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Simone A. Degan

DETECTION OF INAPPROPRIATE CELL PROLIFERATION IN BREAST EPITHELIUM LEADING TO BREAST CANCER

Supervisors:
Dr Anthony C. Woodman
Dr Keith McCarthy
Dr James B. Bristol

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Abstract

Breast cancer is predominantly caused by unrestrained cell proliferation. Proliferation is a complex process mediated by a network of signals that converge to a point called the 'initiation of genome replication' after which either proliferation or cell death could take place. The minichromosome maintenance (MCM) proteins are located at this point and play a pivotal role in regulating DNA replication. The detection of an aberrant level of such proteins can be of use in early breast cancer diagnosis.

The main aim of this thesis was to propose a new system to detect inappropriate cell proliferation in breast epithelium. An *in vitro* model using cancer cell lines was developed to lay the foundation for subsequent studies employing human breast specimens. The application of the *in vitro* findings in breast excisions allowed assessing of the specificity and sensitivity of the biomarkers to ascertain slowly proliferating neoplastic cells. The most striking finding of this study was the abnormal presence of the MCM proteins in tumour compared to normal tissues with a typical pattern of expression unique for the histological classification of the lesion.

The potential of MCM proteins as indicators of cell proliferation defects was further investigated with association studies with Ki-67, Bcl-2 and ER. MCM consistently identified a higher proportion of proliferating cells compared to Ki-67 suggesting that they are interesting markers of the G_1/S-phase. In fact, the MCM proteins start to co-localise in early G_1 whereas Ki-67 is almost absent in this phase. Importantly, MCM proteins could recognise not only the proliferating compartment of the tumour but also those cells with replication potential.

Based on these findings, the novel MCM biomarkers can be helpful in identifying both malignant and potentially malignant breast tissues. This feature can be useful in predicting patients at risk of tumour progression.

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Nomenclature

a.a.	Amino Acids
ALH	Atypical Lobular Hyperplasia
AMV	Avian Myeloblastosis Virus
BLASTn	Basic Local Alignment Search Tools for Nucleotide
BLAT	Basic Local Alignment Tool
bp	Base Pairs
BSA	Bovine Serum Albumin
CDK	Cyclin Dependent Kinase
cDNA	Complementary DNA
CHAPS	3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonic acid
CKI	Cyclin-dependent Kinases Inhibitors
DAB	3,3'-Diaminobenzidine Tetrahydrochloride
DCIS	Ductal Carcinoma *In Situ*
DFF	DNA Fragmentation Factor
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribose Nucleic Acid
dNTP	Deoxynucleoside Triphosphate
EDTA	Ethyl Diamine Tetetric Acid
EGF	Epidermal Growth Factor
EGFR	Epidermal Growth Factor Receptor
ER	Estrogen Receptor
FCS	Foetal Calf Serum
FNA	Fine Needle Aspiration
FP	Forward Primer
H&E	Haematoxylin and Eosin
IHC	Immunohistochemistry
JNK/SAPK	c-Jun N-terminal protein Kinase/Stress-Activated Protein Kinase
LC	Lobular Carcinoma
LCIS	Lobular Carcinoma *In Situ*

LCM	Laser Capture Microdissection
MAPK	Mitogen-Activated Protein Kinases
MCM	Minichromosome Maintenance Proteins
Mcm	Minichromosome Maintenance Protein (individual component of the complex)
Mdm2	Murine Double Minute 2
$MgCl_2$	Magnesium Chloride
MI	Mitotic Index
mRNA	Messenger Ribonucleic Acid
NaCl	Sodium Chloride
NaOH	Sodium Hydroxide
NCBI	National Centre for Biotechnology Information
NST	No Specific Type
OD	Optical Density
ORC	Origin Recognition Complex
PBS	Phosphate Buffer Saline
PCNA	Proliferating Cell Nuclear Antigen
PCR	Polymerase Chain Reaction
post-RC	Post-Replicative Complex
PR	Progesterone Receptor
pre-RC	Pre-Replicative Complex
Rb	Retinoblastoma susceptibility protein
RNA	Ribonucleic Acid
RP	Reverse Primer
RT	Room Temperature
RT-PCR	Reverse Transcriptase Polymarase Chain Reaction
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
Taq	Bacterium *Thermus Aquaticus*
TBS	Triphosphate Buffer Saline
TG	Tumour Grade
T_m	Melting Temperature
TRIS	Tris[hydroxymethyl]aminomethane
V	Volts
VLI	Visual Labelling Index

Genes are represented in *italics* in the text.

1 Introduction

1.1. BACKGROUND

In 2002 breast cancer accounted for 12.838 deaths among women in the United Kingdom (Cancer Research UK Mortality, 2004). Although there has been a steady fall in the mortality during the last decade, the incidence of breast cancer still remains high, accounting for the highest amount of the total cancer burden (Cancer Research UK Incidence, 2004a; World Health Organization, 1950-1999). The increased incidence of the disease recorded over the past thirty years is one of the major concerns in the healthcare community. However, much of the increase in incidence is explained by increased detection from mammographic screening since the 90's. Indeed, the proportion of women aged ≥ 40 having mammograms has increased significantly since 1987 (Smith *et al.,* 2003). Nonetheless, mammographic screening misses a significant number of cancers and the efficacy of screening has been the subject of much recent debate (Olsen & Gotzsche, 2001; Lynge *et al.,* 2003; Yasmeen *et al.,* 2003; Green & Taplin, 2003). After mammographic detection biopsies are used to establish a conclusive typologic diagnosis. In this context molecular-based techniques could provide more sensitive means of early detection. Ideally a screening test should predict the probability that a malignancy will ultimately develop. Time-consumption is the major constraint of molecular-based techniques applied to breast lesions. This is in part due to the histological heterogeneity of the breast tissue, which makes difficult to obtain reproducible test results from different samples, although this problem can sometimes be alleviated by testing for the expression of additional genes or proteins. This heterogeneity of the breast epithelium limits the possibility of a careful investigation compared with other tumour tissues such as bladder, lung or skin that are themselves more homogenous. In addition, a myriad of uncontrollable factors can be involved in

the progression from normal to neoplastic cell and the cancer employs a complex repertoire of tricks to escape both normal cell growth and cell death. Consequently, there is the need to investigate new molecular tools to increase the reliability in detecting the cancer at its earliest stage and to lower the false-negative rate that is currently about 10% (Shah *et al.,* 2003; Pargaonkar *et al.,* 2003). In clinical practice the pathologist assesses tubule formation, mitotic count and nuclear pleomorphism to assess the histological grade of the tumour. Thereafter, tumour markers are employed *ad hoc* to aid the diagnosis. However, clinicians are well aware that some patients with every indication of cure succumb to the disease and some predicted to have recurrence and to die do not. The final consequence is a difficult, prolonged and in some cases annoying process that requires various tumour markers not only to detect the lesion but also to assess the level of cancer.

1.2. DEFINITION OF THE PROBLEM

Predicting markers are needed to reduce the spread of the cancer to neighbouring tissues, lymph nodes and distant parts of the body. In mammary cancer, however, there are two problems related to the detection and recurrence of the tumour.

The first is the identification of a specific and sensitive biomarker to be routinely employed in the clinical setting. Prognostic factors such as tumour size, tumour grade, histology, hormone status receptor, age, axillary nodal status, ploidy and proliferation index are commonly employed to identify breast cancer. Despite the availability of these indicators high risk patients can not be recognised with adequate accuracy. With the rapid advancement in the understanding of molecular biology of cancer, various biomarkers have been identified as diagnostic and prognostic factors for breast lesions. However, many women have distinctive risk factors for breast cancer like family history, proliferative breast diseases and previous diagnosis of other malignancy that challenge the efficacy of the most known molecular markers. An example is *BRCA1* (breast cancer) gene which encodes a tumour suppressor that induces breast cancer. As a large gene with about 100.000 bp *BRCA1* is liable to mutations, reducing its reliability in developing an accurate diagnostic test (Nowak, 1994). Other examples include Ki-67 and proliferating cell nuclear antigen (PCNA) which are both probes of cell proliferation in tumours. It has been hypothesized that Ki-67 may not always be

necessary for cell proliferation and its expression seems to be influenced by nutritional cell status (Verheijen *et al.,* 1989; Baisch & Gerdes, 1987). On the other hand, the relationship between PCNA expression and cell proliferation in some neoplastic forms is controversial. Further PCNA expression can be influenced by external factors such as fixation time (Hall *et al.,* 1990b; Rowlands *et al.,* 1991).

The second problem is to avoid false-negative and false-positive information that could mislead the clinician. Although immunohistochemistry (IHC) is the most adopted procedure in pathology, the main disadvantages of immunohistochemical assays lie in the variation in the sensitivity of assays between different laboratories and different methods of interpretation of the results (Rhodes *et al.,* 2000).

This thesis is the first attempt to disclose the potentiality and reliability of a set of new biomarkers, belonging to the minichromosome maintenance proteins family (MCM). These biomarkers are analysed using both immunoreactive and gene transcription techniques in different kinds of breast cancers.

1.3. MOLECULAR ASPECTS OF INAPPROPRIATE CELL BIRTH IN BREAST CANCER

1.3.1. Introduction

Cancer is a complex and unpredictable disease. The mechanism that triggers the transformation of a normal cell into a cancer cell is called tumorigenesis. During tumorigenesis cancer cells proliferate because of an imbalance between cell growth and cell death (Corn & El-Deiry, 2002). Molecular advances performed in the last two decades have demonstrated that cancer is the consequence of a series of mutations in the genes that directly control cell origin and cell death (Vogelstein & Kinzler, 1993; Lengauer *et al.,* 1998). The cancer cell appears to be self-sufficient in growth signalling, insensitive to antigrowth signals, unaffected by cellular senescence, resistant to cell death and able to invade and metastasize (Hanahan & Weinberg, 2000). In this scenario the normal cellular functions are incorrectly regulated. Cellular functions can be categorised in cell signalling, cell proliferation, cell differentiation and cell death and are linked in the development of neoplasia. A neoplasm is a clone of cells that differ from other tissues by their autonomous growth and somatic mutations. What would be limiting growth conditions for normal cells are not for neoplastic cells. A number of

these cells acquire further genetic alterations that allow them to ultimately become the predominant subpopulation. Hence, tumour progression is a dynamic process where a series of mutations induces subsequent selection (Kern, 1993).

The intermediate state between normal tissue and neoplasia is called dysplasia or intraepithelial neoplasia (Boone *et al.,* 1999). During this primordial phase of the multistep progression to neoplasm tissues, normal mammalian cells evolve to an atypical organization with changes in cellular and nuclear shape and size. Once more, the fuel that induces this additional and uncontrolled cell proliferation is given by transformations in gene expression profiles (*i.e.* mutations) and epigenetic changes due to transcriptional inactivation (*i.e.* methylation) (Jones, 2003). Therefore, a new challenge in cancer prevention is the identification of new premalignant and early malignant phase molecular markers.

1.3.2. Altered cellular functions in cancer

There are biomolecules in cells that regulate proliferation, differentiation, survival and cell death functions. Failure of the regulation of these steps leads to altered phenotype and consequently to cancer. To comprehend how abnormal cell growth happens, it is necessary to understand how cellular functions are controlled in normal cells and how they are deregulated in tumour cells.

1.3.2.1. Cell signalling and regulation of cell growth

In tumorigenesis a cascade of events guides the outcome of cell proliferation and differentiation. In multi-cellular organisms control of cell proliferation is of paramount importance and is driven by a complex network of signals. Breakdown of these signalling pathways are the driving force of tumorigenesis (Pawson, 1995). The continuum of growth factor stimulation or inhibition can be summarised in endocrine, juxtacrine and intracrine regulation of growth. Endocrine mode of action allows hormones to travel great distances from their site of production sending growth signals to other tissues through the blood vessels. The process of juxtacrine regulation is capable of delivering membrane-bound signals to a receptor of an adjacent cell. In intracrine regulation of growth the factor interacts and delivers the stimuli to its receptor within the cell (Fedi *et al.*, 2000). Thus, from receptors located at surface cell level

signal transduction pathway transmits the stimuli to the nucleus causing changes in gene transcription, protein trafficking, cell adhesion and metabolism (Pawson, 1995).

The most outlined receptors involved in the cell cycle, cell migration, cell metabolism and survival, cell proliferation and differentiation belong to the receptor tyrosine kinase family (RTKs). RTKs are targeted by extracellular growth factors (*i.e.* mitogens) such as epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) (Aaronson, 1991; Schlessinger, 2000). The major point of convergence of the impulses arriving from the RTKs is located in the Ras proteins which activate the cascade of events leading to nuclear signalling (Fedi *et al.,* 2000). The activation of Ras protein consists of very short-lived signals which need to be converted into longer-lasting stimuli capable of reaching the nucleus in order to induce cell proliferation or differentiation (Alberts *et al.,* 1994a).

In the pathogenesis of cancer the best characterised signal transduction module is the RTK→Ras→Raf→Mek→MAPK pathway (reviewed by Salh *et al.,* 1999), as shown in Figure 1.3-1. Mitogen-activated protein kinases (MAPKs) are relevant regulatory proteins that transduce the various extracellular signals into intracellular events and provide the physical link from the cytoplasm to the nucleus (Stadheim & Kucera, 2002). MAPKs comprise extracellular signal-regulated kinase (ERK) and c-Jun N-terminal protein kinase/stress-activated protein kinase (JNK/SAPK) (Figure 1.3-1).

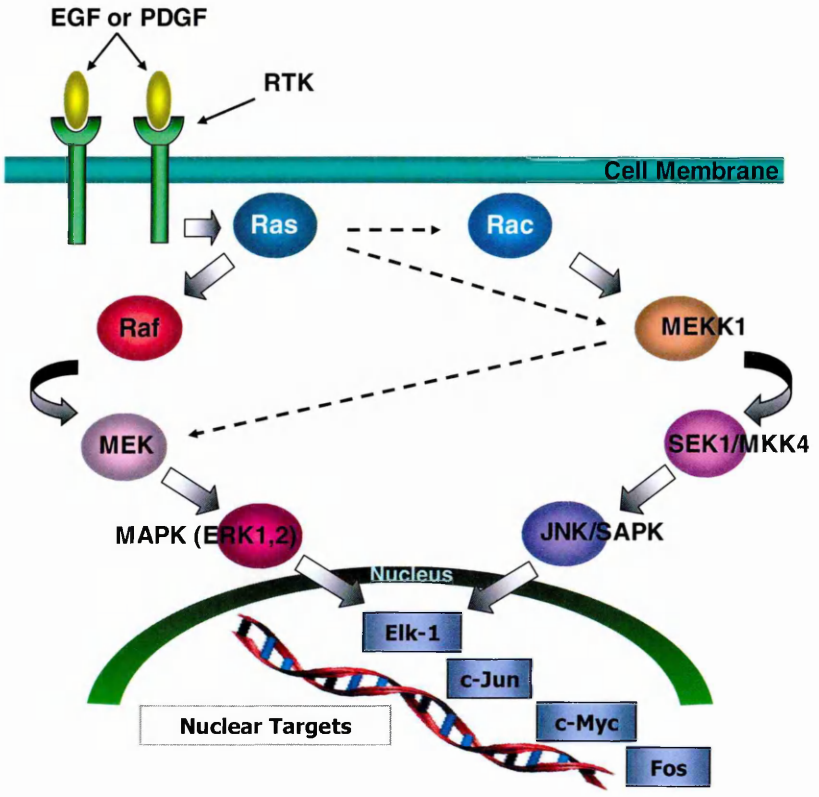


Figure 1.3-1 Inhibition or activation of nuclear targets by different pathways. Overexpression of the ERK cascade induces the activation of transcription factors such as the oncogene c-Myc. The balance between the ERK cascade and JNK/SAPK may be important in regulating cell proliferation or apoptosis (dashed arrows represent cross-talk between the two pathways). Abbreviations: PDGF, platelet-derived growth factor; EGF, epidermal growth factor; RTK, receptor tyrosine kinase family.

These two distinctive signal transduction pathways activate different nuclear targets and show different functions. In many types of cells ERKs, for instance, are associated with cell proliferation, whereas JNK/SAPKs mediate stress and induction of apoptosis (Xia *et al.*, 1995; Le-Niculescu *et al.*, 1999; Stadheim & Kucera, 2002). Moreover, the balance between JNK/SAPK and ERK activities may be important in determining whether a cell survives or undergoes apoptosis (Xia *et al.*, 1995). It has been proposed that disregulation resulting in over-expression of the ERK cascade induces tumorigenesis in breast cancer (Sivaraman *et al.*, 1997; Salh *et al.*, 1999). There is good evidence that the ERK signalling cascade can also mediate a deficiency of oxygen in cells (hypoxia), promoting malignant progression by altering gene expression (Song *et al.*, 2001). Therefore, the recognition of mitogenic pathways and molecular

switches specific for a tumour cell might allow identification of new targets for tumour-specific signal therapy (Liebmann, 2001).

The progression of the cell through the division cycle is consequently quenched when the sum of the total signals that reach the nucleus is sufficient to allow the cell to divide. Once the new born cell is created it starts to proliferate and differentiate with the inherited genetic material of the mother cell.

1.3.2.2. Cell proliferation

The mechanisms that underlie cell proliferation, both in tumour and normal cells, are very similar. However, abnormal cell proliferation, that is the increase in tumour cell number, is a prerequisite in tumorigenesis. A successful cell division sequence implies the orderly and unidirectional switch from one phase to the next phase of the cycle ensuring that each phase occurs only once per cycle. A series of regulatory circuits enforce a sequence of checkpoints (*i.e.* cell cycle transition points). Thus, some events must end before others have initiated. There are two different classes of regulatory circuits, termed intrinsic and extrinsic. The intrinsic circuit is responsible for the specific ordering of the cell cycle events. Extrinsic mechanisms are induced by environmental conditions or when a defect in the cycle is detected (Andreeff *et al.*, 2000; Elledge, 1996).

Cell proliferation is delineated in the cell cycle. In normal autosomal cells the cell cycle consists of four phases: G_1, S, G_2 and M, as depicted in Figure 1.3-2.

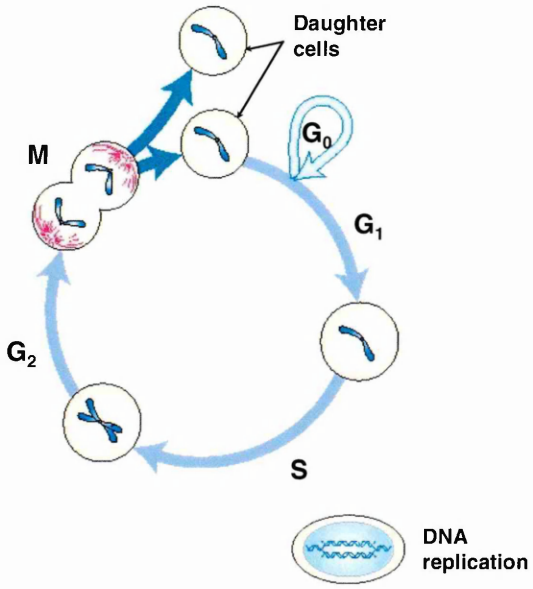


Figure 1.3-2 Events in the cell cycle of proliferating eukaryotic cells.
Non-proliferating cells in vertebrates leave the cell cycle to enter in the latent state (G_0). In proliferating cells G_1 is considered the period following mitosis, signing the birth of the cell. It follows the DNA synthesis phase (S) at the end of which a replicated chromosome consists of two daughter DNA molecules with chromosomal proteins respectively (chromatids). G_2 ends with the onset of mitosis (M) during which the mitotic spindle form spaces out the sister chromatids. Cytokinesis is marked by the division of the cytoplasm and the two daughter cells are created. G_1, S and G_2 are referred to as interphases in most of the non-proliferating cells in vertebrates. Adapted from Lodish *et al.*, 2003b.

In G_1 (gap) protein and RNA synthesis is always in a standby state. During this phase the cell continues to monitor its surroundings and if positive signals from the environment, as well as internal signals, persist to arrive the cell enters irrevocably the latter part of the G_1 phase for subsequent cell division. In the S (synthetic) period new DNA is synthesised. Another gap (*i.e.* G_2) initiates when the newly duplicated chromosomes condense. In the M (mitosis) phase the cell divides and the replicated material is then segregated into the two daughter nuclei. Normal non-dividing cells are also capable of resting in a latent state, named G_0. In this quiescent state the cells are either prompted for cell division, when there is the need to maintain or replace tissue, or discontinued when the required growth is completed.

Tumour cells, however, present aberrations that lead to inactivation of these cell cycle checkpoints transforming them into malignant phenotypes that proliferate without

control. These cells apparently divide *ad infinitum* and seem to have limitless replicative life span (Campisi, 2000).

Molecular defects that induce atypical cell proliferation can occur at various levels. This is the case in the over-expression of the epidermal growth factor receptor (EGFR) that is associated with a variety of carcinomas, including breast (Tang *et al.,* 2000; reviewed by Lu *et al.,* 2001). Alternatively, limiting growth factors may not be required because tumour cells produce their own or mutations that alter the signalling mechanisms may bypass growth factor dependence (Andreeff *et al.,* 2000). In addition, altered forms of normal cellular genes (proto-oncogenes) and inactivated forms of genes that normally restrain growth and induce apoptosis (tumour-suppressor genes) can activate spontaneous growth (Slamon *et al.*, 1984; Lodish *et al.*, 2003a).

The most important checkpoint defects in tumour cells include: (i) inappropriate cyclins regulation, (ii) aberrations of the retinoblastoma (*Rb*) tumour-suppressor gene, (iii) mutations of the *p53* gene and (iv) amplification and/or over-expression of the mouse double minutes (*Mdm2*) gene.

(i) The cell cycle passages are driven by the previously mentioned kinases complexes. These are intracellular enzymes formed by catalytic subunits, the cyclin-dependent kinases (CDKs), and regulatory subunits, the cyclins (*i.e.* cyclin A, B, D and E), that give specificity (Elledge, 1996; Molinari, 2000). Another level of regulation, also controlled by growth factor signals, is the presence of a protein family called cyclin-dependent kinases inhibitors (CKIs) that block the activation of CDKs. In transformed cells the D- and E-type cyclins (*e.g.* D1, E2) are over-expressed deregulating the control of the cell division cycle and the related chromosomal regions have been reported to be amplified in certain tumour including breast cancer (Bartkova *et al.,* 1994; Hamel & Hanley-Hyde, 1997; Geradts & Ingram, 2000; Payton *et al.*, 2002). Two distinct groups of CKIs have been identified. The first class inhibits multiple CDKs such as $p21^{C1P1}$ (p21), $p27^{KIP1}$ (p27) and $p57^{KIP2}$ (p57). The second class specifically blocks cyclin D1/CDK4 or 6 and includes $p16^{INK4A}$ (p16), $p15^{INK4B}$ (p15), $p18^{INK4C}$ (p18) and $p19^{INK4D}$ (p19) (Coats *et al.*, 1996; Sherr, 1996; reviewed by Sherr & Roberts, 1999) (Figure 1.3-3). Therefore, normal signal growth depends on a finely tuned and extremely regulated balance between classes of inhibitors and inducers. However, genetic mutations that promote unregulated passage from G_1 to S checkpoints

of the cell cycle are oncogenic and changes in only one component of this complex regulatory system is all that is necessary to destabilize normal growth control and set the step for cancer.

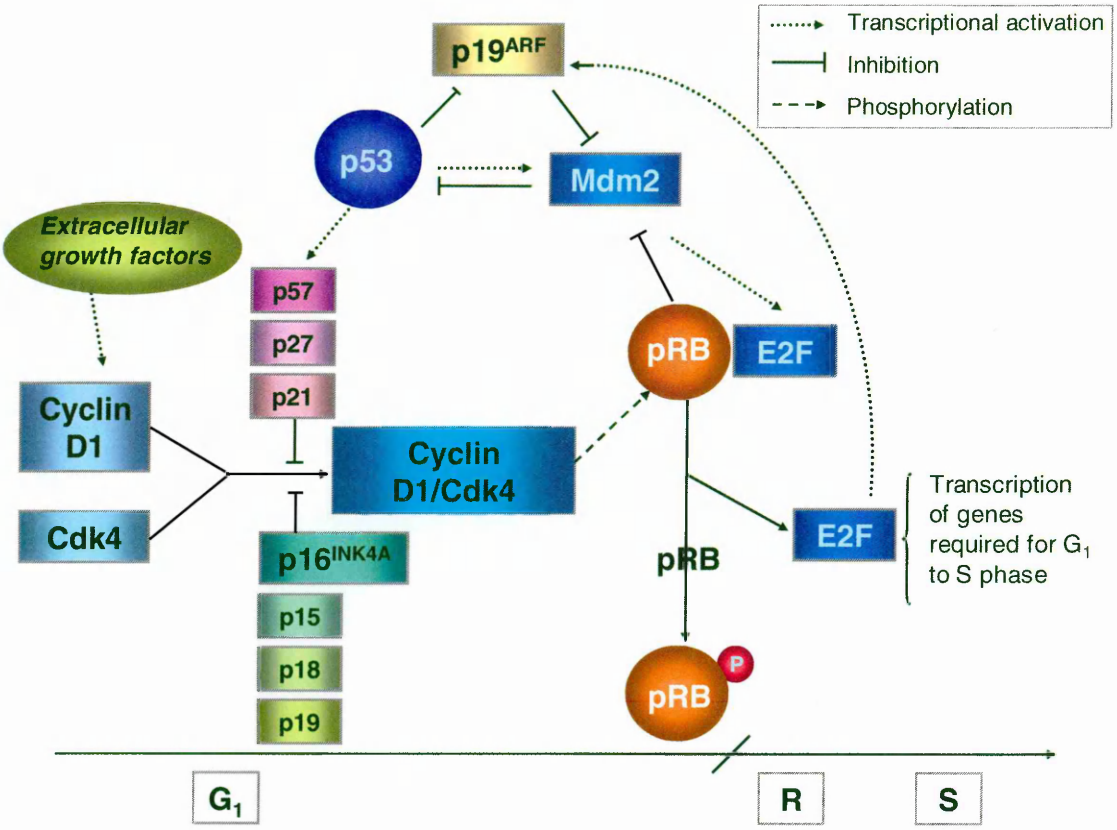


Figure 1.3-3 Positive and negative regulators of cellular proliferation involved in G_1/S progression of the cell cycle. Mitogenic signals promote the assembly of active CDKs containing either CDK4 or CDK6 (not shown). The cyclin D- and E-dependent kinases contribute sequentially to pRb phosphorylation, inhibiting its ability to repress E2F family members and activating genes required for entry into S-phase. Another level of regulation is induced by the CKIs (*e.g.* p21, p27, p57 and p16, p15, p18, p19) that block the activation of CDKs. The tumour suppressor gene *p53* encodes the protein p53 which is involved in growth regulatory processes or, alternatively, promotes apoptosis, differentiation and senescence. p53 also promotes the transcriptional activation of the CKIs. The Mdm2 gene product acts in a feedback loop to limit the levels and action of p53. Abbreviations: Rb (or pRb), retinoblastoma protein; Mdm2, mouse double minutes.

(ii) One of the most important regulators involved in cell proliferation is the retinoblastoma-susceptibility gene (*Rb*) located on chromosome 13q14. In normal cells in response to mitogenic signals inactivation of Rb occurs by phosphorylation which causes the release of the transcription factor E2F (Bartek *et al.*, 1997). After Rb phosphorylation the mitogens stimulate the DNA synthesis and cell proliferation takes place. However, how Rb regulates cell proliferation is only superficially clear. Rb

family members include the related proteins p107 and p130. These proteins are thought to recruit the histone deacetylases (HDACs) to the protein E2F, thereby deacetylating the histones causing the repression of gene transcription and consequently the inhibition of the cell cycle progression (reviewed by Mulligan & Jacks, 1998; Narita *et al.,* 2003). The inactivation of Rb, therefore, is a *sine qua non* condition for cell proliferation. On the other hand, being an inactivator Rb has a central role in tumour development due to its role in regulating cell death. Recent studies have shown that tumours need to preserve Rb which in turn antagonizes cell death signalling (Chau &Wang, 2003). In addition, Rb can regulate the apoptotic function of p53 by binding to Mdm2, therefore preventing Mdm2 from targeting p53 for degradation (Hsieh *et al.,* 1999). It has been proposed that the Rb-E2F pathway can also regulate the human BRCA1 expression because of the presence in the *BRCA1* promoter of a conserved E2F region which is similarly regulated by E2F1 (a member of the E2F family) and Rb (Wang *et al.,* 2000). Rb mutations have been found in a variety of osteosarcomas, small cell carcinoma of the lung, in carcinoma of the breast, bladder, prostate, kidney, liver and leukaemia (Marsh *et al.,* 1998; Andreeff *et al.,* 2000).

(iii) The transcription factor *p53* is the most frequently mutated gene in human cancer. More than 50% of cancers including breast, lung and colon contain mutation of *p53* gene (Bartek *et al,* 1990; Prives & Hall, 1999). *p53* plays a pivotal role in the cellular response to an array of stress stimuli such as DNA damage, nucleotide and growth factor depletion, hypoxia, oncogenic activation and temperature shock (Ljungman, 2000). Aberrations of this tumour-suppressor gene result in loss of function that directly contributes to the altered phenotype of cancer cells. Loss of function of p53 occurs as a consequence of missense mutations and allelic loss. These traits may occur in either the early genesis of cancer or in the multistep progression of cancer (Prives & Hall, 1999). In general, there are two copies of all genes on autosomes such that the defect in one allele may be masked by the wild type allele and both copies of tumour-suppressor genes must be deactivated in order to trigger tumorigenesis (Beaudet *et al,* 1998). However, p53 is an exception because its functional configuration is tetrameric and one aberrant allele is sufficient for the loss of the wild type function (reviewed by Blagosklonny, 1997). The downstream targets of p53 are inappropriately regulated with consequent loss of their normal functions. For instance, loss of p53 can

deactivate cell death, arrest growth arrest in G_1 and/or control genomic instability (Lewin, 2000a).

(iv) Mdm2 is a cellular oncoprotein that has different effects on p53. The Mdm2 gene product acts in a feedback loop to limit the levels and action of p53. While the Mdm2 protein regulates the p53 protein at the level of its activity, the p53 protein regulates the *Mdm2* gene at transcriptional level (Wu *et al.,* 1993). Honda *et al.,* (1997) have provided evidence that Mdm2 may serve as an E3 ubiquitin ligase that causes rapid proteosomal degradation of p53. Secondarily, Mdm2 binds to p300 which is a coactivator required by p53 to function as transcription factor, inducing p53 breakdown (Grossman *et al.,* 1988). Interestingly, the ARF tumour-suppressor protein encoded by the INK4A/ARF locus (human $p14^{ARF}$, murine $p19^{ARF}$) is implicated in regulating p53 functions. The $p19^{ARF}$ binds to the p53/Mdm2 complex inhibiting the degradation of p53 by Mdm2. Thus, another cell cycle checkpoint control pathway can be defined within the functional interrelation between $p19^{ARF}$, Mdm2 and p53 and abnormalities of this pathway play an essential role in cell proliferation and neoplastic transformations (Lewin, 2000a; Andreeff *et al.,* 2000; Eymin *et al.,* 2003).

Although cancer cells use the same cell cycle machinery of normal cells, the cell cycle checkpoints in tumour cells are relaxed. This deregulation is the consequence of inappropriate mitogenic signals that converge to the cell cycle checkpoint that control the G_0/G_1 to S phase transition and activate the CDKs and the initiation of genome replication. Influencing the passage of this checkpoint has a major effect on proliferation of both normal and tumour cells affecting the rate of cell division and the growth fraction. Among the variety of genes affecting signal transduction *Rb* and *p53* are the most frequently mutated in human cancers (Andreeff *et al.,* 2000).

1.3.2.3. Cell differentiation

In normal cells growth and differentiation are often reciprocally exclusive. In other words a cell must stop dividing to proceed with differentiation (Lewin, 2000a; Kubista *et al.,* 2002). The majority of tumours show abnormalities at cell differentiation level such as in anaplasia (dedifferentiation). The anaplasia of tumours is characterised by loss of the normal process of differentiation. This feature can provide insights into the aetiology and prognosis of tumours and characterises the level of tumour

malignancy. In adult tissues the dissimilarities in phenotype arise from differences in gene expression rather than in gene content (Alberts *et al.,* 1994b; Corn & El-Deiry, 2002). Although gene expression programmes are instituted during early embryogenesis, they are sequentially altered as the development progresses. Hence, the expression of specific gene products can mark both the proliferation and the stage of cell differentiation, but again, this is dependent on the level of gene expression of that particular gene. In early embryos, for example, a gene expression programme is set to allow the cells to proliferate vigorously. During this phase the cells secrete factors that increase blood supply for nutrient requirement and produce enzymes that are able to degrade the basement membrane (Bouck *et al.,* 1996). Shortly after proliferation differentiation begins. These mechanisms are also present in tumour cells (*i.e.* angiogenesis). In adult organisms mutations or activated parts of embryonic programmes for gene expression or deactivated parts of the adult programme can produce a cell with features of malignant phenotype (reviewed by Andreeff *et al.,* 2000). These concepts are particularly important in cancers. In adults some cells are terminally differentiated which means that they have lost the ability to proliferate. A common trait of malignant cancer cells, however, is their inability to differentiate terminally (Corn & El-Deiry, 2002). Hence, mutations or particular conditions that interfere with differentiation result in an unbalanced proliferation and uncontrolled growth of the tissue.

The malignant phenotype of cancerous cells may arise either from genetic alteration of the sequence in the genome (*i.e.* mutations) or from epigenetic events (Greek for 'upon' genetics) that may change the hereditable state of gene expression without alteration of the genome such as DNA methylation (Rountree *et al.,* 2001; Garinis *et al.,* 2002). In the majority of the cases before differentiation the cells must undergo one or more rounds of DNA replication. Changes in DNA methylation are frequently introduced in DNA replication. DNA methylation in human cells involves addition of a methyl group on the carbon/5' position of the cytosine ring. Dense methylation of promoter regions is associated with both compacted chromatin structure and regulation of gene expression (Ruddon, 2000; Laird, 2003). Gene expression is presumably affected by DNA methylation because the transcriptional regulatory proteins that bind to DNA are different from those that bind to unmethylated DNA. The

result is a transcriptional silencing of the gene (*e.g.* tumour-suppressor genes) with consequent probable neoplastic transformation of the tissue (Zheng *et al.*, 2000; Asada *et al.*, 2003).

As tumour cells grow they can release glycoproteins and other products similar to those of foetal tissues. The detection of these products in body fluids or in serum can aid in diagnosis and follow-up of the patient. In breast cancer examples include estrogen receptor (ER) and α-lactalbumin (reviewed by Andreeff *et al.*, 2000; Forster *et al.*, 2002). These alterations in the serum are particularly important in predicting the relapse of the neoplasm. Yet, the specificity of these markers for a given neoplasm can be poor because increments can also occur with inflammatory and benign conditions or different kind of neoplasms. Hence, it is evident that a greater knowledge of the molecular basis for the control of cellular differentiation will help not only with more accurate tumour diagnosis and prognosis but also to delineate therapies for regulating tumour growth by altering the state of differentiation.

1.3.2.4. Apoptosis

In a healthy organism there is an integrated collection of differentiated cells which maintain the balance between life and death. Some cells are irreplaceable while others are sacrificed after the completion of their functions and others live until they will be replaced by another generation. Cells that do not fulfil their instituted destiny have catastrophic consequences on an organism. Programmed cell death (PCD) or apoptosis (Greek for 'dropping off' or 'falling off') is the complementary mechanism to cell proliferation that ensures homeostasis in the tissues. Apoptosis was first described as a unique form of cell death distinct from necrosis (Whyllie *et al.*, 1971; Whyllie *et al.*, 1980). Apoptosis can be considered the last phase of a cell's destiny and it is estimated that 50 to 70 billion cells perish each day in the average adult because of PCD (Reed, 1999). This process must be very well regulated since defects in the apoptotic machinery will lead to increased cell survival which may contribute to neoplastic cell transformations. Hence, extended cell survival also harbours genetic instability and accumulation of mutations (Andreeff *et al.*, 2000).

The major consequences of PCD on the cells are at morphological (*e.g.* cell shrinkage, membrane blebbing, pyknosis, chromatin condensation) and biochemical

levels (*e.g.* DNA fragmentation into distinct ladders, degradation of cell cycle regulatory protein such as Rb) (Tang & Porter, 1996; reviewed by Kohler *et al.*, 2002). Cell death is the result of the activation of caspases, a family of eight members that have a catalytic cysteine proteinase that cleaves substrates after aspartic acid (asp) residues. Among caspases, caspase-3 is the most commonly required for apoptosis which is upstream activated by caspases-8, as shown in Figure 1.3-4.

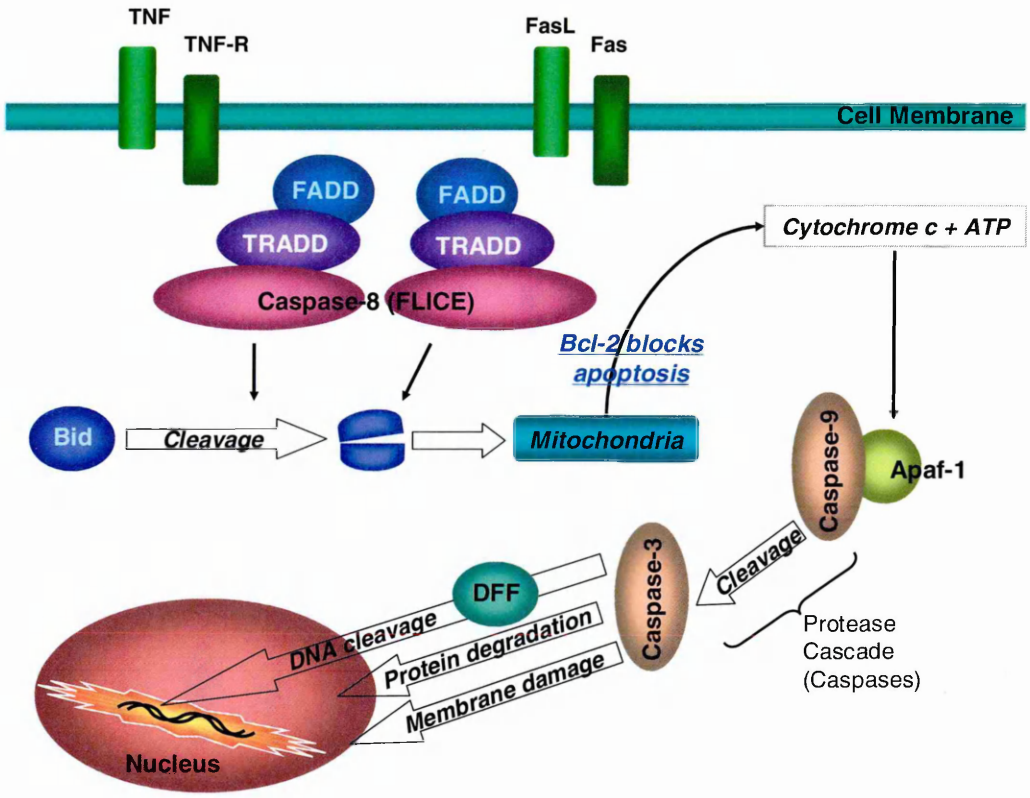


Figure 1.3-4 Schematic representation of the apoptosis pathway.
Apoptosis may be trigged by various stimuli. A common pathway involves the Fas, the receptors TNF and the activation of caspases-8 (FLICE) by oligomerization at an activated surface receptor. Caspase-8 cleaves Bid which triggers the release of cytochrome 'c' from the mitochondria. The cytochrome 'c' causes Apaf-1 to oligomerize with caspases-9. The activated caspases-9 cleaves Pro-caspases-3 whose two subunits form the active protease. Caspase-3 cleaves one subunit of the DFF dimmer. The other subunit activates a nuclease that degrades DNA. The cleavage of these targets leads to cell death. The pathway is inhibited by Bcl-2 at the stage of release of cytochrome 'c'. Bcl-2 protein overcomes apoptosis and its function is to protect the cell from apoptosis (Jacobson *et al.*, 1993; Lewin, 2000b). Abbreviations: TNF, tumour necrosis factor; FLICE, Caspase-8 like interleukin-1 beta converting enzyme; FADD, Fas (TNFRSF6) associated via death domain; DFF, DNA fragmentation factor. Adapted from Wong & Choi, 1997.

When cells fail to undergo apoptosis the escaped cells adversely affect the organism. These cells become resistant to apoptosis, as it is in cancer, creating a dire

situation for the organism. During this process the cancer cells proliferate abnormally, continue irreversibly to evade apoptosis and often dedifferentiate. This abnormal growth is accompanied by a breakdown of normal tissue architecture and invasion into the basal lamina and stromal tissues.

Taken together an intriguing network of genes governs cell proliferation, differentiation and apoptosis. If one of these genes is being silenced, a cross-talk of signals is generated to induce the activation of others genes to accomplish and deliver the initial input instituted in the cell, whether it is a cancer cell or not. This mechanism works similarly in normal and tumour cells and this is the reason why it is difficult to detect in advance inappropriate cell growth and to provide new targets for cancer therapy.

1.3.3. Early cancer detection and diagnosis

Despite the advances in cancer treatments only modest gains in survival rate have occurred in the past decades, particularly for those patients with distant metastasis. Survival is relatively good when cancers are diagnosed at an early stage (Miller *et al,*. 1993; Ratsch *et al.,* 2001; reviewed by Etzioni *et al.,* 2003). For many years public-health organizations have promoted the importance of both mammographic screening and systematic, regular breast self examination for the early detection of breast cancer. However, recent evidence has strongly challenged the validity of both of these screening tests (Olsen & Gotzsche, 2001; Thomas *et al.,* 2002). Despite their usefulness and importance these methods still fall short of providing the best in early detection. Therefore, in order to reduce the growing cancer burden the most promising approach is to investigate new methodologies for the early detection of cancer in order to identify the lesion while it is still confined to the organ of origin (Figure 1.3-5).

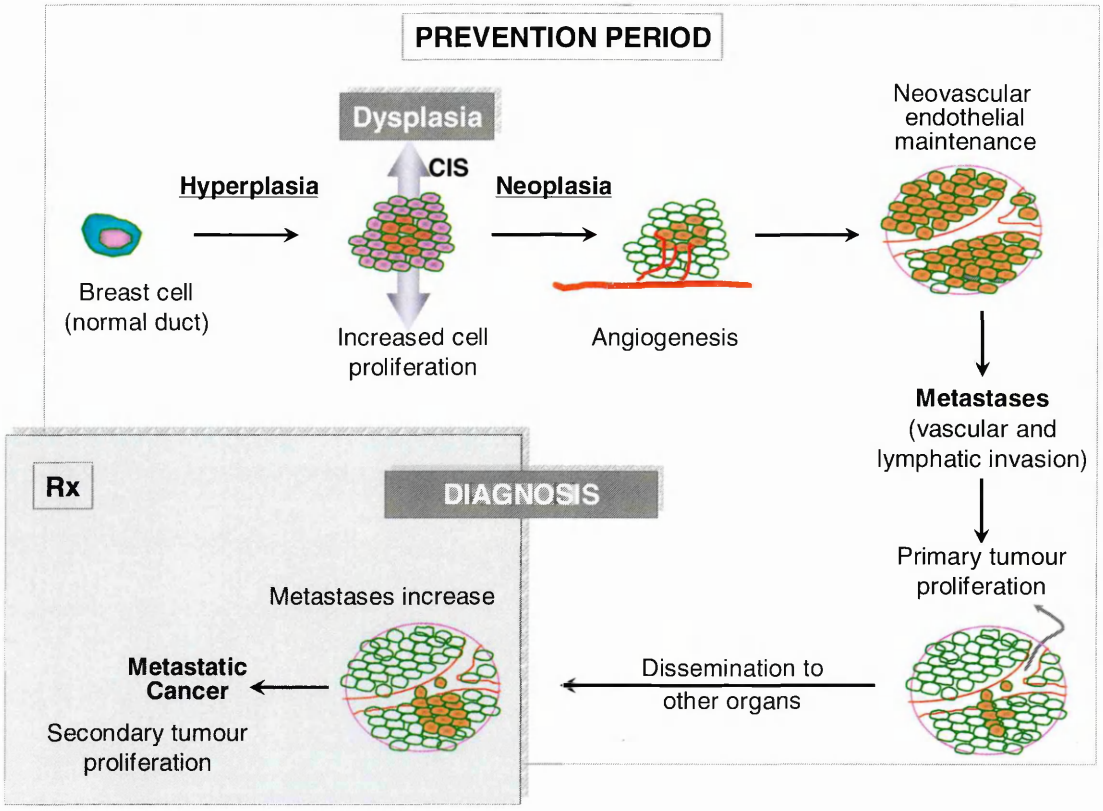


Figure 1.3-5 Temporal progression of breast cancer and stages of tumour development. Diagnosis and treatment of cancer (Rx) occur generally late in the course of disease. At this time, the majority of patients have obvious or occult metastases. Acquisition of the invasive and angiogenic phenotypes occurs very early, in some cancers probably more than 5 to 10 years prior to presentation (Liotta & Khon, 2000). Breast cancers arise from the ductal epithelium of the breast; these lesions are presumptive progenitors of the tumour and can be ordered into a pathway of increasing aberrancy, beginning with hyperplasia and progressing to dysplasia and CIS. Of these, a subset of CIS lesions switches on angiogenesis, telling that angiogenic-CIS is an intermediate stage between CIS and invasive cancer (Hanahan & Folkman, 1996). The tumour cell (metastases) leaves the primary site of tumour travelling to a distant site via the circulatory system causing a violation of normal tissues boundaries (Woodhouse *et al.,* 1997; Liotta & Khon, 2001). A pivotal point in cancer detection lies between hyperplasia and neoplasia, called dysplasia; the dysplastic cells may remain in cell cycle due to deregulation of normal controls over cell proliferation. A common feature to most neoplasms is their rapid proliferation rate compared to normal tissues (Freeman *et al.,* 1999). Abbreviations: CIS, carcinoma *in situ*.

For early detection a screening test should satisfy some practical requirements in order to be effective. The test, for example, should be able to discriminate healthy individuals from cancer cases with a high degree of accuracy (low false-negative and low false-positive rates). The tumour should be detected before the disease progresses to an advanced stage when treatment is less effective, as shown in Figure 1.3-5. The test should distinguish between aggressive and less harmful lesions and, ultimately, it

should be inexpensive and well accepted by the population that is targeted for screening (Etzioni *et al.,* 2003).

The diagnosis, particularly for breast cancer, may be suspected from the medical history. An example includes breast cancer risk assessment for women who plan to participate in an annual mammographic screening programme using the Gain model. This formula estimates the absolute risk and is based on information retrieved from the medical history (*e.g.* age at menarche and menopause, date of the last menstrual period) (Costantino *et al.,* 1999; Armstrong *et al.,* 2000). As previously mentioned, diagnosis also includes physical examination, mammography and more recently ultrasonography which has been suggested to increase the diagnostic yield (Flobbe *et al.,* 2003). However, the result of each of these methodologies ultimately requires confirmation based on the interpretation of histologic tissue sections after biopsy.

Biopsy is the removal of living tissue for histologic diagnosis which is based on cellular and structural features. Unlike cytologic diagnosis (*e.g.* fine-needle aspiration (FNA)), biopsy can diagnose invasion. The most accurate method of histologic diagnosis is the use of stained paraffin-embedded permanent sections to be analysed by light microscopy (Donegan, 2002; Damiani & Eusebi, 2002). This approach is widely applied to investigate the axillary lymph node status which is the most powerful predictor of recurrence and survival in breast cancer (Wong *et al.,* 2002; Choi *et al.,* 2003). More recently, some investigators have proposed that if the sentinel lymph nodes (SLNs) are examined more thoroughly with additional reverse transcriptase polymerase chain reaction (RT-PCR) more micrometastases would be detected and the overall sensitivity of detection would be improved (Kataoka *et al.,* 2000; Ramirez *et al.,* 2001; Branagan *et al.,* 2002). This advance has also been proposed for a less invasive diagnostic technique such as FNA suggesting that the investigation of the fine-needle aspirates in regional melanoma metastases by RT-PCR is a superior method for sensitivity than FNA cytology, particularly when very small or necrotic lesions are examined (Voit *et al.,* 1999).

In conclusion, although advances in diagnostic technology have improved the detection and staging of cancer, the current detection methods present limitations (*e.g.* mammography) (Fong *et al.,* 1999). As will be discussed in this thesis one intriguing approach is the use of multiple diagnostic markers, because single markers typically

lack the sensitivity and the specificity that is required for mass screening (Etzioni *et al.*, 2003).

1.3.4. Biological markers of cell proliferation in breast cancer

The major directions of research in tumour biology are in the overlapping areas of tumour growth and cancer invasion. Tumour growth can be assessed with a number of cell cycle markers, some of which also belong to the category of oncogenes. Yet, tumour growth is induced by a variety of growth factors and hormones and is associated with angiogenesis and the microenvironment of the host tissue (Liotta & Khon, 2001). The cell proliferative activity is a commonly studied parameter to assess tumour status and prognosis. The most important cell proliferation markers investigated in breast cancer are outlined below.

Mitotic index and thymidine labelling index – Mitotic index (MI) represents the number of mitoses per specified number of high-power microscopic fields (hpf), usually 10 fields in routine sections. Mitotic activity index has been reported to be the best single predictor of prognosis, being correlated with a decreasing cancer-specific survival (Baak *et al.*, 1985; Genestie *et al.*, 1998). Analysis of the MI does not require any particular preparation and is frequently found in reports from the pathologists. The mitotic count is also taken into account in the Bloom & Richardson grading system, as modified by Elston & Ellis (1991), to assess, for example, the tumour grade of invasive carcinoma. The thymidine labelling index (TLI) measures the percentage of cells in the S-phase of the cell cycle. The proliferative cell compartment can be investigated by measurement of DNA synthesizing properties through incorporation of the thymidine analogue bromodeoxyuridine (BrdU) in DNA synthesizing (S-phase) cells. Slices of fresh tumour tissue are incubated with tritiated thymidine and the cells synthesising DNA incorporate the radioactive label. The tissue sections are coated with photographic emulsion for one week and developed. Silver grains are found in the emulsion overlying the cells that incorporate the radioactive thymidine. High values of TLI are associated with high MI, poor histologic differentiation and young age (Sugg & Donegan 2002). The TLI has not been adopted in routine analysis mainly because it is a complex procedure. Besides, recent trends in breast cancer detection have limited the availability of fresh breast biopsy tissue. In general, mitotic activity and thymidine

analysis have shown that rapid cell cycle kinetics is an indicator of a poor prognosis (Porter-Jordan & Lippman, 1994).

S-Phase fraction and Ploidy – The synthetic phase fraction (SPF) represents the percentage of total cells in the S-phase and indicates the proliferative activity and aggressive behaviour of the tumour and, hence, bears prognostic significance. The average SPF is about 8%. Higher or lower percentages classify the rate of proliferating tumours (Sugg & Donegan 2002). Using flow cytometry with laser-stimulated fluorescence it is possible to establish whether the DNA content of an individual cell is normal (diploid) compared with the control. It is also possible to discriminate the fraction of cells that are actively synthesizing DNA. In G_0 or in G_1 normal diploid cells possess two matched sets of chromosomes in homologous pairs, thus having two copies of each autosomal genetic locus. Cells with twice the normal DNA content are in either the G_2 or in the early mitotic phase (M), while cells in the S-phase contain intermediate amounts of DNA. In this last case the DNA content in the cell is referred to as aneuploid. Aneuploidy is generally associated with large tumour size with high nuclear grade. This method, however, presents some limitations. As for the TLI, to obtain reliable results this procedure requires fresh frozen tissue samples and the tissues should contain at least 20% of the tumour (reviewed by Sugg & Donegan 2002). Suspensions of diploid and near-diploid tumour cells containing a high content of non-neoplastic cells are not suitable for accurate tumour S-phase calculation and the method needs accurate standardization and quality control during the procedure (Ewers *et al.,* 1992; Wenger *et al.,* 1998).

Ki-67 and **MIB-1** – Ki-67 is a nuclear protein found in all phases of the cell cycle except in G_0 and G_1 (Gerdes *et al.,* 1984). Localization studies have shown that Ki-67 is very dynamic during the cell cycle (reviewed by MacCallum & Hall, 2000). During interphase Ki-67 is located primarily in nucleolar and peri-nucleolar regions and appears to be associated with condensed chromatin. Despite its use in histopathology for assessing the growth fraction of a given cell population, the cellular functions of the Ki-67 protein are still the subject of investigation (Scholzen *et al.,* 2002). The protein appears to be required for cells to progress through the cell cycle and is a constituent of the compact chromatin. An analysis of the nucleic acid and the deduced amino acid showed numerous short sequence stretches that correspond to defined motifs, including

nuclear targeting signals, protein kinase consensus sites and a potential 'adenosine triphosphate/guanosine 5'-triphosphate (ATP/GTP) binding site motif A' (*i.e.* P-loop: phosphate-binding loop, a common motif in ATP- and GTP-binding proteins) (Schluter *et al.,* 1993). In addition, more recent findings showed that the carbon (C) terminus of Ki-67 interacts with the heterochromatin protein 1 family and plays an important role in higher-order chromatin organization and in the establishment and maintenance of heterochromatin domains (Scholzen *et al.,* 2002). The Ki-67 protein also contains a fork-head-associated (FHA) domain (*i.e.* phosphothreonine-binding modules) which is a common feature in several proteins and known to be involved in cell-cycle control. Consequently, Ki-67 protein could interact with phosphorylated proteins through its FHA domain (Pike *et al.,* 2003).

All these considerations suggest that Ki-67 protein is involved in the protein interaction network that drives cell division cycles and plays a role in cellular proliferation. Therefore, the association of Ki-67 expression with the cell cycle and its absence from non-cycling cells and cells undergoing DNA repair have made antibodies to Ki-67 useful tools for the characterization of cellular proliferation in physiological and pathological material.

The percentage of nuclei staining for Ki-67 indicates the proliferative compartment which has been shown to be correlated with the growth fraction of proliferating cells (Brown & Gatter, 1990; Ellis *et al.,* 1996; Ostrowski *et al.,* 2001). A high score of Ki-67 is associated with poor histologic differentiation (poor prognosis) and with lymph node metastasis and inversely correlates with estrogen receptor (ER) status (Gasparini *et al.,* 1991; reviewed by Porter-Jordan & Lippman, 1994). The MIB-1 is a murine monoclonal antibody that reacts with the Ki-67 nuclear antigen expressed by proliferating cells. When limited tumour tissue is available MIB-1 is an alternative to flow cytometry and permits the use of the assay with fixed tissues (Ostrowski *et al.,* 1995; Masood *et al.,* 1998). Opinions about the reliability of Ki-67 as marker of the proliferative activity differ. Some authors suggest that possible staining with antibody (MIB-1/Ki-67) positively correlates with tumour size and proliferation and MIB-1/Ki-67 expression predicts the prognosis (Ostrowski *et al.,* 1995; Keshgegian & Cnaan, 1995; MacGrogan *et al.,* 1997 Ostrowski *et al.,* 2001; Klorin & Keren, 2002). Others suggest that Ki-67 is not essential for cell proliferation, as it is altered by external

factors such as nutrient deprivation, and emphasise the superiority of MI over MIB-1 (Baisch, & Gerdes, 1987; Verheijen *et al.,* 1989; Lehr *et al.,* 1999). Non-proliferating cells may also retain the antigen for a significant period of time, and the expression may be undetectably low at the onset of DNA replication (van Dierendonck *et al.,* 1989). Because of these discrepancies which may partly be due to methodological differences the clinical usage of Ki-67 has remained relatively limited (Freeman *et al.,* 1999).

Proliferating cell nuclear antigen/Cyclin – The PCNA is a proliferation-associated nuclear protein which is mainly expressed during the S-phase of the cell cycle. It appears to play a role in DNA replication possibly as co-factor of DNA polymerase-δ (Bravo *et al.,* 1987). Immunoreactivity of PCNA can be performed in routinely processed tissues providing that uniform standards of fixation length are maintained (Rowlands *et al.,* 1991; Haerslev *et al.,* 1996). Data in the literature indicate that PCNA can be used as an index for the estimation of cell proliferation (Aaltomaa *et al.,* 1992), but in some case heterogeneity in expression may be expected (reviewed by Porter-Jordan & Lippman, 1994). Higher prognostic significance of SPF by flow cytometry and of the histopathological parameters (*i.e.* tumour size and histological grade) compared to PCNA has also been reported (Gasparini *et al.,* 1994; Cummings *et al.,* 1993; Haerslev *et al.,* 1996). In addition, in some forms of neoplasia including breast the relation between PCNA expression and cell proliferation does not seem to be maintained with deregulation or over-expression of the marker probably induced by autocrine growth factor influence on PCNA gene expression (Hall *et al.,* 1990b).

Steroid hormone receptors – Intracellular steroid hormone receptors are represented by estrogen receptor (ER) and progesterone receptor (PR). About 50% to 85% of invasive breast cancers are ER positive. The ER-positive tumours are usually less aggressive and patients with this characteristic have better overall disease-free survival rates than patients with ER-negative. Since ER and/or PR positivity correlates with a higher degree of differentiation and a lower metastatic potential of the cancer (*i.e.* low nuclear grade), there usually is an association with low proliferative indices (*i.e.* low SPF, low TLI, and low Ki-67 expression) (Margolese *et al.,* 2000). The amounts of ER increase with age and the highest levels are found in postmenopausal patients (Sugg & Donegan 2002). Immunohistochemistry (IHC) is the most practical method of measuring ER and is based on visual estimates of the percentage of the cells stained and

the intensity. There is, however, a large variation in the cut-off level (percentage of malignant cells stained) for definition of ER-positive in immunohistochemical assays. The major problem involved in ER analysis in paraffin-embedded tissue is a significant loss of immunoreactivity compared to sections from frozen tissue. This can in part be avoided by modification of the immunohistochemical technique using enzyme pre-treatment and other amplification systems (Andersen, 1992). The estrogen mechanism of action has been studied *in vitro* and *in vivo*. Estrogen binds to the ER which in turn binds to specific enhancer DNA regions and regulates gene transcription (Fishman *et al.,* 1995). A study conducted on human breast cancer cells MCF-7, T47D and ZR-75-1 treated with estrogen pointed out the regulatory effect of estrogen on transcription of the proto-oncogenes *c-myc* and *c-fos* (Davidson *et al.,* 1993). The expression of ER and PR determines the responsiveness of tumours to endocrine therapy (*e.g.* Tamoxifen that is an anti-estrogen), and the absence of these receptors is usually a sign of poor prognosis (Yaghan *et al.,* 1998). In a recent study it has been reported that taking into account the PR status along with ER status improves prediction of benefit from endocrine treatment among patients with primary breast cancer (Bardou *et al.,* 2003).

HER-2/*neu* (c-erbB-2) – The HER2/*neu* oncogene (also known as c-erbB-2) is located on chromosome 17q. It encodes the HER2/neu protein ($p185^{HER2}$) which is an 185kDa transmembrane glycoprotein with tyrosine kinase activity and a member of the EGFR family (Akiyama *et al.,* 1986; Schecter *et al.,* 1984; Gullick, 1990). The amplification of the gene results in over-expression of the receptor. Gene amplification and protein over-expression have been associated with DNA aneuploidy, high cell proliferation rate, negative assays for nuclear protein receptors for estrogen and progesterone, p53 mutation, topoisomerase IIα amplification and alterations in a variety of other molecular biomarkers of breast cancer invasiveness and metastasis (reviewed by Ross *et al.,* 2003). The protein receptor is measured by IHC whereas gene over-expression is detected by fluorescence *in situ* hybridization (FISH). Differently to most IHC assays, the assessment of HER-2/*neu* status is semiquantitative. HER-2/*neu* is expressed in all breast epithelial cells (Ross *et al.,* 2003). About one third of breast cancer cases over-express HER-2/*neu* (Sugg & Donegan 2002). Over-expression is mostly associated with *in situ* carcinoma, especially comedo type (Bose *et al.,* 1996). The frequency of HER-2/*neu* amplification seems to be highly correlated with tumour

grade and ductal versus lobular status; when present in lobular cancer it is a significant adverse prognostic factor (Rosenthal *et al.,* 2002). Stark *et al.* (2000) also reported a greater risk of developing invasive breast cancer for patients with low level of HER-2/*neu* over-expression in benign breast disease biopsies. More intriguingly, it has been proposed that HER-2/*neu* plays an important role in disease initiation rather than progression of ductal carcinomas (Allred *et al.,* 1992).

Epidermal growth factor receptor – As previously mentioned EGFR is associated with a variety of carcinomas, including breast. EGFR is a large transmembrane tyrosine kinase cell surface receptor that binds EGF and the transforming growth factor-alpha (TGFα). Over-expression or over-stimulation of EGFR results in increased cell proliferation (Sugg & Donegan 2002). Studies of EGFR have been restricted by the need for an antibody that recognizes the receptor tyrosine kinase, not the carbohydrate component of the receptor. This is because tumour cells frequently develop altered glycosylation that may interfere with the measure of the receptor (reviewed by Porter-Jordan & Lippman, 1994). Correlation between EGRF expression and its value as prognostic factor has been a matter of debate in breast cancer. Some studies report its clinical significance (Schroeder *et al.,* 1997; Tsutsui *et al.,* 2002) whereas others report its limited value as prognostic indicator (Noguchi *et al.,* 1994; Ferrero *et al.,* 2001).

p53 and **Bcl-2** – The *p53* tumour-suppressor gene is located on chromosome arm 17p13 and is a negative regulator of cell proliferation and its functions have been previously discussed. From the clinical point of view it is strongly associated with other indicators of high tumour proliferation rate such as poor nuclear grade, HER-2/neu protein over-expression, aneuploidy and high SPF and ER negativity (reviewed by Sugg & Donegan 2002). The p53 protein is detected by IHC on 36% to 46% of clinical breast cancers (reviewed by Porter-Jordan & Lippman, 1994). The major problem, however, for assays of p53 protein expression is the lack of uniformity in the method used. It is difficult to recommend the incorporation of this marker in routine clinical use (Isaacs *et al.,* 2001). The Bcl-2 protein functions as blocker of apoptosis. Over-expression of Bcl-2 has been associated with breast carcinoma and other tumours. The immunoreactivity of Bcl-2 is normally restricted in the cytoplasm of the breast cancer cells. Bcl-2 expression in invasive breast carcinomas is associated with good prognostic

markers (*i.e.* low tumour grade, low tumour proliferative activity, p53-immunonegativity and ER-immunopositivity) (Yang *et al.,* 1999; Ioachim *et al.,* 2000). Similar results on FNA have been found by IHC on 130 patients from primary breast carcinomas (Bozzetti *et al.,* 1999). Loss of Bcl-2 protein is generally correlated with high proliferation rate (van Slooten *et al.,* 1988; Rehman *et al.,* 2000; Siziopikou & Schnitt 2000). This negative correlation indicates that Bcl-2 does not proliferate cells, but seems to prolong the life span of epithelial cells allowing proliferation, differentiation and morphogenesis to proceed (Lu *et al.,* 1996; Strasser *et al.,* 1997). In addition, Uhlmann *et al.* (1996) reported that a deletion of a non-conserved region of human Bcl-2 permits continued cell proliferation. In contrast, Mandal & Kumar (1996) demonstrated that over-expression of Bcl-2 resulted in protection of MCF-7 (a breast cancer cell line) cells from the growth-inhibitory effects of butyrate (a potent growth inhibitor). In conclusion, low Bcl-2 protein expression correlates with high proliferation rate, although it may also be involved mechanistically on cellular proliferation. Hence, it is becoming increasingly evident that some genes needed for proliferation and transformation may play a double role in cell growth and cell death.

1.4. MINICHROMOSOME MAINTENANCE PROTEINS AS BIOLOGICAL MARKERS OF CANCER

1.4.1. Introduction

As discussed above, eukaryotic cell transformations rely on stimulation by mitogens through an intricate network of signals that are transferred from the outer cell space to the cell membrane and to the nucleus. These signalling pathways can control cell proliferation, quiescence, differentiation, cellular senescence and PCD, and they converge to a point that is the initiation of genome replication. The core of this convergence point is the assembly of pre-replicative complexes (pre-RCs) resulting in chromatin being 'licensed' for DNA replication in the subsequent S-phase of the cell cycle (reviewed by Ritzi & Knippers, 2000). DNA replication is the primary event that regulates cellular and viral proliferation and failure of mammalian cells to regulate their proliferation cycle leads to cancer. In fact, amplification of genes by over-replication of some regions of DNA is one of the critical mechanisms by which cancer cells become resistant to drug therapy. Yet, the early phase of DNA replication is one of the

fundamental steps in the cell cycle for the inheritance of genetic material and any mutations occurring at this phase will inevitably be transferred to the new daughter cell. Mutations are the primary genetic alterations in cancer progression and are a result of DNA replication. Moreover, DNA replication is the consequence of a number of signals that reflect parameters such as cell size, nutritional status, cell-cell communication and DNA damage (Kearsey & Labib, 1998). In order to avoid over-replication the initiation events must be restricted to only once in a single division cycle ensuring that exactly two copies of each segment of chromosomal DNA are produced. Hence, the hypothesis of this thesis is based on the analysis of (i) certain components of the DNA replication machinery, specifically the minichromosome maintenance proteins (MCM) which are directly involved in cell proliferation, and (ii) the level of MCM expression which determines the proliferative status of the cell.

1.4.2. Regulation of genome duplication in eukaryotic cells

Eukaryotic cells have developed elaborate regulatory fail-safe mechanisms to ensure that DNA replication occurs only once in each cell cycle. The transmission of genetic information from one cell to another requires the precise duplication of the DNA during the S-phase and the faithful segregation of the resultant sister chromatids during mitosis. In the majority of eukaryotic cells these two actions are normally dependent on each other. Hence, the replication of the genome and mitosis occur in alternative, oscillating cycles (Stillman, 1996). Research on eukaryotic replication initiation has its foundation in the study of yeasts. This is why most of the components of genome initiation have names that tell their yeast background. This is the case of the Cdc6 protein, which was discovered in yeast mutants defective in the cell division cycle and the MCM proteins which, when mutated, are defective in circular minichromosome maintenance (*i.e.* mitotically stable plasmid that contains a chromosomal origin of replication) (Ritzi & Knippers, 2000; Maine *et al.,* 1984). There is evidence that the counterparts of the yeast genes are evolutionarily conserved among all eukaryotes and the basic mechanisms of replication initiation are very similar in all eukaryotic cells (reviewed by Tye, 1999). In multicellular organisms, however, the regulation of cell division requires a further level of complexity because cells divide not only to reproduce themselves but also to ensure the correct formation of tissues and organs, that

necessitate differential regulation of growth. Most of the cells in mammalian tissues exit the cell division cycle to enter into different 'out-of-cycle' states (Stoeber *et al.,* 2001). In fact, normal somatic cells have a limited replicative lifespan both *in vitro* and *in vivo* and can undergo only a finite number of cell divisions after which they enter into a senescent state (Andreeff *et al.,* 2000). In this senescence phase cells remain viable indefinitely although they fail to initiate DNA replication in response to mitogens and this mechanism protects higher eukaryotes (*e.g.* mammals) from developing cancer (Campisi, 2000). The way that mammalian cells re-gain proliferative ability and re-enter the cell division cycle is largely unknown. However, one answer may be in the regulation of genome replication. Every proliferating human cell has to copy accurately the same information in the space of a few hours during the cell cycle. Eukaryotic chromosomes, however, are too large to replicate from a single origin (*i.e.* replication start site). Thus they contain multiple origins, each responsible for only the replication of a relatively small part of the genome (Diffley, 2001). The replication proceeds through two replicative forks that move in opposite directions from multiple chromosomal sites, the origins. The number of origins in a eukaryote like yeast is about four hundred whereas in metazoans they may be tens of thousands (Toone *et al.,* 1997). During S-phase thousands of replication forks are involved in this process which must be extremely well co-ordinated to ensure a low error rate since no section of DNA must be left un-replicated or replicated more than once. This mechanism ensures that the correct gene copy number is maintained as cells proliferate. Any defect in this regulatory system may harbour inappropriate cellular proliferation and, if so, the incorrect information is inevitably propagated to the next generation of cells.

1.4.2.1. Initiation of DNA synthesis requires the participation of various proteins

Depending on the genome size the rate of DNA synthesis is regulated by the activation of an appropriate number of initiation events that are relative to the rate of cell division and developmental stage. The proposed model for chromosome replication in eukaryotes has been illustrated with experiments on fusion cells. The chromatin structure of DNA replication origins oscillates between a pre-RC in G_1 phase and post-RC in S, G_2, and M phases (Santocanale & Diffley, 1996). The pre-RC is inherited with the chromosomes into the two daughter cells. However, G_1 cells do not have the S-

phase activator (*e.g.* cyclin-CDK) and initiation of DNA replication does not occur. When the initiation occurs the pre-RC is disrupted and a second state takes place, the post-RC. Both replication complexes contain the ORC (origin recognition complex) which hold six polypeptides that are all essential for cell division and for the initiation of DNA replication. The ORC complex physically interacts with the MCM2-7, a family of six proteins that are the key players in regulating the initiation of DNA replication. In doing so ORC acts as a landing platform for the pre-RC that is formed only at certain stages of the cell cycle (Stillman, 1996). There are numerous other protein factors involved in this regulatory process. Some of these factors have been recently identified to participate in the assembly of the pre-RC. These include Cdc6, Cdt1 and Mcm10. Studies conducted *in vitro* have addressed that Cdc6 is an essential determinant of origin specificity in DNA replication also in metazoan species (Mizushima *et al.,* 2000). Both Cdc6 and Cdt1 are chaperones that facilitate the recruitment of the hexameric MCM complex to all the replication origins that are then licensed to start replication (reviewed by Lei & Tye, 2001). Mcm10 is a replication initiator factor that interacts with MCM2-7 and like ORC it remains associated with the origins during all phases of the cell cycle (reviewed by Tye, 1999; Homesley *et al.,* 2000). Other components are the protein kinase Cdc7-Dbf4 and the Cdc45 component. The Cdc7-Dbf4 complex is recruited to the origin during the G_1 phase and induces phosphorylation of the MCM complex during the subsequent S-phase. The Cdc45 factor is responsible for disassociation of the MCM helicase (*i.e.* enzyme that uses the hydrolysis of ATP to unwind the DNA helix at the replication fork) from Mcm10 and for the loading of DNA polymerase α onto the chromatin (Lei & Tye, 2001; Uchiyama *et al.,* 2001) (Figure 1.4-1).

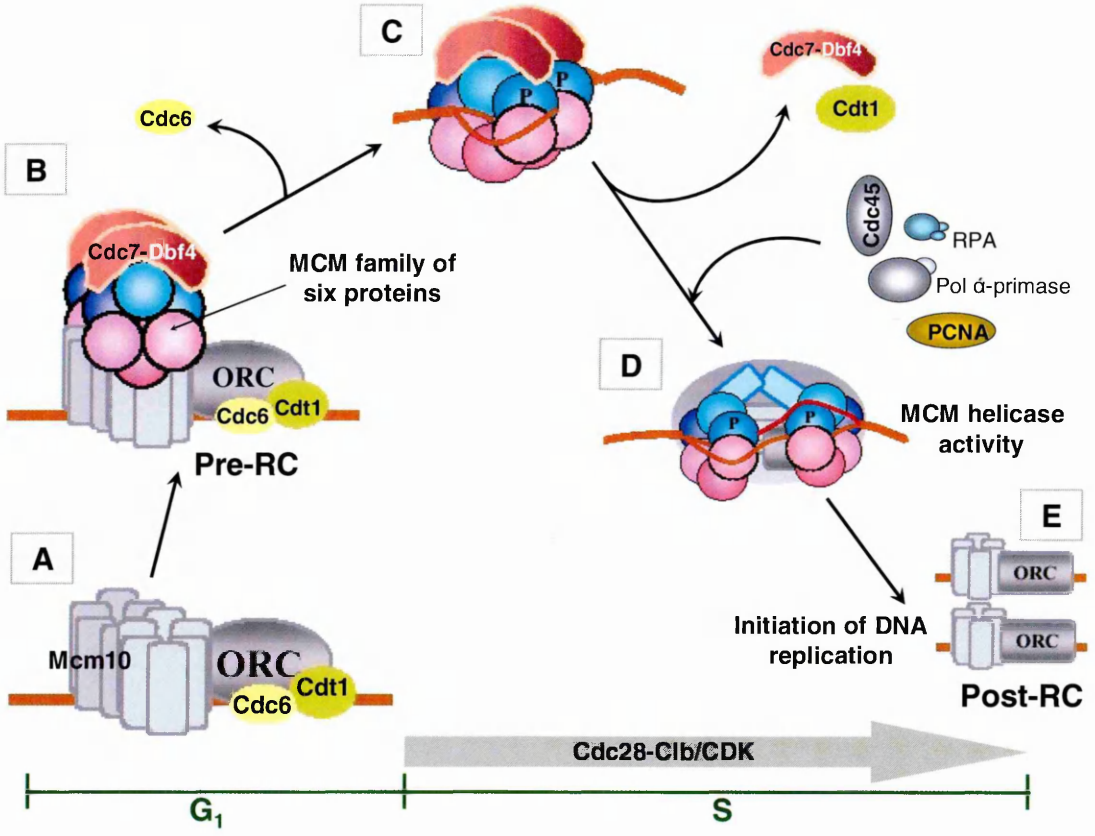


Figure 1.4-1 MCM and the two states for the initiation of DNA synthesis.
(A) ORC acts as a platform, it is required on the DNA in order to recruit Cdc6 and Cdt1 for the assembly of the pre-RC, the first state. Cdc6, Cdt1 and Mcm10 are all loaded to replication origins. (B) Cdc6 and Cdt1 allow the assembly of MCM hexamers on the replication origins. MCM proteins are sequentially assembled into the pre-RC or 'licensing' complex that is essential for the initiation of DNA replication. As soon as the MCM proteins are recruited, Cdc6 is released just before the S phase. MCM protein complex starts gradually to dissociate from chromatin. The gradual release is important in the sequence of events because replicating chromatin that lacks the MCM complex is not competent for DNA replication (Ritzi & Knippers, 2000). (C) In S phase the complex Cdc7-Dbf4 phosphorylates the MCM units and their phosphorylation is controlled locally at individual origins. (D) MCM complex is converted into the enzymatically active helicase and the ring-shaped structure becomes linked to DNA (reviewed by Lei & Tye, 2001). CDKs and the phosphorylation of MCM complex allow the recruitment of Cdc45 which in turn permits the disassociation of the MCM helicase from Mcm10 anchor. The disassociation starts the melting of DNA and the recruitment of RPA, PCNA, DNA polymerase α and primase to the origins for the initiation of the DNA synthesis. (E) ORC conformation changes being induced by the melting of dsDNA before the replication origins assume a post-RC, the second state. After DNA replication the Cdc28-Clb/CDK complexes block the reconstitution of the pre-RC and re-initiation of DNA replication (Lei & Tye, 2001). This may be prevented by the binding of the Clb-CDK complexes either to the Cdc6 proteins, to the ORC or to both (reviewed by Stillman, 1996). Abbreviations: RPA, single-stranded DNA binding replication protein A; PCNA, proliferating nuclear antigen; Cdc, cell division cycle; Clb/CDK, cyclin B/cyclin-dependent kinase; pre-RC, pre-replicative complex; post-RC, post-replicative complex. Adapted from Lei & Tye, 2001.

1.4.3. The MCM protein family

The MCM complex includes six proteins: Mcm2, Mcm3, Mcm4, Mcm5, Mcm6 and Mcm7. A new member isolated from HeLa cells has recently been found. This is the MCM8 protein which deserves further investigation (Johnson *et al.,* 2003). The MCM proteins in higher eukaryotes were originally identified in a murine protein related to the budding yeast *Saccharomyces cerevisiae* called Cdc46/Mcm5 and the isolation of the human P1 protein, homologous to Mcm3 (reviewed by Kearsey & Labib, 1998). MCM are located in the nucleus throughout the cell cycle in the majority of organisms (reviewed by Tye, 1999). Analysis of the Mcm3 nuclear localization sequence (NLS) (*i.e.* a sequence that targets a protein to the nucleus) indicates that the NLS is essential for the functions of Mcm3. Analogue studies on the fission yeast *Schizosaccharomyces pombe* indicated the presence of two NLS in Mcm2 necessary for nuclear targeting of other MCM subunits (Pasion & Forsburg, 1999). There are data indicating that in the normal nucleus they are present in equimolar quantities (*i.e.* 1:1:1:1:1:1) (Tye, 1999; Adachi *et al.,* 1997). Interestingly, Tsuruga *et al.* (1997) found that expression of Mcm2 and 5 was lower than Mcm3 and 7 in the G_0 phase, suggesting that they are not present in stoichiometric amounts throughout the cell cycle. This data indicates that the MCM members may play a distinct role in the regulation of the cell cycle. The six members are the most studied although another two proteins have the same related name: the Mcm10 protein previously discussed and Mcm1. Briefly, Mcm1 is a transcription factor that can act as an activator or a repressor according to the cofactor it interacts with. It is involved in the transcriptional regulation of the expression of some *MCM* genes such as *Mcm7* (Fitch *et al.,* 2003). However, both Mcm10 and Mcm1 have no sequence homology to the MCM2-7 protein family. As illustrated in Figure 1.4-2, a comparison of the amino acid sequence of MCM2-7 reveals that the size of the family members extends from 776 to 1017 amino acids, with Mcm5 the shortest sequence and Mcm6 the largest. There are several conserved regions along the proteins but the largest sequence is sheared in a long central area of approximately 200 amino acids with similarities to a nucleotide-binding fold. This stretch has facilitated the identification of other higher eukaryotic homologues (Kearsey & Labib, 1998; Tye, 1999) (Figure 1.4-2).

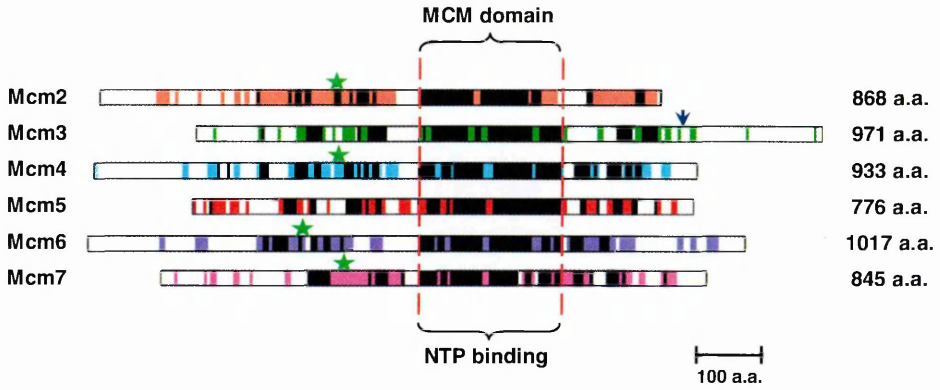


Figure 1.4-2 Features of MCM proteins structure.
Sequence conservation among *S. cerevisiae* MCM proteins of ca. 200 a.a. which is the nucleoside triphosphate-binding motif. Black bars represent regions conserved between *S. cerevisiae* MCMs and the single MCM protein of *Methanobacterium thermoautotrophicum*. Coloured bars represent regions conserved between yeast and mammalian MCMs of the same class. Abbreviations: a.a., amino acids; NTP, nucleoside triphosphate; NLS, nuclear localization sequence for Mcm3 (arrow), not shown for Mcm2; stars represent the zinc-finger-type motif. Adapted from Kearsey & Labib, 1998; Tye & Sawyer, 2000.

For this homology, MCM2-7 family have been identified in all eukaryotes from yeasts to humans, classifying them as a superfamily of proteins with six different classes. In fact, the identification of the six members in higher eukaryotes has been possible because of their evolutionary conservation (*e.g.* cross-reactivity of antibodies between species), their ubiquitous abundance in proliferating cells and their physical association within the group.

1.4.4. Structure and biochemical properties of MCM proteins

According to a work performed by Sherman *et al.* (1998) the MCM complex is formed by the assembly of two distinct subcomplexes, as shown in Figure 1.4-3. Specific roles are emerging for these two subcomplexes and their behaviour appears to be conserved across eukaryotic systems including mouse and human. The first subcomplex contains the Mcm(3+5) proteins and forms a tightly associated dimer that can be easily removed from other MCM. The second subcomplex is made by Mcm(4+6+7) with which Mcm2 is weakly associated (Sherman & Forsburg, 1998; Sherman *et al.*, 1998) (Figure 1.4-3).

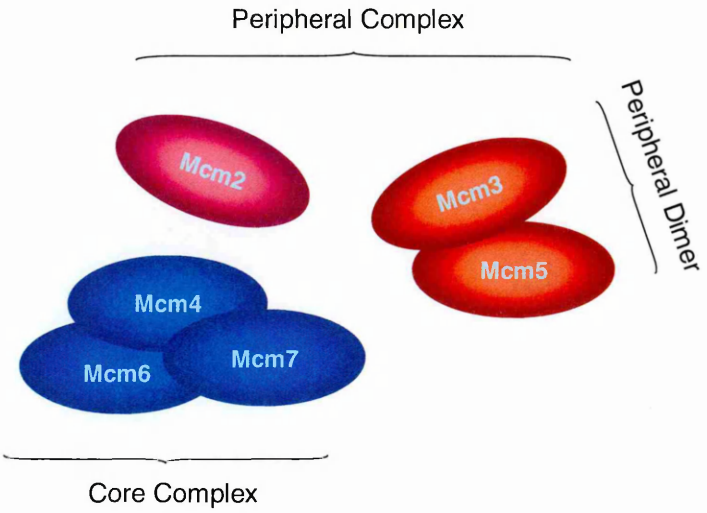


Figure 1.4-3 Proposed model for MCM protein interaction.
The heterohexameric MCM complex appears to be composed by sub-complexes. The core complex is composed by Mcm(4+6+7) which are tightly bound to one another. Mcm(3+5) forms the peripheral dimer and together with Mcm2 is called the peripheral complex. The Mcm2 subunit is not tightly bound to the core, but has a greater affinity than the Mcm(3+5) dimer. Adapted from Sherman *et al.*, 1998.

To shed light on the interactions between members of the MCM family, Ishimi *et al.* (1996) purified the tetrameric complex Mcm(2+4+6+7) from HeLa cell extracts by affinity chromatography based on the electrostatic interactions of the negatively charged Mcm2 and the positively charged histone H3 protein (*i.e.* a protein complexed to DNA in chromatin and chromosomes). However, the Mcm(3+5) peripheral dimer does not show any affinity for the H3 substrate. These experiments suggested that Mcm2 may be released from the core complex Mcm(2+4+6+7) to activate its DNA helicase activity at the onset of DNA replication. Since the interaction between Mcm2 and the histone H3 is strong and specific it may be required in the chromatin binding of MCM protein complexes prior to the initiation of DNA replication or it may be required for the chromatin assembly process during DNA replication. Interestingly, the tetrameric complex Mcm(2+4+6+7) lacks DNA helicase activity in the presence of Mcm2, suggesting that Mcm2 may play a different role in the initiation of DNA replication from the other MCM proteins (Ishimi *et al.*, 1998). This notion may be in agreement

with the results of Maiorano *et al.* (2000) who have speculated that the formation of the active form of the complex Mcm(4+6+7) takes place only after formation of the nuclear membrane, when S-phase CDKs are active and initiation of DNA replication is allowed. In addition, the proteins of this tetrameric complex may act together, probably by virtue of the zinc-finger-type motif they contain which is involved in the structural role of mediating protein-protein interactions.

There are reports suggesting that the helicase activity of the MCM complex is switched on by a structural change in the MCM2-7 complex. From the purification of HeLa cell extracts, the above mentioned trimer of Mcm(4+6+7) was generated. A hexamer containing two trimers forms a ring-shaped structure of about 27 nm in diameter, which could contain central cavity, as shown in Figure 1.4-4.

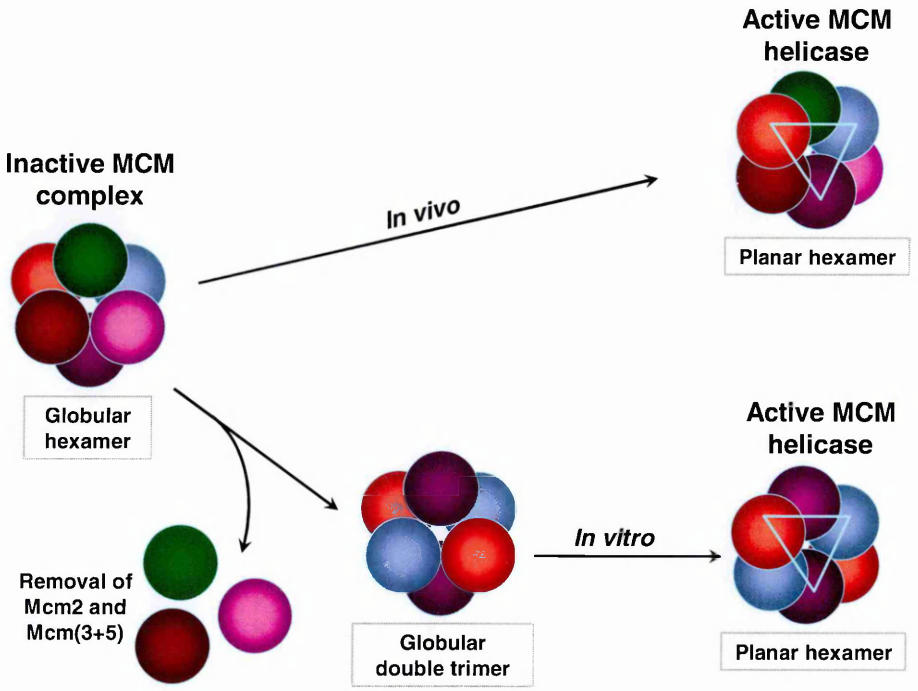


Figure 1.4-4 Proposed pathways associated with MCM helicase activity during DNA replication. Electronic microscope imaging showed that the MCM complex has a globular shape of about 27 nm diameter which could contain central cavity. Two structural models for the hexameric MCM complex have been suggested both involving a conformational change from a globular to a planar structure. *In vivo* helicase activation requires chemical modification of the double trimer as well as phosphorylation of Mcm2 by the complex Cdc7-Dbf4. These modifications lead to the active catalytic core of MCM helicase necessary for the melting of DNA. *In vitro* involves the removal of Mcm2 and Mcm(3+5) from the inactive complex. The subunits removed are replaced by non-catalytic subunits of Mcm(4+6+7) (represented by the triangle). Adapted from Tye, 1999 and Tye & Sawyer, 2000.

This double trimer is able to unwind about 30 base pairs for its helicase and ATPase activity (Tye & Sawyer, 2000). MCM helicase is a hexamer that contains a trimer of Mcm(4+6+7) called the catalytic core which may be involved in the initiation of DNA replication as a DNA unwinding enzyme (Ishimi, 1997). Further, biochemical analysis confirmed the helicase activity of this subgroup and described a possible role of Mcm4 in the single-stranded DNA binding activity of the complex (You *et al.,* 1999). Mcm2 and Mcm(3+5) are the regulatory subunits that negatively regulate the MCM helicase activity. There is evidence that the MCM complex binds to chromatin as a complex and experiments have been conducted both *in vivo* and *in vitro* to create a model for the assembly of the active hexameric MCM helicase from a non active state. *In vivo* investigations suggested that there is no removal of the complex Mcm2 and Mcm(3+5) although the activation still requires the phosphorylation of Mcm2 by the complex Cdc7-Dbf4 and chemical modification of the double trimer (Tye & Sawyer, 2000) (Figure 1.4-4). *In vitro* studies in yeast and human indicated that for the MCM helicase to become active it requires the removal of Mcm2 and Mcm(3+5) from the inactive form, and this is probably due to the phosphorylation of Mcm2 by the complex Cdc7-Dbf4 at the G_1-to-S phase transition (Lei *et al.,* 1997) (Figure 1.4-1). The regulatory subunits removed are replaced by non-catalytic subunits of Mcm(4+6+7). Thus, phosphorylation plays an essential role in the regulation of MCM activities as well as the oligomerization states in the MCM2-7 complex and is essential for the melting of DNA at the origin and the recruitment of Cdc45 and DNA polymerases (reviewed by Lei *et al.,* 2002).

1.4.5. Roles of MCM proteins in DNA replication and cell proliferation

The MCM protein family is essential for assembling the pre-RC at replication origins. However, they seem to be implicated in more roles. Biochemical studies suggest that the complexes convert from an origin assembly factor to a replicative helicase, and that they shift with the replication fork. Other studies also associate the MCM proteins with maintenance of genome integrity and response to damage. In studying cancer they also appear to be directly correlated with cellular proliferation. Their potentially important role in growth regulation of human tissues and their respective tumours is currently a subject of high interest. In the end, these proteins may

be multi-purpose factors that have various roles beyond their essential function in DNA replication. Some of these roles will be discussed below.

1.4.5.1. Requirement for MCM in DNA replication licensing

The MCM2-7 complex is the pivotal component of the pre-RC that primes replication origins for the initiation of DNA synthesis. Once the cluster of MCM proteins is loaded in the early G_1 phase to the chromatin its essential role during the S-phase is to give rise to DNA replication. Thus, the chromatin becomes 'licensed' for replication (Forsburg & Nurse, 1994; Todorov *et al.,* 1994; Chong *et al.,* 1995; Kubota *et al.,* 1995; Aparicio *et al.,* 1997; Tanaka *et al.,* 1997; Kearsey & Labib, 1998; Tye, 1999; Madine *et al.,* 2000; Lei & Tye, 2001). By generating *MCM* mutants a number of studies have tried to clarify the role of MCM proteins. Labib *et al.* (2000) showed that depletion of MCM proteins after initiation irreversibly blocks the progression of the replication forks. Hence, MCM complex is loaded at origins prior to initiation and is indispensable for elongation. Each component of the MCM family is essential for the initiation and elongation of the replication forks and, therefore, become essential for cellular growth. Despite their structural and functional conservation, none of the MCM proteins can substitute for functions of another. The MCM complex can thereafter be considered a complex of six non-identical subunits with different roles inside the family.

The *MCM* genes are expressed in a cell cycle-specific mode and the level of the transcripts reach a peak at M/G_1 or G_1 phase. Consequently this oscillation induces DNA replication once-per-cell-cycle, but this notion is controversial with their abundant and constitutive presence in proliferating cells. A possible explanation that sheds light on these contradictory properties of the MCM family is the possible multiple role of these proteins. According to recent studies it appears that MCM proteins are involved not only in DNA replication but also in other cellular activities including transcription. There is evidence that individual members of the MCM2-7 family may undergo a number of post-translational modifications including polyubiquitination and acetylation. In addition, studies reported that individual members of the MCM2-7 family are also localised at non-origin regions and that these proteins may not act together (reviewed by Fitch *et al.,* 2003). An example is Mcm7 protein which was the first suspected protein

among the group of having capabilities of regulating gene expression. As previously mentioned, Mcm1 is involved in the transcriptional regulation of *Mcm7* gene. Fitch *et al.* (2003) found that a cofactor of Mcm1 in this regulation is Mcm7 which may play a dual role in DNA replication by acting not only as part of the active MCM helicase but also regulating its own expression.

1.4.5.2. Requirement for MCM proteins in cell proliferation

The regulation of pre-RC functions is a powerful mechanism for the control of cell proliferation in tissues of multicellular organisms. MCM proteins play a critical role in this regulation since DNA replication must precede each cell division. Various studies prove their requirement for cell proliferation. The expression of the *DmMcm2* gene in *Drosophila* embryos relates to a pattern of rapidly dividing cells and its inactivation inhibits cellular proliferation. Besides, early embryo development is supported by maternal *MCM* transcripts which are not present when the cells undergo differentiation. The spectrum of proliferation activity, that MCM proteins exert, is in a certain manner conserved throughout different organisms. In yeast the Mcm3 expression in growing cells is high but is down-regulated in starved or quiescent cells. Analysis in mammalian cells presents a similar pattern. The human *Mcm3* gene is likewise low in growth arrested human fibroblasts (reviewed by Tye, 1999). Other works, also on mammalian cells, showed that both the levels of MCM proteins and *MCM* mRNA are low in serum-starved G_0 cells or in differentiate cells (reviewed by Kearsey & Labib 1998). Hence, the implication of the abundance of MCM proteins in cell proliferation is valuable, and their expression may be an indicator of neoplasticity that may be employed in the diagnosis of cancers.

1.4.6. MCM as biomarkers of cells with replication potential

In cancer cells the fundamental growth regulatory mechanisms, that permit cells to withdraw from the proliferative cell cycle into quiescent or differentiated out-of-cycle states, are defective. MCM proteins are among the key factors involved in these mechanisms and the hypothesis to use them in tumour pathology is nowadays gaining significance. Indeed, in non-neoplastic tissues MCM protein expression is normally confined to the proliferative compartment following a pattern similar to other

proliferation markers such as the proliferation-related antigen Ki-67 (Todorov *et al.,* 1998; Stoeber *et al.,* 2001). MCM proteins, however, do not seem to be like conventional proliferation markers which have been valuable in the differential diagnosis of smooth muscle tumour, grading of soft tissue sarcomas and prediction of metastases in thin melanomas, but they have not made a deep impact on tumour pathology and prognosis (Alison *et al.,* 2002). A number of studies indicate the higher expression of MCM proteins detected in tumour tissues compared with Ki-67 antigen (Freeman *et al.,* 1999; Tan *et al.,* 2001; Wharton *et al.,* 2001; Osaki *et al.,* 2002; Ishimi *et al.,* 2003). In particular, Todorov *et al.* (1998) described different cases of breast cancer showing 80% of positive nuclei for Mcm2 and only 10% for Ki-67, while other cases showed 25% immunopositivity for Mcm2 and 20% for Ki-67. The reason for these findings may be found in the heterogeneity of the breast tumours. This heterogeneity may vary according to the number of cells that are actively proliferating (*i.e.* Ki-67 positive and Mcm2 positive) and the cells that are not proliferating but with the potential to exit the out-of-cycle state and start proliferating (*i.e.* Ki-67 negative but Mcm2 positive). Although normal tissue also expresses MCM proteins, their presence is lower than in tumour cells by virtue of the higher proliferation status of the tumour cells (Todorov *et al.,* 1998; Rodins *et al.,* 2002). In fact, terminally differentiated permanent tissues such as neurones myocardial and skeletal muscle cells have the 'license to replicate' withdrawn. Therefore, differentiation and DNA replication licensing seem to be mutually exclusive processes in self-renewing and permanent human tissues. However, their sensitivity is so high that in some normal tissues such as pre-menopausal breast MCM expression is higher than Ki-67 and may identify mammary gland progenitor cells (Stoeber *et al.,* 2001). Such huge variance suggests that MCM proteins may be useful as a marker of dysplasia and may also be used as a pre-cancer marker, detecting those cells licensed to enter the cell cycle that might be missed by conventional immunostaining (Freeman *et al.,* 1999; Kodani *et al.,* 2001). In conclusion, the adoption of the MCM protein family in the pathology diagnosis or cancer management could be helpful in cancer detection and may help to identify those patients most at risk of tumour progression.

1.5. CANCER OF THE BREAST

1.5.1. Introduction

It has been previously mentioned that breast cancer ranks as the most common malignancy affecting women today and the second leading cause of cancer-related deaths in the UK. Despite major advances that have been made in the past two decades in understanding the molecular genetics and the clinical nature of the lesion, the problem still remains and has probably become more complex. This disease has reached epidemic proportions in western countries and it has now been estimated that one in nine women will develop breast cancer in their lifetime in Britain (Cancer Research UK Incidence, 2004b). Because of the magnitude of this problem on public health and the desire to reduce the impact of the disease, breast tumours are among the most studied cancers. Each new discovery or advance has given rise to new questions and further issues to be resolved. However, these advances are the fuel necessary to translate scientific discoveries into clinical practice with the objective of improving detection, treatment and more importantly prevention of breast cancer. It is axiomatic that the application of good scientific information into clinical practice is pivotal in improving cancer management. This thesis is an attempt to understand, assess and translate into practice the scientific importance of MCM proteins as an incremental tool for the clinician to reduce the total cancer burden of the breast among women.

1.5.2. Etiologic factors involved in breast cancer

The causes of breast cancer are complex and involve interactions between environmental and genetic factors. There are many factors that may influence a woman's chance of getting breast cancer. It is the combination of multiple factors and the genetic background that influence the risk of the tumour to develop. These factors can broadly be classified into extrinsic factors, related to exogenous or environmental conditions, and intrinsic factors which are connected to endogenous, physiological and genetic conditions.

1.5.2.1. Extrinsic factors

In this group the incidence of breast cancer varies according to the presence or absence of the following risk factors.

Gender and **age** – The two most prominent factors are being a woman, and getting older. This disease is 100 to 200 times more common in women than in men and this ratio is quite steady all over the world. Most women who get breast cancer in the UK are over the age of fifty. Around 80% of breast cancer occurs in post-menopausal women with a peak at the age of fifty to fifty-four (Cancer Research UK Incidence, 2004b).

Demographic factors and **ethnicity** – Breast cancer more often occurs among women of the upper social classes. The current trend of postponement of motherhood is also more pronounced in urban areas. As a consequence, breast cancer is more common in women with late first birth (thirty years and above) and in spinsters (Rao *et a.l,* 1994; reviewed by Brinton *et al.,* 2002). In addition, breast cancer is more frequent in nulliparous than in parous women, and full-term pregnancy at younger age reduces the incidence of the disease (Kelsey & Horn-Ross, 1993; Rao *et a.l,* 1994; Lambe *et al.,* 1996). Difference in breast cancer stage, treatment and mortality rate varies with race and ethnicity. Different ethnic groups have different incidence of breast cancer, Asian-Pacific women have a lower incidence of breast cancer than women in Northern Europe. Migrant studies indicate that when low-risk groups (*e.g.* China, Japan) migrate to high-risk regions (western countries), their incidence of breast cancer increases to approach that of the native-born population and is affected by the time interval since migration (reviewed by Brinton *et al.,* 2002). The incidence of the disease is also higher among white than black women and environmental and life style components may play a role in this diversity (Gordon, 2003; Li *et al.,* 2003b).

Exogenous hormones – The most important hormones that may play a role in breast cancer are oral contraceptives and menopausal hormones. The association of breast cancer with the first category is not proven, yet. The modern composition of oral contraceptives has changed considerably over the years. A recent population study, however, reports that their use is associated with risk of lobular carcinoma but not ductal carcinoma (Newcomer *et al.,* 2003). Because of the benefits of hormone replacement therapy (HRT), such as a reduced risk of cardiovascular disease and

osteoporosis, this is increasingly used. It has been calculated that the modest increasing risk of breast cancer is outweighed by the benefits of the treatment. However, in a recent study with a million of women, Beral *et al.* (2003) reported that HRT is associated with an increased risk of breast cancer with a higher effect for estrogen-progestogen combinations.

Additional factors that have appeared as risk factors in some but not all studies include dietary factors such as high fat diet, cigarette smoking, scarce physical activity and environmental exposures (*e.g.* chemicals, pollution, toxic waste dumps) (Frazier *et al.,* 2003; Terry & Rohan, 2002; reviewed by Brinton *et al.,* 2002).

1.5.2.2. Intrinsic factors

This group incorporates factors that are less or not controllable by individual women.

Hereditary factors – A family history of breast cancer constitutes the strongest known risk factor for development of the disease. Women with a first-degree relative affected by the disease have approximately a doubling of risk to be afflicted by breast cancer compared to women without such family history (reviewed by Brinton *et al.,* 2002). The family form of the disease, however, accounts for only 5-10% of the total cancer incidence. Non-familial cases are considered sporadic and represent the majority of the incidence (Charpentier & Aldaz, 2002). Because of this fact there have been numerous studies on the identification of cancer susceptibility genes (*i.e.* inherited component) and studies on tumour progression (*i.e.* somatic component). Relevant consideration has received the breast cancer susceptibility genes to prevent cancer development. These genes are the tumour suppressor genes with the function to regulate cell growth and normally suppress tumour development, but when both alleles are mutated or deleted they favour tumour formation. These mutated cancer genes are unique and can be inherited increasing the chance of getting cancer. The best known examples are the breast cancer genes *BRCA1* and *BRCA2*. A woman who has a germline mutation of *BRCA* genes has a lifetime risk of developing breast cancer of 60-80% (Brinton *et al.,* 2002). Other examples include *p53* and the putative tumour-suppressor gene *PTEN* (phosphatase and tensin homology). *p53* is somatically mutated in 25-45% of primary breast carcinomas, and most of the women with mutated form of

PTEN gene have neoplasia and almost half progress to carcinoma of the breast (reviewed by Charpentier & Aldaz, 2002).

Endogenous hormones – There is a strong correlation between the female hormone estrogens and the development of mammary cancer. All the factors that increase a woman's lifetime exposure to estrogen increase cancer incidence. Examples include earlier age at menarche, later age at menopause and postmenopausal obesity. In women and in laboratory experiments ovarian ablation reduces dramatically the risk of breast cancer (Margolese *et al.,* 2000). Endogenous estrogen is required for both proliferative activity and carcinogenesis. Other hormones considered to play a role in breast cancer are prolactin which stimulates cellular proliferation and androgens, a major endogenous precursor of estrogen that may indirectly increase the estrogen levels or cell proliferation (reviewed by Brinton *et al.,* 2002).

Gene-environment interactions – Both genetic and environmental factors are known to be involved in the development of breast cancer. Interactions between the specific genetic makeup and exposure to specific extrinsic factors can cause genotypic polymorphism (*i.e.* allelic variants). The polymorphism's action would confer increased tumour susceptibility. Such interactions would produce increased risks in individuals with the right combination of factors. This explains why some individuals develop cancer, while others do not, after exposure to carcinogens (Charpentier & Aldaz, 2002).

In conclusion, breast cancer aetiology could be explained by a combination of factors such as an inherited predisposition to develop cancer and to accumulate new mutations and extrinsic exposure to carcinogenic agents. This may also explain the etiologic heterogeneity present among women. The identification of the factors involved in tumour development will positively contribute to prevent the disease.

1.5.3. Breast cancer biology

Normal breast growth and development are regulated by a complex interaction of hormones, growth factors and protein kinases. Some hormones and growth factors are secreted by the mammary gland itself and may have autocrine roles; others are produced by stromal cells generating paracrine control on epithelial cells. Among these factors, hormones signals and ratio have a dramatic effect on breast mammary development and

cancer initiation. These hormones include estrogens from the ovaries, progesterone, androgens, glucocorticoids, prolactin, thyroid hormone, insulin, EGF and TGFα. As the normal breast tissue is regulated by these hormones and their receptors, the malignant breast cells also express receptors for many of these hormones as indicated by various investigations both *in vitro* and *in vivo* (reviewed by Margolese *et al.,* 2000).

Among these hormones estrogen is actively involved in carcinogenesis. Estrogen is actually the broad term for any of three similar hormones: estradiol, estrone and estriol. Of these three, estradiol is the most powerful and is present in the greatest amount. Estrogen is a modulator of cellular growth and differentiation, its major target is the mammary gland and it diffuses freely across the cellular membrane. Estrogen exerts its proliferative cellular functions by interacting with ER and induces the expression of many other gene products, including progesterone receptor (PR), through two specific intracellular receptors ERα and ERβ which act as hormone-dependent transcriptional regulators (Osborne *et al.,* 2000). Moreover, estrogens not only act as mitogens but also as a survival factor for ER-positive cells. It has been proposed that estrogen may promote cell survival by regulating the anti-apoptotic Bcl-2 expression which is more often expressed in ER-positive cells (Miller *et al.,* 2002). There is, however, an ER paradox in breast cancer. Despite the role of estrogen in cell proliferation ER and cell proliferation expression (*e.g.* Ki-67) are almost mutually exclusive in the normal human breast (Clarke *et al.,* 1997). Nonetheless, this negative association may be lost in some cases of breast cancers where a variable percentage of proliferating cells are ER-positive (Clarke *et al.,* 1997; Shoker *et al.,* 1999). In addition, ER-positive breast cancer cells are less metastatic compared to ER-negative cells challenging the common opinion that estrogen may be detrimental in mammary cancer (reviewed by Verheul *et al.,* 2000). In the clinical practice ER-positive breast tumours tend to grow more slowly and are usually more differentiated, so that this discrimination is used to select patients that may receive endocrine therapy and to assess patient survival and recurrence (Margolese *et al.,* 2000).

Growth regulation of breast cancer is influenced, therefore, by hormones and growth factors. The balance between growth stimulators and growth inhibitors produced by the tumour indicate the malignant potential of the cancer. As a consequence, this interaction triggers a cascade of intracellular signals (*e.g.* MAPKs)

that activate or repress a number of genes involved in cellular proliferation and/or PCD of both epithelial and mesenchymal cells with the final effect of disturbing the normal control growth mechanisms over uncontrolled cell division.

1.5.3.1. Pathogenesis of breast cancer

The heterogeneous nature of breast cancer is an obstacle in developing a model of progressive genetic changes occurring from normal to cancer cells. In contrast to most of the tissues of the body, the mammary gland does not reach a state of terminal differentiation during the phases of development. In fact, mammary tissue growth is stimulated by specific estrogen cycles. At birth the gland consists only of a primary duct and may secrete the so termed 'witch's milk' induced by the mother's hormones that cross the placenta during pregnancy. Ensuing menarche the gland is subject to various cycles of proliferation, differentiation and regression that relate to reproduction and gestation. Most of these changes are regulated by estrogen and progesterone hormones. Estrogen induces mammary differentiation of the duct system, and progesterone plays a role in lobular development. At puberty in the mammary gland complex hormonal interplay occurs, resulting in growth with elongation and ramification of the ducts and maturation of the adult female breast. Estrogen in this phase plays a critical role. At the onset of sexual maturity the ductal development stops. Following the cyclic hormonal stimuli of the menstrual cycle, however, the end bud epithelial tissue still remain subject to proliferation and PCD (Miller *et al.,* 2002).

Modern molecular biology has identified a number of genes whose products are involved in the regulatory process of breast tissue growth and that are responsible for tumorigenesis if genetic lesions occur (*e.g.* gene amplification, gene deletion, point mutation, loss of heterozygosity, chromosomal rearrangement, aneuploidy). These genes include the breast cancer genes (*i.e. BRCA1* and *BRCA2*), the oncogenes (*e.g.* HER-2/*neu*) and the tumour-suppressor genes (*e.g. p53, Rb*), many of which have already been discussed. The combination of estrogenic hormones, growth factors and the above genes give rise to the malignant phenotype in breast cancer. As family history is a major risk factor in breast cancer, it is worth examining some of the most important genes involved in the emergence and progression of breast cancer including *BRCA*, *PTEN* and the ataxia telangiectasia mutated (*ATM*) genes.

***BRCA1/BRCA2* Genes** – Early linkage studies indicated that *BRCA1* gene is present on chromosome 17 and predisposes individuals to early-onset of breast cancer (Hall *et al,*. 1990a). The locus was termed *BRCA1* and mapped to chromosome region 17q21 (Chamberlain *et al.,* 1993; Miki *et al.,* 1994). Subsequent genetic mapping allowed the identification of another gene linked to breast cancer, the *BRCA2* located on chromosome 13q12-13 (Wooster *et al.,* 1994). More recently a new linkage analysis reported a new candidate, the *BRCA3* locus, linked to breast cancer susceptibility on 128 high-risk breast cancer families with no identified *BRCA1* or *BRCA2* mutations, indicating the existence of more susceptibility loci (Thompson *et al.,* 2002). Yet, the exact functions of *BRCA1* and *BRCA2* remain to be elucidated although their mutations are associated with high incidence of breast cancer. Among the possible functions postulated, both genes may be involved in DNA repair due to the possible association with the DNA repair protein RAD51 (Scully *et al.,* 1997). The gene products BRCA1 and BRCA2 are also possible players in transcription-coupled repair of oxidative DNA damage (Gowen *et al.,* 1998). In addition, BRCA1 may act as an ubiquitin-ligase activity in response to the interactions with the protein BARD1 (BRCA1 associated RING domain 1), exerting the functions of tumour suppressor and coordinating DNA repair, damage and other cellular functions (Wu *et al.,* 1996; Ayi *et al.,* 1998; Chen *et al.,* 2002) (see Figure 1.5-1). As a consequence, mutations of these breast cancer susceptibility genes could lead to missed DNA repair and may also lead to mutations in other genes.

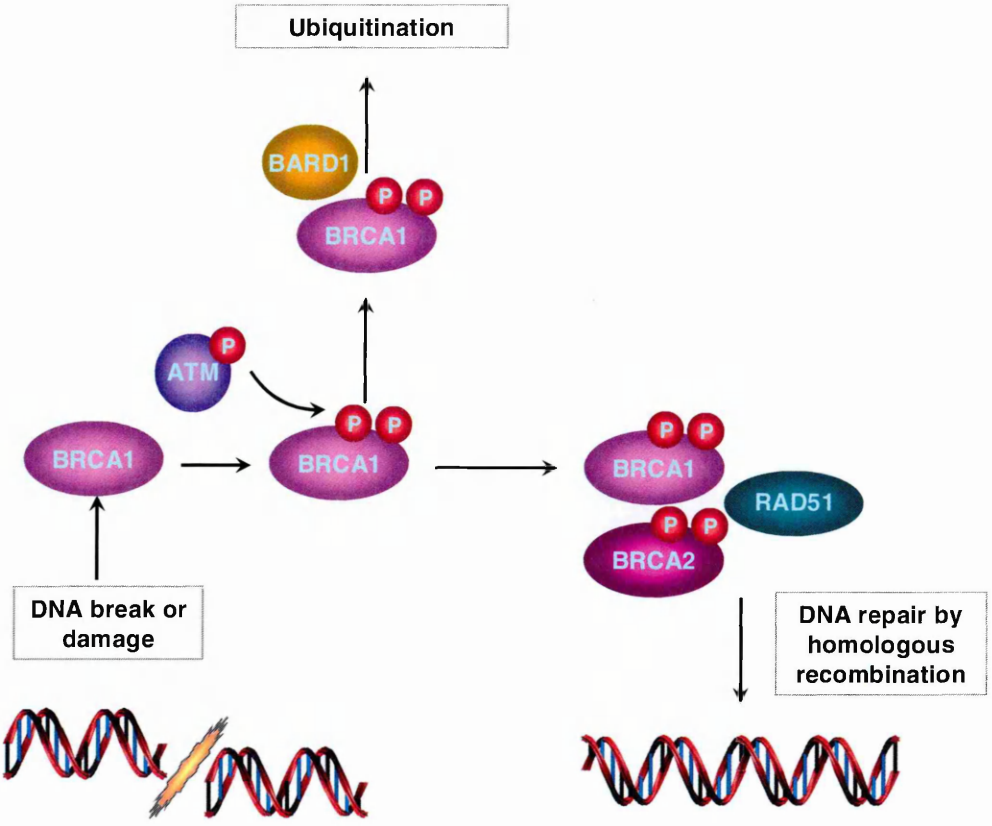


Figure 1.5-1 Possible nuclear functions of BRCA1, BRCA2 and ATM proteins. BRCA1 and BRCA2 may be involved in fixing double strand DNA after damage or breakage. ATM protein is required to phosphorylate BRCA1 which in turn can complex with BRCA2 and the repair protein RAD51, with subsequent activation of DNA repair by homologous recombination (Cortez *et al.*, 1999; Chen *et al.*, 2000). BRCA1 may also interact with BARD1 protein acting as an ubiquitin-ligase (*i.e.* a proteolytic system of eukaryotic cells involved in the selective degradation of misfolded or damaged proteins in the DNA repair process). Ubiquitination defects affect cellular processes such as cell cycle progression, cell differentiation, PCD, the response to DNA damage, DNA repair and transcriptional regulation (reviewed by Brzovic *et al.*, 2003). Abbreviations: BRCA1, associated RING domain 1; ATM, ataxia telangiectasia mutated gene product. Adapted from Studzinski & Harrison, 2002.

***PTEN* Gene** – The putative tumour suppressor gene *PTEN* has been identified on chromosome 10q23 and is responsible for the familial predisposition of Cowden's disease, and breast cancer is a component of this rare syndrome (Liaw *et al,*. 1997). The *PTEN* gene encodes a protein tyrosine phosphatase (*i.e.* an enzyme that removes phosphate groups from tyrosines) and shows homology with tensin (*i.e.* a protein that interacts with actin filaments and links the internal skeleton of the cell with the external surroundings) (Hlobilkova *et al.*, 2000). The most important role attributed to PTEN protein is the negative regulation of cell survival by causing apoptosis and/or G_1 cell cycle arrest by antagonising protein tyrosine kinases (reviewed by Eng, 2002). Studies

with cell lines found that *PTEN* appears to be mutated in human cancers, including breast cancer cell lines, probably regulating cell invasion and metastasis through interactions at focal adhesions (*i.e.* specialised regions of the cell membrane that ensure the extracellular matrix is firmly connected to the actin cytoskeleton) (Li *et al.,* 1997). Although somatic mutations in the *PTEN* gene appear to be quite rare in breast cancers, most of the women with mutations of this gene develop breast neoplasia and nearly half progress to carcinoma (reviewed by Charpentier & Aldaz, 2002).

***ATM* Gene** – Ataxia telangiectasia (AT) is an inherited autosomal recessive disorder characterised by progressive neuronal degeneration, immune defects and predisposition to malignancy including breast cancer. The importance of the mutated form (*ATM* gene) in contributing to breast cancer is a continuing matter of debate. FitzGerald *et al.* (1997) reported that there was no clear association between *ATM* mutations and genetic predisposition to early-onset of breast cancer. Nevertheless, epidemiological studies of AT families showed that AT heterozygote women had an increased risk of breast cancer (five times higher) with estimations that 1% of the population are AT heterozygote (reviewed by Angele & Hall, 2000; reviewed by Khanna, 2000). The *ATM* gene is located at 11q22-23 and the ATM protein product has been shown to play an essential role in various cellular processes such as meiosis, DNA repair, cell cycle check point control and immune development (Xu *et al.,* 1996) (Figure 1.5-1).

In conclusion, breast cancer is the result of complex and heterogeneous combinations of genetic alterations with significant pitfalls that contribute to the development of the malignant clone. It is thereafter difficult to draw a genetic progressive model of the changes associated with breast cancer.

1.5.4. Histopathology of normal and neoplastic breast

The mammary glands are derived from modified sweat glands of ectodermal origin (*i.e.* gives rise to epidermis and neural tissue). In the adult female mammary glands are composed of about fifteen to twenty lobes which are branched tubuloalveolar secretory units. Dense fibrous-connective tissue divides the lobes which are subdivided into multiple lobules located at the deepest end of the duct system, each of which is made up of multiple acini. A loose connective tissue embeds these structures which is

rich in capillaries, whereas the interlobular spaces are sustained by dense fibrocollagenous tissue. Each lobule ends in scores of tiny bulbs and milk originates from them. The milk flows out to the nipple through the ducts (*i.e.* lactiferous ducts). The nipple is an area in the centre of the breast areola made by keratinised stratified squamous epithelium. Before the nipple there is a dilatation: the lactiferous sinus (Figure 1.5-2).

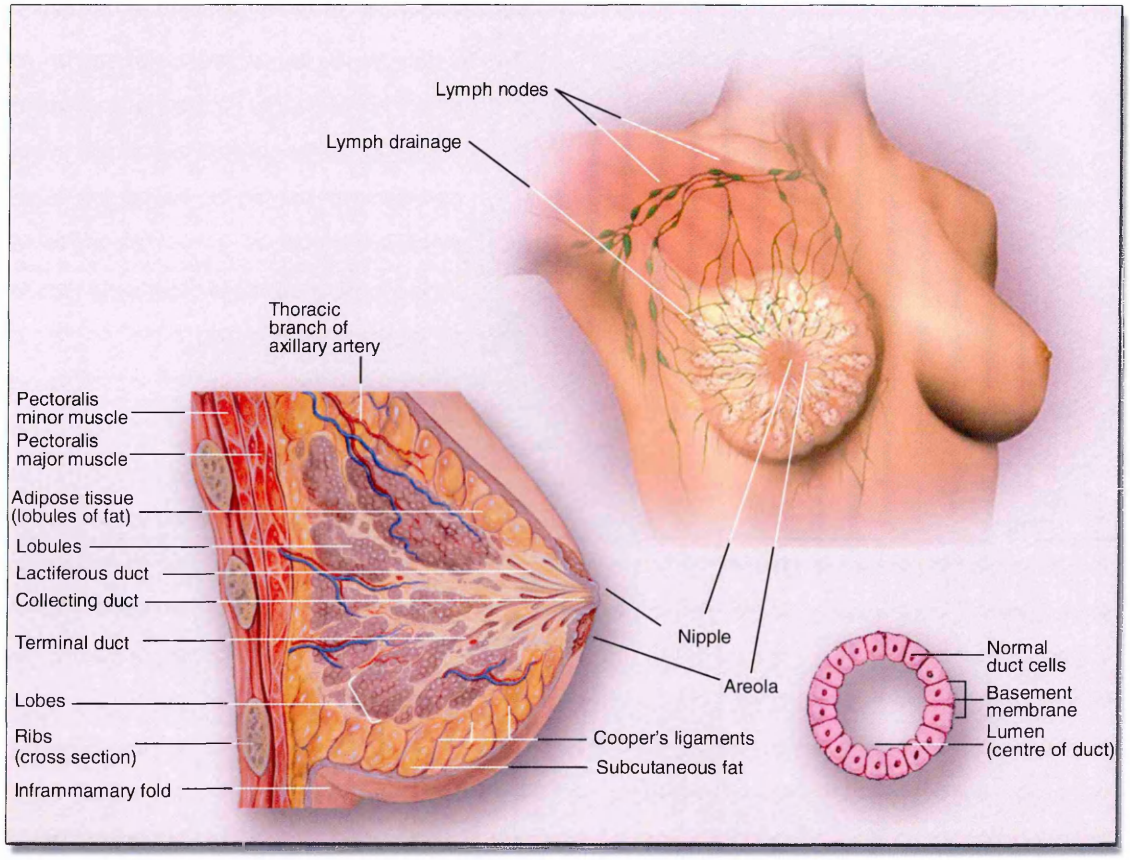


Figure 1.5-2 Normal breast architecture and histology.
The breast is shown from an anterior view (above) and in sagittal section (left). The anterior view shows the mammary ducts radiating out from the nipple and terminating in milk lobules, the sites where the milk is produced. The lymphatic vessels (showed in green) move from the breast through the axillary lymph nodes under the armpit over the collar bone to the thoracic duct. This lymphatic system is the major pathway for the breast to drain toxins. Invasiveness appears when the basement membrane of the ducts or lobules is broken (*e.g.* invasive ductal or lobular carcinomas) and the metastatic cells invade the surroundings migrating by vascular and lymphatic Invasion to distant organs.

The mammary gland architecture indicates the heterogeneity of this organ. Isolated mammary lobules can also be located outside the normal anatomic borders of the breast parenchyma, like in the nipple or in the axilla. An example is the axillary tail of Spence (*i.e.* an extension of the mammary gland along the inferolateral edge of the

pectoralis major toward the axilla) which may explain the presence of some primary breast cancers in the axillary region.

1.5.4.1. Preinvasive and hyperplastic breast lesions

Preinvasive breast lesions are neoplastic proliferations confined to the mammary structures of the gland (*i.e.* ducts, lobules). As with the pathogenesis of breast cancer, the histological stages of breast cancer have been difficult to identify. These represent progressive important changes starting with ductal hyperproliferation, progressing into *in situ* and subsequently into invasive carcinoma, and culminating in metastatic lesions (reviewed by Polyak, 2002) (Figure 1.5-3).

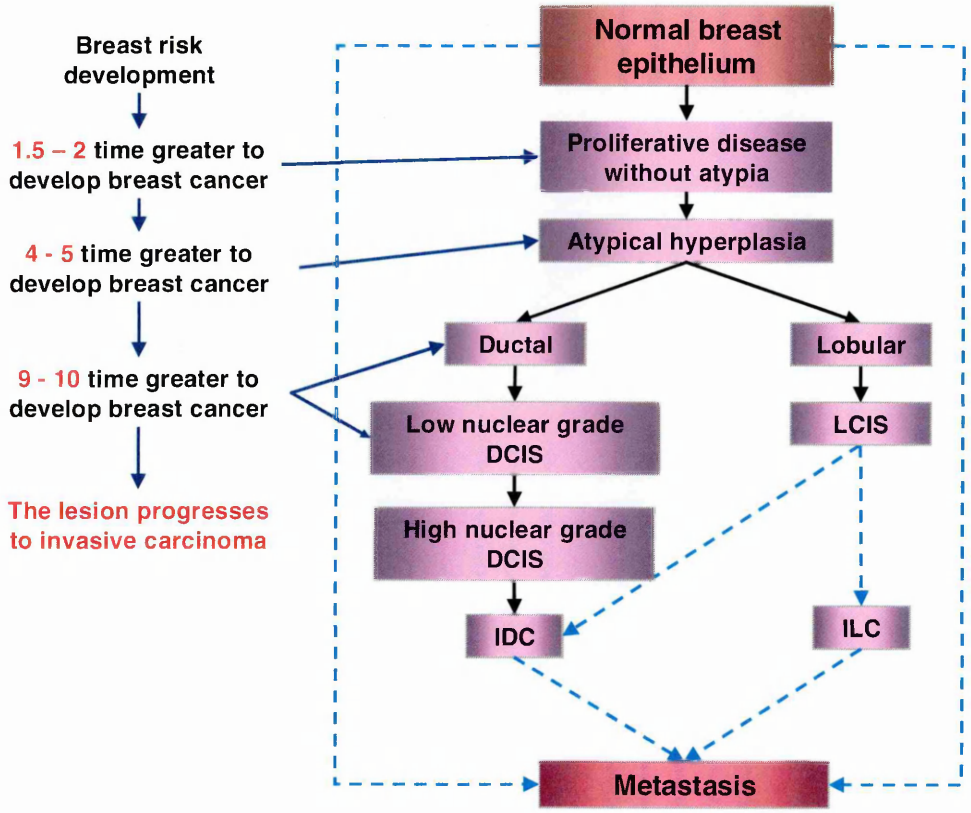


Figure 1.5-3 Model of histopathological stages of breast cancer progression. In proliferative breast cancer the progression from normal breast tissue to cancer tissue is marked by progressive important changes of the breast epithelium. DCIS is considered to be precursor of invasive carcinoma. Women presenting DCIS after biopsy analysis have a higher risk to develop the invasive component. There may be a direct transformation from normal to neoplastic cells without intermediate passages (dashed lines). The risk to develop invasive carcinoma after a diagnosis of LCIS is small but possible (dashed lines) (reviewed by Charpentier & Aldaz, 2002). On the left of the diagram is represented the corresponding estimated risk of breast cancer compared to the general population. Proliferative lesions without atypia are associated with a 1.5 - 2 fold increase in risk, while atypical hyperplasias are

associated with 4 - 5 fold increase in risk of developing breast cancer (reviewed by Schnitt, 2003). Abbreviations: DCIS, ductal carcinoma *in situ*; IDC, invasive ductal carcinoma; LCIS, lobular carcinoma *in situ*; ILC, invasive lobular carcinoma. Adapted from Charpentier & Aldaz, 2002.

Among the non-invasive breast lesions ductal carcinoma *in situ* (DCIS) is the most common and believed to be the essential precursor lesion to invasive carcinoma, followed by lobular carcinoma *in situ* (LCIS). The most important histologic features that distinguish DCIS from LCIS are the size of the cells, smaller in LCIS with a cytoplasmic vacuole, and the tendency of DCIS to have cells more cohesive and forming gland-like or papillary structures. DCIS is frequently a non-palpable lesion which is detected by mammography after the identification of the so called associated microcalcifications (*i.e.* tiny calcium deposits that can indicate cancer). DCIS accounts for 20% of screening-detected cancers and half will be associated with invasive breast cancer by the time of clinical recurrence (Elmore *et al.,* 2003; Damiani & Eusebi, 2002; Recht *et al.,* 1998). There are different subtypes of DCIS which have been classified according to the structural appearance of the pattern such as comedo, cribriform, solid and micropapillary types. In the comedo forms neoplastic cells fill the TDLUs (terminal duct lobular units from which most breast carcinomas take place) and present a central necrotic core. The cribriform type of DCIS is characterised by a lacy pattern of cellular arrangement that fills entirely the TDLUs. The solid variant indicates the neoplastic cells that fill the TDLUs but without manifesting necrosis. In the micropapillary type the proliferating neoplastic cells are organised in a fern like pattern that replace the epithelium coating the ducts (Damiani & Eusebi, 2002). In less aggressive types of DCIS the tumour remains in the TDLUs and causes dilatation and distortion of the TDLUs, whereas in more aggressive types of DCIS the tumour leaves the TDLUs and grows into the lumen of the normal acini (Tot *et al.,* 2002a) (Figure 1.5-4).

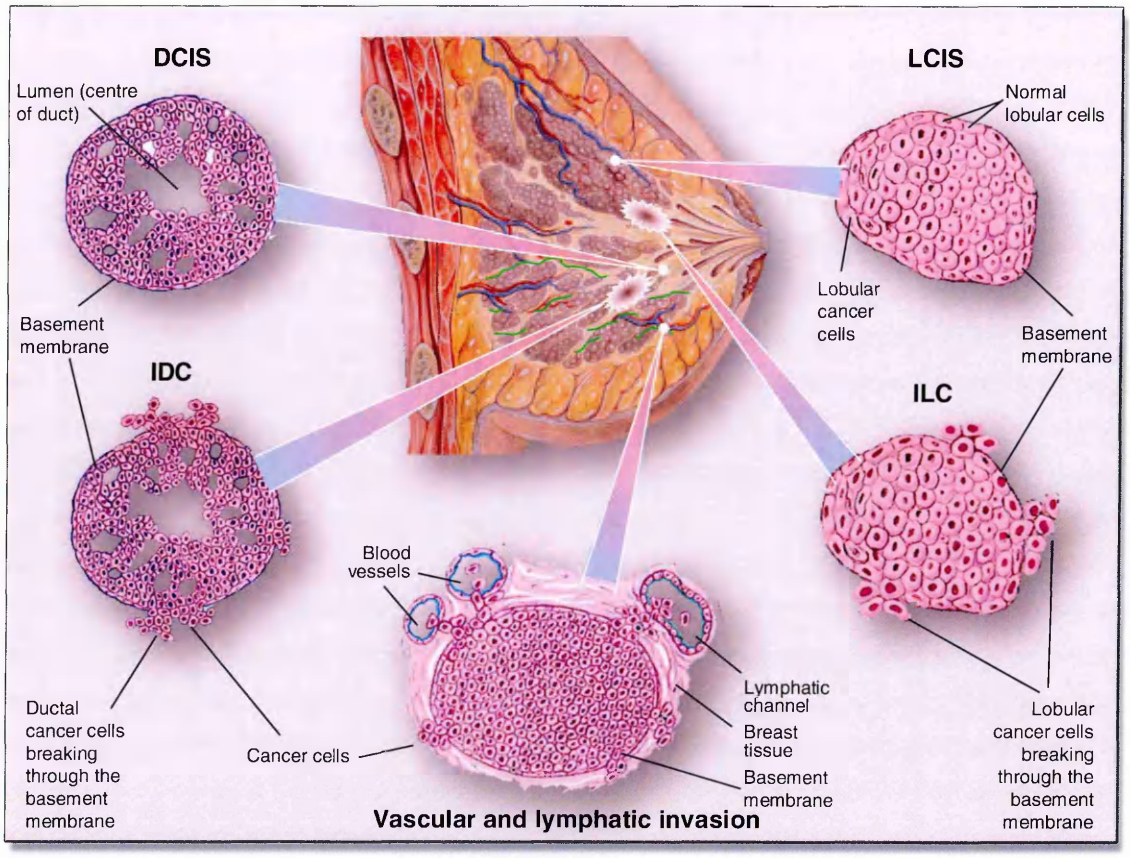


Figure 1.5-4 Histological types of breast carcinomas.
The most important difference between non invasive and invasive forms of breast carcinomas regards the ability of neoplastic cells to start proliferating outside the basement membrane. The spreading of the malignant phenotype induces further expansion of the microinvasion and the neoplasia become invasive carcinoma. Abbreviations: DCIS, ductal carcinoma *in situ*; IDC, invasive ductal carcinoma; LCIS, lobular carcinoma *in situ*; ILC, invasive lobular carcinoma.

The other more insidious type of preinvasive carcinoma is LCIS which is silent and not detectable by mammography because it is not associated with stromal fibrosis and microcalcifications. Because it lacks both clinical and mammographic signs and is mostly an occasional finding in breast biopsies which are performed for other reasons, it has been problematic to calculate the incidence of LCIS among women. Nonetheless, in a big population study Li *et al.* (2003a) observed that the proportion of breast cancers with a lobular component increased from 9.5% in 1987 to 15.6% in 1999 probably due to an increased accuracy in breast cancer screening over the time. Although the risk to the woman of developing invasive cancer after a diagnosis of LCIS is considered, in general, to be small some studies have controversially demonstrated an increased risk of invasive carcinoma after a diagnosis of LCIS (reviewed by Lishman & Lakhani, 1999) (Figure 1.5-4).

Other breast lesions with an increased risk of breast cancer include usual ductal hyperplasia (UDH), atypical ductal hyperplasia (ADH) and atypical lobular hyperplasia (ALH). While UDH is usually considered to correspond to a benign proliferation of ductal epithelial cells, ADH may represent the primordial clonal neoplastic expansion of these cells (Gong *et al.,* 2001). ALH is similar to LCIS but less extensive usually confined to a given lobular unit. Its detection is considered to be incidental after microscopic analysis because there is no clinical or mammography manifestation. These lesions are important because lobular carcinoma is a precursor lesion, partly analogous to ductal carcinoma *in situ* and the risk of developing invasive cancer for women with ALH is four times higher than that in the general population and eight times higher for women who also have a family history of breast cancer (Dmytrasz *et al.,* 2003).

1.5.4.2. Invasive breast carcinomas

If the breast neoplastic cells start proliferating outside the basement membrane the tumour initially shows microinvasion, and later frank invasion. The most common types of invasive carcinomas are infiltrating (invasive) ductal carcinoma (IDC) and invasive lobular carcinoma (ILC) (Figure 1.5-4). IDC takes origin in the milk ducts of the breast and penetrates the wall of the duct, invading the adipose tissue of the breast and other regions of the body. It is the most common type of breast cancer, representing approximately 75% to 80% of breast cancer diagnoses. Most of these carcinomas express the phenotype ranging between well to poorly differentiated adenocarcinomas. If IDC does not fulfil the characteristics of any other variety of carcinomas of the breast, it is called no specific type (NST). IDC NST appears as a solid tumour and its size can range from a few millimetres to several centimetres (Damiani & Eusebi, 2002). ILC is the second variant of invasive carcinomas and begins in the milk glands (lobules) of the breast, but often diffuses to other regions of the breast, and accounts for 10% to 15% of breast cancers. Patients with ILC are at higher risk of developing multifocal and contralateral breast cancer (reviewed by Charpentier & Aldaz 2002).

1.5.5. Prognostic factors and histological grading of breast cancer

Prognostic factors provide information about the behaviour of the tumours, allowing the clinician to identify the prognosis (projected outcome) and to decide the most effective treatment for the patient. In the routine clinical practice, prognostic factors are assessed by staining of the sections with hematoxylin and eosin (H&E). These factors include: tumour grade, tumour size and presence of metastatic lymph nodes. The mitotic count and the hormone status receptor are also considered prognostic factors along with the tumour type and the proliferation markers that have already been extensively discussed in the previous sections.

Tumour grade – The malignancy grade of the invasive carcinoma can be assigned by histologic grading. The tumour's histologic grade is determined after obtaining a sample of breast cells taken from a breast biopsy, lumpectomy or mastectomy. Various grading systems have been proposed to assign a histologic grade to a patient's cancerous breast tumour, but the most commonly used is the Scarff-Bloom-Richardson system, as modified by Elston & Ellis (1991). The classification is based on the arrangement of the cells in relation to each other. The features taken into account are: (i) the percentage of cancer cells composed of tubular structures represented by the lumina surrounded by one or more layers of epithelial cells (tubule formation), (ii) how closely they resemble normal breast cells (nuclear grade) and (iii) how many of the cancer cells are in the course of dividing (mitotic activity). To each of these features is given a score ranging from 1 to 3, where 1 indicates slower cell growth and 3 indicates faster cell growth (Table 1.5-1).

Table 1.5-1 Scarff-Bloom-Richardson grading system, as modified by Elston & Ellis (1991).
It is difficult to have a completely uniform system of grading for breast carcinomas because of the wide variety of histologic cell types. However, the cell types and the invasiveness of the cancer help to predict the biologic behaviour of the cancer. The outlined below grading system considers the histologic characteristics of the breast carcinoma. To each group is given a score ranging from 1 to 3. The scoring for the mitotic counts depends on the make of the microscope used; they are generally counted in ten fields at the periphery of the tumour where there is the highest proliferation activity (Elston & Ellis, 1991; Verhoeven & Marck, 1993).

Feature	Score
Tubule formation (% of carcinoma composed of tubular structures)	
Majority of tumour (>75%)	1
Moderate degree (10 - 75%)	2
Little or none (<10%	3
Nuclear pleomorphism	
Small, regular uniform cells	1
Moderate increase in size and variation	2
Marked nuclear variation	3
Mitotic counts (per 10 high power fields)	
Up to 9 mitoses	1
10 to 19 mitoses	2
20 or more mitoses	3

The scores of each of the cell's features (tubule formation, nuclear grade, mitotic activity) are then added together to obtain a final sum that will range from 3 to 9. The overall tumour grade is then allocated according to Table 1.5-2.

Table 1.5-2 Output of tumour grade according to Elston & Ellis (1991).
The tumour grade is calculated by adding the scores of Table 1.5-1. Grade I is considered the lowest with a good prognosis and the highest overall survival. Grade III is considered the worst with a poor prognosis and the lower overall survival.

Score	Grade	Description	5-year survival	7-year survival
3 - 4 - 5	Grade I (lowest)	Well-differentiated breast cells that generally appear normal and are not growing rapidly, the cancer is arranged in small tubules	95%	90%
6 - 7	Grade II	Moderately differentiated breast cells with characteristics between Grade 1 and Grade 3 tumours	75%	63%
8 - 9	Grade III (highest)	Poorly differentiated breast cells that do not appear normal and are like to grow and spread more aggressively	50%	45%

This grading system, however, is used for invasive cancers but not for *in situ* cancers. For its heterogeneity DCIS, for instance, needs to be stratified by grading and subtyping. Traditionally, the classification of DCIS has been based on architectural patterns (*i.e.* comedo, cribriform, solid, micropapillary subtypes). However, the most important histopathologic prognostic factors used to grade DCIS are the nuclear grade along with the presence of central necrosis. The nuclear grade is assessed similarly to invasive carcinomas and necrosis is assessed according to the presence or absence of central necrosis. Ideally both nuclear grade and necrosis should be assessed like in the proposed Van Nuys classification which takes into account both of these features (Silverstein *et al.,* 1995) (Table 1.5-3).

Table 1.5-3 Histopathologic prognostic factors to assess DCIS.
DCIS is not a homogeneous disease and shows different grade of malignant potential. The most used parameters to assess DCIS are the nuclear grade and the necrosis. The Van Nuys classification system incorporates both the nuclear grade, as it is used for the invasive forms, and the presence of central necrosis in the lumen of the ducts and acini.

Feature	Description	Score/Grade
Nuclear grade	Low: monomorphous, small nuclei without or with very few mitoses and no or few apoptotic bodies	1
	Intermediate: moderately enlarged and polymorphous nuclei with few mitoses and few apoptotic bodies	2
	High: polymorphous and large nuclei with high mitotic rate and many apoptotic bodies	3
Necrosis	Absent	1
	Punctuate/non-zonal	2
	Extensive/zonal (*e.g.* correspond to the type of necrosis seen in comedo DCIS)	3
Van Nuys classification	Score 1 or 2 nuclei and no necrosis	Grade I (lowest)
	Score 1 or 2 nuclei with zonal necrosis	Grade II
	Score 3 nuclei with or without necrosis	Grade III (highest)

Tumour size and **metastatic lymph nodes** – The size of the tumour is directly related to a patient's prognosis. In general, the larger the tumour is, the lower the chances are for effective treatment and long-term patient survival (Tot *et al.,* 2002b). The size of the tumour represents the largest diameter of the largest invasive focus and is one of the three parameters used to assess the stage of the tumour in the TNM classification system (*i.e.* primary tumour size, regional lymph nodes, distant

metastasis) (Greene *et al.,* 2002; Singletary *et al.,* 2002). The presence of metastasis in axillary lymph nodes is another powerful prognostic factor and is important for deciding the most appropriate therapeutic treatment. In general, the presence of involved lymph nodes and periglandular infiltration are negative prognostic factors. Sentinel lymph node biopsy is a new technique used to investigate the presence of axillary lymph node metastases (Tot *et al.,* 2002b). Sentinel lymph node biopsy has nowadays become a common procedure in the early-stage of breast cancer and there is a direct correlation between the tumour size and the frequency and probability of axillary lymph node metastases (Tubiana & Koscielny, 1990) (Table 1.5-4).

Table 1.5-4 The stage of breast cancer in prognosis.
Tumour size, lymph node status and the extent to which the tumour has spread are the prognostic factors used to classify the stage of the breast tumour. From American Joint Committee in Cancer: AJCC cancer staging manual, ed 6, New York, 2002, Springer, pp 255-281 (Greene *et al.,* 2002).

Stage	Description	Lymph node involvement	Metastases (spread)
0	No evidence of primary tumour or contained and not spread beyond the breast ductal system (*i.e.* DCIS, LCIS, Paget's disease of the nipple)	No	No
I	Tumour less than 2 cm in greatest dimension	No	No
II	Tumour between 2 - 5 cm in greatest dimension	No or in same side of breast	No
III	Tumour more than 5 cm in greatest dimension	Yes or in same side of breast	No
IV	Tumour of any size with possible extension to skin involvement, pectoral and chest wall fixation, and axillary or internal mammary nodal involvement, fixed, but without distant metastases	Not applicable	Yes

In conclusion, by assessing the main prognostic factors the different variations of breast cancer can be categorised into three groups: (i) breast cancer with good prognosis (*e.g. in situ* carcinomas, tubular and mucinous carcinomas, tumours smaller than 1.5 cm), (ii) breast cancer with intermediate prognosis and (iii) breast cancer with poor prognosis (*e.g.* tumour with metastases, larger than 2 cm).

1.6. AIM OF THE RESEARCH

Breast cancer progression is the result of a multistep process which can be broadly attributed to the transformation of normal cells through the steps of hyperplasia, premalignant change and *in situ* carcinoma. The aberrant growth of the breast epithelium characterises cancer progression. The main goal of this thesis is to contribute to the control of breast cancer through early detection of the cell proliferation defects that favour tumour development. The following aim was identified in order to achieve this goal:

To investigate the diagnostic potential of MCM proteins in distinguishing between patients with malignancies and benign proliferative lesions in order to lead to their possible use in aiding the diagnosis of breast cancer in district general hospitals.

1.7. OBJECTIVES OF THE RESEARCH

In order to accomplish the project aim, the objectives of the thesis were defined as follow:

1. the establishment of an *in vitro* model in order to test the feasibility of MCM proteins as biomarkers to be used as indicators of cellular proliferation on human tumour derived breast cancer cells and to confirm that cycling cells in culture express MCM proteins;
2. the development of a set of oligonucleotides to be able to work simultaneously to detect the mRNA transcripts of *Mcm2*, *Mcm5* and *Mcm7*;
3. the development of an efficient IHC procedure to be employed for optimising the immunoreactivity of the markers in human tissue *in vivo*;
4. the evaluation of MCM proteins *in vivo* and the assessment of the sensitivity of the markers not only in tumour tissues but also in comparison with associated non-involved tissues in order to prove that quiescent/differentiated breast tissue shows low levels of MCM proteins expression;
5. the profile comparison of MCM proteins with the proliferative marker Ki-67, the oncoprotein Bcl-2 and the steroid hormone receptor ER;
6. the production of statistical data to assess the behaviour, the reliability and whether the analysis of MCM proteins expression is of diagnostic value such that these

markers can be employed to determine cell proliferation in heterogeneous types of breast lesions.

These objectives will each be outlined through the dissertation in three dedicated chapters as described in the next section.

1.8. OUTLINE OF THE THESIS

The organisation of this thesis is structured as follows:

Chapter 1 – The primary objective of this chapter is to introduce the reader to the study chapters of the thesis with a clear and analytic image of the importance of cellular proliferation in cancer diagnosis. This chapter is divided into four sections. The first section starts discussing the current state of breast cancer and the problems related to the early diagnosis of the lesions. It also analyses the motivations for the research. The second section explains the molecular aspects of human neoplasms with particular emphasis on the mechanisms involved in the progression from normal cells into cancer. Inappropriate cellular proliferation is the constant line of direction as it culminates in tumour. The limitations and usefulness of the most employed proliferation markers are also discussed. The third section is a synopsis of the regulation of genome duplication in eukaryotic cells. It explains the overall hypothesis beyond MCM proteins in terms of why MCM proteins were chosen, their mechanism of action, their requirement for cellular proliferation and, more importantly, their unique potentiality as tumour markers. The fourth section analyses the aetiology of breast cancer and gives a background of the breast biology and anatomy. Ultimately, it explains the histopathology of the common neoplasms of the breast, the breast tumour classification and the prognostic factors that are considered in the clinical practice. These features will commonly be recalled through the analysis of the experimental results.

Chapter 2 – This is the first chapter of the research studies and it describes how the research was approached. In fact, the application of MCM proteins *in vivo* requires a platform which had to be built through a preliminary *in vitro* investigation. For this purpose the chapter evaluates the possible use of commercially available antibodies for Mcm2, Mcm5 and Mcm7 proteins in human derived cancer cells and explains why and how this model has gained the access to be transferred to human samples. It also

delineates the development of the set of oligonucleotides to be employed together for the analysis of the *MCM* transcripts in the cell lines.

Chapter 3 – This chapter is the core of the thesis. It discusses the problems encountered in transposing the *in vitro* findings into real women's breast cancer specimens. It investigates the behaviour and the interactions of Mcm2, Mcm5 and Mcm7 protein expression considered individually and as a pool of markers both in tumour and associated non-involved tissues in a heterogeneous population of patients. This chapter also studies the *MCM* mRNA transcripts expression and explains the strategies employed to extract RNA from fresh breast tissue. Secondly, but not less importantly, the chapter gives particular emphasis to the applicability of these methodologies in the clinical routine.

Chapter 4 – This is the final study chapter. In order to be clinically useful the data obtained from the new MCM tumour markers investigated have been compared with the markers that are routinely used in pathology. In this chapter there is a direct comparison of MCM proteins with the most employed cell proliferation marker, Ki-76. In addition, a comparison with the oncoprotein Bcl-2 and ER status for all the cohort of patients investigated was performed which has never been previously done in the literature.

Chapter 5 – In an effort to catalogue this large body of studies the final discussion recalls and analyses as a whole the findings supported by the statistical analysis in order to reveal the potentiality of the MCM biomarkers as diagnostic tools. This discussion represents the bridge to translate this scientific study into the histopathology practice.

Chapter 6 – This is the final chapter of the thesis with concluding remarks about achievements, conclusions as well as proposals for further work.

2 *Evaluation of MCM proteins* in vitro

2.1. INTRODUCTION

The aim of this chapter is to look for patterns of MCM proteins expression *in vitro* using human derived breast cancer cells which will subsequently be employed on the evaluation of the human tumour specimens. To provide insight into the MCM proteins and mRNA transcript expressions, the breast cancer cell line ZR-75-1 and the normal breast cell line 184-B5 were investigated. A third cancer cell line RT-112 derived from human bladder cancer was used as positive control to compare the pattern of expression of the *MCM* transcripts in a carcinoma cell line of different origin than breast. Cell lines were employed because of their ease of growth in a relatively short period of time. Moreover, cell lines allow extraction of substantial quantities of RNA. This was necessary to perform a number of tests in order to select the most specific primers for the transcriptional studies. A careful analysis of the structure of the proteins has shown the homologue sequence conservation of about 200 a.a. localized in the central region of the MCM proteins family (Figure 1.4-2, Chapter 1, Section 1.4.2.) (Kearsey & Labib, 1998; Tye, 1999). These similarities among the proteins gave rise to the idea of using multiple markers RT-PCR. This technique was adopted not only to reduce the time of the analysis to one single operation but, more importantly, to increase the overall sensitivity and specificity of the RT-PCR assay for the gene transcripts (Bostick *et al.*, 1998). With this concept it was possible to challenge the use of the markers as a pool. Because of the heterogeneity of tumour markers expression single markers may be masked or missing in the sample whereas by using multiple marker assays it is possible to increase the chance of examining differential expressions.

The protein analysis was carried out using commercially available monoclonal antibodies raised against Mcm2, Mcm5 and Mcm7. These were chosen as they were

available at the time when the research started. To begin with, the initial immunohistochemical analysis of the proteins was qualitative and, after the optimisation of the protocol, quantitative as well. Subsequently, it was necessary to prove the specificity of the antibodies using western blotting. Through this assay it was possible to assess the size of the proteins picked up by the antibodies, confirming that the antibodies used were capable of binding the epitopes of the proteins of interest in a specific manner.

2.2. MATERIALS AND METHODS

A detailed list of all the buffer formulations employed in the assays can be found in Appendix A.

2.2.1. Cell culture

Three cell lines, all human in origin, were used for MCM analysis: (i) the breast cancer cell line designation ZR-75-1 (CRL-1500), (ii) the chemically transformed normal breast cell line designation 184-B5 (CRL-8799) (ATCC, Manassas, VA, USA), and (iii) the bladder carcinoma cell line (RT-112). The first cell line is derived from a malignant ascitic effusion in a 63-year-old Caucasian female affected with ductal carcinoma. The cells have been characterised to be human malignant mammary epithelium in origin (Engel *et al.,* 1978). The second cell line (*i.e.* 184-B5) is derived from normal mammary tissue obtained from a mammoplasty in a 21-year-old female. The third cell line, RT-112 (accession number ECACC 85061106), is a human bladder carcinoma cell line derived from a female patient of unknown age and was used for an additional assessment of *MCM* mRNA transcript expression.

The cell line 184-B5 was bought immortalised from the American Type Culture Collection (ATCC) and was ready to be cultured in a modified medium as will be described in the following paragraph. Immortalisation was carried out by the depositor and obtained by treatment with benzo[a]pyrene, a potent mutagen and carcinogenic agent which induces immortalisation but not malignancy (Stampfer & Bartley, 1985) (Appendix A). The cancer cell line ZR-75-1 was also bought from ATCC and was ready to be cultured in medium. The cells were originally maintained by the depositors (Engel *et al.,* 1978) in serial culture for over 2.5 years and through 68 passages as

described in Appendix A. The RT-112 bladder cancer cell line was available in our laboratory and, as for the breast cancer cell line the cells were ready to be propagated. The bladder cancer cells were in the beginning maintained by Marshall *et al.* (1977) as described in Appendix A.

All cell lines were grown from samples stored at –196°C under liquid nitrogen. The ZR-75-1 cells were cultured with RPMI-1640 modified medium supplied by ATCC containing 10% of heat inactivated FCS (foetal calf serum) and supplemented with 100 µl of penicillin and streptomycin solution per 500 ml bottle to avoid bacteria contamination. Differently, the 184-B5 cells were cultured on MEGM (mammary epithelial growth medium) serum free composed of 85% of medium containing 10% of FCS and 5% of DMSO (CC-3150) (Cambrex, NJ, USA/BioWhittaker, Wokingham, UK). The MEGM medium (500 ml) was also supplemented with 2.0 ml of bovine pituitary extract, 0.5 ml of EGF, 0.5 ml of gentamicin sulphate and amphotericine B, 0.5 ml of insulin, 0.5 ml of hydrocortisone (CC-4136) (Cambrex) and 1 ng/ml of cholera toxin. The RT-112 cells were grown in Dulbecco's modified eagle's medium (DMEM) (Sigma-Aldrich, MO, USA, code No: D6421) supplemented as in ZR-75-1. Cultures of all cell lines were grown at 37°C in 5% CO_2 in air atmosphere and maintained through passages when they reached 70-80% growth confluence. The subcultivation ratio was about 1:3 to 1:4 (typically double time of circa 80 hours for ZR-75-1 and RT-112 and five to six days for 184-B5 using a T75 flask).

The subculture procedure required Trypsin-EDTA solution treatment to remove the adherent monolayer of cells from the flask (sterile PBS with 5 ml of 1x Trypsin-EDTA concentrate). The mixture was then incubated at 37°C for 7-10 minutes for the cell lines ZR-75-1 and RT-112 and about 15 minutes for the cell line 184-B5 until detachment could be detected. The cells in suspension were removed from the flask and placed into a 5 ml centrifuge tube containing 5 ml of worm media, supplemented with 10% of FCS, in order to inactivate the enzymatic activity of trypsin. The vial was centrifuged at 218 xg at 4°C for five minutes and the supernatant resuspended in about 1 ml of media which was split and transferred into three new flasks containing the pre-warmed media for further subculturing.

2.2.2. MCM transcriptional assays

The methods used for the investigation of the transcriptional expression of *Mcm2, 5* and *7* were carried out through five phases. The first phase was the design of the primers for the mRNA transcripts bearing in mind the use of the multiple markers RT-PCR. The mRNA extraction and cDNA production for RT-PCR represent the second and the third phase respectively. The fourth phase describes the optimised procedure for the application of the primers designed. The last phase explains the procedure employed to assess the specificity of the primers and exclude the amplification of unspecific RT-PCR products from genomic DNA.

2.2.2.1. Primer design

The procedure of designing primers considered two important factors, the capability of the primers to avoid the synthesis of non-specific products (*i.e.* mispriming) and the primer-dimer formation (*i.e.* complementarity to other primers). These effects are more pronounced in RT-PCR than in PCR because of the single-stranded structure of cDNA. To start with, the sequence of *Mcm2, 5* and *7* was retrieved from the National Centre for Biotechnology Information (NCBI) and a number of primer pairs for each marker were developed using dedicated software (Giegerich *et al.,* 1996). The most important parameters set to prevent non-specific binding were: (i) primer length (between 18 and 24 bp to obtain the maximum sequence specificity), (ii) annealing temperature (equal or similar for the primers), (iii) G/C clamps content (the 3' end of the primer should be able to form G/C clamps, that is several consecutive G/C or C/G base pairs between the 3' end of the primer and the template DNA) and (iv) length of PCR product (the optimum size is 100 - 500 bp for conventional PCR). To check the specificity the primers were controlled with the basic local alignment search tools for nucleotide (BLASTn, NCBI) (Altschul *et al.,* 1997). Only primers with low sequence similarities were chosen. The primers selected were also checked for the specificity to amplify cDNA (complementary to mRNA) and not genomic DNA. This assessment was carried out initially using the fast sequence alignment tool BLAT (Kent, 2002) and thereafter by direct analysis of the selected primers using genomic DNA extracted from the cell line ZR-75-1. For *Mcm2* the GenBank accession number was NM_004526; for *Mcm5* the GenBank accession number was NM_006739.

For *Mcm7*, initially, the gene promoter region (GenBank accession number AB004270) was analysed to generate the primers. Later the *Mcm7* transcript variant 1 mRNA was sequenced (GenBank accession number NM_005916). A subsequent analysis with BLASTn and BLAST2 (NCBI) of the primers used confirmed the homologous areas in the two sequences (*i.e.* the extent to which the two sequences are invariant). This indicated that the primers used were also able to amplify the new entry accession with the same degree of accuracy. The exon boundaries and the respective positions within the sequences were determined using BLAT (Assembly July 2003) (http://genome.ucsc.edu/).

2.2.2.2. mRNA extraction

The mRNA was isolated from frozen cells. The cells were grown in suspension in T75 flasks (*i.e.* 75 cm^2 area) until 80% confluent. Once the media was removed the cells were detached using a cell scraper (Nalge Nunc, NY, USA) in 10 ml of fresh sterile PBS previously added to the flask. The resulting cells suspended in PBS were transferred into a centrifuge tube to be spun at 218 xg at 4°C for 5 minutes. Once the supernatant was discharged the cells were resuspended in sterile PBS (1 ml) and placed in a 2 ml γ-irradiated tube. The vial was then spun again as above and the supernatant discharged leaving the pellet intact. The pellet was put at –80°C for further mRNA extraction. The extraction was carried out using the micro-fastrack 2.0 kit (Invitrogen, Paisley, UK) following exactly the manufacturer instructions. Briefly, the buffer containing the RNase and protein degrader was preheated and added to the frozen pellet. The lysate was incubated at 45°C for 20 minutes to allow protein degradation. DNA precipitation was obtained adding NaCl to the mixture and shearing the DNA using an 18 gauge needle. This solution was then incubated with oligo(dT) cellulose to which mRNA binds, allowing it to be separated from the bulk of total RNA, and transferred to a column. Repeated washes of the oligo(dT) with binding buffer were performed to obtain an optical density of the flow-through $\leq$ 0.05 at OD_{260} (for ZR-75-1 OD_{260}=0.012, for 184-B5 OD_{260}=0.009) before eluting the mRNA. The mRNA precipitation was obtained using sodium acetate and ethanol with further snap freezing in liquid nitrogen. After centrifugation the ethanol was removed and the mRNA was

resuspended in 10 µl of elution buffer. The small volumes obtained did not allow quantification of mRNA.

For ZR-75-1 cell line an additional experiment was performed treating the mRNA with DNase to degrade any possible DNA. For this purpose the DNA-free kit was used (Ambion, Huntingdon, UK). In summary, in an eluted sample of 5 µl of mRNA 0.1 volume (0.5 µl) of 10x DNase 1 Buffer and 1 µl of DNase 1 were added. The solution was incubated at 37°C for 30 minutes. To inactivate the DNase agent and to remove divalent cations, which can catalyse heat-mediated degradation of RNA, 5 µl of inactivation agent were added and the solution was incubated at RT for 1 minute. To pellet the DNase inactivation reagent centrifugation at 10.000 xg for 1 minute was performed. The RNA was then transferred to a new tube ready to be used for cDNA production.

2.2.2.3. Synthesis of first strand cDNA by reverse transcriptase

First-strand cDNA was synthesized from mRNA employing the cDNA cycle kit (Invitrogen) according to the manufacturer specifications. Cell extracted mRNA (2 µl) was mixed with 9.5 µl of water and 1 µl of oligo(dT) primer. This solution was incubated at 65°C for 2 minutes and at RT for 2 minutes. The following reagents were added: 1.0 µl RNase inhibitor, 4.0 µl 5x RT buffer, 1.0 µl dNTPs, 1.0 µl sodium pyrophosphate and 0.5 µl AMV RT. This mixture was incubated at 42°C for 60 minutes for the cDNA synthesis.

2.2.2.4. PCR assay

The reverse transcribed cDNA were employed in a standard PCR using the platinum TaqPCR DNA polymerase kit (Invitrogen). This kit provides an automatic 'hot start' that reduces non-specific amplification in PCR during the initial temperature cycle. The oligonucleotides were synthesised by MWG AG Biotech (Bedfordshire, UK) and the concentrations were as following: *Mcm2*, FP 58 pmol/µl, RP 74 pmol/µl; *Mcm5*, FP 83 pmol/µl, RP 54 pmol/µl; *Mcm7*, FP 65 pmol/µl, RP 57 pmol/µl. The components of the master mix were added following the order described in Table 2-1.

Table 2-1 Components and volumes of the master mix for PCR assay.

Master mix components	Volume
10x Buffer	5 µl
dNTP	1 µl
50mM $MgCl_2$	1.5 µl
Forward Primer	1 µl
Reverse Primer	1 µl
cDNA	2 µl
Taq	0.5 µl
Total	12 µl
Nuclease Free Sterile H_2O	18 µl
Total	30 µl
10x PCR Enhancer solution	5 µl
Nuclease Free Sterile H_2O	15 µl
Total	50 µl

After a brief spin the vials were placed in the thermocycler which was set with the PCR conditions indicated in Table 2-2.

Table 2-2 PCR conditions to obtain the amplified products for *Mcm2, 5* and *7*.

Phases	Temperature & time	Number of cycles
Start	95°C for 2 minute	1 cycle
Denature	94°C for 1 minute	32 cycles
Anneal	61°C for 1 minute	32 cycles
Extend	72°C for 2 minute	32 cycles
Final denature	94°C for 1 minute	1 cycle
Final anneal	61°C for 1 minute	1 cycle
Final extension	72°C for 5 minute	1 cycle
	4°C Hold	

The RT-PCR products were resolved under agarose gel electrophoresis in a 1.2% gel and a molecular weight marker (100 bp ladder, Promega, Southampton, UK) ranging from 100 bp to 1500 bp was used to assess the correct size of the products. Positive controls were included using β-actin which is a very abundant protein in

eukaryotic cells detectable by PCR amplification of its mRNA. The following primers were used: sense primer 5'-CTAGAAGCATTTGCGGTGGAC-3' and anti-sense primer 5'-TGACGGGGTCACCCACACTGT-3' with a concentration of 40 pmol/µl for the FP and 70 pmol/µl for the RP. The expected PCR product length was ~764 bp. To ensure that no contamination was present negative controls were employed by omitting the cDNA in the master mix which was substituted by an equal volume of nuclease free H_2O.

2.2.2.5. Assay for the specificity of the oligonucleotides

Genomic DNA was extracted from ZR-75-1 cells that were previously cultured, as indicated for the extraction of mRNA. The DNA mini kit (Qiagen, Crawley, UK) was employed according to the regulations of the manual. Once scraped in T75 flasks the suspended cells in PBS were placed in a centrifuge tube and spun for 5 minutes at 300 xg at 4°C. The pellet was resuspended again in PBS to a final volume of 200 µl to which 20 µl of protease and 20 µl of buffer AL were added. This mixture was incubated at 56°C for 10 minutes. Purification of the genomic DNA was carried out adding ethanol (96-100%) followed by a brief vortexing and centrifugation. DNA was adsorbed onto the silica-based membrane with selective binding properties to DNA during centrifugation for 1 minute at 6000 xg. Two different wash buffers, AW1 and AW2 supplied by the manufacturer, were used to purify the eluted DNA in order to remove any residual contaminants without affecting DNA binding. The column with the DNA bound to the substrate was then placed into a clean 1.5 ml microcentrifuge tube and 200 µl of buffer AE was added. The mixture was incubated at RT for 1 minute followed by centrifugation at 6000 xg for 1 minute.

The quality of DNA extracted was estimated by absorbance spectra between 260 and 280 nm and the ratio was 1.75 (optimal Qiagen range 1.7÷1.9) indicating highly purified DNA free from contaminating protein with a yield of 238 ng DNA/µl. Five different dilutions of genomic DNA with nuclease free sterile water were made to a final concentration of: 100 ng, 10 ng, 1ng, 0.1 ng and 0.01 ng in order to find the amount of DNA in which the primers can detect the gene sequence. The master mix components (with the exclusion of the cDNA) and the PCR conditions of the amplification were as indicated above.

2.2.3. MCM protein assays

Protein expression was investigated using IHC employing antibodies raised against Mcm2, 5 and 7. The first part of this section describes the optimisation of the protocol to obtain a constant and reliable pattern of expression of the proteins. For the immunoblotting assay in spite of the normal breast cell line 184-B5 matched macroscopically and microscopically normal tissues from surgically resected specimens were employed as normal control because the 184-B5 cell line appeared to be positive for the *MCM* gene transcripts investigated.

2.2.3.1. Immunohistochemical assay

A general protocol was developed for the immunohistochemical localisation of the proteins which was performed using monoclonal mouse anti-human antibodies clone CRCT2.1, CRCT5.1 and DCS-141.1 (Novocastra, Newcastle, UK) raised against Mcm2, Mcm5, and Mcm7 respectively on paraffin-embedded sections cut onto aminopropyltriethoxysilane coated slides. Paraffin sections were obtained from a pellet of cells grown on media as indicated above. The paraffin blocks were then cut to 4μm slices using a microtome. This procedure was adopted as the antibodies do not work with frozen sections, such as those derived from a cell line. The paraffin slides were dewaxed in histoclear (Fisher Chemicals) and taken through a series of ethanol passages to water (Appendix B) and then subjected to microwave antigen retrieval in citrate buffer at pH 6.0 (2.1 g citric acid/1 litre distilled H_2O; to adjust the pH 0.2 M NaOH were added) (Appendix A).

Antigen retrieval was required on paraffin sections because of the possible artefacts caused by the formalin during fixation of the cells on the slides. This technique allows breaking the formalin-induced cross-linked bonds between epitopes and unrelated proteins, thus allowing a better penetration of the antibody and the accessibility of the epitopes. During this procedure the energy provided favoured the breaking of some of the bonds formed during fixation, thus increasing the number of positive cells available and the intensity of reactions. However, to avoid protein denaturation by excessive heating temperature and time needed to be assessed very accurately. Thereafter, the optimum microwave time for one litre of citrate buffer was 9 minutes from cold at 800 W. The oven was then set to 50% of the power level and the

optimum time for this second cycle was 8 minutes. To calculate power and timing for this second phase it was necessary to check the slides periodically in order to avoid melting of the samples. After the treatment cooling was allowed to take place slowly inside the microwave for about 20 minutes. Following washes in TBS, endogenous peroxidase activity was inhibited by incubation in 3% w/v hydrogen peroxide/methanol for 10 minutes. Sections were then washed in TBS and blocked with 20% normal rabbit serum (Dako, Ely, UK) for 20 minutes. The antibodies were diluted with a solution of TBS containing 1% of normal serum in order to reduce excessive background. 100 µl of antibody solution (1:20) was added to each section and the slides were incubated at RT for 60 minutes. Following washes in TBS the slides were incubated for 30 minutes with rabbit anti-mouse biotinylated secondary antibodies (Dako). To develop the chromogen stain a strepavidin-horseradish peroxidase (Dako) was used with the substrate diaminobenzidine tetrahydrochloride (DAB) (Sigma-Aldrich) (Appendix A).

Finally, the sections were lightly counterstained in haematoxylin (Sigma-Aldrich) to give the appropriate contrast to the nuclei of the cells. In addition, human placenta was used as positive control to compare and to scale the patterns of expression of the cancer cells. Placenta and normal tissues were obtained from the Departments of Breast Surgery and Histopathology at Cheltenham General Hospital, Cheltenham, UK. For all the samples a negative control was performed by omitting the primary antibody.

2.2.3.2. Assay for the specificity of the antibodies

Protein extraction was performed with CHAPS lysis buffer (Sigma-Aldrich) (Appendix A). The cells from which the protein was extracted were cultured as above and scraped after adding PBS/protease inhibitor (1 ml of protease inhibitor in 50 ml of PBS; ICN Biomedicals, Basingstoke, UK). The solution with the cells was removed to a 15 ml centrifuge tube and centrifuged at 554 xg for 10 minutes at RT to obtain a pellet of cells which was resuspended with 1.5 ml of PBS/inhibitor. This solution was transferred to an eppendorf tube and centrifuged at 12073 xg for 15 minutes. The supernatant obtained was resuspended in CHAPS, snap frozen, thawed and incubated on ice for 60 minutes before centrifugation as indicated above but for 30 minutes. The supernatant consisting of the protein extract was used for protein quantification and stored at –80°C for western blotting analysis.

Protein concentrations were determined by Bradford protein assay (Bio-Rad, Hercules, CA, USA). The dye was diluted 1:5 with distilled H_2O and 1.5 ml was added to the samples and vortexed. Six dilutions of a protein standard were obtained using an IgG protein (1 mg/ml, Bio-Rad) ranging from 0 to 1000 μg protein/ml. After incubation at RT for 10 minutes a differential colour change of a dye occurred in response to the different concentrations of protein absorbance which was read at 595 nm using plastic cuvettes. The final concentration of protein was calculated using the standard curve created and subsequent multiplication by the dilution factor.

The Western blotting analysis was carried out with the same antibodies used for IHC and equal amounts of proteins (63 μg/10 μl) were loaded onto SDS gel for separation (6%). A high molecular weight marker was included to measure the proteins' mass (Cruz Markers, Santa Cruz Biotechnology, CA, USA). The gel was run initially at 75 V for 15 minutes followed by 1 hour at 100 V to allow the loading dye added to the samples to reach the bottom of the gel (Appendix A). The fractionated proteins were transferred to a nitrocellulose membrane (Immobilon-P, Millipore Corporation, Bedford, MA, USA) prepared following the manufacturer recommendations. The blotter was run at 44 mAmps for 55 minutes. Non specific protein-binding sites in the membranes were blocked by a 5% solution of non-fat dry milk in PBS and then incubated overnight at 4°C with the monoclonal antibodies diluted 1:25 for Mcm2 and Mcm7 and 1:15 for Mcm5. A peroxidase-conjugated anti-mouse secondary antibody (Sigma-Aldrich) was used to detect specific reactivity (1:1000 dilution, 1 hour at RT) followed by detection with the enhanced chemiluminescence method (ECL detection system). The film (Hyperfilm, ECL, Amersham UK) was exposed for 1.5 hours before development (developer Kodak, fixer Sigma-Aldrich).

2.2.3.3. Quantification and statistical analysis

The immunoreactive expression of the proteins investigated was scored from 0 (negative) to 5 (strong positive) for both average of staining intensity and labelling indices as percentage of stained cells on the slide in accordance with the method currently in use at the Department of Histopathology at Cheltenham General Hospital, UK. Blind double observer assessment (K.M. and S.D.) by visual light microscopy at low (100 x) and high power (400 x) was employed for the quantification of the stained

sections. Specifically, the intensity of staining was grouped in: 0, 1+, 2+, 3+, 4+, 5+ and the labelling indices for the percentage of cells stained were: 0 (0), 1 (1-4%), 2 (5-24%), 3 (25-49%), 4 (50-74%), 5 (>75%) (McCarthy, 2004). Statistical analysis was carried out using Statistica software (StatSoft, OK, USA). To explore the differences between the three antibodies the Student's two-tailed *t* test was used to determine the level of significance. In addition, the non-parametric Wilcoxon matched pairs signed-ranks test was performed to analyse differences between the samples that may not be normally distributed. A p-value <0.05 was taken as significant.

2.3. RESULTS

2.3.1. Transcriptional studies

2.3.1.1. Computational analysis

The computational analyses identified the best pairs of primers chosen from a first list of 200 and a second list of 8 primers (Appendix C). From this second list a number of primers were tested directly by RT-PCR. The best primers were those represented in Table 2-3.

Table 2-3 Primers specifications.
This list represents the best pairs of primers selected among the list of about 8 primers chosen from a bigger list of 200 generated (Appendix C). Only those with the highest score and with zero T_m difference between the forward and reverse primer were selected and checked with BLASTn and BLAT.

GenBank accession	Marker	Forward primer	Reverse primer
NM_004526	*Mcm2*	5'-TATGTCCAGCGGCACCCTGTCA-3'	5'-GGTGGACCCTCTCCTTGGCGTA-3'
NM_006739	*Mcm5*	5'-ATCGCCAAGGCTGGGATCACCA-3'	5'-CCGCAGGGCCTCCTCCACA-3'
NM_005916	*Mcm7*	5'-TCCCAGCCCCAAGGGTCTAGGA-3'	5'-GGTGGACTGTGGCCGGCCAA-3'
AB004270			

The BLAT analysis revealed that while the primers generated for *Mcm2* and *Mcm5* were located in two different exons of the sequence those for *Mcm7* were in the same exon. This implies that *Mcm2* and *Mcm5* primers do not amplify genomic DNA (the band for *Mcm2* would eventually expect to be about 2200 bp and would not normally be included in the electrophoresis gel or be easily recognisable). Therefore, a

no-RT control was not necessary because the primer sequence of *Mcm2* and *Mcm5* spans more than one intron. By omitting the reverse transcriptase from the reaction the no-RT control generates no signal if the primers are specific for the cDNA and do not amplify genomic DNA. Conversely, the *Mcm7* primers would possibly be able to amplify not only cDNA but also genomic DNA within the same size band. To minimise this problem purification of poly(A) mRNA and/or DNase treatment to remove DNA was performed. Ideally, *Mcm7* primers would have required a new design to span at least one intron to ensure that the product detected would have been from RT-PCR and no-genomic DNA, but the *Mcm7* transcript variant 1 mRNA was sequenced later when the majority of samples were already analysed, as will be described in Chapter 3 (Section 3.3.1.1.). However, if the amplification of genomic DNA had occurred, also after DNA treatment, this would have contributed to the intensity of the band minimally. Nevertheless, a no-RT control for the *Mcm7* PCR products should have been used to definitely prove the integrity of signals. The BLAT results indicating the alignments between human mRNAs in Genbank and the genome are shown in Figure 2-1 together with the exon boundaries. The most important features of the nucleotides and primers selected are summarized in Table 2-4.

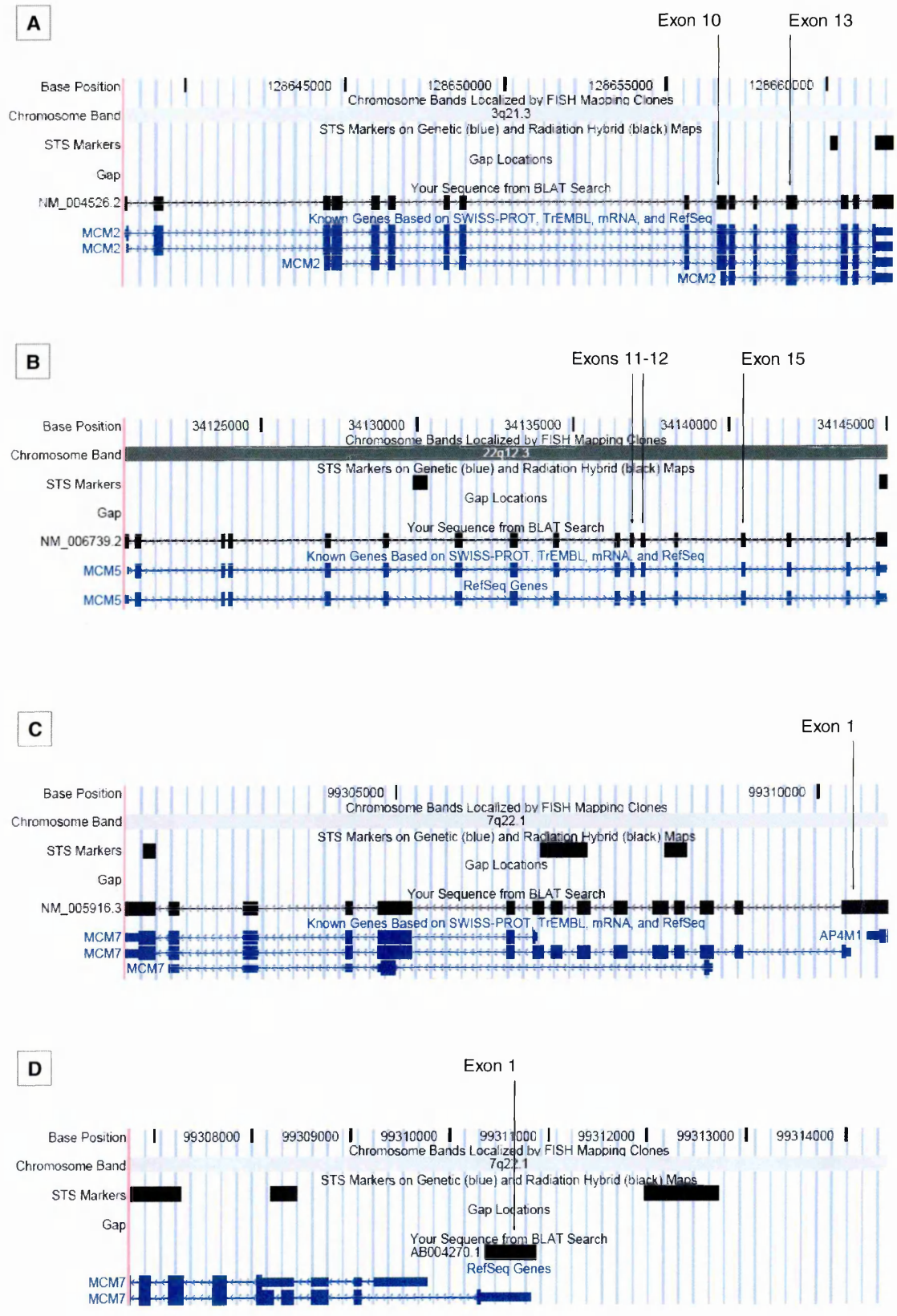

A
Exon 10
Exon 13
Base Position
128645000
128650000
128655000
128660000
Chromosome Band
Chromosome Bands Localized by FISH Mapping Clones
3q21.3
STS Markers
STS Markers on Genetic (blue) and Radiation Hybrid (black) Maps
Gap
Gap Locations
NM_004526.2
Your Sequence from BLAT Search
Known Genes Based on SWISS-PROT, TrEMBL, mRNA, and RefSeq
MCM2
MCM2
MCM2
MCM2
B
Exons 11-12
Exon 15
Base Position
34125000
34130000
34135000
34140000
34145000
Chromosome Band
Chromosome Bands Localized by FISH Mapping Clones
22q12.3
STS Markers
STS Markers on Genetic (blue) and Radiation Hybrid (black) Maps
Gap
Gap Locations
NM_006739.2
Your Sequence from BLAT Search
Known Genes Based on SWISS-PROT, TrEMBL, mRNA, and RefSeq
MCM5
RefSeq Genes
MCM5
C
Exon 1
Base Position
99305000
99310000
Chromosome Band
Chromosome Bands Localized by FISH Mapping Clones
7q22.1
STS Markers
STS Markers on Genetic (blue) and Radiation Hybrid (black) Maps
Gap
Gap Locations
NM_005916.3
Your Sequence from BLAT Search
Known Genes Based on SWISS-PROT, TrEMBL, mRNA, and RefSeq
MCM7
MCM7
MCM7
AP4M1
D
Exon 1
Base Position
99308000
99309000
99310000
99311000
99312000
99313000
99314000
Chromosome Band
Chromosome Bands Localized by FISH Mapping Clones
7q22.1
STS Markers
STS Markers on Genetic (blue) and Radiation Hybrid (black) Maps
Gap
Gap Locations
Your Sequence from BLAT Search
AB004270.1
RefSeq Genes
MCM7
MCM7

Figure 2–1 BLAT genome browser.
The BLAT alignment tool aligns sequences with 95% or greater similarity and allows determining the exon structure of a gene. From top to bottom of each section (A, B, C, D) the alignments between human mRNA tracks in Genbank and the genome are represented (*e.g.* alignment of mRNA NM_4526 (A) and the human gene *Mcm2*). Aligning regions (*i.e.* exons) are shown as black boxes connected by lines representing introns. The arrowheads on the connecting intron lines indicate the direction of transcription (*e.g.* arrowheads in C and D are from left to right indicating the negative chromosome strand). The gene promoter region (D) showed the alignment with *Mcm7* which matched the NM_005916 sequence with 100% of identities (C). (Section D is 1.5x zoomed out). A: NM_004526; B: NM_006739; C: NM_005916; D: AB004270.

Table 2-4 Features of the nucleotides and of the selected primers.
Abbreviations: NM_, RefSeq accession for mRNA; Chrom., chromosome; FP, forward primer; RP, reverse primer; bp, base pairs.

GenBank accession	Chrom. number and location	Chrom. strand	Number of bp	Total exon number of the gene	Exon location FP	Exon location RP	RT-PCR product size (bp)
NM_004526	3q21	+	3453	16	10	13	500
NM_006739	22q13.1	+	2548	17	11/12	15	500
NM_005916	7q21.3-q22.1	-	2821	15	1	1	407
AB004270	7q21	-	505	1	1	1	407

2.3.1.2. RT-PCR and genomic DNA analysis

The results of the RT generated cDNA from ZR-75-1 following purification of poly(A) mRNA indicated the presence of three predicted bands. On agarose gel electrophoresis these bands correspond to a product size of 500 bp for both *Mcm2* and *Mcm5* (Figure 2-2, lanes 1 and 2) while for *Mcm7* the product size was 407 bp (Figure 2-2, lane 3) and for *β-actin* about ~764 bp. (Figure 2-2, lane 4).

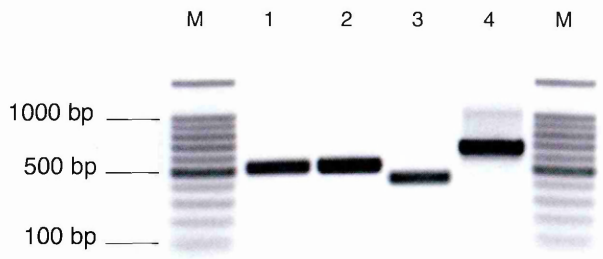


Figure 2–2 Agarose gel of the selected primers. The sample electrophoretic gel for ZR-75-1 illustrates the relative abundance of *MCM* mRNAs amplified by RT-PCR. The sizes of the bands in the gel correspond to those calculated by computational analyses which for *Mcm2* and *Mcm5* are 500 bp whereas for *Mcm7* the product size is about 407 bp. Lane M: marker of 100 bp scale; lane 1: *Mcm2*; lane2: *Mcm5*; lane 3: *Mcm7*; lane 4: *β-actin* (reference). H_2O as negative control is not shown in this electrophoresis gel, it was performed during the PCR reaction and included in a separate gel.

The genomic DNA results were completely in agreement with the computational analysis. In fact, the best sets of primers chosen for *Mcm2* are flanking a region that contains more than one intron (*i.e.* 3 introns spanning between exon 10 and 13) and consequentially they are amplifying a band of about 2200 bp with genomic DNA concentration of 100 ng (Figure 2-3, lane 2) and 10 ng (Figure 2-3, lane 3). The primers amplifying *Mcm5*, however, were not showing genomic DNA amplification at all confirming that the exon location of the forward primer anneals at a splice junction (*i.e.* half of the primer is in the last sequence of exon 10 and the other half is in the first sequence of exon 11), as shown in Figure 2-3, lanes 8-12. On the other hand, the primers for *Mcm7* were not spanning any intron and genomic DNA amplification occurred as predicted, as it is shown in Figure 2-3. However, this occurred only at high genomic DNA concentration of 100 ng (lane 14) and 10 ng (lane 15). Therefore, these quantities are less likely to alter the PCR results if working either with extraction of pure poly(A) mRNA or after DNase treatment.

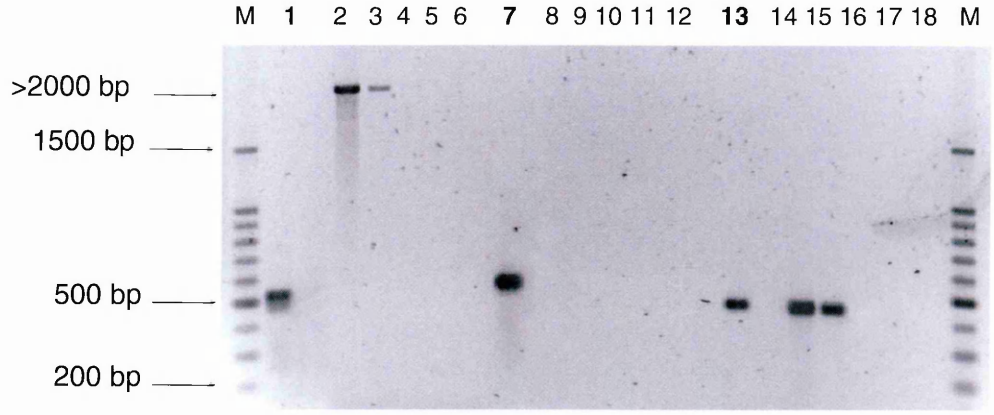


Figure 2–3 Genomic DNA analysis.
Bands in lanes 1, 7 and 13 represent the control using first strand cDNA generated by RT form ZR-75-1. Genomic DNA analysis for *Mcm2* with concentration of DNA: 100 ng (lane 2), 10 ng (lane 3), 1 ng (lane 4), 0.1 ng (lane 5) and 0.01 ng (lane 6) were used to find the amount of DNA that the primers can detect in the gene sequence. The two bands in lanes 2 and 3 have a product size of about 2200 bp which corresponds to genomic DNA amplification. *Mcm5* primers did not give rise to any band since the forward primer anneals at slice junctions so that genomic DNA can not be amplified (same genomic DNA concentration as above for lanes 8-12). *Mcm7* amplifies not only the cDNA complimentary to mRNA but also the genomic DNA with same product size (same genomic DNA concentrations as above for lanes 14-18). However, these primers can detect the genomic DNA only at high concentration of: 100 ng (lane 14) and 10 ng (lane 15). Lane M: marker of 100 bp scale.

The same experiment previously performed on poly(A) mRNA extracted from ZR-75-1 (Figure 2-2) was carried out after DNase treatment to test any difference between the two methods. These results did not show huge dissimilarities to those obtained without DNase treatment (Figure 2-4). This indicated that the extraction methods to separate mRNA from the bulk of total RNA without DNA contamination could be considered reliable.

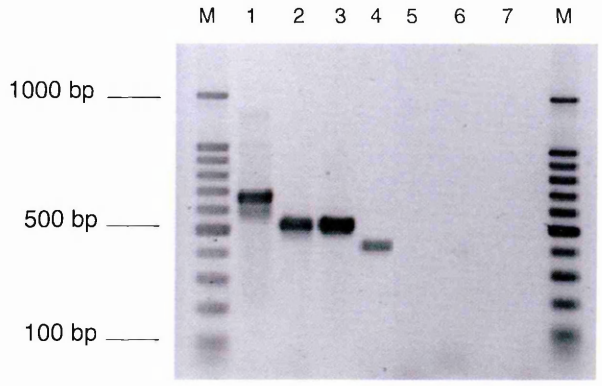


Figure 2–4 Agarose gel after DNase treatment. The expression of the transcripts is similar to the one previously obtained without DNase treatment. Lane M: marker of 100 bp scale; lane 1: *β-actin* (reference); lane2: *Mcm2*; lane 3: *Mcm5*; lane 4: *Mcm7*; H_2O in place of cDNA in the master mix was used as negative control simultaneously for: lane 5: *Mcm2*, lane 6: *Mcm5* and lane 7: *Mcm7*.

The cell line 184-B5 is not anaplastic and was not thought to express the *MCM* gene transcripts or to express them to a much less extent than in cancer cells. However, it showed to be positive after carrying out RT-PCR. In this analysis two different mRNA extractions were performed from ZR-75-1 to compare variations within the cell line. There were no real differences between the two extractions from ZR-75-1 analysed (Figure 2-5, lanes 1-2, 4-5 and 7-8), indicating very low or no variability for these cancer cells. However, some differences could be detected for the transcripts expressed by 184-B5 cell line. By visual inspection in this cell line *Mcm2*, *Mcm5* and *Mcm7* were less expressed than in ZR-75-1 (Figure 2-5 lanes 3, 6 and 9). However, these differences could not be considered meaningful to allow this cell line to be used as negative control for *MCM* expression. Hence, matched macroscopically and microscopically normal tissues were employed as normal control because they showed to be nearly negative after RT-PCR analysis (Chapter 3, Section 3.3.1.2.).

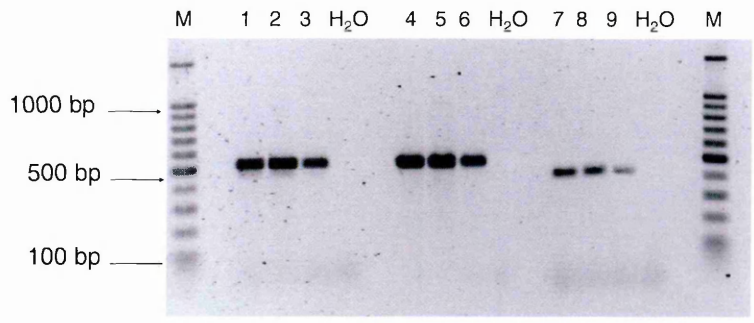


Figure 2–5 Ethidium bromide staining comparison of ZR-75-1 and 184-B5. No significant differences were observed by comparison of the breast cancer cell line and the normal breast cell line. Only *Mcm7* from normal showed a weaker band compared to the tumour cell line. Bands in lanes 1, 2, represent the control (ZR-75-1) for *Mcm2*; lanes 4, 5 for *Mcm5*; lanes 7, 8 for *Mcm7*. Bands in lane 3, represents *Mcm2* from 184-B5; lane 6 *Mcm5* from 184-B5; lane 6 *Mcm7* from 184-B5. Lane M: marker of 100 bp scale. *β-actin* control was substituted by ZR-75-1. H_2O in place of cDNA in the master mix was used as negative control simultaneously for *Mcm2*, *Mcm5* and *Mcm7*.

The RT-112 cancer cells were used as positive control to assess not only the sensitivity of the primers but also the variability of the transcripts in tumours of different nature. Overall the *MCM* transcript expressions can be considered in alignment with those of ZR-75-1 and 184-B5.

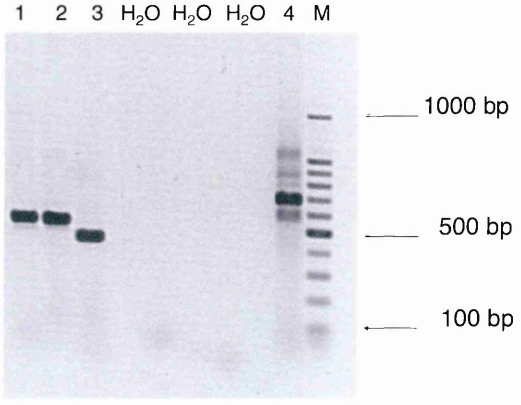


Figure 2–6 Ethidium bromide staining of RT-112 bladder cancer cells. These cells were used to assess the variability of the transcripts in a different kind of tumour. All three transcript profiles could be similarly expressed. *β-actin* (lane 4) was used as reference band. Numbers were rounded up to the nearest whole number. Lane 1: *Mcm2*; lane 2: *Mcm5*; lane 3: *Mcm7*; lane 4: *β-actin* (reference); M: marker of 100 bp scale. H_2O in place of cDNA in the master mix was used as negative control simultaneously for *Mcm2*, *Mcm5* and *Mcm7*.

2.3.2. Translational studies

2.3.2.2. Immunohistochemistry

The immunohistochemical staining of the paraffin sections with the antibodies against Mcm2, 5 and 7 proteins revealed that the majority of the nuclei of the tumour cells were abundantly expressing the pre-replicative proteins (Plate 2-1 A, B and C). This suggests that cycling cells in culture over-express the pre-replicative proteins. Following the assessment of 10 replicates (n=10) the differences in intensity of the immunoreactions were significant only for Mcm7 which stained the nuclei of the cells less strongly than Mcm2 and Mcm5 ($p<0.05$) (Table 2-5). Importantly, non-significant differences were observed among Mcm2, 5 and 7 for the percentage of the cells stained. This indicates that the proteins are expressed in similar proportion in the human derived breast cancer cells. Nevertheless, Mcm7 was about 10% less expressed even though this difference was not significant (Figure 2-7). The background expression of the markers was also observed in placenta used as positive control, but the proportion of cell stained was assessed to be about 60% lower than in ZR-75-1 along with a weaker staining strength (Plate 2-1, D, E, F).

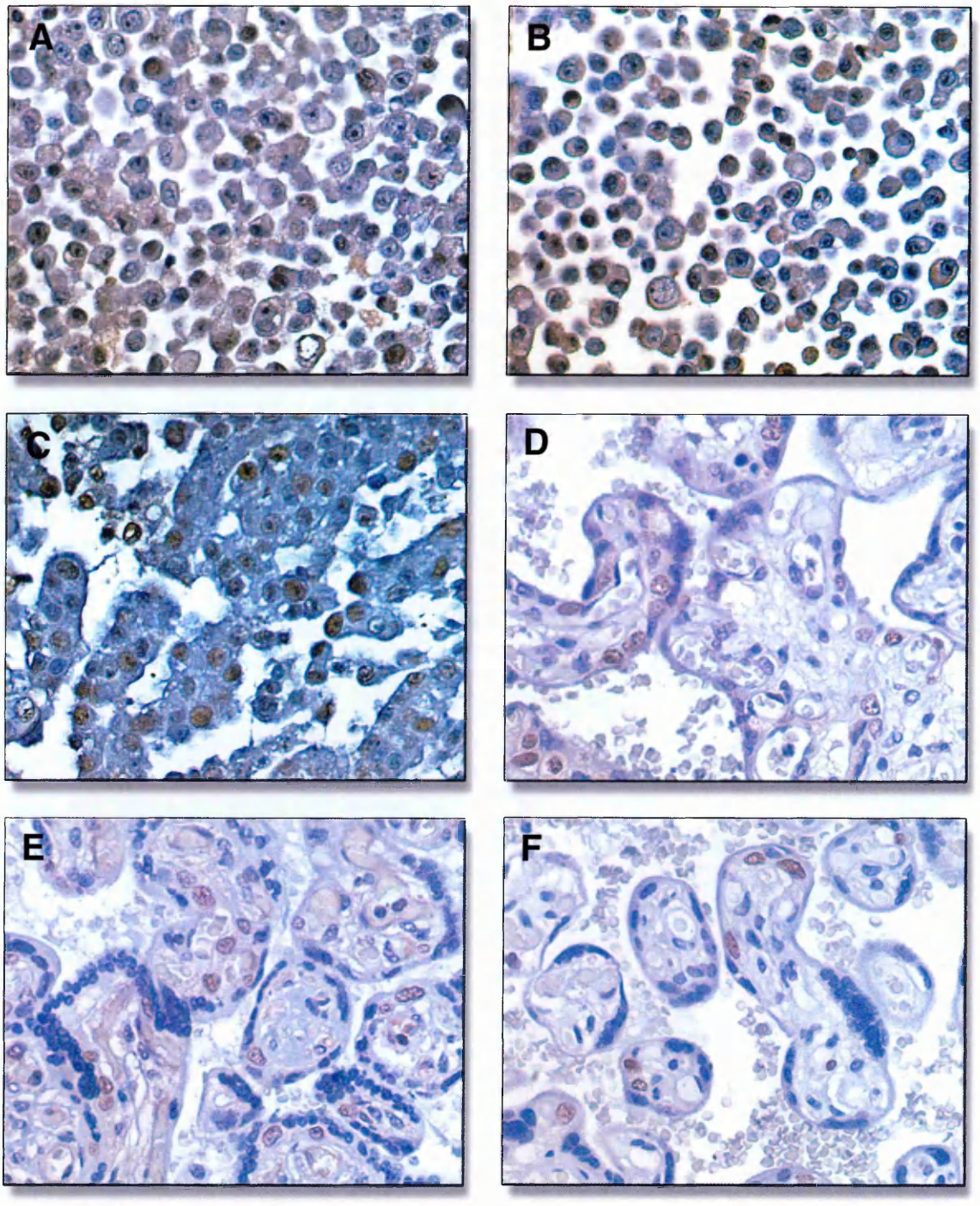


Plate 2-1 Comparison of staining by antibodies anti-Mcm2, 5 and 7 (A, B, C) in parallel sections of the human breast cancer cell line ZR-75-1 and human placenta Mcm2, 5 and 7 (D, E, F). The majority of the cancer cells showed a strong constant pattern of MCM proteins mainly localized in the cell nuclei suggesting that these cultured cells are undergoing aberrant proliferation. This feature of the cells, indicating inappropriate cellular proliferation, could be employed to assess mirrors of proliferation in malignant tissues. Staining for MCM proteins in placenta was occasional. All objective lens magnifications were 400x.

Table 2-5 Percentage and intensity of staining for Mcm2, 5 and 7 in ZR-75-1.
The intensity of the stain and the percentage of cells stained ranges from 0 (absent) to 5 (strong). The intensity of stain was higher for Mcm2 and Mcm5 by comparison with Mcm7 ($p<0.05$) for both the paired difference t-test and the Wilcoxon matched pairs test. No significant differences among the Mcm2, 5 and 7 were observed for the proportion of cells stained. Number of assessed observations 10 (n=10). (Abbreviation: SEM, standard error of mean).

Descriptive statistic (n=10)	Mcm2 mean range		Mcm5 mean range		Mcm7 mean range	
	Stain strength	% cell stained	Stain strength	% cell stained	Stain strength	% cell stained
Mean	3.6	4.3	3.4	4.2	2.7	3.9
Range (labelling index)	2-5	3-5	3-4	3-5	2-4	3-4
Range (labelling index %)		25-100		25-100		25-74
SEM	0.30	0.26	0.16	0.25	0.21	0.23

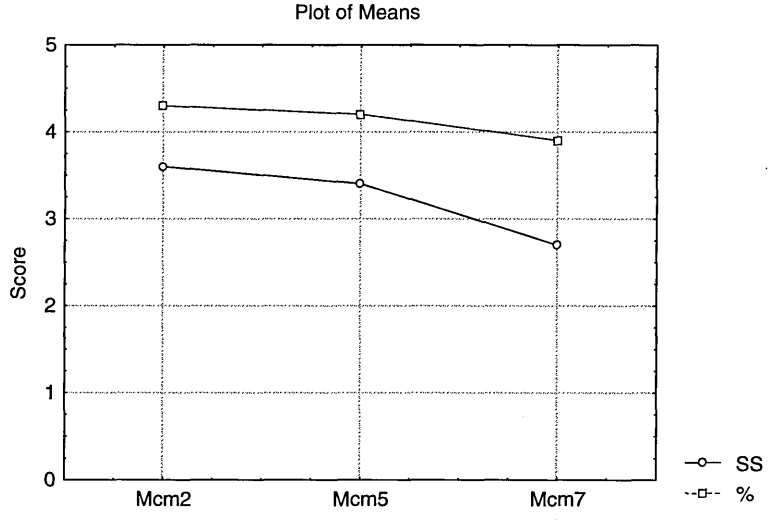


Figure 2–7 Plot of means by direct comparison of the three antibodies. The antibodies raised against MCM proteins generated similar patterns for both the parameter analysed (*i.e.* intensity of staining (SS) and percentage of cells stained (%)). Score: 0 (absent); 5 (strong).

2.3.2.3. Western blotting

The Western blot analysis confirmed the immunostaining motif, indicating not only the presence of the proteins in the cancer cell line but also the specificity of the antibodies employed. In Figure 2-8 Mcm7 and Mcm2 were invariably expressed in all the human breast cancer cells and in all the tests performed. In particular, Mcm7

showed a band size of about 85 kDa (lanes 1 and 2) and by comparison their intensity was slightly stronger than the other bands. A similar pattern was detected for Mcm2 which showed a band size of about 125 kDa (lanes 7 and 8). Despite the increased concentration of the antibody (1:15) Mcm5 was invariably expressed and the band is visible with a protein size of 90 kDa though weaker than the other two (lanes 4 and 5). These results indicate the ubiquitous expression of the proteins in the cancer cells. On the contrary, Mcm2, 5 and 7 proteins were never markedly expressed in the non-involved breast tissues (lanes 3, 6 and 9). Interestingly, a band size of approximately 70 kDa blotted with Mcm2, appeared in lane 9 of Figure 2-8. This band could be due either to proteolysis of Mcm2 protein or to a truncated protein generated by a nonsense mutation about 2/3 of the way from the 5′ end of the *Mcm2* gene.

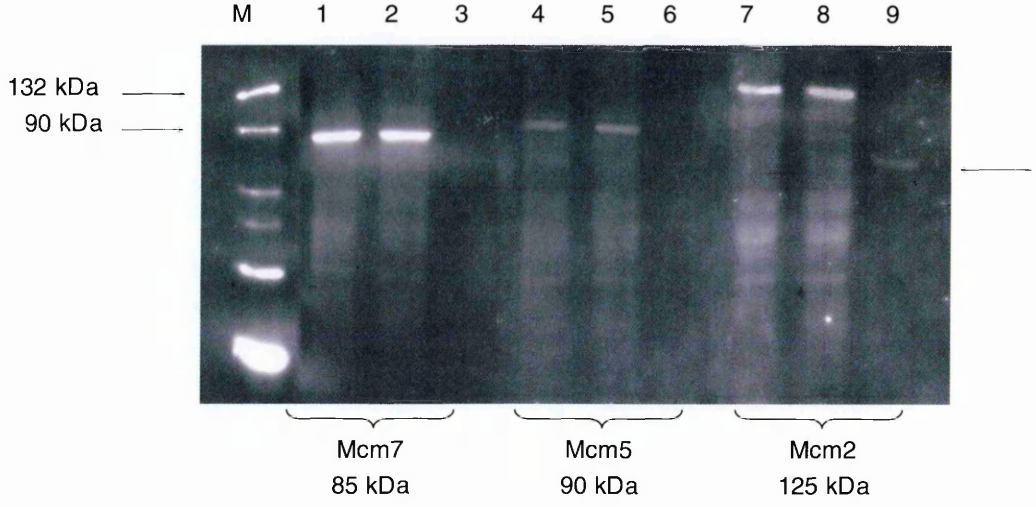


Figure 2–8 Immunoblotting in tumour cells and non-involved tissues.
SDS-PAGE analysis for the expression of Mcm2, 5 and 7 in ZR-75-1 and non-involved breast tissue. Although with different band intensities the breast cell line expresses all the three proteins. Specifically the protein size for MCM7 (lanes 1 and 2) is approximately 85 kDa. Mcm5 is expressed in the cell line at a molecular mass of about 90 kDa (lanes 4 and 5). The Mcm2 band size is about 120 kDa (lanes 7 and 8). Matched normal tissue did not show the bands for Mcm7 (lane 3) and Mcm5 (lane 6) but a band in line 9 (arrow) of about 70 kDa is present which may be caused by either proteolysis product of Mcm2 or a truncated protein generated by a nonsense mutation about 2/3 of the way from the 5′ end of the *Mcm2* gene. Lane M: molecular weight marker.

2.4. DISCUSSION

This chapter has examined the expression profiles of both the *MCM* mRNA transcripts and the MCM proteins in human derived cancer cells to optimise the techniques for the subsequent *in vivo* investigation. The results suggest that *Mcm2*, *Mcm5* and *Mcm7* genes are expressed in ZR-75-1 and 184-B5 cell lines. From this type of experimentation it cannot be ascertained which *MCM* is the most expressed. These results suggest that there may be some dissimilarity in expression though they need to be confirmed by more sensitive methods (*e.g.* northern blot). It appears that the variability among the three transcripts is perhaps less consistent in the bladder cancer cells (RT-112) (Figure 2-6). A consideration arises from these results. The differences in expression among the three transcripts can be considered relatively constant in the breast cells indicating that *Mcm5* may be the most highly expressed of the genes followed by *Mcm2* and *Mcm7* in both cell lines. The reason *Mcm5* may be the most highly expressed of the genes remains to be elucidated. In fact, the functionally related *MCM* genes are likely to have similar expression patterns when they prime DNA replication. In addition, *MCM* mRNA levels reach a peak at the G_1 phase suggesting that *MCM* transcription is periodic in cycling cells (Tsuruga *et al.,* 1997; Kearsey & Labib, 1998). A simple explanation is that while individual MCM proteins may be present in excess the assembled complex that binds to replication origins is present in limiting and nearly similar quantities. This hypothesis could also suggest different individual roles of some of the MCM family components beyond their DNA replication licensing one. Differences in expression could also be caused by the specificity of the primers selected for cDNA generation. However, this hypothesis did not find any correlation with both the BLAST and the BLAT search for the possible product alignments of the primers. In fact, this analysis showed high homologies to all the three gene sequences of *Mcm2*, *5* and *7*. Moreover, the same computational analyses showed that if less specificity could be attributed to *Mcm7* for the position of the exons boundaries, this un-specificity could not be given to *Mcm2* and *5* (Figure 2-1). In addition, non-specific genomic DNA binding of the primers was proved by the double analyses carried out with poly(A) mRNA and DNase treatment of the RNA extracted from both cell lines (Figures 2-2, 2-4 and 2-5). As outlined, these differences in expression are relatively constant in all the experiments.

From the PCR products it could be hypothesised that all cell lines are actively proliferating. These results are in agreement with previous experiments. In fact, *MCM* mRNA levels go down dramatically when cells are differentiated and increase in proliferating cells (Kearsey & Labib, 1998). Furthermore, Tsuruga *et al.* (1997) demonstrated that the *Mcm5* gene is almost not expressed in the G_0 state. This notion is consistent with the opposite high expression of the *MCM* mRNA transcripts in 184-B5. These cells may not enter the G_0 phase because of anchorage independent growth due to their immortalisation once exposed to benzo[a]pyrene. A topic for future research could be the adoption of FACS analysis to confirm that the majority of these cells are in G_1 or in S transition phase of the cell cycle.

Cell synchronisation studies of the cell lines to analyse the gene expression and protein content at various stages of the cell cycle could have been performed. The use of cell cycle inhibitors would have allowed to discriminate between the differential expressions of the proteins in the specific cell cycle phases and to confirm the presence of the MCM proteins investigated in early G_1 phase of the cell cycle. This analysis could also have been extended to the simultaneous investigation of Ki-67. However, this study was not performed since this idea arose at a late stage of the research.

The IHC results have confirmed that the monoclonal anti-Mcm2, 5 and 7 antibodies provide consistent and reliable staining in routinely fixed tissues. It is, however, important to notice that the microwave antigen retrieval procedure needs to be accurate to avoid a decreased sensitivity of the antibodies. In agreement with other authors immunostaining with the monoclonal antibodies yielded predominantly nuclear staining (Todorov *et al.,* 1998; Freeman *et al.,* 1999; Tan *et al.,* 2001) (Plate 2-1). The results obtained were easy to interpret and the staining intensity was very similar for Mcm2 and 5, though it was weaker for Mcm7 ($p<0.05$). This difference may be due to the optimisation of the protocol rather than a lower functional affinity of the Mcm7 antibody. In fact, on paraffin sections the optimal dilutions of primary antibodies are not only those marked by a peak in staining intensity. Hence, the protocol took into account also the presence of minimal background in order to obtain maximal signal-to-noise ratios. Besides, all three antibodies were exposed to the same heating conditions. Thus, the differences in variation of intensity can not be attributed to prolonged heat that, in different conditions, may affect the intensity of staining. More importantly the

mean for the percentage of cells stained did not show any significant variation for Mcm2, 5 and 7 which ranged from 25% to 100% for all of them (n=10) (Table 2-5). Both the results of the Student's *t* test and the non-parametric Wilcoxon matched pairs test were in agreement suggesting that these markers could be employed *in vivo*.

Finally, the immunoblotting experiment confirmed the specificity of the monoclonal antibodies raised against MCM proteins used for IHC. Specifically, the antibodies were reacting with the protein extracts from ZR-75-1 with a band size of about 125 kDa, 90 kDa and 85 kDa respectively for Mcm2, 5 and 7 without signs of cross-reactivity (Figure 2-8). This is in agreement with data previously published concerning the human molecular weights of MCM proteins separated by mass in SDS-PAGE gel (Kearsey & Labib 1998; Ishimi *et al.,* 1996). The power of this test lies not only in the simultaneous detection of the molecular protein mass but also in the specific protein by means of its antigenicity. Although the first observation was satisfied the second one deserves some consideration.

It appears from the gel that there is discordance between the signal intensity of Mcm5 by Western blotting and IHC. This lower intensity may represent less proteins expressing Mcm5. A possible explanation for this discordance between detection by IHC and that by Western blot is degradation of Mcm5 during the preparation of extracts for Western blotting regardless of the use of protease inhibitors in the extraction medium. Controversially, it could appear that Mcm7 is more expressed by Western blot than that observed by IHC. In this case the level of Mcm7 in the cancer cell may be increased but below the threshold of detection by IHC. In any case, extraction and concentration of MCM proteins by IHC from cancer cells allowed detection by Western blotting.

On the other hand, the normal tissues lack detectable expression of bands of the same molecular weight. These results are in agreement with others reported in the literature (Todorov *et al.,* 1998; Freeman *et al.,* 1999). This indicated that these proteins were not ubiquitous in the normal tissues. However, for Mcm2 a band corresponding to about 70 kDa was represented on the gel although it was weak (Figure 2-8, lane 9). The nature of this band is not entirely clear but there are two possible explanations for this non-specific band. First, it could be the consequence of proteolysis of Mcm2. It has been previously reported that phosphorylation of MCM proteins is

essential in the regulation of the activities of this complex (Tye & Sawyer, 2000; Lei *et al.*, 2002). Schwab *et al.* (1998) proposed that protein cleavage may be the way to remove these proteins from potential replication sites. Thus, the protein product may be represented by this weak band. The second hypothesis may be represented by a truncated protein possibly generated either by a nonsense mutation, which induces premature stop codons, or by a truncated mRNA. Whether a nonsense mutation or not this truncated protein probably lacks the antibody recognition sites by IHC. If this hypothesis is true, this could indicate genetic instability which is the fuel for the myriad of mutations that harbour tumour initiation and progression.

In conclusion, this *in vitro* study has confirmed the specificity of *Mcm2* and *Mcm5* primers to detect the mRNA transcripts. Further, it has shown that the MCM monoclonal antibodies can be used to assess clinical breast specimens. The techniques described in this chapter will be employed in the *in vivo* study for assessing the applicability of the pre-replicative protein complex to the diagnosis of breast cancer.

3 Evaluation of MCM proteins in human breast specimens

3.1. INTRODUCTION

A major problem in breast cancer detection and surveillance is the complex normal histological architecture. In the mammary gland the ducts and lobules are normally supported by stroma consisting of fibrous tissue and a high percentage of fat. However, in cancer this tissue loses its normal architecture and often contains inflammatory infiltrates (*e.g.* lymphocytes and monocytes) and fibrosis. In addition, cancer cells create a modified microenvironment of the host tissue developing their own blood supply system (Liotta & Khon, 2001). As a result, it is difficult to isolate large quantities of tumour cells for analysis from clinical samples because samples are often composed of normal cells, cellular debris and free substrate (*e.g.* DNA, RNA and proteins) (Sidransky, 2002). As a consequence, dysplastic changes may be very focal and the biopsy may not sample dysplasia due to sampling error. A representative example of normal breast histology and neoplastic tissue is shown in Plate 3-1.

The scope of this chapter is to correlate malignant change with the expression of Mcm2, 5 and 7 proteins. Therefore, MCM protein expression was analysed in a variety of breast lesions using protein and nucleic acid based techniques. A correlation between the gene expression of *Ki-67*, a marker of cell proliferation commonly used in breast pathology, and the *MCM* investigated was not studied because the idea to investigate *Ki-67* came when all the fresh tissues had already been used for RNA extraction. However, correlation studies between Ki-67 and MCM protein expression were performed, as described in Chapter 4, because of the availability of the paraffin embedded tissues.

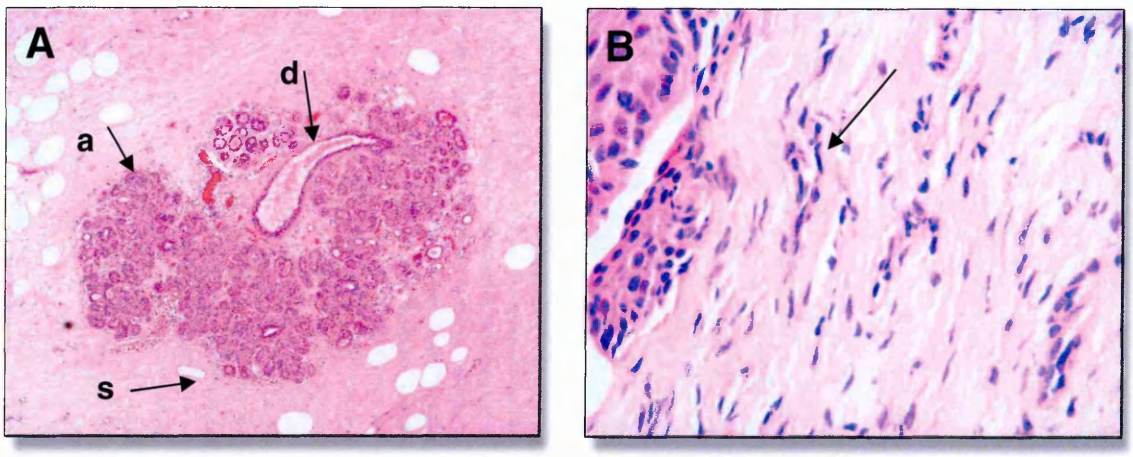


Plate 3–1 Normal breast histology (A) and infiltrating carcinoma (B). Normal breast tissue is lined by the ductal epithelial cells forming the lumen (d), the cells forming the acinus (a) and the surrounding stroma (s). Cancerous monomorphous cells in infiltrating breast tissue form nests of cells which are 2 to 3 layers thick and form primitive glandular spaces. The tumour spreads into the surrounding stroma (arrow in B). Objective lens magnifications 50x (A) and 100x (B).

By comparison with the previous chapter that evaluated MCM proteins *in vitro*, different methodologies were employed for the transcriptional analysis of the *MCM* mRNA transcripts. The procedure described in Chapter 2 (Section 2.2.2.2.) for mRNA extraction in the cell lines failed to be useful for the breast specimens because of the histologic heterogeneity of the mammary tissue. Hence, a number of methods were tested, but all gave a wide variability in the results which was again caused by the complexity of the breast tissue. In addition, testing and optimisation of these techniques wasted significant tissue material. Moreover, the analyses for the transcripts were only possible on tissues obtained from breast mastectomies which comprised the minority of the total number of cases which consisted mainly of local excisions or core biopsies. In the end, the technique that gave the most consistent results was laser capture microdissection (LCM). This technique dissected the homogenous population of cells of interest while keeping not only their morphology intact but also preserving the nucleic acids and proteins. This is particularly important since in solid tumour specimens the percentage of cells of interest may represent only 5% (Simone *et al.*, 1999). On the contrary, conventional nucleic acid extraction methods do not allow obtaining selective tumour extraction and very little material is obtained which is often contaminated with heterogeneous material that decreases the sensitivity of the test. Thus, this can confuse the information in the specimen. The different sensitivity

between the conventional approaches in extracting genetic material from the cancer cells and LCM will also be highlighted in this chapter.

For the translational studies the protocol previously optimised for IHC needed further refinement. This was not necessary for the tumour tissues but it was required for the normal control tissues. This analysis compared the pattern of expression with tumour specimens and assessed the applicability of MCM markers for this *in vivo* study. The majority of the normal specimens had a very high content of fibrofatty material along with a lower proportion of cells (~70-90% less), as shown in Plate 3-2, and were susceptible to melting after microwave pre-treatment. Western blotting analyses were not carried out because of the lack of tissues.

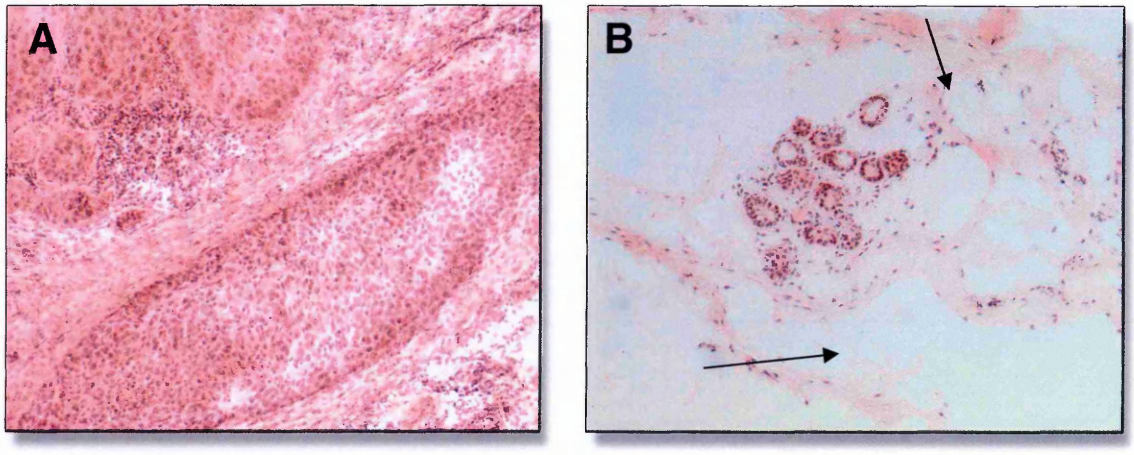


Plate 3–2 Breast tumour tissue compared with histologically normal tissue (H&E).
By comparison with the tumour tissue (case of invasive DC associated with solid DCIS grade III) (A) the normal breast tissue (B) is mostly composed of fibrofatty material (arrows) and contains a lower percentage of cells. These tissues were subjected to ease melting after thermal treatment necessary for IHC and were particularly difficult to cut with the cryostat. All objective lens magnifications were 100x.

3.2. MATERIALS AND METHODS

3.2.1. Patients and clinical specimens

The surgically resected carcinoma specimens were derived from 65 women undergoing either elective mastectomy or lumpectomy at the Department of Breast Surgery at Cheltenham General Hospital, Cheltenham, UK. The specimens were collected from July 2001 to May 2003. All tissues were obtained from patients having given informed consent in accordance with the Gloucestershire Health Authority (NHS) Local Research Ethics Committee (approval number 00/142G) (Appendix E).

Histopathological grading was performed by the Department of Histopathology at Cheltenham General Hospital according to the Scarff-Bloom-Richardson system as modified by Elston-Ellis, (1991) (Chapter 1, Section 1.5.5., Tables 1.5-1 and 1.5-2). In addition, matched macroscopically and microscopically normal tissues taken at least 4 cm from primary tumour were employed as control (n=65).

Surgical resected tissues were fixed in 10% buffered neutral formalin solution and embedded in paraffin; 4 μm consecutive sections were cut on a rotary microtome and mounted on glass slides. As shown in Plate 3-3, fresh specimens from mastectomies were promptly collected in the surgical theatre and immediately transferred to the Department of Histopathology for immediate cutting of the fresh tumour and histologically normal tissues which were then placed in 1.5 mm RNase and DNase free tubes. These specimens were promptly put under liquid nitrogen to preserve the nucleic acids and stored at –80°C for further RNA extraction.

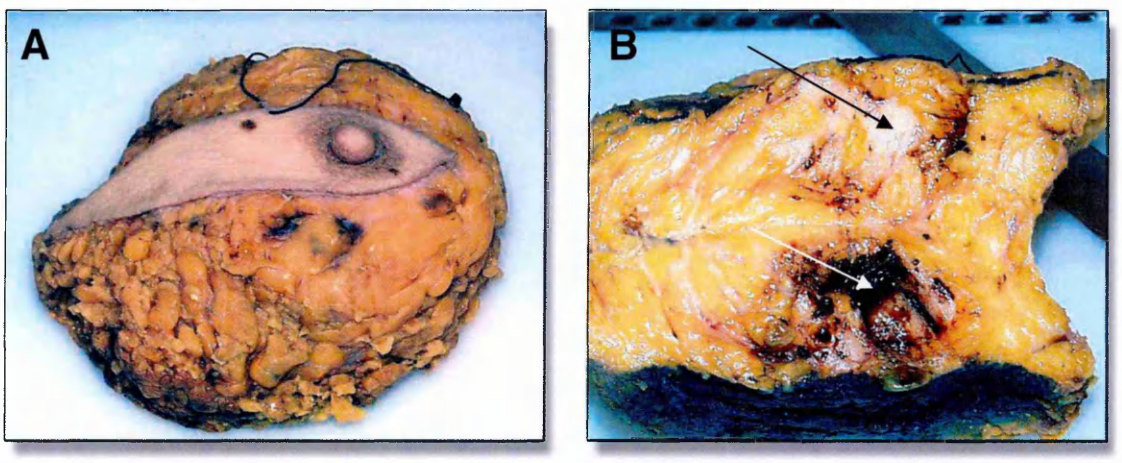


Plate 3–3 Macro of breast tumour.
In order to avoid RNA deterioration, the surgical specimen was immediately sectioned and put under liquid nitrogen until stored at –80°C for further RNA extraction. Tumour excision (A) and breast macro (B). Tumour localisation is represented by the arrows in (B).

The clinicopathological characteristics of the patient series are contained in Table 3-1. Among the 53 cases of invasive breast ductal carcinomas 41 were associated with DCIS, and both cases of invasive lobular carcinomas also contained LCIS. There were 4 cases of invasive carcinoma showing both DCIS and LCIS. A single case of phyllodes tumour was analysed; phyllodes tumours are very different from usual breast carcinomas because they behave like sarcomas (*i.e.* cancers of fibrous and supportive

tissues in the body). There was one case of mucinous tumour where the grade was not assessable (although they are normally grade I or II).

Table 3–1 Clinicopathological data for the breast cancer tumour studied.
Among the 53 cases of invasive DC 41 were associated with DCIS, whereas both cases of invasive LC were showing LCIS. The grade of one of the two mucinous cases was not assessable. Abbreviations: DC, ductal carcinoma; LC, lobular carcinoma; DCIS, ductal carcinoma *in situ*; LCIS, lobular carcinoma *in situ*; C: carcinoma.

Histology	Age	Grade				n
	Median (range)	I	II	III	NA	
Invasive DC	59 (36-86)	9	29	15		53
DCIS	60.5 (59-62)		1	1		2
Invasive LC	59.5 (53-66)		2			2
Invasive C, DCIS, LCIS	54.5 (54-63)	1	2	1		4
Invasive C (mucinous)	76 (69-83)		1		1	2
LC	64	1				1
Phyllodes tumour	53			1		1
Combined data	59 (36-86)	11	35	18	1	65

3.2.2. MCM transcriptional assays

Two methods were employed to extract RNA from tumours and histologically normal tissues. The first method consisted of extracting total RNA followed by mRNA separation. The second method employed laser microdissection to capture the cells of interest to be used for extracting the RNA which was then subject to DNase treatment to avoid DNA contamination.

3.2.2.1. RNA extraction from frozen breast tissues

The major problem of tissue thawing in the absence of ribonuclease inhibitors is RNA degradation. To overcome this, all the procedures were carried out using RNase- and DNase-free plastic ware. Scalpels, tweezers and forceps were washed with detergent and baked at 210°C for 4 hours before use. Work surfaces, pipetters and equipment was wiped using RNaseZap (Ambion, Huntingdon, UK) and disposable gloves were changed frequently. The tissue samples were prepared using two methods: (i) the tissue was embedded in OCT embedding fluid (Bright Instruments, Cambridge, UK) and from each sample 100-200 8 μm sections were taken using a cryostat set at –

28°C to aid subsequent homogenization (full details of the procedure are described in Appendix D) and (ii) the tissue was pulverized in a pool of liquid nitrogen using a mortar for further homogenisation.

In both methods Tri-reagent solution (Sigma-Aldrich) was immediately added to the tissue to inhibit the degradation of RNA. All the procedures were done according to the manufacturer recommendations. In short, the homogenisation was performed adding 1 ml of Tri-reagent solution (sample average weight ~75 mg) and sheared using an 18 gauge needle. The mixture was centrifuged at 12.000 xg for 10 minutes at 4°C to remove the insoluble material consisting of fat. The layer of fatty material on the surface of the mixture was removed. The clear supernatant containing the RNA and protein was then transferred to a fresh tube. To ensure complete dissociation of nucleoprotein the sample was left for 5 minutes at RT and 0.2 ml of chloroform was added to perform phase separation. The mixture was vortexed vigorously and kept at RT for 15 minutes, then spun as above for 15 minutes at 4°C. The upper colourless phase representing the RNA was transferred to a new tube and 0.5 ml of isopropanol was added to allow precipitation of RNA. Again, the complex was kept at RT for 10 minutes and spun as above for 10 minutes. The supernatant was removed and the RNA pellet washed by adding 1.3 ml of 75% ethanol. The mixture was vortexed and centrifuged at 7.500 xg for 5 minutes at 4°C. At this point the RNA solution was added with 63 µl of 5 M NaCl at RT and further mRNA extraction was performed with the micro-fastrack 2.0 kit (Invitrogen) as described in Chapter 2 (Section 2.2.2.2.).

3.2.2.2. Tissue preparation by laser capture microdissection (LCM)

Twenty neoplastic and twenty histologically normal sections for each case were cut at 8 µm using a cryostat set as above (Appendix D). The sections were placed in DNase- and RNase-free slides provided with the HitoGene frozen section staining kit (Arcturus, Engineering Inc., Mountain View, CA). Slides were transferred in dry ice and stored at -80°C. For each case tissue integrity and quality were checked by staining one slide in every 4 with H&E. Slides with visible folded or wrinkled sections were discarded as recommended by the protocol. During all the phases particular attention was given to the sections to avoid thawing which would have deteriorated and decreased the nucleic acid yield. Slides were prepared for LCM after being treated with

the staining solution provided with the HitoGene kit (Arcturus). In summary, 4 slides at a time from the same case were placed at RT for 30 seconds and stained and dehydrated as previously described (Mikulowska-Mennis *et al.,* 2002) following exactly the sequential steps recommended by the manufacturer instructions through passages in different concentrations of ethanol (full details of the procedure are outlined in Appendix D). Once the slides were dehydrated, after xylene treatment in a fume hood with adequate ventilation, they were placed and kept in desiccant drying pearls (Fluka, Sigma-Aldrich) to avoid RNA deterioration by moisture contamination until laser microdissection occurred.

A laser pulse PixCell IIe (Arcturus) was used for microdissection of the stained sections. The dehydrated tissue sections were overlaid with a thermoplastic membrane mounted on optically transparent caps (CapSure Macro, Arcturus). The breast cancer epithelium, the surrounding stroma and the normal epithelial or stromal cells undergoing microdissection were identified and targeted through microscopic visualization. The previously H&E stained sectioned tissues helped with the identification of the neoplastic cells of the tumour specimens. In general, the size of the nuclei (normally slightly larger than in normal cells) and monomorphic nuclei in size and shape were identified for capture. This technique allowed capturing the cells of interest by focal melting of the membrane through laser activation. Microdissection was multiregional and, when appropriate, within the invasive compound and the *in situ* component of the section. Attention was given to the procurement of the cell avoiding encroachment of adjacent non-neoplastic cells and surrounding stroma. The laser was set according to the histologic features of the sections within the following ranges: laser spot size 7.5-30 μm, pulse power 85-100 mW, pulse width 0.5-5.0 ms and threshold voltage 150 mV. Each laser pulse was estimated to capture approximately 5-20 cells depending on the diameter of the beam used. These capture rates were multiplied by the number of laser pulses used in the capture experiment to estimate the number of cells captured (~1000). The selective transfer and recovery of breast cancer tissue after LCM is shown in Plate 3-4.

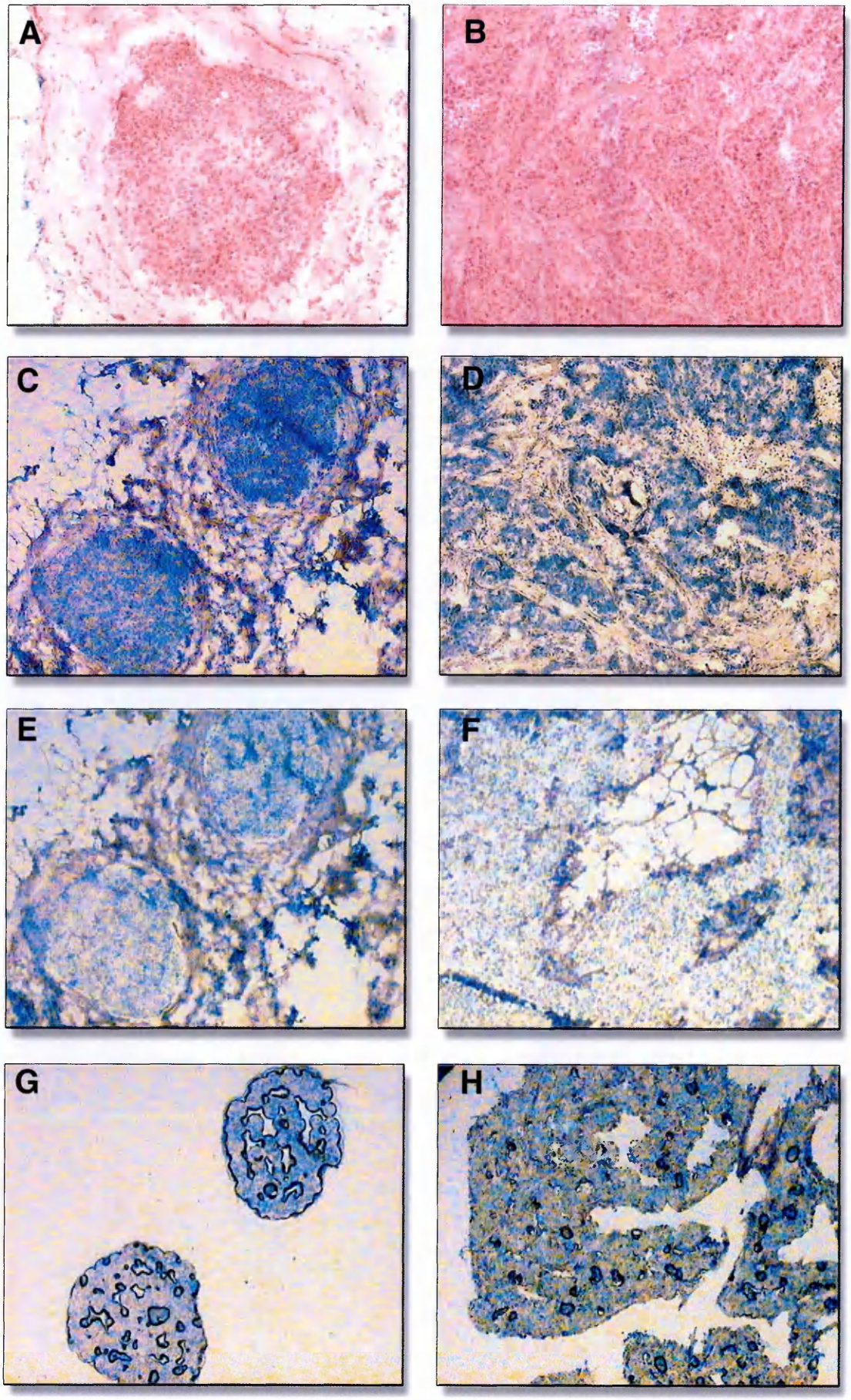

A
B
C
D
E
F
G
H

Plate 3–4 LCM from breast cancer specimens.
Captured cells were dehydrated and stained to help recognise the malignant cells. Sections were inspected after H&E staining (A, B). Cells were captured from the ducts (A, C, E, G) (case of invasive DC grade III associated with DCIS high nuclear grade) and/or from the invasive tumour compartment when appropriate (B, D, F, H) (case of invasive DC grade III). C, D: before laser shot; E, F: after laser shot; G, H: cells captured and fixed to the cap.

3.2.2.3. RNA extraction after LCM

Four 0.5 ml microcentrifuge tubes were pre-loaded with 1 µl of RNase inhibitor (RNaseOUT, Invitrogen) and 50 µl of extraction buffer (XB) and kept on ice (PicoPure RNA isolation kit, Arcturus). The extraction buffer is a GITC based buffer *(i.e.* guanidine isothiocyanate) which is a powerful chaotropic agent necessary for the sample lysis and homogenization prior to RNA extraction. Soon after microdissection the LCM caps with the captured cells were placed on the above tubes. The caps and tubes were put upside down to allow the buffer to cover the cap entirely. The assembled caps and tubes were then placed into an incubation oven at 42°C for 30 minutes. Following incubation the assembly was centrifuged at 800 xg for 2 minutes to collect the cell extract into the microcentrifuge tube and the cap was discharged. All the RNA isolation phases were carried out following the manufacturer recommendations in the protocol for use with CapSure Macro LCM Caps (Arcturus). The purification columns were pre-conditioned pipetting 250 µl of buffer (CB) onto the columns, incubated at RT for 5 minutes and centrifuged at 16.000 xg for 1 minute. The tubes with the cell extract were loaded with 50 µl of 70% ethanol and mixed. This mixture was then centrifuged at 100 xg for 2 minutes immediately followed by a second centrifugation at 16000 xg for 30 seconds. The membrane with the RNA was subject to a first wash with 100 µl of wash buffer (W1) and centrifuged at 8000 xg for 1 minute. To avoid interference from possible DNA contamination DNase treatment was performed (Qiagen ref: 79254), as described in Appendix D. After DNase treatment two additional washes were carried out directly on the membrane of the purification column to purify the RNA. These washes consisted of pipetting 100 µl x2 of wash buffer (W2) followed by centrifugation at 8000 xg for 1 minute and 16000 xg for 2 minutes respectively. The washed purification columns were transferred to new DNase- and RNase-free tubes and 15 µl of elution buffer was added to each one. To recover the purified RNA incubation for 1 minute at RT was allowed followed by centrifugation at 1000 xg and 16000 xg for 1 minute each. Because of the small volumes of RNA

extracted, RNA quantification was not possible and this was a limitation of this study. The purified RNA was thus ready to be used for cDNA preparation.

3.2.2.4. Synthesis of first strand cDNA by reverse transcriptase

First strand cDNA preparation by reverse transcription was performed as described in Chapter 2 (Section 2.2.2.3.). Tissue extracted RNA (7 µl) was mixed with 4.5 µl of water and 1 µl of oligo(dT) primer. By comparison with the *in vitro* study the cDNA synthesis was further optimised due to the low-abundant messages obtained from breast tissues and an additional reverse transcriptase step was performed for all the cDNA preparations. In brief, 0.5 µl of AMV reverse transcriptase (cDNA cycle kit, Invitrogen) was added to the solution previously incubated which was spun briefly and incubated at 42°C for an additional hour. After incubation the solution was spun briefly and placed on ice for the PCR assay.

3.2.2.5. PCR assay

The cDNA synthesised was used as indicated in Chapter 2 (Table 2-2; Section 2.2.2.4.). However, the most important difference compared with the *in vitro* study was a higher volume of cDNA employed in order to obtain constant results. Specifically, the cDNA volume employed for the tissues which was subject to Tri-reagent RNA extraction was 5 µl in a total volume of 25 µl of master mix PCR. Whereas 2 µl of cDNA was included in the master mix components for the tissues from which the RNA was extracted by LCM (Table 3-2). The PCR conditions were as described in Chapter 2 (Section 2.2.2.4.). The negative control consisted of an equal volume of water substituted for the volume of the RNA in the RT reaction and was always performed. The no-RT control was not undertaken because of the limited volumes of RNA extracted from the fresh breast tissues (see also Chapter 2, Section 2.3.1.1.). To prevent amplification from genomic DNA DNase treatment was carried out so that if contamination had occurred this would have contributed minimally to the band signal in the electrophoresis gel.

Table 3–2 Components and volumes of the PCR master mix for breast tissue mRNA transcripts assay after RNA extraction by LCM.

Master mix components	Volume
10x Buffer	2.5 µl
dNTP	0.5 µl
50mM $MgCl_2$	0.8 µl
Forward Primer	0.5 µl
Reverse Primer	0.5 µl
cDNA	2 µl
Taq	0.3 µl
Total	7.1 µl
Nuclease Free Sterile H_2O	7.9 µl
Total	15 µl
10x PCR Enhancer solution	2.5 µl
Nuclease Free Sterile H_2O	7.5 µl
Total	25 µl

3.2.3. MCM protein assay

3.2.3.1. Immunohistochemical assay

The IHC analysis was carried out employing one antibody each time to avoid cross contamination. For the pre-treatment phase of IHC the paraffin sections were dewaxed and cleared in histoclear and then rehydrated in a graded ethanol series (5 minutes x2 washes in 100%, 90% and 70%, respectively) (Appendix B). To unmask all the antigenic proteins the slides were subjected to antigen retrieval (maximum 25 slides per batch), as indicated in Chapter 2 (Section 2.2.3.1.). However, the times set for microwave treatment were different for the histologically normal sections. For these sections the initial treatment in 1 litre of citrate buffer of 9 minutes from cold at 800 W was followed by a half power cycle of 5 minutes (it was 8 minutes for tumour sections). This showed to be the optimal thermal treatment in such sections with high content of fibrofatty tissue. Finally, the slides were dehydrated through a series of ethanol passages as indicated above but in reverse order, cleared in histoclear and mounted for microscopic assessment. Positive controls (placenta) and negative controls (by omitting the primary antibody) were performed. The microscopic images were acquired by an

AxioCam Zeiss camera supported by the software AxioVision Viewer attached to a Zeiss light microscope.

3.2.3.2. Quantification and statistical analysis

The scores for the protein expression in terms of staining intensity and labelling indices were described in Chapter 2 (Section 2.2.3.3). This assessment procedure is that in use at the Department of Histopathology at Cheltenham General Hospital (UK) (McCarthy, consultant histopathologist and Head of the Histopathology Department, Cheltenham General Hospital). Scoring was performed by two independent observers (K.M. and S.D.). Positivity for each antigen was determined by counting the number of positive nuclei. Approximately 1000 cells were counted in each case in at least 5 representative high-powered microscopic fields across the slide. The selection of fields was made at random. That is the first high power field containing a significant number of tumour cells was taken as a starting point. Thereafter, the microscope stage was moved at random away from this point. Contiguous fields were not necessarily used because breast carcinomas are by their very nature heterogeneous in cellular content. Moreover, because some are focally necrotic and many have abundant fibrous tissue stroma, contiguous fields may well contain no or few tumour cells. Therefore, only fields containing a significant number of tumour cells were assessed.

The assessment of staining intensity is not a fully quantitative method of assessing protein expression and is open to subjective variation. However, the degree of subjective variation was minimised by employing two observers using the same methods of field selection. The observers worked blindly of each other's results. Also, comparison with an internal control (*i.e.* normal breast epithelium) was made.

The use of a scoring system such as from 0 to 3 or 5 rather than giving an absolute percentage of cells stained is often applied in histopathology (Allred *et al.,* 1993; Hsu *et al.,* 1998; Biden *et al.,* 1999; Fuqua *et al.,* 2003; McCarthy, consultant histopathologist, 2004). According to McCarthy (consultant histopathologist, 2004) and Toner (consultant histopathologist, Cheltenham General Hospital, 2004) the absolute percentages scoring system is often considered unsuitable because of the difficulties of counting the cells in sections of breast tumour. The use of staining intensity and labelling indices as a semi-quantitative method was reported in a number of studies

(O'Malley *et al.,* 1996; Joki *et al.,* 2000; Zimmermann *et al.,* 1999; El-Sheikh *et al.,* 2001; Lee *et al.,* 2002; Hammock *et al.,* 2003; Zhigang & Wenlv, 2004;). At Cheltenham General Hospital this method of assessing IHC is also used to appraise Her-2/neu over-expression in breast carcinoma (McCarthy, consultant histopathologist, 2004). However, the IHC results of the current research will be discussed in term of labelling index expressing the percentages of cells stained by the antibodies.

For the labelling indices an interobserver reproducibility test was performed by comparing the results of the first observer (K.M.) with those of the second observer (S.D.). The interobserver variability was determined by calculating the mean and the standard deviation for each pair of readings (*i.e.* data from the first observer and the second observer). Thereafter, by dividing the mean of the standard deviations by the mean of the means the percentages indicating the variability were obtained. Specifically, for the tumours the interobserver variation figures were as follows: Mcm2, 6.5%; Mcm5, 6.4%; Mcm7, 6.9%. For the normal tissues the values calculated were: Mcm2, 7.1%, Mcm5, 6.3%, Mcm7, 8.3%. These values were approved by the above mentioned consultant histopathologists and the medical statistician (Foy, Cheltenham General Hospital, 2004).

The distribution of MCM proteins scores on tumour tissues showed a degree of positive skew. Hence, to test the differences of the markers across specimen classes (tumour grade I, II and III) for each stain (Mcm2, 5 and 7) the analysis of variance (ANOVA) as well as the non-parametric Kruskal-Wallis test were employed. To explore the differences between the staining characteristics (*i.e.* intensity of stain and percentage of cells stained) represented by the labelling indices given for Mcm2, 5 and 7 both the parametric pared Student t-test and the non-parametric Wilcoxon matched pairs signed-ranks test were performed. Because of a different degree of positivity only the pared Student t-test and the non-parametric Wilcoxon matched pairs test were carried out for the histologically normal specimens. For the percentage of tumour cells expressing MCM proteins the Pearson correlation coefficient and the non-parametric Spearman's rank correlation coefficient were used to examine correlations between tumour grades and markers and across markers. Parametric and non-parametric tests were employed for a meaningful analysis of the data because the normal distribution postulation accepted for the parametric tests may not be satisfied in all cases. Whereas

parametric tests have more statistical power, the non-parametric tests do not require any assumptions on the distribution of the population and may better reflect the nature of the clinical measurements. However, this means that the 'p' value tend to be higher, making it harder to detect real differences as being statistically significant. For all the statistical tests a two-sided p-value <0.05 was considered statistically significant.

3.3. RESULTS

3.3.1. Transcriptional studies

3.3.1.1. Expression of the human MCM *transcripts in frozen tissues*

The level of *MCM* mRNA expression following about ten analyses for each of the 5 different cases investigated (five for tumour and five for the matched normal tissues) showed inconsistent results. Only one lesion, classified with invasive DC grade II, displayed a number of detectable alterations in expression of the transcripts. These results indicated that the RNA extraction method used was not capable of retrieving sufficient quantities of RNA from the breast tissues for consistent PCR amplification. This observation also suggests that the heterogeneous nature of the breast tissue may be a cause of inconsistent extraction of the genetic material. Figure 3-1 is a representative example and shows the high variability of expression after a number of extractions from the same case of invasive DC grade II. The gel suggests that *Mcm2* mRNA transcripts may to some extent expressed with a band of about 500 bp (lanes 3 and 5). The discrepancy between the two bands intensity is clearly visible. Moreover, the majority of lanes do not show any fragment amplified by the cDNA generated from the multiple RNA extracted. The weak or absent bands could be caused by RNA degradation or RNA absence following extraction. However, this could not be proved since this experiment lacked the presence of the housekeeping gene (*e.g.* β-actin) which would have shown the presence and the integrity of the RNA extracted from the sample.

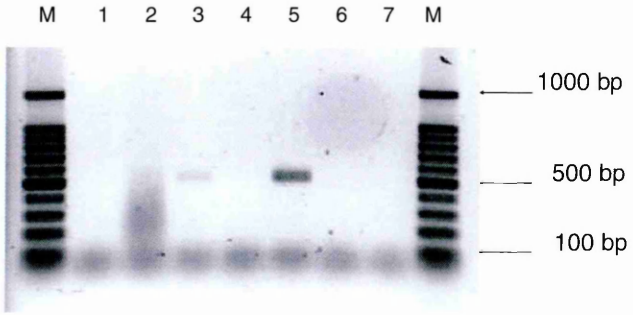


Figure 3–1 Expression of Mcm2 transcripts in breast tumour specimen after RT-PCR. This agarose gel represents the inconsistent results obtained using cDNA synthesised from the RNA extracted by Tri-reagent and subsequent mRNA purification. Various extractions were carried out (6) from the same tumour specimen of a case of invasive DC grade II. Bands in lanes 1, 2, 4 and 6 were absent. A faint band is in lane 3 and a visible band is in lane 5 with a product size of about 500 bp. This experiment may indicate that the methodology applied is not specific and that the heterogeneous composition of the breast tissue could be the cause of this variability. Lane 7: H_2O in place of cDNA in the master mix was used as negative control; lane M: marker of 100 bp scale.

The other tumour cases investigated gave either negative or poor results. Occasionally, there were bands expressed but these were too faint to be considered even marginally positive. Overall, these analyses did not show any repeatability; hence they can not be considered reliable. Of interest is that the weak bands observed were obtained extracting the RNA from the powder of tissues obtained by pestling the specimen in a pool of liquid nitrogen. This may suggest that the tissues were less prone to release the RNase (responsible for degrading the nucleic acid) due to temperature variations induced by repetitive thawing which could be caused by a number of transfers of the tissues which were necessary for the preparation when using the cryostat. In addition, the tweezers used for placing the slices of tissue (~100-200) in the tubes may have been contaminated with RNase, although wiped and cleaned frequently.

The *MCM* transcript levels in histologically normal tissues were never markedly visible. However, after a number of extractions a band of about 500 bp appeared from one case of excised normal tissue retrieved from a patient that was diagnosed with invasive DC grade II. This 500 base fragment of expected size represents *Mcm5* and is visible in lane 7 of Figure 3-2. On the contrary, no bands were visible in the gel for the tumour tissue of the same patient. However, the intensity of the band appeared to be weaker than that of ZR-75-1 which was used as positive control (Figure 3-2, lane 8).

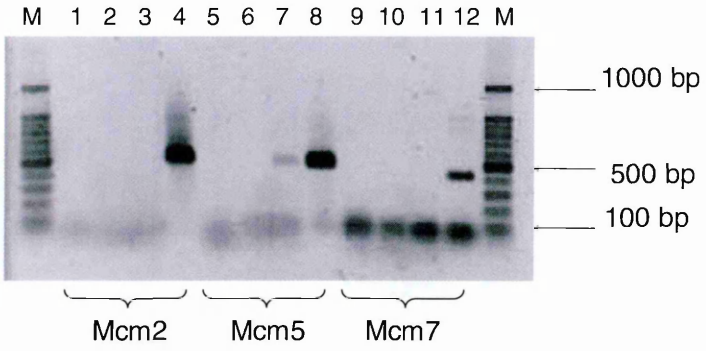


Figure 3–2 mRNA *MCM* transcript levels of histologically normal and tumour specimens. Ethidium bromide staining of tumour and normal tissue taken from a patient diagnosed with invasive DC (grade II). The gel shows a weak band of about 500 bp in lane 7 expressing *Mcm5* in normal tissue. A different extraction in lane 6 was not showing the PCR product suggesting that the variability of the test may depend on the heterogeneous nature of the breast tissue which can influence the RNA extractions. Lanes 1, 5 and 9: tumour tissue; lanes 2, 3, 6, 7, 10 and 11: non-involved tissue; lanes 4, 8 and 12: ZR-75-1 used as control; M: marker of 100 bp scale. H_2O in place of cDNA in the master mix was used as negative control simultaneously but is not shown because the image was cropped on the right.

3.3.1.2. Expression of the human MCM *transcripts after LCM transfer*

To establish a specific, sensitive and reproducible RT-PCR methodology for the analysis of the *MCM* mRNA levels in breast specimens it was necessary to optimise the primers and cDNA concentrations. By comparison with the *in vitro* study carried out with the cell lines, there was no need to adjust reaction temperatures and times. To determine the analytical sensitivity after LCM and to obtain accurate PCR reproducibility of the results 12 different experiments for each breast specimen were carried out (tumour plus associated histologically normal tissue). However, of the five patients investigated following the extraction from the frozen sections (*i.e.* Tri-reagent) only the tissues from two patients were available because most of the tissues retrieved from the mastectomies had already been used for the Tri-reagent RNA extractions.

With the exception of the initial variability encountered with the optimisation of LCM and RNA extraction, all the subsequent experiments gave similar results. The repeated analysis of three cases of infiltrating DC associated with DCIS confirmed that *MCM* transcripts are expressed in neoplastic cells. Two 500 bp fragments of the expected size were amplified from the mRNA fractions for *Mcm2* and *Mcm5* whereas a 407 bp fragment was amplified for *Mcm7*. However, some of the transcripts were

variably expressed in tumour specimens, as shown in Figure 3-3. Overall, these results indicate that *MCM* mRNAs are more abundant in neoplastic cells than in the majority of normal tissues analysed, as it will be later observed. In addition, the amplified bands shown for the two patients in lanes 5-12 confirmed the higher reliability of extracting RNA after LCM technique than from frozen tissues where no comparable base fragments of any expected size were observed.

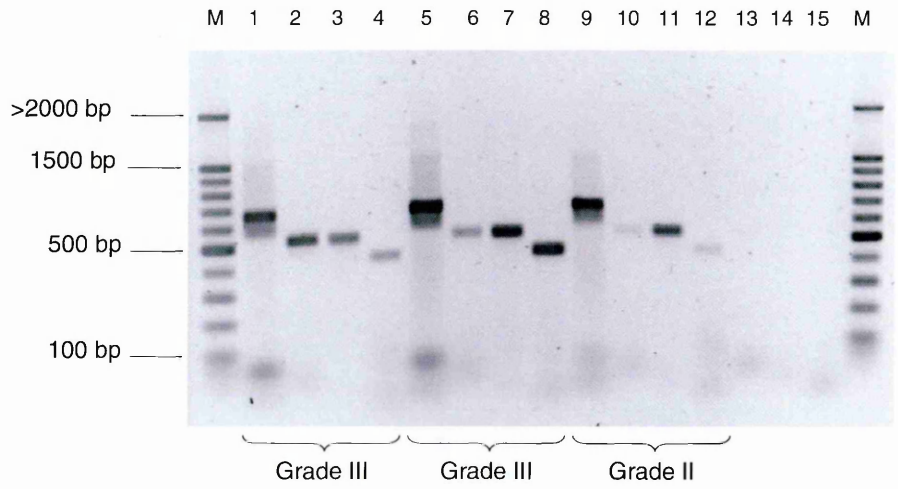


Figure 3–3 Abundance of *MCM* mRNA in neoplastic breast cells captured by LCM. Ethidium bromide staining of tumour breast specimens from 2 cases diagnosed with invasive DC associated with DCIS grade III (lanes 2-4 and 6-8) and 1 case of the same histology classified with grade II (lanes 10-12). Mcm2 (lanes 2, 6 and 10) and Mcm5 (lanes 3, 7 and 11) show bands of about 500 bp, whereas Mcm7 (lanes 4, 8 and 12) shows a band of about 407 bp. The *MCM* transcripts following LCM transfer are variably expressed in all cases. After a number of RNA extractions by LCM transfer the results were showing no significant variations of the transcript expressions. Lanes 1, 5 and 9: *β-Actin* (reference); lanes 13, 14 and 15: H_2O in place of cDNA in the master mix was used as negative control simultaneously for *Mcm2, Mcm5* and *Mcm7* respectively; M: marker of 100 bp scale.

The same analysis was carried out in order to understand the level of transcript variation in associated normal tissues. Only one case of normal tissue obtained from the same patient previously analysed (Figure 3-3, lanes 9-12) showed two 500 base fragments corresponding to *Mcm2* and *Mcm5*. For the other cases no amplification product was visualized on the gel. Visual inspection showed that *Mcm2* was more expressed in this sample compared with the tumour tissues (Figure 3-4, lanes 2 and 3). This may suggest that the cells were starting proliferation, as it may occur in normal mammary tissue due to its particular biology, or alternatively that the tissue may not be

entirely normal. In addition, a similar band was observed of about 500 bp for *Mcm5* using Tri-reagent extraction method from frozen tissues, as shown in Figure 3-2 (lane 7). For this reason this case was also used for Western blotting analysis, as previously reported in Chapter 2 (Figure 2-8, Section 2.3.2.3.).

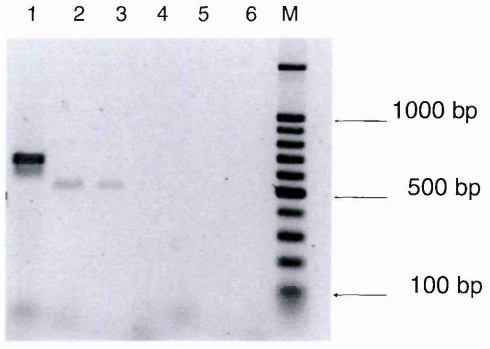


Figure 3–4 *MCM* transcript levels of normal breast tissue after LCM transfer. One case of associated normal tissues showed positivity for Mcm2 (lane 2) and Mcm5 (lane 3) with the expected band size of 500 bp, indicating that specific transfer of the cells to the film occurred. *β-Actin* (lane 1) gene amplification from the same cDNA sample was used as reference. Lanes 4, 5 and 6: H_2O in place of cDNA in the master mix was used as negative control simultaneously for *Mcm2, Mcm5* and *Mcm7* respectively; M: marker of 100 bp scale.

3.3.2. Translational studies

3.3.2.1. Expression of MCM proteins in neoplastic and normal breast tissues

Immunoreactivity of Mcm2, 5 and 7 was well-defined in the nuclei of the cancer cells. The tumour cells were stained in the invasive compartment of the sections as well as in the ducts when associated with DCIS. However, in a number of cases there was an observed variability of both intensity of staining and proportion of cells stained. A comparison of the different immunoreactivity of the tumour cells to MCM antibodies in relation to the tumour grade (III, II and I) is shown in Plates 3-5, 3-6 and 3-7. Infiltrating tumour cells always stained with the MCM antibodies following a similar staining pattern as shown in Plates 3-5 (A), 3-6 (A) and 3-7 (A) where the same case classified with tumour grade III was investigated. Plate 3-6 (arrows in E) shows an example of DCIS of intermediate grade associated with grade I invasive tumour. The proliferative fraction is particularly visible in the neoplastic cells that are breaking the basement membrane and are invading the surrounding stroma as well as in the duct with

the *in situ* component. Very rarely, however, tumours showed minimal or no staining for MCM proteins suggesting that these particular tumours may grow as a result of decreased apoptosis rather than increased cellular proliferation.

There was a variable intensity of staining in tumour lesions as observed by the wide range of both intensity of stain (Mcm2: 1-5, median 3; Mcm5 and Mcm7: 1-4, median 2) and labelling index (Mcm2, 5 and 7; 1-4, median 2) for all the three markers (Tables 3-3 and 3-4). Specifically, the intensity of staining increased with the tumour grade from I to III, being significantly stronger for Mcm2 than Mcm5 and Mcm7 ($p<0.001$) (Figure 3-5 A, B and C). Grade III tumours demonstrated a significantly higher MCM labelling index than grade II and I ($p<0.001$), as shown in Figure 3-6. Exploring the differences between markers the paired Student's t-test showed that Mcm2 stained a higher percentage of cells than Mcm7 ($p<0.001$) and the differences between Mcm5 and Mcm7 could be considered nearly significant ($p=0.041$) since the non-parametric test showed a non-significant trend ($p=0.067$). A similar proportion of cells were stained by Mcm2 and Mcm5 antibodies and there were no significant differences between the two groups ($p=0.208$). The ANOVA test demonstrated that the differences of means across specimen classes of all the three groups (Mcm2, 5 and 7) were similar ($p<0.099$) indicating that, taken together, the three markers were staining a similar percentage of cells. No significant interactions (tumour grade/markers) were observed within the markers either for the intensity of staining ($p<0.940$) or for the labelling indices ($p<0.663$) suggesting a similar behaviour of MCM antibodies (Figures 3-7 and 3-8).

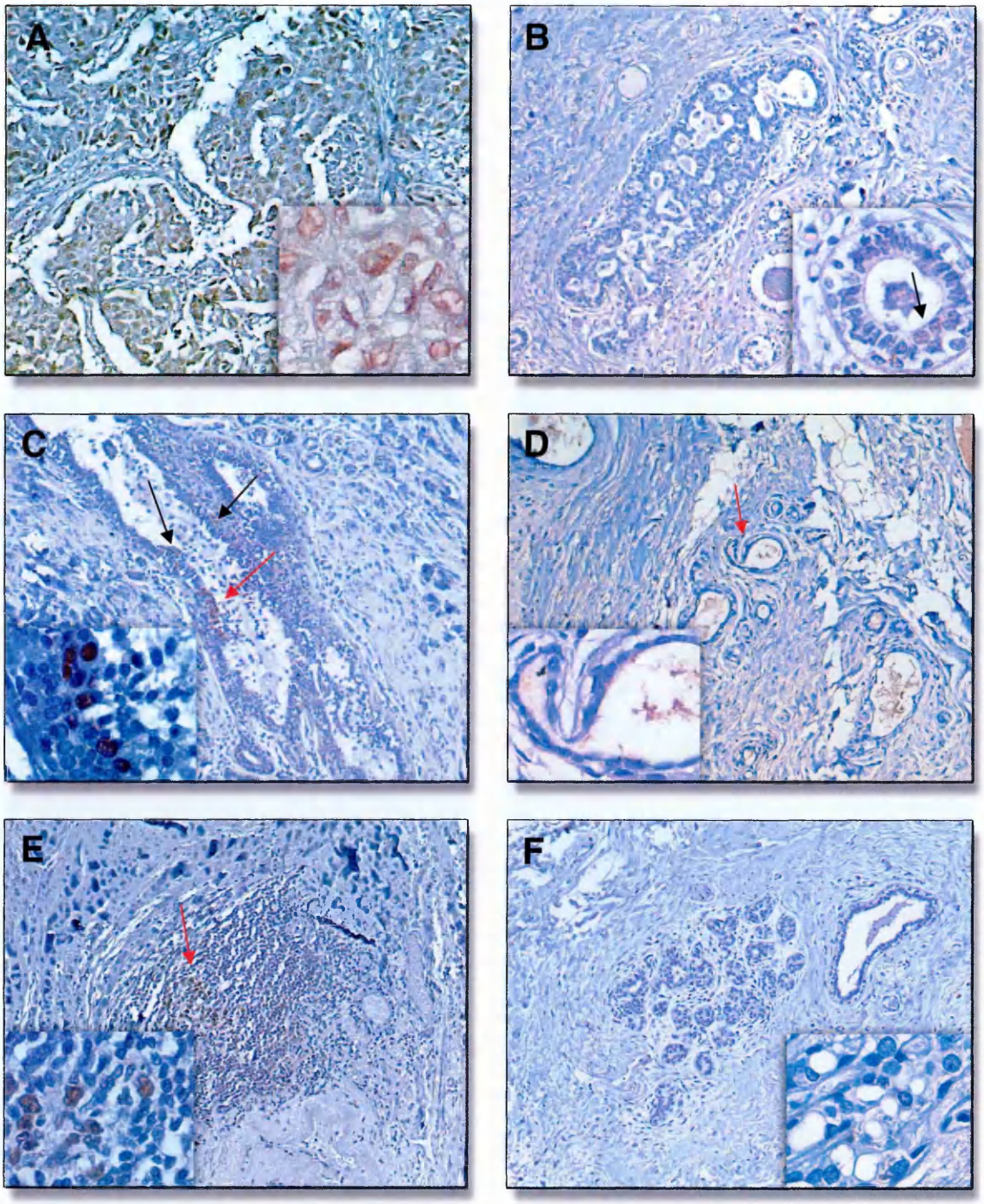


Plate 3–5 IHC for Mcm2 in neoplastic and matched normal breast sections from cases with different tumour grades. Three representative cases of invasive DC classified with tumour grade III (A), II (C) and I (E) are compared with the associated normal tissues taken at least 4cm from the site of the tumour (B, D, F). IHC demonstrated a strong nuclear pattern in all tumour specimens (arrows in A, C and E). The proportion of cells expressing the proliferative markers was shown to decrease from grade III to I ($r=0.64$ $p=0.000$, Figure 3-6**)** with a labelling index ranging 1-74% of cells stained (Table 3-4). Staining for Mcm2 in normal tissue was occasional and weak (arrow in B) within a range of about 0-24% of cells stained (Table 3-5). Differences in both intensity of stain and proportion of immunoreactive cells between tumour and matched normal tissues were always very significant ($p<0.001$). Red arrows represent the zoomed areas when applicable. All objective lens magnifications were 100x and 400x in zoomed windows.

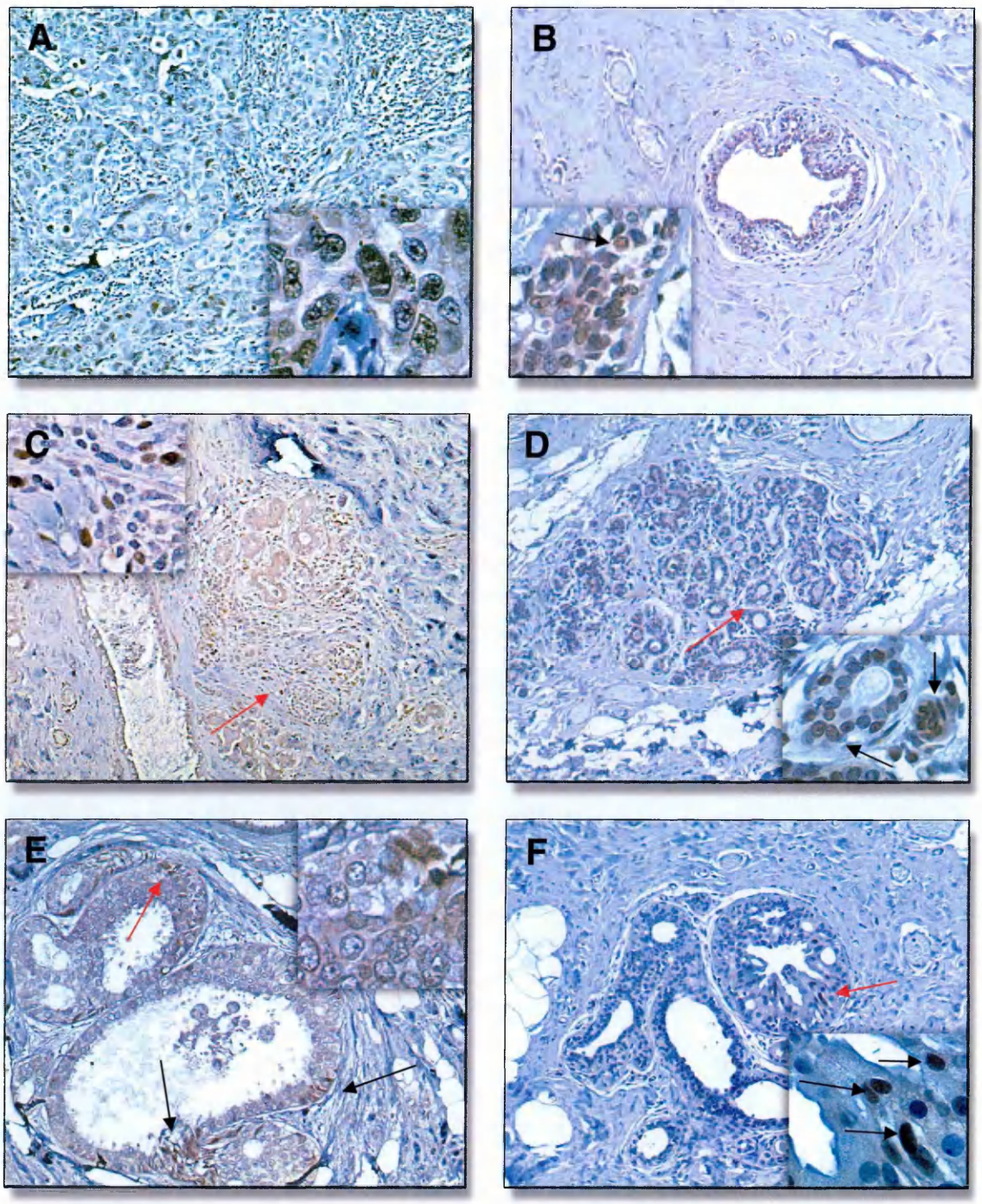


Plate 3–6 IHC for Mcm5 in neoplastic and matched normal breast sections from cases with different tumour grades. Three representative cases of invasive DC (same as Plate 3-5) classified with tumour grade III (A), II (C) and I (E) are compared with the associated normal tissues taken at least 4cm from the site of the tumour (B, D and F). E shows the DCIS component associated with grade I invasive tumour. IHC demonstrated a strong nuclear pattern in all tumour specimens. The proportion of cells expressing the proliferative markers was shown to decrease from grade III to I ($r=0.50$ $p=0.000$, Figure 3-6) with a labelling index ranging 1-74% of cells stained (Table 3-4) (arrows in E represent a smaller percentage of cells stained compared with A and C). Staining for Mcm5 in normal tissue was detected (arrows in B, D and F). Although background noise was observed in these sections (*e.g.* D) probably caused by fixation, the proportion of cells stained ranged 0-24% with the highest median for all associated tumour grades of 1.00 by comparison with Mcm2 (0.00) and Mcm7 (0.50) antibodies (Table 3-5). The proportion of cells stained in normal tissues was similar for all tumour grades (Table 3-5). Differences in both intensity of stain and proportion of immunoreactive cells between tumour and matched normal tissues were always very significant ($p<0.001$). Red arrows represent the zoomed areas when applicable. All objective lens magnifications were 100x and 400x in zoomed windows.

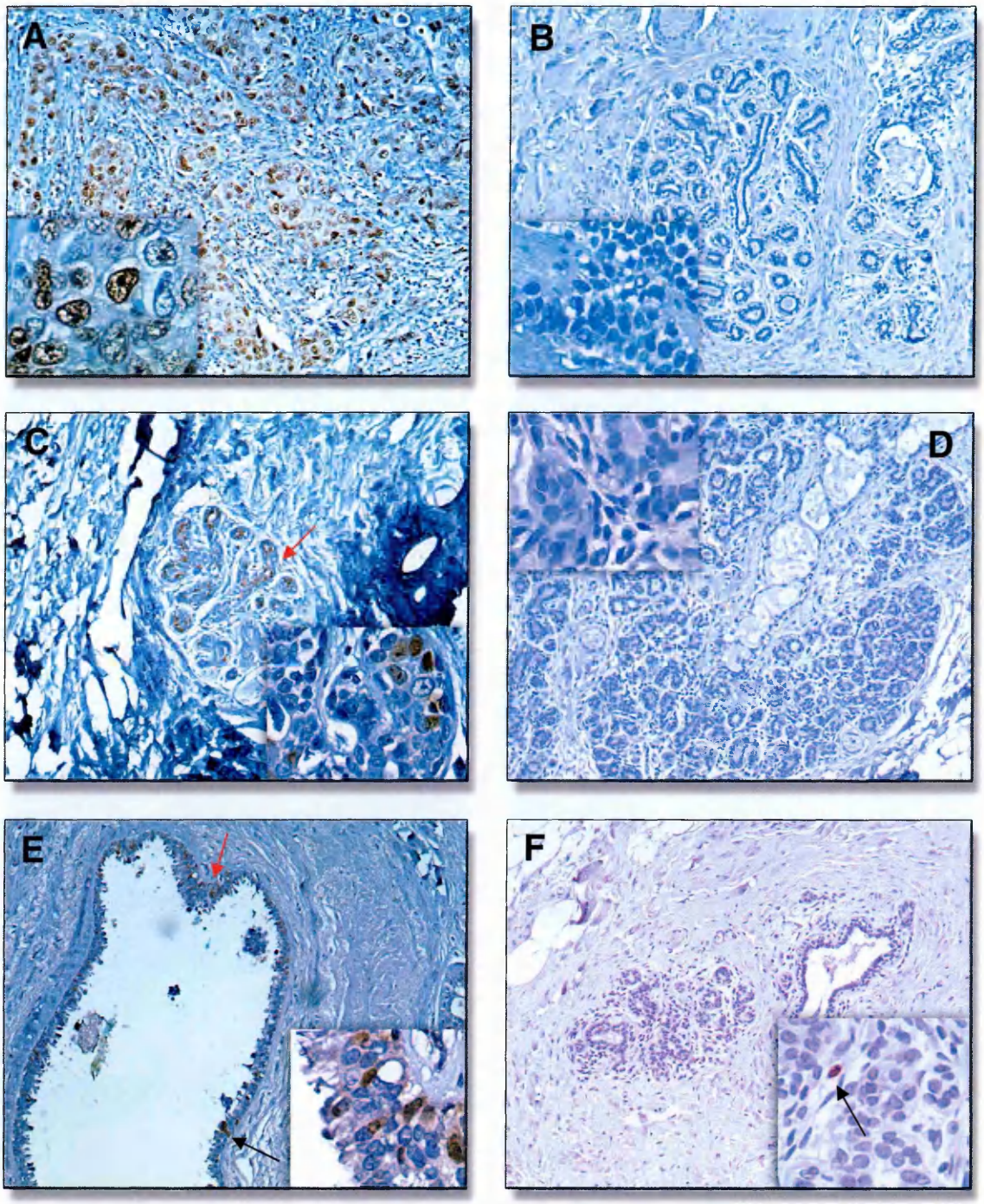


Plate 3–7 IHC for Mcm7 in neoplastic and matched normal breast sections from cases with different tumour grades. Three representative cases of invasive DC (same as Plates 3-5 and 3-6) classified with tumour grade III (A), II (C) and I (E) are compared with the associated normal tissues taken at least 4cm from the site of the tumour (B, D and F). IHC demonstrated a strong nuclear pattern in all tumour specimens. The proportion of cells expressing the proliferative markers was shown to decrease from grade III to I ($r=0.61$ $p=0.000$, Figure 3-6) with a labelling index ranging 1-74% of cells stained (Table 3-4) (arrows in E represents a smaller percentage of cells stained compared with A and C). Staining for Mcm7 in normal tissue was occasional (arrow in F) within a range of about 0-24% of cells stained (Table 3-5). Differences in both intensity of stain and proportion of immunoreactive cells between tumour and matched normal tissues were always very significant ($p<0.001$). Red arrows represent the zoomed areas when applicable. All objective lens magnifications were 100x and 400x in the zoomed windows.

Table 3–3 Descriptive statistic of stain intensity for Mcm2, 5 and 7 in breast tumour specimens.
The intensity of stain ranges from 0 (absent) to 5 (very strong). The ANOVA test showed that there were significant differences in staining intensity among the three markers (p<0.001) [(a)] and that the intensity background increased with the tumour grade [(b, c, d)]. The paired Student's t-test confirmed that Mcm2 intensity was always significantly stronger (p<0.001) [(e,f)] than Mcm5 [(e)] and Mcm7 [(f)]. No significant differences were observed between Mcm5 and 7 (p=0.464) [(g)]. No significant interactions (tumour grade/markers) were observed (p<0.940) indicating a similar trend in the staining characteristics among the markers (ANOVA). Values are shown correct to 2 decimal places. NA (*i.e.* mucinous tumour) was scored 2 for all the three markers. Abbreviations: NA, tumour grade not assessable; SEM, standard error of mean.

Descriptive statistic	Mcm2[a]					Mcm5[a]					Mcm7[a]				
	Total	Grade				Total	Grade				Total	Grade			
		I[b]	II[c]	III[d]	NA		I[b]	II[c]	III[d]	NA		I[b]	II[c]	III[d]	NA
Mean	2.88[e,f]	2.45	2.86	3.22	-	2.09[e,g]	1.73	2.03	2.44	-	2.18[f,g]	1.73	2.06	2.72	-
Median	3	3	3	3	-	2	1	2	2	-	2	1	2	3	-
Range	1-5	1-4	1-5	1-5	-	1-4	1-4	1-4	1-4	-	1-4	1-3	1-4	2-4	-
SEM	0.13	0.34	0.16	0.26	-	0.11	0.30	0.14	0.18	-	0.12	0.27	0.16	0.18	-
Cases	65	11	35	18	1	65	11	35	18	1	65	11	35	18	1

[a] p-value<0.001 for ANOVA and Kruskal-Wallis test.
[b, c, d] p-value<0.001 for ANOVA and p-value=0.000 for Kruskal-Wallis test.
[e] p-value<0.001 for Student t-test and Wilcoxon matched pairs test.
[f] p-value<0.001 for Student t-test and Wilcoxon matched pairs test.
[g] p-value=0.464 for Student t-test and p-value=0.522 for Wilcoxon matched pairs test.

Table 3–4 Descriptive statistic of percentage of cells stained for Mcm2, 5 and 7 in breast tumour specimens.
The percentage of stained cells ranges from 0 (0% cells stained) to 5 (75%-100% cells stained). The ANOVA test showed that the differences of means across specimen classes of all the three groups were not significant (p<0.099) [(a)] indicating that the three markers were staining a similar percentage of cells. However, exploring the differences between markers the paired Student's t-test showed that Mcm2 (p<0.001) [(f)] and Mcm5 (p<0.05) [(g)] stained a higher percentage of cells than Mcm7 [(f,g)], though the latter comparison (Mcm5 against Mcm7) [(g)] was rejected by the non-parametric Wilcoxon matched pairs test (p=0.067). The number of cells expressing all the markers was significantly increased with the tumour grade (p<0.001) [(b, c, d)]. No significant interactions (tumour grade/markers) were observed (p<0.663) indicating a similar behaviour of MCM antibodies against the proportions of cells stained (ANOVA). All Pearson correlation coefficients were very significant: TG *vs* Mcm2 r=0.64 (p=0.000), TG *vs* Mcm5 r=0.50 (p=0.000), TG *vs* Mcm7 r=0.61 (p=0.000); Mcm2 *vs* Mcm5 r=0.60 (p=0.000), Mcm2 *vs* Mcm7 r=0.73 (p=0.000), Mcm5 *vs* Mcm7 r=0.54 (p=0.000). The Spearman's rank correlation coefficient gave a comparable estimate with similar levels of significance. Values are shown correct to 2 decimal places. NA (*i.e.* mucinous tumour) was scored 2 for all the three markers. Abbreviations: TG, tumour grade; NA, tumour grade not assessable; SEM, standard error of mean.

Descriptive statistic	Mcm2[a]					Mcm5[a]					Mcm7[a]				
	Total	Grade				Total	Grade				Total	Grade			
		I[b]	II[c]	III[d]	NA		I[b]	II[c]	III[d]	NA		I[b]	II[c]	III[d]	NA
Mean	2.58[e,f]	1.72	2.43	3.44	-	2.46[e,g]	1.82	2.37	3.06	-	2.26[f,g]	1.64	2.09	3.00	-
Median	2	2	2	4	-	2	1	2	3	-	2	2	2	3	-
Range (labelling index)	1-4	1-3	1-4	2-4	-	1-4	1-3	1-4	1-4	-	1-4	1-3	1-4	2-4	-
Range (labelling index %)	1-74	1-49	1-74	5-74	-	1-74	1-49	1-74	1-74	-	1-74	1-49	1-74	5-74	-
SEM	0.11	0.19	0.12	0.18	-	0.10	0.30	0.11	0.17	-	0.10	0.20	0.10	0.14	-
Cases	65	11	35	18	1	65	11	35	18	1	65	11	35	18	1

[a] p-value<0.099 for ANOVA and p-value=0.112 for Kruskal-Wallis test.
[b, c, d] p-value<0.001 for ANOVA and Kruskal-Wallis test.
[e] p-value=0.208 for Student t-test and p-value=0.259 for Wilcoxon matched pairs test.
[f] p-value<0.001 for Student t-test and Wilcoxon matched pairs test.
[g] p-value=0.041 (p<0.05) for Student t-test and p-value=0.067 for Wilcoxon matched pairs test.

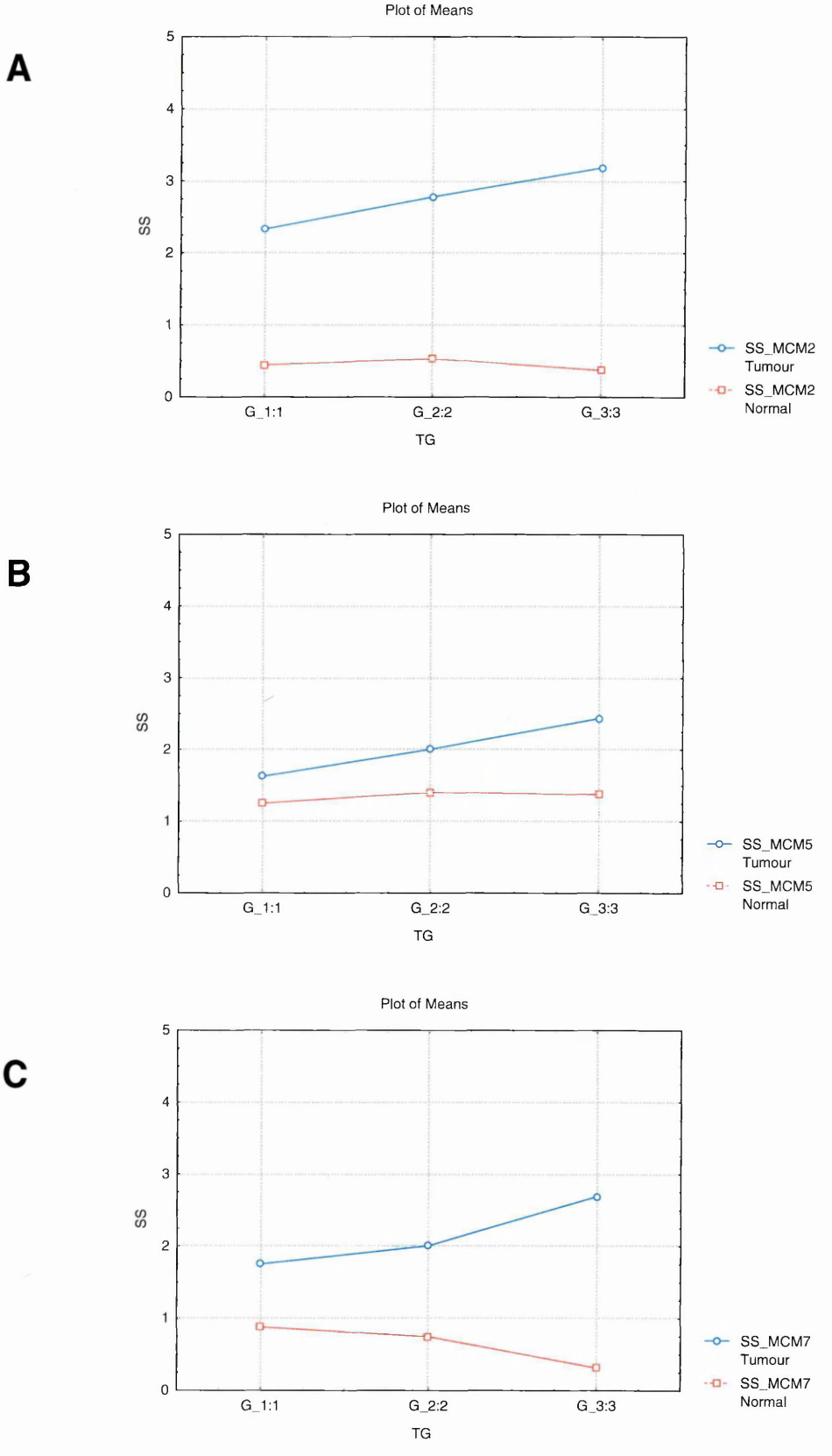
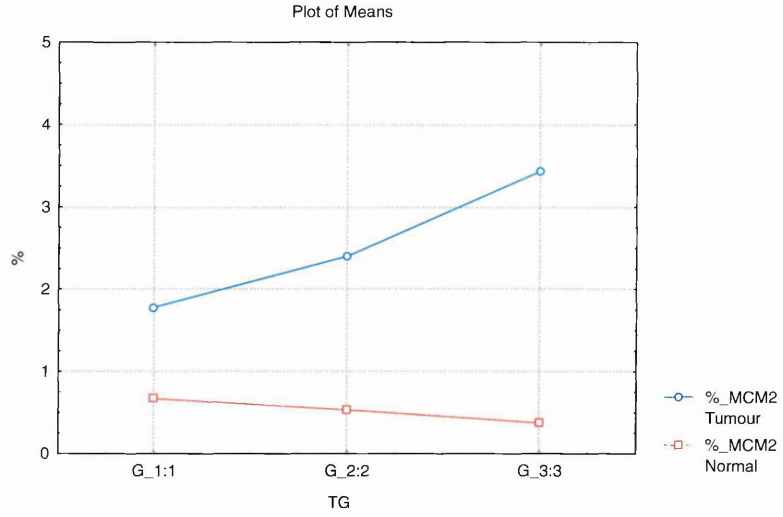
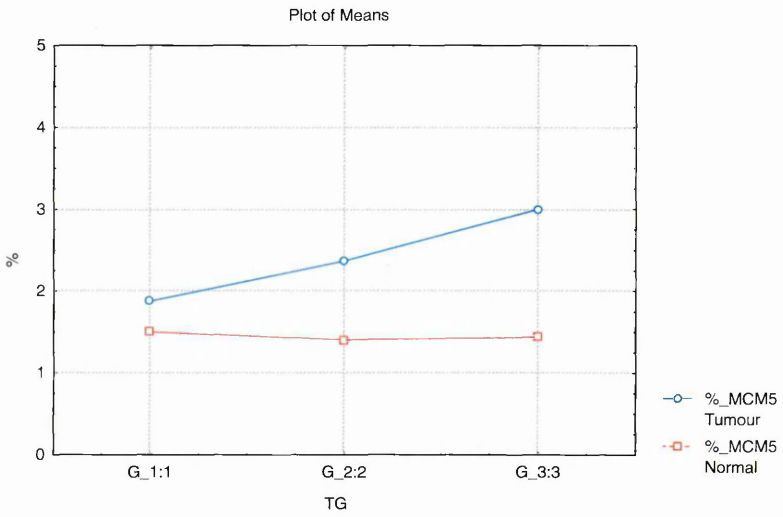


Figure 3–5 Intensity of staining for Mcm2 (A), Mcm5 (B) and Mcm7 (C) in relation to the TG in neoplastic specimens (blue line) and histologically normal tissues (red line). Abbreviations: SS, stain strength; TG, tumour grade.

A



B



C

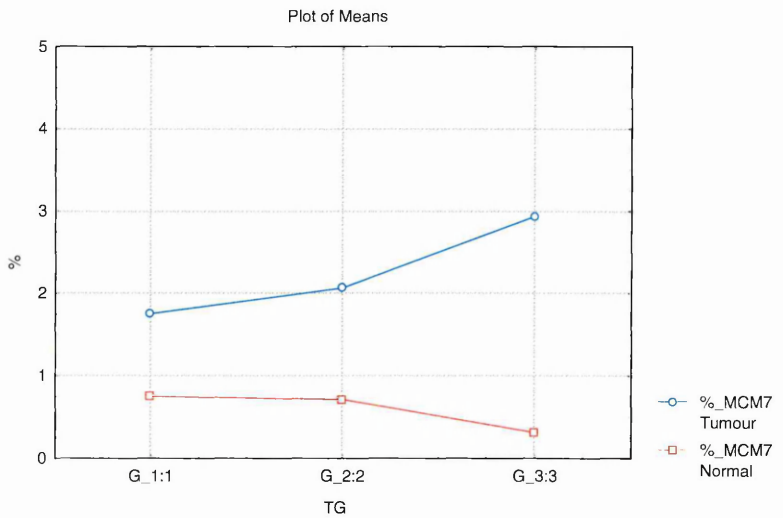


Figure 3–6 Proportion of cells reacting to Mcm2 (A), Mcm5 (B) and Mcm7 (C) in relation to the TG in neoplastic specimens (blue line) and histologically normal tissues (red line). Abbreviations: %, percentage of cells stained; TG, tumour grade.

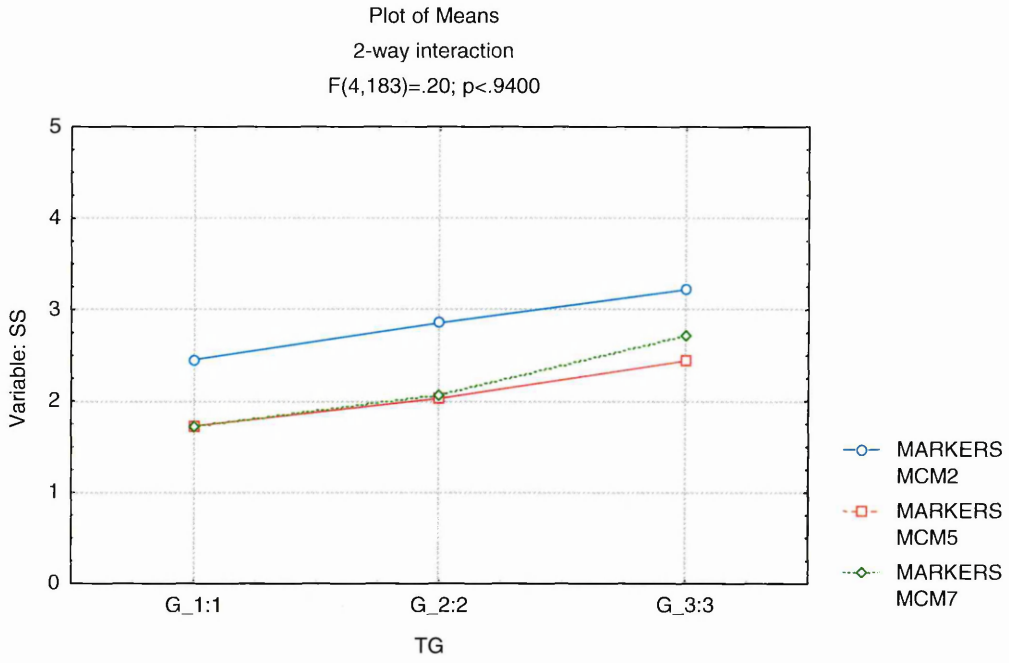


Figure 3–7 Interactions tumour grade/markers for intensity of staining.
No significant interactions were observed (p<0.940) indicating similar trend of the staining characteristics of the markers (ANOVA). Abbreviations: %, percentage of cells stained; TG, tumour grade.

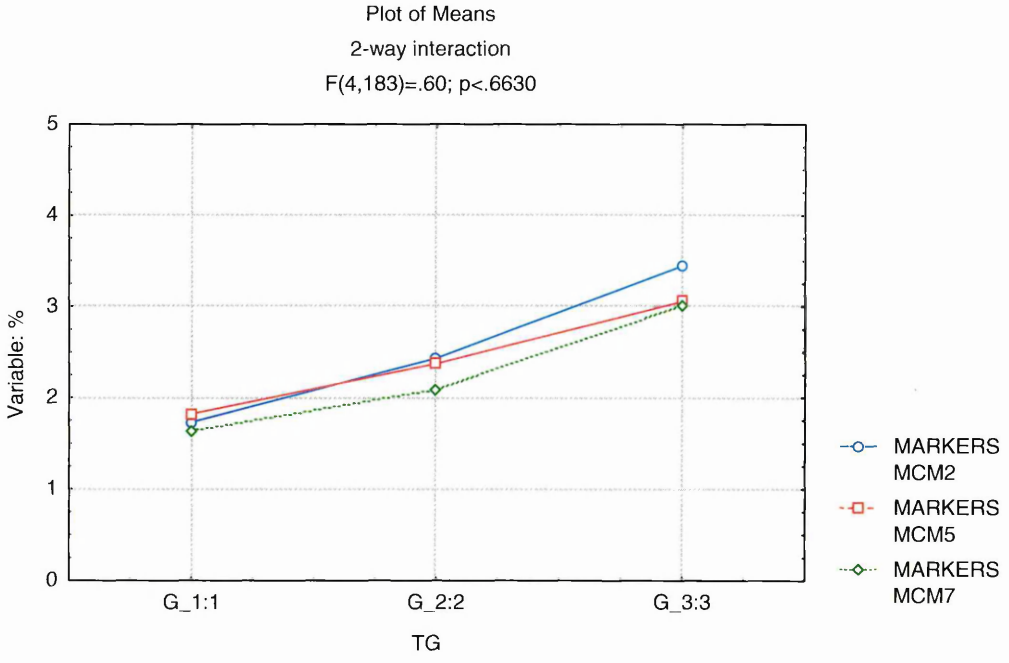


Figure 3–8 Interactions tumour grade/markers for percentage of cells stained.
No significant interactions were observed (p<0.663) indicating a similar behaviour of MCM antibodies against the proportions of cells stained (ANOVA). Abbreviations: %, percentage of cells stained; TG, tumour grade.

A scatterplot, shown in Figure 3-9, suggested that the relationship between MCM labelling indices and tumour grade was very significant and could be modelled as linear. The highest correlation with the tumour grade was for Mcm2 labelling index (r=0.64; p=0.000) followed by Mcm7 (r=0.61; p=0.000) and Mcm5 (r=0.50; p=0.000) (Table 3-4). This indicates that the aberrant expression of the pre-RC follow a positive skew which increases with the tumour grade.

The scatterplots used to examine the relationships between Mcm2, 5 and 7 labelling indices showed that the frequency of the data points were in most cases close to the line generated by the linear equations (Figure 3-10). Although very significant the weakest correlation was between Mcm5 versus Mcm7 labelling indices (r=0.54; p=0.000) (Figure 3-10 C) (Table 3-4). Overall, the correlations among MCM labelling indices were always positive and strong suggesting that MCM proteins act as a complex.

In all cases the correlation coefficients were also confirmed by the non-parametric Spearman's rank analysis which gave comparable estimates with the same level of significance.

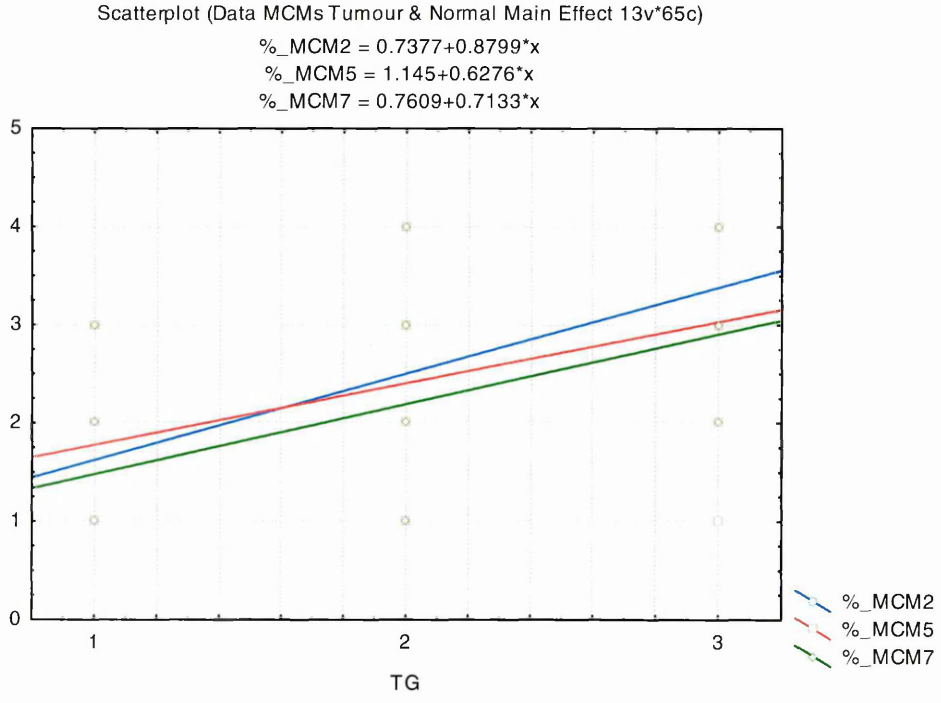
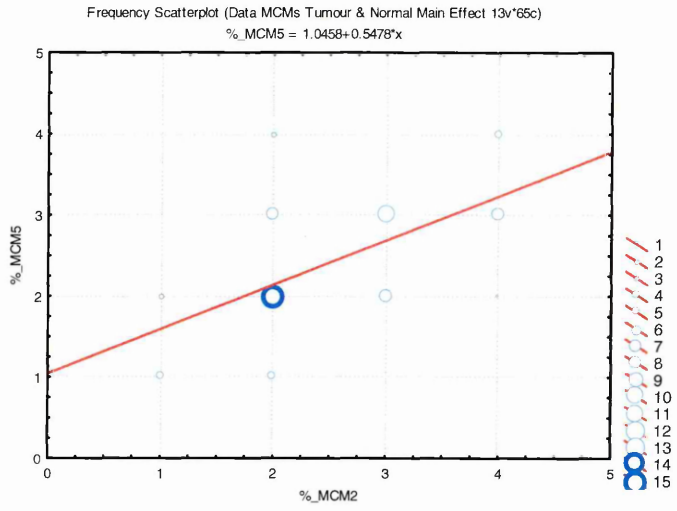
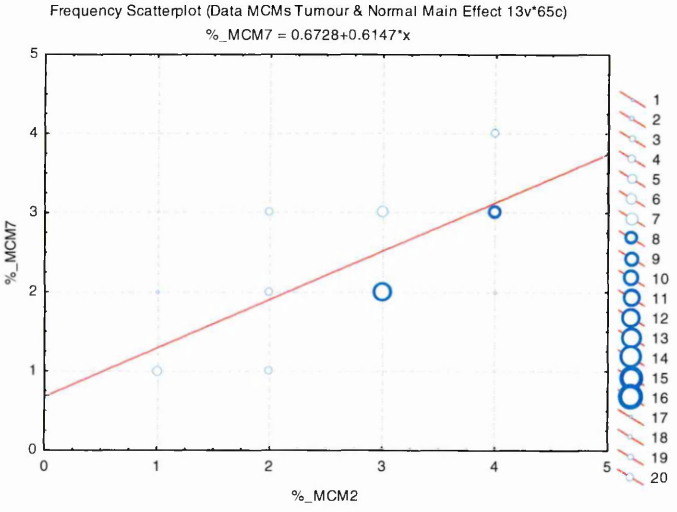


Figure 3–9 Scatterplot of tumour grade *vs* MCM labelling indices.
The correlation between TG and MCM labelling indices was always very significant and could be considered linear (p=0.000). The above regression equations describe the relationships of these data. Abbreviations: %, percentage of cells stained; TG, tumour grade.

A



B



C

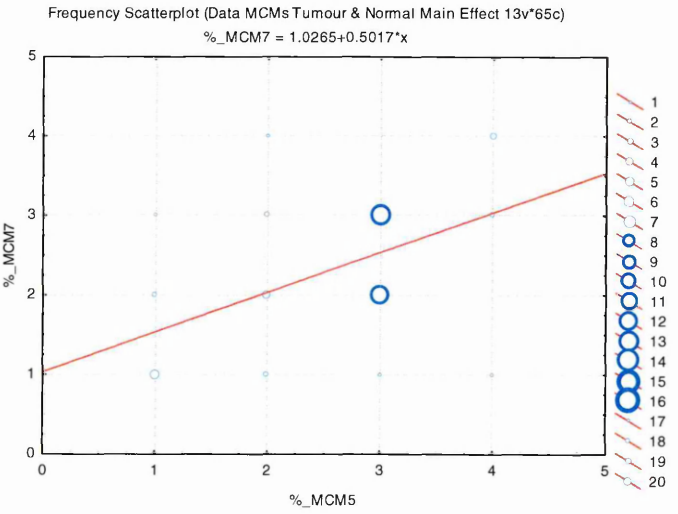


Figure 3–10 Frequency scatterplots of Mcm2 *vs* Mcm5 (A), Mcm2 *vs* Mcm7 (B) and Mcm5 *vs* Mcm7 (C) labelling indices. The regression equations on the top of the diagrams describe the relationships of these data. Abbreviations: %, percentage of cells stained by the antibody.

The immunohistochemical staining of paraffin sections from matched normal tissues with MCM antibodies was scanty and occasional compared with the neoplastic lesions, as shown in the above Plates 3-5, 3-6 and 3-7 (B, D and F). As observed in the neoplastic lesions, when staining was present this affected only the nuclei. In general, the intensity of staining in normal specimens was significantly weaker than in the tumour specimens ($p<0.001$) (Table 3-5) (Figure 3-5). In addition, most of the normal cases showed a significantly lower percentage of MCM proteins expression which was about two fold below the threshold of detection than in cancer specimens ($p<0.001$) (Table 3-5) (Figure 3-6).

The only exception was observed for Mcm5. The expression of Mcm5 in normal specimens was higher compared to Mcm2 and Mcm7 but still not considered to be abnormally elevated when compared with the malignant tissues. Although background noise was detected in the slides stained with anti-Mcm5 antibody (Plate 3-6), which was probably due to the fixation time, the ducts (Plate 3-6 F) and various mammary luminal epithelial acinar cells were expressing the marker (Plate 3-6 D). The proportion of cells stained ranged 0-24% with the highest median for all associated tumour grades of 1.00 by comparison with Mcm2 (0.00) and Mcm7 (0.50), as shown in Table 3-5. These results may indicate that the presence of Mcm5 probably goes further than the normal role of this protein as a signature component of the pre-RC.

A total of seven, ten and nine cases respectively for Mcm2, 5 and 7 were not assessable because of the absence of cells on the slides after microwave treatment of the paraffin sections. The Pearson correlation coefficient displayed negative and no significant relationships of the labelling indices of Mcm2 ($r=-0.09$; $p=0.495$), Mcm5 ($r=-0.02$; $p=0.889$) and Mcm7 ($r=-0.26$; $p=0.060$) with the histological grade (Figure 3-6) (Table 3-5).

Table 3–5 Descriptive statistic of stain intensity percentage of cells stained for Mcm2, 5 and 7 in histologically normal breast specimens.
The intensity of stain ranges from 0 (absent) to 5 (very strong) and the percentage of stained cells ranges from 0 (0% cells stained) to 5 (75%-100% cells stained). The highest labelling index for the intensity of background was 2 for all the markers. The stain strength of Mcm5 was always significantly higher ($p<0.001$) than Mcm2 [(a)] and Mcm7 [(c)]. Similar results emerged through the analysis of the labelling indices expressing the percentage of cells reactive to Mcm5 ($p<0.001$) which were higher by comparison with Mcm2 [(d)] and Mcm7 [(f)] though the highest score was still 2. Pearson correlation coefficients between tumour grade and labelling indices were negative and not significant: TG *vs* Mcm2 r=–0.09 (p=0.495), TG *vs* Mcm5 r=–0.02 (p=0.889) and TG *vs* Mcm7 r=–0.26 (p=0.060). The Spearman's rank correlation coefficient gave similar estimates with similar levels of significance. Values are shown correct to 2 decimal places. NA (*i.e.* mucinous tumour) was scored 0 for both stain strength and proportion of cells stained for all the three markers. Abbreviations: NA, tumour grade not assessable; SEM, standard error of mean.

Descriptive statistic	**Mcm2**					**Mcm5**					**Mcm7**				
Intensity of stain strength	**Total**	**Associated Grade**				**Total**	**Associated Grade**				**Total**	**Associated Grade**			
		I	**II**	**III**	**NA**		**I**	**II**	**III**	**NA**		**I**	**II**	**III**	**NA**
Mean	0.47[a,b]	0.45	0.53	0.38	-	1.34[a,c]	1.25	1.40	1.38	-	0.62[b,c]	0.88	0.74	0.31	-
Median	0.00	0.00	0.00	0.00	-	1.00	1.00	1.00	1.00	-	0.50	1.00	0.00	0.00	-
Range	0-2	0-1	0-2	0-1	-	0-2	0-2	0-2	0-2	-	0-2	0-1	0-2	0-1	-
SEM	0.75	0.18	0.11	0.13	-	0.83	0.25	0.10	0.15	-	0.94	0.23	0.14	0.12	-
Percentage cells stained															
Mean	0.50[d,e]	0.67	0.53	0.38	-	1.40[d,f]	1.50	1.40	1.45	-	0.59[e,f]	0.75	0.71	0.31	-
Median	0.00	0.00	0.00	0.00	-	1.00	0.20	1.00	1.50	-	0.50	1.00	1.00	0.00	-
Range (labelling index)	0-2	0-2	0-2	0-1	-	0-2	0-2	0-2	0-2	-	0-2	0-1	0-2	0-1	-
Range (labelling index %)	0-24	0-24	0-24	0-4	-	0-24	0-24	0-24	0-24	-	0-24	0-4	0-24	0-4	-
SEM	0.82	0.29	0.11	0.13	-	0.84	0.26	0.10	0.16	-	0.87	0.16	0.13	0.12	-
Specimens not assessable	7	2	3	2	-	10	3	5	2	-	9	3	4	2	-
Cases	65	11	35	18	1	65	11	35	18	1	65	11	35	18	1

[a] p-value<0.001 for Student t-test and Wilcoxon matched pairs test.
[b] p-value=0.118 for Student t-test and p-value=0.145 for Wilcoxon matched pairs test.
[c] p-value<0.001 for Student t-test and Wilcoxon matched pairs test.
[d] p-value<0.001 for Student t-test and Wilcoxon matched pairs test.
[e] p-value=0.260 for Student t-test and p-value=0.312 for Wilcoxon matched pairs test.
[f] p-value<0.001 for Student t-test and Wilcoxon matched pairs test.

3.4. DISCUSSION

The work in this chapter evaluated the spatial expression patterns of the Mcm2, Mcm5 and Mcm7 proteins of both neoplastic and matched normal human breast specimens. The results suggest that the cells from the proliferative compartment of the tumour tissues had the MCM proteins up-regulated. In contrast, differentiated cells such as those of normal tissues were accompanied by the disappearance of the components of the pre-RC. Similarly, the gene expression analysis indicated that the mRNA *MCM* transcripts levels were expressed in tumour specimens and were almost absent in matched normal tissues.

The immunohistochemical assessment evaluated both staining intensity and labelling indices as percentage of cells stained by the antibodies because the appraisal of both parameters can be of aid for further patient treatment (McCarthy, consultant histopathologist, 2004). However, this discussion concentrates on labelling indices. The MCM labelling indices showed that the proteins investigated were present in similar proportions in neoplastic sections belonging to the same tumour grade (I, II and III) (Table 3-4 and Figure 3-8). In addition, the labelling indices always showed a positive correlation across the markers (Figure 3-10). This evidence supports the notion that in proliferating cells each protein of the MCM complex (MCM2-7) is present in a similar amount when they prime the replication origins for the initiation of DNA synthesis (Tye, 1999; Adachi *et al.,* 1997). Abnormalities of growth factors, cell cycle control proteins and the proteins that regulate apoptosis have a dramatic effect on tumour growth. As a component of the pre-RC and because MCM are present throughout the cell cycle, their ubiquitous expression represent the point of convergence of numerous signalling pathways involved in cell growth (Stoeber *et al.,* 2001). Hence, the hypothesis that aberrant MCM labelling indices may provide information about the biology and the proliferative status of the tumour. This study also showed that in the series analysed there was a good correlation between the proportions of cells stained for MCM antibodies and the tumour grades ($p=0.000$) (Figure 3-9). This relationship is important since it helps to identify the level of differentiation of the tumours and aids the diagnosis. In fact, poorly differentiated breast carcinomas such as those of tumour grade III or those with extended invasion were more likely to show high levels of MCM proteins in the nuclei of the cells. Contrarily, in grade I tumours few and less stained

nuclei were positive for MCM proteins (Figures 3-5 and 3-6; E in Plates 3-5, 3-6 and 3-7) and in grade II the percentage of nuclei stained was about intermediate (C in Plates 3-5, 3-6 and 3-7). This suggests that the prognostic value of histological grading of tumours is marked by the differences in their proliferation rate (Aaltomaa *et al.,* 1992). A similar correlation between Mcm2 and tumour grade was previously reported in oligodendrogliomas and renal cell carcinomas (Wharton *et al.,* 2001; Rodins *et al.,* 2002).

Here, the study has also extended the analysis of MCM proteins expression from neoplastic lesion to histologically normal tissues. Normal tissues were taken from the same cases that provided the test tissues for various practical reasons. (i) Operative procedures are only rarely carried out on normal breast tissue. The only potential source would have been reduction mammoplasty specimens but it is unlikely that enough specimens would have been available via this route. Also there are potential problems with this, in that the age range of women undergoing reduction mammoplasty is significantly different to that developing breast carcinoma. (ii) If tissues from a different range of cases were to be used, there are inevitably differences in the degree of fixation and there are likely to be differences in the pattern and intensity of staining. In the extreme, immunohistochemical staining may even fail because of inadequate fixation. According to McCarthy (consultant histopathologist, 2004), these potential differences can be minimised by the use of control tissue from the cases providing the test tissues.

It is acknowledged that there are problems with this protocol. The main one is that the tissue taken as a control may not be entirely normal. Whilst it is conceivable that a 'field change' may occur in the breast epithelium surrounding a tumour, the probability of this was minimised by only using breast tissue that was morphologically entirely normal, as judged by Dr. K. McCarthy an experienced consultant histopathologist. Moreover, the lack of multifocality and bilaterality in ductal carcinoma makes it unlikely that such a 'field change' occurs; it is recognised, however, that in the case of lobular carcinoma such 'field changes' are possible.

By comparison with the tumour sections the immunohistochemical analysis consistently showed that the antibodies against Mcm2, 5 and 7 did not identify the same proliferative fractions of cells in matched normal tissues (Figure 3-6). The

immunoreactivity was generally restricted to the nuclei of a small number of mammary luminal epithelial acinar cells representing the population of proliferating cells. Loss of the MCM complex occurs in differentiating cells that have lost the capability to proliferate (Endl *et al.,* 2001). In all of the matched normal cases examined MCM nuclear staining was about two fold lower than in tumours ($p<0.001$). This result suggests that the proliferative components are closely regulated in normal breast tissues. Similar findings have also been reported by other studies where the normal proliferative compartment was examined in a range of tissues with MCM antibodies (Todorov *et al.,* 1998; Freeman *et al.,* 1999; Ishimi *et al.,* 2003). Of particular interest, strikingly higher proportions of cells in the normal fractions were expressing Mcm5 (mean=1.40) than Mcm2 (mean=0.50) and Mcm7 (mean=0.62) (Table 3-5). This observation is consistent for all the normal series analysed and raises a number of issues. Firstly, the dissimilarities within definite population of cells in normal sections could be partially explained because certain tissues may be licensed to replicate and have the potential to exit the 'out-of-cycle' state, but rarely synthesise DNA (Stoeber *et al.,* 2001). Hence, the cells stained for MCM proteins may be in a transitional condition between replication licensing and the resting phase (B, D and F in Plates 3-5, 3-6 and 3-7). This could be explained by the particular nature of the breast epithelium, where the cells are subject to a special adaptation since they undergo rapid and periodic cell expansion and loss. Secondly, the evidence that in normal tissues Mcm5 is the most expressed of the MCM analysed may indicate that some components of the complex (MCM2-7) are present at higher concentration in cells when they are in the 'out-of-cycle' state and do not proliferate. If so, these data suggest that there are elements of the MCM complex that, though normally present throughout the cells cycle, are biologically up-regulated but below the level reached when they are in the active form during G_1-to-S phase transition. Following a semiquantitative assessment of Mcm5 protein levels, Freeman *et al.* (1999) reported that Mcm5 was similarly expressed in normal and cancer cells from uterine cervix, breast and large intestine. Indeed, proteins such as Mcm5 may have additional roles other than DNA replication licensing but this remains to be elucidated. Interestingly, analysing the data from the tumour specimens (Table 3-4) Mcm5 was still the most abundantly expressed in well-differentiated tumour tissues (n=11). Although the labelling index was higher than in normal sections, this parallel

expression of Mcm5 in specimens of low tumour grade (I) may also be linked to an additional role that goes beyond the helicase activity of the protein when complexed with the other MCM components.

The RT-PCR analysis indicated that *Mcm2, 5* and *7* mRNA were more expressed in breast lesions than in matched normal tissues. The enhanced synthesis of Mcm2, 5 and 7 proteins in tumour samples may be explained by the relative high abundance of mRNAs. The transcriptional study, however, examined a small range of tissues for the expression of the three *MCM* mRNAs. This is because the majority of the tissue obtained from the mastectomies had been exhausted in different extraction methods, as mentioned in Section 3.2.2.1.. This was a limitation of this part of the study. Further, RNA extraction from frozen tissues failed to be of use because of the inconsistency of the results obtained. Only after the optimisation of LCM was it possible to overcome this variability which appeared to be caused by the high heterogeneity of the breast clinical samples. In fact, all the repeated experiments carried out with the cells transferred by LCM from different sections of the same sample were analogous in results. Hence, this observation points out that this technology is more sensitive and specific for investigating the mRNA profiles obtained from excised breast specimens.

The PCR analysis suggested that the transcripts appeared more marked in tumour grade III than II (Figure 3-3). The assessment of the RNA integrity along with more sensitive methods such as southern blot or silver-stained polyacrylamide gel (compared to ethidium bromide staining) would have lead to more faithful, detailed and discriminatory information about the expression of the genes and these methods are suggested to confirm these differences. However, it should be noted that within the same specimen the bands of the three transcripts were almost never similar (Figure 3-3). Nevertheless, the present results suggest that the MCM molecule present in the smallest proportion could be the limiting factor for DNA replication initiation, as was also proposed by Tye (1999). In fact, a certain proportion of these subunits may exist in a free form separated from the MCM complex (Tsuruga *et al.,* 1997). The present data also indicate that there is not a specific subunit of the complex that is always present in the lowest amount which is the limiting factor. Therefore, this brings evidence that the signature of the active MCM form could be the presence of the subunits coexpressed in similar proportions. In a recent study Yoshida & Inoue (2003) showed that despite the

enhanced expression of Mcm7, the DNA replication activity was not changed in Epstein-Barr virus. This again may suggest other implications of the free form of proteins in tumour mass formation or in proliferative tissues. It would be of interest to know why some of the pre-RC components are biologically more expressed than others, assuming they have to be present in similar quantities when they prime the replication origins in proliferating cells. Additional studies on the entire complex (MCM2-7) are needed to further clarify this molecular aspect that may contribute to the growth of cancer cells by facilitating genome replication.

An abnormal pattern of *MCM* expression was not reported in normal tissues except in one case which showed minimal levels of *Mcm2* and *5* (Figures 3-2 and 3-4). Nonetheless, the positivity of this specimen was below the threshold of expression of the parent tumour tissue. This may indicate that either the tissue was starting cellular proliferation or part of the lesion excised was closed to the normal one or again the two molecules were present in a free form probably not complexed for the helicase activity. In any case, though minimal, an expression was detected that could trigger inappropriate cell proliferation. This case may therefore suggest that the sensitivity of these markers may be employed for early diagnosis of the cancer. It is unlikely, however, that gene expression analyses using RT-PCR would be routinely employed in the histopathology laboratories in hospital districts for a number of reasons. This includes the possibility of contamination and degradation of RNA under inappropriate temperature conditions that could decrease the yield and effectiveness of the RNA extraction, the cost of the method and the lack of personnel with appropriate technical expertise.

In conclusion, the immunohistochemical results indicated that Mcm2, 5 and 7 protein expressions can estimate the cellular growth fraction in paraffin embedded tissues of breast carcinomas and this facilitates the differentiation between tumour and the matched normal tissues and the assessment of the tumour grade. It might be possible that the stage of breast carcinoma may well be correlated with the MCM protein expression but these data were not available. This correlation could be considered in further studies.

4 Expression of Ki-67, Bcl-2 and ER: a comparative study with MCM proteins

4.1. INTRODUCTION

The studies in this thesis have concentrated so far on elucidating the sensitivity and specificity of the novel MCM biomarkers in identifying the proliferation status of the cells in human derived cell lines and in a population of heterogeneous breast tumours. The purpose of this chapter is to evaluate MCM diagnostic performance with the existing clinical markers of cancer routinely employed in breast pathology. Thus, Ki-67, Bcl-2 and ER have been investigated on the same series of breast carcinomas previously analysed in Chapter 3 with Mcm2, 5 and 7.

A direct comparison with Ki-67 would determine whether the use of MCM biomarkers would more accurately reflect inappropriate cellular proliferation and would have more discriminatory value for the differential diagnosis of breast lesions and for the detection of early breast cancer. For its versatility the cell cycle related molecule Ki-67 is among the most employed in the clinical diagnosis of breast diseases. This proliferation-associated marker detects a nuclear antigen only in proliferating cells and is present in all phases of the cell cycle except in G_0 and G_1 (Gerdes *et al.*, 1984) (Chapter 1, Section 1.3.4.).

The comparison of MCM proteins with Bcl-2 and ER has not been characterized in the literature before. This analysis will delineate the behaviour of MCM proteins expression in the breast proliferative lesions. The oncoprotein Bcl-2 was chosen because in tumours its over-expression is often associated with low proliferation rate (van Slooten *et al.*, 1998; Rehman *et al.*, 2000; Siziopikou & Schnitt 2000). The intracellular steroid hormone receptor ER was chosen not only because it is the most commonly used molecular prognostic marker in breast cancer, but also for its positive correlation with a higher degree of differentiation (Margolese *et al.*, 2000; reviewed by

Sugg & Donegan 2002). Hence, an inverse association of the proliferative proteins with Bcl-2 and ER would additionally clarify the functional potentiality of MCM proteins to discriminate the level of cellular proliferation in breast cancer.

This chapter also extends the investigation to normal breast tissue from cancer patients and compares the different patterns of expression of Ki-67 between normal and tumour specimens. The Bcl-2 and ER expressions in normal tissues have not been reported because a comparison with the tumour sections is inappropriate. In fact, in a non-pregnant and non-involuting female Bcl-2 protein is expressed in the normal ductal epithelia of the mammary gland, albeit not deregulated such as in ductal carcinomas (Kumar *et al.,* 2000). Moreover, ER-positive cells are often found within the normal luminal epithelial cells of the breast lining ducts and lobules (Speirs *et al.,* 2002). The pattern of these two markers is different in tumour and normal breast but the aim of the comparison was to validate the biological activity of the proliferative markers with the regulatory proteins Bcl-2 and ER.

By comparison with the previous chapters, a different protocol was used for IHC since an automated immunostaining system was employed. Important features of an automated system are constant staining quality and consistent results. Hence the effectiveness of the MCM manual immunostaining method was compared with the automated system. Although both staining strength and labelling indices expressed as percentage of cells stained have been assessed, the discussion concentrates on the second parameter as in Chapter 3.

The transcriptional studies were not performed because of the scarcity of breast tissues from which to retrieve additional RNA for RT-PCR. As in Chapter 3, the statistical analysis included both parametric and non-parametric tests. This is because parametric tests are more robust and sensitive than non-parametric methods. However, the non-parametric tests take into account the non-Gaussian distribution in clinical data.

4.2. MATERIALS AND METHODS

4.2.1. Clinical specimens

The same patient population (n=65) previously studied with the MCM proteins was investigated in this work. The clinicopathological data of the patients are outlined in Table 3-1 (Chapter 3, Section 3.2.1.). In an attempt to compare the same fractions of

cells within different antibodies immunostaining, the paraffin-embedded breast tumour sections and the macroscopically and microscopically matched normal tissues were obtained by sequential cutting from the same paraffin blocks previously used. The tissues were embedded in paraffin and cut 4 μm thick, as described in Chapter 3 (Section 3.2.1.). The sections were, as far as possible, wrinkle-free as recommended by the manufacturer instructions to assist the automated staining (Dako, ChemMate code No. K 5001).

4.2.2. Antibodies

For the immunohistochemical detection of Ki-67 the rabbit anti-human polyclonal antibody was employed for labelling the cells expressing the protein (Dako, code No. A 0047). The Bcl-2 antigen was demonstrated using the monoclonal mouse anti-human antibody clone 124 (Dako, code No. M 0887). The monoclonal mouse anti-human ER clone 1D5 was used to assess the ERα positive nuclear antigen (Dako, code No. M 7047). Binding of the primary antibodies was assessed by biotinylated secondary antibodies goat anti-rabbit and goat anti-mouse, respectively (Dako, ChemMate code No. K 5001).

4.2.3. Immunohistochemical assay

The paraffin sections were dewaxed in xylene (Fisher Chemicals) and rehydrated through graduated ethanol to water, as described in Chapter 3 (Section 3.2.3.1. and Appendix B). For all the antibodies microwave antigen retrieval pre-treatment was carried out according to the settings in use at the Department of Histopathology at Cheltenham General Hospital. Antigen retrieval was performed by microwaving the sections (maximum 20 slides per batch) in citrate buffer at pH 6.0 (same composition as in Chapter 2, Section 2.2.3.1.) for 20 minutes from cold at 800 W. The slides were allowed to cool down slowly inside the microwave for 20 minutes.

Optimum primary antibody dilutions were predetermined using known positive and negative control tissues. The primary antibodies were diluted in ChemMate diluent buffer (Dako, code No. S 2022) as follows: Ki-67 1:400; Bcl-2 1:100; ER 1:200. Subsequent steps of the immunostaining procedure were performed using the DAKO

Autostainer (Universal Staining System) at room temperature. The automated steps are showed in Table 4-1.

Table 4–1 Sequential steps and incubation times set for the automatic immunohistochemical staining of Ki-67, Bcl-2 and ER.

Immunostainer sequential phases	Incubation time (minutes)
1. Buffer Rinse	
2. Primary antibody (Ki-67, Bcl-2, ER)	60
3. Wash buffer	2
4. H_2O_2 blocking solution	10
5. Wash buffer	2
6. Secondary antibody (biotinylated)	45
7. Wash buffer	5
8. Tertiary detection reagent (HRP):	45
9. Wash buffer	2
10. DAB substrate	2 times x 5
11. H_2O Rinse	

For the washing steps the Dako ChemMate buffer concentrate was diluted 1:10 in distilled H_2O for use (code No. K 5006). The non-specific binding sites were blocked with the Dako ChemMate peroxidase-blocking solution (code No. S 2023). Finally, the incubation with biotinylated secondary antibodies, the tertiary detection (after incubation with strepavidin-horseradish peroxidase) and the incubation with DAB chromogen were performed with the Dako ChemMate kit (code No. K 5001). Sections were then lightly counterstained with Mayer's hematoxylin (Dako, code No. S 3309), dehydrated through an ethanol series followed by exposure to xylene and coverslipped.

Negative controls for each case included omission of the primary antibody and incubation with Dako ChemMate antibody diluent (Code No. S 2022). Positive controls (per each IHC run) for Ki-67 and Bcl-2 primary antibody staining included normal appendix, whereas for ER a known breast carcinoma was employed.

4.2.4. Quantification and statistical analysis

For all the markers the immunoreactivity was scored by the relative intensity of staining and by counting the number of positive stained cells and was expressed as a

percentage of the total tumour cells (≥ 1000), as described in Chapter 3 (Section 3.2.3.2.). As in the assessment of Mcm2, 5 and 7 labelling indices, areas of high labelling were chosen for the assessment of Ki-67, Bcl-2 and ER. Thereafter, for Ki-67 the results were expressed as a labelling index representing the percentage of positively stained nuclei out of the total number of nuclei counted in the representative microscopic fields. Similarly, for ER the number of nuclei was counted to allow statistical comparison. However, to discriminate positive from negative cases the cutoff level of one nucleus stained for ER was considered, as in common use at the Department of Histopathology at Cheltenham General Hospital. Cytoplasmatic staining was considered for Bcl-2 and followed the same criteria as for the others markers. No cutoffs were introduced for Bcl-2 and Ki-67 expression. An interobserver variability test was performed as described in Chapter 3 (Section 3.2.3.2). For Ki-67 tumour cases the interobserver variation was 5.9%, whereas for the normal cases was 8%. For Bcl-2 and ER a variation of 5.4% and 3.9% was observed respectively. These values were considered satisfactory by the consultant histopathologist (McCarthy, 2004) and the medical statistician (Foy, 2004).

Statistical analysis was performed employing parametric (ANOVA, Student t-test and Pearson correlation coefficient) and non-parametric tests (Kruskal-Wallis test, Wilcoxon matched pairs test and Spearman's rank correlation coefficient) as delineated in Chapter 3 (Section 3.2.3.2.). The survival curve for prognosis evaluation was not calculated because of the limited number of years of surveillance. To test the predictive power of the proliferative markers to distinguish between tumour and normal tissues and to analyse if the tumour grade can be predicted by the markers a multivariate logistic regression analysis using the Chi-square model was performed, as suggested by the medical statistician at Cheltenham General Hospital (Foy, medical statistician, 2004). The Bcl-2 and ER markers were also tested to understand if they could predict the grade of the tumour. A value of $p<0.05$ was considered significant for all the statistical tests.

4.3. RESULTS

4.3.1. Expression of Ki-67 in tumour and non-neoplastic breast tissues

The Ki-67 expression was always confined to the cell nuclei of the positive cells which are expected to proliferate. The intensity of staining varied from weak to very intense both within and between tumours and the positive nuclei were in the majority of cases identifiable although with considerable intratumoural variation. Hence, there was a wide range of tumour immunoreactivity in the cases studied (Plate 4-1). The proliferation marker stained the invasive tumour cells (Plate 4-1 A) as well as the areas of DCIS (Plate 4-1 C). Interestingly, most of the poorly differentiated breast carcinomas (grade III) showed an almost twice as high proliferation labelling index than in well-differentiated (grade I) and moderately differentiated (grade II) lesions (Table 4-2). Overall, the analysis of variance showed that the percentage of proliferating cells was significantly increasing with the tumour grade from I to III ($p<0.001$) (Table 4-2 and Figure 4-1). This trend was not displayed for the intensity of staining (Table 4-2 and Figure 4-2). A frequency scatterplot showed that the majority of cases were close to the line generated by the regression equation which describes the positive association between Ki-67 labelling index and tumour grade (Pearson $r=0.46$; $p=0.000$), as represented in Figure 4-3.

Such pattern of expression was not observed in the benign cells that exhibited occasional immunoreactivity to Ki-67, albeit usually to a much less extent than the grade I tumours (Plate 4-1 B, D and F). Background staining, when present, was in most cases low and showed by few cells never being cytoplasmatic (Plate 4-1, arrows in D and F). Both parametric and non-parametric tests indicated a very significant difference in staining intensity and mean labelling indices between tumour and normal tissues ($p<0.001$) (Table 4-2). This suggests a lower proliferation rate in normal breast tissues compared with matched tumours. A total of seven cases were not assessable because of the absence of cells on the slides after microwave treatment of the paraffin sections.

No significant correlation was seen between Ki-67 and tumour grade in normal sections ($r=0.16$; $p=0.223$) (Table 4-2). This trend was further confirmed by the similar estimates calculated with the non-parametric test.

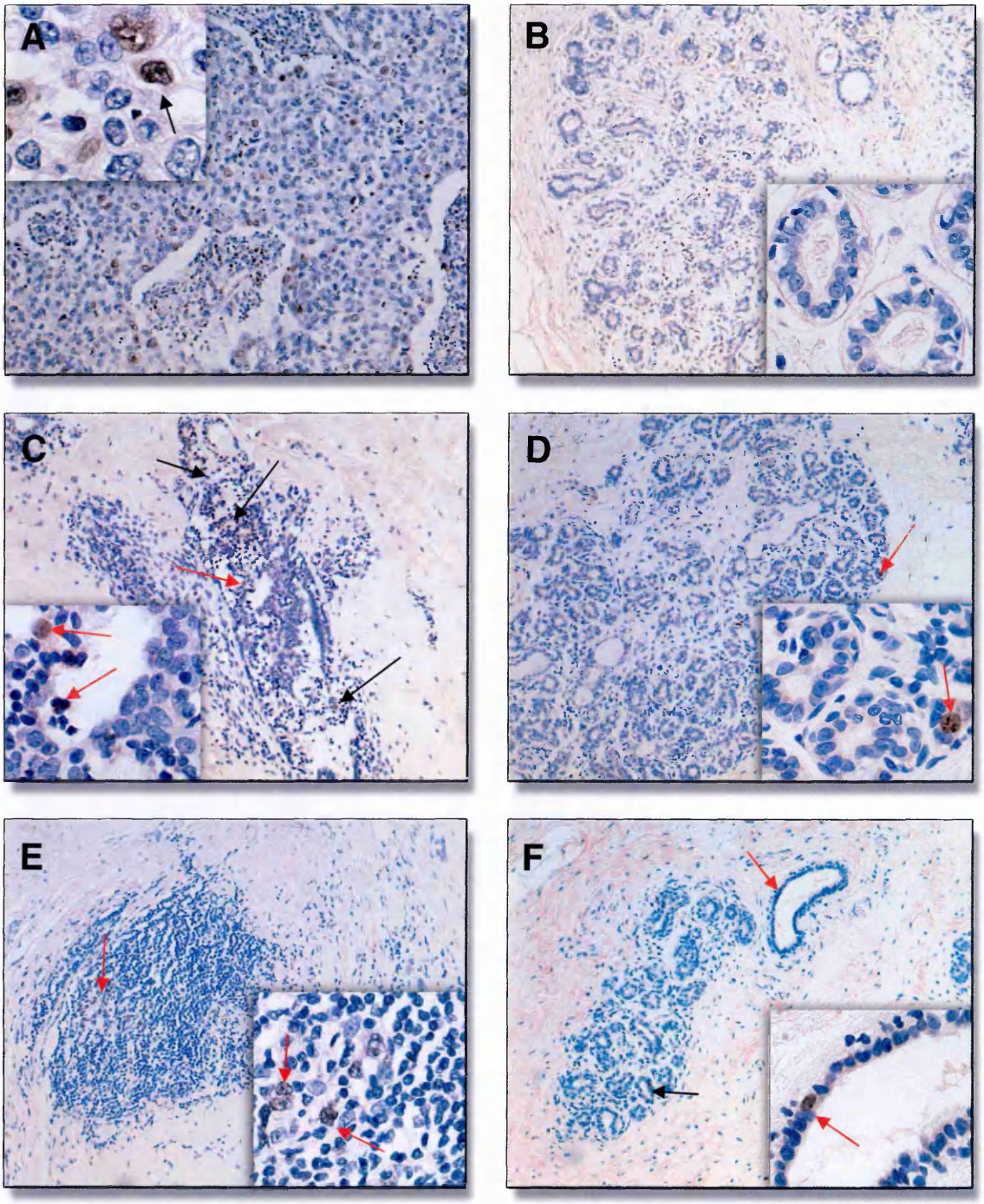


Plate 4–1 IHC for Ki-67 in neoplastic and matched normal breast sections from cases with different tumour grades. Three representative cases of invasive DC (same as for Plates 3-5, 3-6 and 3-7 in Chapter 3, Section 3.3.2.1.) classified with tumour grade III (A), II (C) and I (E) are compared with the associated normal tissues (B, D and F). IHC demonstrated a nuclear pattern in all specimens. The proportion of cells expressing the proliferative markers was shown to decrease from grade III to I ($r=0.46$; $p=0.000$) (Figure 4-1) with a labelling index ranging 0-74% of cells stained (Table 4-2). Arrows in E show the smaller number of cells stained compared with A and C. Staining for Ki-67 in normal tissue was occasional (arrows in D and F). The proportion of cells stained ranged 0-49% with a lower median value (median=1) compared to the tumours (median=2) (Table 4-2). Differences in both intensity of stain and proportion of immunoreactive cells between tumour and matched normal tissues were always very significant ($p<0.001$). All objective lens magnifications were 100x and 400x in zoomed windows (red arrows represent the zoomed areas).

Table 4–2 Descriptive statistic of stain intensity and percentage of cells stained for Ki-67 in tumour and normal breast adjacent to tumours specimens. The intensity of stain ranges from 0 (absent) to 5 (very strong) and the percentage of stained cells ranges from 0 (0% cells stained) to 5 (75%-100% cells). The paired Student's t-test and the non-parametric Wilcoxon matched pairs test confirmed that Ki-67 intensity and percentage of cells stained in tumour sections were always significantly stronger ($p<0.001$) [a, c] than normal [a, c]. The ANOVA test showed a significant effect of the tumour grade ($p<0.001$) [b, d] for the background intensity and the labelling index indicating that these parameters increased with the tumour grade. This trend was further confirmed by the significance of the Pearson correlation coefficients for tumour: TG *vs* Ki-67 labelling index $r=0.46$ ($p=0.000$). The correlation coefficient for the normal sections was weak and not significant: TG *vs* Ki-67 labelling index $r=0.16$ ($p=0.223$). The non-parametric Spearman's rank correlation coefficient gave a comparable estimate with similar levels of significance. Values are shown correct to 2 decimal places. NA (*i.e.* mucinous tumour) was scored 1 for stain strength and 2 for number of cells stained. Abbreviations: NA, tumour grade not assessable; SEM, standard error of mean.

Descriptive statistic	Ki-67 Tumour					Ki-67 Normal				
Intensity of stain strength	**Total**	**Grade**				**Total**	**Associated Grade**			
		I	**II**	**III**	**NA**		**I**	**II**	**III**	**NA**
Mean	2.06[a]	1.90[b]	1.69[b]	2.95[b]	-	1.08[a]	1.00	1.06	1.21	-
Median	2	2	2	3	-	1	0.5	1	1	-
Range	0-5	0-4	0-3	0-5	-	0-4	0-4	0-3	0-4	-
SEM	0.15	0.31	0.18	0.32	-	0.14	0.42	0.16	0.35	-
Percentage cells stained										
Mean	1.60[c]	1.18[d]	1.31[d]	2.39[d]	-	0.84[c]	0.60	0.85	1.00	-
Median	2	1	1	2.5	-	1	0.5	1	1	-
Range (labelling index)	0-4	0-3	0-3	0-4	-	0-3	0-2	0-3	0-2	-
Range (labelling index %)	0-74	0-49	0-49	0-74	-	0-49	0-24	0-49	0-24	-
SEM	0.12	0.23	0.13	0.24	-	0.10	0.22	0.13	0.23	-
Specimens not assessable	-	-	-	-	-	7	1	2	4	-
Cases	65	11	35	18	1	65	11	35	18	1

[a] p-value<0.001 for Student t-test and Wilcoxon matched pairs test.
[b] p-value<0.001 for ANOVA and p-value=0.004 for Kruskal-Wallis test.
[c] p-value<0.001 for Student t-test and Wilcoxon matched pairs test.
[d] p-value<0.001 for ANOVA and p-value=0.000 for Kruskal-Wallis test.

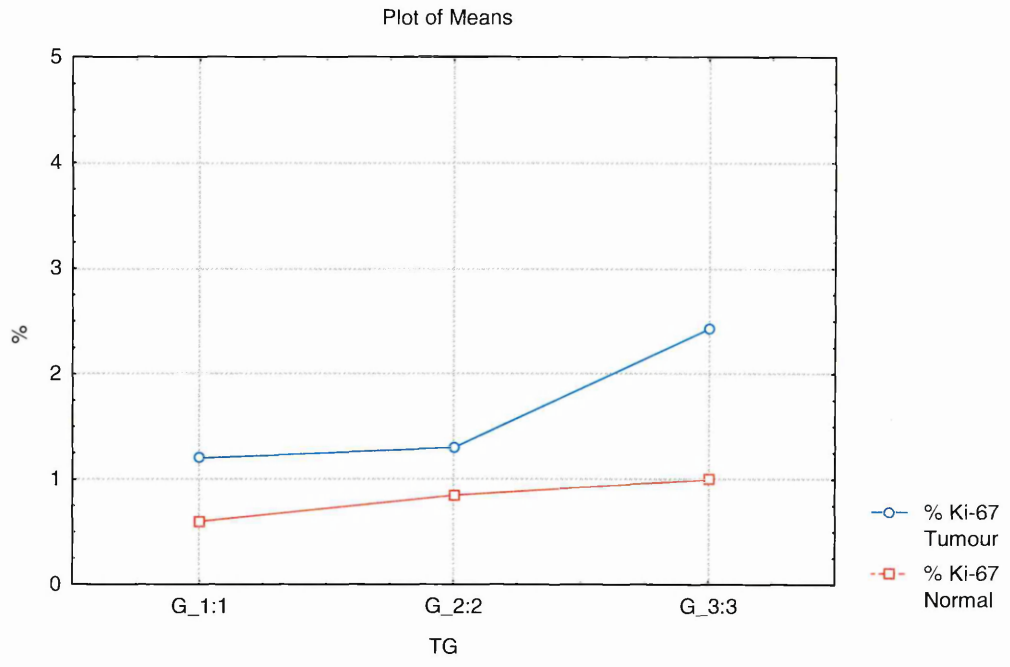


Figure 4–1 Proportion of cells reacting to Ki-67 in relation to the TG in neoplastic specimens (blue line) and histologically normal tissues (red line). Abbreviations: %, percentage of cells stained; TG, tumour grade.

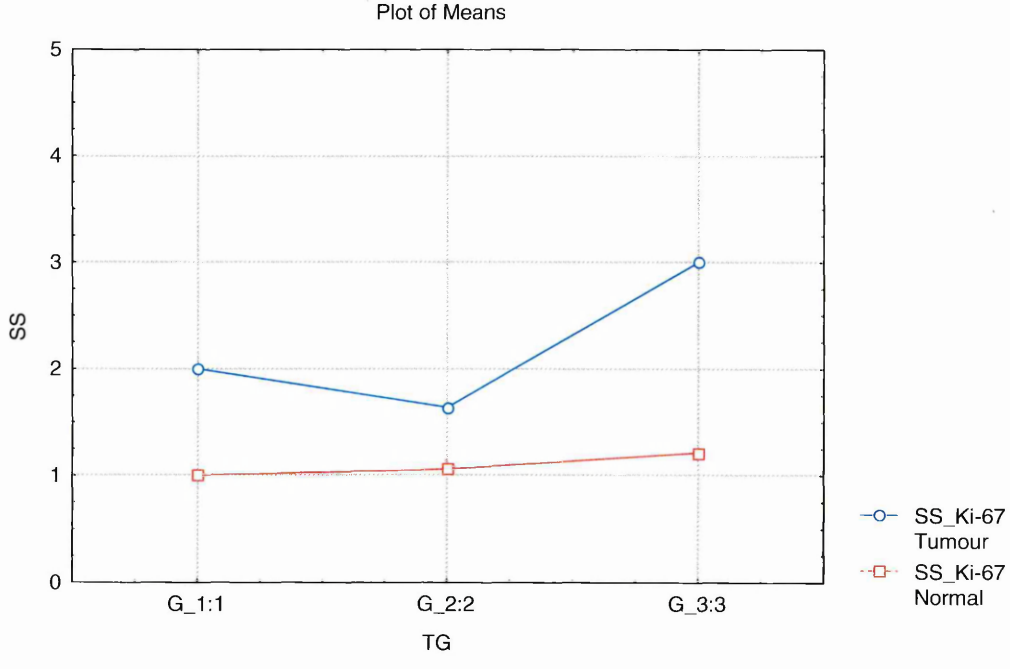


Figure 4–2 Intensity of staining for Ki-67 in relation to the TG in neoplastic specimens (blue line) and histologically normal tissues (red line). Abbreviations: SS, stain strength; TG, tumour grade.

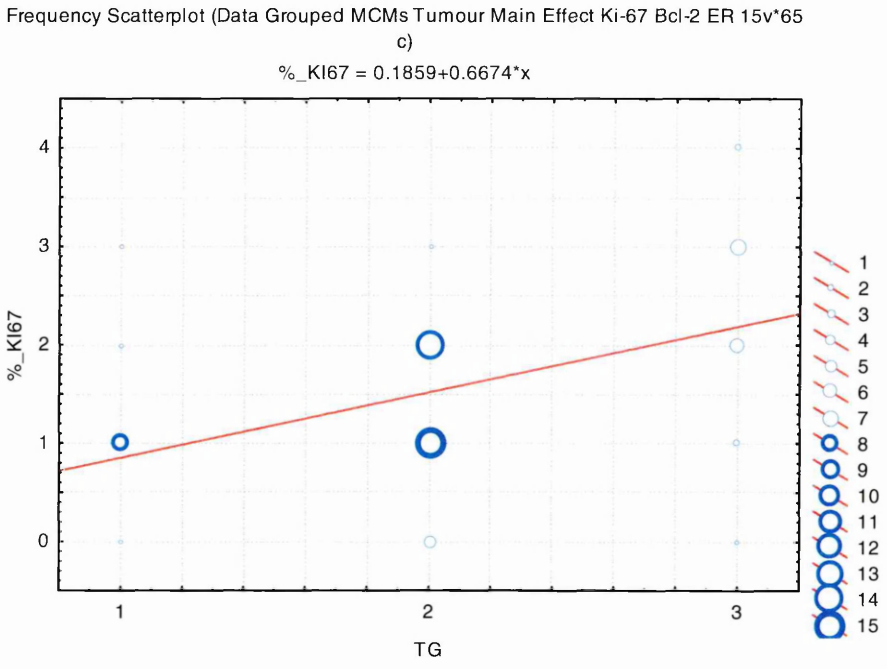


Figure 4–3 Frequency scatterplot of Ki-67 labelling index *vs* tumour grade. The regression equation on the top of the diagram describes the positive relationships of these data. Abbreviations: %, percentage of cells stained by the antibody; TG, tumour grade.

4.3.2. Expression of Bcl-2 in tumour breast tissues

As expected the Bcl-2 immunostaining was cytoplasmatic, sometimes with a granular appearance due to the presence of the oncoprotein in the mitochondria (Plate 4-2). Bcl-2 immunoreactivity was observed, though not frequently, in monomorphous cells of the invasive breast carcinoma, as shown in Plate 4-2 A. Moreover, epithelial cells of large and small ducts were noted to have abundant cytoplasmatic Bcl-2 (Plate 4-2 B). Stain strength displayed some variability across tumour specimens of the same grade and cells were less likely to have such a strong pattern in higher grades (Table 4-3 and Figure 4-4). In some cases almost all the cells of the invasive compartment were positively stained by the antibody (Plate 4-2 C), whereas in others the invasive cells were rarely stained (Plate 4-2 A). In general, the sections were homogenously stained with little or absent background noise. A higher labelling index for the percentage of cells stained by Bcl-2 was identified in well-differentiated tumours (grade I) (Figure 4-4). The negative correlation with increasing histologic grades was further confirmed by the Pearson and the Spearman's rank correlation coefficient (Pearson r=–0.37; p=0.003) (Table 4-3).

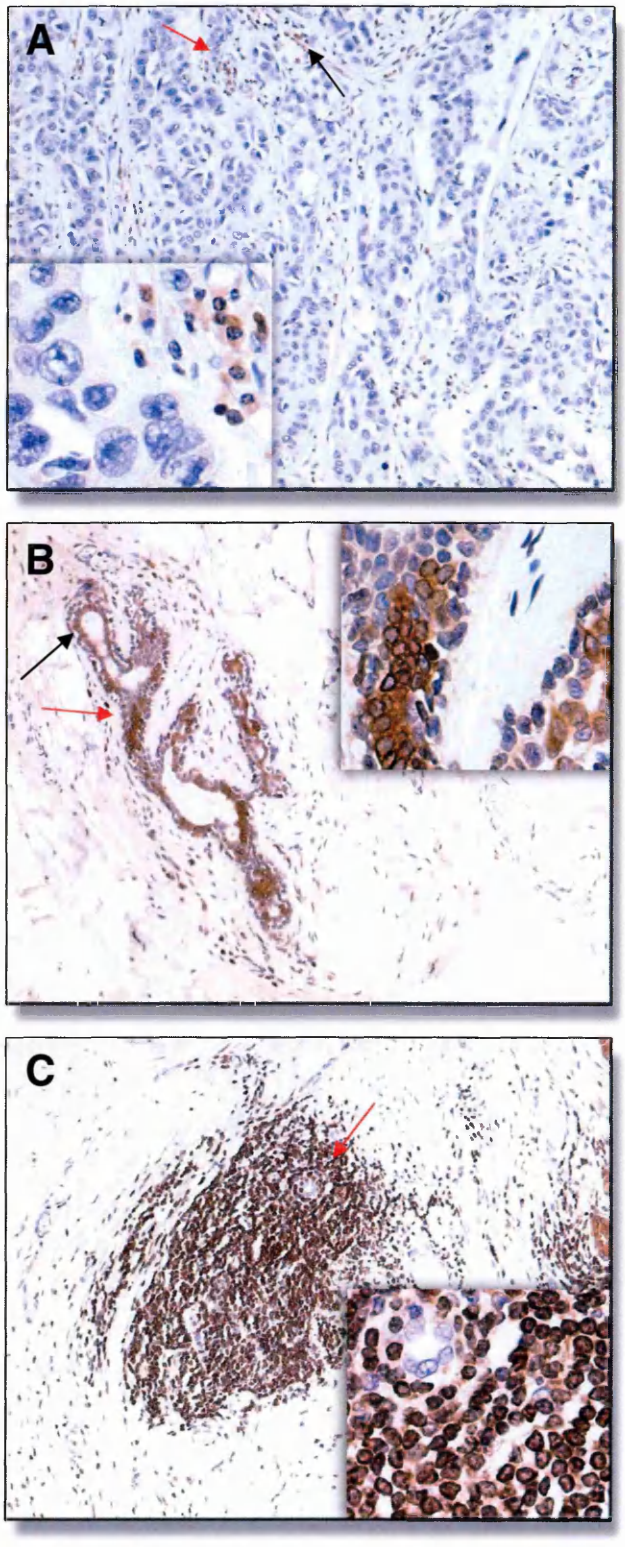


Plate 4–2 Immunohistochemical staining with anti-Bcl-2 antibody.
Three representative cases of invasive DC (same as for Plates 3-5, 3-6 and 3-7 in Chapter 3, Section 3.3.2.1.) classified with tumour grade III (A), II (B), and I (C). Bcl-2 immunostaining showed a characteristic intracellular distribution in the cytoplasmic and/or cell membranes. The differences in the mean labelling indices for the cases with tumour grade III (A) were about 49% lower than grade I (Table 4-3).

Table 4–3 Descriptive statistic of stain intensity and percentage of cells stained for Bcl-2 in tumour specimens. The intensity of stain ranges from 0 (absent) to 5 (very strong) and the percentage of stained cells ranges from 0 (0% cells stained) to 5 (75%-100% cells). The ANOVA test showed a significant effect of the tumour grade for the background intensity ($p<0.025$) [(a)] which was however rejected by the non-parametric Wilcoxon matched pairs test ($p=0.139$). Tumour grade main effect was significant for the labelling indices ($p<0.05$) [(b)] indicating that the percentage of cells stained for Bcl-2 followed a negative trend when associated with the tumour grade. The significance of the correlation coefficients confirmed this negative association with the tumour groups: TG *vs* Bcl-2 labelling index $r=-0.37$ ($p=0.003$). The non-parametric Spearman's rank correlation coefficient gave a comparable estimate with the same level of significance. Values are shown correct to 2 decimal places. NA (*i.e.* mucinous tumour) was scored 3 for stain strength and 4 for number of cells stained. Abbreviations: NA, tumour grade not assessable; SEM, standard error of mean.

Descriptive statistic	**Bcl-2**				
Intensity of stain strength	**Total**	**Grade**			
		I	**II**	**III**	**NA**
Mean	2.82	3.36[a]	2.91[a]	2.27[a]	-
Median	3	3	3	2.5	-
Range	0-4	3-4	1-4	0-4	-
SEM	0.14	0.15	0.14	0.37	-
Percentage cells stained					
Mean	3.28	3.73[b]	3.51[b]	2.5[b]	-
Median	4	4	4	2.5	-
Range (labelling index)	0-4	2-4	1-4	0-4	-
Range (labelling index %)	0-74	5-74	1-74	0-74	-
SEM	0.15	0.20	0.16	0.37	-
Specimens not assessable	-	-	-	-	-
Cases	65	11	35	18	1

[a] p-value=0.025 for ANOVA and p-value=0.139 for Kruskal-Wallis test.
[b] p-value=0.004 for ANOVA and p-value=0.017 for Kruskal-Wallis test.

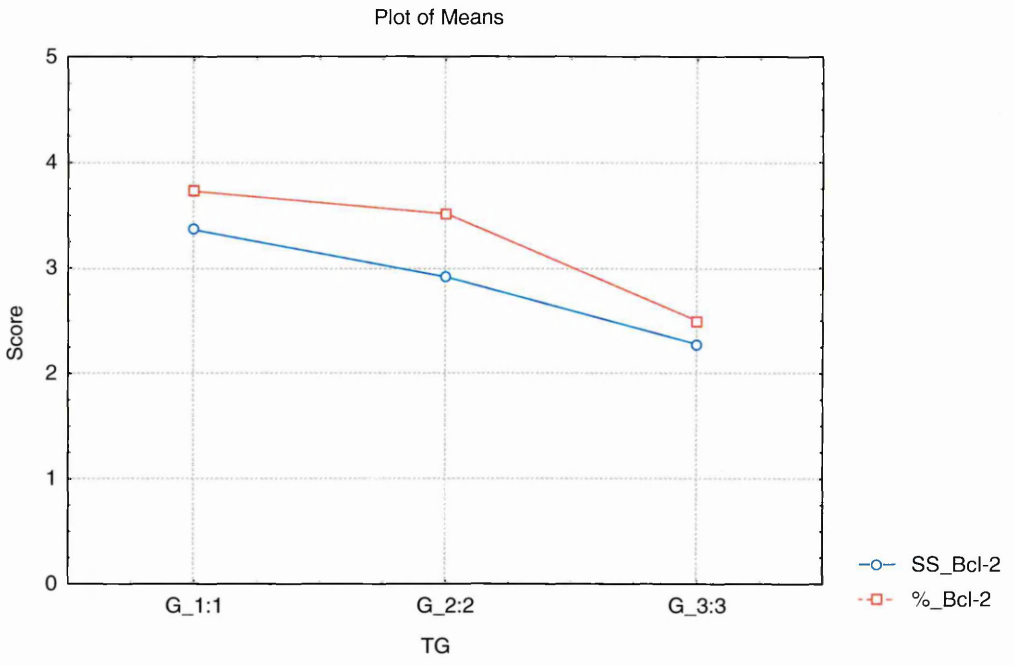


Figure 4–4 Intensity of staining (blue line) and labelling index (red line) for Bcl-2 in relation to the TG in neoplastic specimens. Abbreviations: SS, stain strength; %, percentage of cells stained; TG, tumour grade.

4.3.3. ER expression in tumour breast tissues and association with Bcl-2

The immunohistochemical staining for ER demonstrated a prominent nuclear signal in non-malignant epithelial cells within lobules and ducts (Plate 4-3). Variable staining intensity was detected albeit less evident in the samples belonging to the well-differentiated tumours (grade I) (Table 4-4). Both intensity of staining and percentage of cells expressing ER were expressed to a less extent in grade III tumours than grade II and grade I (Figure 4-5). This behaviour was expected as indicated by the trend of ER labelling expression which was negatively correlated to the tumour grade. The correlation coefficient was weak but still significant indicating the negative relationship of ER with the histologic grade of tumours (r=–0.28; p=0.023) (Table 4-4).

Agreement between Bcl-2 and ER was further investigated with parametric and non-parametric tests. A frequency scatterplot to examine this relationship showed the positive association between the labelling indices of the two markers. This correlation was not very strong probably due to the wide spread of the distribution points, but significant for all statistical tests (r=0.33; p=0.007) (Figure 4-6).

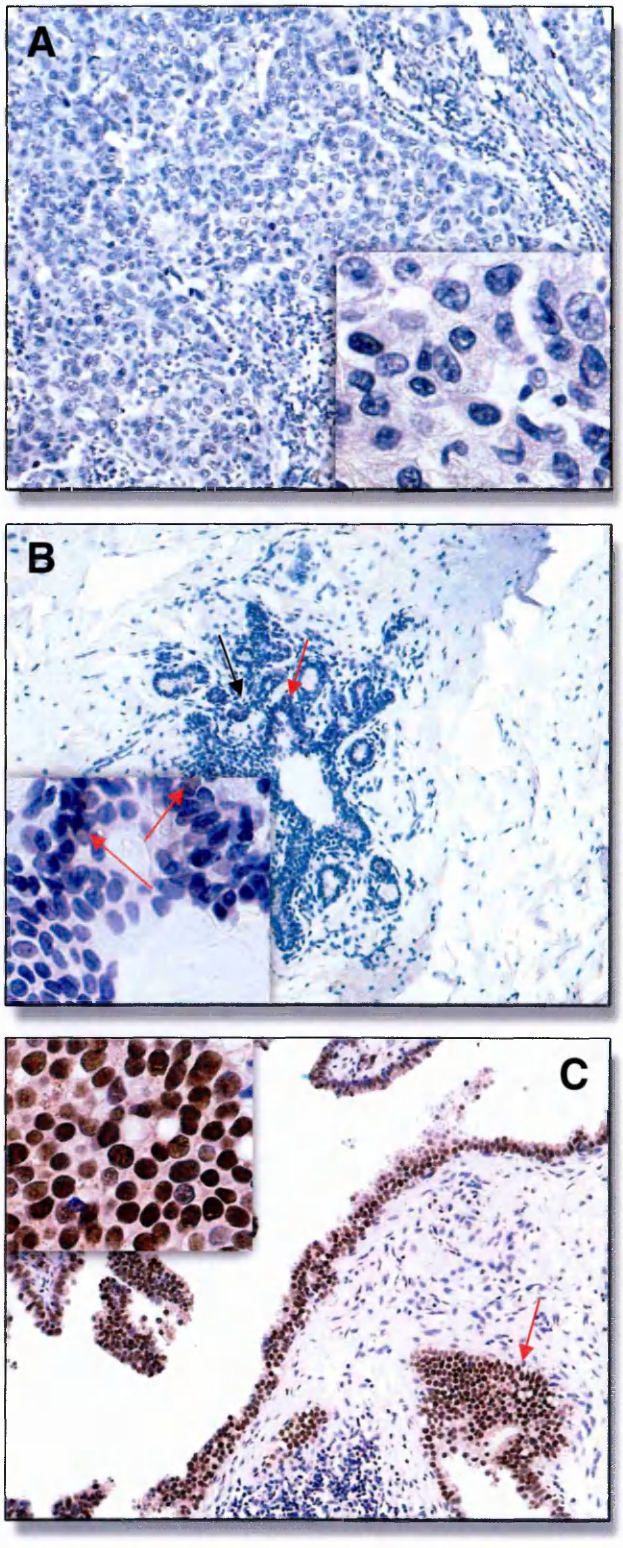


Plate 4–3 Immunohistochemical staining with ER antibody.
Three representative cases of invasive DC (same as for Plates 3-5, 3-6 and 3-7 in Chapter 3, Section 3.3.2.1.) classified with tumour grade III (A), II (B), and I (C). Sections from primary breast tumours displayed a positive nuclear pattern with the labelling index associated with well-differentiated specimens (grade I) (C) (Table 4-4). In B and C a variation in staining intensity is shown.

Table 4–4 Descriptive statistic of stain intensity and percentage of cells stained for ER in tumour specimens. The intensity of stain ranges from 0 (absent) to 5 (very strong) and the percentage of stained cells ranges from 0 (0% cells stained) to 5 (75%-100% cells). The ANOVA test showed a non-significant effect of the tumour grade for the background intensity ($p<0.061$) [(a)] and for the labelling indices ($p<0.063$) [(b)] indicating no significant differences within the three group grades. The correlation coefficients confirmed the negative association with the tumour groups: TG *vs* ER labelling index $r=-0.28$ ($p=0.023$). The non-parametric Spearman's rank correlation coefficient gave a comparable estimate with the same level of significance. Values are shown correct to 2 decimal places. NA (*i.e.* mucinous tumour) was scored 1 for stain strength and 2 for number of cells stained. Abbreviations: NA, tumour grade not assessable; SEM, standard error of mean.

Descriptive statistic	**ER**				
Intensity of stain strength	**Total**	**Grade**			
		I	**II**	**III**	**NA**
Mean	2.57	3.73[a]	2.51[a]	2.05[a]	-
Median	3	4	3	2	-
Range	0-5	2-5	0-5	0-5	-
SEM	0.23	0.30	0.32	0.50	-
Percentage cells stained					
Mean	2.23	3.19[b]	2.20[b]	1.72[b]	-
Median	2	3	2	1	-
Range (labelling index)	0-5	2-4	0-5	0-5	-
Range (labelling index %)	0-100	5-74	0-100	0-100	-
SEM	0.20	0.26	0.27	0.45	-
Specimens not assessable	-	-	-	-	-
Cases	65	11	35	18	1

[a] p-value=0.061 for ANOVA and p-value=0.095 for Kruskal-Wallis test.
[b] p-value=0.063 for ANOVA and p-value=0.065 for Kruskal-Wallis test.

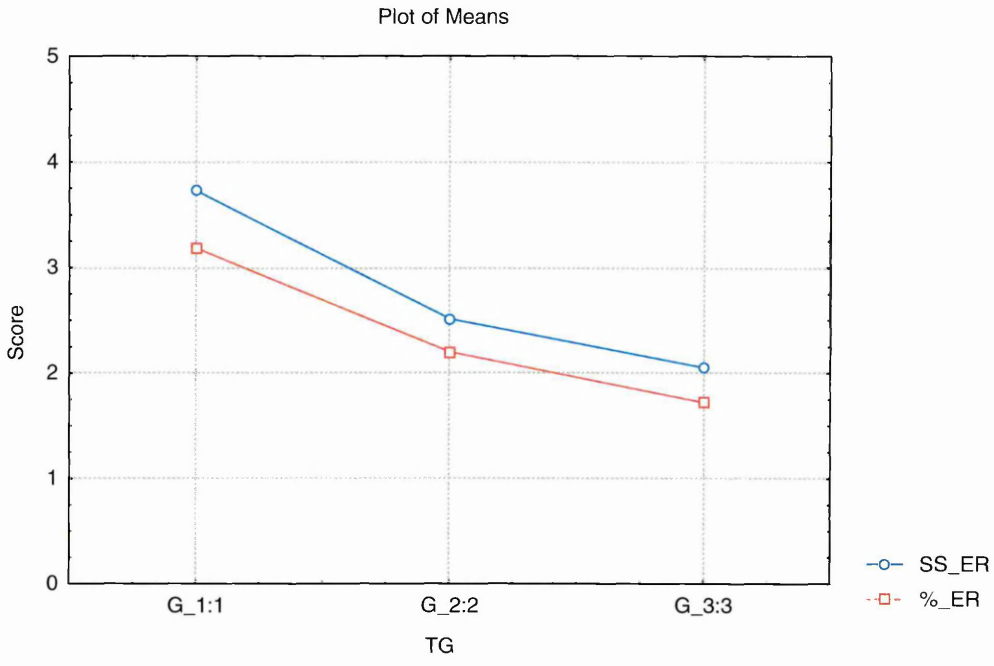


Figure 4–5 Intensity of staining (blue line) and labelling index (red line) for ER in relation to the TG in neoplastic specimens. Abbreviations: SS, stain strength; %, percentage of cells stained; TG, tumour grade.

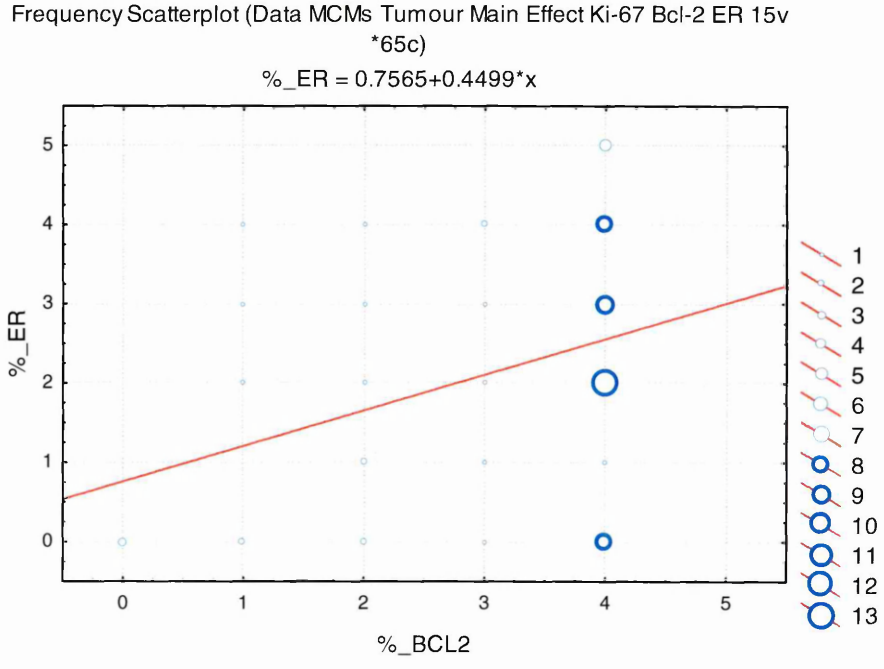


Figure 4–6 Frequency scatterplot of Bcl-2 *vs* ER labelling indices.
The regression equation on the top of the diagram describes the positive relationships of these data. Abbreviations: %, percentage of cells stained by the antibody.

4.3.4. MCM and Ki-67 proliferative indices comparison

It was previously reported that the proliferating tumour cells within the invasive compartment and ducts stained positive for both Mcm2, 5 and 7 and Ki-67. However, following a side-by-side comparison there were significant differences with regards to the proportion of cells stained between the MCM investigated and Ki-67. The means and ranges of the MCM expression profiles of each tumour group are tabulated in Table 3-4 (Chapter 3, Section 3.3.2.1.). Three representative cases of different tumour classes (grades III, II and I) are shown in Plate 4-4 where the same tissue spot is stained with the MCM and Ki-67 antibodies. In the majority of cases the number of cells stained for Ki-67 was lower than those stained by the MCM antibodies. The semi-quantitative immunoperoxidase assessment of MCM proteins showed marked differences between the labelling indices when compared with Ki-67 (Table 3-4 and Table 4-2). Specifically, the Student t-test indicated that the proportions of cells stained respectively for Mcm2, Mcm5 and Mcm7 were 61% (p=0.000), 54% (p=0.000) and 41% (p=0.000) more expressed than for Ki-67 (Figure 4-7). The non-parametric Wilcoxon matched pairs tests were totally in agreement with these conclusions. There was, however, variable staining for all the proliferative markers. For instance, some staining variability was observed within single lesions, with regions more stained than others. The intensity of staining also presented some variability within the same region of the lesion. Overall, the level of variability was similar for the proteins of the pre-RC and Ki-67.

The ANOVA test revealed that there were no significant interactions ($p<0.605$) between the proliferative proteins (tumour grade/marker), thus suggesting a similar trend of expression across markers in relation to the tumour grade (Figure 4-8). From this graph it is also possible to summarise the differences in expression across markers. For the well-differentiated tumours (grade I) the most expressed marker was Mcm5. This pattern of expression may suggest an interesting role of this marker for the early diagnosis of breast cancer (arrows in Plate 4-4 E and F).

A series of scatterplots examined the relationships between MCM proteins and Ki-67. In particular, the strongest association was between Mcm7 and Ki-67 (Pearson r=0.48; p=0.000) (Figure 4-9 C) followed by Mcm2 (Pearson r=0.43; p=0.000) (Figure 4-9 A) and both correlations were confirmed by the non-parametric tests. However, the

Pearson correlation coefficient between Mcm5 and Ki-67 was weak and not significant ($r=0.22$; $p=0.082$) but this trend was instead confirmed by the significance of the non-parametric Spearman's rank correlation coefficient ($p=0.045$) (Figure 4-9 B).

Normal breast adjacent to tumour tissues did not express the proliferative markers to the same extent as in neoplastic sections. Among all the markers Mcm5 was the most highly expressed with a labelling index of 66% higher than Ki-67 and immediately followed by Mcm7 and Mcm2, as shown in the boxplot in Figure 4-10.

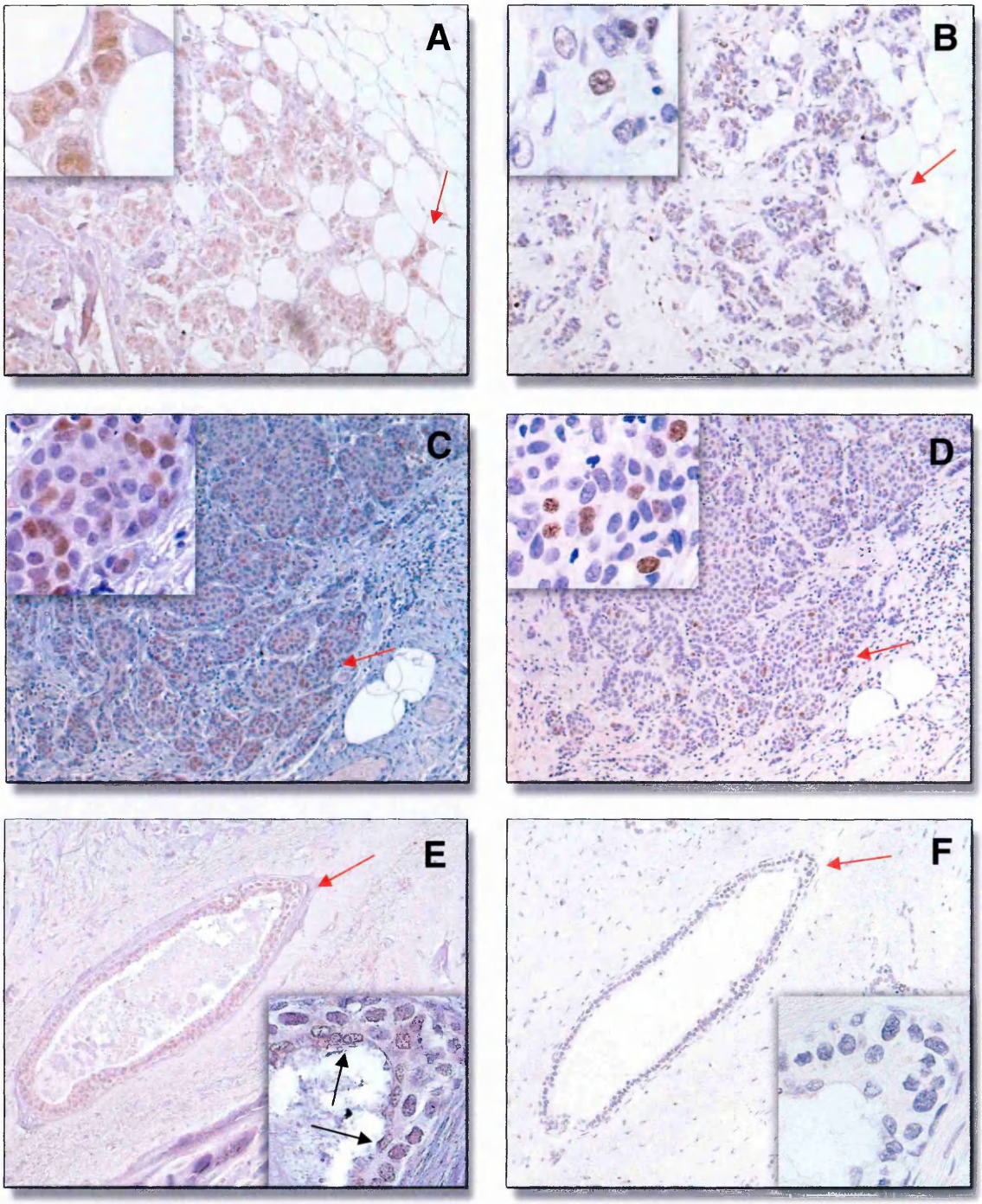


Plate 4–4 Comparison of staining by anti-MCM proteins and anti-Ki-67 antibodies in parallel sections of invasive DC of different grades. Mcm2 (A) and Ki-67 (B) in invasive DC grade III. Mcm7 (C) and Ki-67 (D) in invasive DC grade II. Mcm5 (E) and Ki-67 (F) in invasive DC grade I with no significant *in situ* component. The differences between MCM protein expression and Ki-67 can be easily identified mostly at the invasive edge of tumours. MCM antibodies detected a higher number of proliferative cells than Ki-67 antibody, both in poorly differentiated and moderately differentiated tumours (p=0.000). The same trend was observed in well-differentiated tumours (p=0.000) (E and F) where a pattern of expression in the cells underlying the epithelium (arrows) is visible, although weak. All objective lens magnifications were 100x and 400x in zoomed windows (red arrows represent the zoomed areas).

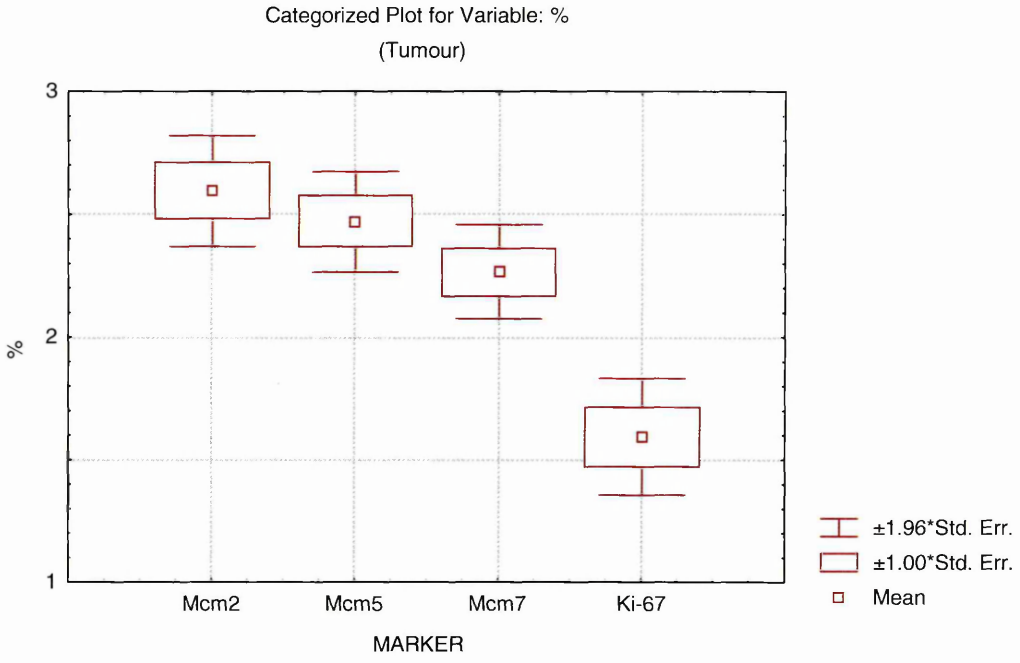


Figure 4–7 Boxplot of MCM proteins and Ki-67 labelling indices for the proportion of cells stained in neoplastic specimens (n=65). The score scaling (%) has been reduced (1-3) to allow better visualisation of the precision of the sample mean represented by the width of the standard error confidence interval (*i.e.* the 95% confidence interval or the 68% confidence interval). Abbreviations: %, percentage of cells stained.

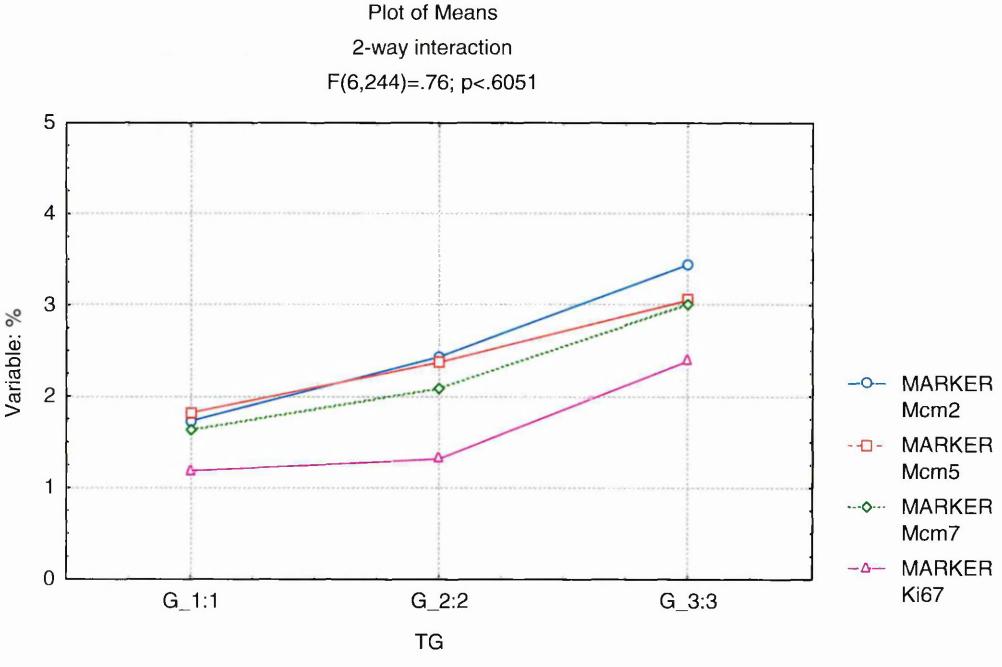
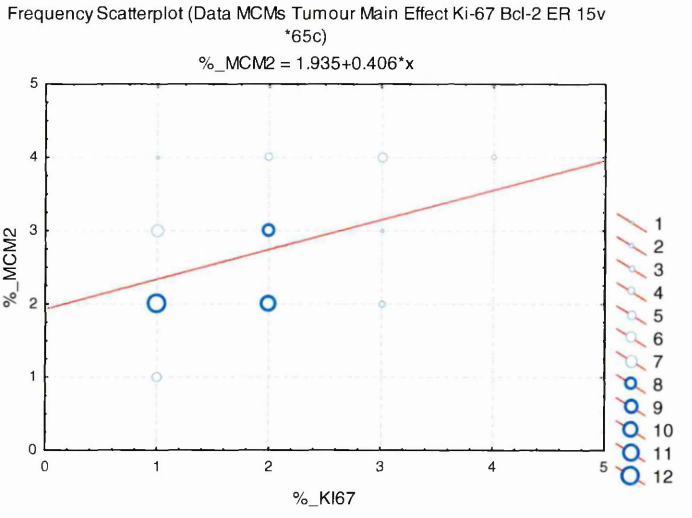
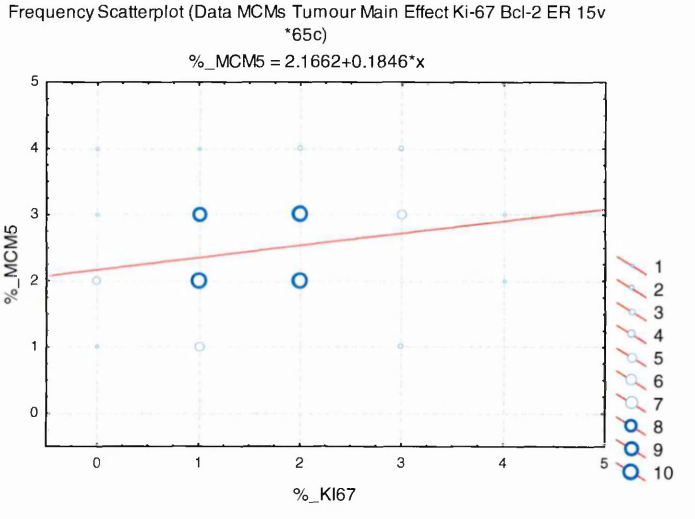


Figure 4–8 Interactions tumour grade/markers for percentage of cells stained. No significant interactions were observed ($p<0.605$) indicating a similar behaviour of the proliferative markers against the proportions of cells stained (ANOVA). Abbreviations: %, percentage of cells stained; TG, tumour grade.

A



B



C

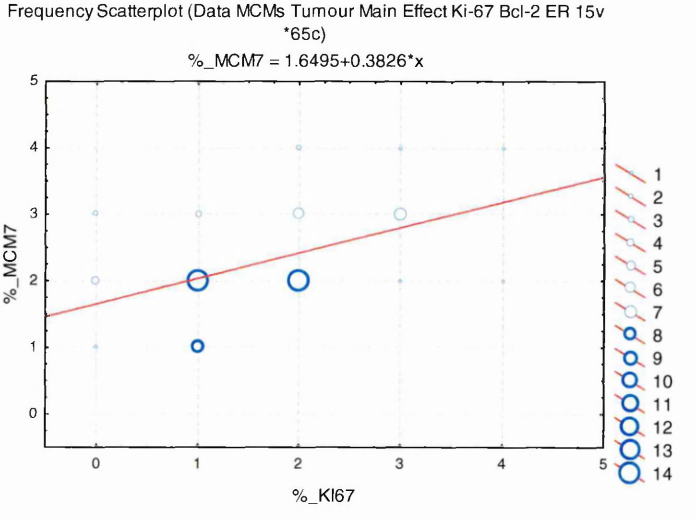


Figure 4–9 Frequency scatterplots of Mcm2 *vs* Ki-67 (A), Mcm5 *vs* Ki-67 (B) and Mcm7 *vs* Ki-67 (C) labelling indices in tumour. The regression equations on the top of the diagram describe the relationships of these data. Abbreviations: %, percentage of cells stained by the antibody.

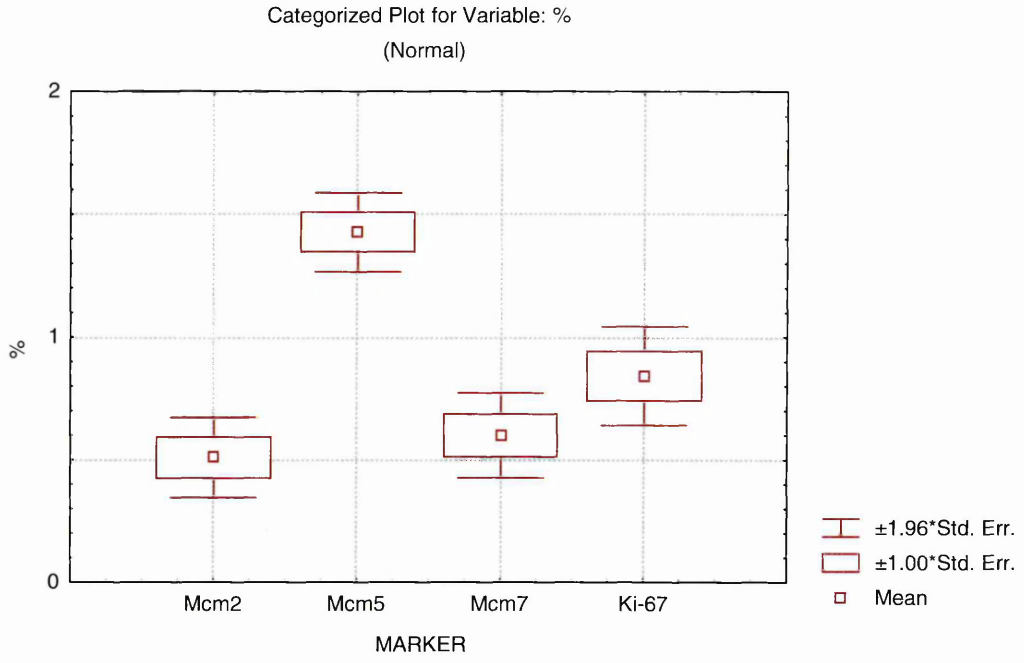


Figure 4–10 Boxplot of MCM proteins and Ki-67 labelling indices for the proportion of cell stained in normal specimens (n=65). The score scaling (%) has been reduced (0-2) to allow better visualisation of the precision of the sample mean represented by the width of the standard error confidence interval (*i.e.*, the 95% confidence interval or the 68% confidence interval). Abbreviations: %, percentage of cells stained.

4.3.5. Relationship of the proliferative markers to Bcl-2 and ER and to histological grade

All tumour cases (n=65) studied in this work were assessable for the proliferative markers Mcm2, 5, 7, Ki-67 and for Bcl-2 and ER. Histologic tumour grade was evaluated in 64 cases. MCM proteins and Ki-67 presented different trends of negative correlation with Bcl-2 and ER. Specifically, Mcm2 displayed a weak negative relationship with Bcl-2 (r=–0.23; p=0.069) and ER (r=–0.17; p=0.187). For Mcm5 no significant correlation was seen either for Bcl-2 (r=0.03; p=0.803) and for ER (r=0.01; p=0.930). Mcm7 also displayed a weak correlation with Bcl-2 (r=–0.24; p=0.053) and no association with ER (r=–0.04; p=0.670). A significant inverse association was instead observed between Ki-67 and Bcl-2 (r=–0.35; p=0.004) but not with ER (r=–0.10; p=0.438). These results were also validated by the non-parametric tests. Interestingly, there were five cases (n=1 case, grade III; n=3 cases, grade II; n=1 case, grade I) that were co-expressing all the three MCM proteins and ER but not Ki-67.

In addition, a comparison of the correlation indices of the markers demonstrated their pattern of expression in relation to the grade of the tumours and so their biological activities to cellular proliferation and differentiation. The scatterplot in Figure 4-11 summarises these relationships. Following the distribution of the cases the regression equations drew linear associations of the markers to the tumour grade. Specifically, the proteins of the pre-RC investigated and Ki-67 were positively linked to the grade being their expression higher from grade I to III (p=0.000). The almost overlapping relationships of MCM are as a consequence of their close labelling indices as previously discussed in Chapter 3 (Section 3.3.2.1.). On the contrary, both Bcl-2 (p=0.003) and ER (p=0.023) showed a significant negative correlation with cellular tumour grade as a result of their lower expression in grade III than II and I (Figure 4-11).

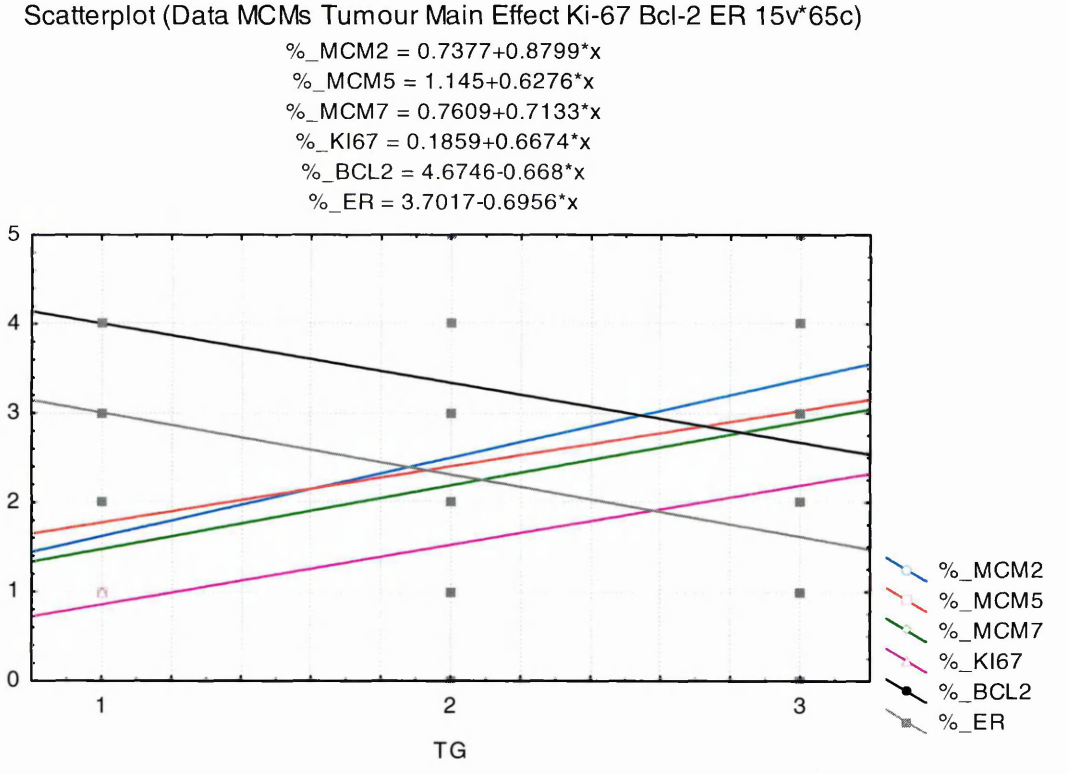


Figure 4–11 Scatterplot of tumour grade *vs* antibodies labelling indices.
The positive correlation between TG and MCM/Ki-67 labelling indices were always significant and could be considered linear. Proliferation markers (Pearson p=0.000): Mcm2 (r=0.64), Mcm5 (r=0.50), Mcm7 (r=0.61), Ki-67 (r=0.46), Bcl-2 (r=–0.37; p=0.003) and ER (r=–0.28; p=0.023); similar correlation coefficients were calculated with the Spearman rank sum for MCM and Ki-67 (p=0.000) and for Bcl-2 (p=0.006) and ER (p=0.028). The above regression equations describe the relationships of these data. Abbreviations: %, percentage of cells stained; TG, tumour grade.

4.3.6. Multivariate regression analysis to predict lesion and tumour grade

The logistic regression analysis showed that in general the combination of the proliferative markers (*i.e.* MCM and Ki-67) has more predictive power to discriminate between normal and tumour cases than the single marker (Table 4-5). From Table 4-5 it may appear that Mcm2 alone is a better predictor than the four markers together but this happens because missing data have reduced the number of cases (n) used in the analysis of the four markers. With an accuracy of 90% the four markers predicted 45 cases as normal (only 5 cases were predicted as tumour) and with a percentage of 91% they predicted 59 tumour cases (only 6 cases were predicted as normal). When the markers were analysed separately the best predictors are Mcm2 and Mcm7 (Table 4-5).

Table 4–5 Multivariate logistic regression for normal (0) and tumour samples (1).

Mcm2, Mcm5, Mcm7, Ki-67	**Normal (0) *vs* Tumour (1)**			n=115
Observed	***Predicted (0)***	***Predicted (1)***	***% correct***	***p value***
0	45	5	90	p<0.001
1	6	59	91	
Mcm2				n=123
0	54	4	93	p<0.001
1	6	59	90	
Mcm5				n=120
0	29	26	53	p<0.001
1	8	57	88	
Mcm7				n=121
0	51	5	91	p<0.001
1	9	56	86	
Ki-67				n=123
0	47	11	81	p <0.001
1	32	33	51	

The multivariate analysis also considered the power of the markers at predicting the tumour grade I and the associated normal cases (Table 4-6). Although a small number of cases was compared, the prediction looks promising. When the markers are analysed together they are significantly predicting normal cases with a percentage of 75% (6 over 8 cases) and tumour cases with a percentage of 91% (10 over 11 cases). As before the number of cases (n) differs slightly because of missing data regarding the

normal tissues. When the markers are studied individually the highest reliability was shown by Mcm2 (Table 4-6; $p<0.05$).

Table 4–6 Multivariate logistic regression for normal (0) and tumour grade (TG) I (1).

Mcm2, Mcm5, Mcm7, Ki-67	**Normal (0) *vs* TG I (1)**			n=19
Observed	***Predicted (0)***	***Predicted (1)***	***% correct***	***p value***
0	6	2	75	p <0.001
1	1	10	91	
Mcm2				n=20
0	7	2	78	p<0.05
1	4	7	64	
Mcm5				n=19
0	1	7	13	p=0.42
1	0	11	100	
Mcm7				n=19
0	8	0	100	p<0.05
1	5	6	55	
Ki-67				n=21
0	5	5	50	p=0.06
1	1	10	91	

From the comparison between tumour grade I and II it appears that the markers failed to accurately predict the tumour grade in all cases (*i.e.* low percentages). Moreover, the Chi-square significance of Mcm7 ($p<0.05$) is useless because all cases are predicted to be tumour grade II (Table 4-7). The results in Table 4-7 are in contrast with the analysis of the means in Table 3-4 (Chapter 3) and Table 4-2 which showed that the markers significantly increased with the tumour grade I and II. This suggests that the differences highlighted previously were not marked enough to make these four markers useful predictors between grade I and II.

Table 4–7 Multivariate logistic regression for tumour grade (TG) I (1) and II (2).

Mcm2, Mcm5, Mcm7, Ki-67	**TG I (1) *vs* TG II (2)**			n=46
Observed	***Predicted (1)***	***Predicted (2)***	***% correct***	***p value***
1	4	7	36	p=0.052
2	2	33	94	
Mcm2				n=46
1	4	7	36	p<0.05
2	2	33	94	
Mcm5				n=46
1	6	5	55	p<0.05
2	1	34	97	
Mcm7				n=46
1	0	11	0	p<0.05
2	0	35	100	
Ki-67				n=46
1	0	11	0	p=0.06
2	0	35	100	

A good predictive power was observed when tumour grades II and III were compared. Again, the best prediction is shown when the proliferative markers are used in combination (Table 4-8). When considered independently the best predictor of tumour grade was Mcm7 followed by Mcm2 (Table 4-8).

Table 4–8 Multivariate logistic regression for tumour grade (TG) II (2) and II (3).

Mcm2, Mcm5, Mcm7, Ki-67	TG 2 (2) *vs* TG 3 (3)			n=53
Observed	***Predicted (2)***	***Predicted (3)***	***% correct***	***p value***
2	32	3	91	p<0.001
3	5	13	72	
Mcm2				n=53
2	33	2	94	p<0.001
3	7	11	61	
Mcm5				n=53
2	33	2	94	p<0.001
3	14	4	22	
Mcm7				n=53
2	29	6	83	p<0.001
3	3	15	83	
Ki-67				n=53
2	34	1	97	p<0.001
3	9	9	50	

Table 4-9 represents the prediction analysis of Bcl-2 and ER. It appears that both markers are not good predictors of tumour grade in all cases. An individual analysis for Bcl-2 and ER was also performed but resulted to be either no significant or useless in predicting different tumour grades. Hence, the results are not shown. The combination of the four proliferative markers along with Bcl-2 and ER were also analysed. Since the results were similar to those in Tables 4-7 and 4-8 they are not reported. Overall these results suggest that Bcl-2 and ER have no predictive power on tumour grade discrimination.

Table 4–9 Multivariate logistic regression forBcl-2 and ER for tumour grade (TG) I (1) and II (2) and III (3).

Bcl-2, ER	TG I (1) *vs* TG II (2)			n=46
Observed	***Predicted (1)***	***Predicted (2)***	***% correct***	***p value***
1	0	11	0	p=0.111
2	3	32	91	
Bcl-2, ER	**TG 2 (2) *vs* TG 3 (3)**			n=53
2	30	5	86	p<0.05
3	10	8	45	

4.4. DISCUSSION

An accurate analysis of the proliferative markers is important to evaluate and predict the rate of growth in breast carcinomas. In breast cancer the altered expression of Bcl-2 and ER has been described in the literature. However, the correlation between their levels of expression and their differential association with MCM proteins had yet to be evaluated. The aim of this study was to assess not only the Ki-67 proliferating nuclear antigen, the Bcl-2 anti-apoptotic protein and the steroid hormone receptor ER by immunohistochemistry on various breast carcinomas but also to evaluate their relationships with Mcm2, 5 and 7. After this comparison, the specificity and sensitivity of the MCM proteins investigated was addressed.

The comparison with Ki-67, an established marker of cell cycle entry, was undertaken to determine whether the use of MCM proteins have more power to detect the proliferative status of the breast epithelium. Ki-67 antibody specifically reacts with a nuclear antigen present in proliferating human cells since it appears to be involved in the protein interaction network that drives the cell division cycles (reviewed by Li *et al.,* 2004). Assessment of immunoreactivity of Ki-67 antigen is one of the most frequently used methods for studying cell proliferation in cancer because of its correlation with the growth fractions of a given cell population (Ellis *et al.,* 1996; Goodson *et al.,* 1998; Ostrowski *et al.,* 2001). In the cases studied here Ki-67 labelling indices represented as percentage of cells stained showed a significant association with Mcm2 and Mcm7 ($p=0.000$) and Mcm5 ($p=0.045$) (Figure 4-9). Similar findings were recently reported for the association between Mcm2 and Ki-67 (Chatrath *et al.,* 2003; Bukholm *et al.,* 2003). In addition, Mcm2, 5 and 7 labelling indices were considerably higher ($p=0.000$) than those of Ki-67 (Table 3-4, Chapter 3, Section 3.3.2.1. and Table 4-2). This pattern of expression has also been shown in other systems. Antibodies against MCM proteins effectively labelled the proliferative fractions of cells in a number of dysplastic epithelia and malignant tissues, whereas Ki-67 was not able to evaluate the same proportions of proliferative cells with the same accuracy (Freeman *et al.,* 1999). Similarly, Kato *et al.* (2003) demonstrated the capability of Mcm2 to identify a higher percentage of proliferating cells in esophageal squamous cell carcinoma than Ki-67. It would have been interesting to analyse breast dysplastic tissues using the MCM proliferative markers and Ki-67 in order to appraise the effectiveness of MCM in distinguishing

dysplasia from normal tissue and neoplasia. This analysis was not performed due to the unavailability of such tissues but it is suggested for further studies.

It is doubtful whether Ki-67, unlike MCM proteins, reliably labels all the proliferative fractions of cells in the tissues. This conclusion can be reached for a number of reasons. The ubiquitous nuclear protein Ki-67 may be down-regulated in proliferating cells by nutritional deprivation which may occur regionally in solid tumours (Baisch, & Gerdes, 1987). In MCF-7 human breast cancer cells, van Dierendonck *et al.* (1989) reported that non-proliferating cells could also retain the antigen for a significant period of time, and in tumour cells with longer G_1 phase duration the undetectably low expression of the antigen can be deceptive for the detection of proliferating cells. Besides, there is evidence to suggest that Ki-67 is involved in ribosome biosynthesis; therefore, it may not be the key molecule directly responsible for cellular proliferation (Verheijen *et al.,* 1989; MacCallum & Hall, 2000). Above all, the pattern of Ki-67 expression shows some variations through the cell cycle, being predominantly expressed during S, G_2 and M phases (Gerdes *et al.,* 1984; Verheijen *et al.,* 1989). Consequently, the likelihood of detecting cellular proliferation by immunohistochemistry using Ki-67 may vary. This suggests that the Mcm2, 5 and 7 positive cells that were negative for the cell cycle marker Ki-67 were presumably in early G_1. However, this hypothesis needs further studies. The application of, for instance, confocal microscopy or co-immunoprecipitation techniques to examine protein-protein interactions could show the co-localisation of the MCM proteins and the absence or presence of Ki-67 within the cell. Such investigation is suggested for further studies in order to give additional evidence that some cells not positive for Ki-67 can be positive for the MCM confirming the higher sensitivity of the pre-replicative proteins at detecting cellular proliferation defects.

The analysis of variance showed that the labelling indices of the MCM investigated and the Ki-67 antibody increased with progression to poorly histologically differentiated carcinoma, and this increase was significant ($p<0.001$). The histology grade of breast carcinoma may reflect the proliferative capacity of the tumour cells since it considers tubule formation, nuclear pleomorphism and, more importantly, mitotic frequency. In fact, the relationship found for all the proliferative markers with the grade, as a measure of differentiation, was positive, very significant ($p=0.000$) and

consistent with previous authors (Weidner *et al.*, 1994). However, following a comparison of the labelling indices of all the proliferative markers across tumour grades the MCM proteins analysed appeared to be associated with cellular proliferation independently of Ki-67 (Figure 4-8). Therefore, MCM may provide a measure of the growth fraction of cells distinct from that offered by Ki-67. As pictured in Plate 4-4 and Figure 4-8 the lower pattern of expression of Ki-67 in all histological grades may denote a diminished capability of this marker to show early abnormalities of the breast tissue growth. Thus, this indication supports the hypothesis of a higher sensitivity of the MCM proteins in detecting early molecular changes in cellular proliferation especially during the transition from benign cellular atypia to frank malignancy. Although this concept is applicable for all tumour grades it may be particularly important for the diagnosis of well-differentiated tumours (Plate 4-4 E and F) where the detection of a small neoplasm is often difficult in breast epithelium. Once more, in support of this hypothesis the proteins of the pre-RC serve as a point of convergence for all the signalling pathways that transmit the command of cell proliferation, differentiation, senescence, quiescence or cell death, and MCM are located at this convergence point. Hence, it is important for early detection of abnormal cell proliferation that MCM proteins are strategically located upstream of cellular proliferation. In addition, MCM antibodies do not detect cells undergoing DNA repair, which has been shown to be a feature of PCNA (reviewed by Hall *et al.*, 1990b). Therefore, these results taken together indicated that Mcm2, 5 and 7 were more effective at demonstrating abnormal cycling cells than Ki-67 in breast epithelium.

The immunostained estimation based on the percentage of positive cells labelled by the antibodies in adjacent histologically normal breast was partially in agreement with previous works. Stoeber *et al.* (2001) identified about seven times higher proportions of Mcm2, and ten times of Mcm5 proliferating cells in normal pre-menopausal breast than Ki-67. Conversely, in this study Mcm2 (mean=0.50) was observed to be less expressed than Ki-67 (mean=0.84) whereas the labelling index of Mcm5 (mean=1.40) was much higher than Ki-67 (66%) (Table 3-5, Chapter 3, Section 3.3.2.1. and Table 4-2). In this work Mcm7 (mean=0.59) showed similar proportions of cells decorated by Mcm2 in normal tissues (Table 3-5, Chapter 3, Section 3.3.2.1.). The different histology of the normal breast may have influenced the expression of the

proliferative markers comparing the above work and the present one. In fact, in this population study the median age was 59 (36-86) with the majority of patients in post-menopausal phase (Table 3-1, Chapter 3, Section 3.2.1.). Therefore, it may be thought that the different cell kinetics in mammary gland of older women could have influenced the expression profile of the markers since the breast epithelium undergoes hyperplasia or involution in response to hormonal regulation. Following the comparison with Ki-67, Mcm5 in normal tissue was still the more expressed. As discussed in Chapter 3 (Section 3.4.), this certainly is in line with the hypothesis that Mcm5 may play additional functions other than being part of the signature of the pre-RC. However, only further investigations may shed light on this hypothesis.

The oncoprotein Bcl-2 is normally employed as an apoptotic indicator since it is the only oncogene that acts by inhibiting apoptosis rather than directly increasing cellular proliferation (Beenken & Bland, 2002). Excess of Bcl-2 promotes cell survival by inhibiting apoptosis (Chapter 1, Section 1.3.2.4.). As expected the predominant intracellular distribution was cytoplasmic and in the membrane because of Bcl-2 presence in the inner mitochondrial membrane (Hockenbery *et al.,* 1990). In this study Bcl-2 displayed a negative correlation with increasing histologic grade ($r=-0.37$; $p=0.003$) (Figure 4-11) possibly indicating the presence of a rapid cell turnover. Exploring the differences across markers, this study showed that the spatial expression pattern of the oncoprotein had a weak inverse correlation only with Mcm2 ($r=-0.23$) and was not significant. Associations with Mcm5 and 7 were found, but they were not significant either. However, the correlation with Ki-67 was stronger and very significant ($r=-0.35$; $p=0.004$). The negative correlation of Bcl-2 with tumour grade and the relationship between Ki-67 and Bcl-2 in breast cancer are certainly in line with other findings (van Slooten *et al.,* 1998; Yang *et al.,* 1999; Rehman *et al.,* 2000; Siziopikou & Schnitt 2000). Intriguingly, the present results of MCM and Bcl-2 are controversial since MCM are markers of cellular proliferation as Ki-67 is considered to be. The low or absent association of the MCM proteins investigated with Bcl-2 may possibly be explained by the higher sensitivity of MCM. In other words, if there is presence of Bcl-2 and concomitant low or similar presence of MCM proteins this may indicate the existence of a population of cells where PCD has been stopped by Bcl-2 and MCM proteins are ready for DNA replication to enter proliferation. Indeed, cells

that express Bcl-2 and MCM may represent an interesting subset. In light of these findings it would be possible, for instance, to identify preinvasive lesions with different behaviour that would be difficult to observe with other proliferative markers such as Ki-67, which showed a stronger correlation with Bcl-2. Indeed, Ki-67 would be absent in this subset of cells. In a model of breast cancer progression, examples include intraductal carcinoma *in situ* that may represent the link between well and poorly differentiated breast lesions as proposed by Viacava *et al.* (1999). Interestingly, Borner (1996) suggested a function of Bcl-2 in promoting cell survival in slow proliferating tumour cells, reporting that Bcl-2 over-expressing cells tend to accumulate in G_0/G_1 phase. This could partially explain the missed correlation of Bcl-2 with MCM proteins for this subset of cells since, as previously mentioned, MCM proteins start to co-localise in early G_1 phase (Chapter 1, Figure 1.4-1 and Section 1.4.2.1), while Ki-67 is almost absent in this phase of the cell cycle (Gerdes *et al.*, 1984). Undoubtedly, these data support the indication that cell death and cell proliferation are somewhat linked. In fact, it is increasingly clear that there are genes necessary for proliferation which are associated with apoptosis as well. This is the case of c-Myc which over-expression plays a double role; it increases the rate of cell death and cell proliferation (Zornig & Evan, 1996). Interestingly, this is also the hypothesis proposed by Burhans *et al.* (2002) concerning the possible role of the origin licensing in regulating cell death other than only regulating DNA replication.

This study further investigated the positivity of the steroid hormone receptor ER in the same series of breast specimens. The nuclear positivity of ER was associated with Bcl-2 with particularly strong significance ($r=0.33$; $p=0.007$) (Figure 4-6). This correlation is in line with other works (Yang *et al.*, 1999; Rehman *et al.*, 2000; Ioachim *et al.*, 2000; Park *et al.*, 2002). This association suggests that estrogen may regulate Bcl-2 in breast cancer. In fact, anti-estrogens (*e.g.* tamoxifen) have been shown to induce cell death in breast cancer by down-regulation of Bcl-2 without altering Bax (a pro-apoptotic protein) or p53 (reviewed by Kumar *et al.*, 2000). A weak, albeit significant, correlation of ER with increasing tumour grade was shown in this work ($r=-0.28$; $p=0.023$) (Figure 4-11). This was probably caused by the random scatter of points of ER generated by the variability of the labelling indices across tumour grades. Conversely, ER did not show strong correlations with the proliferative markers as

reported by others employing Ki-67 (Yang *et al.*, 1999; Rehman *et al.*, 2000). This means that in the series analysed here various tumour cases displayed the proliferative markers along with some level of expression of ER. However, in an earlier work Clarke *et al.* (1997) showed that about two-thirds (n=19) of the total number of breast cancer cases analysed (n=25) were expressing ER and Ki-67, whereas a dissociation between the two markers was seen in normal breast epithelium. In addition, Shoker *et al.* (1999) showed that a variable percentage of proliferating precancerous cells co-expressed ER, suggesting that this pattern could represent important modifications during the pathogenesis of breast cancer. ER positive cells may also have an autocrine way to respond to secreted growth factors. It has also been demonstrated that steroid receptors are not down-regulated during cell proliferation because they are expressed in separate cells (reviewed by Clarke, 2003).

The fact that the down-regulation of Ki-67 protein followed the decrease in proliferative activity, while the down-regulation of MCM proteins was belated, indicated that Ki-67 is still a valid proliferative marker in cancer. However, this study showed the higher sensitivity of the MCM proteins since these proteins more frequently stained the neoplastic cells than Ki-67 antibody. This higher sensitivity may be paralleled by a lower specificity of MCM proteins. If this is the case, the use of multiple markers could overcome this drawback. For example, among all the proliferative markers analysed this study displayed a higher presence of Mcm5 in tumour grade I and in associated normal tissues belonging to the same grade. This result may be seen in two different ways. Mcm5 may be more sensible at detecting premalignant cells since the labelling indices in tumour grade I (mean=1.82) are higher than those in normal tissues (normal associated to grade I: mean=1.50) (Tables 3–4 and 3–5, Chapter 3, Section 3.3.2.1.). Conversely, being that the values of the means are relatively close, Mcm5 may be considered not to be highly specific at detecting premalignant lesions and, as previously hypothesised, that is why it could be involved in playing a part in additional roles rather than being only a DNA replication co-factor. Hence, this shows the importance of using multiple markers for the early diagnosis of breast cancer and for the detection of distant metastasis. In this mode, sensitivity may not be compromised by specificity and it would possibly be more powerful to portrait the growth dynamics of the heterogeneous breast epithelium otherwise not obtainable

by staining for a marker alone. Further investigation of the value of Mcm5 labelling index as a diagnostic indicator of early tumour development is warranted.

The advantages of using a combination of markers are also suggested by the multivariate logistic regression analysis performed. The proliferative indicators (*i.e.* MCM and Ki-67) showed a higher power to predict both the tumour from the associated normal cases and the grade of the tumour lesions when employed in combination than alone (Tables 4-5, 4-6, 4-7 and 4-8). When the markers were studied singularly, Mcm2 and Mcm7 showed a better tumour predictive power (Table 4-5). From the combined regression analysis (*i.e.* MCM and Ki-67), shown in Table 4-6, a good prediction was observed between tumour grade I and the associated normal tissues although the number of cases compared was small (n=19). Singularly, Mcm5 and Ki-67 were the worst predictors (Table 4-6) and this can be related to their higher presence in normal tissues as shown in the analysis of the means in Figure 4-10. A high percentage of prediction was observed when the combination of factors was tested for tumour grade II and III (Table 4-8; $p<0.001$), but not for grade I and II (Table 4-7; $p=0.052$). The comparison between the results in Table 4-7 and the differences of the means summarised in Table 3-4 (Chapter 3) and Table 4-2 indicates that the MCM and Ki-67 are not useful predictors of grade I and II. At last, Table 4-9 shows that Bcl-2 and ER are not good tumour grade predictors because of the low percentages correctly predicted. Overall, these multivariate logistic analyses suggest that the combination of Mcm2, 5, 7 and Ki-67 may help to predict tumours from the associated normal tissues and may facilitate the prediction of the tumour grade. Individually the more powerful proliferative predictors are Mcm2 and Mcm7. However, further trials employing multivariate analysis using a higher number of cases are definitely indicated to better understand the predictive nature of these markers.

A survival curve could have added information about the effectiveness of MCM as prognostic factors since the proliferative fraction correlates with increasing malignancy and inversely with prognosis in various types of tumour (Wharton *et al.*, 2001). This analysis was not possible because the data were collected prospectively. This investigation could be carried out in the future upon the availability of the data.

In conclusion, in breast carcinoma the Mcm2, 5 and 7 proteins were constantly more expressed than the nuclear protein Ki-67 making them potential markers for

cancer diagnosis. The behaviour of Bcl-2 in relation to the proliferative markers suggests that tumour growth is the result of the balance between the proportion of cells licensed to proliferate and the cells designated to die. Immunopositivity of ER was related to good prognostic markers such as low tumour grade. Nevertheless, it was not directly associated to the proliferative markers possibly by virtue of its role as modulator of cell proliferation and differentiation.

5 Final discussion

5.1. INTRODUCTION

Breast cancer progression is the result of a multistep process where the cumulative effect of genetic alterations leads to the transformation from normal to premalignant to frankly malignant tissue and finally metastasis. Advanced breast cancer diagnosis is more likely to be associated with breast cancer death. Hence, the utmost premise for breast cancer screening is to detect the lesion before it becomes palpable. Inappropriate cellular proliferation is the hallmark that characterises cancer progression. However, the mechanism that underlies cell proliferation is very similar both in normal and in early cancerous cells, making it difficult to discriminate benign from malignant cells.

An intriguing network of signalling pathways drives the activation of some genes to accomplish and deliver the initial command instituted in the cells. These signalling pathways can control cell proliferation, quiescence, differentiation, cellular senescence and apoptosis, and they converge to a point that is called the 'initiation of genome replication'. The core of this convergence point is the assembly of pre-RC resulting in the chromatin being 'licensed' for DNA replication in the subsequent S-phase of the cell cycle (reviewed by Ritzi & Knippers, 2000). Defects often occur in the proliferative pathways that converge on origin licensing. This convergence point is strategically located downstream of these proliferative responses and an aberrant level of the components involved in DNA replication would allow the prompt detection of abnormal cell proliferation. This is particularly important when considering pre-neoplastic cells or cells with replication potential that might be missed by conventional immunostaining. The centre of the initiation of genome replication is the sequential assembly at replication origins of the initiation factors representing the pre-RC.

Fundamental platforms for the pre-RC are the MCM proteins actively involved in DNA replication licensing that have been studied.

The primary goal of this research was to study aberrant cell proliferation in breast epithelium. This would contribute to the control of breast cancer through the use of the novel MCM proteins.

5.2. APPROACH AND DISCUSSION

The research started with the development of an *in vitro* model using cancer cell lines to optimise the methodologies for the subsequent studies on human breast specimens (Chapter 2). The members of the MCM family complex investigated were Mcm2, Mcm5 and Mcm7 because of the availability of the commercial antibodies at the time when the research started. These techniques after further optimisation were applied to a population of 65 patients using the above replication factors (Chapter 3). Then, parallel comparisons with other markers (Ki-67, Bcl-2 and ER) normally in use in the clinical histopathology of breast cancer were performed (Chapter 4). Statistical analyses were undertaken to analyse the results and to evaluate the diagnostic value of the MCM proteins. The findings have been discussed in detail at the end of each chapter. Yet a few considerations need to be addressed in this final discussion since it is important to cluster these findings in light of the MCM applicability as a potential tool for early breast cancer diagnosis.

The *in vitro* study presented in Chapter 2 investigated the *MCM* transcripts in three cell lines which were ready to be propagated, two breast cell lines (one normal and one tumour) and a bladder cancer cell line. The latter was used as control. All three cell lines were found positive for the mRNA *MCM* transcripts analysed (Figures 2-2, 2-5 and 2-6; Chapter 2). Therefore, the normal cell line was considered unsuitable to perform the gene expression comparison between tumour cell and normal breast. It is possible that the positivity of the normal breast cell line was caused by the immortalisation procedure. These results are in alignment with previous work by Kearsey & Labib (1998). To better understand the differential expression of the *MCM* in the different phases of the cell cycle, cell synchronisation studies are suggested as mentioned in Chapter 2. This analysis could also be extended to the simultaneous investigation of *Ki-67*.

The gene expression analysis in Chapter 3 using fresh breast specimens showed that the most reliable of the techniques investigated to extract RNA (*i.e.* RNA extraction from frozen breast tissues and LCM) is LCM. Although the number of fresh specimens retrieved and analysed was limited the results obtained were quite constant. This technique, however, is expensive, needs trained personnel and each experiment needs to be confirmed by a second repetition making its use unsuitable as a routine procedure in the district hospital.

The labelling indices expressed as percentages of cells stained by the MCM antibodies were always higher in tumour than in the associated normal sections. As explained in Chapter 3 (Section 3.4.), the possibility that the control tissues were not entirely normal was minimised by choosing sections that were morphologically entirely normal as judged by an experienced consultant histopathologist. In all the normal controls examined Mcm2, 5 and 7 labelling indices were about two fold lower than in tumours ($p<0.001$). Significant differences were also observed across tumour grades which were classified according to the Scarff-Bloom-Richardson system, as modified by Elston-Ellis (1991). The analysis of variance showed that the labelling indices were always increasing with progression to poorly histologically differentiated carcinomas ($p<0.001$). This pattern of expression applied also to Ki-67 but to a much lower extent (Chapter 4). By comparison the labelling indices of Mcm2, 5 and 7 were always consistently higher than Ki-67 ($p=0.000$) (Chapter 4, Section 4.3.4.). The simplest explanation of this lower expression of Ki-67 could lie in the Ki-67 presence in S, G_2 and M phase of the cell cycle but not in G_1, as reported by Gerdes *et al.* (1984) and Verheijen *et al.* (1989). This possibly explains why MCM proteins may be the right candidates to be considered as markers of the G_1 and S-phase since they start to co-localise in early G_1 being loaded by the Cdc6 proteins and exert the DNA replication regulation in S-phase (Figure 1.4–1, Chapter 1, Section 1.4.2.1.). In other terms, MCM proteins could recognise not only the proliferating compartment of the tumour but also those cells that, despite not proliferating, may be competent to re-enter the cell division cycle and start proliferating. This subset of cells would not express Ki-67 but would express the MCM proteins. This may explain the higher sensitivity of the MCM biomarkers.

This sensitivity of the MCM proteins can be of interest when looking for a reliable marker for early breast cancer screening. Ideally a reliable marker is one with the features of high sensitivity and specificity. This ideal marker could reduce not only the false-positive but also the false-negative rate adding precious information to the clinician for diagnosis and treatment. As Sidransky (2002) suggested, however, there is a trade-off between sensitivity and specificity. High sensitivity does not parallel well with specificity, with a probable final false result. MCM proteins might represent a balanced compromise. During the early stages of tumour progression, tumour cells proliferate slowly and often reside in a prolonged G_1 phase and do not frequently enter the S-phase (Stoeber *et al.,* 2001). Thus, the presence of MCM proteins in this phase would identify them as sensitive biomarkers with a high degree of specificity. This hypothesis could be studied using protein co-localisation techniques to determine the simultaneous presence of the MCM but not of Ki-67, as mentioned in Chapter 4 (Section 4.4.).

When screening for breast cancer or cancer, in general, the use of multiple markers would allow the portrayal of the growth dynamics of the heterogeneous breast epithelium in a more precise way not obtainable by staining for a marker alone. This is demonstrated in the multivariate predictive analysis discussed in Chapter 4 (Section 4.3.6.) where the use of a combination of markers (*i.e.* MCM and Ki-67) was shown to be superior in predicting breast lesions from normal tissues and, in general, in differentiating the tumour grades.

The analysis of the labelling indices for tumour grade I and normal tissue associated to grade I tumour suggests that Mcm5 protein expression was the most expressed compared to Mcm2, 7 and Ki-67 (Table 3-4 and 3–5, Chapter 3, Section 3.3.2.1.; Table 4-2, Chapter 4, Section 4.3.1.). As previously mentioned, these potential differences need further studies. It may be difficult to shed light on this possible high presence of Mcm5. The MCM2-7 protein family acts as a heterohexameric complex when the MCM members are in the active helicase form (Tye & Sawyer, 2000). In addition, previous works suggest that the family components are present in similar amounts when they prime the replication origins (reviewed by Kearsey & Labib, 1998, Ley & Tay, 2001). However, it may be possible that taken independently some component of the family is present in excess. Therefore, an additional role could be

hypothesised for Mcm5 besides the replication licensing one. For instance, Mcm5 could act as a transcriptional regulator for the other components of the protein family as it has been reported for Mcm7 by Fitch *et al.* (2003). On the contrary, the component present in the minimal amount could be the limiting factor when DNA replication initiates. In terms of sensitivity and specificity Mcm5 might be considered highly sensitive in detecting the proliferative compartment of cells, when comparing normal tissues and grade I tumours. Conversely, it could also be that Mcm5 is a less specific marker because of the relatively small difference of the labelling indices between these two groups (Tables 3-4 and 3–5, Chapter 3, Section 3.3.2.1.). The predictive multivariate regression analysis validates the latter hypothesis (*i.e.* lower specificity) as shown in Table 4-6 (Chapter 4; Section 4.3.6.) where, though the number of cases analysed is relatively small, the predictive power of Mcm5 was low and not significant.

This work further extended the analysis to the cytoplasmatic oncoprotein Bcl-2 and the steroid hormone receptor ER. This novel correlation study was important to assess the functional potentiality of the MCM proteins. Bcl-2 and ER are indicators of well-differentiated tumours and are likely to be down-regulated in moderately as well as poorly differentiated carcinomas (van Slooten *et al.,* 1998; Rehman *et al.,* 2000; Siziopikou & Schnitt 2000; Margolese *et al.,* 2000; reviewed by Sugg & Donegan 2002). Interestingly, the strongest negative association with Bcl-2 was shown for Ki-67 ($r=-0.35$; $p=0.004$) (Chapter 4, Section 4.3.5.), whereas the relationships with Mcm2, 5 and 7 were poor and non significant. This behaviour may also be explained in terms of sensitivity. In fact, although at a very low level this poor or absent correlation indicated a co-expression of the MCM proteins and Bcl-2, this co-expression was less likely to occur for Ki-67 and Bcl-2. This subset of cells that co-expressed the proliferative MCM markers and the oncoprotein suggested the hypothesis that MCM could allow identifying preinvasive forms otherwise not detectable using Ki-67. Importantly, it has been shown that Bcl-2 promotes cell survival in slow proliferating cells being up-regulated in G_0/G_1 phase (Borner, 1996). This could further explain the stronger negative association with Ki-67 in light of its absence in G_1 phase of the cell cycle.

The nuclear positivity of ER was significantly correlated with Bcl-2 ($r=0.33$; $p=0.007$) (Chapter 4, Section 4.3.3.) but not with all the proliferative markers. Indeed, the cutoff level of one nucleus stained for ER played a role in this correlation study.

Although no association study has been reported between MCM and ER, Clarke *et al.* (1997) found a high proportion of breast tumours expressing ER and Ki-67. This could be explained by the fact that the steroid receptors are not down-regulated during cell proliferation since they can be expressed in different cells (reviewed by Clarke, 2003).

In conclusion, this work has concentrated on the potential diagnostic use of the MCM proteins. The present findings suggest that Mcm2, 5 and 7 proteins may be interesting markers for detecting neoplastic and pre-neoplastic cells to aid breast cancer diagnosis.

6 Conclusions and further work

6.1. CONCLUSIONS

This thesis has focused on the MCM proliferative proteins as indicators of cellular proliferation defects in breast cancer. The protein expression findings suggest that these signature components (*i.e.* Mcm2, 5, 7) of DNA replication are promising biomarkers for early cancer diagnosis and can aid the grading of breast cancer. Hence, targeting proteins directly regulating DNA replication may represent a more accurate way of reflecting tumour proliferation.

The comparative analysis with Ki-67 which is the most used marker of cellular proliferation in breast histopathology showed that Mcm2, 5 and 7 proteins are more frequently detectable in proliferating cancer cells. This suggests that the pre-replicative MCM proteins may be able to recognise not only the proliferative compartment of the lesions but also those cells resting in a non-proliferative state and potentially competent to start proliferating. However, this hypothesis needs to gain further evidence employing different methodologies such as double label confocal microscope and/or immunoprecipitation, as discussed in Chapter 4. The multivariate regression analysis suggests that the combination of Mcm2, 5, 7 and Ki-67 protein expression has more predictive power than the single marker alone in discriminating between normal and malignant tissue and between tumour grades.

As stated in Chapter 3 a drawback of the immunohistochemistry assessment used in this study is the subjectivity of the observations. Thus, to increase uniformity two observers were employed. For further experimental trials it is suggested to increase the number of observers to minimise human error.

6.2. FURTHER WORK

The detection of breast cancer is a broad subject especially when looking at the proliferation activity of the cells. The findings of this thesis have highlighted areas for further work:

1. To extend the investigation to the other components of the MCM protein family (*i.e.* Mcm3, Mcm4 and Mcm6). This work could be carried out initially using cell lines to determine the co-expression of all the components of the heterohexameric complex in specific phases of the cell cycle such as in G_1 phase. This could be achieved by using cell cycle inhibitors to synchronise the cells. Subsequently the analysis could investigate the presence of the six proteins in human specimens to assess their effectiveness in detecting cell proliferation defects using these proteins both individually and in combination.

2. To investigate other components of the pre-RC such as Cdc6 proteins and to correlate them with MCM. The Cdc6 proteins are present in early G_1 and functionally interact with the ORC and load the MCM proteins. This could be achieved as indicated in point number one of this section and extended to include Ki-67 and PCNA. Such analysis would allow assessing the reliability of each of these components at detecting cellular proliferation defects.

3. To study any additional role of the components of the MCM protein family, besides the replication licensing one. For example, this was the case for Mcm5, as suggested by this research. This could be investigated using correlation studies after having induced either Mcm5 protein expression or protein repression using antisense or RNA interference (RNAi) procedures.

4. To analyse dysplastic breast tissues for the MCM proteins. As previously described, dysplasia is an intermediate state between normal tissue and neoplasia. Such investigation could give further evidence on the superiority of these markers compared to conventional markers in identifying pre-cancer lesions.

5. To extend the investigation of MCM to specimens obtained by carrying out less invasive techniques than breast lumpectomy or mastectomy. This is the case of fine needle aspiration (FNA) which is normally performed for the diagnosis of breast cancer. FNA consists of collecting sample fluid from a breast cyst using a syringe

or of removing clusters of cells from a solid mass. More invasive procedures are collecting the sentinel lymph nodes or breast biopsies (*e.g.* core biopsy samples from primary breast tumours). These are solid tissues that are stained in paraffin-embedded permanent sections.

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Appendixes

APPENDIX A

Buffers solutions

PBS	1 PBS tablet (Sigma-Aldrich) 500 ml of distilled H_2O in a beaker Autoclave to sterilise.
TBS (20x)	121.1 g of Trizma (TRIS) 175.3 g of NaCl ~60 ml of HCl 9.8 M to reach a final pH of 7.6 To a final volume of 1 litre of distilled H_2O.
TBS (1x)	50 ml of TBS (20x) To 950 ml of distilled H_2O.

Immortalization of 184-B5 and establishment of ZR-75-1 and RT-112 cell cultures

184-B5	The cell line was bought immortalized after treatment by Benzo[a]pyrene. The following induction of transformation has been performed by Stampfer & Bartley, 1985 as mentioned in the ATCC data sheet: briefly, benzo[a]pyrene exposure occurred to sub-confluent primary cultures of HMEC. Five ml of medium containing 1 ug of Benzo[a]pyrene per ml dissolved in 2.5 ul of dimethyl sulfoxide was placed on the cells for 24 hr periods. Control cells received the dimethyl sulfoxide alone (Stampfer & Bartley, 1985).
ZR-75-1	The cancer cell line was bought ready to be cultured with RPMI-1640. The depositor of the cell line, Engel *et al.,* 1978, performed the establishment of the culture as following: briefly, the cancer cell pellets were resuspended in culture medium at 36°C to form a dense suspension and seeded in 75-sq cm plastic flasks, cultures were maintained with minimal population and twice-weekly feeding until resultant monolayer were passaged 2:1, 47 days after the initiation of the culture, two subsequent 1:1 passages were made at weekly intervals after which 1:2 passages weekly became routine (Engel *et al.,* 1978).
RT-112	The cancer cell line was available in the laboratory and ready to be cultured with RPMI-1640. Marshall *et al.,* 1977 performed the derivation of the cancer cell line as follow: briefly, the cancer cells

were maintained in Dulbecco's modified eagle's medium supplemented with 10% of FCS and passaged at 1:10 split ratios when they reached confluence, the double time of the cells was 24-36 hours and appeared not to have a finite life-span (Marshall *et al.*, 1977).

Protein extraction solutions

Protease inhibitor

The kit comprises four vials (EDTA, pepstatin, leupeptin and AEBSF) to be resuspended in 100 ml of distilled H_2O.

CHAPS lysis buffer

20 mM of TRIS at pH 8.0
0.15 M NaCl
5 mM EDTA
310 g mg of CHAPS
1 ml protease inhibitor
To a final volume of 50 ml of distilled H_2O.

IHC solutions

DAB

0.14 g DAB
440 ml H_2O_2
200 ml TBS (1x)

Citrate buffer

2.1 g of citric acid
To 1 litre of distilled H_2O in a beaker
0.1 N of NaOH to reach the final pH of 6.0.

Western blotting

Main gel (6%)

2.5 ml of 1M TRIS
2 ml of 30% Bis/Acrylamide
100 µl of 10% SDS
50 µl of 10% Ammonium Persulphate
10 µl of TEMED
5.35 ml of distilled H_2O.

Stacking gel (4%)

1.25 ml of 0.5M TRIS
650 µl of 30% Bis/Acrylamide
50 µl of 10% SDS
25 µl of 10% Ammonium Persulphate
10 µl of TEMED
3.05 ml of distilled H_2O.

Loading buffer

5 ml of 0.5M TRIS
5 ml of Glycerol
10 µl of 10% SDS
5 ml of 1% Bromophenol blue

	25 ml of distilled H_2O.
Electrophoresis buffer (10x)	30.3 g of TRIS 144 g of Glycine 100 g of SDS To a final volume of 1 litre of distilled H_2O.
Transfer buffer	2.905 g of TRIS 1.465 g Glycine 0.5 g of SDS 100 ml of Methanol To a final volume of 1 litre of distilled H_2O.

APPENDIX B

Paraffin embedded sections treatment

De-waxing	5 minutes incubation x2 washes in Histoclear (or xylene) 5 minutes incubation x2 washes in graded 100% ethanol 5 minutes incubation x2 washes in graded 90% ethanol 5 minutes incubation x2 washes in graded 70% ethanol.
Re-hydration	Sections rinsed in distilled H_2O until required.
Dehydration	5 minutes incubation x2 washes in graded 70% ethanol. 5 minutes incubation x2 washes in graded 90% ethanol 5 minutes incubation x2 washes in graded 100% ethanol 5 minutes incubation x2 washes in Histoclear (or xylene).

APPENDIX C

Primers computational data sheets

Primer Calculation Results

Mcm2 NM_004526

Query ID	_997978530_17683
User email	s.degan.s00@cranfield.ac.uk
Project description	Breast Cancer Research

8 best Pairs (of max. 11891)

ID	Forward Primer	Reverse Primer	Qual.	Prod. Len.	T_mDiff.	FPPos. RPPos.
1	CTCCTCGCAGATCTGGTGGACA	CAAGGTCCACTCCCTGCTGACA	886	500	0	1186 1686
2	ACCTCCAGCCCTGGCCGTGA	GGCCGTGGCTGTCGACGTGA	886	500	0	109 609
3	AGCCCAAAAACCCAGGTGGCAA	GGGCCAGCATCTCGTCCTGGA	886	500	0	1502 2002
4	TATGTCCAGCGGCACCCTGTCA	GGTGGACCCTCTCCTTGGCGTA	886	500	0	1669 2169
5	CCAGTCAGAGGGAGGCAGCAGA	TGTGGTTGGTGATGCGGTCGTA	886	500	1	314 814
6	GGACTTGGCAGCCAGGGAGCA	CTGGCTTGCAGCTGTCCACCA	886	500	1	699 1199
7	TCAACGTGCTCTTGTGCGGAGA	TTGTTGCTGGGGTGGTGTCTGA	886	500	0	1544 2044
8	ACAGCTGCAAGCCAGGAGACGA	GAACCAGGCCCCAGCCTCCAA	886	500	0	1205 1705

Progress output for your primer calculation is here.

Primers Profile - Data Sheet

Query ID	_997978530_17683
User email	s.degan.s00@cranfield.ac.uk
Project	Breast Cancer Research

Pair	Priming Sites
#4	

	Pair Data
Product Size	500
T_m Difference	0
Quality	886

	Forward Primer Data	Reverse Primer Data
Sequence	TATGTCCAGCGGCACCCTGTCA	GGTGGACCCTCTCCTTGGCGTA
GC Content	59	64
Position	1669	2169
Degeneracy	0	0
3' GC	50	50
3' Degeneracy	0	0
T_m	66.4555	66.4848
Quality	517	517
Location	1669	2169

Primer Calculation Results *Mcm5* NM_006739

Query ID	_1001934001_11337
User email	s.degan.s00@cranfield.ac.uk
Project description	Breast Cancer Research

8 best Pairs (of max. 8063)

ID	Forward Primer	Reverse Primer	Qual.	Prod. Len.	T_mDiff.	FPPos. RPPos.
1	ATCGCCAAGGCTGGGATCACCA	CCGCAGGGCCTCCTCCACA	886	500	0	1486 1986
2	CGGCCGCTGGGATGAGACGA	CCTGGCTGGTGAAGCCCTCCA	886	500	0	1557 2057
3	CGGCCGCTGGGATGAGACGAA	CCTGGCTGGTGAAGCCCTCCA	886	500	0	1557 2057
4	GGGCTATGCCCTGCCCAGGAA	TTTCGGGAGCCCCCAAAGAGCA	886	500	0	648 1148
5	CGGCTGTCAGCAGAGGCTGCA	GCGTGGCGAGTCCATGAGTCCA	886	500	0	1798 2298
6	GTCGGACGCCAGCCCTTCCA	GTGTCCACCTGGATGCCCAGGA	886	500	0	468 968
7	GGGGCCCGTCAGCACGAGA	GGGAGGCAGAGGTCCCAGCAA	886	500	0	1854 2354
8	AGCTGCAGAGGCGCTTCAAGGA	GTCCAGCCTGATCTGTGTTGCA	885	500	3	170 670

Progress output for your primer calculation is here.

Primers Profile - Data Sheet

Query ID	_1001934001_11337
User email	s.degan.s00@cranfield.ac.uk
Project	Breast Cancer Research

Pair	Priming Sites
#1	

	Pair Data
Product Size	500
T_m Difference	0
Quality	886

	Forward Primer Data	Reverse Primer Data
Sequence	ATCGCCAAGGCTGGGATCACCA	CCGCAGGGCCTCCTCCACA
GC Content	59	74
Position	1486	1986
Degeneracy	0	0
3' GC	50	50
3' Degeneracy	0	0
T_m	66.8291	65.8885
Quality	517	517
Location	1486	1986

Primer Calculation Results

Mcm7 NM_005916

Query ID	_1002118118_5759
User email	s.degan.s00@cranfield.ac.uk
Project description	Breast Cancer Research

8 best Pairs (of max. 203)

ID	Forward Primer	Reverse Primer	Qual.	Prod. Len.	T_mDiff.	FPPos. RPPos.
1	TCGCCCCTTCCCAGCCCCAA	CTGAGAATCTCCGCGCGGTGGA	848	429	0	48 477
2	TCCCAGCCCCAAGGGTCTAGGA	CTGAGAATCTCCGCGCGGTGGA	844	421	0	56 477
3	TCGCCCCTTCCCAGCCCCAA	GGTGGACTGTGGCCGGCCAA	841	415	0	48 463
4	TCGCCCCTTCCCAGCCCCAA	ACTGTGGCCGGCCAACCGAA	838	410	1	48 458
5	TCCCAGCCCCAAGGGTCTAGGA	GGTGGACTGTGGCCGGCCAA	837	407	0	56 463
6	TCCCAGCCCCAAGGGTCTAGGA	ACTGTGGCCGGCCAACCGAA	834	402	0	56 458
7	TCGCCCCTTCCCAGCCCCAA	GGCCAACCGAAATTGGCGCGAA	833	399	0	48 447
8	TCCCAGCCCCAAGGGTCTAGGA	GGCCAACCGAAATTGGCGCGAA	829	391	1	56 447

Progress output for your primer calculation is here.

Primers Profile - Data Sheet

Query ID	_1002118118_5759
User email	s.degan.s00@cranfield.ac.uk
Project	Breast Cancer Research

Pair	Priming Sites
#5	

	Pair Data
Product Size	407
T_m Difference	0
Quality	837

	Forward Primer Data	Reverse Primer Data
Sequence	TCCCAGCCCCAAGGGTCTAGGA	GGTGGACTGTGGCCGGCCAA
GC Content	64	70
Position	56	463
Degeneracy	0	0
3' GC	50	50
3' Degeneracy	0	0
T_m	66.8924	67.4819
Quality	517	517
Location	56	463

APPENDIX D

Miscellaneous protocols

Tissue sectioning for RNA extraction from frozen breast tissues

RNase- and DNase-free plastic ware was used. Scalpels, tweezers and forceps were washed with detergent and baked at 210°C for 4 hours before use. Work surfaces pipetters and equipments were wiped using RNaseZap (Ambion, Huntingdon, UK) and disposable gloves were changed frequently.

Frozen tissues were removed from the 2ml vials and placed in the cryostat chucks. The cryostat was pre-cooled at -28°C. The tissue was embedded in OCT embedding fluid (Bright Instruments, Cambridge, UK) to aid subsequent homogenization to 100-200 8µm sections which were taken from each block of breast tissue.

Section cut from each specimen were placed into a 2ml γ-irradiated tube. Sections for subsequent use were stored at –80°C.

Tissue sectioning for RNA extraction for LCM

Same procedure as above, but each 8µm section was placed in a RNase- and DNase-free glass slide. Folded or wrinkled sections were discarded as recommended by the protocol. Sections for subsequent use were stored at –80°C.

Slide preparation prior LCM

RNase-free techniques were used as described above and recommended by the manufacturer (Arcturus).

Staining and Dehydration: four slides at a time were placed into the slide jars provided with the kit (HitoGene kit).

1. Slides were removed from –80°C freezer and were placed at RT for 30 seconds.
2. 100µl of the HistoGene Staining Solution was placed to cover the tissue section for 20 minutes.
3. Slides were placed in slide jar containing distilled H_2O for 30 seconds.
4. Slides were placed in slide jar containing 75% ethanol for 30 seconds.
5. Slides were placed in slide jar containing 95% ethanol for 30 seconds.
6. Slides were placed in slide jar containing 100% ethanol for 30 seconds.
7. Slides were placed in slide jar containing xylene for 5 minutes.
8. Slides were allowed to dry up in the hood for 5 minutes.

Slides were placed in fresh desiccant until LCM.

DNase treatment after LCM

Kit used for DNase treatment: Qiagen, catalogue No. 79254.

1. 5 µl DNase I Stock Solution were pipetted to 35 µL Buffer RDD (provided with RNase-Free DNase Set). The solution was mixed by gently inverting.
2. The 40 µl total DNase incubation solution was pipetted and mixed into the purification column membrane and incubated at RT for 15 minutes.
3. The 40 µl PicoPure RNA Kit Wash Buffer 1 (W1) was pipetted into the purification column membrane and centrifuged at 8000 xg for 15 seconds.

APPENDIX E

Ethical approval

Gloucestershire Health Authority NHS

Victoria Warehouse
The Docks
Gloucester
GL1 2EL

Tel: (01452) 300222
Fax: (01452) 318800

Direct Tel: (01452) 318864
Direct Fax: (01452) 318868

Please contact: Hazel Moynihan
(Administrator, Gloucestershire LREC)

Our Ref: 00_142G(10_7).doc

10 July 2001

Dr Keith McCarthy
Consultant Histopathologist
Dept. of Histopathology
Cheltenham General Hospital
Sandford Road
Cheltenham
GL53 7AN

Dear Dr McCarthy

Study No 00/142G : Investigation of molecular abnormalities in breast cancer: the role of interactions between duct secretions and breast epithelium & the use of molecular markers in increasing the accuracy of tumour cytodiagnosis

Thank you for sending me the amended Consent Form and Patient Information Sheet for the above study. We are now able to give you full approval to proceed with the above study. Can you please send a copy of the Consent Form with the changes I suggested in my email of 6 July 2001, in order to keep our files up to date.

The Committee draws your attention to:

a) It is the responsibility of the investigator to notify the LREC immediately of any information received by him/her, or of which he/she becomes aware which would cast doubt upon, or alter, any information contained in the original application, a later amendment application or verbal resume submitted to the LREC. The committee should be informed immediately if this information would raise questions about the safety and/or continued conduct of the research.

b) The need to comply with the Data Protection Act 1998.

c) The need to comply with the Research Governance Framework for Health and Social Care (Department of Health 2001). Further information regarding this document can be obtained from Gloucestershire Research & Development Support Unit on 01452 318864.

d) The need to refer proposed amendments to the protocol to the LREC for further review and to obtain LREC approval thereto prior to implementation (except only in cases of emergency where the welfare of the subject is paramount).

e) The requirement to furnish the LREC with details of the progress of the research project periodically (usually annually) and **failure to do this could result in approval to continue with the study being withdrawn.** Please also inform us of the conclusion and outcome of the research project and inform the LREC should the research be discontinued or any subject withdrawn altogether.

f) You will be required to supply the name and hospital number of all patients entered into your trial. This is so the Clinical Records Officer can mark them as 'trial subjects' as the trust will be required to retain these for longer than usual to fulfil the requirements of new government legislation.

g) It is the responsibility of the person conducting any Trial to ensure that all professional staff and management of NHS Trusts involved are notified that it is taking place.

A list of the members of the Gloucestershire LREC may be supplied if required.

Please indicate your agreement to comply with the requirements outlined in this letter by signing both copies of this letter and returning one to Hazel Moynihan.

Yours sincerely

Hazel Moynihan

pp **Dr M J Richards**
Chair, Gloucestershire LREC

I agree to comply with the requirements outlined in this letter.

Signed

13.vii.00
Date

Patient consent form

East Gloucestershire NHS Trust
Department of Histopathology
Cheltenham General Hospital
Sandford Road
Cheltenham
GL53 7AN
Tel: 01242 274075
Fax: 01242 226255

Centre Number:

Study Number:

Patient Information Number for this trial:

CONSENT FORM

Title of Project: Analysis of molecular abnormalities in breast cancer: the role of breast duct secretions in early breast cancer and the use of molecular analysis in increasing the diagnostic accuracy of fine needle aspiration.

Name of Researchers: Mr J Bristol, Dr K McCarthy, Dr A Woodman

Mrs S Scarrott, Mrs S Kendall and Mrs A Thomas (Breast Care Nurses, CGH ext 3216, bleep 677).

1. I confirm that I have read and understand the information sheet dated --/--/-- (version - - -) for the above study ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time without my medical care or legal rights being affected. ☐
3. I am willing to allow access to my medical records but understand that strict confidentiality will be maintained. The purpose of this is to check that the study is being carried out correctly. ☐
4. I agree to take part in the above study. ☐
5. After the study, I wish tissue used for the study (but not for diagnosis) to be destroyed. ☐

Name of patient	Date	Signature
Name of person taking consent (if different from researcher)	Date	Signature
Researcher	Date	Signature

1 for patient; 1 for researcher; 1 to be kept with hospital notes

Patient information sheet

Patient Information Sheet

Study: Investigation of Molecular Abnormalities in Breast Cancer: the Role of Breast Duct Secretions and the Use of Molecular Abnormalities to Improve the Diagnostic Accuracy of Fine Needle Aspiration .

Purpose of the Study: We wish to gain a better understanding of the early events in the development of breast lumps by studying the possible of role of breast duct secretions. We also wish to study the different genetic abnormalities that are present in benign and malignant lumps so that they can be used to make the results of the needling of breast lumps diagnostically more accurate

Why me? We are hoping to include all patients having breast lumps removed at Cheltenham General Hospital.

Who is organising the Study? This study is a collaborative project between the Departments of Surgery and Pathology at Cheltenham General Hospital, and Cranfield University.

What will happen to me if I take part? You will notice no difference in the way that you are investigated and treated. We will only use material that has been obtained for routine investigation and diagnosis in the normal way, and only after all routine investigations and analyses have been performed.

Are there any disadvantages in taking part in this trial? No.

What are the possible risks in taking part? There are no risks at all.

What are the possible benefits in taking part? It is unlikely that there will be any direct benefits to you, but this study will help to increase our knowledge of how breast cancer starts and will also directly benefit future patients with breast lumps by improving our ability to make accurate and early diagnosis.

Is my doctor being paid for including me in this study? No.

Confidentiality – who will know I am taking part in the study? All information which is collected about you during the course of this study will be kept strictly confidential. Any information which leaves the hospital will be anonymised so that you cannot be recognised from it.

Ethics Committee Approval: This study has been approved by Gloucestershire Local Research Ethics Committee.

Contact for further information: If you have any questions with regard to this trial, please contact either Sue Kendall, Sue Scarrott or Angela Thomas, Breast Care Nurses.

We would like to take this opportunity to thank you for agreeing to participate in this study.

12/03/01 Version 2.

APPENDIX F

Published work

Degan, S. A., McCarthy, K., Bristol, J. B., Annis, M., Chan, H. Y., and Woodman, A. C. Evaluation of inappropriate cell proliferation in clinical breast specimens with Minichromosome Maintenance Proteins. *Proceedings of the American Association for Cancer Research.* (2004).

Degan, S. A., McCarthy, K., Bristol, J. B., and Woodman, A. C. Gene and protein expression of Minichromosome Maintenance Proteins in breast cancer. *Proceedings of the American Association for Cancer Research.* (2003).

Degan, S. A., McCarthy, K., Bristol, J. B., and Woodman, A. C. The Detection of Genetic Abnormalities in Breast Epithelium leading to Breast Cancer. *Proceedings of the World Conference on Breast Cancer*, Victoria, BC, Canada. (2002).

Degan, S. A., McCarthy, K., Bristol, J. B., Annis, M. Chan, H. Y., and Woodman, A. C. Altered expression of Minichromosome Maintenance Proteins in breast epithelium: potential use for prevention and early diagnosis in breast cancer. (*Submitted*).

Degan, S. A., McCarthy, K., Bristol, J. B., Annis, M. Chan, H. Y., and Woodman, A. C. The diagnostic significance of the replicative Mcm2, 5 and 7 proteins in ductal carcinoma in situ of the breast. (*In preparation*).