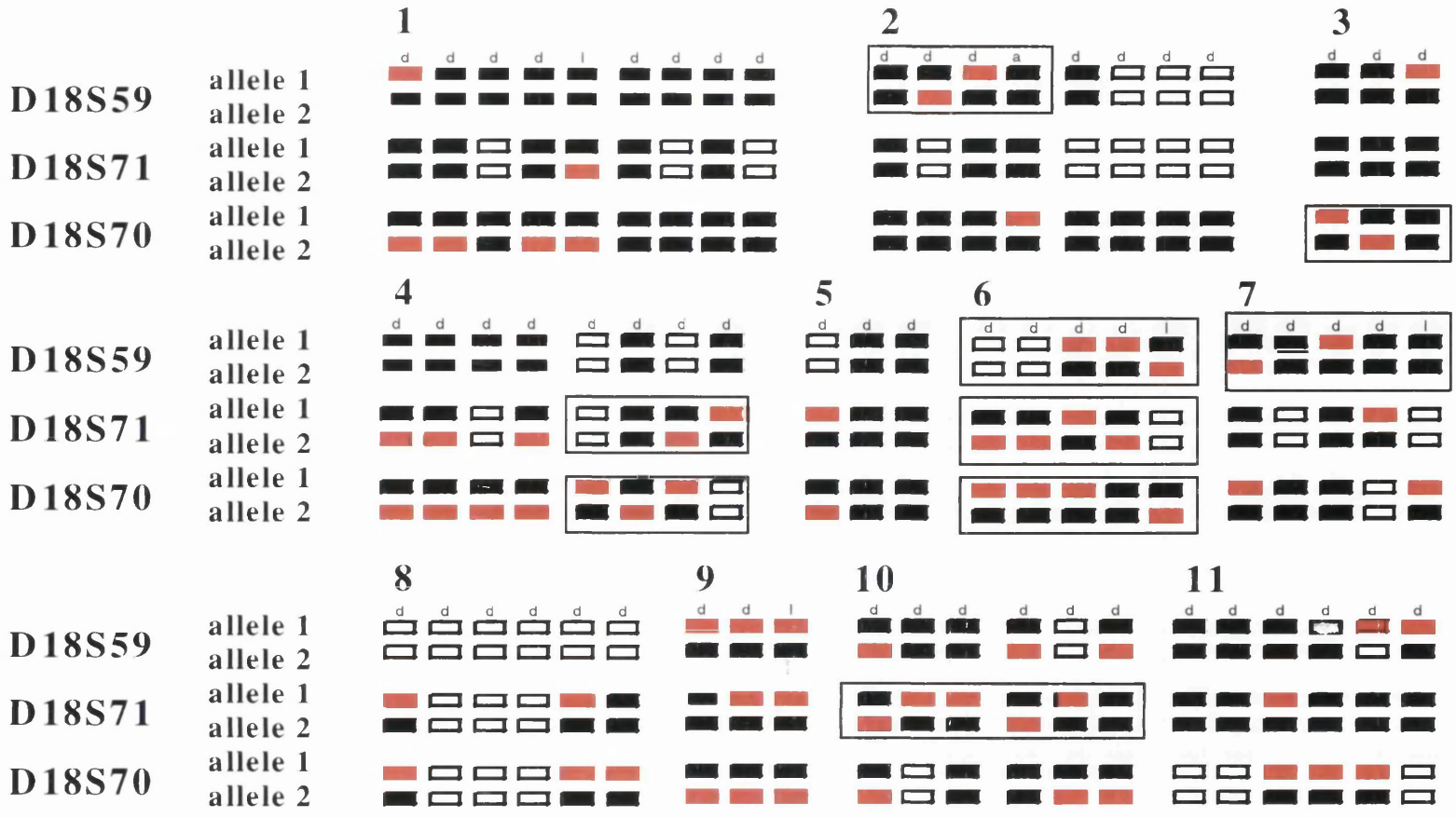


Figure 4. **Schematic Representation of Allelic Imbalances in DCIS**

(d - DCIS ; I - invasive)

A feature of the heterogeneity between ducts was the frequent observation that spacially related ducts lost opposite alleles for the same polymorphic marker, (Figure 5; examples are boxed). This phenomenon has been reported by other authors but at a low frequency (Stratton et al. 1995).

6.4. Discussion.

Interphase cytogenetics previously demonstrated that whole chromosome 18 loss was a feature of DCIS. This has now been complemented by microsatellite analysis which has shown regional chromosome 18 alterations in DCIS. The frequency of these allelic imbalances were far higher than previously reported regional chromosome 18 changes. The factors which contributed to the high frequencies of allelic imbalances observed are firstly, the precise microdissection of ducts with DCIS which reduces to a minimum the potential for normal cellular contamination which would mask allelic loss. Secondly, examination of multiple foci of DCIS within each section has revealed that allelic imbalances are not uniformly present in all portions of a tumour and would be missed or underestimated if only one area of DCIS had been analysed.

The markers on the p-arm of chromosome 18 (D18S59 &D18S71) demonstrated a frequency of allelic imbalance of 45% and 39% respectively. The p-arm of chromosome 18 has not been subjected to a great deal of analysis before in DCIS or invasive breast cancer. However, despite this, the frequency of alterations are high suggesting that the p-arm of chromosome 18 should be revisited in breast cancer and other tumours as a possible region of importance.

In contrast to the p-arm of chromosome 18, the q-arm has been the subject of intensive research in many tumours and indeed chromosome 18q has been implicated to contain tumour suppresser genes. One such gene is DCC, which has been isolated and cloned from 18q21 and has been found to be deleted in a number of tumours but most commonly in colonic carcinoma (Devilee et al.

1991). LOH / allelic imbalance at this locus has also been observed in invasive breast cancer ranging from 16% to 43% (Devilee et al. 1991). Hence DCC is thought to play an important role in the evolution of breast cancer.

18q21 also harbours a tumour suppresser gene, DPC4 / Smad1, which has been implicated in a number of tumours including pancreatic, ovarian, lung and head & neck (Hahn et al. 1996). Recent analysis has also shown that there is a high frequency of allelic imbalance in this region in invasive breast cancer also, outwith the locus of DCC (Schenk et al. 1996).

However, perhaps the most promising area on 18q that may harbour a suppresser gene is 18q23 (Huang et al. 1995). Our data demonstrates a frequency of allelic imbalance on 18q23, which includes this locus, of 63% in DCIS which is in concordance with analysis of this locus in invasive disease, with allelic imbalance in this area being detected in up to 43% of patients with breast cancer (Devilee et al. 1991). In addition, the rate of allelic imbalance of 18q23 in ovarian cancer is reported to be 60%, greater than that of the DCC gene, hence this suggests that 18q23 may contain another tumour suppresser gene common to both breast and ovarian cancer (Kerr J.et al et al. 1992). The high frequency of allelic imbalance in DCIS also suggests that this region may be involved in an early stage of progression and that the alterations observed in this region are not just an epiphenomenon as a result of the genetic instability induced by the malignant phenotype.

A striking feature of the pattern of allelic imbalance in DCIS was the marked genetic heterogeneity between ducts, (Figure 5). The analysis of the multiple foci of DCIS raises several issues. Firstly, the identification of similar alterations to chromosome 18 in DCIS and invasive tumour from the same section, (Figure 3; section 9) provides further evidence for the clonal evolution of DCIS to invasive cancer. Secondly, the demonstration of different alterations to chromosome 18 in different foci of DCIS provides evidence of subclonal evolution within a section of DCIS.

This hypothesis of subclonal evolution is demonstrated in (Figure 6), which is a representation of the genetic alterations to the three individual ducts from section 3. A number of key chromosomal alterations must be attained during the development of a tumour to reach the malignant phenotype. The allelic imbalance at 18q23 present in ducts 1 and 2, although affecting different alleles may be important in the evolution of that subclone. This could be via a gene dosage effect which confers a growth advantage on that clone thus selecting for that clone with the 18q23 alteration to evolve and diversify further and possibly gain further critical chromosomal alterations which may breach the biological barrier of invasion. Hence, the alteration to 18q23 will be present at a high frequency in a number of subsequent subclones and may well be associated with further alterations to chromosome 18. Although present at a lower frequency, the alteration to the p-arm in duct 3 may also provide a growth advantage which allows the progression along a different pathway of subclone evolution.

Indications from the interphase cytogenetic analysis of PDWA suggest that chromosome 18 alterations occur early in the evolution of breast cancer progression, hence alterations to key genes on chromosome 18 could prove to be the target of future preventative therapy for breast cancer. The process of subclone evolution is clearly complex even for one chromosome, however, the technique of microdissection and the analysis of multiple foci of DCIS may well result in the detection of genetic alterations critical for a subclone to develop the potential to invade.

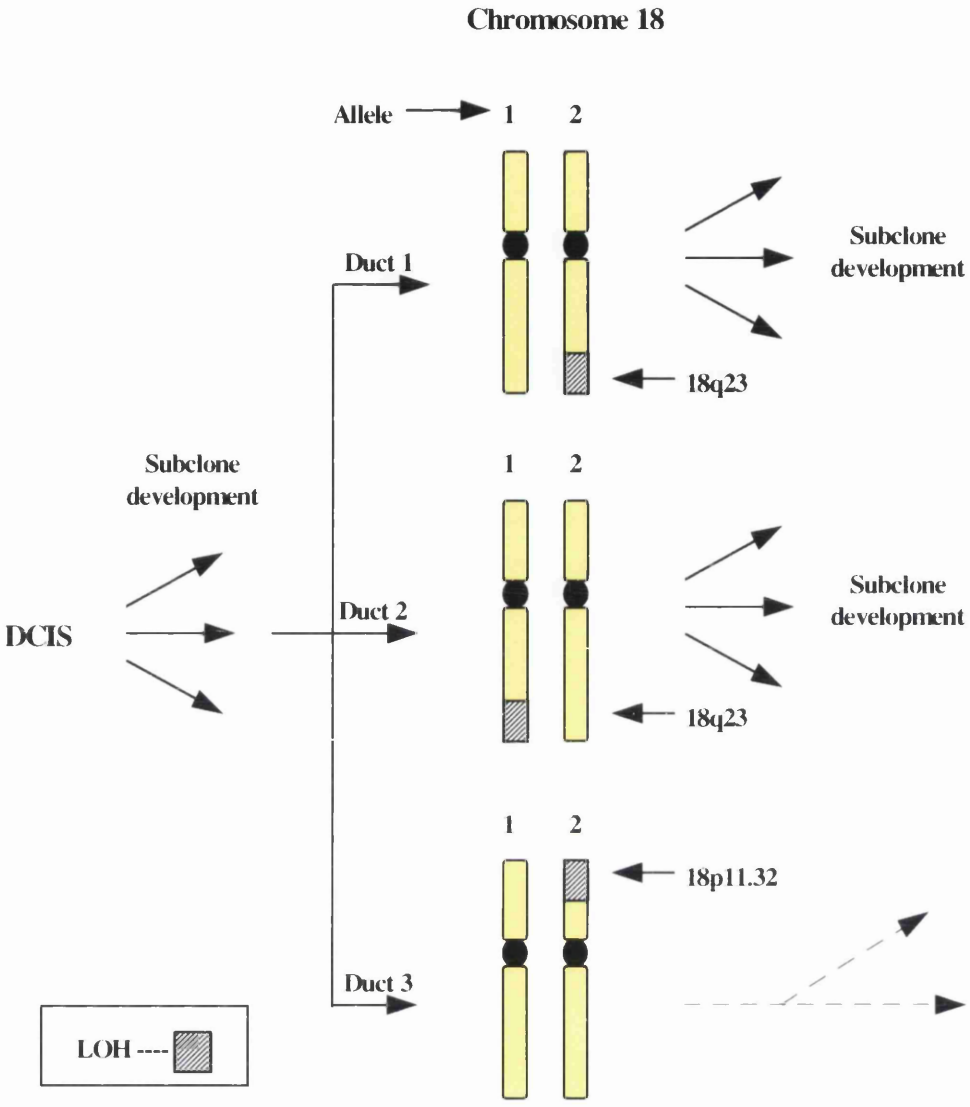


Figure 6. **Subclonal evolution of DCIS**

DISCUSSION

Chapter 7.

7. General Discussion

In recent years we have seen improvements in the multidisciplinary treatment of breast cancer which has led to modest improvements in disease free interval and survival for stage I and stage II invasive breast cancer. However, the incidence of breast cancer continues to increase and will continue to do so unless the biological nature of the disease is understood and novel therapeutic strategies can be developed both to treat and prevent breast cancer.

In an attempt to alter the natural history of breast cancer by diagnosing it as early as possible, before the tumour has developed a potential to metastasise, mammographic screening was introduced. This has led to an explosion in the diagnosis of all forms of pre-invasive breast lesions. Advances in molecular biology and recombinant DNA technology have provided the tools to analyse both breast cancer and its potential precursors in an attempt to determine the critical genetic alterations that lead to the invasive phenotype.

As you might expect the majority of genetic investigation has been conducted on invasive disease and more recently ductal carcinoma in-situ, which has provided strong evidence that DCIS is a non-obligate precursor of breast cancer. However, little is known about the genetics of early breast cancer progression.

The aim of this study was to reconstruct the molecular evolution of breast cancer progression with particular emphasis on the critical pre-invasive steps. Interphase cytogenetics allowed the identification of chromosomal alterations common to a subpopulation of PDWA and DCIS, providing for the first time genetic evidence that DCIS and thus invasive breast cancer are genetically similar to a group of benign lesions that are known to convey a higher risk of breast cancer to the individual. This provides important evidence to

substantiate the hypothesis that a normal breast epithelium can evolve through a number of sequential genetic alterations to invasive carcinoma, (Figure 1). The presence of similar genetic alterations in PDWA and DCIS suggests that crucial initiating or transforming changes occur early in breast cancer progression. The pattern of genetic alterations identified in PDWA and DCIS also sets an agenda for the future investigation of pre-invasive breast disease. For instance the huge increase in frequency of chromosome 3 and 17 alterations between PDWA and DCIS suggests that those chromosomes harbour genes critical in the progression of a benign precursor to one with a high potential to transform into invasive breast cancer.

One such gene is ERBB2 (Ch17) which is amplified in a proportion of DCIS and invasive cancers but not in PDWA. Hence, the amplification of this oncogene may be associated with the progression of a subpopulation of PDWA to DCIS. However, only approximately 30% of DCIS show amplification of this oncogene. The subsequent analysis of initial and recurrent DCIS samples revealed that ERBB2 amplification was not crucial to the development of invasive recurrences following initial wide excision of pure DCIS. These findings imply that there are a number of pathways of sequential gene alterations that can lead to invasive cancer one of which involves chromosome 17 and the amplification of ERBB2. It therefore appears that alterations to chromosome 17, including ERBB2, are not involved in the final common pathway to invasion.

The demonstration that the progression of PDWA to DCIS was also associated with an increased frequency of chromosome 3 alterations was of particular interest because our group have just mapped the human telomerase gene, (hTR), to chromosome 3q26.6. and have demonstrated amplification of this gene in a number of solid tumours.

The reactivation of telomerase activity, which maintains telomere length in dividing cells, conveys immortality to those cells and may well be a fundamental feature of cancer progression. It therefore seems reasonable to

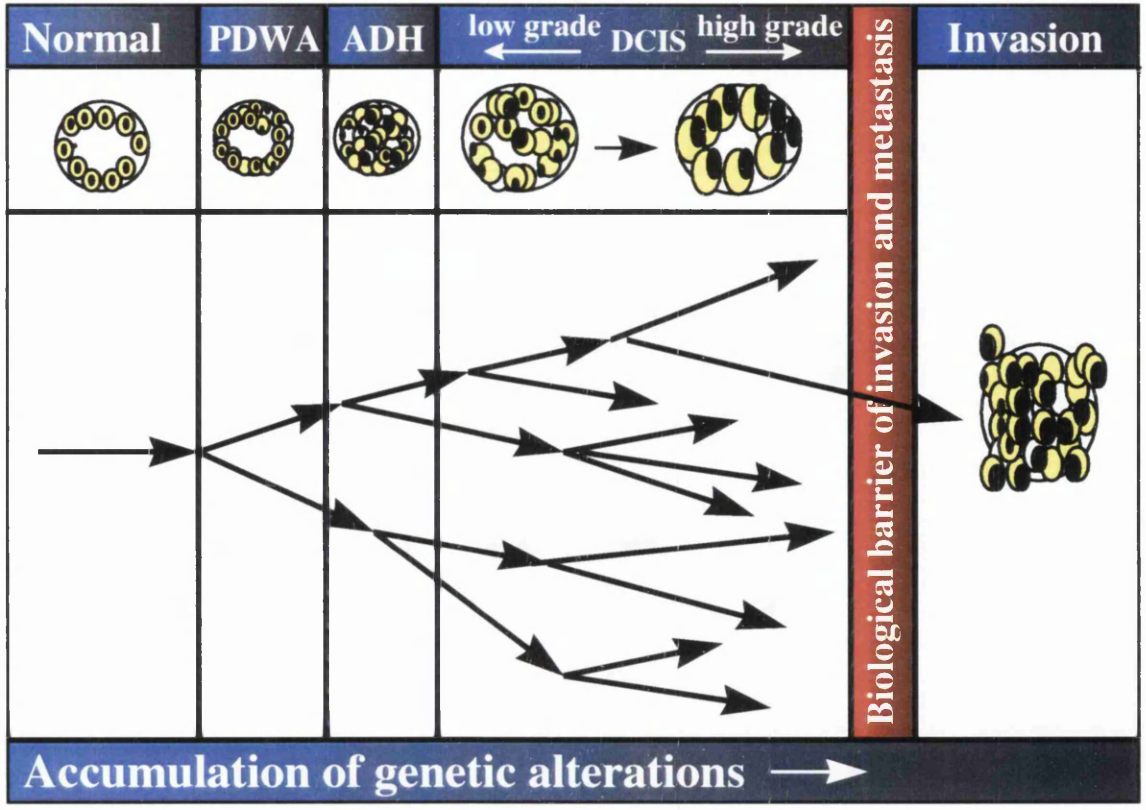


Figure 1. **Sub-clonal evolution of Breast Cancer progression.**

hypothesise that the chromosome 3 alterations identified in PDWA and DCIS could include amplification of the hTR gene locus resulting in the reactivation of telomerase and the establishment of a small subpopulation of PDWA which evolves to DCIS as a result.

Future analysis of breast cancer and pre-invasive breast disease will determine the significance of these observations, however the discovery of the telomerase gene on a chromosome known to be altered in breast cancer and pre-invasive lesions provides the opportunity to develop new therapies for breast cancer. For example, an 'anti-telomerase' agent. Indeed, if the function and interactions of the promoter region of this gene can be elucidated, the opportunity exists to develop novel therapies to prevent the amplification of the gene and therefore potentially prevent breast cancer progression.

The interphase cytogenetic analysis of PDWA and DCIS indicated that chromosome 18 alterations occurred at a high frequency in both pre-invasive lesions suggesting that genetic alterations to chromosome 18 are an early event in the sequential genetic progression to invasive breast cancer or that one particular pathway to invasion involves alterations to chromosome 18 genes. The study subsequently demonstrated regional alterations to chromosome 18 in DCIS, pinpointing 18q23 as a region likely to harbour a new tumour suppresser gene or genes.

Those regional changes on chromosome 18 were subject to extensive intratumour variation or heterogeneity, implying that a section of DCIS is composed of multiple subclones of DCIS, each with differing genetic alterations seeking the correct combination of genetic alterations that will allow that particular clone to breach the biological barrier of invasion. Those divergent clones of DCIS are thought to have evolved from PDWA, therefore in theory PDWA should also be composed of subclones, less genetically mature than those of DCIS. However, FISH was not sensitive enough to detect intratumour variation in this study.

The objective of this study was to provide scientific evidence for the hypothesis that breast cancer can evolve from benign precursors by identifying genetic alterations common to those benign lesions and breast cancer. The results of the study have accomplished this and indeed identified certain chromosomes and chromosomal regions that may harbour genes critical to early breast cancer progression. The results of this study together with other groups analyses of DCIS and invasive breast cancer provide compelling genetic evidence that the invasive phenotype is a result of one or more subclones of cells derived from a normal breast epithelium which has evolved and diverged from related clones of cells on its path toward invasion, (Figure 1). Those multiple clones are arbitrarily called PDWA, ADH and DCIS based on their light microscopic appearances, however they all represent the same biologically evolving clones of cells at different stages in their maturity.

REFERENCES

Chapter 8.

References

Aldaz, C.M., Chen, T., Sahin, A., Cunningham, J. and Bondy, M. Comparative allelotype of in situ and invasive human breast cancer: high frequency of microsatellite instability in lobular breast carcinomas. Cancer Research 55 (1995) 3976-3981.

Allred, D.C., Clark, G.M., Molina, R., Tandon, A.K., Schnitt, S.J., Gilchrist, K.W., Osborne, C.K., Tormey, D.C. and McGuire, W.L. Overexpression of HER-2/neu and its relationship with other prognostic factors change during the progression of in situ to invasive breast cancer. Human Pathology 23 (1992) 974-979.

Allred, D.C., O'Connell, P. and Fuqua, S.A. Biomarkers in early breast neoplasia. [Review]. Journal of Cellular Biochemistry - Supplement 17G (1993) 125-131.

Alpers, C.E. and Wellings, S.R. The Prevelence of Carcinoma In Situ in Normal and Cancer Associated Breasts. Pathology 16 (1985) 796-807.

Anderson, E.D. Familial versus sporadic breast cancer. Cancer 70 (1992) 1740-1746.

Anderson, T., J., Alexander, F.E. and Roberts, M.M. Edinburgh trial of screening for breast cancer: mortality at seven years. Lancet 335 (1990) 241-246.

Barnes, B., Johnson, G., Duhig, T., Craston, A., Cohen, E.P. and Gendler, S.J.The locus of (PEM) tumour antigen on chromosome 1q21 shows a high frequency of alterations in human primary breast cancer. International Journal of Cancer 45 (1990) 431-435.

Bartek, J., Lukas, J. and Strauss, M. Unrestricted cell cycling and cancer. Nature Medicine 1 (1995) 1245-1248.

Bartow, S.A., Pathak, D.R., Black, C.W., Key, C.R. and Teaf, S.R. Prevelence of Benign, Atypical, and Malignant Breast Lesions in Populations at Different Risk for Breast Cancer. Cancer 60 (1987) 2751-2760.

Bellamy, C.O., McDonald, C., Salter, D.M., Chetty, U. and Anderson, T.J. Noninvasive ductal carcinoma of the breast: the relevance of histologic categorization. Human Pathology 24 (1993) 16-23.

Beral, V. and Hermon, C. Breast cancer mortality rates are levelling off or beginning to decline in many western countries: analysis of time trends, age-cohort and age -period models of breast cancer mortality in 20 countries. British Journal of Cancer 73 (1996) 955-960.

Bieche, I and Lidereau, R. Genetic Alterations in Breast Cancer. Genes, Chromosomes & Cancer (1995) 227-251.

Blair, P.B., Bern, H.A., Faulkin, L.J. and DeOme, K.B. Development of mammary tumours from hyperplastic alveolar nodules transplanted into gland free mammary fat pads of female C3H mice. Cancer Research 19 (1959) 515-520.

Broders, A.C. Carcinoma in situ contrasted with benign penetrating epithelium. JAMA 99 (1932) 1670-1674.

Brown, J., Cole, P. and MacMahon, B. Etiology of human breast cancer. Journal of the National Cancer Institute 50 (1973) 21

Bzsuch, H., Senie, R. and Howe, H. Early abortion and breast cancer risk in women under the age of 40. International Journal of Epidemiology 18 (1989) 300

Callahan, R., Lidereau, R. and Ali, I.U. Reduction to homozygosity of genes on chromosome 11 in human breast neoplasia. Science 238 (1987) 185-188.

Callahan, R., Lidereau, R. and Ali, I.U. The presence of two members of c-erbA receptor gene family (c-erbA beta and c-erbA2) in the smallest region of somatic homozygosity on chromosome 3p21 - p25 in human breast carcinoma. Journal of the National Cancer Institute 81 (1989) 1815-1820.

Callahan, R., Champeme, M.H., Campbell, G., Lidereau, R. and Cropp, C.S. Loss of hereozygosity on chromosomes 17 and 18 in breast cancer: 2 additional regions identified. Proceedings of the Association of American Physicians 87 (1990) 7737-7741.

Cardiff, R.D. and Morris, D.W. The multistep model of mouse mammary tumour development. Advances in viral oncology 7 (1987) 123-140.

Carter, D.C., Steel, C.M., Wylie, A.H., Wallace, M., Morris, R.G. and Thompson. Allele loss from 5q21 (APC / MCC) and 18q21 (DCC) and DCC mRNA expression in breast cancer. A.M. British Journal of Cancer 68 (1993) 64-68.

Chen, Y., Dong, J. and Li, C. [Amplification and overexpression of c-erbB2 in human breast cancer]. [Chinese]. Chung-Hua Chung Liu Tsa Chih [Chinese Journal of Oncology] 17 (1995) 16-19.

Colditz, G.A., Stampfer, M.R. and Willett, W. Dietary fat and risk of breast cancer. New England Journal of Medicine 316 (1987) 22

Cole, P., MacMahon, B. and Trichopoulos, D. Menopause and breast cancer risk. Journal of the National Cancer Institute 48 (1972) 605

Cole, P., Hsieh, C. and Trichopoulos, D. Age at first birth and breast cancer risk. International Journal of Cancer 31 (1983) 701

Collins, F.S. BRCA1 - Lots of mutations, lots of dilemmas. New England Journal of Medicine 334 (1996) 186-188.

Cordon-Cardo, C., Fair, W.R., Reuter, V., Presti, J. and Dalbagni, G. Genetic alterations in bladder cancer. Lancet 2 (1993) 469-471.

Cornelisse, C.J., Keipers-Dijkshoorn, N., van Sloun, P., van Vleit, M. and Devilee, P. Allelotype of human breast carcinoma : A second major site of loss of heterozygosity is on chromosome 6q. Oncogene 6 (1991a) 1705-1711.

Cornelisse, C.J., Keipers-Dijkshoorn, N., van Sloun, P., van Vleit, M. and Devilee, P. Frequent somatic imbalance of marker alleles for chromosome 1 in human breast cancer. Cancer Research 51 (1991b) 1020-1025.

Cornelisse, C.J. and Devilee, P. The genetics of human breast cancer. Cancer 9 (1990) 605-630.

Devilee, P., van Vleit, M., Kuipers-Dijkshoorn, N., Pearson, P.L. and Cornelisse, C. Somatic Genetic Changes to Chromosome 18 in Breast Carcinomas:Is the DCC gene involved? Oncogene 6 (1991) 311-315.

Devilee, P., Hansen, B., Sutherland, G., Callen, D.F., Keipers-Dijkshoorn, N., Moerland, E. and Cleton-Jansen, A.M. At least two different regions are involved in alleilic imbalance on chromosome arm 16q in breast cancer. Genes, Chromosomes & Cancer 9 (1994a) 101-107.

Devilee, P., Schuuring, E., van de Vijver, M.J. and Cornelisse, C. Recent Developments in the Molecular Genetic Understanding of Breast Cancer. Critical Reviews in Oncogenesis 5 (1994b) 247-270.

Dixon, J.M. and Blamey, R.W. ABC of breast cancer. BMJ 81 (1994) 1076-1079.

Dupont, W.D., Parl, F., Hartmann, W.H., Brinton, L.A., Winfield, A.C., Worrell, J.A., Schuyler, P.A. and Plummer, W.D. Breast Cancer Risk Associated with Proliferative Breast Disease and Atypical Hyperplasia. Cancer 71 (1993) 1258-1265.

Dupont, W.D. and Page, D.L. Risk Factors for Breast Cancer in Women with Proliferative Breast Disease. New England Journal of Medicine 312 (1985) 146-151.

Early Breast Cancer Trialists' Collaborative Group: Systemic treatment of early breast cancer by hormonal, cytotoxic or immune therapy. Lancet 339 (1992) 1-15.

Early Breast Cancer Trialists' Collaborative Group: Effects of Radiotherapyand Surgery in Early Breast Cancer. An Overview of Randomised Trials. New England Journal of Medicine 333 (1995) 1444-1455.

Escot, C., Peters, G.N., Dickson, C., Callahan, K. and Lidereau, R. Amplification of the int-2 gene in primary human breast cancer. Oncogene 2 (1988) 285-291.

Evans, H.J., Forrest, A.P., Elder, P.A., Steel, C.M. and McKay, J. Allele loss on short arm of chromosome 17 in breast carcinomas. Lancet (1988) 1384-1385.

Fearon, E.R. and Vogelstein, B. Alleleotype of colorectal carcinomas. Science 244 (1989) 207-211.

Fearon, E.R. and Vogelstein, B. A Genetic Model for Colorectal Tumourigenesis. Cell 61 (1990) 759-767.

Feldman, J. and Gardner, B. Are Positive Axillary Nodes in Breast Cancer Markers for Incurable Disease? Annals of Surgery 218 (1993) 270-278.

Friedman, M., A., Phillips, P., H. and Abrams, J., S. Meeting highlights: A reappraisal of Research Results for the Local Treatment of Early Stage Breast Cancer. Journal of the National Cancer Institute 87 (1995) 1837-1845.

Frykberg, E.R. and Bland, K.I. Overview of the biology and management of ductal carcinoma in situ of the breast. [Review]. Cancer 74 (1994) 350-361.

Fujii, H., Szumel, R., Marsh, C., Zhou, W. and Gabrielson, E. Genetic progression, histological grade, and allelic loss in ductal carcinoma in situ of the breast. Cancer Research 56 (1996) 5260-5265.

Gad, E., Day, N.E., Duffy, S.W., Fagerberg, G. and Tabar, L. Update of the Swedish two-county program of mammographic screening for breast cancer. Radiologic Clinics of North America 30 (1992) 187-210.

Gelb, S., Mushinshki, M. and Seidman, H. Probability of eventually developing or dying of breast cancer. Cancer Research 7 (1985) 123-128.

Hahn, S.A., Schutte, M., Hoque, A.T., Moskaluk, C.A., da Costa, L.T., Rosenblum, E., Weinstein, C.L., Fischer, A., Yeo, C.J., Hruban, R.H. and Kern, S.E. DPC4,a Candidate Tumour Suppressor Gene at Human Chromosome 18q21.1. Science 271 (1996) 350-353.

Hankey, B.F., Feuer, E.J. and Miller, B.A. The significance of the rising incidence of breast cancer in the United States. Important Advances in Oncology 1 (1994) 193-200.

Harrison, M., Magee, H.M., O'Loughlin, J., Gorey, T.F. and Dervan, P.A. Chromosome 1 aneusomy, identified by interphase cytogenetics, in mammographically detected ductal carcinoma in situ of the breast. Journal of Pathology 175 (1995) 303-309.

Hayward, N.K., Walters, M.K., Palmer, J. and Walker, G.J. A genetic model of melenoma tumourigenesis based on allelic losses. Genes, Chromosomes & Cancer 12 (1995) 134-141.

Heim, S., Bardi, G., Limon, J., Jin, Y.T. and Pandis, N. Interstitial deletion ofm the short arm of chromosome 3 as a primary chromosome abnormality in breast cancer. Genes, Chromosomes & Cancer 12 (1995) 173-185.

Hellman, S. Natural History of Small Breast Cancers. Journal of Clinical Oncology 12 (1994) 2229-2234.

Holland, R., Peterse, J.L., Millis, R.R., Eusebi, V., Faverly, D., van de Vijve, MJ and Zafrani, B. Ductal carcinoma in situ: a proposal for a new classification. [Review]. Seminars in Diagnostic Pathology 11 (1994) 167-180.

Hoppe, R., Tucker, M. and Hancock, S. Breast cancer after treatment for Hodgkin's disease. Journal of the National Cancer Institute 85 (1993) 25

Howell, A., Ponder, B.A.J., Asbury, D., McPherson, K., Fentiman, I.S. and Evans, D.G.R. Familial breast cancer. BMJ 308 (1994) 183-187.

Huang, T.H., Yeh, P.L., Martin, M.B., Straub, R.E., Gilliam, T.C., Caldwell, C.W. and Skibba, J.L. Genetic alterations of microsatellites on chromosome 18 in human breast carcinoma. Diagnostic Molecular Pathology 4 (1995) 66-72.

Hubbard, A.L., Doris, C.P., Thomson, A.M., Chetty, U. and Anderson, T., J. Critical Determination of the Frequency of C-ERBB-2 Amplification in Breast Cancer. British Journal of Cancer 70 (1994) 434-439.

James, A., Mitchell, ELD., Menasce, L., Varley,JM. Comparitive Genomic Hybridisation of ductal carcinoma in-situ of the breast: identification of regions of DNA amplification and deletion in common with invasive breast cancer. Oncogene 14 (1997) 1059 - 1065.

Janzon, L., Aspegren, K. and Andersson, I.T. Mammographic screening and mortality from breast cancer. The Malmo mammographic screening trial. BMJ 297 (1988) 943-948.

Kerr J.et al, Leary J and Chenevix-Trench G: Frequent loss of heterozygosity on chromosome 18 in ovarian adenocarcinoma which does not always include the DCC gene locus. Oncogene 7 (1992) 1059-1065.

Knudson, A.G. Antioncogenes and human cancer. Proceedings of the National Academy of Sciences of the United States of America 90 (1993) 10914-10921.

Krag, D.N. and Alex, J.C. The gamma probe guided resection of radiolabelled primary lymph nodes. Surgical Oncology 5 (1996) 33-41.

Krasne, D.L., Evans, S.W., Morton, D.L., Turner, R.R., Dale, P.S. and Giuliano, A.E. Improved axillary staging of breast cancer with sentinal lymphadenectomy. Annals of Surgery 222 (1995) 394-401.

Ku, N., Balducci, L., Yeatman, T., Cox, C., Lyman, G.H. and Albertini, J.J. Lymphatic mapping and sentinal node biopsy in the patient with breast cancer. JAMA 276 (1996) 1818-1822.

Lagios, M.D. and Page, D.L. Radiation therapy for in situ or localized breast cancer [letter; comment]. New England Journal of Medicine 329 (1993) 1577-8; discussion 1578.

Lakhani, S.R., Collins, N., Stratton, M.R. and Sloane, J.P. Atypical ductal hyperplasia of the breast: clonal proliferation with loss of heterozygosity on chromosomes 16q and 17p. Journal of Clinical Pathology 48 (1995) 611-615.

Lakhani, S.R., Slack, D.N., Hamoudi, R.A., Collins, N., Stratton, M.R. and Sloane, J.P. Detection of Alleillic Imbalance Indicates That a Proportion of Mammary Hyperplasia of Usual Type are Clonal, Neoplastic Proliferations. Laboratory Investigation 74 (1996) 129-135.

Legault-Poisson, S., Redmond, C. and Fisher, B. Postoperative chemotherapy and tamoxifen compared with tamoxifen alone in the treatment of positive-node breast cancer patients aged 50 years and older with tumours responsive to tamoxifen: Results from the NSABP-16. Journal of Clinical Oncology 8 (1990) 1005-1018.

Lennington, W.J., Jensen, R.A., Dalton, L.W. and Page, D.L. Ductal carcinoma in situ of the breast. Heterogeneity of individual lesions. Cancer 73 (1994) 118-124.

Lidereau, R. and Bieche, I. Two distinct regions involved in 1p deletion in human primary breast cancer. Cancer Research 53 (1993) 1990-1994.

Liscia, D.S., Gaglia, P., Canale, L., Bernardi, A., Venesio, T. and Merlo, G.R. Loss of heterozygosity on chromosome 17p13 in breast carcinomas identifies tumours with high proliferation index. American Journal of Pathology 140 (1992) 215-223.

Liu, E., Thor, A., He, Ljung, B.M. and Benz, C. The HER2 (ERBB2) Oncogene is Frequently Amplified in In-Situ Carcinomas of the Breast. Oncogene 7 (1992) 1027-1032.

Lobo, R., Ross, R. and Bernstein, L. The effects of moderate physical activity on menstrual cycle patterns in adolescence:Implications for breast cancer prevention. British Journal of Cancer 55 (1987) 681

London, S.J., Connolly, J.L., Schnitt, S.J. and Colditz, G.A. A Proepective Study of Benign Breast Disease and the Risk of Breast Cancer. Journal of the American Medical Association 267 (1992) 941-944.

Ludwig Breast Cancer Study Group: Randomised trial of chemo-endocrine therapy, endocrine therapy and mastectomy alone in post-menopausal patients with operable breast cancer and axillary node metastases. Lancet 1 (1984) 1256-1260.

Micale, M.A., Visscher, D.W., Gulino, S.E. and Wolman, S.R. Chromosomal aneuploidy in proliferative breast disease. Human Pathology 25 (1994) 29-35.

Miller,WR., Dixon,JM. . ABC of breast cancer. Prognostic factors. BMJ 81:10(1994) 1573-1576.

Muleris, M., Almeida, A., Gerbault-Seureau, M., Malfoy, B. and Dutrillaux, B. Identification of amplified DNA sequences in breast cancer and their organization within homogeneously staining regions. Genes, Chromosomes & Cancer 14 (1995) 155-163.

Munn,KE., Walker,RA., Menasce, L., Varley, JM. Allelic imbalance in the region of the BRCA-1 gene in ductal carcinoma in situ of the breast. British J. of Cancer 73 (1996) 636-639.

Murphy, D.S., Hoare, S.F., Going, J.J., Mallon, E.E., George, W.D., Kaye, S.B., Brown, R, Black, D.M. and Keith, W.N. Characterization of extensive genetic alterations in ductal carcinoma in situ by fluorescence in situ hybridization and molecular analysis. Journal of the National Cancer Institute 87 (1995) 1694-1704.

Nakamori, S., Kasumi, F., Sakamoto, G., Akiyama, F. and Sato, T. Accumulation of genetic alterations and progression of primary breast cancer Cancer Research 51 (1991) 5794-5799.

Nakamori, S., Sakamoto, G., Kasumi, F., Akiyama, F., Yamakawa, T., Tanigami, A. and Sato, T. Allelotype of breast cancer : Cumulative allele losses promote tumour progression in primary breast cancer. Cancer Research 50 (1990) 7184-7189.

Nakamura, T., Fukutomi, T., Callen, D.F. and Tsuda, H. Allele loss on chromosome 16q24.2qpter occurs frequently in breast cancer irrespectively of differences in phenotype and extent of spread. Cancer Research 54 (1994) 513-517.

Nowell, P. The clonal evolution of tumour cell populations. Science 194 (1976) 23-28.

O'Connell, P., Pekkel, V., Fuqua, S., Osborne, C.K. and Allred, D.C. Molecular genetic studies of early breast cancer evolution. [Review]. Breast Cancer Research & Treatment 32 (1994) 5-12.

Orza, M., Berlin, J. and Longnecker, M. A metaanalysis of alcohol consumption in relation to breast cancer. JAMA 260 (1988) 642

Osteen, R.T. and Harris, J. Patients with early breast cancer benefit from effective axillary treatment. Breast Cancer Research & Treatment 5 (1985) 17-21.

Page, D.L., Dupont, W.D., Rogers, L.W. and Rados, M.S. Atypical Hyperplastic Lesions of the Female Breast. Cancer 55 (1984) 2698-2708.

Page, D.L., Watson, P. and Marcus, J.N. Hereditary Breast Cancer. Cancer 77 (1996) 697-709.

Page, D.L. and Dupont, W.D. Anatomic Markers Of Human Premalignancy and Risk of Breast Cancer. Cancer 66 (1990) 1326-1335.

Page, D.L. and Dupont, W.D. Anatomic indicators (histologic and cytologic) of increased breast cancer risk. [Review] [57 refs]. Breast Cancer Research & Treatment 28 (1993) 157-166.

Palli, D., Roselli, M., Simoncini, R. and Bianchi, E. Benign Breast Disease and Breast Cancer: A Case Control Study Cohort in Italy. International Journal of Cancer 47 (1991) 703-706.

Parkinson, E.K. Do Telomerase Antagonists Represent a Novel Anti-Cancer Strategy? British Journal of Cancer 73 (1996) 1-4.

Perren, T.J. Adjuvant therapy for operable breast cancer; more answers, new questions. British Journal of Cancer 71 (1995) 1142-1144.

Radford, D.M., Fair, K.L., Phillips, N.J., Ritter, J.H., Steinbrueck, T., Holt, M.S. and Donis-Keller, H. Allelotyping of ductal carcinoma in situ of the breast: deletion of loci on 8p, 13q, 16q, 17p and 17q. Cancer Research 55 (1995) 3399-3405.

Riou, G., Spielmann, M., Terrier, M.J., Barrios, M. and Guerin, M. Overexpression of either c-myc or ERBB2 protooncogenesin human breast cancer.Correlation with poor prognosis. Oncogene 3 (1988) 21-31.

Rodigues-Canteras, R., Delago-Rodriguez, M. and Silleros-Arena, M. Menopausal hormome replacement therapy and breast cancer. A metaanalysis. Gynaecology 79 (1992) 286

Romieu, L., Berlin,J., Colditz, G. Oral contraceptives and breast cancer: Review and metaanalysis. Cancer 66 (1990) 2253 - 2259

Rosen, P.P. Proliferative breast "disease". An unresolved diagnostic dilemma [comment]. [Review]. Cancer 71 (1993) 3798-3807.

Saunders, G.F., Anderson, E.D., Tsihira, H. and Genuardi, M. American Journal of Human Genetics 45 (1989) 73-84.

Schenk, M., Leib-Mosch, C., Schenk, I.U., Jaenicke, M., Indraccolo, S., Saeger, H.D., Dallenbach-Hellweg, G. and Hehlmann, R. Lower Frequency of Allele Loss on Chromosome 18q in Human Breast Cancer than in Colorectal Tunours. Journal of Molecular Medicine 74 (1996) 155-159.

Schnitt,SJ., Connelly, JL., Tavassoli, FA., Fechner, RE., Kempson, RL., Gelman, R. Interobserver reproducibility in the diagnosis of proliferative breast lesions using standard criteria. Am. J. of Surg. Pathology 16 (1992) 1095-1097.

Sellberg, G., Olsson, H., Alm, P., Zhang, Q.X. and Borg, A. The retinoblastoma gene in breast cancer : Allele loss is not correlated with loss of gene protein expression. Cancer Research 52 (1992) 2991-2994.

Shapiro, S. Determining the efficacy of breast cancer screening. Cancer 63 (1989) 1873-1880.

Sherman, G., Howe, G. and Miller, A.B. Mortality from breast cancer after irradiation during fluoroscopic examinations of patients being treated for tuberculosis. New England Journal of Medicine 321 (1989) 1285

Silverstein, M.J., Poller, D.N., Waisman, J.R., Colburn, W.J., Barth, A., Gierson, ED, Lewinsky, B., Gamagami, P. and Slamon, D.J. Prognostic classification of breast ductal carcinoma-in-situ. Lancet 345 (1995) 1154-1157.

Smith, H.S., Dollbaum, C. and Chen, L.C. Proceedings of the National Academy of Sciences of the United States of America 86 (1989) 7204-7207.

Smith, H.S., Moore, D., Goldman, E.S., Ljung, B.M., Kuriso, W. and Chen, L.C. Journal of the National Cancer Institute 84 (1992) 506-510.

Soder, A.I., Hoare, S.F., Muir, S., Going, J.J., Parkinson, E.K. and Keith, W.N. Amplification, Increased Dosage and In-Situ Expression of the Telomerase RNA gene in Human Cancer. Oncogene 41 (1997) 1013-1021.

Stratton, M.R., Collins, N., Lakhani, S.R. and Sloane, J.P. Loss of heterozygosity in ductal carcinoma in situ of the breast. Journal of Pathology 175 (1995) 195-201.

Trent, J.M., Dalton, W.S., Meltzer, P.S. and Guan, X.Y. Identification of cryptic sites of DNA sequence amplification in human breast cancer by chromosome microdissection. Nature Genetics 8 (1994) 155-161.

Tsuda, H., Fukutomi, T. and Hirohashi, S. Pattern of Gene Alterations in Intraductal Breast Neoplasms Associated with Histological Type and Grade. Advances in Brief 1 (1995) 261-267.

U.K.Trial of Early Detection of Breast Cancer Group: First results on mortality reduction in the UK Trial of Early Detection of breast cancer. Lancet 2 (1988) 411-416.

Veronesi, U., Banfi, A., Valagussa, P., Rossi, A. and Bonadonna, G. The CMF programme for operable breast cancer with positive axillary nodes. Cancer 39 (1977) 2904-2915.

Waldman, F.M. and Kallioniemi, A. Detection and mapping of amplified DNA sequences in breast cancer by comparitive genomic hybridisation. Proceedings of the National Academy of Sciences of the United States of America 91 (1994) 2156-2160.

Weinstat-Saslow, D., Merino, M.J., Manrow, R.E., Lawrence, J.A., Bluth, R.F., Wittenbel, K.D., Simpson, J.F., Page, D.L. and Steeg, P.S. Overexpression of cyclin D mRNA distinguishes invasive and in situ breast carcinomas from non-malignant lesions [see comments]. Nature Medicine 1 (1995) 1257-1260.

Weiss, N.S., Daling, J.R. and Malone, K.E. Oral contraceptives in relation to breast cancer. Epidemiology Review 15 (1993) 80

Wenngren, E., Olsson, H., Zhang, Q.X. and Borg, A. Chromosome 1 alterations in breast cancer Alleilic loss on 1p and 1q is related to lymphogenic metastases and poor prognosis. Genes, Chromosomes & Cancer 5 (1992) 311-320.

Willett, W., Veronesi, U., Lippman, M.E. and Harris, J.R. Breast cancer. New England Journal of Medicine 327 (1992) 319-328.

Wiseman, R.W., Barrett, J.C., Cochran, C., Iglehart, J.D., Marks, J.R., Soederkvist, P. and Futreal, P.A. Detections of frequent loss on proximal chromosome 17q in sporadic breast carcinoma using microsatellite length polymorphisms. Cancer Research 52 (1992) 2624-2627.

Zafrani, B. and Dutrillaux, B. Characterisation of chromosome anomalies in human breast cancer. Cancer Genetics & Cytogenetics 49 (1990) 203-217.