Discovery and Restoration of Aberrant Nuclear Structure and Genome Behaviour in Breast Cancer Cells

A Thesis Submitted for the Degree of Doctor of Philosophy by:

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Abstract

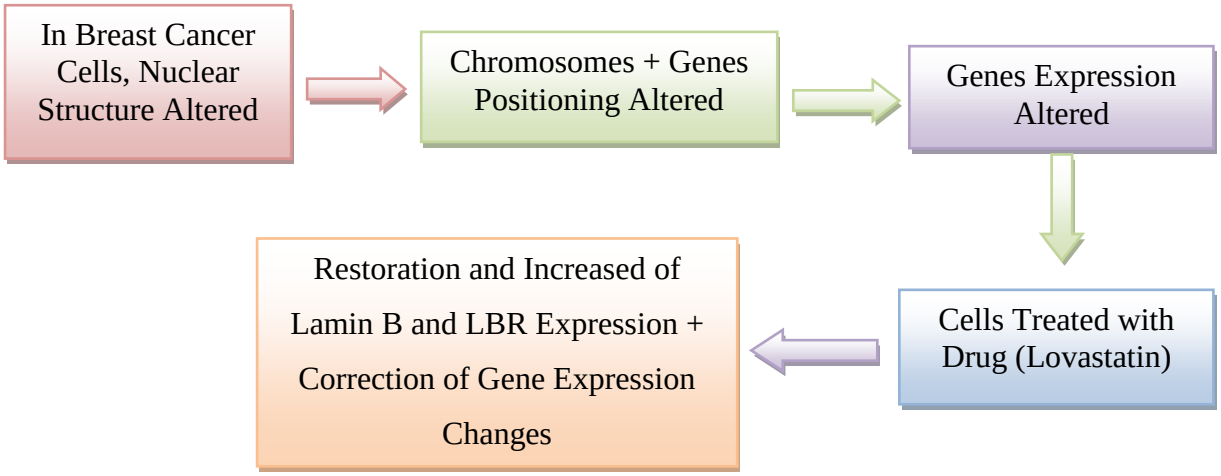
The eukaryotic interphase nucleus is well organised and the genome positioned non-randomly. Nuclear structure is an important regulator of genome behaviour and function. Genome organisation and nuclear structure are compromised in diseases such as cancer and laminopathies. This study was to find out and to determine if there is any functional relationship between nuclear structure and genome mis-organisation in cancer cells. I have assessed the presence and distribution of specific nuclear structural proteins (A-type, B-type lamins and its receptor LBR, many of their binding proteins such as MAN1, LAP2α, LAP2, and Emerin and other nuclear proteins (PML, Nucleolin, and Ki67) using indirect immunofluorescence. From this study, it is found that the nuclear structure of breast cancer cells is often altered. The most severely affected proteins are the nuclear lamins B1 and B2 and they found as large foci within the nucleoplasm with little LBR expression to localise the lamin B. I also assessed the chromosome positioning (HSA 7, 10, 11, 14 and 17) and gene positioning (*AKT1, CCND1, HSP90AA1, EGFR, ERRBB2/HER2* and *PTEN*) in breast cancer cell lines (T-47D, GI-101, Sk-Br-3 and BT-474) and in normal breast cell lines (MCF-10A) using 2D-FISH technique. I also assessed the position of the genes in nuclei and correlated with gene expression using qRT-PCR. Breast cell lines have treated with a drug named lovastatin and it was found that the cells have restored LBR expression and localisation of lamin B, leading to altered gene positioning and changed expression of breast cancer genes.

Since the drug (lovastatin, 12 μM/48 hours) affects the prenylation as a post-translation modification process and lamins B biosythensis, it is found that B-type lamins and its receptor expression and distribution were improved and increased in expression by 2-fold in expression levels in the most affected cells (T-47D, and BT-474) compared to the normal cells (MCF-10A) and these cells also showed abnormal nuclei and dead cells. When

analysing the nuclear positioning of the genes (*AKT1*, *HSP90AA1* and *ERRBB2/HER2*), it is found that *AKT1* was positioned periphery in BT-474 and T-47D cells and interiorly in the normal cells (MCF-10A) before treatment whereas the same gene was positioned periphery in T-47D and MCF-10A cells and interiorly in BT-474 after treatment with lovastatin. It is also found that *HSP90AA1* was positioned periphery in MCF-10A and T-47D cells and interiorly in BT-474 cells before and after treatment (no change). Moreover, *ERRBB2/HER2* gene was positioned periphery in T-47D and BT-474 cells and interiorly in MCF-10A cells before treatment whereas the same gene was positioned periphery in MCF-10A and T-47D cells and interiorly in BT-474 after treatment with the same drug. Regarding *LMNB1*, *LMNB2*, and *LBR* genes, the study focussed only on their expression levels and no work has done on their chromosome positioning as well as gene position before and after treatment. These three genes were over expressed when assessed by measuring the relative and fold changes in expression. Therefore, it is suggestive that 2D-FISH experiment to assess their localisation and their specific chromosome territories is required.

The results shown in this thesis demonstrate the importance and roles of nuclear architecture specifically nuclear lamins and the integral nuclear membrane proteins (B-type lamins and LBR) in mediating correct genome organisation and function. The breast normal (immortalised cells) and cancerous cell lines showed different nuclear structures as lamin B affect the position of specific target chromosomes and genes. These results will strength the finding that the nuclear lamina is a significant nuclear structure which associates, organises, and regulates numerous vital nuclear processes and the stability of the genome.

To Summarise This Work:



Declaration

I hereby declare that the work presented in this thesis has been performed by me unless stated.

Mai M. Hassan Ahmed

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I dedicate this work to my parents.

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Abbreviations

%.....Percentage

Δ……...……Delta

µg……Microgram

µl……..……..…..Microlitre

µM……...…...…….……..Micromolar

µm……..……Micrometre

2D……..…..……....Two Dimensional

A……..…….…..Adenine

AF……...……..Activation Function

AKT……..……Protein Kinase

Anacardic Acid……..…….. 6-nonadecyl Salicylic Acid

APL…….…… Acute Promyelocytic Leukaemia

BAF…..……..…….Barrier to Autointegration Factor

BC……..……..……...……..Breast Cancer

BRCA1……..…..…Breast Cancer Tumour Suppressor Gene

BSA……...……...…..…….Bovine Serum Albumin

CAAX…….....…Cysteine, Aliphatic, Aliphatic, Any of Several Residues

CB……..……..……..……..Cajal Bodies

CDC2 ……..……..Cyclin Dependent Cyclin2

CDK……....…….……...……..Cyclin -Dependent Kinase

cm……...……Centimetre

CML……...……Chronic Myeloid Leukaemia

CMV…….…….…….. Cytomegalovirus

CO_2…….……..……..……Carbon Dioxide

CT……..……...Chromosome Territories

DAPI……..…….....4, 6-Diamidino-2-Phenylindole

DBD……..……..…….DNA Binding Domain

ddH_2O……...……...…….……..Double Distilled Water

DFC……..……...……...……Dense Fibrillar Centres

DMEM..……. ……..…..…..Dulbeco's Modified Eagles Medium

DMSO……..…..Dimethyl Sulphoxide

DNA……...…….Deoxyribouleic Acid

DOP……...…….……Degenerative Oligonuleotide Primer

DPBS……...……...……...Dulbecco's Phosphate Buffered Saline

DSBs…….Double Strand Breaks

dUTP……...……..……Deoxyuridine Trinucleotide Phosphate

E2……..……..……..……..…Estrogen

E2F……..……..……..…Cell Cycle Transcription Factor

EAF1……..……..……...…… Transcription Factor

ECM……...……..……..……Extracellular Matrix

EDTA……...……...……...……...…Ethylenediaminetetra Acetic Acid

EGF……..……..……..Epithelial Growth Factor

EGFR……..……..…….. Epidermal Growth Factor Receptor

EDMD……..…….Emery-Dreifuss Muscular Dystrophy

ER……..……..…..……Estrogen Receptor

ErbB2…….……Erythroblastic Leukaemia Viral Oncogene Homolog 2

ERE……..……..……..…….Estrogen Response Element

FAS……..……...……..……. Fatty Acid Synthase

FBS……...……..…….…….……Fetal Bovine Serum

FC……Fibrillar Centres

FCS……Fetal Calf Serum

FISH……Fluorescence *In Situ* Hybridisation

FITC…… Fluorscein-Isothiocyanate

FSH……Follicle-Stimulating Hormone

FTI……Farnesyltranseferase Inhibitor

G……Guanine

g……Grams

G1……Cell Cycle Gap1 Phase

GC……Granular Component

HAT……Histone Acetyltransferase

HATI……Histone Acetyltransferase Inhibitor

HBV……Hepatitis B Virus

HC…… Hydrocortisone

HER2……Human Epidermal growth factor Receptor 2

HGPS……Hutchinson-Gilford Progeria Syndrome

HIV1……Human Immunodeficiency Virus Type 1

HR……Homologous Recombination

HRT……Hormone Replacement Therapy

HSA……*Homo sapien* Autosome

HSP……Heat Shock Protein

HSV-1…… Herpes Simplex Virus Type-1

ICMT……Isoprenylcysteine Carboxyl Methyltransferase

IF……Intermediate Filament

IGCs……Interchromatin Granule Clusters

IMPs……..……...……..Integral Membrane Proteins

INK……...…….……...……..Inhibitor Kinase

INM……...……...……...…Inner Nuclear Membrane

Kb……..…..……..…….……..Kilo Base

KCl.....Potassium Chloride

KDa……..……..…Kilo Dalton

KIP……..……...…...……..Kinase Inhibitory Protein

L……...…...…….…….Litre

LAP1……..……..…..……..Lamin Associated Polypeptide 1

LAP2……...……..……..…..Lamin Associated Polypeptide 2

LBD…….....……Ligand Binding Domain

LBR…..…….....……....Lamin B Receptor

LEM……..……...……..…..……Lamin, Emerin, and MAN1

LH……..……..……..……Luteinising Hormone

LMNA……..……....…….……..*Lamin A* gene

LMNB1……..……..…..……...…*Lamin B1* gene

LMNB2……...…….....……..……..*Lamin B2* gene

M: A……...……...…..…….……Methanol: Acetone

M……..…….……...…Molar

mAb……..…..…....……..…….Monoclonal Antibody

MAC……..……..……Macintosh Programme

MAPK……...…...…..Mitogen-Activated Protein Kinase

MARs……..…….……Matrix Attachment Regions

MDM2……...……...…..……*Murine Double Minute 2* Oncogene

MEK……..……....…MAPK/ Extracelluar Protein Kinase Kinase

mg……...……...…….……...…Miligram

miRNA……..……...…..……..……*Micro*RNA gene

ml……..……...…….….Mililitre

mM……..……..……..…….Milimolar

mm……..……..……..……..milimetre

MMPs……...……...…… Matrix Metalloproteinases

mRNA……....……...…….……Messenger RNA

MW……..……..……..……...Molecular Weight

Na.....Sodium

NA…….……...……...……..Not Available

NBs……..……Nuclear Bodies

NCS……..…....New Born Calf Serum

NE……...…….…Nuclear Envelope

NHEJ……...……..……. Non-Homologous End-Joining

NLS……..…. Nuclear localisation signal

NM I……...…….…….Nuclear Myosin I

NM……...….…….Nuclear Membrane

nm……..……...…..Nanometre

NMPs……..…...……....……Nuclear Matrix Proteins

NORs……...…..……Nucleoli Organising Regions

NPCs……...……...…….…….Nuclear Pore Complexes

º C……...……...…..……..Degree Centigrade

OC……..……....…….Ovarian Cancer

ONM…….....…….Outer Nuclear Membrane

p53……...…...……...……..Nuclear Protein

PBS……..…….……Phosphate Buffer Saline

PCNA……..Elongation Factor

PCP……...……..Partial Chromosome Paint

PCR……..……..…..……Polymerase Chain Reaction

PI3K……..……..…… Phosphatidylinositol 3-Kinase

PIPK……....…….……..Phosphotidylinositol Phosphate Kinase

PML……..…..……..……...Promyelocytic Leukemia

PNC……...……...…….……...…..…. Perinucleolar Compartment

Pol I……..…….…….……RNA Polymerase I

Pol II……...……...RNA Polymerase II

Pol III……...……..……..…..……RNA Polymerase III

PR……...……....……Progesterone Receptor

pRB……..……...Retinoblastoma Protein

PSQ…….…….Penicillin, Streptomycin, and L-Glutamine

PTEN…….....……Phosphatase and Tensin Homolog

PTF…..……..……..Transcription Factor

Rad51……...……..Homologous Recombination Protein

RAF……..…..…….Tyrosine Kinase Pathway

RAS…….……Tyrosine Kinase Pathway

Rce 1…….....…….……..…..Ras Converting Enzyme 1

RFC……...…….. Elongation Factor

RNA……....……Ribonucleic Acid

RPMI…….……...…..Medium Developed at Roswell Park Memorial Institute

rRNA……..……..……..……..Ribosomal RNA

RT……...……...……...……Room Temperature

S……..……..……..……...Cell Cycle DNA Synthesis Phase

SMN……..……..……..……Survival of Motor Neuron

snoRNA……..……..……..……..Small Nucleolar RNA

snRNA……..……..……..……...Small Nuclear RNA

SRC……..Steriod Receptor Co-activator

SSC……...……Sodium Saline Citrate

SUMO-1……...…….Small Ubiquitine-Like Modifier

T……...……...……..…….Thymine

TBE……..…….……...Tris, Boric Acid and EDTA

TF……..…….…….Transcription Factor

TGF……..……...……Tumour Growth Factor

TNF……..……...…..……Tumour Necrosis Factor

UV…….....……...….Ultraviolet

v/v……..……Volume/Volume

w/v……..……..……..Weight/Volume

WCP……..….……Whole Chromosome Paint

α……..…….Alpha

β…….……...…….Beta

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Chapter 1: Introduction

1. Introduction:

1.1 Incidences and Risk Factors in Breast Cancer:

Breast cancer (BC) is the most common cancer of women worldwide and is the second leading cause of cancer death in women after lung cancer, with around 50,000 women and around 400 men diagnosed each year. It is a multifactorial disease caused by genetic, lifestyle, and environmental factors (World Health Organisation (WHO) and Breakthrough Breast Cancer Websites, April 2013). The official website of the American Cancer Society shows the female breast cancer incidence rates began to decrease in 2000 then start to fall more about 7% in 2002. It has been found that there was a higher link between hormone replacement therapy (HRT) and breast cancer risk and mortality as the use of estrogen and progestrone is associated with increased breast cancer incidence (Chlebowski *et al.*, 2013). The death rates from breast cancer have been fallen since 1989 especially in women less than 50 years old. These decreases might be due to earlier detection through screening and increased awareness, as well as improved treatment (American Cancer Society Website, February 2013). Five out of six women diagnosed with breast cancer survive for at least five years (Breakthrough Breast Cancer Website, 2013).

Studies suggest that the risk factors for BC may include duration of exposure to female hormones (early or late menopause), reproductive factors (late age of first pregnancy), dietary and low physical activity (obesity and high fat diet), ionising radiation during breast development, environmental factors due to geographical variation are of greater importance than genetic factors, chronic use of hormone replacement therapy (HRT), and genetic inheritance (family history) of BC (McPherson *et al.*, 2000).

1.1.1 Steroid Hormones and Their Receptors:

Progesterone and estrogen (17β-estradiol, E2) are ovarian steroid hormones and are essential co-regulators of the female reproductive activities as well as having non reproductive effects, which are induced by gonadotrophines, luteinising hormone (LH) and follicle-stimulating hormone (FSH), or other factors and genes. These effects are involved in development, differentiation, and maintaining the cardiovascular health by promoting blood clotting, bone integrity by its effect to minimise the loss of calcium from bones as well as other organs such as brain and pancreas (Edwards, 2005), in addition to their role in ageing (Pike *et al.*, 1993). Normally, these hormones and their receptors have the ability to co-regulate the normal development and function of the ovary, the uterus and the mammary gland in addition to their role in breast and ovary tumourgenesis (Lydon and Edwards, 2009). At puberty, the hypothalamus induces the ovary to secrete estrogen and this will induce the development of mammary gland. This development is associated with proliferation of the epithelial cells and mammary ducts. In pregnancy, progesterone is synthesised by the corpus luteum and placenta to stimulate the growth of lobuloalveolar mammary structures for secretion (Lapidus *et al.*, 1998).

In BC, the mechanism by which estrogen can induce the cellular changes is through binding directly to the estrogen receptor (ERα and ERβ, encoded by *ESR1* and *ESR2* genes, respectively) in the cell nucleus or indirectly to ER on the cell surface membrane (Nilsson *et al.*, 2001). Furthermore, ERα and ERβ differ in their tissue distribution and ligand binding characteristics (Katzenellenbogen and Katzenellenbogen, 2000). The complex that binds directly to estrogen response element (ERE) sequences in the promotor of DNA sequence (Figures 1.1 and 1.2) resulting in production of co-regulatory proteins (co-activators or co-repressors) (Demay *et al.*, 1995). These receptors are composed of three interacting

functional domains: (1) NH_2-terminal domain that encodes a ligand-independent activation function (AF1, involved in protein-protein interactions and transcriptional activation of target gene expression) and is a very active domain in the stimulation of gene expression. (2) DNA binding domain (DBD) that contains two zinc fingers and plays an important role in receptor dimerisation and binding of receptors to specific DNA sequences. (3) COOH terminal, and ligand binding domain (LBD, AF2) (Figure 1.1) that mediates ligand binding, receptor dimerisation, nuclear translocation and transactivation of target gene expression (Nilsson *et al.*, 2001). Although both receptors have identical DBDs, they differ in their abilities to activate different estrogen-responsive genes. The activity of each activation function (AF) of ER is cell and promotor dependent (McInerney and Katzenellenbogen, 1996). These differences suggest the efficacy of different ligands interaction through the two ER subtypes (Katzenellenbogen and Katzenellenbogen, 2000).

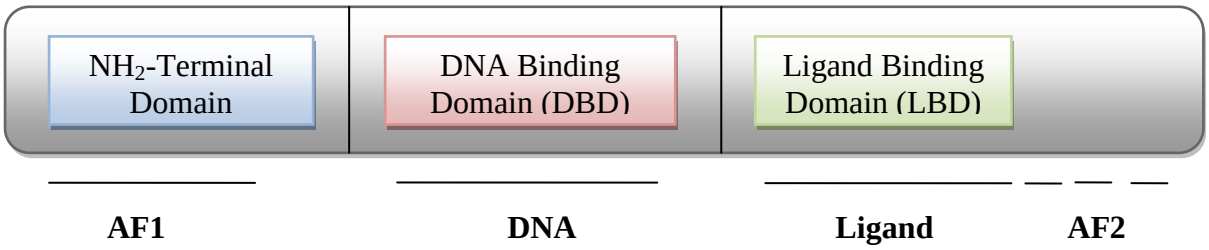


Figure 1.1: The Structure and Functional Domains of Estrogen (E2) Nuclear Receptors
The figure shows ER subtypes are composed of three interacting functional domains (AF1, DB, and AF2 domains) and their role in transcription and gene expression. The AF1 domain contains the NH2 terminus where transcription factors interact. The DNA binding domain contains the two-zinc finger structure that binds to DNA, and the ligand domain contains the ligand binding domain as well as the AF2 domain that directly binds co-activator proteins. Both nuclear receptors have identical DBDs but they differ in AF1 and AF2 efficacy interaction to ligands (By Mai H. Ahmed, 2010 based on Nilsson *et al.*, 2001).

However, the mechanism of functional domains interaction can be achieved through their binding indirectly to receptors (ERα) on the cell surface membrane resulting in increased levels of calcium and activation of kinases (Nilsson *et al.*, 2001; Deroo and Korach, 2006). Different studies suggest that estrogen and progesterone are implicated in BC and ovarian cancer (OC) development via their binding to ER and PR, respectively that stimulates cell proliferation and increase in cell division and DNA synthesis (Wittliff, 1984). Gonadotrophines are also implicated in the aetiology of OC since they stimulate steroidogenesis and increase the estrogen levels produced by ovary (Deroo and Korach, 2006). This may increase the risk for replication errors (double strand breaks and changes in daughter chromosomes) which may result in mutations that disrupt normal cellular processes such as apoptosis, normal proliferation or DNA repair mechanisms. In addition, estrogen metabolism leads to the production of toxic products that may directly damage DNA resulting in mutations. Mutation or aberrant expression of the regulators may affect their normal function and disrupt the normal development of the mammary gland and ovary which may lead to BC and OC, respectively. Since PR is induced by ER, its presence is a marker of functional and active ER. This supports the idea that the tumour is poorly differentiated when the ER and PR in BC patients are inactivated or lost. In addition, ER-positive tumours that lack PR in BC patients are less to responsive to hormonal therapies than those express both receptors (Lapidus *et al.*, 1998).

1.1.2 Other Cell Signalling Pathways:

Clearly, estrogen and progestrone hormones have an important role in BC and OC via ERs and PRs. In the absence of estrogen, other signalling pathways can modulate and co-regulate ER by tyrosine phosphorylation (Katzenellenbogen and Katzenellenbogen, 2000). This can be achieved by protein kinases, STAT, growth factors, cytokines, neurotransmitters, and cell

cycle regulators (Demay *et al.*, 1995; Buettner *et al.*, 2002). These have been demonstrated to contribute directly to oncogenesis by stimulating cell proliferation and preventing apoptosis. Therefore, activation of these regulators participating in oncogenesis through up-regulation of genes encoding apoptosis deregulators and cell cycle regulators such as *EGFR*, *PTEN*, *AKT1*, *HSP90AA1*, *HER2*, *cyclins D1*/*D2*, and *c-Myc* (Buettner *et al.*, 2002). Thus, the identification and understanding of these targets, that play an important role in diagnosis, prognosis, and therapeutics, are exciting new area of studying the biological roles of signalling responses (Lemoine, 1994; Deroo and Korach, 2006; Sárvári *et al.*, 2010). In addition, E2 and progesterone, the role of epidermal growth factor receptor (EGFR) and ERBB2 is that to stimulate and promote cell growth, survival, proliferation, and suppresses cellular apoptotic. HER-2 act as an oncogenic product, phosphatase and tensin homolog (PTEN) expression in OC and cyclin D1 activation during G1 are involved in cell cycle regulation (Lakhani *et al.*, 2002; Bell, 2005; Drosten *et al.*, 2010) (Figure 1.2). Furthermore, not only E2 regulates and activates phosphatidylinositol 3-kinase (PI3K)/AKT signalling pathway in tumour cells that poorly expresses ER and proliferate out of control (Figure 1.2) but also Erk, IGF and insulin (Guo *et al.*, 2007; Drosten *et al.*, 2010; Miah *et al.*, 2012). Moreover, the synthesis of heat shock proteins (HSPs) or stress proteins in BC cells are induced by environmental factors, pathological conditions, or physiological stresses (Whitesell and Lindquist, 2005). HSPs play an important role in the control of the cell cycle and tumourgenesis (Figure 1.2). HSP90α is involved in the regulation of steroid receptors (ER) by binding to these receptors and activation of other proteins such as src-family and kinases to induce BC cells proliferation (Yano *et al.*, 1999; Miah *et al.*, 2012). Blockage of these pathways and cascades may become a potentially effective way to control tumour progression especially in ER-negative which shows no response to endocrinal therapy.

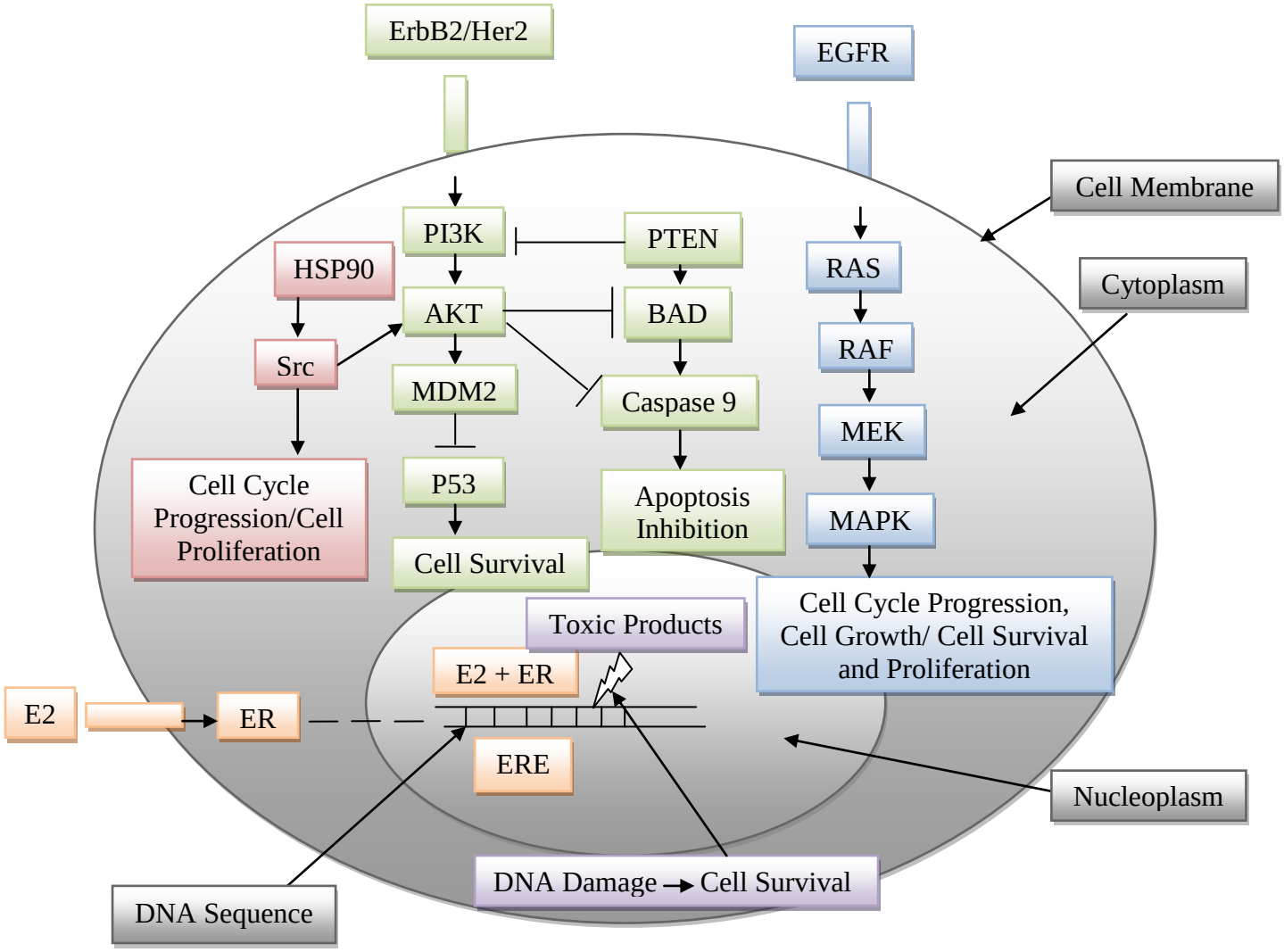


Figure 1.2: Cell Survival and Proliferation Induced By Different Cell Signalling Pathways

The carton displays the binding of estrogen (E2) to estrogen receptor (ER) via ER elements in the nucleus to regulate transcription and gene expression. This figure also shows interesting genes that are capable of inducing cell proliferation in the absence of ER and are involved in breast cancer progression. A number of other signalling pathways can modulate and co-regulate (co-activators or co-repressors) ER by phosphorylation. Activation of the RAS/RAF/MEK/Erk and PI3K/AKT as well as inhibition of PTEN and caspase 9 pathways induce signal transduction to cell cycle progression and cell survival. Moreover, toxic products from E2 metabolism may directly damage DNA resulting in mutation and replication errors. This will lead to cell survival, cell cycle progression and proliferation.

However, the loss of estrogen or its receptor contributes to the development of various diseases such as osteoporosis, neurodegenerative diseases, insulin resistance, stroke and coronary artery diseases, and obesity (Edwards, 2005) as well as *E2* gene dysregulation are responsible for the mechanisms by which these cancers occur through activation of other genes involved in cancer progression such as breast cancer (Figure 1.2). Since the expression and activity of PR depends on ER transcriptional activity, it has been shown that PR expression has strong correlation with prognosis in BC than ER expression alone because progesterone contributes to early stages of breast tumourgenesis (Lydon and Edwards, 2009). Clearly, BC phenotype is dependent on ovarian steroids and matrix metalloproteinases (MMPs) activity which are also implicated in mammary epithelial transformation either through steroid hormones, tumour growth factor β1 (TGFβ1), or extracellular matrix (ECM) remodelling. This will lead to increased morphogenesis, hyperplasia, and tumours of these organs (Simian *et al.*, 2009).

1.1.3 The Principal Target Agents for Breast Cancer Therapy:

The developments in molecular technologies have allowed researchers to gain a better understanding and improvement of the mechanisms in breast cancer (BC) transformation and progression. In general, the aim of targeted therapy is to target specific tumour cells leading to improved treatment with limited toxicity. BC is the most common female tumour with an increased morbidity in patients at different stages with different prognosis (Gasparini *et al.*, 2005). In the last few years, an improved understanding of molecular pathways involved in BC growth and progression allowed researchers for the identification of interesting targets that can be inhibited by new generations of anticancer drugs.

1.1.4 Targeting Farnesylation by Lovastatin:

Farnesylation is an essential step for activation and maturation of several proteins involved in cellular and nuclear organisation, apoptosis, gene transcription and cell proliferation. The 3-hydroxy 3-methylglutaryl-CoA reductase inhibitor, lovastatin, is used widely to treat hypercholesterolemia and has been shown to have cell cycle-specific effects (Holstein and Hohl, 2001). Furthermore, lovastatin causes growth inhibition and induces apoptosis in cancers. Activation of Ras oncogene is also farnesyltransferase dependent. However, continuous activation of Ras protein can result from growth factor stimulation. Lovastatin can inhibit Ras farnesylation and therefore, induce antiproliferative effects as a result from the inhibition of Ras activity (Schafer and Rine, 1992; Rowinsky *et al.*, 1999; Johnston, 2001; Chatterjee and van Golen, 2011). Other intracellular targets include nuclear lamin proteins, lamins A and B (Farnsworth *et al.*, 1989), modulation of the PI3-K/AKT pathway (Du *et al.*, 1999) or GTP-binding proteins that regulating cell adhesion or motility (Liu *et al.*, 2000; Chatterjee and van Golen, 2011) are involved in this inhibition. In 1999, Du *et al.* showed that the apoptotic effects of lovastatin in Ras transformed cells and they found that lovastatin disrupted a signal that is crucial for survival of malignant cells, but not normal cells, if the PI3-K/AKT pathway is inactivated (Du *et al.*, 1999). These results seem positive for clinical applications of lovastatin to induce the apoptotic responses, however there are still questions that need to be answered such as the biological dose, tumour cell types, stages of the disease, and toxcicity.

1.1.4.1 Interphase 3-hydroxy-3 methylglutaryl-Coenzyme A Reductase (HMG-CoA Reductase) Inhibitor (Lovastatin) Treatment: *Ras* Family Genes: Maturation and Activation Mechanism:

The *ras* family genes have been identified as potential targets for therapeutic purposes because of somatic mutations in different human cancers. They are mutated in many cancerous cells (Downward, 2003; Graham and Olson, 2007). The enzyme farnesyltransferase is involved in post-translational modification of the Ras proteins by adding a farnesyl group to the Ras protein at C terminus. This permits the Ras protein to be translocated to the cell surface membrane, allowing the protein to be involved in signalling pathways for increased proliferation and inhibition of apoptosis. The class of farnesyltransferase inhibitors is designed to block farnesylation and prevent the mature Ras signalling and thus inhibit cell proliferation and facilitate apoptosis (Whyte *et al.*, 1997; Johnson and Heymach, 2004). The association of mutant forms of Ras protein with a variety of human cancers has increased strong interest in therapies based on inhibiting oncogenic Ras signalling pathway. Attachment of Ras proteins to the plasma membrane is required for effective Ras signalling and this is initiated by the enzyme farnesyltransferase (Graham and Olson, 2007).

HMG-CoA reductase is an enzyme that is responsible for the conversion of HMG-CoA to mevalonate to participate cholesterol biosynthesis (Alberts, 1988). Lovastatin binds to this enzyme and prevents the synthesis of cholesterol (Figure 1.3). Since lovastatin is specific for farnesylated proteins such as Ras, it is therefore expected to have less toxicity. Inhibitors such as lovastatin are specific for Ras converting enzyme (Rce1) and isoprenylcysteine carboxyl methyltransferase (ICMT), both enzymes are required for prenylation and for lamins to be mature and active, and expected to have more toxicity as lovastatin can modify all *CAAX*

proteins. However, Rho protein, one of Ras superfamily, might function normally under conditions where Rce1 and/or ICMT are blocked, making these more attractive targets for drugs. However, because the major limitation of lovastatin is alternative prenylation by geranylgeranyltransferase (Whyte *et al.*, 1997), it has been suggested that the addition of an Rce1 or ICMT inhibitor might not overcome the effects of alternative prenylation. Therefore, a prenylated protein might be unable to be transported to the nuclear membrane in Rce1 or ICMT deficient cells (Michaelson *et al.*, 2005; Malhas *et al.*, 2007; Christiansen *et al.*, 2011). Both Rce1 and ICMT are required for membrane targeting of farnesylated but not geranylgeranlyated GTPases and this indicate the importance of post-prenylation of CAAX proteins (Whyte *et al.*, 1997; Martin *et al.*, 2009; Christiansen *et al.*, 2011).

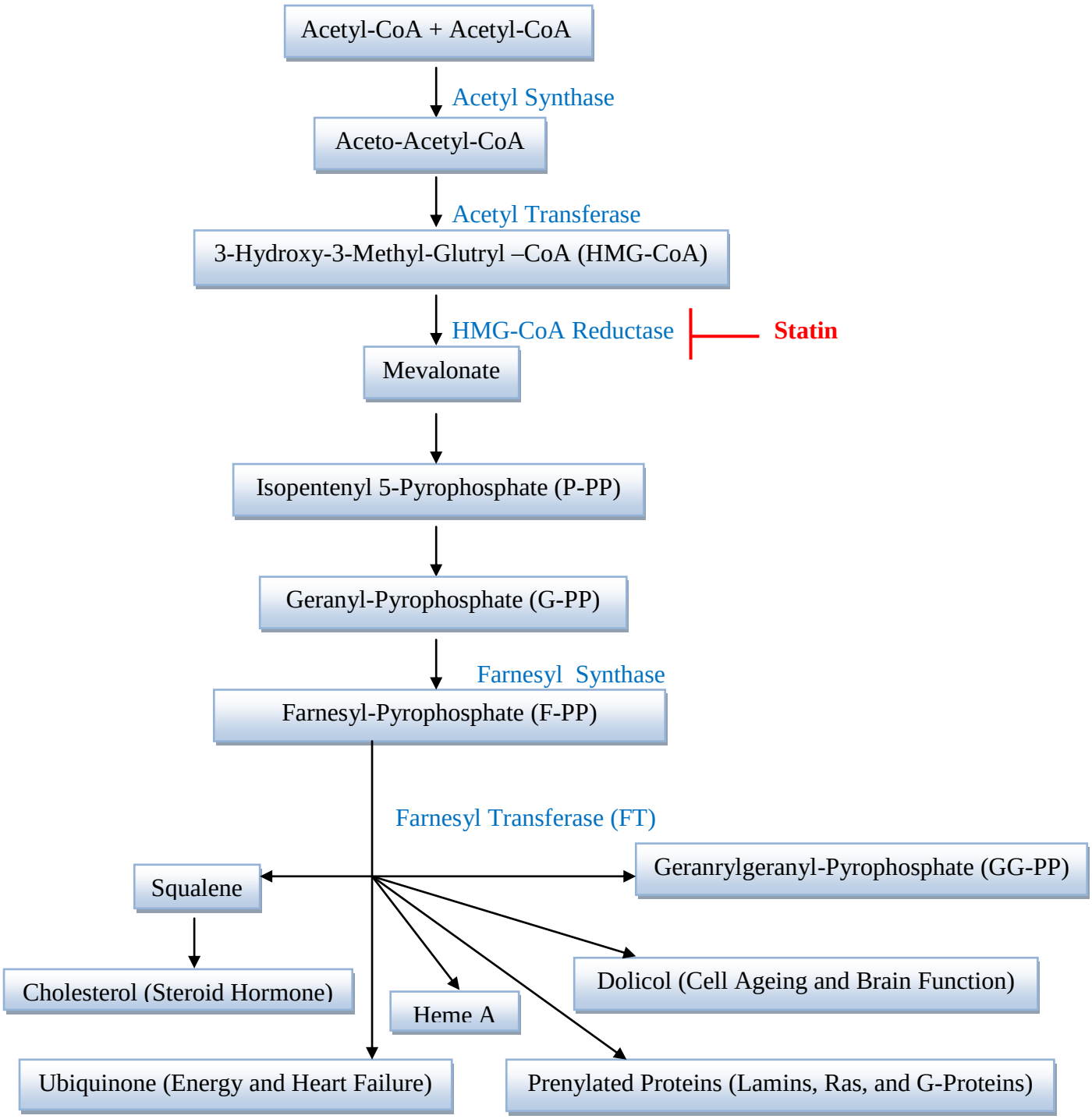


Figure 1.3: Schematic Presentation of the Isoprenoids Pathway
This pathway can be inhibited by Statin (Lovastatin) Treatment to inhibit HMG-CoA Reductase and prevent Cholesterol Synthesis, inhibit Ras Activation, and Increase Lamin B Expression. Other Inhibitors such as Farnesyltransferase Inhibitors (FTIs) and Biphosphonates can Target the Pathway at Farnesyltransferase (FT) and Farnesyl-PP Synthase Enzymes, Respectively.

1.2 Nuclear Structure and Disease:

The eukaryotic cell nucleus is an important regulator for a variety of functions and processes, including gene expression, DNA replication during cell cycle, apoptosis, DNA repair, transcription events, ribosome assembly and maintaining the integrity of genes (Garagna *et al.*, 2001; Prokocimer *et al.*, 2006; Parnaik, 2008; Linnemann and Krawetz, 2009; Peric-Hupkes and van Steensel, 2010). To perform these functions efficiently, the nucleus is well compartmentalised by nuclear structure, providing a high degree of spatial organisation (Cremer and Cremer, 2001; Parada *et al.*, 2004; Malhas *et al.*, 2007; Peric-Hupkes and van Steensel, 2010). These structures include the nuclear envelope, nuclear lamina, nuclear matrix, nucleoli, and nuclear bodies. In laminopathies (diseases caused by mutation in A-type and B-type lamin proteins, Section 1.2.3.6.1) and cancers, this organisation and nuclear functions are altered (Zink *et al.*, 2004; Meaburn *et al.*, 2007a; Mehta *et al.*, 2011). These alterations could be important to characterise as cancer biomarkers at different stages. Previous studies have shown that the nuclear matrix composition, in cancerous cells, can be different from normal cells (Prokocimer *et al.*, 2006). This finding has been useful in characterising cancer biomarkers and has helped to diagnose the disease earlier. In addition, these biomarkers are used for early detection and prognosis of human cancers. Thus, these alterations and differences in nuclear structure of the cancerous cells can be used in the development of new anticancer drugs (Agrelo *et al.*, 2005; Prokocimer *et al.*, 2006; Willis *et al.*, 2008). Treatment with anticancer drugs may lead to the restoration and improvement of nuclear structure and a reversion of cancer phenotype thereby identifying therapeutic agents.

1.2.1 The Nuclear Matrix:

The nuclear matrix, nuclear scaffold, or nucleoskeleton is a dynamic RNA-protein complex and thought to be a network of fibrous proteins that consist of nuclear lamina inside the cell

nucleus providing structural framework for organising chromatin, nuclear structures, and regulating nuclear DNA metabolism (Hozák *et al.*, 1995; Spencer *et al.*, 2000) (Figure 1.4). It was first described by Berezney and Coffey in 1974 using rat liver nucleus which consists of nuclear matrix. This matrix is 98.2% protein, 0.1% DNA, 1.2% RNA, and 0.5% phospholipid. The nuclear protein matrix is composed of three acidic polypeptide fractions with a molecular weight range from 60–70 kDa. (Berezney and Coffey, 1974). It is highly dynamic structure and involved in the regulation of gene expression and supporting major nuclear functions (Barboro *et al.*, 2002; Martin *et al.*, 2009). Different regions of the genome interact with the nuclear matrix and they are called matrix attachment regions (MARs). Telomeres and centromeres also attach to the NM. The spatial arrangements in the nucleus are maintained by attachments to nuclear matrix proteins (NMPs) network and RNAs (Fey *et al.*, 1986; Hozák *et al.*, 1995). The nuclear matrix binds to nuclear proteins and supports their assembly into functional macromolecules involved in nuclear processes such as transcription, RNA splicing, regulating gene expression and DNA replication (Bissell *et al.*, 1999; Nickerson, 2001). Furthermore, it can participate in chromatin organisation via matrix attachment regions (MARs). MAR-binding proteins bind MARs and can be influenced by stress. The activity of MAR-binding proteins, p114, was found only in human cancers especially breast carcinoma and in poorly differentiated tissue rather than well differentiated carcinoma (Yanagisawa *et al.*, 1996). This protein may play an important role in breast tumourigenesis and is effective as a diagnostic and prognostic marker. The down regulation and mislocalisation of genes is caused by the loss of MAR-binding protein in malignant cells (Woynarowski, 2004). Therefore, MARs and MAR-binding proteins are attractive therapeutic targets in cancer and promising for further drug discovery.

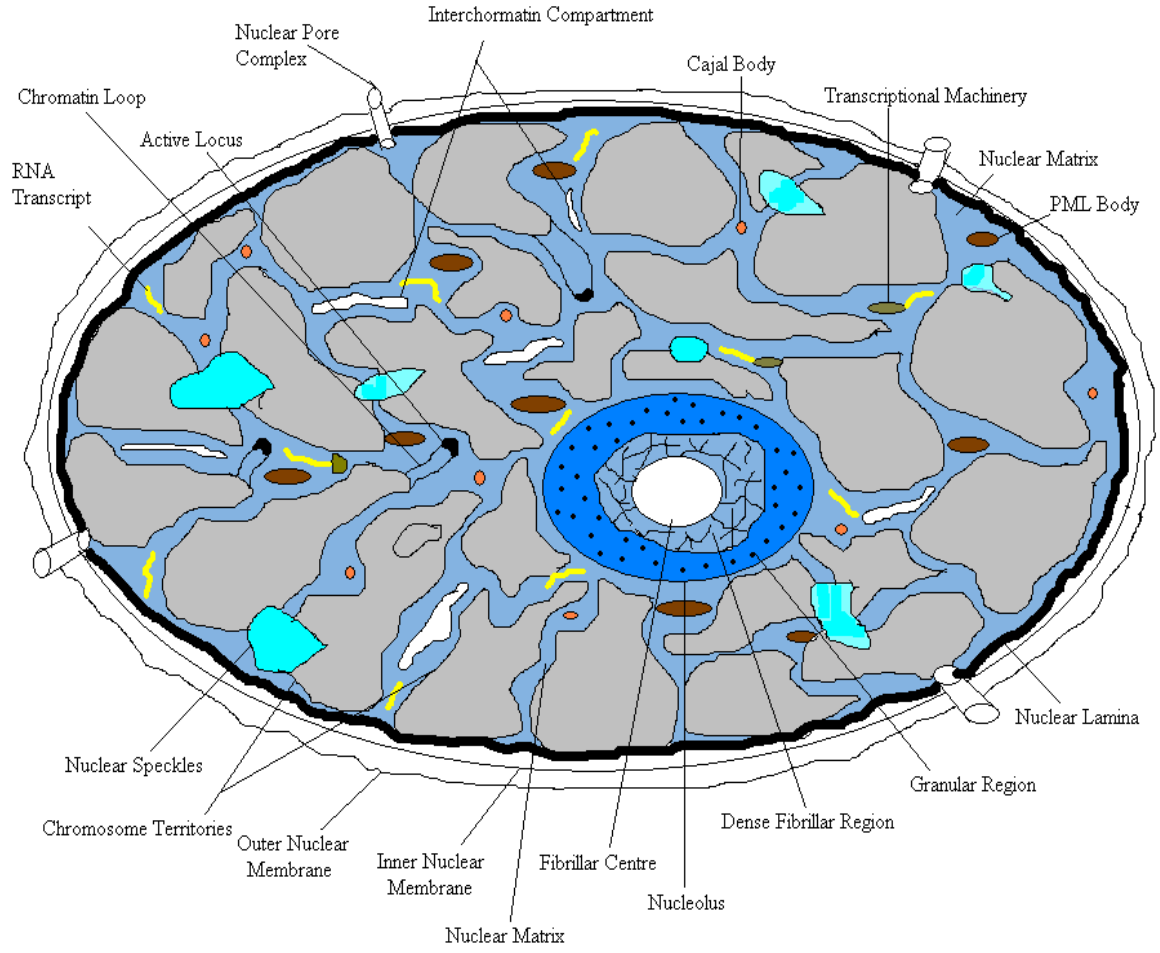


Figure 1.4: Mammalian Interphase Nuclear Architecture
Cartoon of nucleus is displaying the genome organisation and nuclear structure that contribute to the overall interphase nuclear architecture. The figure shows an overview of essential nuclear substructures that contains chromosome territories (CTs) and interchromatin (IC) space, nuclear pore complexes (NPCs), the inner and outer nuclear membrane (INM and ONM), and the nuclear lamina (NL) at the nuclear periphery. In addition to, chromatin loops with active loci going to transcription machineries and RNA transcripts that are extended from chromosome territories to that share transcription sites (van Steensel and Dekker, 2010). A diverse set of nuclear bodies (NBs) (site for post-transcriptional and processing of RNA modification in the nucleus and for other nuclear functions such as apoptosis, cell cycle regulation, and viral infection control (Gall, 2000)), such as speckles, paraspeckles, the perinucleolar compartment, Cajal bodies (CBs), or PML bodies are found in the interchromatin space. The spatial organisation of chromosomes is regulated by interchromosomal contacts, intermingling, and by interactions of specific chromosomal loci with nuclear 'landmarks' such as the nuclear envelope (NE), nuclear matrix, and the nucleolus with its three main components: the fibrillar centres (FC), the dense fibrillar component (DFC), and the granular component (GC) (Maggi and Weber, 2005). Therefore, the nucleus is highly organised machinery that is required for proper nuclear functions and processes.

1.2.2 Nuclear Envelope (NE):

The nuclear envelope (NE) is composed of two lipid bilayers and is made up of the nuclear membrane (NM) and other proteins, with an inner and an outer nuclear membrane (INM and ONM). The INM and ONM are studded by the nuclear pore complexes (NPCs) that mediate and regulate molecules movement between the nucleus and the cytoplasm and are composed of multiple proteins (nucleoporins) (Rout *et al.*, 2000) (Figure 1.6). The NM encloses the contents of the nucleus and separates the genetic material from the cytoplasm and serving as a barrier to prevent macromolecules from diffusing between the nucleoplasm and the cytoplasm (Chi *et al.*, 2009). The ONM is continuous with endoplasmic reticulum (ER) and ribosomes; and the INM faces the nuclear interior, chromatin and nuclear lamina (Aaronson and Blobel, 1975; Gruenbaum *et al.*, 2005; Guttinger *et al.*, 2009; Roux *et al.*, 2009; Zwerger *et al.*, 2010). The integral membrane proteins, found in the INM, include lamin B receptor (LBR), lamin associated polypeptides 1 and 2 (LAP1 and LAP2), emerin, MAN1 and the nesprins (Zhang *et al.*, 2001; Roux *et al.*, 2009; Postel *et al.*, 2011). The interaction between the nuclear lamina and chromatin is mediated through these integral nuclear membrane proteins at the nuclear edge (Morris, 2001). The space between the INM and ONM is called the perinuclear space or lumen and is 10-50 nanometres (nm) wide (Rout *et al.*, 2000; Chi *et al.*, 2009; Roux *et al.*, 2009) (Figure 1.5).

1.2.2.1 The Nuclear Lamina:

1.2.2.1.1 Introduction to Nuclear Lamins:

The nuclear lamina is a polymeric protein meshwork of 10 nm filaments (Gerace, 1986; Lin and Worman, 1997). It is subjacent to the INM and is a complex network of type V intermediate filaments (IF) proteins, the nuclear lamins (Fisher *et al.*, 1986; Moir *et al.*, 2000b). The lamina provides the mechanical and structural support for the nuclear membrane

(NM) and anchoring sites for chromosomes and nuclear pores (Aebi *et al.*, 1986; Lehner *et al.*, 1986; Gerace, 1988; Goldman *et al.*, 1992; Bridger *et al.*, 1993; Moir *et al.*, 2000a/b; Malhas *et al.*, 2007; Parnaik 2008; Martin *et al.*, 2009; Roux *et al.*, 2009; Kubben *et al.*, 2010; Peric-Hupkes and van Steensel, 2010; Dittmer and Misteli, 2011; Helfand *et al.*, 2012). The nuclear lamina is composed of A-type and B-type lamins and lamins binding proteins (Gerace and Blobel, 1980). The interaction between lamins, chromatin and transcription factors at the inner nuclear membrane is more specific. The lamina forms associations with heterochromatin, histones and lamin binding proteins such as emerin, LAP2α and LAP2β, lamin B receptor (LBR, p58) and MAN1 (Figure 1.6). This interaction is important to regulate transcription and gene expression.

1.2.2.1.2 Lamin Genes, Structures and Expressions:

Lamin genes, found in all metazoan, are divided into A-type lamins which are regulated and expressed in many differentiated cells. A-type lamins are 70 and 60 kilodaltons (Gerace and Blobel, 1980; Goldberg *et al.*, 2008b), are encoded by the *LMNA* gene located on chromosome 1q21.2-q21.3 (Wydner *et al.*, 1996; Lin and Worman, 1997) and are generated by alternative splicing to produce different isoforms, lamins A, AΔ10, C and C2, which are all found in different cell types (Foster and Bridger, 2005; Martin *et al.*, 2009). Lamin C2 is restricted only to spermatocytes during rat spermatogenesis whereas, AΔ10 is found only in a few carcinoma cell lines (Alsheimer and Benavente, 1996). AΔ10 results from the deletion of exon 10 (Hutchison, 2002) and is localised at the nuclear envelope (Broers *et al.*, 1999). The *LMNA* gene contains 12 exons. Exon 1 codes for the N-terminal head domain and the first part of the central rod domain of this gene. Exon 2-6 code for the central α-helical rod domain. Exons 7-9 code for the C-terminal tail domain for lamin A and lamin C. The nuclear localisation signal (NLS) is contained in exon 7. Exon 10 contains a splicing site to generate

lamin A and lamin C. Exon11 and 12 are specific for lamin A and coding for the CAAX box of pre-lamin A (Lin and Worman, 1993). In mammalian cells, lamins A and lamins C share the first 566 amino acids and differ by length of 98 and 6 amino acids, respectively at the C-terminus (Gerace, 1988; Lin and Worman, 1995; Sasseville and Raymond, 1995).

There are three types of B-type lamins (67 kilodaltons) in mammals: lamin B1, lamin B2 and lamin B3. Lamins B1 and lamins B2 are regulated and expressed in both embryonic and somatic cells during development whereas lamins B3 is regulated and expressed only in spermatocytes (Gerace and Blobel, 1980; Furukawa and Hotta, 1993; Lin and Worman, 1995; Alsheimer and Benavente, 1996; Furukawa and Kondo, 1998; Hutchison, 2002; Goldberg *et al.*, 2008b). Lamin B1 is encoded by *LMNB1* and lamin B2 and lamin B3 are encoded by *LMNB2* genes on chromosomes 5q23.2-q31.3 and 19p13.3, respectively (Lin and Worman, 1995; Wydner *et al.*, 1996; Lin and Worman, 1997; Dechat *et al.*, 2008). *LMNB1* gene contains 11 exons. Exon 1 codes for the N-terminal head domain and the first part of the central rod domain of this gene. Exon 2-6 code for the central α-helical rod domain. Exons 7-11 code for the C-terminal tail domain of lamin B. The nuclear localisation signal (NLS) is contained in exon 7. Exon11 contains a specific sequence for the CAAX box lamin B for post-translational farnesylation (Lin and Worman, 1993) (Figure 1.5).

The lamins contain a central α-helical coiled-coil rod domain of 350 amino acids flanked by non-helical N-terminal head (4 KDa) and C-terminal tail (20-30 KDa) domain with a globular structure (Aebi *et al.*, 1986; Gerace, 1986, 1988; Stuurman *et al.*, 1998) and four subsegments (1A, 1B, 2A, and 2B) able to form coiled coils. These α-helical segments are separated by linkers, L1, L12 and L2 (Parry and Steinert, 1999; Bridger *et al.*, 2007). This rod domain is responsible for the dimerisation of the lamin proteins and is also required for interactions of

the lamin dimers into the polymerised structure to form the nuclear lamina (Stuurman *et al.*, 1998). The roles of the head and tail domains IF in nuclear lamins structure, assembly and network formation *in vivo* and *in vitro* more systematically. N-terminal head domain promotes lateral association into filaments and C-terminal tail domain controls lateral assembly so that it terminates at 10 nm filaments level and this will participate in the formation of IF network through their binding to chromatin. The head to tail overlap gives rise to filaments thickenings (Gerace, 1988; Stuurman *et al.*, 1998). The nuclear localisation signal (NLS) between the central rode and C-terminal tail domain is required for the transport of lamins into the nucleus (Dechat *et al.*, 2008). Lamin dimers are stable under high pH and salt conditions (Gerace, 1986; Bridger *et al.*, 2007). In addition, A-type lamins form foci within the nucleoplasm that are distinct from peripheral lamina (Bridger *et al.*, 1993; Hozak *et al.*, 1995; Goldman *et al.*, 2002), as do B-type lamins which are associated with sites of DNA replication (Moir *et al.*, 1994; Sasseville and Raymond, 1995).

1.2.2.1.3 Lamins Biosynthesis and Assembly:

Lamin classification is based on their sequence homology, expression, biochemical properties, and localisation during mitosis (Gerace and Blobel, 1980; Aebi *et al.*, 1986; Gerace, 1988). Nuclear lamins differ from cytoplasmic IF proteins such as vimentin in their central α-helical rod domain which is 40-50 amino acids longer than cytoplasmic IF proteins. Lamins reveal strong homologies in their α-helical rod domain and contain a structural similar to immunoglobulin fold (Ig-fold) within their C-terminal tail domain (Herrmann and Aebi, 2004; Dechat *et al.*, 2008).

Pre-lamin B contains a CAAX box motif at the carboxyl-terminal (Cysteine, Aliphatic, Aliphatic, any of several residues) (Lutz *et al.*, 1992) for processing and maturation, post-

translation modifications to be more mature and active. The post-translational modifications and the formation of mature and active lamins through farnesylation by adding a farnesyl group to the cysteine residue of the C-terminal (via farnesyltransferase) (Figure 1.4) that promotes pre-lamin B to be associated to the nuclear membrane (Beck *et al.*, 1990). Pre-lamins B undergo cleavage to loose the last three amino acids by endoproteolysis on the AAX amino acids using Ras converting enzyme 1, Rce l (FACE2 in humans). In order for lamins B to be mature and active, lamins B at the C-terminus is carboxymethylated (via isoprenylcysteine carboxyl methyltransferase, ICMT) (Beck *et al.*, 1990; Corrigan *et al.*, 2005; Prokocimer *et al.*, 2009). After lamin Bs have completed their post-translation modification process then accumulated within the nucleus as isoprenylated proteins.

After B-type lamin localisation, pre-lamin A undergoes the post-translational modifications and the formation of mature and active lamins through farnesylation by adding farnesyl group to the C-terminal (farnesyltransferase) (Figure 1.5). Furthermore, lamin A undergo cleavage to loss the last three amino acids by endoproteolysis on the AAX amino acids using ZMPSTE24 (FACE1 in humans). Zinc metalloproteinase (ZMPSTE24, FACE1) is involved in the post-translational proteolytic process of carboxy terminal residues of farnesylated pre-lamin A to form mature lamin A. Once the farnesyl group is lost, lamin A becomes incorporated into the nuclear lamina. If the farnesyl group is not cleaved, the protein remains associated with the nuclear envelope and is not released and inserted into the nuclear lamina (Holtz *et al.*, 1989). In order for lamin A to be mature and active, lamin A at the C-terminal is carboxymethylated (isoprenylcysteine carboxyl methyltransferase, ICMT) (Beck *et al.*, 1990; Corrigan *et al.*, 2005; Prokocimer *et al.*, 2009). The next stage is followed by additional cleavage resulting in a loss of 15 amino acids and the farnesyl group at the C-terminal using ZMPSTE24 (FACE1) (Sasseville and Raymond, 1995; Naetar and Foisner, 2009; Prokocimer

et al., 2009). This final step for post-translational modification processes is thought to be important for lamin A maturation and incorporation into the lamina. However, the difference between processing of lamins A from that of lamins B is a further endoproteolytic cleavage of 15 amino acids from the C-terminal (Sasseville and Raymond, 1995) (Figure 1.5). These steps are essential for stable association of lamins with the nuclear periphery as well as their role in DNA synthesis, DNA replication, RNA processing and gene expression. Moreover, these steps of processing and modification are important and critical for lamins localisation that means blocking the farnesylation, will inhibit lamins post-translation processing.

Lamin C and lamin C2 do not undergo any post-translational modifications and have no CAAX box motifs at their C-terminus (Fisher *et al.*, 1986; Beck *et al.*, 1990; Vergnes *et al.*, 2004; Malhas *et al.*, 2007) (Figure 1.5). Furthermore, Lamin C2 (52 KDa) shows its similarities with lamin C because it has a sequence identical to that of lamin C except that the N-terminal segment, containing the head and the alpha-helical coil 1A domains, is replaced with a short non-alpha-helical stretch of amino acids. In mice, lamin C2 was found to be specifically expressed in germ cells (Furukawa *et al.*, 1994).

Interestingly, inhibition of lamin farnesylation does not inhibit their incorporation into the lamina. For example, lamin C that lack 98 amino acids in their C-terminal and only have 6 amino acids lacking a CAAX box become incorporated into the nuclear lamina (Dechat *et al.*, 2008). Different studies showed that pre-lamin A is modified and cleaved with a half-life of 90-100 minutes in order for assembly into the lamina (Sasseville and Raymond, 1995). Their assembly starts with the formation of coiled coil dimers which is associated with head to tail overlap. Whether this occurs during translation or post-translation is unknown. During interphase, the lamins are localised in the nuclear envelope resulting in a rim at the nuclear

periphery and become dephosphorylated and polymerised at the nuclear periphery during telophase (Burke and Gerace, 1986; Foisner and Gerace, 1993). During mitosis, A-type lamins are disassembled into monomers (Gerace and Blobel, 1980 and Prokocimer *et al.*, 2009) and become associated with mitotic chromosome surfaces. A-type lamins are found in a soluble form and have a neutral isoelectric point whereas B-type lamins are bound to nuclear membrane vesicles and participate in nuclear envelope assembly and disassembly (Gerace and Blobel, 1980; Burke and Gerace, 1986; Gerace, 1988; Georgatos *et al.*, 1997; Prokocimer *et al.*, 2009). During prophase, after the disassembly of lamins, A-type lamins become dispersed throughout the cytoplasm and lose their association with chromosomes. The lamins remain disassembled until the nuclear envelope begins to reform during early telophase and when they start to associate with the surface of chromosomes and this assembly of lamins is completed by late telophase and after the nuclear rim appearing in early G1 when chromatin decodensation has occurred (Gerace, 1986). The disassembly is due to phosphorylation and depolymerisation into a monomeric form which is a reversible process from dimeric or tetrameric forms (Gerace and Blobel, 1980; Georgatos *et al.*, 1997). Moreover, the changes in the structure during the various phases of the cell cycle are identical for all cells of the same differentiated state (Blobel, 1985). These processes allow nuclear structure and genome to reorganise and reform in the daughter cell nuclear membranes.

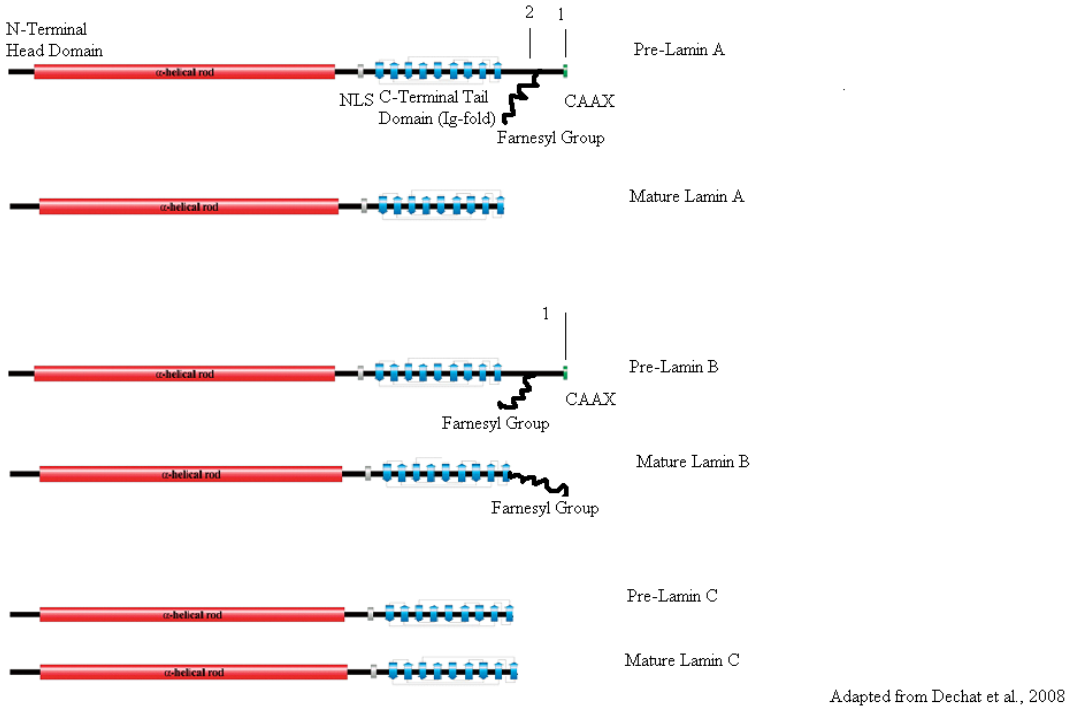


Figure 1.5: The Structure and Biogenesis of Lamins in Normal Cells
The steps of processing lamin B and modifications, through farnesylation by Farnesyltransferase, endoproteolysis by ZMPSTE24 (FACE1) or Rce1 (FACE2) and carboxymethylation by ICMT, are essential for activation and maturation, stable association of lamins with the nuclear periphery, DNA synthesis, DNA replication, RNA processing and gene expression, and lamins localisation into the lamina. Excluding, Lamin A again undergoes second cleavage that requires ZMPSTE24 to remove the last 15 amino acids (Dechat *et al.*, 2008). Since lamin C lacks this CAAX motif, it does not undergo these post-translational modifications and their localisation at the nuclear envelope depends on lamin A (Vaughan *et al.*, 2001). Thus, blocking the farnesylation will inhibit lamins post-translation processing (Dechat *et al.*, 2008). ^{1}|=First Cleavage Site and ^{2}|= Second Cleavage Site.

1.2.2.1.4 Lamin Foci and Functions:

Lamins are not only components of the lamina but also found in the nucleoplasm. The nuclear A-type lamins and B–type lamins can also be found in internal foci within the nucleoplasm (Goldman *et al.*, 1992; Lutz *et al.*, 1992; Bridger *et al.*, 1993; Moir *et al.*, 1994; Prokocimer *et al.*, 2006). Studies have shown that lamin A is found within the nucleoplasm during G1 phase of the cell cycle forming internal foci structures (Bridger *et al.*, 1993; Hozak

et al., 1995; Sasseville and Raymond, 1995). Non-prenylated pre-lamin A accumulat in intranuclear foci suggesting these foci act as storage sites (Lutz *et al.*, 1992). Lamins B foci are prominent in mid and late S-phase and co-localised with sites of DNA replication. Lamins B1 play an important role in DNA replication, cellular ageing, and stress responses (Sun *et al.*, 2010).

Both lamin A and lamin B do not co-localise with each other and each one has its own localisation (Moir *et al.*, 1994). The role of this localisation within the nucleoplasm is unknown, but it may be these foci considered as sites for lamins isoprenylation, lamins unable to bind to the lamina or may be associated with sites for DNA replication (Stuurman *et al.*, 1998). The foci can associate with transcription factories (TFs) and retinoblastoma (pRB) proteins to induce cell proliferation. pRB dependent E2F activity is also involved in organising nuclear structure, gene expression, maintaining nuclear integrity, DNA replication, DNA repair, apoptosis, transcription process, genome organisation, and cell cycle control and regulation (La Thangue, 1995; Weinberg, 1995; Garrett, 2001; Naetar and Foisner, 2009).

Nuclear lamin A and C are present in different cell organisms can interact with polynucleosomes and mitotic chromosome (Lin and Worman, 1993). However, down-regulation and instability of nuclear lamins play important roles in the infection cycles of viruses and some cancers (Dechat *et al.*, 2008). Different expression between nuclear lamins A and lamins B support the idea of their alternative functions during development and differentiation. The absence of lamin A/C from undifferentiated cells is responsible for the deformation of the nuclei in these cells. For example, lamin A is present in late embryos but not expressed in blastocysts whereas lamin A/C is expressed in oocytes (Prather *et al.*, 1989;

Bridger *et al.*, 1993). Foster *et al.*, in 2007, have shown that A-type lamins and B-type lamins are present at the nuclear envelope in early porcine embryos and that lamin A is also found in large intranuclear aggregates in two-cell to eight-cell embryos but is lacking from later embryonic stages (Foster *et al.*, 2007).

1.2.3 Lamins and Nuclear Functions:

1.2.3.1 Lamins and DNA Replication:

The precise regulation of DNA replication is fundamental to the preservation of intact genomes during cell proliferation. The higher order chromatin structures and the spatial architecture of replication sites contribute to the replication of specific regions of the genome at precise times of S phase (Maya-Mendoza *et al.*, 2012). From the literature, the nuclear matrix, a platform for network proteins including lamins, is required for efficient DNA replication. The DNA is found to be associated with the nuclear matrix regions (Berezney and Coffey, 1975). Several enzymes which participate in DNA replication are also attached to the nuclear matrix such as histone transferases, DNA polymerases, nucleases, DNA primases, DNA helicases, single strand DNA binding proteins, and topoisotopomerases (Jackson and Cook, 1993; Jackson *et al.*, 1996; Chagin *et al.*, 2010). Other studies reveal that replication sites, these anchored by B-type lamins (Hassan and Cook, 1993; Hozak *et al.*, 1993; Hozak *et al.*, 1994; Moir *et al.*, 1994; Okamoto *et al.*, 2011). This attachment to the nuclear matrix is important for the initiation of replication (Djeliova *et al.*, 2001; Radichev *et al.*, 2005; Chagin *et al.*, 2010). However, origins of replication are not associated with the nuclear matrix throughout the cell cycle; they only associate with the structure in late G1 and released in early S phase (Djeliova *et al.*, 2001). Most DNA synthesis occurs in specific dense structures (replication factories) attached to the nuclear matrix. These factories appear at the end of G1 phase and quickly become active when S phase progresses, they increase in

size and decrease in number. These factories are a subset of nuclear bodies; they change in their characteristics and contained DNA polymerase I (Ellis *et al.*, 1997). The involvement of lamins in DNA replication requires correct lamins organisation. When this lamin organisation disrupted, it results in nuclei that do not replicate their DNA (Ellis *et al.*, 1997; Goldman *et al.*, 2002).

Other studies suggest that lamins play a more direct role in DNA synthesis since the organisation of initiation factors was not altered by lamin disruption. This suggests that alteration of lamin organisation does not affect the initiation phase of DNA replication (Spann *et al.*, 1997; Moir *et al.*, 2000a). During S phase, lamins colocalise with proliferating cell nuclear antigen (PCNA), a factor that is required for the elongation phase of DNA replication, at sites of replication (Moir *et al.*, 1995). Furthermore, the normal localisations of both PCNA and RFC, another factor that is required for the elongation phase of DNA replication, are altered when lamin organisation is disrupted (Moir *et al.*, 2000a). Therefore, disruption of nuclear lamin organisation blocks the elongation phase of DNA replication (Spann *et al.*, 1997; Moir *et al.*, 2000a). Nuclear lamins may form a nucleoplasmic scaffold upon which the active elongation complexes required for DNA synthesis are assembled. However, other experiments have suggested that nuclear lamins may only be required to initially organise replication factors and once this occurs, lamins may no longer be required for DNA synthesis (Ellis *et al.*, 1997). For understanding this hypothesis, this will require both the identification and characterisation of additional factors that interact with nuclear lamins during DNA synthesis.

1.2.3.2 Lamins and Transcription:

It is important to understand that the nuclear matrix provides the foundation for transcription factories. RNA polymerase I and II are mediated by active transcription and attached to the nuclear matrix (Jackson *et al.*, 1996; Iborra *et al.*, 1996; Chakalova and Fraser, 2010). Therefore, transcriptionally active genomic regions are associated with the nuclear matrix while inactive regions are not (Ciejek *et al.*, 1983; Gerdes *et al.*, 1994). Transcription factors such as estrogen receptor (Alexander *et al.*, 1987; Metzger and Korach, 1990), steroid hormone receptor (Barrack, 1987), and corticosteroid receptor (van Steensel *et al.*, 1991) are nuclear matrix associated. Furthermore, elements required for ribosomal assembly, pre mRNA processing and splicing including nucleophosmins and spliceosomes, are also attached to the nuclear matrix (Zeitlin *et al.*, 1987; Mattern *et al.*, 1996).

Functional nuclear matrix is required to support transcription and this process is perturbed when lamins are missing or mutant. The presence of mutant lamin A inhibits the activity of RNA polymerase II (Spann *et al.*, 2002) whereas depleting lamin B1 disrupts RNA synthesis (Tang *et al.*, 2008). In *Xenopus*, lamin B3 has a direct role in the assembly of a replication competent nucleus (Goldberg *et al.*, 1995). Cancer cells that lose their differentiated phenotype express lower levels of lamin A (Venables *et al.*, 2001). The specific function of lamins in transcription remains unknown. However, transcriptionally active genes and transcription factors have been identified in specific sites on the nuclear matrix. More specifically, Oct-1, a repressor of the collagenase gene, colocalises in the nuclear periphery with lamin B (Imai *et al.*, 1997). The finding that the retinoblastoma protein (pRB) interacts with A-type lamins *in vivo* and *in vitro* supports the role of lamins in transcription regulation (Mancini *et al.*, 1994; Ozaki *et al.*, 1994; Kennedy *et al.*, 2000). Rb represses the transcription of genes through its interactions with E2F (Kaelin, 1999). Interestingly, the

disruption of lamin organisation also alters the distribution of the TATA box binding protein, a component of transcription factor TFIID, which is required for polymerase II activity and transcription initiation. These results indicate that lamins may interact with the basal transcriptional machinery and provide a scaffold for the assembly or stabilisation of active transcription complexes. Lamin binding proteins (LAP2β) have also been implicated in transcription through their interaction with E2F transcription factor and chromatin (Gant *et al.*, 1999). In mammalian cells, GCL, essential for germ cell formation and localised to the nuclear envelope, co-localises with LAP2β to the nuclear envelope and interact with the E2F transcription factor. This interaction can reduce the transcriptional activity of the E2F. LAP2β is also capable of reducing the transcriptional activity of the E2F. Co-expression of both LAP2β and GCL with the E2F complex resulted in a reduced transcriptional activity equal to that produced by the pRB protein (Gant *et al.*, 1999; Nili *et al.*, 2001). The binding between LAP2β proteins that are reduced E2F activity during cell cycle and B-type lamins that are involved in DNA replication, thus LAP2β might also be involved in DNA replication either directly or indirectly by affecting lamina assembly during cell cycle (Foisner, 2003).

The interaction of lamins, lamin binding proteins, and transcription factors raises the possibility of a common defective mechanism in diseases and cancers. This mechanism could be the regulation of gene expression, which is normally dependent on correct interactions between transcription factors, chromatin and proteins of the nuclear envelope.

1.2.3.3 Lamins and DNA Repair:

The nuclear matrix is thought to act as a platform for the regulation of cell cycle progression and DNA repair. In addition, its role in chromatin modelling, replication and transcription. DNA repair occurs at the nuclear matrix where homologous recombination proteins are

anchored such as Rad51. Spatial organisation of the genome represents an important role in the regulation of nuclear functions. Mutations in the *LMNA* gene or changes in its expression are associated with defects in DNA replication, transcription and repair, as well as alterations in epigenetic modifications of chromatin (Hutchison and Worman, 2004). Defects in A-type lamins are associated with a whole variety of degenerative disorders, premature ageing syndromes and cancers and this supports the hypothesis that these proteins are considered as the guardian of the genome. A-type lamins control transcription and degradation of proteins with important roles in cell cycle regulation and DNA double strand breaks (DSBs) repair (Warren and Shanahan, 2011) by non-homologous end-joining (NHEJ) and homologous recombination (HR). Importantly, the proteins that are binding by A-type lamins such as RB family members and BRCA1 accelerate tumour suppressor functions and their loss is associated with cancer susceptibility (Redwood *et al.*, 2011). Repair of damaged DNA is critical for maintenance of genomic stability. DSBs are harmful to the genome, leading to mutations, loss of genomic material, and translocations if not properly repaired.

1.2.3.4 Lamins and Apoptosis:

Apoptosis (programmed cell death) is a process that is required for the normal development and homeostasis of tissues and can also be activated in response to cancer, viral infection, or stress (Gruenbaum *et al.*, 2000). Apoptosis is characterised by reduction in nuclear size and chromatin condensation which is accompanied by chromatin fragmentation. The nucleus fragments into small parts containing highly condensed chromatin. Interestingly, lamin assembly may also be involved in triggering apoptosis, as inhibition of the assembly of lamin B induces cell death (Steen and Collas, 2001). The nuclear lamins are the first proteins identified as caspase targets which begins before DNA cleavage or chromatin condensation are detected (Gruenbaum *et al.*, 2000) and the cleavage of the lamins is used as a marker for

caspase activation and the induction of apoptosis. The single caspase cleavage site of lamin A was mapped to aspartic acid 230 (Takahashi *et al.*, 1996). This residue lies in the 2B region of the central rod domain, which is one of the most highly conserved regions in all known intermediate filament proteins. This domain is critical for polymerisation of IF proteins into higher order structures (Stuurman *et al.*, 1998). The cleavage of lamins by caspases probably serves to disassemble the lamina during apoptosis and this disassembly may be essential for nuclear apoptosis to be completed successfully (Okinaga *et al.*, 2007). Lamin degradation appears to play a critical role in shutting down vital nuclear processes during apoptosis.

1.2.3.5 Lamins and Viral Infection:

There is increasing evidence that lamins play a role in viral infection. The replication cycle of cytomegalovirus (CMV) and herpes simplex virus type-1 (HSV-1). Both members of the *Herpesviridae* family, assemble their capsids and encapsidate their DNA in the nucleus, while the final steps in the maturation of virions takes place in the cytoplasm (Mettenleiter *et al.*, 2006). The nucleocapsids exit from the nucleus in a two-step process involving primary envelopment at the INM and fusion of their primary membrane with the ONM to facilitate their movement into the cytoplasm. In order to gain access to the INM, the nuclear lamina has to be destabilised by phosphorylation and disassembly of the lamins specifically in locations where viruses are accessing the INM (Radsak *et al.*, 1991). Lamin A and its binding proteins (BAF, LAP2α, and emerin) have also been implicated in viral infections. BAF is involved in the incorporation of retroviral cDNA into the genome of host mammalian cells (Segura-Totten and Wilson, 2004) and LAP2α stabilises this association. Emerin is required for the efficient integration of viral cDNA into the host genome in HIV1 infections in primary macrophages (Jacque and Stevenson, 2006). It is obvious that lamins and several of their binding proteins are involved in distinct processes of the infection cycles of various viruses.

1.2.3.6 Lamins and Diseases:

Mutation or mis-organisation in lamin and other binding protein genes disturbs the nuclear activities and functions and may lead to laminopathies such as X-linked Emery-Dreifuss Muscular Dystrophy (X-EDMD) and some cancers. Mutations in B-type lamins are considered to be lethal and very few defects have been reported in the expression. In mice, the expressed mutated lamin B1 have more severe effects and they die from respiratory failure and prenatal lethality in addition to abnormalities in other organs (Vergnes *et al.*, 2004; Martin *et al.*, 2009; Chi *et al.*, 2009). This supports the idea that lamin B1 is required during development and associated with sites of DNA replication in early S-phase and not G1 (Moir *et al.*, 1994; Vergnes *et al.*, 2004; van Steensel and Dekker, 2010). Lamin B is required to anchor peripheral chromosomes and any disruption of this interaction may lead to chromosomal relocation and up regulation of genes expression on these chromosomes during interphase (Malhas *et al.*, 2007). In the central nervous system, lamin B1 expression is crucial for oligodendrocyte development and myelination (Lin and Fu, 2009). Mutations in *LMN* genes lead to genetic diseases that affect a number of different tissues such as muscle, adipose, and neuronal tissues. The interactions of lamins with INM proteins, chromatin, and various regulatory factors are considered to be the basis of the role of lamins in cellular processes and tissue specific signalling pathways (Parnaik, 2008).

1.2.3.6.1 Laminopathies:

Diseases caused by mutations in genes encoding nuclear lamins or lamin binding proteins are termed “laminopathies”. They are diverse range of tissues specific diseases. *LMNA* gene can have different mutations in different regions resulting in different laminopathies, this highlights the variety of processes and functions that A-type lamin are involved in disease include Emery-Dreifuss Muscular Dystrophy (EDMD) that is caused by mutations in lamin A

and emerin and affects skeletal muscles, tendons and heart whereas, Progeria is caused by mutations in lamin A and also affect similar tissues skin, hair, fat, muscle, bone and cardiovascular system (Gruenbaum *et al.*, 2005). Mutations in emerin can cause Limb-Girdle muscular dystrophy (Hutchison *et al.*, 2001; Dechat *et al.*, 2008). Heterozygous MAN1 (*LEMD3*) mutations cause osteopoikilosis, Buschke-Ollendorff syndrome, and melorheostosis, characterised by increased bone density (Hellemans *et al.*, 2004). This pathological effect may result from impaired MAN1 regulation of Smad signalling, which is important for bone development. With respect to lamin B regulation, the over-expression of B-type lamin knock-out mice die soon after birth (Vergnes *et al.*, 2004). The over-expression of B-type lamins cause autosomal dominant leukodystrophy (Barraquer Syndrome) (Padiath *et al.*, 2006). LBR, that regulates sterol reductase, also binds lamin B and is required for correct nuclear shape and reorganises chromatin in differentiating neutrophils (Hoffmann *et al.*, 2002). Heterozygous LBR mutations cause Pelger-Huet anomaly, a benign autosomal dominant syndrome characterised by abnormal nuclear shape and chromatin organisation in blood granulocytes (Hoffmann *et al.*, 2002). Greater loss of LBR protein correlates with more severe phenotypes, including developmental delay, epilepsy, and skeletal abnormalities (Hoffmann *et al.*, 2007). Mutations that significantly reduce the sterol reductase activity of LBR are linked to autosomal recessive Greenberg's skeletal dysplasia, characterised by lethal skeletal and visceral anomalies (Waterham *et al.*, 2003).

1.2.3.6.2 Summary of Diseases that are Associated with *LMNA* Mutations:

Some of the examples of laminopathies with preferential involvement of skeletal and cardiac muscle, range from muscular dystrophies to cardiac defects such as in Emery-Dreifuss muscular dystrophy (EDMD), Muscular dystrophy limb-girdle type 1B (LGMD1B), and Cardiomyopathy dilated 1A (CMD1A); lipodystrophies, which specifically affect adipose

tissues such as familial partial lipodystrophy (FPLD); neuropathy disorders, which affect the motor and sensory neurons of the peripheral nervous system such as Charcot-marie-tooth disease (CMT); systemic laminopathies, which are heterogeneous disorders involving multiple tissue systems such as mandibuloacral dysplasia (MAD), Restrictive Dermopathy (RD), Heart-Hand syndrome, and mutations associated with premature ageing disorders. Mutations affecting amino acids 1 to 566 affect both lamin A and C isoforms, whereas mutations found in the carboxy-terminal 566 to 664 amino acids are specific to the lamin A isoforms such as Hutchinson-Gilford progeria syndrome (HGPS) and a typical Werner syndrome (WRN) (Dittmer and Misteli, 2011).

1.2.4 Lamin Binding Proteins:

The roles of lamins are mediated by interactions with numerous lamin binding proteins both at the nuclear periphery and/or in the nucleoplasm. There are many inner nuclear membrane (INM) proteins bind to lamins either directly or indirectly. For example, lamins bind *in vitro* to INM proteins: emerin, MAN1, LBR, LAP1, LAP2β, nesprin, otefin, and SUN1 (Foisner, 2003). Lamins can also bind non integral proteins including chromatin, histone (H2A/H2B) (Höger *et al.*, 1991), transcription factors (E2F), RNA polymerase II transcription machinery (Mattout-Drubezki and Gruenbaum, 2003), pRb (Dorner *et al.*, 2007), BAF (Barrier to Autointegration Factor) (Furukawa, 1999), LAP2α, extracellular signal-regulated kinase (Erk), nuclear actin, and proteins of the nuclear pore complex such as nucleoporin (Nup153) (Vlcek *et al.*, 2001; Mattout-Drubezki and Gruenbaum, 2003; Dechat *et al.*, 2008; Olins *et al.*, 2010). The actual *in vivo* interactions between lamins, DNA, chromatin and the INM remain unclear. However, interaction must be specific and reversible to allow nuclear growth and disassembly during mitosis. After mitosis and during interphase, the reorganisation of nuclear lamina and its assembly at the nuclear periphery is elicited by the IMPs. Most of

these proteins can bind directly to lamin A, lamin B or both (Wilson and Foisner, 2010) (Figure 1.6).

1.2.4.1 Lamin Associated Proteins (LAP):

Several integral membrane (IMPs) proteins known as lamin binding proteins are found at the nuclear periphery and this include Lamin Associated Proteins 1 and 2 (LAP1 and LAP2). LAP1 (LAP 1A, 1B, and 1C) are found at the nuclear membrane and require high concentrations of monovalent salts and nonionic detergents to extract them from nuclei (Senior and Gerace, 1988; Foisner and Gerace, 1993). They arise by alternative splicing, and interact with the genome (Foisner and Gerace, 1993; Pyrpasopoulou *et al.*, 1996). LAP1C has a transmembrane domain and is localised in the nucleoplasm (Martin *et al.*, 1995). LAP2α lacks a transmembrane domain, localises to the nuclear interior and specifically binds lamin A (Harris *et al.*, 1994). LAP2β has the specificity to bind B-type lamins and heterochromatin as well as to different other histones, such as HP-1 (Foisner and Gerace, 1993; Harris *et al.*, 1994; Bridger and Bickmore, 1998). This binding is responsible for attaching the nuclear lamina to the INM. LAP2α has N-terminal nucleoplasmic domain containing phosphoacceptor sites for CDC2 kinase and a C-terminal hydrophobic domain which codes for transmembrane segments. The physiological role of LAP2α and lamin A complexes bind directly to pRB and are involved in the pRB activity of E2F/pRB target gene (Furukawa, 1994; Wilson and Foisner, 2010). LAP2β has N-terminal domain residing in the nucleoplasm and C-terminal domain localised in the lumen between the INM and the ONM. This protein can bind HA95 which is a chromatin protein involved in DNA replication (Martins *et al.*, 2003; Wilson and Foisner, 2010). LAP2 has critical roles in genetic disorders and hematopoietic malignancies. LAP2 is widely over-expressed in diverse digestive tract cancers and this increase the motility of cancer cells. LAP2β is over-expressed in gastric cancer

tissues. Knockdown of LAP2β did not affect the proliferation of most digestive tract cancer cells except pancreatic cancer cells whereas; over-expression of LAP2β increased motility of gastric and pancreatic cancer cells (Kim *et al.*, 2012) (Figure 1.6).

1.2.4.2 Lamin B Receptor:

Lamin B Receptor (LBR or p58, 58 kDa) is an integral membrane protein of the interphase nuclear envelope with a structural function interacting with chromatin and lamins, and an enzymatic function as a sterol reductase (Worman *et al.*, 1990; Ye and Worman, 1994). It has eight transmembrane receptors with functional domains. The LBR required high KCl/Triton-X-100 to be extracted from the nuclear envelope (Worman *et al.*, 1990; Ye and Worman, 1994; Meier and Georgatos, 1994; Ye and Worman, 1996; Wydner *et al.*, 1996; Silve *et al.*, 1998; Olins *et al.*, 2010). It is a receptor to lamin B. Human LBR contains 615 amino acids (aa), the N-terminus (~208aa) resides in the nucleoplasm binding to both lamin B and heterochromatin with these interactions disrupted during mitosis. The N-terminal region also contains three DNA-binding motifs that are found in gene regulatory proteins and histones, suggesting that the LBR may additionally play a role in gene regulation and/or chromatin organisation (Worman *et al.*, 1990). While the C-terminus (~407aa) resides within the inner nuclear membrane, it remains connected with B-type lamins during mitosis, exhibits sterol Δ^{14}-reductase activity and is involved in the conversion of 7-dehydrocholestrol to cholestrol (Ye and Worman, 1996; Silve *et al.*, 1998; Olins *et al.*, 2010). LBR is a substrate for several protein kinases including p34 and p58 kinase (Ye and Worman, 1994). LBR, LAP1A and 1B and LAP2 bind to lamin B whereas LAP1A and 1B again bind both to lamin A. LBR also binds directly to heterochromatin (HP1α and HP1γ); which is thought to participate in gene silencing and play important role in nuclear membrane-chromatin interaction as well as its interaction with HA95, LAP2β, and emerin (Ye and Worman, 1994; Pyrpasopoulou *et al.*,

1996; Ye and Worman, 1996; Olins *et al.*, 2010) (Figure 1.6). Heterozygous *LBR* mutations cause nuclear hyposegmentation in neutrophils (Pelger Anomaly), while homozygous mutations cause prenatal death with skeletal defects and abnormal sterol metabolism (Greenberg Dysplasia). It has remained unclear whether the lethality in Greenberg dysplasia is due to cholesterol defects or altered nuclear morphology (Hoffmann *et al.*, 2002; Waterham *et al.*, 2003; Clayton *et al.*, 2010). Therefore, *LBR* mutations can affect sterol reductase activity, causing lethal Greenberg dysplasia but not Pelger anomaly. These findings separate the metabolic from the structural function and indicate that the sterol reductase activity is essential for human intrauterine development. In addition, LBR mutation can lead to other abnormalities including alopecia and hydrocephalus (Shultz *et al.*, 2003; Clayton *et al.*, 2010). In cancer, the lack of LBR at the NM in papillary thyroid carcinoma might be linked to *LBR* gene mutation or alterations in the expression of LBR (Fischer *et al.*, 1998).

1.2.4.3 Emerin:

Emerin was first identified from the gene mutation on chromosome Xq28 causing X-linked recessive disease Emery-Dreifuss muscular dystrophy (X-EDMD). The emerin gene encodes a 254 amino acid type II integral membrane protein. Structural analysis predicts that emerin contains a transmembrane region at the C-terminus and a large hydrophilic N-terminal domain with multiple putative phosphorylation sites (Bione *et al.*, 1994). In addition, emerin contains the LEM (LAP2, Emerin, and MAN1) domain that is common to a number of integral membrane proteins of the inner nuclear membrane, INM (Vaughan *et al.*, 2001). Emerin is an IMP that has some homology to LAP2. It binds to transcription factors such as E2F/RB, actin, nesprin and also binds to lamin A and lamin C (Figure 1.6). In cells lacking functional A-type lamins, emerin is mislocalised to the endoplasmic reticulum (Sullivan *et al.*, 1999; Burke and Stewart, 2002; Holaska *et al.*, 2002; Dahl *et al.*, 2008). Thus, its correct

nuclear localisation requires A-type lamins. Emerin colocalises with lamin proteins *in vitro* and *in vivo* (Hutchison *et al.*, 2001; Vaughan *et al.*, 2001). The specificity of this binding depends on its phosphorylation by CDC2 kinases during interphase and mitosis (Haque *et al.*, 2006).

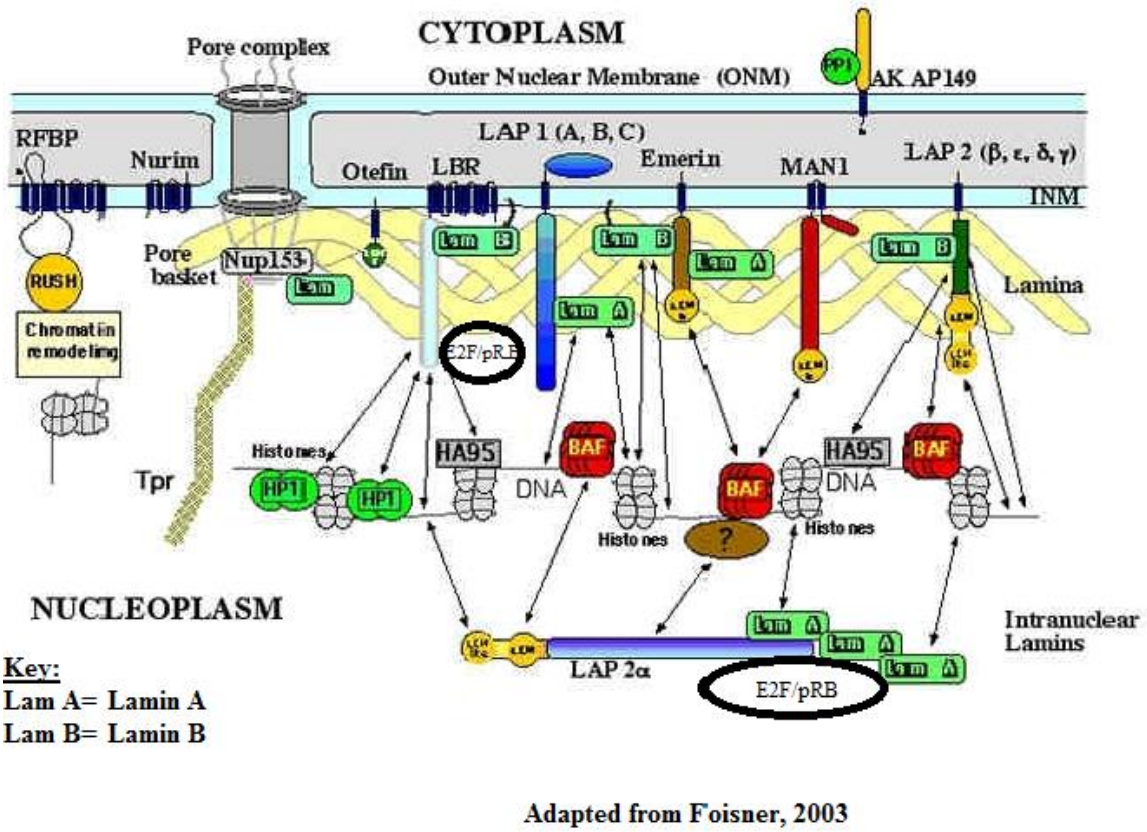
1.2.4.4 MAN1:

The "MAN antigens" are polypeptides recognised by autoantibodies from a patient with a collagen vascular disease and localised to the nuclear envelope. MAN1 is a 82.3 kDa protein. The *MAN1* (*LEM3*) gene is located on human chromosome 12q14 (Lin *et al.*, 2000). A number of experiments show that MAN1 is an IMP of the INM and shares the LEM domain with LAP2β and emerin (Hellemans *et al.*, 2004; Mansharamani and Wilson, 2005). The protein sequence analysis reveals that MAN1 shares a conserved globular domain of approximately 40 amino acids, associated with other inner nuclear membrane proteins, lamin A, lamin B1, LAP2β, and emerin (Lin *et al.*, 2000; Mansharamani and Wilson, 2005). MAN1 is located in the nuclear membrane and has a short C-terminus. MAN1 serves as a membrane anchor and play roles in nuclear positioning by connecting the nuclear membrane with the cytoplasm as well as its interaction with Smad proteins to regulate bone development (Haque *et al.*, 2006). Over-expression of MAN1 results in inhibition of R-Smad phosphorylation, nuclear translocation, and repression of transcriptional activity of transforming growth factor beta (TGF-β) (Pan *et al.*, 2005; Ishimura *et al.*, 2006). It has been shown to regulate TGF-β by interacting with receptor-associated Smads. However, the *in vivo* roles of MAN1 have not been fully characterised. In 2006, Ishimura and his co-workers showed that MAN1 regulates vascular remodeling by analysing MAN1-deficient embryos lacking the Smad interacting domain. MAN1-deficient embryos die at midgestation because of defects in embryonic vasculation. They have revealed a novel role for MAN1 in angiogenesis and provided the

first evidence that vascular remodeling can be regulated at the INM through the interaction between MAN1 and Smad proteins (Hellemans *et al.*, 2004; Lin *et al.*, 2005; Mumm *et al.*, 2007) (Figure 1.6).

1.2.4.5 LEM Domain Proteins:

LEM complex (belong to **L**AP, **E**merin and **M**AN1 domain family) is important in the interaction with chromatin and/or lamins and other proteins to regulate signalling transcription. LEM interacts directly with BAF (dsDNA binding protein, 10 kDa) and lamin A and/or lamin B and thus mediates chromatin interactions. Thus, BAF is required to segregate and enclose chromosomes within the nuclear envelope and assemble the nuclear lamina (Furukawa, 1994; Margalit *et al.*, 2005). LEM is a group of proteins which share a motif of ~40 amino acids in length (Lin *et al.*, 2000). There is a large family of proteins called nesprins which is a part of LEM complex family and interact with heterochromatin regions of the genome as well as SUN1 proteins in the lumen that connect the cytoplasm and the nucleoplasm (Broers *et al.*, 2006). Lamin A but not lamin C, lamin B1 or lamin B2 interacts with SUN1/2 in the lamina while in the cytoplasm; nesprin is thought to bind to actin. This interaction creates nesprin-SUN complexes connecting the cytoskeleton to nuclear components (Haque *et al.*, 2006; Roux *et al.*, 2009) (Figure 1.6).



Adapted from Foisner, 2003

Figure 1.6: Dynamic Connections of Nuclear Envelope Proteins, Nuclear Lamina and Lamin Binding Proteins to Chromatin and the Nuclear Matrix

The figure displays the nuclear envelope (NE) that consists of inner nuclear membrane (INM), outer nuclear membrane (ONM), and the nuclear lamina which is a filamentous scaffold structure underneath the INM. The lamina consists of A-type lamins and B-type lamins and integral nuclear membrane proteins (IMPs) such as lamin B receptor (LBR), MAN1, emerin, and various isoforms of lamina-associated polypeptides 1 (LAP1, A, B, and C) and LAP2 (β, ε, δ, and γ). Lamins, LAP2, emerin and LBR interact with DNA and/or chromosomal proteins such as histones, BAF, HP1 and HA95, and provide a complex dynamic link between the peripheral lamina and nucleoplasm structures and chromatin fibers for chromatin organisation and gene expression. In addition, components of nuclear pore complexes (NPCs), such as Nup153 and translocated promotor region (Tpr), link the nuclear envelope to the nuclear interior and involved in mRNA transport and transcription repression of telomeric genes. Furthermore, intranuclear complexes of A-type lamins and LAP2α are likely involved in higher order chromatin organisation throughout the nucleus. Moreover, lamin A associate directly with retinoblastoma protein (pRb), which binds E2F and represses transcription of S-phase-specific genes during cell cycle. These interactions are regulated in a spatial manner during the cell cycle and are responsible for the dynamic nuclear and chromatin structure organisation, in DNA replication, gene transcription, cell cycle progression, and apoptosis. This figure is adapted from Foisner, 2003 (Foisner, 2003).

1.3 Genome Organisation:

1.3.1 Chromatin Loops are Selectively Anchored Using Scaffold/Matrix-Attachment Regions:

It is clear that the nuclear envelope, specifically nuclear lamins, forms part of a major important nuclear structure which is the nuclear matrix (Figures 1.4 and 1.6). The nuclear matrix is composed of two main nuclear parts; the interior nuclear matrix and peripheral nuclear lamins (Nickerson, 2001). Numerous modifications of biochemical pathways start signal transductions for accurate physiological conditions, therefore the term nucleoskeleton (Jackson and Cook, 1988). The visualisation of chromatin attached to the nucleoskeleton initiated the discovery of looped DNA at least 10-30 μm long or 30-90 kilobases which are responsible for correct replication and transcription (Cook and Brazell, 1975; Paulson and Laemmli, 1977) as it is where RNA polymerases cluster and loop the DNA (Cook, 2002). Therefore, it considered to be the active site for replication, repair, transcription, organisation, and integrity.

The biological significance of nuclear scaffold/matrix-attachment regions (S/MARs) remains a subject of interest. Understanding S/MAR behaviour relies on the physical functions and chromatin loop anchors. In 2004, Heng *et al.* found that S/MARs functioned as the mediators of loop attachment and they were used in a selective and dynamic fashion (Heng *et al.*, 2004). The genome interacts with the nuclear matrix proteins at specific DNA sequences known as matrix attachment regions (MARs). These proteins include nuclear lamins, A-type and B-type lamins, are involved in associations and interactions of the nuclear matrix proteins and the genome. A-type lamins have been shown to associate with telomeres (Raz *et al.*, 2006). The loss of these proteins disrupts telomere localisation and maintenance (Gonzalez-Suarez

et al., 2009). However, many of these regions are discovered but still they are poorly characterised (Bode *et al.*, 2000).

1.3.2 The Network of the Nuclear Lamina Proteins Bind to Chromatin and Histones:

The dynamic nature of the NE is well illustrated at mitosis when the NE breaks down and reassembles around daughter chromosomes in interphase. Disassembly of the NE at prophase correlates with phosphorylation of proteins of the chromatin, nuclear membranes and lamina, leading to soluble lamin A/C form while B-type lamins remain in a membrane-bound form (Gerace and Blobel, 1980). In telophase, lamins are dephosphorylated and repolymerised (Ottaviano and Gerace, 1985). Proteins of the INM, lamina and chromosomes are extensively and strongly interconnected. LBR and LAP2β also bind chromatin via interactions with heterochromatin protein HP1 (Ye and Worman, 1996) and the small DNA-binding protein BAF (Furukawa, 1994). LAP2α also interacts with lamins A/C (Dechat *et al.*, 1998) while H2-type histones bind lamins A/C and B *in vitro* (Goldberg *et al.*, 1999). Thus, the chromatin provides multiple anchoring sites for the NE. HA95, also called HAP95 or NAKAP95, is a novel nuclear protein (Orstavik *et al.*, 2000; Seki *et al.*, 2000; Westberg *et al.*, 2000). HA95 exhibits high homology to the human nuclear A-kinase (PKA) anchoring protein, AKAP95 (Eide *et al.*, 1998) and like AKAP95, HA95 harbors two zinc fingers (Orstavik *et al.*, 2000) and co-localises with AKAP95 in interphase and at mitosis. To support this view, HA95/HAP95 was recently shown to interact with RNA helicase A and enhance expression of the constitutive transport element (CTE), an RNA element involved in nuclear export of retroviruses and DNA replication (Westberg *et al.*, 2000). Furthermore, HA95 is a chromatin and nuclear matrix associated protein implicated in the regulation of NE-chromatin interactions. HA95 directly interacts with the lamina and with a subset of integral proteins of the INM for chromatin condensation and assembly of nuclear membranes. However, HA95 is

a central element of the chromatin and the nuclear matrix implicated in regulating NE-chromatin interactions during the cell cycle.

Both lamins and lamin binding proteins bind directly to chromatin, histones, DNA polymerase II, transcription factors, and DNA (Zink *et al*, 1999). Peripheral chromatins are considered to be epigenetically silent and inactive (Mattout-Drubezki and Gruenbaum, 2003). These interactions are thought to be the basis of a tethering mechanism that direct specific chromosomes or chromosome regions to the periphery. However, there is a problem with this study; that lamins and lamin binding proteins (lamin A and LAP2α) can be found within the nucleoplasm. LAP2α-lamins A/C complexes in the nucleoplasm have been implicated in the regulation of gene expression. They bind chromatin proteins and chromatin modifying enzymes, and can thus participate in epigenetic control pathways. Furthermore, binding of lamins A/C complexes to specific transcription factors and repressors may directly affect their transcriptional activity. LAP2α-lamins A/C also regulate pRB/E2F and influence cell cycle progression and differentiation, which could have important implications for molecular mechanisms of laminopathic diseases (Dorner *et al*, 2007). To investigate these interactions, researchers have identifed the specificity of this binding and they found that some histone such as H2A and H2B sub-types and chromatin bind specifically to the coiled-coil rod domain. The tail domains of mammalian lamins C, B1, and B2 also bind to chromatin and histones. This lamin-histone interaction could be involved in specifying the high avidity attachment of chromatin to the nuclear envelope *in vivo* (Höger *et al.*, 1991; Taniura *et al.*, 1995) (Figure 1.6).

The lamins and the lamin binding proteins, LAP2β and LBR have been described to bind to DNA or to interact with chromatin via histones, BAF, and HP1 chromodomain proteins,

respectively, and may provide anchorage sites for chromatin fibers at the nuclear periphery (Gotzmann and Foisner, 1999; Foisner, 2003). Nuclear envelope proteins also bind chromatin; LBR binds lamin B, heterochromatin via histones H3/H4 as well as HP1α and HP1γ (Pyrpasopoulou *et al.*, 1996) as well as binds LAP2β through BAF. BAF is a soluble protein that can also bind to histones and DNA and plays a role in the higher order structure of nucleoproteins (Olins *et al.*, 2010). BAF has specificity of interaction with histones (H1) as well as its association with NE through LAP2α. Based on its interactions with histones and DNA, it is suggested that BAF might bind nucleosomes *in vivo* (de Oca *et al.*, 2005; Greer and Shi, 2012) (Figure 1.6). It is clear this binding of NE proteins with chromatin is important and required for NE assembly after mitosis. It is interesting that emerin and LBR appear to assemble at different positions on mitotic chromosomes suggesting that some of interphase chromatin organisation has affinity interaction at the end of mitosis. In addition, MAN1 binds directly to the DNA through its carboxyl terminal domain whereas LAP2β binds to chromatin proteins HA95 to restrict its association with the nuclear envelope (Martins *et al.*, 2000 and 2003). Several nuclear envelope proteins not only bind to chromatin but also bind chromatin modification enzymes such as LBR-specific kinase and histone deacetylase (HDAC3) for H4 deacetylation and can bind LAP2β (Pyrpasopoulou *et al.*, 1996; Somech *et al.*, 2005). Emerin binds directly to HDAC3 and recruits HDAC3 to the nuclear periphery. This binding stimulates the catalytic activity of HDAC3 and inactivates H4K5 acetylation, protein involved in chromatin formation at the nuclear periphery (Demmerle *et al.*, 2012). Therefore, nuclear lamina and chromatin are supposed to provide mechanical stability for nuclear structure to form a platform for most metabolic nuclear processes and to organise chromatin within the nucleoplasm and thus regulate gene expression at the chromatin structure and spatial levels.

1.3.3 Spatial Genome Organisation is Tissue Specific:

Genomes are organised into individual chromosomes and each chromosome occupies a territory in cell nuclei. This non-random positioning of territories is according to their gene density or the cell's transcriptional or differentiation status (Foster and Bridger, 2005). Gene density is highlighted by the positions of HSAs 18 and 19 territories has been observed (Croft *et al.,* 1999; Boyle *et al.,* 2001). These chromosomes are similar in DNA content (86 Mb and 72 Mb, respectively) and 2.6 % and 2 % of the physical length of the genome, respectively (Tanabe *et al.,* 2002) but have contrasting functional and structural characteristics such as they differ in their gene content and replication timing (Croft *et al.,* 1999). Most of HSA19 chromatin belongs to G-light bands, which is gene dense and replicates early during S phase, are located toward the nuclear interior whereas HSA18 chromatin represents G-dark bands, which consists of gene poor chromatin and replicate later, are located close to the nuclear periphery (Croft *et al.,* 1999; Boyle *et al.,* 2001; Zink *et al.,* 2004; Bolzer *et al.,* 2005; Foster and Bridger, 2005). Our knowledge about the structural and functional genome organisation of the cell nucleus is limited and the position of a chromosome territory is determined by its DNA contents and transcriptional status. The nuclear functions are highly organised and localised within the cell nucleus as well as chromatins are organised in loops, containing active areas of the genome (Figure 1.4) within the nuclear matrix (van Driel *et al.,* 1991). This organisation and arrangement of chromatin is responsible for the regulation of gene expression.

The location of a gene within the nucleus is responsible for specific nuclear function such as transcription and splicing (Parada and Misteli, 2002). Heterochromatin contains few genes, replicates during late S-phase, is enriched in specific nuclear proteins and has the ability to suppress the transcriptional activity of active genes. It is believed that the heterochromatic

rich regions of the nuclear periphery are stable. Therefore, the majority of transcription machinery is also found peripherally to regulate the gene expression activity (Deniaud and Bickmore, 2009). Spatial genome organisation is tissue specific (Cremer and Cremer, 2001; Parada *et al.*, 2004; Malhas *et al.*, 2007). For example, human chromosome 5 was preferentially found towards the centre of the nucleus in liver cells, but was predominantly peripheral in lung cells and was located in an intermediate position in lymphocytes (Parada *et al.*, 2004). Furthermore, most cell types shared positioning of some but not other chromosomes. For example, lung cells and liver cells shared the position of chromosomes 12 and 14 but not chromosomes 5 and 6. Furthermore, lymphoblasts and myeloblasts differ in the positioning of HSA 5 (Parada *et al.*, 2004). There is also preferential position of all chromosomes in spermatogenesis. The positioning of sex chromosomes are highly organised at the nuclear edge in primary spermatocytes, whereas, in spermatids, the sex chromosomes with a preferential location in the central region (Foster *et al.*, 2005). This explains the repositioning of chromosome territories in testes sections during spermatogenesis. Moreover, the radial positioning of HSA 12 and 16 was also observed during adipocyte differentiation. There is a close association between HSA 12 and 16 in differentiated adipocytes, an association not observed in preadipocytes. This suggests the translocation of chromosomes 12 and 16 might play a key role in human liposarcomas (Kuroda *et al.*, 2004).

It could be hypothesised that specific chromosomes and/or gene loci would change their nuclear location before or after changes in regulation associated with cell differentiation because it is tissue specific. However, no changes in human chromosomes positioning were observed in both skin fibroblasts and lymphoblasts with the exception of two chromosomes, 8 and 21 (Boyle *et al.*, 2001). Porcine chromosomes have also been demonstrated to change nuclear location during adipogenesis, using mesenchymal stem cells, derived from porcine

bone marrow. These chromosome territories (SSC4, SSC6, SSC12, SSC13, SSC15, and SSC17) were found to alter their nuclear position during the *in vitro* adipogenesis, from the nuclear periphery towards the nuclear interior and found that during *in vitro* adipogenesis chromosome territories decondensed (Szczerbal *et al.*, 2009). The same model was used to assess the chromosome territories during normal spermatogenesis (Foster *et al.*, 2005). This is supporting model of the involvement of spatial genome repositioning in regulating gene expression and the nuclear interior is being an important region of the nucleus for transcriptional activity.

Foster *et al.*, in 2012, have assessed the interphase genome behaviour using porcine cells and tissues. They have positioned individual whole chromosomes in different cell types such as embryonic fibroblast cell line (ESK4), *ex vivo* lymphocytes and *ex vivo* bone marrow Mesenchymal stem cells (MSCs). They found that chromosomes occupy similar interphase nuclear positions regardless of *in vitro* or *in vivo* conditions. The comparisons between chromosome positioning of chromosomes 5, 13, 17 and X in cultured nuclei (during S-phase) and nuclei within *ex vivo* tissue sections, revealed that chromosomes 5, 13 and X occupy peripheral positions, whereas chromosome 17 is localised at more internal nuclear locations. That means nuclear chromosomes 5, 13, 17 and X share nuclear positions between *in vitro* cultured nuclei and *in vivo* nuclei (Foster *et al.*, in 2012).

We can conclude that the positioning of chromosomes within interphase nuclei is tissue specific in the formation of chromosome arrangements among tissues. However, the functional properties of tissue specific spatial genome organisation in gene expression and gene silencing during cell differentiation remain unclear.

1.3.4 Chromatin, Chromosome Territories and Gene Loci within Interphase Nuclei:

Since the human chromosomes are positioned non-randomly during interphase, the genome is well organised and re-organised after mitosis for the correct nuclear functions. Nuclear structure and genome organisation are involved in organising and co-ordinating interphase chromosome positioning and gene loci regulation and expression. Nuclear architecture interacts with itself and anchors the genome. Mis-organisation or perturbing of this nuclear architecture can lead to aberration or alteration in regulating normal chromosome behaviour and increases the cells possibility to diseases such as cancer.

Understanding of chromosome territories behaviour in the interphase nucleus increases the understanding of the differences between cancerous and normal cells. The changes in the nuclear architecture of cancer cells are used in tumour diagnosis for over 140 years. The first patient biopsy was examined in 1860; this led to improvement in understanding these changes. However, diagnosis still relies on the analysis of cell morphology. Specific nuclear markers of cancer include changes in nuclear size and shape, nucleolar alterations, changes in chromatin organisation, aberrant nuclear lamina and alterations to PML (Zink *et al.*, 2004). Nuclear morphological changes in shape are observed in papillary thyroid carcinomas (Fisher *et al.*, 1998) and enlarged nucleoli are associated with small-lung carcinoma (Zink *et al.*, 2004). Moreover, these were some changes observed to heterochromatic foci in cancer and usually associated with gene repression (Hahn *et al.*, 2010). The changes to heterochromatic foci found in knockdown mice lacking the tumour suppressor gene BRAC1 resulted in loss of foci from the nuclear periphery leading to a more diffuse state of foci throughout the nucleus. BRAC1 binds H2A at satellite repeat to maintain heterochromatin structure (Zhu *et al.*, 2011). It is important to understand whether changes in chromatin structure or location cause

deregulation of genes and thus drive cancer. These studies suggest that the loss of a tumour suppressor gene can lead to aberrant chromatin changes then induce cancer.

Specific areas of the genome that are tethered to the nuclear lamina or the nucleolus have been characterised during interphase chromatin (Lodén and van Steensel, 2005; Woodcock, 2006; van Steensel and Dekker, 2010; Fudenberg *et al.*, 2011). In the past decade, there is increased debate in the idea of effects of the gene positioning and organisation. Now it is reasonable to accept the theory of organisation is in fact non-random (Figure 1.7), tissue specific and that individual chromosome occupy discrete regions (territories) (Parada *et al.*, 2004). This explains the idea of genes activities may be affected by its location on chromosomes in the nucleus. Repositioning of chromosome territories (CTs) has been observed in different tissue types. This has also been seen during differentiation (Foster *et al.*, 2007). Interestingly, a recent study showed that the nuclear periphery, a zone of transcriptional repression, gene-poor and inactive chromatin, has an active role in some mammalian genes. This regulation and activation is affected by gene expression level and through their association with different genomic regions and proteins associated with the nuclear pore complexes thus different genome functions (Deniaud and Bickmore, 2009).

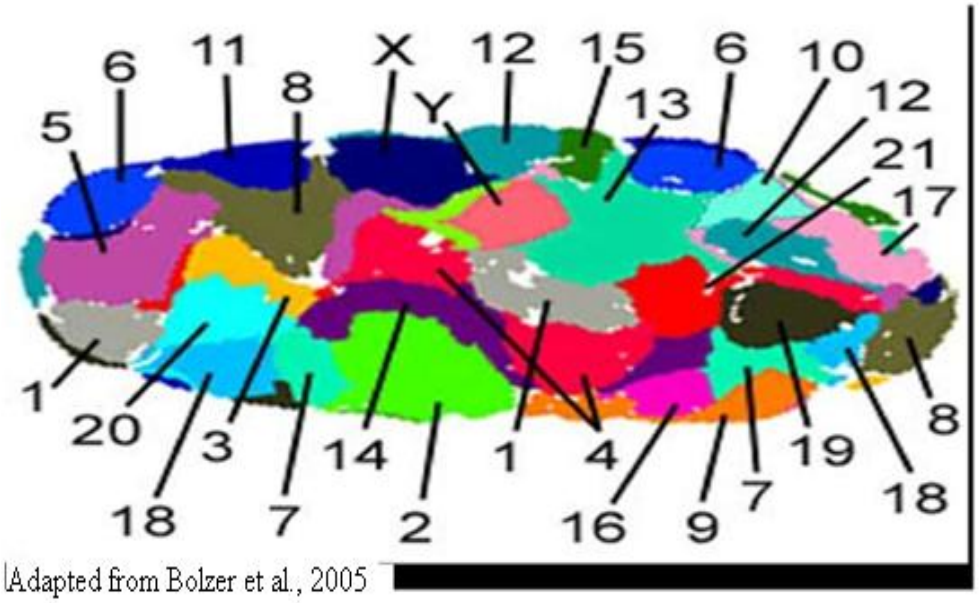


Figure 1.7: Chromosome Territories
The figure shows that 24-Colour 3D FISH chromosome territories are organised in non-random positions in a human fibroblast interphase nucleus (Bolzer *et al.*, 2005).

1.3.4.1 Genome Interactions with Lamina and Nuclear Pore Complex:

The nuclear envelope provides anchoring sites for interphase chromosomes and participates in genome organisation inside the nucleus through two main structures, nuclear lamina (NL) and nuclear pore complexes (NPCs). Both the NL and NPCs are involved in anchoring sites for interphase chromosomes (Blobel, 1985) (Figure 1.6). Different studies have supported this idea. Some genomic loci are preferentially located close to the nuclear envelope, while other loci are found in the nuclear interior (Takizawa *et al.*, 2008; Fedorova and Zink, 2009). Recent genome-wide mapping techniques have begun to provide more global insights into the molecular interactions of chromosomes with components of the nuclear envelope.

Interactions of the genome with the NL have been mapped in *Drosophila* by fusion of the NL to DNA adenine methyltransferase (Dam) from *E. coli*. When expressed in cells, this chimaeric protein is incorporated into the NL. Thus, DNA that is in molecular contact with the NL *in vivo* becomes methylated by the tethered Dam. By this approach, NL interactions have been mapped in detail in *Drosophila*, mouse and human cells (Guelen *et al.*, 2006; Pickersgill *et al.*, 2006; Peric-Hupkes *et al.*, 2008). In all three species, interactions with the NL involve large genomic domains, rather than focal sites. Human genomes have more than 1,000 lamina-associated domains (LADs) with a median size of ~ 0.5-10Mb. LADs are relatively gene-poor, they harbour thousands of genes and most of these genes are transcriptionally inactive (Guelen *et al.*, 2006; van Steensel and Dekker, 2010). The reason could be of a repressive role of the NL in gene regulation. Lamin B in Drosophila causes repression of some NL-associated genes such as testis-specific gene cluster (Shevelyov *et al.*, 2009). Moreover, the NL can cause the down-regulation of some endogenous genes, although this may depend on its genomic integration site (Reddy *et al.*, 2008; Finlan *et al.*, 2008). Furthermore, during differentiation hundreds of genes show altered interactions with NL.

Interactions of the genome with NPCs have been studied by both DamID and Chromatin Immunoprecipitation (ChIP). These techniques employ cross-linking of protein-DNA interactions with formaldehyde, followed by mechanical fragmentation of the DNA and immunoprecipitation using antibodies against NPC proteins (Nups). Genome-wide microarrays were used to identify the immunoprecipitated DNA sequences. In yeast, *Drosophila* and human cells, hundreds of genes are associated with various Nups (Brown *et al.*, 2008a).

1.3.4.2 Genome Interactions with Nucleolus:

The nucleolus is an important organelle to study genome activity, since all RNA polymerases (pol I, II, and III) are involved in the dynamic and regulation of ribosome biogenesis process, which is its main function. The high proliferation activity of tumour cells with high ribosome biogenesis activity exposes the nucleolus as a promising target in cancer biology (Drygin *et al.*, 2009). In addition, cell-type and function-dependent nucleolar localisation of tumour suppressor proteins, such as p53 and MDM2 indicates the role of the nucleolus in carcinogenesis (Sirri *et al.*, 2008). A number of other biological processes such as senescence, RNA modification, cell cycle control and stress are also regulated in the nucleolus and connect it to several functional networks of the cell (Sirri *et al.*, 2008). Furthermore, high chromatin density is found at nucleoli or nuclear periphery, and disruption of nucleoli increases motility of chromatin domains, indicating the role of the nucleolus in chromatin arrangement (Chubb *et al.*, 2002; van Steensel and Dekker, 2010). It was thought that this nuclear compartment harbors only the rRNA-encoding genes, which are transcribed by RNA polymerase I. In order to find other sequences that may interact with nucleoli, a recent study isolated nucleoli from human cells. The associated DNA was then characterised and identified genomic regions named nucleolus-associated domains (NADs). NADs are large genomic segments (median size 750 kb, 97 chromosomal regions) that are highly enriched in centromeric satellite repeats and specific inactive gene clusters, which is consistent with the preferential localisation of centromeres around nucleoli (Stahl *et al.*, 1976 and Németh *et al.*, 2010). Interestingly, the 5S and tRNA genes, which are transcribed by RNA Polymerase III, also preferentially associate with the nucleolus (Németh *et al.*, 2010; van Steensel and Dekker, 2010), suggesting that nucleolar interactions may help to coordinate the expression of specific gene sets. These results demonstrated and confirmed that chromosomal regions interact specifically with the nuclear lamins, NPCs and nucleoli. The

nucleolus can therefore be considered as a model system to investigate functional genome organisation. However, that alteration in the nucleolus might be linked to multiple forms of human disease, including cancer and viral infections. Moreover, multiple genetic disorders have been mapped to genes that encode proteins located in nucleoli under specific conditions such as Werner's syndrome (Marciniak *et al.*, 1998) and fragile X (Tamanini *et al.*, 2000).

1.3.5 Interphase Chromosome Positioning in Cancer:

During disease, the spatial organisation of the genome can be altered. Chromosomal aberrations are the most characteristic of tumour cells. The aberration of genetic material from two or more chromosomes will lead to the cells transformation that acts as a cause of tumourgenesis by fusion or mis-regulation of genes, this is termed chromosomal translocation. This formation is processed by double strand breaks (DSB) and failure in repair mechanisms to eliminate DSBs can lead to mis-position of specific chromosomes (Kanaar *et al.*, 1998; Meaburn *et al.*, 2007b). In addition, the distribution of centromeres and telomeres can be altered in specific cancer cells (Poddighe *et al.*, 1992). Furthermore, chromosome aberrations and type of tumour are considered the most important for diagnosis and prognosis of cancer with chromosomal abnormalities. A number of studies were performed in order to understand the deferential positioning in chromosomes interphase nuclei. Abnormal relocation of chromosome 18 from the nuclear periphery to the interior has been documented in several types of tumour cell lines (Cremer *et al.*, 2003), including melanoma-derived cell line, cervix carcinoma, colon carcinoma, Hodgkin-derived cell line and colon carcinoma metastasis cells (Cremer *et al.*, 2003). Moreover, report supports the idea of the functional correlation between non-random positioning and formation of translocation, for example HSAs 9 and 22 in chronic myeloid leukaemia (CML), (Lukasova *et al.*, 1997) as well as the correlation between tissue specific spatial organisation and tissue specific translocation

(Parada *et al.*, 2004). Furthermore, the nuclear location of chromosomes 10, 18 and 19 were assessed in normal thyroid tissue and compared to adenomatous goiters, papillary carcinomas and undifferentiated carcinomas. There was no difference in chromosome position in the normal and goiter tissue with chromosomes 10 and 18 positioned towards the nuclear periphery; and chromosome 19 in a central location. However, in the papillary carcinoma tissue chromosome 19 was located centrally as in normal cells. Furthermore, in undifferentiated carcinomas all the chromosomes assessed were mislocalised. These findings indicate that alteration of chromosome positioning could be related to DNA amplification and abnormal chromatin features (Murata *et al.*, 2007). Marella *et al.*, in 2009, used normal human WI38 lung fibroblast and MCF10A epithelial breast cells and identified that similar levels of associations were found in the normal cell lines WI38 and MCF10A for chromosomes 1, 4, 11, 12, 14, and 16 whereas a 2-fold increase of chromosome 4 and 16 associations in a malignant breast cancer cell line (MCFCA1a) compared to the related normal epithelial cell line (MCF10A) was found. This demonstrates that chromosome associations are cell type specific and undergo alterations in cancer cells (Marella *et al.*, 2009).

Furthermore, Wiech *et al.*, 2005 analysed chromosome 8 positions in wax embedded pancreatic cancer tissue samples. Their results obtained from non-neoplastic pancreatic cells of randomly selected individuals indicated that the radial arrangement of the chromosome 8 territories as well as their shape (roundness) did not significantly differ between the individuals. In pancreatic tumours, the radial distance indicated the repositioning of chromosome 8 to the nuclear periphery and the decreased roundness of CTs found may reflect the genomic and transcriptional alterations in carcinoma. There were considerable differences between pancreatic tumour and non-neoplastic cells. In non-neoplastic ductal

epithelium of the breast there was a larger distance in the positioning of the centromere 17 and HER2 domains between individuals. In neoplastic epithelial breast cells, the distances between centromere and gene domains were smaller than in non-neoplastic cells. They demonstrated repositioning of the centromere on chromosome 17 to a more internal location (Wiech *et al.*, 2005; Timme *et al.*, 2011). A study by Wiech *et al.*, in 2009 reported repositioning of chromosome 18 during cell differentiation of cervical squamous epithelium towards the nuclear centre whereas, in cervical squamous carcinomas showed a positioning of chromosome 18 towards the nuclear periphery (Wiech *et al.*, 2009).

1.3.6 Interphase Gene Positioning in Cancer:

Nuclear architecture and chromatin structure are important features for the regulation of gene expression and can be altered in cancer. Altered chromosome positioning has been associated with numerous types of cancer in which altered gene expression that lead to malignant transformation of the cell. Individual gene loci are also repositioned during early tumorigenesis (Ferrai *et al.*, 2010). *In vitro* studies of tumourigenesis in early breast cancer showed altered positioning of cancer-associated genes such as AKT1, BCL2, ERBB2, and VEGF loci although no correlation was found between radial redistribution and gene activity level (Meaburn and Misteli, 2008). However, translocation occurs in specific type of cancers that contribute to nuclear positioning of the loci involved (Roix *et al.*, 2003). Furthermore, the intermingling between specific pairs of chromosomes correlates with the frequency of translocations between the same chromosomes that observed after ionizing radiation (Branco and Pombo, 2006). Analyses of single genes close to or at translocation sites have also shown that their nuclear positioning is closer in cell types in lymphoma associated with t(2;5) translocations (Roix *et al.*, 2003).

Meaburn *et al.*, in 2009 studied the repositioning of genes in the nuclei that are involved in breast cancer. From 11 normal human breast and 14 invasive breast cancer tissue specimen, they identified eight genes (*HES5*, *ERBB2*, *MYC*, *FOSL2*, *HSP90AA1*, *AKT1*, *TGFB3*, and *CSF1R*) that have altered their position when they compared their spatial organisation in cancerous tissue and normal, suggesting that the changes are gene specific. It was shown this alteration did not reflect large scale changes in global genome organisation. The repositioning of these genes was not due to genome instability as the genes analysed in this study had no changes in copy number and are cancer type specific (Meaburn *et al.*, 2009). Furthermore, this study is supported by the results of Weich *et al*, 2005 who identified *BCL2* repositioning to the periphery in *BCL2* cervical squamous cell carcinomas but not in *BCL2* cervical squamous epithelial cells (Weich *et al*, 2005).

In breast cancer, the position of a specific gene, HES5, a transcriptional repressor that regulates cell differentiation, could distinguish between a cancerous tissue and a healthy one with almost 100 percent accuracy. Alteration or repositioning of this gene has been associated with tumourgenesis and was identified in several types of breast cancer. This could prove a useful diagnostic tool (Meaburn *et al.*, 2009; Cukierski *et al.*, 2012). The gene repositioning occurs at an early stage of tumourgenesis, however, the gene repositioning did not affect transcription levels (Meaburn and Misteli 2008). Chromatin rearrangement is pivotal mechanism for the control of transcription (Kosak *et al.*, 2002). These finding was observed when Ig heavy (H) and Igκ loci were preferentially positioned at the nuclear periphery in hematopoietic progenitors and pro-T cells but are centrally located in pro-B nuclei. To find out the relationship between position and transcription, the inactive loci at the periphery do not associate with centromeric heterochromatin and their localisation away from the nuclear periphery in pro-B cells, suggested that the IgH locus appears to influence by other factors

such as tissue type. They suggest that this positioning is regulated by transcription and recombination of IgH and Igκ loci during lymphocyte development (Kosak *et al.*, 2002). In addition to gene repositioning in cancer, it was also observed during viral infection.

Therefore, changes in the radial position of specific gene loci in cancer cells could contribute to tumourgenesis but further investigation is still needed. These observations strongly support the idea that the genomic regions influenced by states of gene activity and cell-type specific genome architecture, this can predispose translocations that are characteristic to specific cell types and cancers due to changes in gene expression placing genes in a new location.

1.3.7 Lamins and Cancers:

Alterations to the nuclear lamina are thought to be involved in malignant transformations and cancer processes because of their role as guardian of the genome, their role in regulating basic nuclear activities that are implicated in tumourgenesis, their interactions with cancer gene pathways and their role in chromosomal territories and reorganisation. Thus, lamin expression in cancer cells may serve as a biomarker for diagnosis, prognosis and surveillance. In small cell lung cancer cell lines, lamins A and C levels decreased whereas in non-small cell lung cancer cell lines were both clearly detected. However, levels of B-type lamins were equal in both cell lines (Kaufmann *et al.*, 1991; Broers *et al.*, 1993). Studies in gastrointestinal neoplasms have found reduced or no expression of lamins A and C, and B1 (Moss *et al.*, 1999; Willis *et al.*, 2008). Further, reduced or no expression of lamins A and C in basal cell carcinoma of the skin was found whereas B-type lamins were expressed in all these cells (Venables *et al.*, 2001). Evaluation of lamin expression in prostate cancer was also been assessed and reduced expression of lamins A, C, and B1 was found (Moss *et al.*, 1999). In contrast, there was a significant increase in lamin B1 and no change in lamins A and C in

prostate cancer, the increase in lamin B1 was strongly correlated with the prognostic status of the patient (Coradeghini *et al.*, 2006). In cervical and utrine cancers, lamin A and C were not detected whereas lamins B1 expression was reduced (Moss *et al.*, 1999). There was increased expression of lamins A and C in ovarian cancer from 30 patients and 30 healthy individuals (Hudson *et al.*, 2007). In papillary thyroid carcinoma there was no change found in the expression or localisation of lamins A, C, and B1 or LAP2 and LBR (Fischer *et al.*, 2001). In breast cancer, there was reduced expression in lamins A, C, and B1 (Moss *et al.*, 1999). Moreover, the expression level of lamin B1 correlated positively with tumour stages, tumour sizes, and number of nodules and is clinically useful biomarker for early stages of hepatocellular carcinoma in tumour tissues and plasma (Sun *et al.*, 2010). In this thesis and for the first time, my work evaluated the expression of lamins and lamin binding proteins in normal breast (MCF-10A cell lines) and cancer (T-47D, BT-474, GI-101, and Sk-Br-3) cell lines. Differential aberrations in the presence and distribution of lamins A, C, B1, and B2, LAP2α, LAP2, MAN1, emerin, and LBR whereas, co-localisation was found between lamins and some nuclear proteins such as PML, nucleolin, LBR, and Ki67 (Chapter 4).

1.3.7.1 Lamins and Cancer Pathways:

Tumour suppressor genes such as pRB are in one of the pathways that are involved in the control of tumourgenesis. The nuclear lamins are involved in tumour suppressive pathways that trigger apoptosis or senescence and thus yielding an anti-cancerous effect through protecting this pathway. Lamin alterations allow cancer cells to change the normal control of cell proliferation and cell death yielding a pro-cancerous effect (Vogelstein and Kinzler, 2004). Moreover, the epigenetic silencing of the lamins A and C gene by hypermethylation to CpG island promoter is responsible for the loss of expression of A-type lamins in leukemia and lymphoma (Agrelo *et al.*, 2005). LAP2α and A-type lamins both participate in pRB

dependent E2F repression and loss of LAP2α or A-type lamins in fibroblasts results in accelerated S-phase entry, through loss of pRB activity. Lamin A causes up-regulation of T-plastin which can lead to down regulation of E-cadherin. Plastins are a family of actin binding proteins that implicated in invasion and metastasis (Foran *et al.*, 2006). This is confirmed by Willis *et al.*, in 2008 where they found that the expression of A-type lamins within a tumour was a highly significant risk indicator of tumour related mortality. Expression of lamin A in colorectal cancer (CRC) cell lines promoted invasiveness via up-regulated expression of the actin, which in turn gives rise to down-regulated expression of the cell adhesion molecule E-cadherin. They concluded that expression of A-type lamins in CRC promotes tumour invasiveness through reorganisation of the actin cytoskeleton (Willis *et al.*, 2008). Furthermore, pre-lamin A and mature lamin A have been implicated in regulation of cell cycle control and DNA repair mechanisms whereby they activate a proliferative arrest (Varela *et al.*, 2005; Shalev *et al.*, 2007). The Wnt/β-catenin signalling cascade has been implicated in multiple stages of hematopoietic development. It was proposed that Wnt signalling controls the auto-renewal of hematopoietic stem cells (HSCs). Aberrant of β–catenin signalling during thymocyte development has been found in various epithelial and haematological tumours due to aberrant thymocyte development and reduction in T-cell receptor β rearrangements (Guo *et al.*, 2007). Thus, alterations in composition of lamins within cell nuclei result in loss of proliferative control and hence may be involved in tumour progression (Venables *et al.*, 2001; Burke and Stewart, 2006). The interaction between lamins and chromatin is mediated through other proteins at the lamina, these include lamina associated polypeptide-2 (LAP-2), emerin and MAN1 (LEM), nesprin, barrier to autointegration factor 1 (BAF1) as well as lamin B receptor (LBR) (Morris, 2001; Ostlund and Worman, 2003). These interactions might be involved in heterochromatin formation at the nuclear periphery. Alterations of nuclear structure are often associated with altered

organisation or even loss of heterochromatin. Changes in the nuclear lamina and nuclear shape may affect chromatin territories organisation and gene position, respectively (Zink *et al.*, 2004). This may result in chromosome aberrations and alteration of gene expression and transformation of the cell nucleus.

Table 1.1: Different Nuclear Lamins Expression in Different Cancerous Tissues:

Tissue	**Lamins A/C**	**Lamins B**	**Reference**
(1) Lymphoma	No	Yes	Stadelmann *et al.*, 1990
(2) Non-Small cell Lung Cancer	Yes	Yes	Kaufmann *et al.*, 1991 and Broers *et al.*, 1993
(3) Small cell Lung Cancer	No	Yes	Kaufmann *et al.*, 1991 and Broers *et al.*, 1993
(4) Gastrointestinal Neoplasms	No	No	Moss *et al.*, 1999 Willis *et al.*, 2008
(5) Cervical Cancer	No	Yes	Moss *et al.*, 1999
(6) Skin Cancer	No	Yes	Venable *et al.*, 2001
(7) Prostate Cancer	No	Yes	Coradeghini *et al.*, 2006
(8) Ovarian Cancer	Yes	-*	Hudson *et al.*, 2007
(9) Colorectal Cancer	Yes	-*	Willis *et al.*, 2008
(10) Gastric Carcinoma	No	-*	Wu *et al.*, 2009
(11) Liver Cancer	-*	Yes	Sun *et al.*, 2010

-* = Nothing Mentioned

1.3.8 Changes in Nuclear Structure during Cancer:

The architecture of cell nuclei is often altered in cancerous cells. The types of cancers are associated with characteristic changes and these may provide an important diagnostic feature. Different studies showed that much debate about changes in the cell nucleus structure in cancer diagnosis. One of these changes was observed in nuclear structure variation. This was followed by cytoplasmic features of the cells. These include changes in nuclear lamina and nuclear matrix. These alterations can be characteristic of cancer type and its stage which is might be related to the altered functions of cancer cells (Zink *et al.*, 2004).

1.3.9 The Nuclear Matrix and Cancer:

Since the nuclear matrix is the dynamic scaffold through which cell nucleus is structurally and functionally organised, some of nuclear matrix proteins (NMPs) are restricted to cancer cells or to stages of cancer progression. The NMPs are tissue and cell type specific as well as reflecting the state of cell differentiation and transformation. They can serve as biomarkers for malignant phenotype (Getzenberg *et al.*, 1996). These proteins are tumour specific, for example PC-1, which is only active in the nuclear matrix of prostate cancerous cells and inactivated in normal and benign prostate tissues (Partin *et al.*, 1997). The relationship between NMPs activity and cancer progression has also been reported in acute promyelocytic leukaemia (APL) due to chromosomal translocation (Sukhai *et al.*, 2004) and bladder cancer (van Le *et al.*, 2004). Moreover, NMP66 and c-erbB2 promotors bind nuclear matrix protein present in human breast cancer cells and they induce mitogenesis (Ya-Ian *et al.*, 2005). A number of studies showed that the nuclear matrix interacts with steroid receptors to help modulate cell function (Khanuja *et al.*, 1993). Therefore, the changes in nuclear matrix in cancer cells may affect DNA organisation, DNA replication, gene expression and other nuclear functions.

1.3.10 The Nuclear Lamina and Cancer:

The nuclear lamina is thought to be a principal determinant of the nuclear shape. The nuclear lamina and nucleoplasmic lamin networks are organised in an interconnected domains. These domains have different functions in chromatin organisation and gene regulation (Goldman *et al.*, 2002). The nuclei of most normal cells have regular shape, structures and functions but an irregular nuclear shape, structures and functions are observed in cancerous cells (Zink *et al.*, 2004). These changes are characteristics of different cancer types. For examples, small-cell lung carcinomas lack the proteins of lamin A whereas lamin B expression varied between normal and cancer cells (Broers *et al.*, 1993) and lack of expression of A-type lamins in lymphoma and leukemia has been reported, demonstrating that the *lamin A/C* gene encoding these central components of the nuclear lamin undergoes transcriptional silencing by promoter CpG island hypermethylation (Agrelo *et al.*, 2005). However, lamin A/C proteins are positively involved in malignant behaviour of prostate cancer cells through the PI3K/AKT/PTEN pathway. Lamin A/C may represent a new oncogenic factor and a novel therapeutic target for prostate cancer (Kong *et al.*, 2012). Therefore this may contribute to the structural changes observed in the lamina. However, changes in lamina proteins or nuclear shape, involved in the process of cancer transformation, remain unclear. Chromatin is attached to the inner surface of the nuclear lamina (perinuclear heterochromatin) and the lamina may be involved in transcriptional repression of gene loci (Brown *et al.*, 1997).

1.4 Conclusion:

The nuclear envelope and integral nuclear membrane proteins, A-type and B-type lamins and their binding proteins have a role in maintaining the correct functions of the nucleus and genome organisation. Diseases and cancers can arise from aberrant forms of these proteins and changes in protein amount. Since the genome organisation is disorganised in a number of diseases and cancers, it is important to understand how, why and when this occurs and whether it has an effect on gene expression. My thesis addresses nuclear structure and genome organisation in breast cancer which have revealed interesting findings. Clearly, there is a need for better understanding, diagnosis and treatment of this cancer and how it is important to understand the nucleus for disease control. Understanding the progress would enable the designing of novel therapies and avoid side effects. In addition, the study will help in the fundamental understanding of this cancer.

Chapter 2: Chromosome Territories and Gene Position in Human Breast Cancer Cell Nuclei

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2.1 Introduction:

The spatial organisation of the genome alters during physiological processes such as differentiation and development (Foster and Bridger, 2005). In addition, large scale alterations to spatial organisation of the genome also occur in cancer conditions (Zink *et al.*, 2004; Meaburn *et al.*, 2009) and between quiescence and proliferation (Mehta *et al.*, 2010a).

2.1.1 The Role of Nuclear Envelope (NE) in Genome Organisation, Gene Expression and Genome Stability:

Nuclear architecture is important, since it is capable of maintaining genome organisation, stability, and regulating gene expression. Numerous genes relocate from the nuclear periphery to the nuclear interior upon activation and are hypothesised to interact with pre-assembled sites of transcription (Brown *et al.*, 1997). In contrast to the nuclear interior, the nuclear periphery is considered as less transcriptionally active (Shaklai *et al.*, 2007; Van de Vosse *et al.*, 2011). This is reflected by the preferential association of heterochromatin and gene poor areas of the genome with the NE (Guelan *et al.*, 2008). However, some activated genes in yeast are recruited to the nuclear periphery through interactions with nuclear pore complexes (NPCs), and NPC proteins are capable of preventing the spread of silent chromatin into regions of active chromatin, leading to the idea that NPCs may facilitate the transition of chromatin between transcriptional sites. Thus, the NE might be considered as a discontinuous platform that promotes both gene activation and repression (Van de Vosse *et al.*, 2011). Indeed, some human genes when targeted to the NE still transcribe (Finlan *et al.*, 2008). Many diseases are frequently associated with alterations in the NE. Therefore, the effects of the NE and its effects on chromatin organisation, gene expression, and stability could be the causative agents for many cancers and other diseases such as laminopathies, where the nuclear lamina is aberrantly affected by mutant lamina proteins. The nuclear

lamina is thought to provide an anchoring site for interphase chromosomes and have a role in gene regulation and expression. Recent genome mapping studies and functional experimental data strongly support these roles for the nuclear lamina (Peric-Hupkes and van Steensel, 2010).

2.1.2 The Relationship between Chromosome Territories (CTs), Transcription Sites, and Gene Expression:

There is a functional link between chromatin structure at the level of the nucleosome and gene expression (Jenuwein and Allis, 2001). However, the functional significance of higher-order chromatin structures in transcription remains unclear. Hence, it has been proposed that transcription and RNA processing might occur in a space between territories (interchromosome domain, ICD) compartment in which transcription, RNA processing, RNA transport and splicing of mRNA occur (Verschure *et al.*, 1999; Cremer and Cremer, 2001). In support of this model, specific gene transcripts and components of the splicing machinery have been reported to be found at the border of chromosome territories (CTs) (Zirbel *et al.*, 1993). If transcription occurs close to the surface of chromosome territories, genes should preferentially be found there, and non-coding sequences should be more internal. Kurz *et al.*, 1996, found that coding DNA is found preferentially at the periphery of chromosome territories, whereas a non-coding genomic locus was found predominantly in the interior of the territory. It has been shown that the gene-rich major histocompatibility complex (MHC) lies on large chromatin loops that extend away from the surface of chromosome 6 territory when activated (Volpi *et al.*, 2000). Therefore, most active genes lie on the surface of chromosome territories. However, both early and late replicating DNA that are usually equated with gene-rich and gene-poor domains, respectively, as well as RNA and splicing factors appear to be distributed throughout chromosome territories (Visser *et al.*, 1998).

Similarly, the most GC-rich fraction of the human genome (which has a high gene density) is also distributed throughout the territories (Tajbakhsh *et al.*, 2000). Chromatin fibres containing transcriptionally active DNA may be decondensed at the surface of these sub-domains or extended into the interchromatin spaces and newly synthesised RNA accumulates in the interchromatin spaces inside and around chromosome territories (Verschure *et al.*, 1999). However, it has been found that most expressed genes are indeed located at the surface of chromosome or on loops. It seems there is clear correlation between chromosome territory sub-domains and the expression of tissue specific genes.

As has been discussed in the Introduction; chromosomes and genes are non-randomly positioned in interphase nuclei (Croft *et al.*, 1999; Boyle *et al.*, 2001; Szczerbal *et al.*, 2009; Mehta *et al.*, 2010a). These positions can change in differentiation and disease leading to changes in gene expression (Bourne *et al.*, 2013).

The goal of this chapter is to assess the global spatial genome organisation within cancerous cells during interphase. This is done by determining nuclear positions of human chromosomes and genes. This work demonstrates that chromosomes (7, 10, 11, 14, and 17) and genes (*EGFR, PTEN*, *CCND1*, *AKT1*, *HSP90AA1*, and *ERBB2*/*HER2*) in normal breast (MCF-10A) and breast cancer (T-47D, GI-101, BT-474, and Sk-Br-3) cell lines are positioned non-randomly but in different locations to publish normal chromosome positions and the control cell line. Interestingly, my results showed that *HER2* gene position was altered in all the cancer cells studied which was similar to the work by Wiech *et al.*, in 2005 and Meaburn and Misteli, in 2008. They both found that *HER2* gene changes location in neoplastic breast epithelial cells (Wiech *et al.*, 2005). In addition, Meaburn and Misteli, in

2008, demonstrated that gene position (including *HER2*) had altered in the cancer cells (Meaburn and Misteli, 2008).

2.2 Material and Methods:

2.2.1 Cell Culture:

The five human breast cell lines either immortalised (MCF-10A) or transformed (T-47D, BT-474, Sk-Br-3, and GI-101) were obtained from Dr. Amanda Harvey at Brunel University, London. They were grown and cultured in medium (GIBCO) which is described in tables 1, 2, and 3, supplemented with 10 % fetal calf serum (FCS) and antibiotics at 50 mg⁄ml concentration (Stock: 100X: 10,000 u/ml penicillin, 10,000 µg streptomycin, and 29,2 mg/ml L-Glutamine in a 10 mM citrate buffer (for pH stability)) and maintained in a humidified environment at 37ºC with 5% CO_2. The cells were passaged therize time weekly and the normal cells were passaged twice weekly. For passaging, cells were washed twice with 1X Dulbecco's Phosphate Buffer Saline (DPBS). Cells were harvested by incubation in 0.5% Trypsin-ethylenediaminetetraacetic acid (1X EDTA) phenol red (Ivitrogen) for 3-10 minutes in a humidified environment at 37ºC with 5% CO_2. The trypsin was neutralised by the addition of equal volume of fresh medium. The cellular suspension was distributed into flasks. For indirect immunofluorescence, cellular suspensions were prepared and cells were seeded in 13 mm glass coverslips on plastic petri dishes. The table below summarises the general characteristics and properties of the different cell lines. The cell lines were not tested for Mycoplasm infection as this organism can modify the cell physiology and no bacterial or fungal contamination was observed during this study. This test is essential for quality control in tissue culture laboratory every three month. The cell lines were selected for this study on the basis of several criteria as described below:

(1) MCF-10A cell line:

The human mammary epithelial cell line MCF-10A is non-tumorgenic and immortalised. It is derived from human fibrocystic mammary tissue. This cell line can be engineered using molecular manipulation techniques to allow future studies of malignent progression changes and mechanisms especially signal transduction pathways such as Ras pathway to increase malignancy. These cells are "normal" breast epithelial cells as they are relatively stable and have nearly diploid karyotype (Data not Shown) and are dependent on different growth factors for proliferation. MCF-10A cell line is an excellent model system for understanding epithelial cell biology. The cells were grown in **D**ulbecco's **M**odified **E**agles **M**edium (DMEM/Ham's F12 ((+) L-Glutamine, GIBCO) and supplemented with 10% fetal calf serum (FCS), 5 ml PSQ antibiotics at 50 mg⁄ml concentration (Stock: 100X: 10,000 u/ml penicillin, 10,000 µg streptomycin, and 29,2 mg/ml L-Glutamine in a 10 mM citrate buffer (for pH stability)) and 1.25 ml (µg/ml) Insulin, 500 µl (1 µg/ml) Hydrocortisone (HC) and 20 µl (10 µg/ml) Epithelial Growth Factor (EGF). This cell line was sub-cultured and was split 1:2. Cells were grown on 75cm^2 flasks.

(2) T-47D cell line:

The human ductal breast epithelial tumour cell line T-47D is derived from an invasive ductal carcinoma cell line. The cells were grown in RPMI 1640 medium, developed in 1966 by Moore *et al.* as a modification of McCoy's 5A medium at **R**oswell **P**ark **M**emorial **I**nstitute (www.atlantabio.com), ((-) L-Glutamine, GIBCO) and supplemented with 10% fetal bovine serum (FBS) and antibiotics 5 ml PSQ antibiotics at 50 mg⁄ml concentration (Stock: 100X: 10,000 u/ml penicillin, 10,000 µg streptomycin, and 29,2 mg/ml L-Glutamine in a 10 mM citrate buffer (for pH stability)). This cell line was sub-cultured and was split 1:4. Cells were grown on 25cm^2 flasks.

(3) GI-101 cell line:

The human breast carcinoma cell line GI-101 is derived from a metastasic ductal adenocarcinoma cell line. The cells were grown in RPMI 1640 medium ((-) L-Glutamine, GIBCO) and supplemented with 10% fetal bovine serum (FBS), some additives 625 µl (µg/ml) Insulin, and antibiotics 5 ml PSQ antibiotics at 50 mg⁄ml concentration (Stock: 100X: 10,000 u/ml penicillin, 10,000 µg streptomycin, and 29,2 mg/ml L-Glutamine in a 10 mM citrate buffer (for pH stability)). This cell line was sub-cultured and was split 1:4. Cells were grown on 75cm^2 flasks.

(4) Sk-Br-3 cell line:

The human breast adenocarcinoma carcinoma cell line Sk-Br-3 is a poorly differentiated adenocarcinoma and hypertriploid cell line. Its name came from the Memorial Sloan-Kettering Cancer Centre where it was isolated. The cells were grown in RPMI 1640 medium ((-) L-Glutamine, GIBCO) and supplemented with 10% fetal bovine serum (FBS) and antibiotics 5 ml PSQ antibiotics at 50 mg⁄ml concentration (Stock: 100X: 10,000 u/ml penicillin, 10,000 µg streptomycin, and 29,2 mg/ml L-Glutamine in a 10 mM citrate buffer (for pH stability)). This cell line was sub-cultured and was split 1:4. Cells were grown on 25cm^2 flasks.

(5) BT-474 cell line:

The human ductal breast carcinoma cell line BT-474 is derived from a solid, invasive ductal carcinoma cell line. The cells were grown in RPMI 1640 medium ((-) L-Glutamine, GIBCO) and supplemented with 10% fetal calf serum (FCS) and antibiotics 5 ml PSQ antibiotics at 50 mg⁄ml concentration (Stock: 100X: 10,000 u/ml penicillin, 10,000 µg streptomycin, and 29,2

mg/ml L-Glutamine in a 10 mM citrate buffer (for pH stability)). This cell line was sub-cultured and were split 1:4. Cells were grown on 75cm^2 flasks.

2.2.1.1 Tables 2.1-2.3 Display the Characteristics of Cell Lines:

Table 2.1: Characteristics of Cell Lines I:

	T-47D Cell Line**	MCF-10A Cell Line***
Species	Human, Female (54 Years)	Human, Female (36 Years)
Morphology	Epithelial Cells	Epithelial Cells
Tissue	Breast	Breast
Tumour	Invasive Ductal Carcinoma	Non-tumourgenic, Immortalised (Normal) and arose spontaneously in culture
Culture Medium	RPMI 1640 media, 10% FBS, and 5 ml PSQ*	DMEM/Ham's F12 media, 10% FCS, 5 ml PSQ*, and (Insulin, Hydrocortisone and Epithelial Growth Factor)
Split Ratio	1:4	1:2
Properties	$Tp53^{+}$, PR^{+}, ER^{+}, and $Her2^{+}$. Model of Apoptosis and Drug Resistant**.	Nearly Diploid, and Adherent in growth. Responds to insulin, HC, and EGF. $Cialmucin^{+}$, ER^{-}, PR^{-} and normal expression to $p53^{+}$ and normal $Her2^{+}$ expression***

*PSQ antibiotics at 50 mg⁄ml concentration (Stock: 100X: 10,000 u/ml penicillin, 10,000 µg streptomycin, and 29,2 mg/ml L-Glutamine in a 10 mM citrate buffer (for pH stability)).

**(http://icbp.lbl.gov/breastcancer/list_data.php?id=1, July 2009).

***(http://icbp.lbl.gov/breastcancer/viewline.php?id=37, July 2009).

Table 2.2: Characteristics of Cell Lines II:

	GI-101 Cell Line**	Sk-Br-3 Cell Line***
Species	Human, Female (57 Years) Passed Through a Mouse	Human, Female (43 Years)
Morphology	Epithelial Cells	Epithelial Cells
Tissue	Breast	Breast
Tumour	Metastasis Ductal Adenocarcinoma	Poorly Differentiated Adenocarcinoma
Culture Medium	RPMI 1640 media, 10% FCS, 5 ml PSQ*, and Insulin	RPMI 1640 media, 10% FCS, and 5 ml PSQ*
Split Ratio	1:4	1:4
Properties	ER^-, Does not respond to Estradiol, Model for Metastases mechanisms and Therapy**.	Hypertriploid cells Overexpression of Fatty Acid Synthase (FAS) and Overexpresses Her2/c-erb-2 gene Product, $p53^-$, PR^-, ER^+, and $Her2^+$. Model for FAS mechanism***.

*PSQ antibiotics at 50 mg⁄ml concentration (Stock: 100X: 10,000 u/ml penicillin, 10,000 µg streptomycin, and 29,2 mg/ml L-Glutamine in a 10 mM citrate buffer (for pH stability)).

**(http://www.freepatentsonline.com/5693533.html, July 2009).

***(http://icbp.lbl.gov/breastcancer/viewline.php?id=53, July 2009).

Table 2.3: Characteristics of Cell Lines III:

	BT-474 Cell Line**
Species	Human, Female (60 Years)
Morphology	Epithelial Cells
Tissue	Breast
Tumour	Solid, Invasive Ductal Carcinoma
Culture Medium	RPMI 1640 media, 10% FCS, and 5 ml PSQ*
Split Ratio	1:4
Properties	Adherent Patches of cells, Form Nodules, Aneuploid and some Chromosomes are absent (11, 13 and 22)**. $Tp53^{+}$, PR^{-}, ER^{-}, and $Her2^{+}$.

*PSQ antibiotics at 50 mg⁄ml concentration (Stock: 100X: 10,000 u/ml penicillin, 10,000 µg streptomycin, and 29,2 mg/ml L-Glutamine in a 10 mM citrate buffer (for pH stability)).

**(http://icbp.lbl.gov/breastcancer/viewline.php?id=6, July 2009).

2.2.2 Bacterial Artificial Chromosomes (BACs):

Bacterial Artificial Chromosomes (BACs) were selected using The University of California Santa Cruz (UCSC) Genome Browser website and obtained from Invitrogen. Different BACs were stored at -80ºC.

2.2.2.1 BAC Preparation:

BACs containing *EGFR*, *CCND1*, *PTEN*, *AKT1*, *HSP90AA1* and *ERRBB2*/*HER2* genes, were streaked on a Luria-Bertani (LB) agar plate (1% NaCl, 1% Tryptone, 0.5% Yeast Extract, 1.5% Agar Technical, and 12.5% Chloramphinicol). Each plate was incubated at 37ºC overnight. Single colonies from a plate was used to inoculate 10 ml of LB broth (1% NaCl, 1% Bactotryptone, 0.5% Yeast Extract and 12.5% Chloramphinicol) and then incubated at 37ºC overnight with shaking. In order to make glycerol stocks of BACs clones, 0.5 ml of bacterial culture was placed in specific tubes (used for low temperature) with 0.5 ml glycerol. They were mixed and stored at -80ºC.

2.2.2.2 BACs DNA Extraction:

The rest of culture (9.5 ml) was added to 90 ml LB broth medium and incubated at 37ºC overnight. 100 ml suspension was centrifuged at 1300 rpm for 10 minutes. The supernatant was discarded and the pellet was resuspended in 6 ml P1 solution using vortex (P1=15 mM Tris (pH8) (Fisher Scientific), 10 mM EDTA (Fisher Scientific), and 100 µg/ml RNase A (Sigma-Aldrich)). 6 ml P2 solution was added to this mixture (P2= 0.2 M NaOH (BDH, AnalaR) and 1% SDS (Fisher Scientific)). The suspension containing the DNA was incubated at RT for 5 minutes and followed by addition of 6 ml P3 solution drop by drop with gentle mixing (P3= 3 M CH_3COOK, (Sigma-Aldrich)). This suspension was incubated on ice for 10 minutes followed by centrifuge at 9,500 rpm for 10 minutes at 4ºC. The supernatant that

contained the DNA was transferred to another tube and 16 ml of ice-cold isopropanol was added. These tubes were incubated at -20ºC overnight to allow DNA precipitation. After overnight incubation, these tubes were centrifuged at 9,500 rpm for 15 minutes at 4ºC. The supernatant was discarded and the pellet was resuspended with 10 ml ice-cold 70% ethanol. Again this was centrifuged at 9,500 rpm for 5 minutes at 4ºC. The supernatant was discarded again and the pellet were left to dry at RT. 2 ml of ddH_2O was added to the pellet DNA and then transferred to small eppendroff tubes to be stored at -20ºC.

2.2.2.3 Nick Translation for Labelling the Genes:

This is a labeling technique for DNA labeling of FISH probes. This allows the incorporation of a modified nucleotide conjugated with either biotin, Biotin-16-dUTP, (Invitrogen, red) or digoxigenin, Digoxigenin-11-dUTP, (Roche, green).

Table 2.4: The Concentration of Each BAC Clone and Its Location on Human Chromosomes: The concentration for each gene was measured in ng per 1 µl using nano-drop machine:

Genes	HSAs	BACs ID	Concentration (ng/1 µl)
(1) *ERRBB2*/*HER2*	17q12	CTD-2251J22	78.1
(2) *CCND1*	11q13.3	RP11-266K14	104.7
(3) *EGFR*	7p11.2	CTD-2151I21	321.3
(4) *AKT1*	14q32.33	CTD-3022N7	78.7
(5) *PTEN*	10q23.31	CTD-2553L21	2993.6
(6) *HSP90AA1*	14q32.31	RP11-367F11	3984

2.2.2.3.1 Biotin Labeling: for all six genes and five chromosomes, biotin labeling by nick translation was performed using BioNick^{TM} Labeling System (Invitrogen) and the method as followed: firstly, these concentrations should be transferred into µg to know how many µls that we need (for example 78.1 ng/1 µl = 0.0781 µg/1 µl or 1 µg/X µl and X is the volume of probe). Then, 5 µl 10X dNTP Mix was added to (1) 15 µl, (2) 12 µl, (3) 5 µl, (4) 15 µl, (5) 1 µl and (6) 1 µl DNA from these BACs, respectively then followed by addition of 25 µl, 28 µl, 35 µl, 25 µl, 39 µl and 39 µl ddH_2O, respectively to make it 45µl. 5µl 10X enzyme mix was added to this mixture and then incubated at 16ºC for 2 hours. These tubes were incubated at -20ºC untill the next use.

2.2.2.3.2 Digxogenin labeling: for *AKT1*, *HSP90AA1*, and *ERBB2*/*HER2* genes, digxogenin labeling was performed using the method as followed: 1 µg of template DNA was used. Then, 13 µl, 1 µl, and 13 µl (respectively) was added to 3 µl, 15 µl, and 3 µl ddH_2O to make it 16 µl. 4 µl of Dig-Nick Translation Mix (Roche) was added to the mixture to make it 20 µl. The mixture was then incubated at 15ºC for 90 minutes. The labeled genes were stored at -20ºC and were ready for the next use.

2.2.3 2-Dimensional Fluorescence *In Situ* Hybridization (2D-FISH):

2.2.3.1 Harvesting Cells and Fixation (methanol: acetic acid, 3:1):

After the cells were harvested during cell culture, the cells were placed in an equal volume of medium and spun at 1000 rpm for 5 minutes in the bench centrifuge. The supernatant was removed and the pellet resuspended using hypotonic solution (0.075 M KCl) at room temperature (RT) for 15 minutes. The hypotonic solution was added drop wise with constant agitation. The cells were spun again at 800 rpm RT for 5 minutes in a bench centrifuge. The pellet was resuspended in the remaining supernatant. Then fixation was initiated by adding

methanol: acetic acid, 3:1 drop wise with gentle shaking by hand. The cells with fixative solution were placed on ice for 1 hour and spun at 800 rpm for 5 minutes. The pellet was resuspended again with methanol: acetic acid (3:1) and spun again at 800 rpm for 5 minutes. The fixation procedure was repeated at least 6 times. The cells were checked with light microscopy to determine when the nuclei were free of cytoplasm.

2.2.3.2 Degenerated Oligonucleotide Primer-Polymerase Chain Reaction (DOP-PCR):

2.2.3.2.1 Whole Chromosome Paints Template DNA without Labelling and Labelled with Biotin using DOP-PCR:

The protocol for Degenerated Oligonucleotide Primer-Polymerase Chain Reaction (DOP-PCR) was followed to generate two different rounds of amplifications: the first, the primary amplification products without labeling of different chromosomes 7, 10, 11, 14, and 17. The second, the secondary amplification products were labeled with 20 µl biotin-16-dUTP (1 mM, Roche) was also performed.

Table 2.5: The Master Mix Components for DOP-PCR: This technique was performed on PCR master mix using the DOP primer as follows:

	Without Biotin	**With Biotin**
10X DOP PCR Buffer	10 µl	10 µl
dACGTP (2µM)	10 µl	10 µl
dTTP (2µM)	10 µl	4 µl
DOP-PCR Primer (20 µM)	10 µl	10 µl
Sterilised Water	57 µl	35 µl
DNA Template	2 µl	10 µl
Biotin-16-dUTP (1 mM)	-	20 µl
Taq-KAPA HIFI	1 µl	1 µl
Total	**100 µl**	**100 µl**

Table 2.6: The Cycling Conditions for DOP-PCR Programme: The PCR was performed on a thermocycler machine. The two rounds of amplification were performed. Cycling conditions for DOP-PCR programme were as follows:

Phase	**Step (Cycle)**	**Temperature (°C)**	**Time (Minutes)**
Initial Denaturation	1	94	3
Denaturation	30	94	1
Annealing		62	1
Extension		68	1.5
Final Extension	1	68	8
For Collection		4	∞

2.2.3.2.2 Confirmation of Amplified DNA Products by Agarose Gel Electrophoresis:

Agarose gel electrophoresis was performed using 1% agarose gel (1 g agarose in 100 ml 1X TBE, **T**ris, **B**oric Acid, and **E**DTA (Appendices)) to check that the DNA amplification had occurred. After heating the agarose, 2 µl ethidium bromide (10 mg/ml) was added to the warm gel. The gel was poured into the gel tank with comb and left to set. Then 1X TBE was poured into the tank to cover the gel. The samples were prepared for loading as well as the control (sterilised water instead of DNA). Each sample contained 3 µl of the PCR products, 1 µl sterilised water, and 1 µl loading dye. The DNA ladder (marker, 3 µl) (1 Kb, 50 µg/ml, BioLabs) was loaded into the first lane on the gel. The gel electrophoresis apparatus was run at 80 volts for 40 minutes. Images were captured using the UV Transilliminator to visualise the products. The unlabelled and labeled DOP-PCR products were stored at -20ºC until use.

2.2.3.3 Probe Preparation:

The template probes labeled with biotin (HSAs 7, 10, 11, 14, and 17) were prepared and amplified using DOP-PCR technique and stored at -20ºC. In order to prepare probes for 2D-FISH, 8 µl of probe template or 25 µl of DNA template (200-400 µg of chromosome paint or gene, respectively) was added to 7 µl of C_0t-1 DNA (1 µg/1 µl), and 3 µl of salmon herring sperm DNA, in 1.5 ml eppendroff tube. $1/10^{th}$ of the volume of 3M Na Acetate and 2 volumes ice-cold 100% ethanol were added. This mixture was incubated at -80ºC for at least 30 minutes. After incubation, the probe was centrifuged at 13,000 rpm for 30 minutes at 4ºC. After centrifugation, the excess liquid was removed and the DNA pellet was washed with 200 µl of ice-cold 70% ethanol. This was centrifuged again at 13,000 rpm at 4ºC for 15 minutes and the excess liquid was removed carefully. The pellet was then dried at 37ºC on the hot block or oven until it became transparent. 12 µl of hybridisation buffer was added to the dried

pellet and allowed to dissolve for 2 hours at 50ºC with gentle tapping every 15 minutes or left overnight at RT.

2.2.3.4 Slide Cell Preparation:

In order to prepare slides, they were made damp or steamed. The cells in fixative solution were spun and resuspended in fresh methanol: acetic acid, 3:1 and one drop of cell suspension was placed onto a damp slide to spread the cells. These cells were checked with the light microscope and then incubated for 2 days at RT or 1 hour at 70ºC before denaturing.

2.2.3.5 Probe Denaturation:

The probes that were stored at -20ºC were spun and incubated at 75ºC for 10 minutes on a heat block. Then the probes were placed in a water-bath at 37ºC for 30 minutes - 2 hours for reannealing.

2.2.3.6 Slide Denaturation:

After ageing the slides, they were subjected to an ethanol row 70% ethanol, 90% ethanol and 100% ethanol for 5 minutes each. The slides were then dried on the hot block. After this dehydration, the slides were warmed in an oven at 70ºC for 5 minutes. Meanwhile, formamide solution (70% formamide in 2XSSC, pH 7.0) was placed in a water-bath and heated to 70ºC. The dried slides were placed in the formamide solution (70%) for 2 minutes at 70ºC. Then, slides were immediately placed in ethanol ice-cold 70% for 5 minutes, 90% and 100% ethanol solutions at RT for 5 minutes each to be ready for hybridisation with the probe.

2.2.3.7 Hybridisation:

After preparation and denaturation of probes and slides, hybridisation was performed by adding 10 µl of probe on the slide. This probe was covered with a warmed coverslip and sealed with rubber glue. The probe and the slide were allowed to hybridise in a humidified chamber for 2 days before washing.

2.2.3.8 Washing and Mounting on Slides for 2D-FISH:

Buffer A and B (appendices section) were pre-warmed at 45ºC and 60ºC, respectively. Then the slides were removed from the hybridisation chamber one by one. The rubber glue was removed carefully using forceps and the slides with coverslips were placed in wash buffer A (50% Formamide and 2X sodium saline citrate (SSC), pH 7.0) at 45ºC for 15 minutes with 3 changes of the buffer. Then buffer B (0.1X SSC, pH 7.0) pre-warmed at 60ºC was immediately transferred to water-bath at 45ºC and the slides washed for 15 minutes with 3 changes of buffer. The slides were placed immediately into 4X SSC at RT for 5-10 minutes to cool down. 100 µl of 4% bovine serum albumin (BSA) (Sigma-Aldrich) solution in 4XSSC was added onto the slides and covered with parafilm for blocking for 10 minutes at RT. 100 µl of Cyanine 3 (Cy3) conjugated streptavidin (Amersham Life Sciences) solution in 1% BSA in 4XSSC (1:200 dilution) was placed on the slide in order to detect the labeled probe.

For dual colour FISH, streptavidin-cyanine 3 was mixed with anti-dixogenin antibody (Roche) (diluted 1:50, in 1% BSA in 4XSSC). Then, the slides were incubated at 37ºC in a dark place (oven) for 30 minutes. The slides were washed in 4X SSC with 0.5% Tween-20 (Appendices) for 15 minutes with 3 changes at 42ºC. This was followed by washing the slides again in ddH_2O to remove the excess of 4X SSC-Tween-20. The slides were mounted

in counterstain, Vectashield medium (Vector Laboratories) with 4`, 6-Diamidino-2-Phenyindole (DAPI, 1.5 µg/ml) and covered by glass coverslips. Then mounted slides were visualised and images captured by fluorescence microscopy. DAPI excites at 360 nm and emits at 460 nm when bound to DNA producing a blue fluorescence. The slides were placed on a dark box at 4ºC.

2.2.3.9 Evaluation and Detection by Capturing Images using Microscopy:

Fluorescence microscopy was performed with a microscope (Olympus BX41 with Smartcapture3 Software). All slides were examined under 100X immersion oil (Immersol)™ objective lens and images of genes, chromosomes and both were captured with a camera and previewed on Macintosh Apple Computer (MAC) operated with SmartCapture3 software programme. The slides were stored in a black dark box at 4ºC.

2.2.3.10 Image Analysis for Chromosomes, Genes, and Both:

Images were randomly selected in IPLab Spectrum Software. To analyse chromosome and gene position using the erosion script analysis programme. This programme divides the nucleus (DAPI stain) into 5 shells of equal area. 1^{st} shell is peripheral and 5^{th} shell is interior (Figure, 2.1). The script determines the pixel intensity of DAPI, chromosomes and gene in these five shells. This intensity of the probe signal was normalised by dividing the percentage of probe signal by the percentage of DAPI signal in each shell. For at least 50 nuclei, the normalised intensity was calculated for the 5 shells, the standard error bars of the mean were measured and graphs made using Microsoft Excel software.

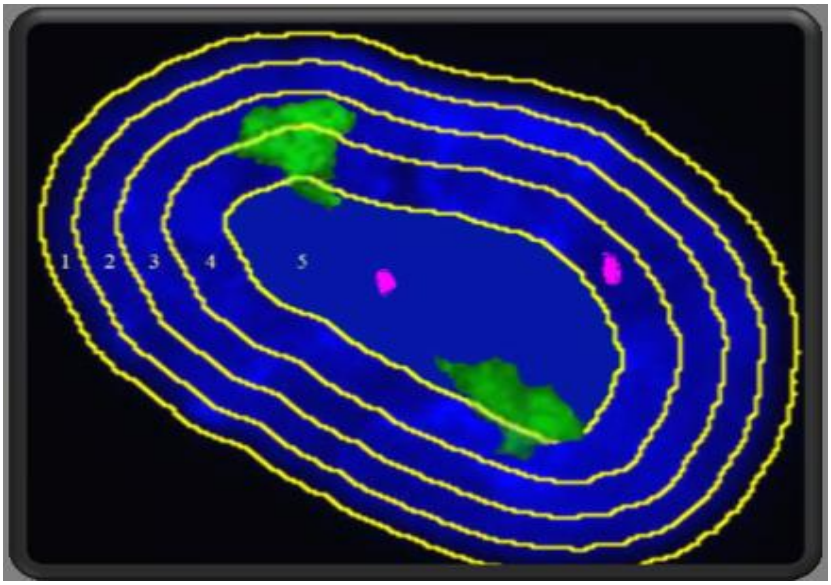


Figure 2.1: Erosion Script Analysis: The Erosion Script Analysis for Positioning of Chromosome Territories and Intensity Measurement
The cell nuclei were fixed with methanol: acetic acid (3:1) and 2D-FISH was performed for whole chromosome paints (Green) or gene signals (Red). Images were captured and analysed using erosion script (Croft *et al.*, 1999). The erosion script programme divides the nucleus into 5 shells of equal area, 1st shell is peripheral and 5th shell is interior. The script measures the signal intensities of probe and DNA in each shell. These probe measurements were normalised.

2.3 Results

From previous studies it is known that the genome is positioned non-randomly during interphase (Bridger and Bickmore, 1998), chromosomes are positioned according to their gene density (Croft *et al.*, 1999; Bridger *et al.*, 2000) or their size (Bolzer *et al.*, 2005).

In this chapter, the work investigated the spatial positioning of chromosomes 7, 10, 11, 14, and 17 in normal breast (MCF-10A) and breast cancer (T-47D, GI-101, Sk-Br-3, and BT-474) cell lines, as well as the positioning of specific genes of interest (*EGFR*, *PTEN*, *CCND1*, *AKT1*, *HSP90AA1*, and *ERBB2*/*HER2*). These chromosomes were chosen because they accommodate the genes of interest that are involved in breast cancer progression. These cell lines were chosen as a model system because they represent different stages of breast cancer. There are no previously published data about chromosome and gene positions in these cell lines.

To assess the positions of these chromosomes and genes in the breast cancer cell line, 2D-FISH was performed, digital images captured and analysed, the data analysed and displayed as histograms with error bars.

2.3.1 Chromosome Territories and Gene Loci in Breast Cancer Cells by 2D-Fluorescence *In Situ* Hybridisation (2D-FISH):

The localisation of human chromosomes within the different cell lines was assessed and representative images are shown in Figure 2.2. It can be seen that not all chromosomes have two chromosome territories many of them have more than one. Gene loci were delineated by

2D-FISH and specific BACs as probes and representative images are shown in Figure 2.3. Again in some cells there are more than the normal two gene loci.

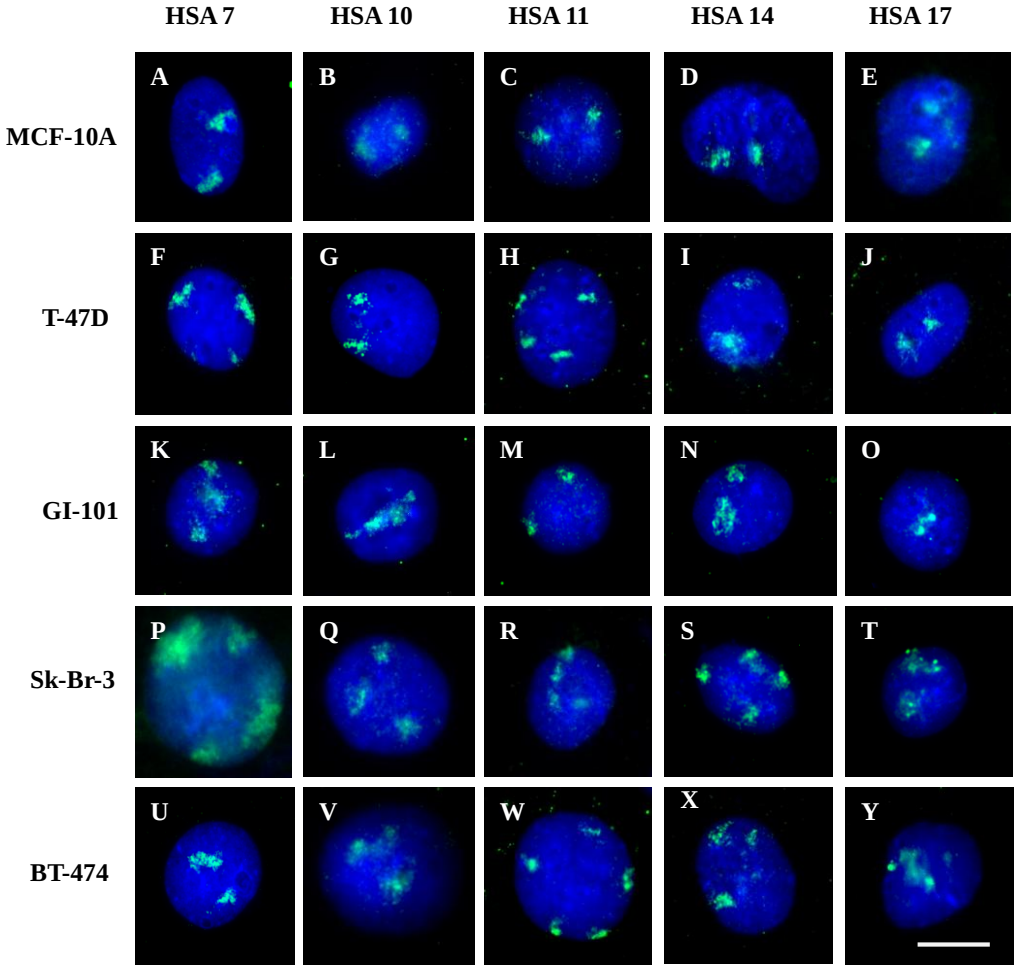


Figure 2.2: (A) Chromosomes Positioning: 2D-FISH was used to analyse the position of human chromosomes 7, 10, 11, 14, and 17 in normal breast (MCF-10A, A-E) and cancer (T-47D (F-J), GI-101 (K-O), Sk-Br-3 (P-T), and BT-474 (U-Y)) cell lines. DNA has counterstained using DAPI (Blue) whereas; chromosome signals can be seen in Red (Cy3-streptavidin) and then converted into green colour to facilitate their analysis. Magnification = X100; Scale Bar = 10µM.

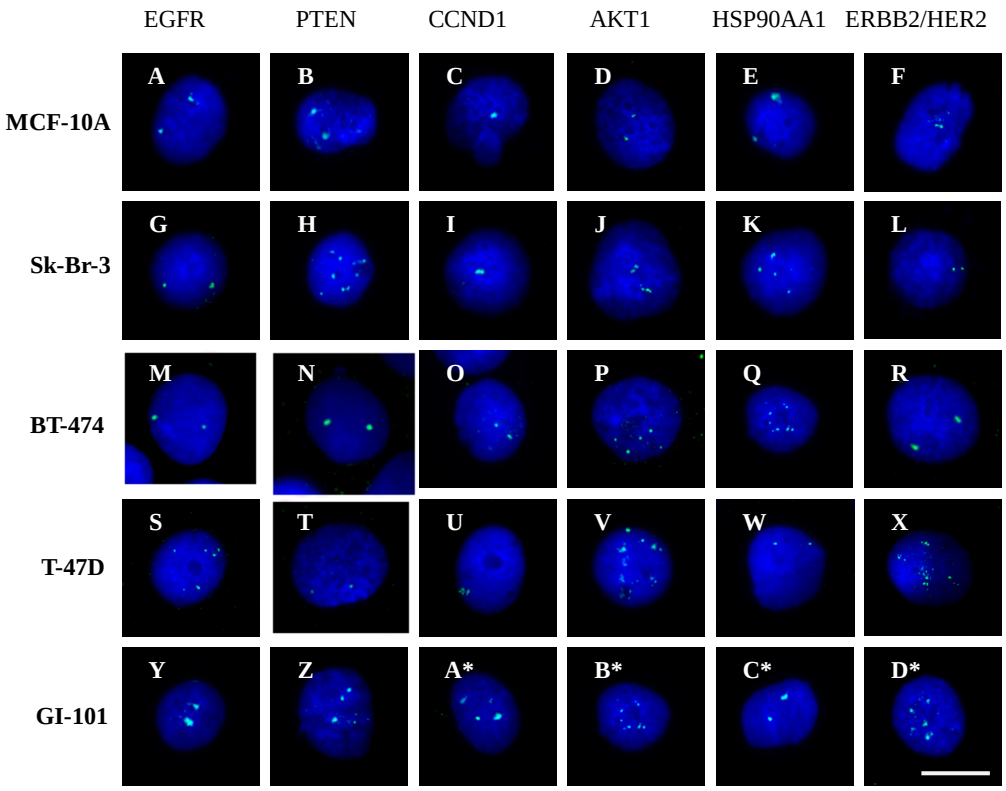
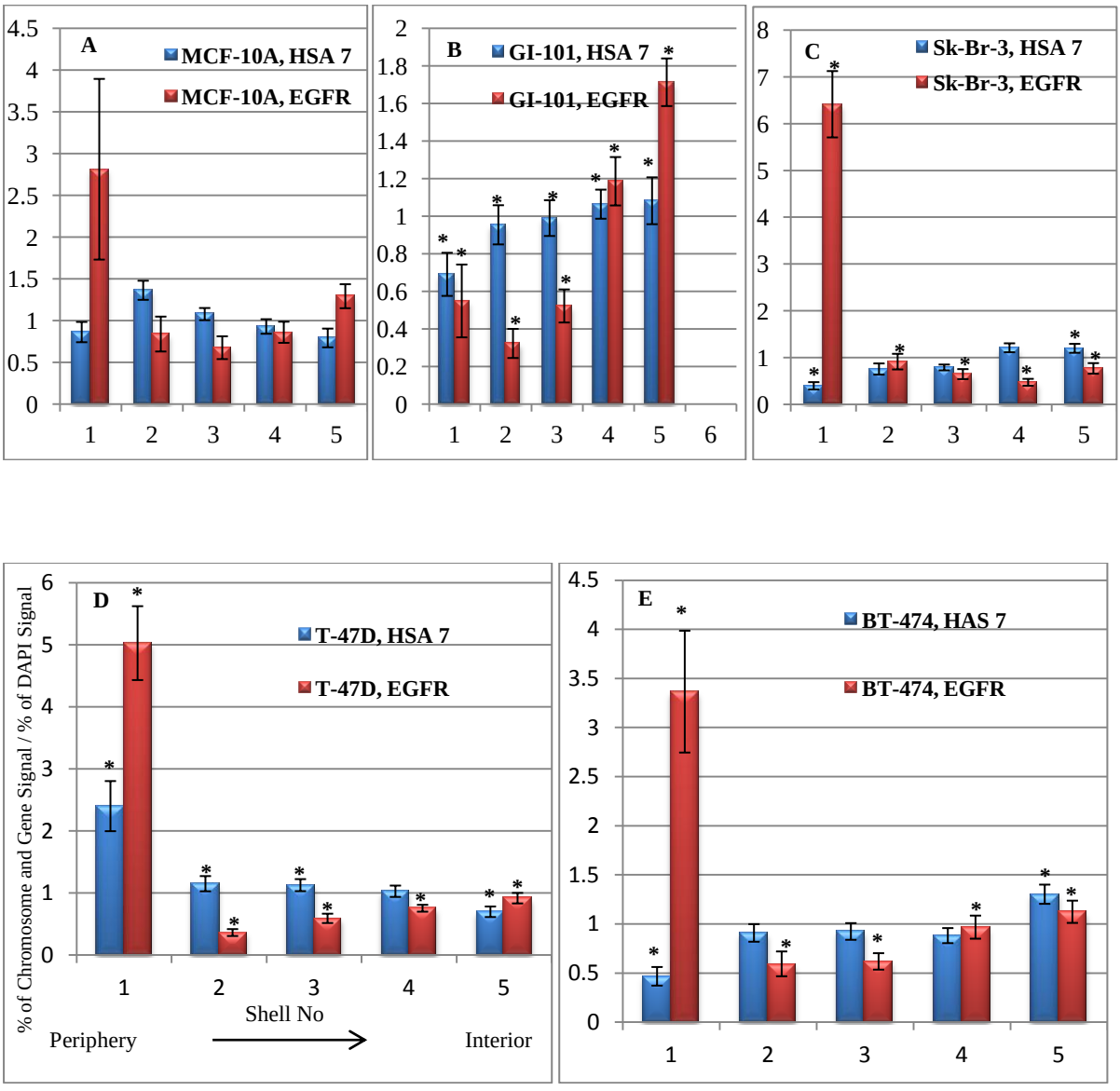
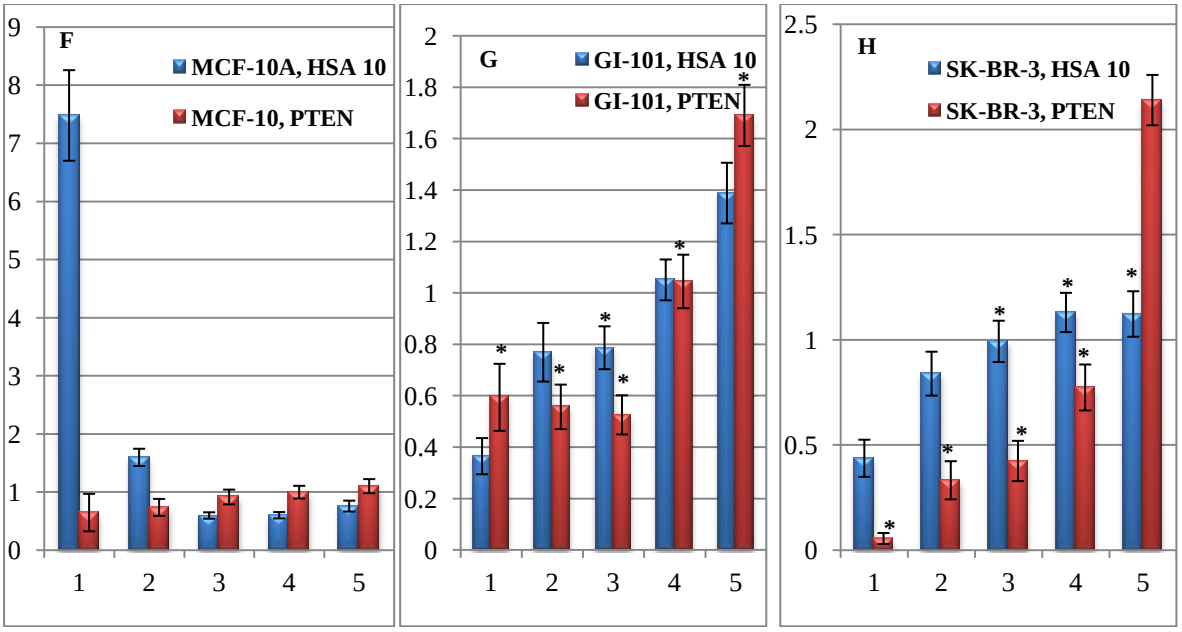


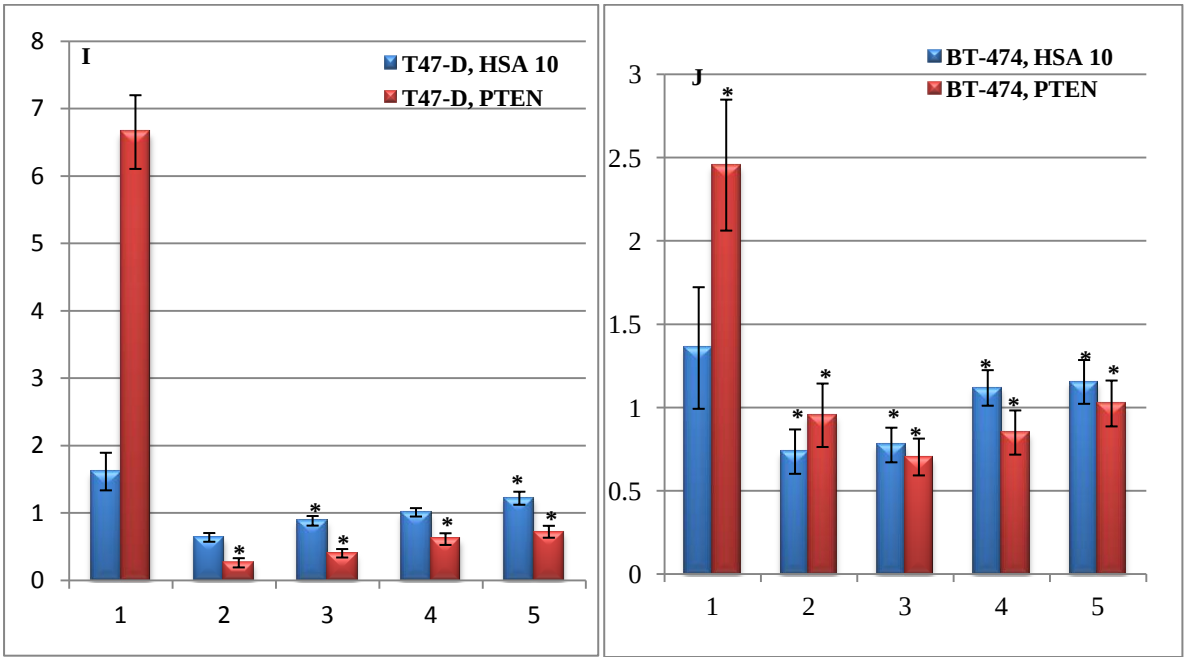
Figure 2.3: (B) Genes Positioning: Using 2D-FISH to analyse the positioning of specific genes *EGFR*, *PTEN*, *CCND1*, *AKT1*, *HSP90AA1*, and *ERBB2*/*HER2* of interest in breast normal MCF-10A (A-F) and cancer T-47D (S-X), GI-101 (Y-D*), Sk-Br-3 (G-L), and BT-474 (M-R) cell lines. DNA has been counterstained using DAPI (Blue) whereas; gene signals can be seen in Red (Cy3-streptavidin) and then converted into green colour to facilitate their analysis. Magnification = X100; Scale Bar = 10µM .

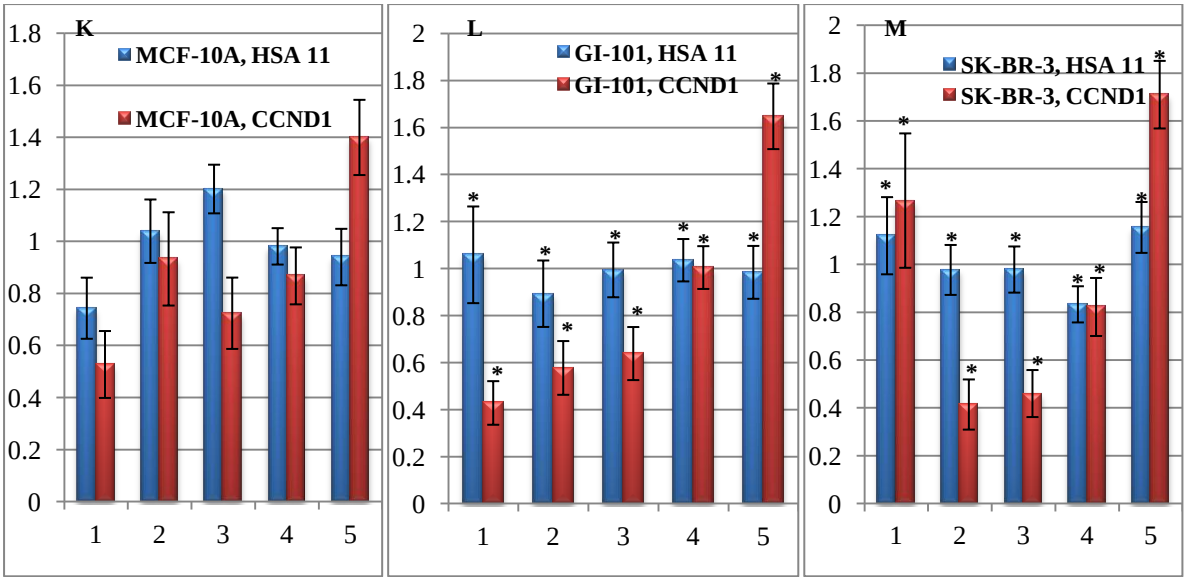
2.3.2 Position Analysis for both Chromosomes and Genes

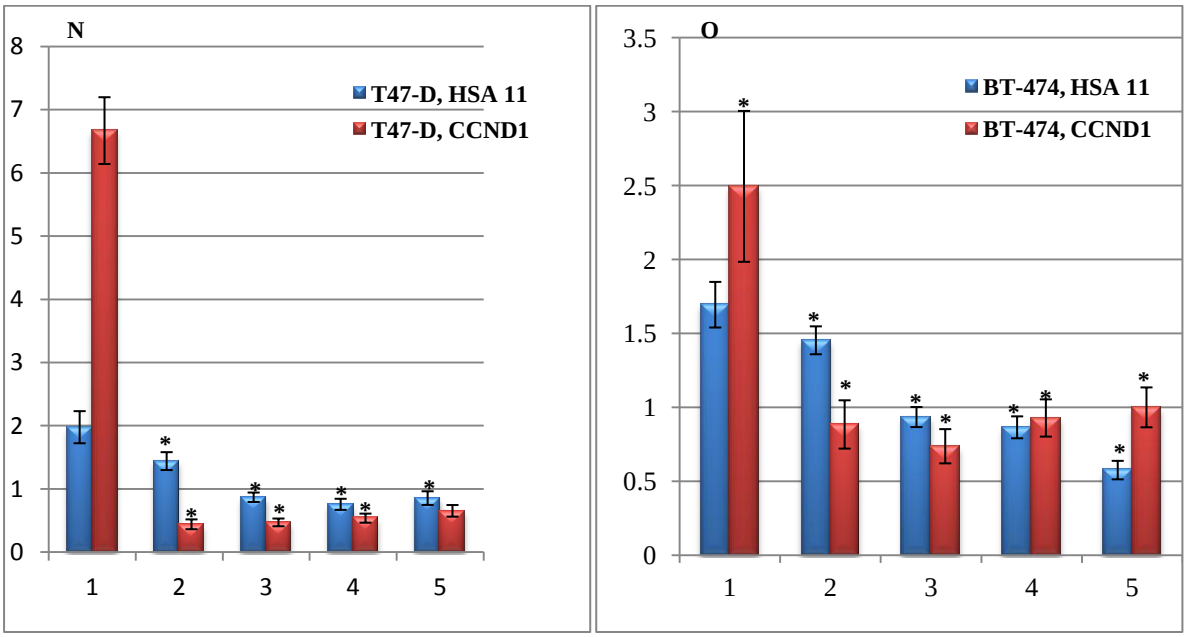
The images collected of the different chromosomes and gene loci were subjected to the erosion analysis script (Croft *et al.*, 1999) Histograms were plotted with individual chromosomes and the genes they host for all the different cell lines (Figure 2.4).

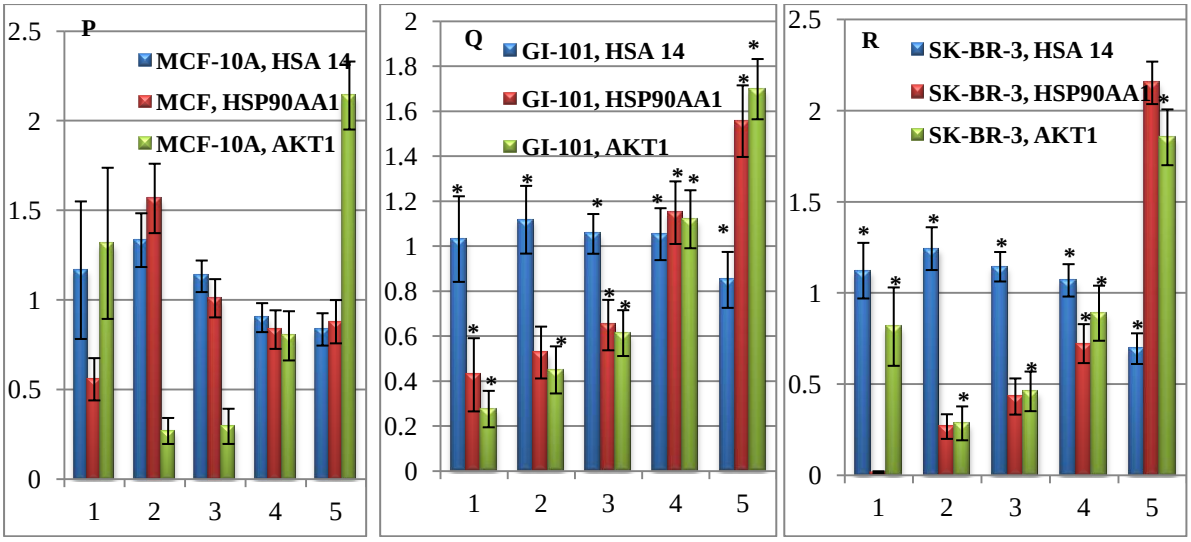


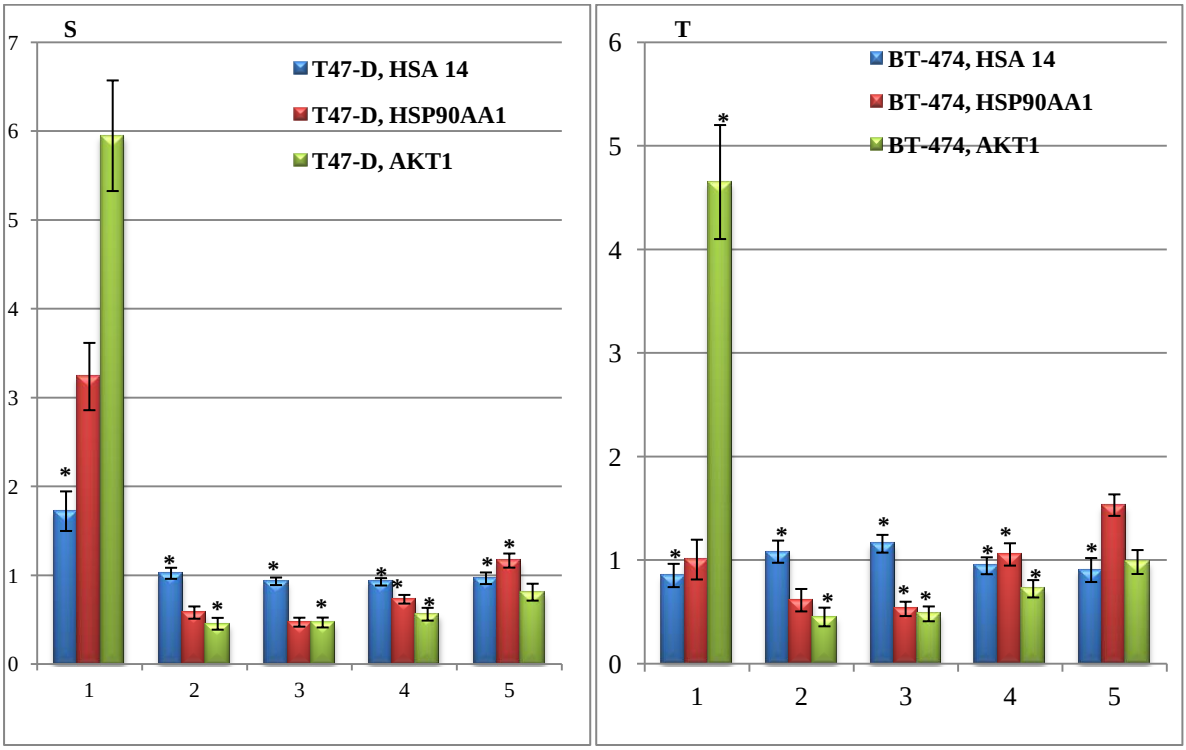

F
MCF-10A, HSA 10
MCF-10, PTEN
G
GI-101, HSA 10
GI-101, PTEN
H
SK-BR-3, HSA 10
SK-BR-3, PTEN


I
T47-D, HSA 10
T47-D, PTEN
J
BT-474, HSA 10
BT-474, PTEN


K
MCF-10A, HSA 11
MCF-10A, CCND1
L
GI-101, HSA 11
GI-101, CCND1
M
SK-BR-3, HSA 11
SK-BR-3, CCND1


N
T47-D, HSA 11
T47-D, CCND1
O
BT-474, HSA 11
BT-474, CCND1


P
MCF-10A, HSA 14
MCF, HSP90AA1
MCF-10A, AKT1
Q
GI-101, HSA 14
GI-101, HSP90AA1
GI-101, AKT1
R
SK-BR-3, HSA 14
SK-BR-3, HSP90AA1
SK-BR-3, AKT1


S
T47-D, HSA 14
T47-D, HSP90AA1
T47-D, AKT1
T
BT-474, HSA 14
BT-474, HSP90AA1
BT-474, AKT1

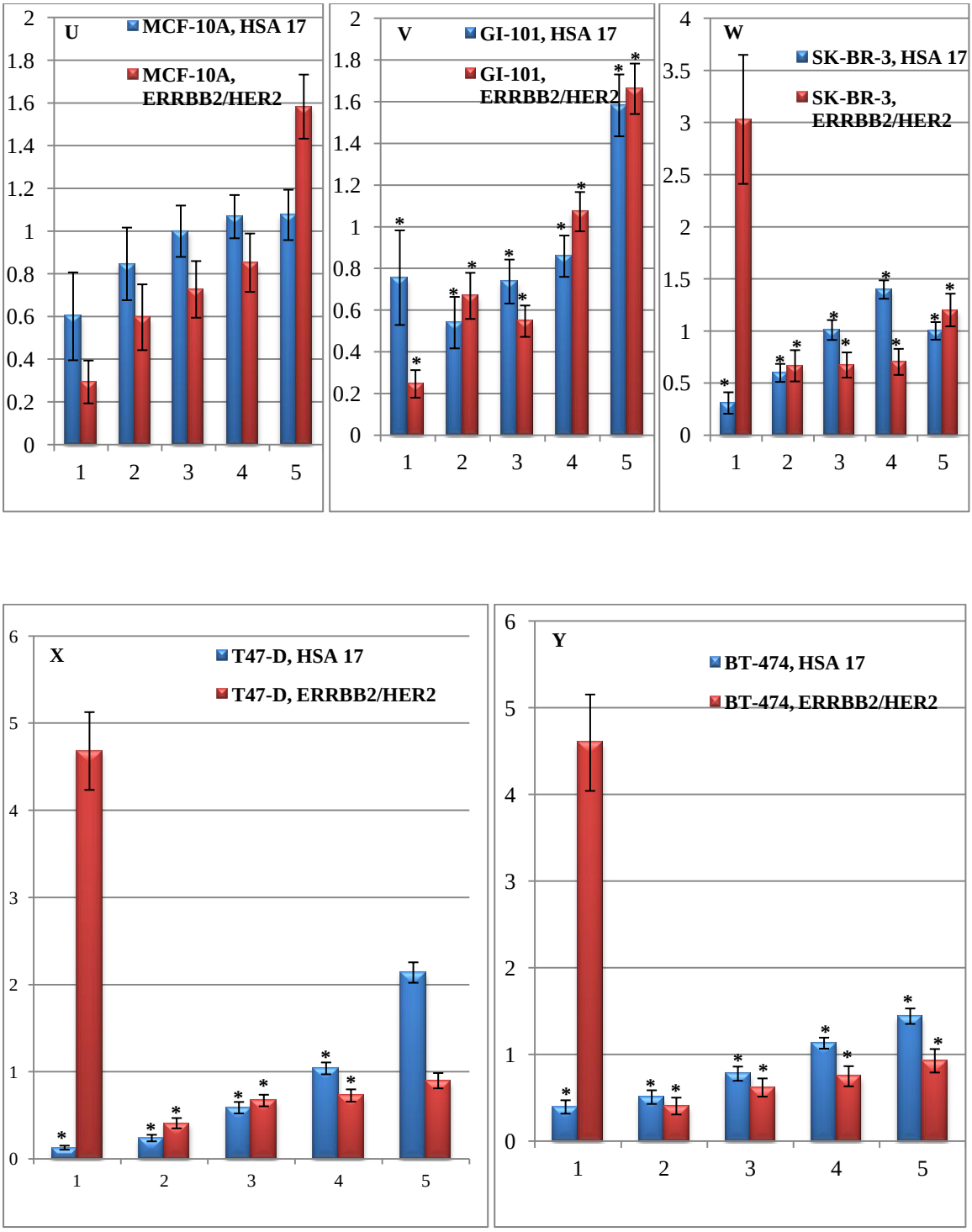


Figure 2.4: Images Analyses for Genes and Chromosomes using 2D-FISH
These histograms show the nuclear localisations of human genes *EGFR, PTEN, CCND1, HSP90AA1* and *AKT1*, and *ERRBB2/HER2* and chromosomes 7, 10, 11, 14 and 17 in normal breast (MCF-10A) and breast cancer (T-47D, GI-101, Sk-Br-3, and BT-474) cell lines. Their positions were assessed by 2D-FISH and analysed by simple erosion analysis. Error bars was measured from the standard error of the mean (SEM). Y Axis = % of Chromosome and Gene Signal / % of DAPI Signal whereas X Axis = the position from periphery to interior (1 to 5).

2.3.3 Statistical Analysis of using Student's *t*-test:

The Student's *t*-test with Excel Software Programme was used to statistically compare the positions of genes and chromosomes within BT-474, GI-101, T-47D, and Sk-Br-3 cells with the normal MCF-10A immortalised cells that were statistically different from each other. The probability-value (*p*-value) in the test was known; $p<0.5$ was considered significant and any less than 0.05 that means there is a significant difference in position and indicated with star and more than 0.5 that means no significant difference in position.

2.3.3.1 Statistical Analyses for Chromosome Position:

Statistical analyses with the Student's *t*-test were also performed for the cancer cell lines compared to the control cell line. All the cell lines show chromosomes in different nuclear locations to the control cells. The chromosome that is most unaffected is HSA 10.

(1) HSA 7:

	BT-474	GI-101	T-47D	Sk-Br-3
Area 1				
Area 2				
Area 3				
Area 4				
Area 5				

(2) HSA 10:

	BT-474	GI-101	T-47D	Sk-Br-3
Area 1				
Area 2				
Area 3				
Area 4				
Area 5				

(3) HSA 11:

	BT-474	GI-101	T-47D	Sk-Br-3
Area 1				
Area 2				
Area 3				
Area 4				
Area 5				

(4) HSA 14:

	BT-474	GI-101	T-47D	Sk-Br-3
Area 1				
Area 2				
Area 3				
Area 4				
Area 5				

(5) HSA 17:

	BT-474	GI-101	T-47D	Sk-Br-3
Area 1				
Area 2				
Area 3				
Area 4				
Area 5				

2.3.3.2 Statistical Analyses Comparing Cancer Cells with the Control Cells for Gene Position:

In order to determine if gene position had changed from the control cells (MCF-10A) in the cancer cells statistical analysis was performed on the data generated for gene loci position. The results are shown in the following charts. Black signifies no significant difference whereas green and red do show differences. Interestingly, most of the genes that haven't changed location are in T47D cells when compared to control cells.

(1) *EGFR*:

	BT-474	GI-101	T-47D	Sk-Br-3
Area 1				
Area 2				
Area 3				
Area 4				
Area 5				

(2) *PTEN*:

	BT-474	GI-101	T-47D	Sk-Br-3
Area 1				
Area 2				
Area 3				
Area 4				
Area 5				

(3) *CCND1*:

	BT-474	GI-101	T-47D	Sk-Br-3
Area 1				
Area 2				
Area 3				
Area 4				
Area 5				

(4) *AKT1*:

	BT-474	GI-101	T-47D	Sk-Br-3
Area 1				
Area 2				
Area 3				
Area 4				
Area 5				

(5) *HSP90AA1*:

	BT-474	GI-101	T-47D	Sk-Br-3
Area 1				
Area 2				
Area 3				
Area 4				
Area 5				

(6) *ERBB2-HER2*:

	BT-474	GI-101	T-47D	Sk-Br-3
Area 1				
Area 2				
Area 3				
Area 4				
Area 5				

The Key:

Black = Indicates no significant difference between the positions with the *p*-value >0.5

Green = Indicates significant difference between the positions with the *p*-value <0.5

Red = Indicates significant difference between the positions with the *p*-value <0.05

From this analysis, it can be seen that the positions of chromosome 7 in T-47D and MCF-10A cells was located at the periphery whereas, it was interior in BT-474, GI-101 and Sk-Br-3 cells. The positions of chromosomes 10 in MCF-10A, T-47D, and BT-474 cells were located at the periphery and at the nuclear interior in Sk-Br-3 and GI-101 cells. The position of chromosomes 11 in MCF-10A cells was intermediate but at the periphery in GI-101, T-47D and BT-474 cells, and in the interior in Sk-Br-3 cells. The positions of chromosomes 14 in T-47D, MCF-10A, GI-101, and Sk-Br-3 cells was located at the periphery whereas, BT-474 cells showed an intermediate localisation. The positions of chromosomes 17 in MCF-10A, Sk-Br-3 T-47D, BT-474 and GI-101 cells was located at the interior (Figure 2.4 and Table 2.4, A).

The position of *EGFR* gene was located at the nuclear periphery in all cell lines except GI-101 cells were located interiorly. *PTEN* gene was located in the nuclear interior in MCF-10A, GI-101 and Sk-Br-3 cells whereas, at the periphery in T-47D, BT-474 cells. *CCND1* gene was positioned interiorly in MCF-10A, GI-101, and Sk-Br-3 cell lines whereas it was at the periphery in T-47D, BT-474 cells. Furthermore, *HSP90AA1* gene was positioned interiorly in BT-474, GI-101, and Sk-Br-3 cell lines whereas it was at the periphery in MCF-10A and T-47D cells. Moreover, *AKT1* gene was localised in the interior in MCF-10A, GI-101, and Sk-Br-3 cells but at the periphery in T-47D and BT-474 cells. *ERRBB2/HER2* gene was located in the interior in MCF-10A and GI-101 cells but at the periphery in T-47D, BT-474, and Sk-Br-3 cells (Figure 2.4 and Table 2.4, B).

Table 2.7 (A and B): Summary of Locations of Human Chromosomes (HSA 7, 10, 11, 14, and 17, Table A) and Genes (*EGFR*, *PTEN*, *CCND1*, *AKT1*, *HSP90AA1*, and *ERBB2*/*HER2*, Table B) in Interphase Nuclei of Normal and Abnormal Breast Cell Lines: The tables below (A and B) display the position of human genes *EGFR*, *PTEN*, *CCND1*, *AKT1*, *HSP90AA1*, and *ERBB2*/HER2 that are located and resided on human chromosomes 7, 10, 11, 14 and 17 territories in Sk-Br-3, GI-101, T47-D and BT-474 breast cancer cell lines occupy altered localisations during interphase nuclei when compared to MCF-10A normal but immortalised breast cell line.

A:

HSA	MCF-10A	T-47D	BT-474	GI-101	Sk-Br-3
7	Periphery	Periphery	Interior	Interior	Interior
10	Periphery	Periphery	Periphery	Interior	Interior
11	Intermediate	Periphery	Periphery	Periphery	Interior
14	Periphery	Periphery	Intermediate	Periphery	Periphery
17	Interior	Interior	Interior	Interior	Interior

B:

Gene	MCF-10A	T-47D	BT-474	GI-101	Sk-Br-3
EGFR	Periphery	Periphery	Periphery	Interior	Periphery
PTEN	Interior	Periphery	Periphery	Interior	Interior
CCND1	Interior	Periphery	Periphery	Interior	Interior
AKT1	Interior	Periphery	Periphery	Interior	Interior
HSP90AA1	Periphery	Periphery	Interior	Interior	Interior
ERBB2/HER2	Interior	Periphery	Periphery	Interior	Periphery

2.3.4 Two Colour FISH for Both Gene and Chromosome Positioning:

From the findings, it was seen that all genes were in the same compartment as their host chromosome as shown in table 2.4 (A and B) in all cell lines except *PTEN* and *AKT1* in MCF-10A, *ERBB2*/*HER2* in T-47D, *EGFR* and *ERBB2*/*HER2* in BT-474, *CCND1*, *AKT1*, and *HSP90AA1* in GI-101, and *EGFR* and *ERBB2*/*HER2* in Sk-Br-3 cell lines. In these cells the genes were not in the same compartment as their chromosome because they must be away from chromosomes on chromatin loops. Therefore, two colour FISH experiments were performed to assess the position of both gene and chromosome in the same nuclei to determine if the genes were colocalised or at a distance from their chromosomes (Figure 2.5 and Table 2.5).

2.3.4 Two Colours FISH for Both Gene and Chromosome Positioning

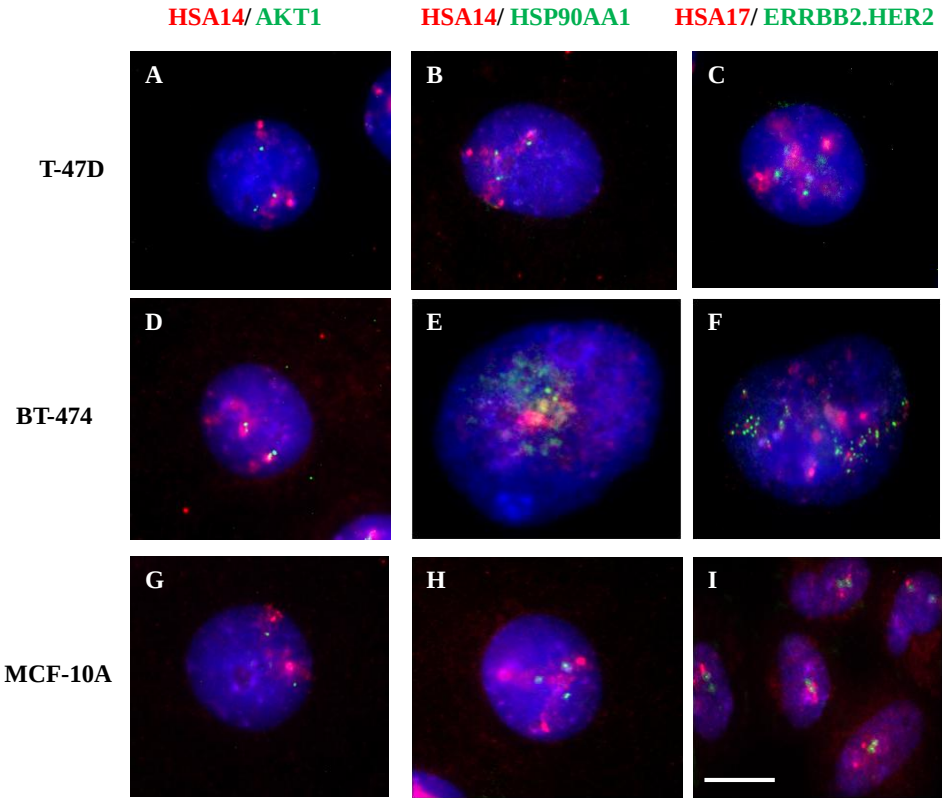


Figure 2.5: Spatial Reorganisation of both Genes and Chromosomes in Breast Cancer and Normal Cell Lines: These images are demonstrating the positions of both human chromosomes (red, Cy3-streptavidin) and genes (green, FITC). These genes were chosen to see their location on/away from chromosomes. They were detected by dual colour FISH technique in normal breast (MCF-10A, G-I) and cancer (T-47D, A-C and BT-474, D-F) cell lines. Nuclei were counterstained with DAPI (blue). Magnification = X100; Scale Bar = 10µM.

Table 2.8: Gene Loci at A Distance from Their Host Chromosomes: The spatial organisation of both genes on/away from chromosomes in breast cancer and normal breast cell lines was assessed. The percentages of copy number of genes (*AKT1*, *HSP90AA1*, and *ERBB2*/*HER2*) that located onto chromosomes (HSA 14 and 17) or found at a distance from their home chromosomes were counted in T47-D, BT-474, and MCF-10A cell lines (Figure 2.5).

(1) T47-D Cell Lines:

	On Chromosome	**At a Distance from Chromosome**
***AKT1*, HSA 14**	60%	40%
***HSP90AA1*, HSA 14**	38%	62%
***ERBB2*/*HER2*, HSA 17**	40%	60%

(2) BT-474 Cell Lines:

	On Chromosome	**At a Distance from Chromosome**
***AKT1*, HSA 14**	100%	0%
***HSP90AA1*, HSA 14**	86%	14%
***ERBB2*/*HER2*, HSA 17**	17%	83%

(3) MCF-10A Cell Lines:

	On Chromosome	**At a Distance from Chromosome**
***AKT1*, HSA 14**	75%	25%
***HSP90AA1*, HSA 14**	57%	43%
***ERBB2*/*HER2*, HSA 17**	40%	60%

The T47D cell line displayed two genes with over 50% of their loci away from their home chromosome. Whereas the other two cell lines, BT474 and MCF10A, had only one gene away from the host chromosome in more than 50% of the cells. This was the same gene in both cell lines; *ERBB2/HER2* which is interesting considering how important this gene is in breast cancer progression.

2.4 Discussion:

To study the organisation of the genome in the nuclei of breast cancer cells, chromosomes and genes were delineated with labeled FISH probes and their nuclear distribution and position assessed.

Figure 2.4 displays the position of human genes *EGFR, PTEN, CCND1, AKT1, HSP90AA1,* and *ERBB2*/HER2 that are accommodated on human chromosomes 7, 10, 11, 14 and 17 territories in Sk-Br-3, GI-101, T47-D and BT-474 breast cancer cell lines. These territories were found in different nuclear locations interphase nuclei when compared to MCF-10A normal immortalised breast cell line. This alteration and difference in position was significant and indicated by green (p-value <0.5) and red (p-value <0.05) colour in the statistical analysis tables. The significance of this alteration and changes in most genes positioning within breast cancer cells requires further investigation. However, the re-positioning of genes in these cells indicates the disruption of genome organisation. These differences in position raise several questions; why are certain chromosomes and genes were affected while others are not? Is this due to involvement of other factors such as transcription factors activity? Or this is cell type dependent? Most importantly has this change in gene position within nuclei affected gene expression profiles?

Whole chromosome territory positions are generally maintained during interphase and these territories have individual loci that can move rapidly over large distances (Mehta *et al.*, 2010a). These results indicate that an aberrant gene position was seen in breast cancer cells when compared to the normal cells. In this study, I found that HSA11 accommodating *CCND1,* occupies an intermediate location in normal cell nuclei whereas *CCND1* was interiorly positioned in the same cell line which is similar result to the work done by Meaburn

et al., 2008; Mehta *et al.*, 2010a. The position of genes was assessed by investigation the position either on or away from chromosomes. In T-47D cells, 60-62% of cell nuclei showed the position of *HSP90AA1* and *ERBB2*/*HER2* genes was away from their chromosomes and 60% of cell nuclei showed the position of *AKT1* gene on chromosome. In BT-474 cells, all cell nuclei showed that *AKT1* gene position on chromosome (100%) whereas *HSP90AA1* gene positioned on chromosome in 86% of nuclei and only 17% of nuclei showed *ERBB2*/*HER2* gene position on chromosome. In MCF-10A cells, 25% of nuclei showed that *AKT1* gene was position away from chromosomes whereas 43% of nuclei showed the position of *HSP90AA1* gene away from chromosome and 40% of nuclei showed *ERBB2*/*HER2* gene was positioned on chromosome. Regarding, the position of genes away from their chromosomes this could suggest that in these cells, the gene loops away from home chromosome. The change in gene position may be due to specific internal nuclear re-organisation and this indicates a change in the activity of the cells and their transcription status.

Often upon transcriptional activation a locus will decondense and move until it associates with PML bodies to maximise transcriptional output or some loci on chromosomes move into large distances to interact with transcription factories to regulate their transcription and expression whereas, some of chromosomes and genomic regions associated with specific structures (nuclear matrix, nuclear lamina, and nucleoli) and then determined their movement (Eskiw *et al.*, 2003; Elcock and Bridger, 2010).

2.4.1 The Concept of Chromosomes Kissing and Gene Regulation in Genome Organisation:

Eukaryotic chromosomes occupy distinct territories in the cell nucleus. These territories interact with other chromosomes to make several interactions between different chromosomal loci via *cis* contacts which have been investigated in a phenomenon called “chromosome kissing” (Cavalli, 2007). These contacts are due to preferential chromosome neighbourhoods and of sharing of transcriptional machineries while others seem to have regulatory functions. Moreover, these contacts are modulated by specific chromatin features of each locus, and they play important roles in the regulation of gene expression. Chromosome kissing events may also be at the origin of chromosomal rearrangements (Cavalli, 2007). Furthermore, gene expression can influence by *cis*-regulatory elements (on the same chromosome) or *trans*-regulatory elements (on different chromosome) that affect gene function (therefore, it might be involved in mechanisms of gene activation or silencing) as well as transcription factors and chromatin-histone complexes. However, the correlation of these components of the nucleus is unclear. Technical advances are now identifying the relationships, locations, and interactions of genes and these regulatory elements in the nucleus are revealing that there is a network within and between chromosomes and genes but to which this network organisation affects gene function is still unclear (Fraser and Bickmore, 2007). Recent studies show that genes on the same chromosome or even on different chromosomes can come together in the nuclear space via loops. It has been hypothesised that functionally related genes 'kiss' at transcription factories to coordinate their expression and discover this romantic concept of nuclear architecture (de Laat, 2006). Figure 2.3 displays the position of multiple copies of the gene at the same place or at the transcription machinery to regulate their expression and activity.

2.5 Conclusion:

Interestingly, there are differences in chromosomal and gene locations between different cells (Figure 2.4 and Table 2.4). The results showed chromosomes 14 and 17 with clear differences with respect to position between cells as well as *AKT1*, *HSP90AA1*, and *ERBB2*/*HER2* genes within MCF-10A, T-47D, and BT-474 cell lines.

When analysing the nuclear positioning of specific genes and their chromosomes in breast cancer cell lines, it is found that the important genes that involved in breast cancer progression are positioned in different localisations as compared to controls. These changes will affect chromatin organisation and gene expression. If this alteration is consistent as seen in Meaburn's studies then it may be extremely useful as a diagnostic biomarker.

Understanding the nuclear behaviour of breast cancer cells will aid in the goal to investigate new pathways involved in expression of interested genes in cancer. The results from this study emphasise the genome organisation and its importance to genomic stability. New directions of monitoring genomic instability could impact on cancer biology, genetics, diagnosis and efficiency of treatment response in medicine. However, the development in molecular and cytogenetic techniques allows us to understand how the nucleus is well organised and how it functions although the mechanistic basis by which alterations in genome organisation, and function lead to cancer is an exciting new area of study.

Chapter 3: Expression of Specific Genes in Human Breast Cancer Cell Lines

3.1 Introduction:

Studies have shown that individual chromosomes have preferential neighbouring territories and the organisation appears to be cell type specific (Nagele *et al.*, 1999; Parada and Misteli, 2002; Parada *et al.*, 2004; Bolzer *et al.*, 2005; Foster and Bridger, 2005; Branco and Pombo, 2006; Meaburn and Misteli, 2008). The arrangement of these chromosome territories (CT) is based on the transcriptional activity. Croft *et al.*, in 1999 and Bridger *et al.*, in 2000, studied the gene rich chromosome 19 and gene poor chromosome 18 and showed the morphology of chromosome territories is linked to their transcriptional status. The eukaryotic cells demonstrate variable ranges in gene expression, because of variable rates in kinetics of gene activation, transcription, and translation. This demonstration has been linked to gene position via spatial variation in the induction, regulation and activation of transcription factors, nucleosomes, and chromatin remodeling complexes (Batenchuk *et al.*, 2011). However, different studies of gene expression in yeast have failed to display this idea (Newman *et al.*, 2006). Moreover, chromosome position can influence the transcriptional activity of genes within it and this will affect their expression (Volpi *et al.*, 2000). Several mechanisms or factors can alter the position of genes for example non-allelic homologous recombination, movement of chromosomes, movement of genes, and movement of nuclear sub-structures such as nucleolus, chromatin fibres, and PML bodies (De and Babu, 2010). These events can contribute to cause specific disease such as cancer.

Individual genes, as well as chromosomal regions, occupy preferential, distinct radial locations within interphase nuclei (Bridger and Bickmore 1998). Transcriptionally active genes can be found at the periphery of chromosome territories (Kurz *et al.*, 1996), within the chromosome territory (Mahy *et al.*, 2002), and they can also loop out from the chromosome territory (Branco and Pombo, 2006) where they can access to the transcriptional machinery.

Moreover, the preferential location of the *ANT2* and *ANT3* genes at the edge of X chromosome territories (Dietzel *et al.*, 1999) suggest a non-random and function-related distribution of genomic regions. In order to understand the relationship between chromosome architecture and its gene activity, Volpi *et al.*, 2000 studied genomic regions of human chromosome 6 during interphase and assessed their responses to transcriptional activation. They used two cell types, fibroblasts and B-lymphoblastoid cells, which express the major histocompatibility complex (MHC) genes upon regulation by interferon-gamma (IFNγ) to assess the effect of transcriptional activation. They found that the MHC genes came out on loops away from chromosome 6 when activated to transcribe. This is what we believe happened to the genes seen away from their home chromosome in chapter 2.

Changes in nuclear position of genes are induced by many factors such as transcriptional status of genes and stress. Upon activation, genes can move away from a CT to a site that is enriched in gene expression machinery. However, gene activation is not always accompanied by movement, as positioning is determined by many factors, including gene structure and the local genomic environment (Mekhail and Moazed, 2010; Geyer *et al.*, 2011**).** Finlan *et al.*, in 2008 investigated the role of gene position and transcriptional suppression at the nuclear periphery in mammalian cells. They relocated two different human chromosomes via the interaction of *Eschericia coli* (*E.coli*) lac operator (*lacO*) to the nuclear periphery with lac repressor (*lacI*) that is fused to the integral INM protein LAP2β. They found that reduction in the expression of some genes close to the *lacO* sites and some other genes that away from *lacO* also appear to be down-regulated by relocalisation of these chromosomes toward the nuclear periphery and this is due to histone deacetylases (HDACs) inactivity. Their data suggest that, during development and differentiation, re-position of genes to the nuclear periphery could be used as a mechanism to modulate the expression of specific genes without

changing expression of their neighbours in vertebrates. Szczerbal *et al.*, in 2009 provide evidence that genes that are to be expressed in adipogenesis move towards the nuclear interior and some move back again when their transcription has ceased. These data and others (Bourne *et al.*, 2013) strongly suggest that the position of a gene in the cell nucleus is involved in regulating gene expression.

The aim of this chapter focuses on the expression of specific genes that are involved in breast cancer susceptibility and progression but they can also play a role in other types of cancer, such as colorectal, ovarian, or prostate. These are the genes that have changed nuclear location in the breast cancer cell lines compared to normal control cells (Chapter 2). These genes showed variant expression within normal breast (MCF-10A) and breast cancer (T-47D, GI-101, BT-474, and Sk-Br-3) cell lines when assessed by qRT-PCR (Figure 3.1). Their expression characteristics are compared to their nuclear position.

3.2 Material and Methods:

3.2.1 Gene Primer Design:

To design primers, the steps were followed using the Invitrogen website:

www.Invitrogen.com → Nucleic Acid Amplification and Expression Profiling → Invitrogen™ Custom Oligonucleotides → Primer Designer Tools → Oligo Perfect™ Designer → Country → Gene Name, Researcher Name, and PCR Detection (mRNA Sequence was copied) → Submit. The primer size was chosen between 18-25 bp and the GC% of primer was chosen between minimum 40 and maximum 60 (Appendices).

3.2.2 Genes Assessed in Breast Cancer Cell Lines:

3.2.2.1 *EGFR* (*Epidermal Growth Factor Receptor Gene*):

Gene Symbol	Gene Location	Gene Description
EGFR; ERBB1; HER1 in humans	located on 7p11.2 (OMIM)	It is a cell surface receptor for members of the epidermal growth factor family (EGF-family) of extracelluar protein and is a transmembrane glycoprotein that constitutes one of four members of the ERBB family of tyrosine kinase receptors (EGFR (ERBB1), HER2 (ERBB2), HER3 (ERBB3) and HER4 (ERBB4)). Binding of *EGFR* to its ligands leads to phosphorylation of receptor tyrosine kinase and activation of signal transduction pathways that are involved in regulating cellular proliferation, differentiation, motility, survival, and tissue development (Wang *et al.*, 2004). Although is present

		in normal cells, *EGFR* is over-expressed in a variety of tumour cell lines (breast, head and neck (HNC) and lung cancers) and has been associated with poor prognosis and decreased survival. Mutations affecting EGFR expression or activity could result in cell proliferation and cancer. PML and p73 are involved in the regulation of *EGFR* expression (Herbst, 2004; Klanrit *et al.*, 2009).

3.2.2.2 *PTEN* (*Phosphatase and Tensin Homology*):

Gene Symbol	Gene Location	Gene Description
PTEN gene	located on 10q23.31 (OMIM)	This gene is identified as a tumour suppressor that is mutated in a large number of cancers at high frequency. Mutations in this gene have been linked to various tumours including renal, glioma, melanoma, endometrial, breast, prostate, lung, bladder, and thyroid (Li and Sun, 1998). It encodes a major lipid phosphatase that antagonises the phosphatidylinositol-triphosphate kinase (PI3K) signaling pathway through its lipid phosphatase activity, resulting in the inhibition of the AKT proto-oncogene and mTOR pathway and induces G1 cell cycle arrest and apoptosis (Eng, 2003). This phosphatase activity

		negatively regulates the mitogen-activated protein kinase (MAPK) pathway. Inactivation or loss of *PTEN* function results in an increased cell survival and uncontrolled cellular proliferation, growth, migration, and survival mediated by these pathways and resulting in neoplasia (Cowden disease) (Katso *et al.*, 2001; Yin and Shen, 2008). PTEN is believed to be an active protein; its activity is dependent on its levels (Blumenthal and Dennis, 2008). Despite this fact, *PTEN* has significant roles in regulating the cell cycle, implicated in apoptosis processes, maintaining of cell size (Huang *et al.*, 1999), maintaining chromosome integrity and stability through the interaction with centromeres and control of DNA repair suggesting that *PTEN* acts as a guardian of genome integrity (Shen *et al.*, 2007), differentiation (Zheng *et al.*, 2008), and in the malignant transformation associated with carcinogenesis. Relatively little is known about the mechanisms that control transcriptional regulation for *PTEN* expression (Pezzolesi *et al.*, 2007).

3.2.2.3 *CCND1* (*Cyclin D1*):

Gene Symbol	Gene Location	Gene Description
CCND1 gene	located on 11q13.3 (OMIM)	It is regulatory subunit of a holoenzyme that phosphorylates and inactivates the pRB and promotes progression through the G1-S phase of the cell cycle dependent on cyclin-dependent kinases, CDKs. In addition, CCND1 has a number of cell cycle and CDK-independent functions. CCND1 associates with and regulates transcription factors, ac as co-activator, and co-repressor that controls histone acetylation and chromatin remodeling proteins. *CCND1* has also roles in cellular growth, metabolism, and cellular differentiation. Amplification or over-expression of *CCND1* plays pivotal roles in the development of several human cancers, including parathyroid adenoma, breast cancer, colon cancer, lymphoma, melanoma, and prostate cancer (Fu *et al.*, 2004).

3.2.2.4 *AKT1* (*V-Akt Murine Thymoma Viral Oncogene Homolog 1*):

Gene Symbol	Gene Location	Gene Description
serine-threonine protein kinase encoded by the *AKT1* gene	located on 14q32.33 (OMIM)	It is inactive in serum-starved primary and immortalised fibroblasts. It was shown that the activation occurs through phosphatidylinositol 3-kinase (PI3K). PI3Ks generate specific inositol lipids implicated in the regulation of cell growth, proliferation, survival, differentiation, and cytoskeletal changes. One of the targets of PI3K lipid products is the protein kinase AKT, or protein kinase B (PKB) (Vanhaesebroeck and Alessi, 2000). In the developing nervous system, *AKT* is a critical mediator of growth factor-induced neuronal survival. Survival factors can suppress apoptosis in a transcription-independent manner by activating the serine/threonine kinase *AKT1*, which then inactivates components of the apoptotic machinery.

3.2.2.5 *HSP90AA1* (*Heat-Shock Protein, 90-KD, Alpha, Class A, Member 1*):

Gene Symbol	Gene Location	Gene Description
HSP90AA1 gene	located on 14q32.31 (OMIM)	It is encoded for HSP90 proteins which are highly important molecules that have key roles in signal transduction, protein folding, protein degradation, and morphologic evolution. HSP90 proteins normally associate with other molecules and play important roles in producing newly synthesised proteins or stabilising denatured proteins after stress. The genome analyses revealed that the human HSP90 family includes 17 genes that fall into four classes. These classes are HSP90AA, HSP90AB, HSP90B, and TRAP and contain 7, 6, 3, and 1 genes, respectively (Chen *et al.*, 2005). There are 2 major cytosolic HSP90 proteins, HSP90AA1, an inducible form, and HSP90AB1, a constitutive form. Other HSP90 proteins (HSP90B1 and TRAP1) are found in endoplasmic reticulum (ER) and mitochondria (Chen *et al.*, 2005 and Nagahori *et al.*, 2010).

3.2.2.6 *ERBB2*/*HER2*:

Gene Symbol	Gene Location	Gene Description
V-ERB-B2 AVIAN Erythroblastic Leukemia Viral Oncogene Homolog 2 (*ERBB2*) Neuroblastoma- or Glioblastoma (*Neu*) Tyrosine Kinase-Type Cell Surface Receptor (*HER2*) The oncogene originally called *NEU*	located on 17q12 (OMIM)	It was derived from rat neuro/glioblastoma cell lines. It encodes a tumour antigen, p185, which is serologically related to EGFR. It is a cell surface receptor of the tyrosine kinase gene family named HER2 and its sequence is very similar to the EGFR. Therefore, its name came from the **H**uman **E**GF **R**eceptor, *HER2* (Coussens *et al.*, 1985). In 1986, Akiyama *et al.*, raised antibodies against a synthetic peptide and they precipitated the *ERBB2* gene product from adenocarcinoma cells and its size estimated to be a 185 kDa glycoprotein with tyrosine kinase activity (Akiyama *et al.*, 1986). Di Fiore *et al.*, in 1987, indicated that *NEU* and *HER2* are both the same as *ERBB2*. In addition, the chromosomal location of the *V-ERBB* oncogene for this protein is similar with the *NEU* oncogene, which suggests that the two oncogenes may be identical (Coussens *et al.*, 1985).

3.2.2.7 *β-Actin* (*ACTB*):

Gene Symbol	Gene Location	Gene Description
Beta-actin gene (*ACTB*)	located on 7p22.1 (OMIM)	It is one of the six different actin isoforms (OMIM) which have been identified in humans and is one of the two non-muscular actins. They are highly conserved proteins (Hanukoglu *et al.*, 1983) that are involved in cell motility, structure and integrity. Alpha actins are a major protein of the contractors. Mutations in this gene have been associated with B-cell Lymphoma (Lohr *et al.*, 2012). Beta-actin is usually used as a control or housekeeping gene to measure the integrity of cells and protein degradation in PCR and Western Blots experiments.

Table 3.1: The List of Primers *EGFR, PTEN, CCND1, AKT1, HSP90AA1, HER2,* and *β-Actin* Genes for qRT-PCR: Forward and reverse primers as well as melting temperature used for each set of primer are displayed in the table below:

Primer Name	Primer Sequence (5` → 3`)	Product Length (bp)	%GC	Tm (°C)
EGFR (F)	CAGCGCTACCTTGTCATTCA	195	50.00	60.01
EGFR (R)	TGCACTCAGAGAGCTCAGGA		55.00	60.01
PTEN (F)	CATAACGATGGCTGTGGTTG	106	50.00	59.99
PTEN (R)	CCCCCACTTTAGTGCACAG		55.00	60.03
CCND1 (F)	GAGGAAGAGGAGGAGGAGGA	237	60.00	59.88
CCND1 (R)	GAGATGGAAGGGGGAAAGAG		55.00	60.01
AKT1 (F)	AGAAGCAGGAGGAGGAGGAG	139	60.00	60.09
AKT1 (R)	CCCAGCAGCTTCAGGTACTC		60.00	60.01
HSP90AA1 (F)	AGACCCAGTCTTGTGGATGG	120	55.00	59.96
HSP90AA1 (R)	ACTCCCCTTTCCCCCTAAAT		50.00	60.01
HER2 (F)	ACAGTGGCATCTGTGAGCTG	148	55.00	60.06
HER2 (R)	CCCACGTCCGTAGAAAGGTA		55.00	59.99
β-Actin (F)	AAGAGAGGCATCCTCACCCT	216		
β-Actin (R)	TACATGGCTGGGGTGTTGAA			

3.2.3 Primer Dilution:

Primers were diluted according to their concentration and each stock was prepared to a final concentration of 1 µg/1 µl. Dilutions were prepared again for each primer (10 µl/100 µl in nuclease free water, Ambion) and then all primers were stored at -20ºC.

3.2.4 Cell Culture:

The five human breast cell lines either immortalised (MCF-10A, normal) or cancerous (T-47D, BT-474, GI-101, and Sk-Br-3) were investigated. They were grown and cultured as in Chapter 2.

3.2.5 RNA Extraction:

Total RNA was extracted from different cell lines, using the GenElute^{TM} Mammalian Total RNA Miniprep Kit (Sigma-Aldrich), according to the manufacturer's instructions. Before starting, 60 ml of absolute ethanol was added to wash solution 2 and 10 µl 2-mercaptoethanol (2-ME) was mixed with 990 µl lysis solution. The cells were then washed once with pre-warmed 1X PBS then 1 ml of lysis solution/2-ME was added to the flask with gentle rocking for 2 minutes to ensure all cells come into contact with the lysis mixture. The lysed cells were pipetted into GenElute Filtration Columns (blue) and centrifuged at maximum speed 16000 rpm for 2 minutes. The filtration columns were discarded and 500 µl of 70% ethanol solution were added to the 2 ml tubes and then vortexed. 700 µl of that mixture (lystae/ethanol) was taken and added to the GenElute Binding Columns and then centrifuged for 15 seconds at 16000 rpm maximum speed. The liquid that came through was discarded and the binding columns were returned to tubes. 500 µl of wash solution 1 was added to the binding columns and then they were centrifuged at maximum speed for 15 seconds. The liquid that passed through was discarded. The columns were transferred to fresh new collection tubes. 500 µl of

diluted wash solution 2 was added to the columns and then they were centrifuged at maximum speed for 15 seconds. The liquid was discarded and another 500 µl of diluted wash solution 2 was added to the columns and then centrifuged for 2 minutes at maximum speed. The columns were centrifuged again for 1 minute at maximum speed to dry after discarding the excess liquid. The binding columns were transferred to a fresh 2 ml collection tubes and then 50 µl of the elution solution were added into the binding columns, again these tubes were centrifuged at maximum speed for 1 minute. This process of elution was repeated. Isolated RNA from each sample was maintained on ice at all times. However, there was no addition or treatment of DNase I solution for this isolated RNA to avoid any genomic contamination but later on these samples showed high validity and integrity.

3.2.6 RNA Analysis (Nano-drop):

The amount of RNA in each sample was measured and quantified by spectrophotometry using nano-drop machine. 1 µl of RNA extraction was added to the pin head on the nano-drop 2000c machine (Thermo Scientific) to measure the quality and concentration of the RNA samples. RNA concentration (ng/µl) was measured and the ratio 260/280 was automatically calculated. Accurate ratio reading is between 1.9 and 2.1. The isolated RNAs were stored at -80ºC until further use for cDNA synthesis and qRT-PCR. MCF-10A cell line showed low cell number and this could be of growth activities such as passage number, confluence, viability, and cell morphology therefore, this could affect the RNA levels in this cell lines.

3.2.7 Complementary DNA (cDNA) Synthesis:

Two master mix groups (1 and 2) were prepared and RNA standardisation was calculated for each cell line (Appendices). Master Mix 1 was composed of 1 µl random primers (oligonucleotides of every possible combination), 1 µl dNTPs mix (10 mM), and 10 µl of both RNA and water solution as calculated on the table below to make up standardised RNA amount in ng. Master mix 2 was composed of 4 µl of 5X first strand buffer (250 mM Tris-HCl, pH 8.3, 375 mM KCl, and 15 mM $MgCl_2$), 1 µl of molecular grade water, and 2 µl of 0.1 M DTT (Dithiothreitol). This was followed by adding 1 µl of Superscript^{TM} II reverse transcriptase enzyme (200 unit/1 µl, Invitrogen).

In order to perform the cDNA synthesis, 2 µl of master mix 1 was added to each sample tube followed by adding dH_2O then mixed. This mixture was heated for 5 minutes at 65ºC on heating block, then centrifuged and placed quickly onto ice. This was followed by the addition 7 µl of master mix 2 to each of the sample tubes with mixing. These tubes were left for 2 minutes at room temperature. Then 1 µl of Superscript^{TM} II reverse transcriptase enzyme was added to each tube, and left at RT for 10 minutes. These tubes were incubated at 42ºC for 50 minutes followed by heating for 15 minutes at 75ºC. The tubes were stored at -20ºC.

Table 3.2: RNA Standardisation Calculation:

The RNA concentrations from each sample were used as shown on the table below. Large samples can use up to 500 ng RNA for cDNA synthesis; smaller concentrations can use 100 ng for cDNA synthesis:

Cell Lines	Concentration of RNA (ng/ µl)	Standard Amount (ng)	RNA Required (µl)	H_2O Required (µl)
	X	**Y**	**Y / X**	**10 – (Y / X)**
(1) T-47D	420.2	500	1.18	8.82
(2) BT-474	284.8	500	1.75	8.25
(3) GI-101	389.8	500	1.28	8.72
(4) Sk-Br-3	718.1	500	0.69	9.31
(5) MCF-10A	59.3	500	8.43	1.57

3.2.8 Reverse Transcriptase - Polymerase Chain Reaction (RT-PCR):

It is considered as the standard method for accurate, sensitive and fast measurement of gene expression (Derveaux *et al.*, 2010). The qPCR reactions for each template were done in small Optical 96-Well Fast Thermal Cycling Plates in triplicate under the standard conditions. The comparative method was used to determine gene expression changes in each sample, using β-Actin as a reference control (Applied Biosystems). All the qPCR mixtures or components were prepared and distributed to 0.2 ml wells. Then the plates were centrifuged before amplification by qPCR machine (ABI machine). All 7 genes and the house keeping gene (β-Actin) were amplified using the cDNAs (samples) generated by reverse transcriptase. The Power SYBR® Green PCR Master Mix was used to perform RT-PCR Assays (Applied

Biosystems). A number of different primers were employed. The component of each reaction (master mix) can be seen on the table below.

Table 3.3: The qPCR Reaction Components Used to Amplify *EGFR, PTEN, CCND1, AKT1, HSP90AA1, HER2,* and *β-Actin* Genes: The Mixture was used to Measure their Fold Change in Expression in Different Cell Lines as Manufacturer's Instructions (Applied Biosystems):

Component from Power SYBR® Green PCR Master Mix	**Total Volume in a 20µl Reaction**
PCR Grade Water (Nuclease Free)	7 µl
2X Power SYBR® Green PCR Master Mix	10 µl
Forward Primer (100nM)	1 µl
Reverse Primer (100nM)	1 µl
cDNA Template	1 µl (end)

The cDNAs were diluted using nuclease free water (1:10) before the experiment started. Then master mix was prepared for each gene and maintained on ice. 19 µl of qPCR master mix was added into each well. 1 µl of cDNA sample or water (negative control) was added to the side then the plate was centrifuged for a few seconds in a micro-centrifuge to collect the cDNA sample into the reaction master mix. β-Actin was used as an internal or endogenous control to ensure cDNA quality and loading accuracy. The expression of each gene was assessed in triplicate. The plate was placed in the qPCR machine with specific programme parameters. The qPCR cycles required for amplification are 40 cycles with annealing temperature 58ºC.

The cycling conditions for relative quantification were: 95°C for 10 minutes; 95°C for 15 seconds, and 58°C for 1 minute for 40 cycles whereas; the cycling conditions for absolute quantification were: 95°C for 15 seconds, 60°C for 15 seconds, and 95°C for 15 seconds for 40 cycles. Specificity of the qPCR products has confirmed by analysis of the dissociation curve. The plates were stored in the fridge for further analyses by agarose gels to verify the band size of each gene and to ensure the quality and integrity of product amplification. The expected size and the absence of nonspecific products were confirmed by analysis of the products on 2% agarose gels.

3.2.9 Confirmation of Amplified Gene Primer Products by Agarose Gel Electrophoresis:

Agarose gel electrophoresis was performed using 2 % agarose gel (2 g agarose in 100 ml 1X TAE, **T**ris, **A**cetic Acid, and **E**DTA, (Appendices)). After heating the agarose gel, 5 µl ethidium bromide (10 mg/ml, Sigma-Aldrich) was added to the warm gel. The gel was poured into a sealed gel container with comb and left to set. When the gel had set, it was placed inside a tank. Then the gel running buffer (1X TAE, Appendices) was poured on the tank to cover the gel. The samples were prepared for loading into the wells as well as to the negative control (sterilised water instead of DNA sample). Each sample contained 15 µl of the qPCR products, and 5 µl 5X loading dye. The DNA ladder or marker, have 12 bands, (15 µl plus and 5 µl 5X loading dye, Appendices) (100 bp, 50 µg/ml, BioLabs) was loaded onto the first lane of the gel then followed by loading other samples (T-47D, BT-474, GI-101, Sk-Br-3, and MCF-10A). The gel electrophoresis apparatus was connected to the power and was run at 100 volts for 60-90 minutes. This is followed by capturing images with the BIO-RAD gel document system to visualise the products and bands on the gel were revealed on a UV-transluminator and were identified with the expected band size using IPLab software (Appendices).

3.3 Results:

Chromosomes territory (HSA 7, 10, 11, 14, and 17) and gene positioning (*EGFR*, *PTEN*, *CCND1*, *AKT1*, *HSP90AA1*, and *HER2*) in human normal breast (MCF-10A) and cancer (T-47D, BT-474, GI-101, and Sk-Br-3) cell lines were determined using 2D-FISH (Chapter 2). Alterations in chromosome and gene position can lead to changes in gene expression. Understanding the signalling pathways of these genes and their involvement in breast cancer progression is an important goal (Chapter 1). Difference in position of chromosomes where these genes resided was observed between different cell lines. Chapter 2 demonstrates the aberrant nuclear positioning of chromosomes as well as some important genes. These are studied in this chapter by qRT-PCR to ascertain their relative change in expression.

It is important to understand the functional relevance of these differential positions of chromosomes and to assess whether the positions of these chromosomes are linked to gene expression and transcription profiles of genes involved in breast cancer progression. Furthermore, these positions were compared with normal but immortalised MCF-10A cell line, for example, *ERBB2*/*HER2*, resided on HSA 17, is located at the nuclear periphery in T-47D, BT-474, and Sk-Br-3 breast cancer cell lines whereas the same gene is positioned in the interior in MCF-10A normal breast and GI-101 cancer cell lines. Chromosome 17 is positioned in the interior in T-47D, BT-474, and Sk-Br-3 breast cancer cell lines whereas the same chromosome is positioned in the interior in MCF-10A normal breast and GI-101 cancer cell lines (referring to chapter 2 to compare the positions of other genes and other chromosomes). Thus, any change in position could lead to change in gene expression.

3.3.1 qRT-PCR Analysis for Selected Genes from Different Cells:

The two most commonly used methods to analyse data from quantitative RT-PCR experiments are absolute quantification (AQ) and relative quantification (RQ). Absolute quantification determines the copy number, usually by relating the PCR signal to a standard curve. Relative quantification is measured by relating the PCR signal of the target group of sample to that of untreated sample such as an untreated control. The $2^{(-\text{Delta Delta } C_T)}$ method is a prefered way to analyse the relative changes (fold change or RQ) in gene expression from quantitative RT-PCR experiments (Livak and Schmittgen, 2001).

In figure 3.1, the graphs show the percentage of relative quantification of gene expressions by fold changes in expression for each gene using qRT-PCR. The levels of expression of all genes studied here are calculated from $2^{-\Delta\Delta C}{}_t$ or relative quantification (RQ) using RQ Manager and Microsoft Excel software.

The equations are:

$\Delta Ct = Ct\ (target) - Ct\ (housekeeping\ gene)$

$\Delta\Delta Ct = \Delta Ct\ (sample) - \Delta Ct\ (normal\ or\ calibrator)$

$RQ = 2^{-\Delta\Delta C}{}_t$

3.3.2 qPCR Analysis for Gene Expression in Breast Cell Lines:

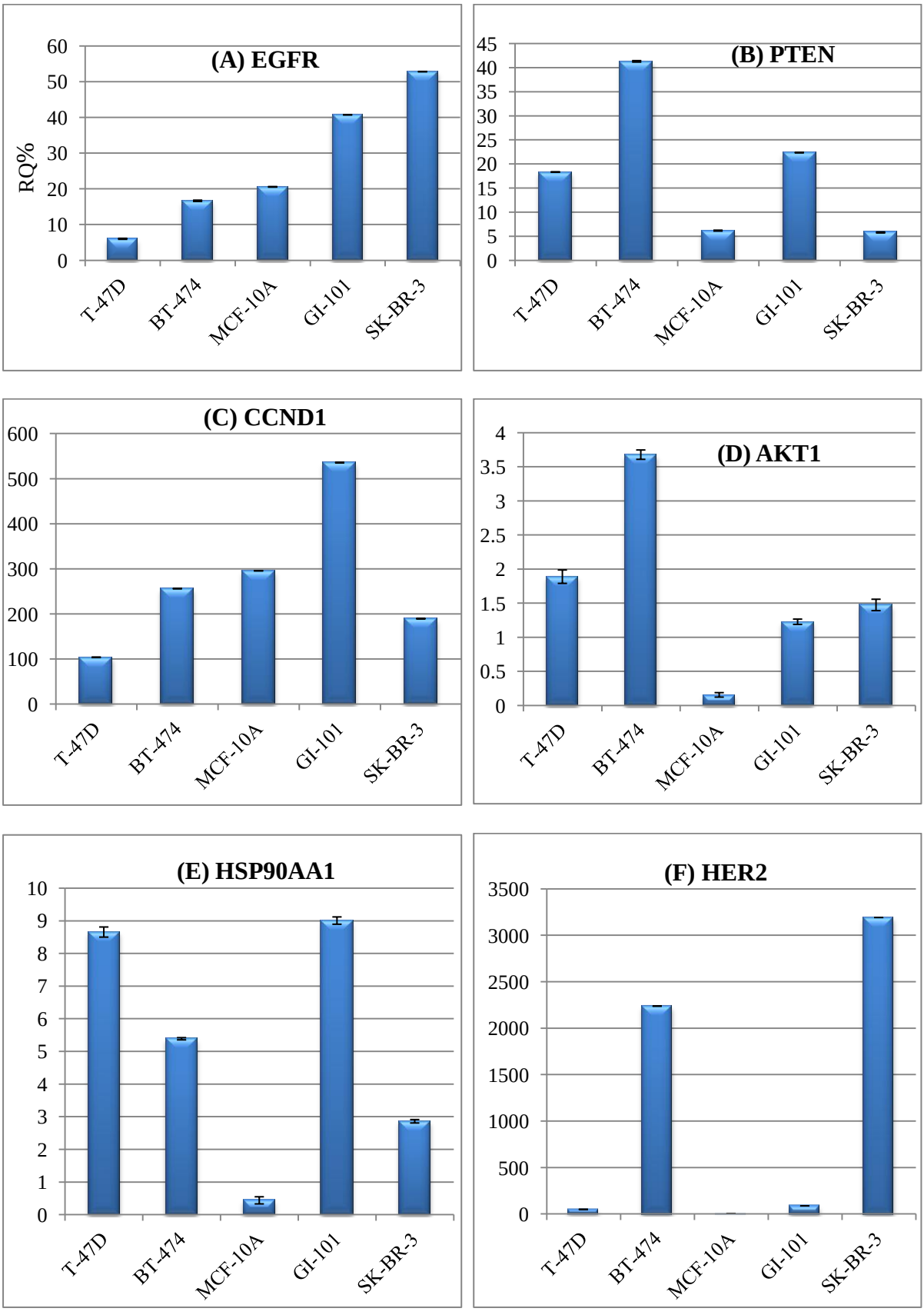
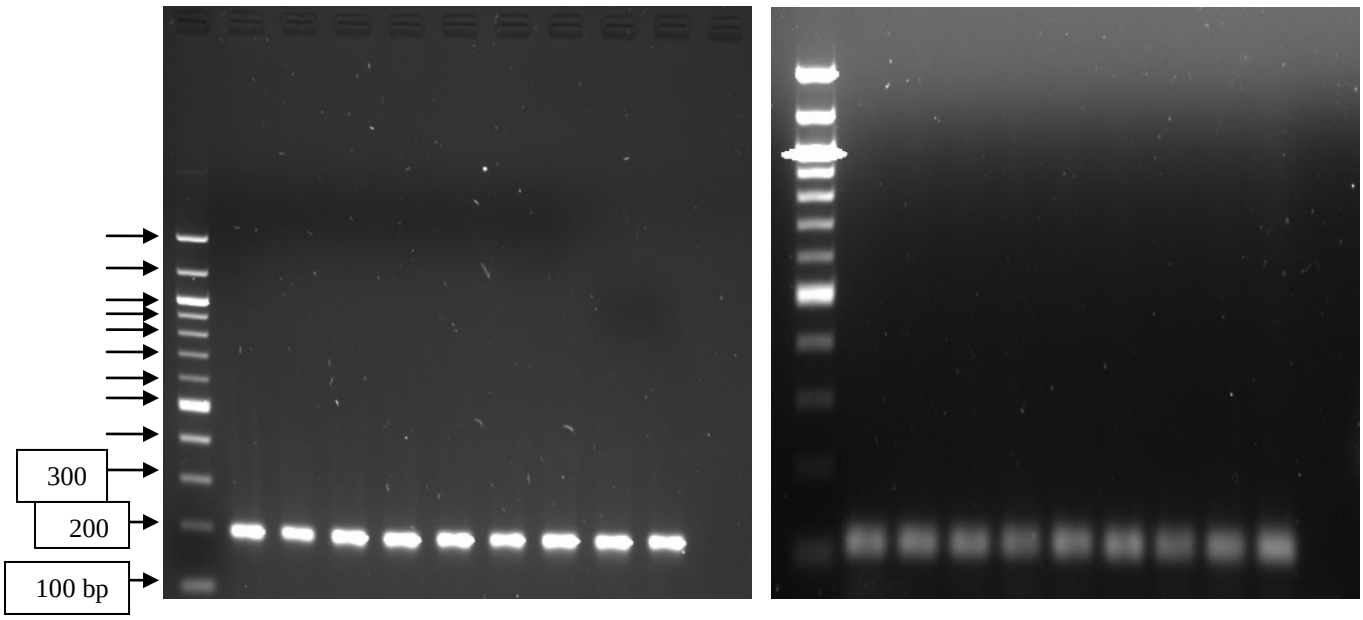


Figure 3.1: Expression Levels of *EGFR, PTEN, CCND1, AKT1, HSP90AA1, HER2,* and *β-Actin* Genes mRNA of Breast Cell Lines using qRT-PCR
These histograms show the percentage of relative quantification (RQ%) of different genes in present of β-actin as a housekeeping gene with no supplement set as 1 (100%) (A-F). The fold changes in different gene expression were assessed between cell lines (MCF-10A, T-47D, BT-474, GI-101, and Sk-Br-3) using RQ Manager and Microsoft Excel Software. Different mRNA levels showed a two-fold increase or decrease change in the gene expression. The expression over 100% is up-regulated and below this percentage is down-regulated. Error bars represent the Standard Error of the Mean (SEM) of three replicates.

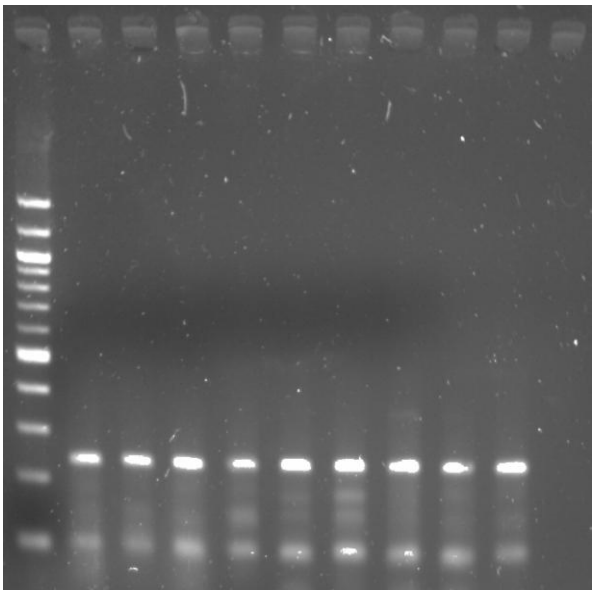
3.3.3 Agarose Gels from qPCR Products:

Figure 3.2 shows the detection of specific gene qPCR products and their molecular sizes (Figure 3.2 and Appendices) by agarose gel electrophoresis. These products are shown after 40 cycles of amplification to evaluate the quality and integrity of these primers.

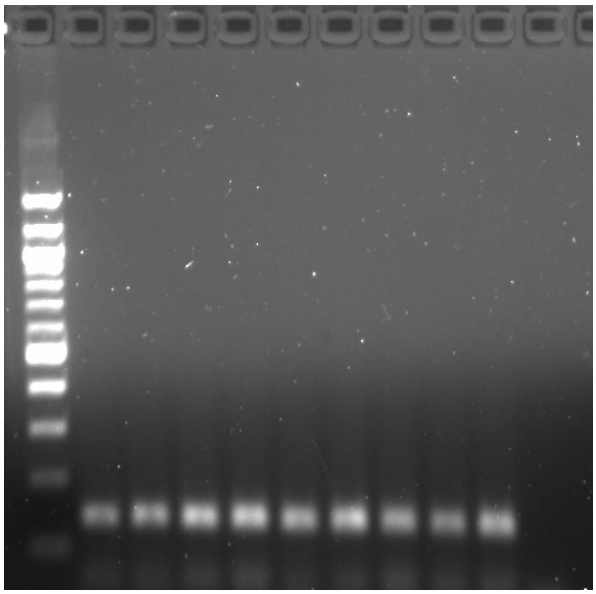
(1) *EGFR* (195 bp): **(2) *PTEN* (106 bp):**



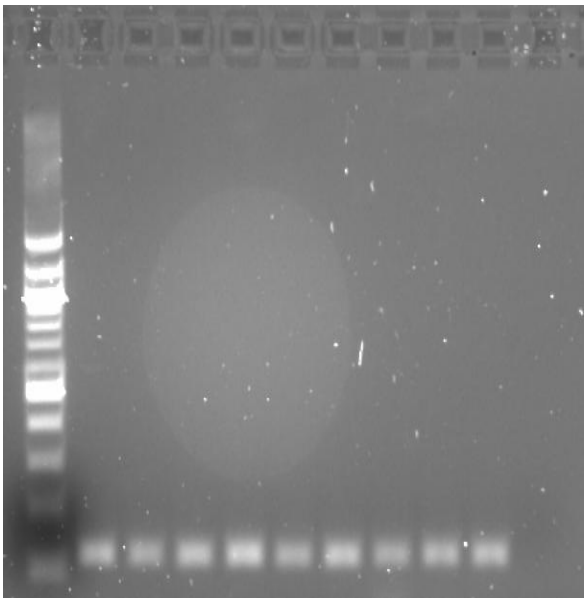
(3) *CCND1* (237 bp):



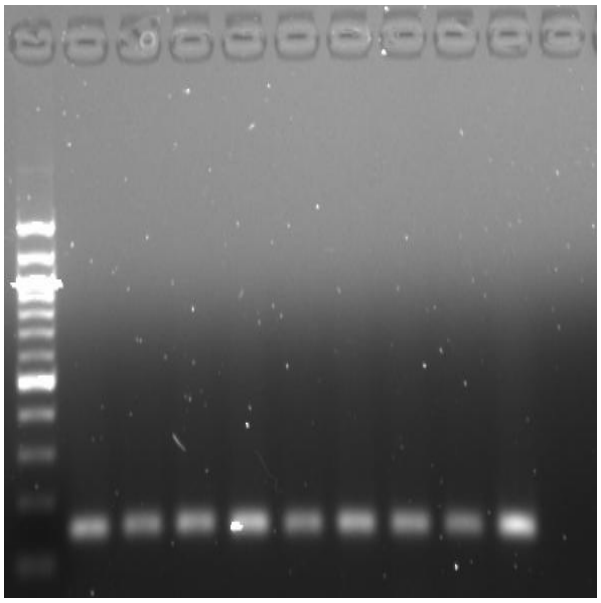
(4) *AKT1* (139 bp):



(5) *HSP90AA1* (120 bp):



(6) *HER2* (148 bp):



(7) *β-Actin* (216 bp):

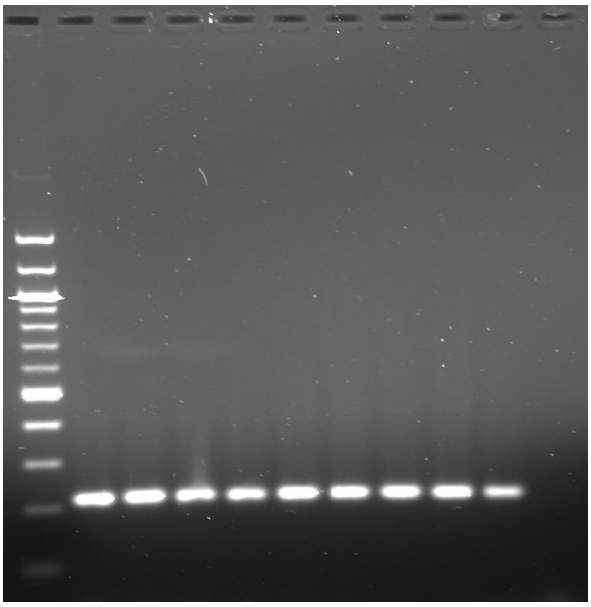


Figure 3.2: Representative Images of Gels Showing the Identification and Specificity of 7 Selected Genes of Interest in Breast Normal and Cancerous Cell Lines, Either Treated or Untreated Cells

Complemetry DNA (cDNA) was synthesised from RNA samples obtained from different cell lines. qRT-PCR experiments were performed using specific primers for each of the 7 target genes as well as to the β-actin as a reference gene. For amplification of each gene, qPCR was also performed in triplicate for positive and negative control at the same time on 96-well plate. To confirm and ensure this amplification has worked properly, the amplicons from each experiment were run on 2% agarose gel. The table above showed the size of the band for each product. Moreover, the dissociation curve (DC) can also confirm this accuracy, efficiency, specificity, and validity of the cDNA (Data not shown). ***The Key:** M= Marker (100 bp Ladder, BioLabs), 1=T-47D, 2=BT-474, 3=GI-101, 4=Sk-Br-3, 5=MCF-10A, 6=T-47D (12uM/48hours), 7=MCF-10A (12uM/48hours, lovastatin), 8=MCF-10A (24uM/48hours, lovastatin), 9=BT-474 (12uM/48hours, lovastatin), and 10=-ve Control (H_2O). Note: Lanes 6-9 include cells treated with lovastatin (see Chapter 5).

3.4 Discussion:

Both chromosomes and genes are organised in a non-random position in interphase nuclei (Croft *et al.*, 1999 and Cremer and Cremer, 2001). Non-random positioning of chromosomal domains relative to each other and to nuclear components is a common feature of eukaryotic genomes. Different studies showed that this organisation of the genome within the nucleus is specific, non-random, dynamic, and changes during cell life span (Bridger *et al.*, 2000 and Meaburn *et al.*, 2007a). This spatial organisation of the genome has been suggested to be the controller of all nuclear processes and functions (Lanctôt *et al.*, 2007).

The distribution of gene loci relative to the nuclear periphery has been linked to both transcriptional activation and repression. Nuclear pores and integral nuclear membrane proteins are key players in the dynamic organisation and regulation of the genome in the nucleus as well as recent advances in our understanding of the molecular networks that organise and regulate genomes at the nuclear periphery led to a further role for non-random locus positioning in DNA repair, remodeling, nuclear function and stability (Mekhail and Moazed, 2010).

In most cells the peripheral chromatin, which includes a large fraction of the heterochromatin, is associated with the nuclear lamina. Previous studies showed that lamin B associates with the chromatin in mammalian cells and control their localisation and gene expression (Malhas *et al.*, 2007). In *Drosophila* cells the centromeres, telomeres, and heterochromatin are almost always associated with the nuclear lamina (Sáez-Va´squez and Gadal, 2010). In addition, in both *Drosophila* and mammalian cells the B-type lamins interact in higher frequencies with gene-poor regions of the genome (Sáez-Va´squez and Gadal,

2010). The mechanisms that control gene activation at the nuclear pore are not well understood.

The hypothesis in this chapter is that the gene expression is changed by alteration of the spatial position of the genes in the cancer cells as compared to the control. Chapter 2 studied the chromosomes and gene positioning which are differentially localised in breast cancer and normal cell lines (Chapter 2). Since the alteration in position of these chromosomes and genes are observed with different cells, together the selection of genes on chromosomes was studied by dual colour FISH and found that some genes were localised on their chromosomes and some are on loops and away from their chromosomes (Chapter 2). In order to study the expression levels of these genes, the transcriptional activity of each gene was assessed by q-PCR as shown in figure 3.1.

Table 3.4: To summarise the comparison and correlation between *EGFR*, *PTEN*, *CCND1*, *AKT1*, *HSP90AA1*, and *HER2* gene positions and their change in expression in each cell line (T-47D, BT-474, GI-101, and Sk-Br-3) when compared with normal cell line (MCF-10A):

	MCF-10A	**T-47D**	**BT-474**	**GI-101**	**Sk-Br-3**
EGFR	Peripheral	↓ Peripheral	↓ Peripheral	↑ Interior	↑ Peripheral
PTEN	Interior	↑ Peripheral	↑ Peripheral	↑ Interior	↓ Interior
CCND1	Interior	↓ Peripheral	↓ Peripheral	↑ Interior	↓ Interior
AKT1	Interior	↑ Peripheral	↑ Peripheral	↑ Interior	↑ Interior
HSP90AA1	Peripheral	↑ Peripheral	↑ Interior	↑ Interior	↑ Interior
HER2	Interior	↑ Peripheral	↑ Peripheral	↑ Interior	↑ Peripheral

The Key: ↑ = up-regulated ↓ = down-regulated

With respect to the hypothesis there are nine situations where genes in the interior are up-regulated as compared to the control and four situations where genes at the periphery are down-regulated. Against the hypothesis there are also nine situations where genes are positioned at the nuclear periphery and are up-regulated with two situations where genes in the interior are down-regulated. So it seems in cancer cells that spatial positioning of genes does not fit with the hypothesis that genes at the nuclear periphery are down-regulated and ones in the interior are up-regulated. In the literature there are a number of examples that show genes in normal cells being down-regulated at the nuclear periphery and *vice versa* for the interior (Bourne *et al.*, 2013). Three genes are up-regulated in all cancer cell lines, *HER2, HSP90AA1* and *AKT1*. All genes were up-regulated in GI-101 cells.

The spatial organisation of the genome can influence gene expression in normal cells. However, cancer cells may be more difficult to fit gene position with expression since there is a more chaotic distribution of the genome. Thus more analysis is required to investigate the link between the position of the gene and its expression in cancer. From these results, the location of genes and chromosomes within different cells might be due to changes in the interaction of chromosomes and genes with the nuclear components (the nuclear lamina, transcription factories, splicing speckles, PML bodies, nucleoli, and Cajal bodies) Chromosomes found at the nuclear periphery and the genes on these chromosomes might be located on the inner site of these chromosomes and away from the nuclear periphery or on loops extending away from their chromosomes into the nuclear interior. It is possible that these changes in position are either of a global re-organisation of chromosomes in breast normal, and cancer, cells or due to other re-positioning events. Other factors may participate in transcriptional activity, regulation, and spatial organisation of the genome such as epigenetic modification.

Transformed and tumour cells exhibit alterations in nuclear morphology as well as in the distribution of genes and regulatory factors. The functional relationships of nuclear structure and gene expression is found to be more related and linked to tumour modifications in nuclear organisation and gene regulation during the progression of cancer (Stein *et al.*, 2000). The finding that changes in gene position are correlated with changes in gene expression mean that if gene position can be controlled then perhaps gene expression can be controlled as well which would open up a whole new field of treatments for cancer patients. The cells in this study do have changes in nuclear morphology and this may lead to a re-organisation of the genome. In diseases of the nuclear lamina nuclei are mis-shapened and genome organisation is affected (Mehta *et al.*, 2011). The next step would be to analyse nuclear structures such as the nuclear lamina to see if they are affected in these cells.

3.5 Conclusion:

In conclusion, my data show that a change in gene positions in breast cancer cells is correlated with a change in gene expression. This alteration in expression is most noticeable in T-47D and BT-474 cell lines in comparison to MCF-10A normal cell lines. The correlation between gene position and expression could probably uncover many of the secrets of non-random genome organisation and its role in gene regulation and expression, genome stability and disease such as cancer.

Chapter 4: Aberrant Distribution and Presence of Nuclear Envelope Proteins in Breast Cancer Cell Lines

Manuscript in prep 2: Hassan Ahmed M; Harvey M; Bridger JM.

4.1 Introduction:

The nuclear envelope, which forms the border of the nucleus in eukaryotic cells, restricts nuclear metabolism activities and helps to organise nuclear structure (Hetzer, 2010). Genetic and cell biological studies indicate that the nuclear lamina, part of the nuclear envelope, is a structural scaffold that provides mechanical strength to the nucleus and helps to maintain nuclear shape. The lamina is also involved in tethering chromatin to the nuclear envelope and therefore this implicates the lamina in the regulation of gene expression. The importance of the lamina in cells is emphasised by recent work showing that over 20 inherited human diseases "laminopathies" are caused by mutations in lamins. Many laminopathies target specific tissues, such as skeletal muscle and heart, adipose tissue, or bone and connective tissue and therefore, these mutations target nuclear envelope proteins (lamins) and affect many cell types (Prokocimer *et al.*, 2009).

Transmembrane proteins of the inner nuclear membrane (INM) are also involved in chromatin regulation and nuclear envelope (NE) function. For example, lamin B receptor (LBR) protein in mammals binds to HP1 which is involved in heterochromatin formation. Moreover, LAP2β, emerin, and MAN1 proteins have LEM domain. This LEM domain binds BAF which is involved in chromosome organisation and nuclear assembly. Indeed, multiple INM proteins, including LEM domain proteins, have been found to directly bind to transcriptional regulators. These proteins have roles in signal transduction at the nuclear envelope (Holaska, 2002).

4.1.1 Lamin B1 is Required for RNA Synthesis and Gene Expression in Human Cells:

A study from Goldman and colleagues provides data to link the organisation of the lamin network to gene expression in somatic mammalian cells (Spann *et al.*, 2002). The loss of lamin B1 is also correlated with a loss of transcriptional activity (Dechat *et al.*, 2008; Malhas *et al.*, 2009). During the early stage of lamin B1 depletion, pre-rRNA synthesis in nucleoli was unaffected. However, at later stages, pre-rRNA synthesis was inhibited and changes in nucleolar morphology seen. This indicates that lamin B1 is required for assembly of active sites of RNA synthesis (Tang *et al.*, 2008). In contrast, reducing the level of lamin A/C had no obvious effects on transcription by either RNA polymerase I or II (Vergnes *et al.*, 2004). Moreover, it is the loss of lamin B1 but not lamin B2 resulted in transcriptional inhibition (Tang *et al.*, 2008).

4.1.2 Defects in Lamin B1 Expression or Processing Affect Interphase Chromosome Position, Gene Expression, and Other Nuclear Functions:

Association of lamin B1 with the nuclear lamina is dependent on processing of its CAAX motif, this reflex the importance of post-translational modifications for stable lamina assembly (Maske *et al.*, 2003). The loss of lamin B1 makes an unstable lamina when CAAX farnyesylation, endoproteolysis, or carboxymethylation does not occur (Chapter 1).

From previous studies, cells with defects in lamin B1 expression or in the CAAX processing machinery exhibit altered gene expression levels, as a result of the absence of processed lamin B1 in the lamina (Malhas *et al.*, 2007). Others found that unprocessed lamin B1 in the nuclear interior plays an important role in regulating gene expression (Kennedy *et al.*, 2000; Hutchison and Worman, 2004). Lamin B1 might associate with the machinery of transcription and RNA processing similar to that observed for lamin A within the

nucleoplasm. However, a more indirect effect via specific transcription factors is suggested by lamin B1 binding to the transcriptional repressor protein, Oct1, (Imai *et al.*, 1997). This binding could lead to regulation of gene expression that is lost when processed lamin B1 is missing at the nuclear periphery. Malhas *et al.*, in 2007 suggested that the presence of up-regulated genes at the nuclear periphery might be due to disruption of peripheral lamin localisation because of the absence of proteolysed lamin B1. Malhas *et al.*, in 2007, found that chromosome 18 is located at the nuclear periphery in three independent wild type (WT) mouse embryo fibroblast populations. This peripheral localisation was observed in negative isoprenylcysteine carboxyl methyltransferase cells (*ICMT*$^{-/-}$), but in both deleted *LMNB* (*LMNB1*$^{-/-}$) and ras converting enzyme 1 (*Rce1*$^{-/-}$) cells, it became centrally located, like chromosome 19. This suggests that farnesylated and proteolysed lamin B1 anchors chromosome 18 to the periphery and that carboxymethylation of lamin B1 is not important for chromosome positioning. The authors also showed that this positioning played an important role in the expression of genes on this chromosome. Chromosome 19, on the other hand, showed a central location in the WT cells and in all the knockouts and none of its genes were up-regulated in both *LMNB1*$^{-/-}$ and *Rce1*$^{-/-}$ cells. Therefore, the genes that are disregulated in the absence of processed lamin B1 are specific to defects on the enzymes that are involved in farnesylation and are not due to abnormality in the nuclear lamina. Furthermore, the change in chromosome position as a result of the absence of lamin B1 or its processing is specific to lamin B1 (Malhas *et al.*, 2007).

From literature, it can be seen that there is clear difference in the roles played by A- and B-type lamins with both lamin protein contributing to the structure of lamin protein network. Lamin B1 is essential to maintain correct levels of gene expression, chromosome position, and other nuclear functions.

4.1.3 The Nucleolus and Other Bodies:

4.1.3.1 The Nucleolus:

The nucleolus is a non-membranous structure within the nucleus and is composed of proteins and nucleic acids. It is considered to be 25% of the total volume of the nucleus. It is involved in the biogenesis and transcription of ribosomal RNA (rRNA). There are three main components of the nucleolar structure: the fibrillar centres (FC), the dense fibrillar component (DFC), and the granular component (GC). Nucleoli are formed around specific genetic loci called Nucleoli Organising Regions (NORs) (Maggi and Weber, 2005; Martin *et al.*, 2009). The NORs are composed of tandem repeats of rRNA genes. The human genome contains more than 200 copies of rRNA genes on five different chromosomes (13, 14, 15, 21, and 22) (Arsuaga *et al.*, 2004). The rRNA gene consists of a promoter and coding sequences (18S, 5.8S, and 28S). The nucleolus is involved in making rRNA, genome organisation and chromosome positioning (Martin *et al.*, 2009). Recent studies demonstrated that lamin B expression is required to maintain the architecture and functional plasticity of nucleoli (Raz *et al.*, 2006; Chi *et al.*, 2009).

4.1.3.2 Promyeolocytic Leukaemia (PML):

Promyeolocytic leukaemia (PML) bodies also known as ND10 domains (Bridger *et al.*, 1998a) and Kremer bodies contain the PML protein which is encoded by *pml* gene (Stuurman *et al.*, 1992; Bernardi and Pandolfi, 2007). PML bodies position non-randomly in the nucleus with respect to chromatin and other sub-nuclear structures. Over-expression of small ubiquitin-like modifier (SUMO-1) prevents stress that induces the disassembly of PML bodies, implicating SUMO-1 as an important factor to regulate PML body integrity, organisation and function. Down regulation of SUMO-1 may be the mechanism to change PML body association with local chromatin domains. PML bodies may provide useful

information for the dynamics and integrity of other important molecular proteins involved in nuclear processes such as transcription, RNA processing, DNA repair and replication ((Dundr and Misteli, 2001; Eskiw *et al.*, 2003; Bernardi and Pandolfi, 2007). They are heterogeneous and dynamic structures implicated in tumour suppression in leukemia and cancer pathogenesis as well as implicated in induction of apoptosis and cellular senescence, inhibition of proliferation, maintenance of genomic stability, DNA repair and antiviral responses (Bernardi and Pandolfi, 2003; Dellaire *et al.*, 2006). They are found near other nuclear bodies such as Cajal bodies, splicing speckles and nucleoli (Bernardi and Pandolfi, 2007). Therefore, PML bodies play a pivotal role in nuclear functions, organisation, and gene expression.

4.1.3.3 The Ki-67 Protein (Proliferation Marker):

The Ki-67 antigen was identified by Gerdes *et al* in 1983 as a nuclear non-histone protein and highly susceptible to protease treatment, in the city of Kiel, Germany (hence "Ki") after immunisation of mice with the Hodgkin's lymphoma cell line L428 (67 refers to the clone number on the 96-well plate in which it was found) (Gerdes *et al.*, 1983). It belongs to a class of antigens associated with the structural organisation of metaphase and anaphase chromosomes, and proteins located near the regions of prenucleolar bodies and nucleoli (van Dierendonck *et al.*, 1989). Furthermore, Ki-67 antigen associates with a fibrillarin-deficient region of the dense fibrillar component of the nucleolus. Integrity of this region is lost following either nucleolar dispersal or nucleolar segregation (Kill, 1996). The *Ki-67* gene is on the long arm of human chromosome 10q25 (Fonatsch *et al.*, 1991). During G1, Ki67 expression is in low levels and this is increased during S-phase and G2 to reach maximum expression during mitosis of cell cycle. Ki67 is associated with the nucleolar region in larger foci as well as with some heterochromatin regions (Gerdes *et al.*, 1984; van Dierendonck *et*

al., 1989; Bridger *et al.*, 1998b; Scholzen and Gerdes, 2000; Urruticoechea *et al.*, 2005). Ki67 is absent in quiescent cells (Gerdes *et al.*, 1984; Gerdes, 1990; Gerdes *et al.*, 1991).

4.1.4 B-type Lamins and Lamin B Receptor are Affected in Human Breast Cancer Cells:

Due to the alterations in gene and chromosome position, as well as gene expression revealed in the breast cancer cell lines it became pertinent to assess the structural integrity of the nuclei in these cells. Thus, the presence and distribution of a range of nuclear envelope proteins was assessed in all the cancer cell lines under study. It was found that nuclear structure was hugely affected in these cells with some cells lacking B-type lamins and their receptor LBR. Cells displayed large aggregates of lamin B inside the nucleoplasm and it was found that these aggregates colocalised, at some level, with PML bodies and nucleoli. With these data collaborators at the London Breast Clinic screened 183 breast samples and found that severity, prognosis were correlated with a decrease in lamin B and LBR. Further survival of women with breast cancer was positively correlated with the extent of lamin B expression (Wazir, Hassan Ahmed; Harvey; Bridger; Sharma and Mokbel (2013), The Clinicopathological Significance of Lamin A, Lamin B and Lamin B receptor mRNA Expression in Human Breast Cancer in preparation).

The goal of this chapter was to understand the presence and distribution of nuclear envelope proteins in breast cancer cell lines. To help this understanding, experiments have assessed and analysed distribution and expression of nuclear envelope proteins including nuclear lamins (lamins A, C, A/C, B1, and B2), lamin B receptor (LBR), lamin binding proteins, PML, Ki67, and nucleolin in breast cancer (T-47D, BT-474, Sk-Br-3, and GI-101) and compared their localisation to normal breast cell line (MCF-10A) using

indirectimmunofluorescence (IIF). This investigation followed by gene expression assessment to *LMNB1*, *LMNB2*, and *LBR* using qRT-PCR. These data reveal aberrant and unequal distribution of these proteins between different cell lines.

4.2 Material and Methods:

4.2.1 Cell Culture:

As per chapter 2, Section 2.2.1.

4.2.2 Indirect Immunofluoresence:

4.2.2.1 Cell preparation:

The human breast cancer (Sk-Br-3, GI-101, T-47D and BT-474) and normal breast (MCF-10A) cell lines were prepared for indirect immunofluorescence. The cells were grown and seeded onto sterile 13 mm glass coverslips in plastic petri dishes. They were grown and cultured under the standard conditions in appropriate medium and supplemented with 10% fetal calf or bovine serum (FC/BS) with/without additives according to the cell lines needs and maintained in a humidified environment at 37ºC with 5% CO_2. The cells were allowed to proliferate for 3 days prior to fixation.

4.2.2.2 Cell Fixation:

Cells on coverslips were prepared for fixation and staining techniques using different fixation procedures. Firstly, the medium was removed and then the cells were washed 3 times with 1X phosphate buffer saline (PBS) to be ready for fixation. There were two different fixation procedures used; (1) cells were fixed with fresh cold methanol: acetone (1:1, v/v) for 10 minutes on ice to precipitate the proteins and permeablise the cells followed by 3 washes in 1X PBS; and the other method (2) cells were fixed with paraformaldehyde 4% (w/v) for 4 minutes at room temperature (RT) followed by 3 washes in 1X PBS and then detergent was added to the dish 1% Triton-X100 in 1X PBS (Sigma-Aldrich) for 10 minutes at RT and then washed again 3 washes with 1X PBS.

4.2.2.3 Primary and Secondary Antibodies Immuno-reactivity:

After fixation, cells were prepared for indirect immunofluorescence. The primary monoclonal (mAbs) and polyclonal antibodies were raised in goats, mice and rabbits. They were diluted in 1% newborn calf serum (NCS, v/v) in 1X PBS according to the manufacturer's instructions. The cells on coverslips were covered with 20 µl of the diluted primary antibody and then incubated at 4ºC overnight, 37ºC for 30 minutes or RT for 1 hour. The primary antibodies that were used at final concentration were; goat anti-Lamin A antibody (diluted 1:100, Santa Cruz Biotechnology), mouse anti-Lamin B1 antibody (diluted 1:50, Abcam), mouse anti- Lamin B2 antibody (diluted 1:500, Abcam), rabbit anti-LAP2α antibody (diluted 1:250, Abcam), goat anti-MAN1 antibody (diluted 1:100, Santa Cruz Biotechnology), mouse anti-Lamin A/C antibody (diluted 1:100, Santa Cruz Biotechnology), rabbit anti-Lamin C antibody (diluted 1:50, Novocastra), goat anti-Emerin antibody (diluted 1:100, Santa Cruz Biotechnology), mouse anti-nucleolin antibody (diluted 1:200, Abcam), rabbit anti-PML antibody (diluted 1:100, Chemicon), rabbit anti-Lamin B receptor antibody (diluted 1:500, Abcam), rabbit anti-Ki67 antibody (diluted 1:1500, Novocastra), mouse anti-LAP2 antibody (diluted 1: 100, Abcam), and mouse anti-Lamin B2 antibody (diluted 1:100, Novocastra). After incubation for 1 hour at RT, the cells on glass coverslips were washed 3 times in 1X PBS and then covered with 20 µl of the diluted appropriate secondary antibody at final concentration using donkey anti-goat (FITC) antibody (diluted 1:50, Jackson), donkey anti-mouse (FITC) antibody (diluted 1:70, Jackson), Cy3 conjugated donkey anti-mouse antibody (diluted 1:100, Jackson), Cy5 conjugated donkey anti-goat antibody (diluted 1:100, Jackson) and swine anti-rabbit (TRITC) antibody (diluted 1:30, DAKO). The cells were incubated for 1 hour at room temperature (RT) in the dark. The cells on coverslips were washed again in triplicate in 1X PBS followed by rinsing in double distilled water (ddH_2O). Single, Dual and Triple colour experiments were performed to observe co-localisations.

4.2.2.4 Mounting Cells:

Cells were mounted onto glass microscope slides using Vectashield (Vecta Labs) mounting medium containing 4`, 6-diamidino-2-phenylindole (DAPI, 1.5 µg/ml). Mounted slides were viewed and images captured by Fluorescence Microscopy. These slides were stored at 4ºC and protected from light in a black box.

4.2.2.5 Evaluation and Detection using Microscopy and Images Analysis:

Fluorescence microscopy was performed with a microscope (Olympus BX41 with Smartcapture 3 Software) with a CCD digital scientific camera. All slides were examined under 100X Olympus UPLAN FLN lens immersion oil (Immersol)™ and images captured with the camera and previewed on Macintosh Apple Computer (MAC) operated by SmartCapture3 programme. Cells were screened to analyse the presence and distribution of LAP2α, MAN1, lamin A/C, lamin C, emerin, lamin A, lamin B1, lamin B2, lamin B receptor, PML, nucleolin, LAP2, and Ki67 proteins.

4.2.3 qRT-PCR:

As per chapter 3, section 3.2.1

4.2.3.1 Gene Description:

4.2.3.1.1 *LMNB1* and *LMNB2* (*Lamins B1* and *B2*, Respectively):

Lamins are reviewed in more details in Chapter 1.

4.2.3.1.2 *LBR* (*Lamin B Receptor*):

Gene Symbol	Gene Location	Gene Description
LBR gene	located on 1q42.12 (OMIM)	It encodes the lamin B receptor, an inner nuclear membrane protein that binds lamin B and has been characterised in avian cells (Worman *et al.*, 1990). The nuclear envelope is composed of the nuclear lamina, the nuclear pore complexes, and the nuclear membranes. The nuclear lamina, a meshwork of intermediate filament proteins termed lamins, may interact directly with the chromatin. Several integral proteins, including lamin B receptor (LBR), of the nuclear inner membrane that may be associated with the lamina and the chromatin have been identified (Ye and Worman, 1994 and Chapter 1).

4.2.3.1.3 *β-Actin* (*ACTB*):

β-Actin was reviewed in details in chapter 3.

Table 4.1: The List of Primers *LMNB1, LMNB2, LBR*, and *β-Actin* Genes for qRT-PCR:

Forward and reverse primers as well as melting temperature used for each set of primer are displayed in the table below:

Primer Name	Primer Sequence (5` → 3`)	Product Length (bp)	%GC	Tm (°C)
LMNB1 (F)	AGGATCAGATTGCCCAGTTG	125	50.00	60.07
LMNB1 (R)	GCGAAACTCCAAGTCCTCAG		55.00	59.99
LMNB2 (F)	AAGGACCTGGAGTCCCTGTT	153	55.00	59.97
LMNB2 (R)	CTTCTCCAGCTGCTTTTTGG		50.00	60.13
LBR (F)	CCGTGAATTAAACCCTCGAA	128	45.00	59.93
LBR (R)	CGCGGTCCTGTATTTTCATT		45.00	59.96
β-Actin (F)	AAGAGAGGCATCCTCACCCT	216		
β-Actin (R)	TACATGGCTGGGGTGTTGAA			

4.2.3.2 Primers Dilution:

As per chapter 3, section 3.2.2 – 3.2.3

4.2.3.3 RNA Extraction:

As per chapter 3, section 3.2.4 - 3.2.6

4.2.3.4 Reverse Transcriptase - Polymerase Chain Reaction (RT-PCR):

As per chapter 3, section 3.2.7 - 3.2.8

4.2.4 Western Blot:

This is an important method that was used to assess the expression and detection of different proteins (lamin B1, lamin B2, LBR, LAP2 isoforms, and MAN1). In this study, it is necessary to mention that the experiment was performed by starting with lysates preparation, loading $5X10^5$ cells per well, bands separated, proteins transferred on nitrocellulose membrane, blocking of the blots, detection of these proteins using commercial primary and secondary antibodies, and then processed with substrate and enhancer to develop colour and visualise the bands using BIO-RAD gel document system supported by IPLAB software.

This experiment was repeated more than 11-12 times by changing the protocol (changes to antibody concentration, marker, blocking solution, timing, detection method, method of lysates preparation, and concentration of loading samples) but it did not work properly (Data not shown).

4.3 Results:

The genetic basis of cancer has been investigated at the structural and genomic levels, and many fundamental questions have begun to be addressed. The molecular basis for the link between nuclear deformation and malignancy has not been determined (Capo-chichi *et al.*, 2011). Nuclear lamins and lamin binding proteins are essential components of nuclear architecture and genome organisation and they are essential for correct gene expression and normal functions of cells and the loss of this organisation or aberrant distribution in lamins, lamin binding proteins, other important nuclear compartments, and genome are associated with cancer.

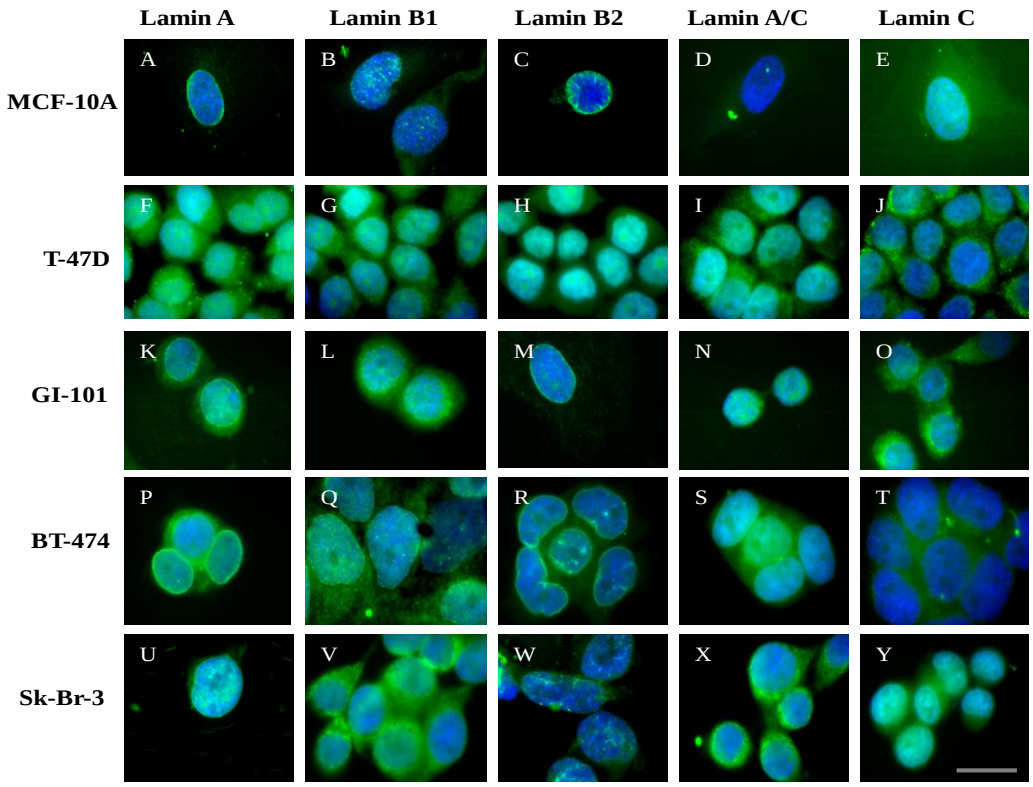
4.3.1 Distribution of Lamins and Lamin Binding Proteins by Indirect Immunofluorescence (IIF):

The study in this chapter aims to assess and analyse the presence and distribution of lamins and lamin binding proteins in human normal breast MCF-10A and breast cancer cell lines T-47D, GI-101, BT-474, and Sk-Br-3. This was determined by indirect immunofluorescence using a range of antibodies (see material and methods). The cell lines were fixed with methanol: acetone (1:1) and paraformaldehyde 4% (Figures 4.1 and 4.2, respectively) and the results showed how the cell nuclei influenced by the type fixation. **Metanol: acetone:** it is organic fixative and perfect fixation that can immoblise the proteins. This type of fixation can remove lipids and dehydrate the cells as well as precipitating proteins on the cellular architecture. **Paraformaldehyde 4%:** it is cross-linking reagent and form intermolecular bridges, thus creating a network of linked antigens which is better than organic solvents (keep or preserve the cell structure). However, this type of fixation may reduce the antigenicity of some cell components and require the addition of Triton-X100 to increase the permeability of cells and allow access of antibodies to inside the cells. Both methods may denature proteins

and this is the reason why I used antibodies against these proteins which is more useful for cell staining.

(1) Cell Fixation with Paraformaldehyde 4%:

Panel (I):



Panel (II):

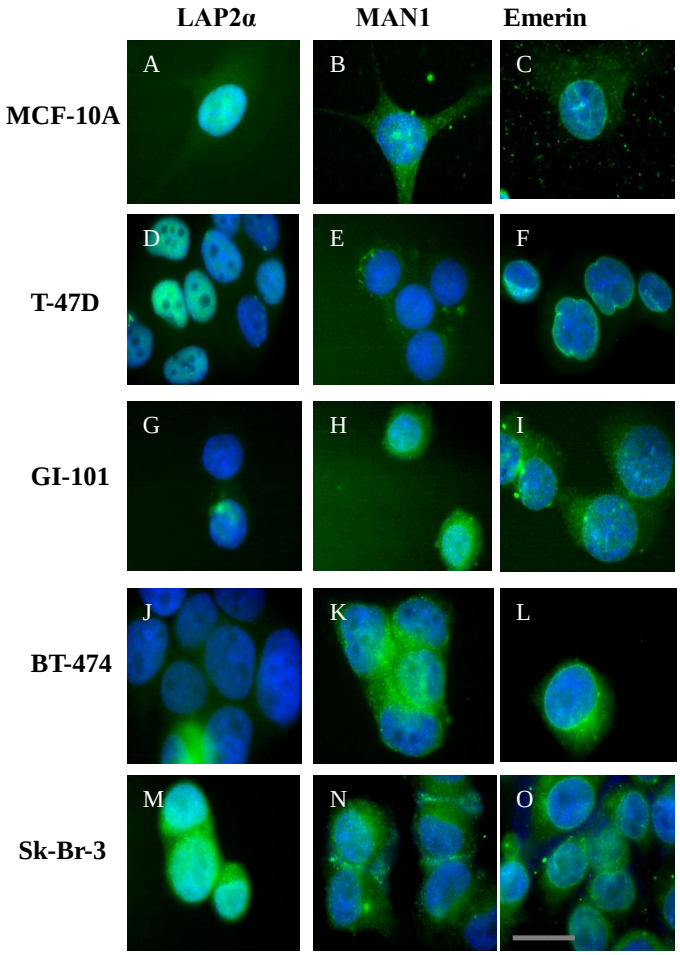
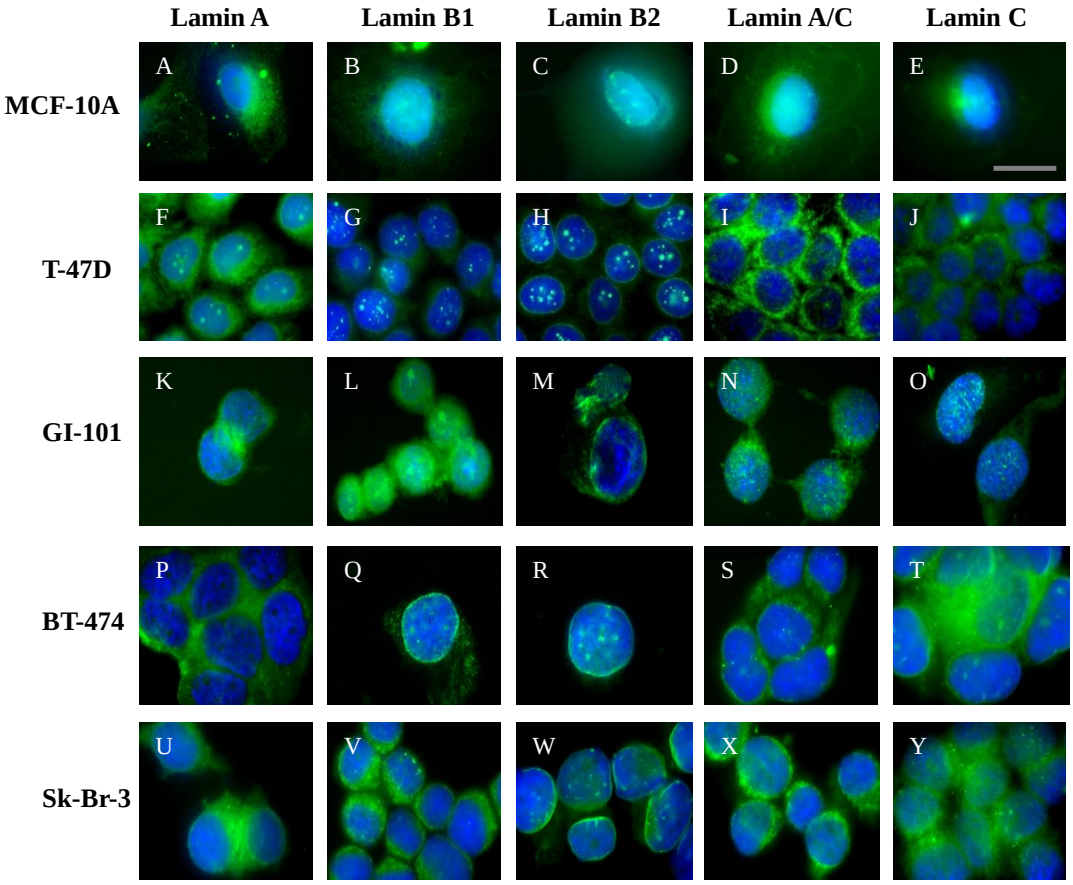

LAP2α
MAN1
Emerin
MCF-10A
T-47D
GI-101
BT-474
Sk-Br-3
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O

Figure 4.1: Different Distributions of Nuclear Lamins and Lamin Binding Proteins: Cells were fixed with 4% paraformaldehyde and lamin and lamin binding protein distributions were processed and determined by indirect immunofluorescence method. The DNA was stained with DAPI (Blue). **Panel (I):** demonstrates the images of MCF-10A cells when immunostained with anti-lamin A, anti-lamin B1, anti-lamin B2, anti-lamin A/C, and lamin C antibodies (A-E), T-47D cells (F-J), GI-101 cells (K-O), BT-474 cells (P-T), and Sk-Br-3 cells (U-Y) with same antibodies. **Panel (II):** displays representative images of MCF-10A when immunostained with anti-LAP2α, anti-MAN1, and anti-emerin antibodies (A-C), T-47D cells (D-F), GI-101 cells (G-I), BT-474 cells (J-L), and Sk-Br-3 cells (M-O) with same antibodies. The secondary antibodies at final concentration were donkey anti-goat (FITC) antibody, donkey anti-mouse (FITC) antibody, and swine anti-rabbit (TRITC) antibody. Cells were mounted using Vectashield mounting medium containing DAPI to facilitate its visualisation and detection, Magnification = X100 and Scale Bar = 10μM.

(2) Cell Fixation with Methanol: Acetone, 1:1

Panel (I):



Panel (II):

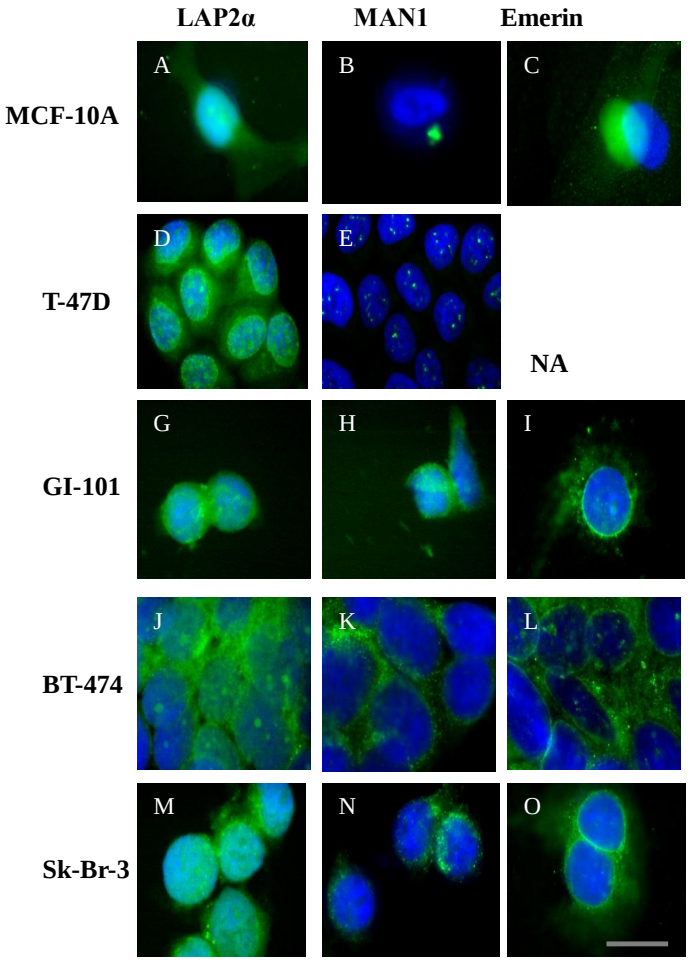

LAP2α
MAN1
Emerin
MCF-10A
T-47D
GI-101
BT-474
Sk-Br-3
A
B
C
D
E
NA
G
H
I
J
K
L
M
N
O

Figure 4.2: Different Distributions of Nuclear Lamins and Lamin Binding Proteins: Cells were fixed with methanol: acetone (1:1) and lamin and lamin binding protein lamins distributions were processed and determined by indirect immunofluorescence method. The DNA stained with DAPI (Blue). **Panel (I):** demonstrates the images of MCF-10A cells when immunostained with anti-lamin A, anti-lamin B1, anti-lamin B2, anti-lamin A/C, and lamin C antibodies (A-E) as well as T-47D cells (F-J), GI-101 cells (K-O), BT-474 cells (P-T), and Sk-Br-3 cells (U-Y) with same antibodies. **Panel (II):** displays representative images of MCF-10A when immunostained with anti-LAP2α, anti-MAN1, and anti-emerin antibodies (A-C), T-47D cells (D and E), GI-101 cells (G-I), BT-474 cells (J-L), and Sk-Br-3 cells (M-O) with same antibodies. The secondary antibodies at final concentration were donkey anti-goat (FITC) antibody, donkey anti-mouse (FITC) antibody, and swine anti-rabbit (TRITC) antibody. Cells were mounted using Vectashield mounting medium containing DAPI to facilitate its visualisation and detection, Magnification = X100 and Scale Bar = 10μM.

4.3.2 Distribution Analyses of Lamins and Lamin Binding Proteins by Indirect Immunofluorescence:

In order to analyse the results from these experiments, 200 nuclei were scored for each antibody, in each cell line. The experiment was performed in triplicate for each sample. The analysis was performed and nuclei scored for different categories rim only, rim and spot, homogenous, spots only - either a few large or many small spots and negative. These proteins should all be found at the nuclear rim except for LAP2α which is found as throughout the nucleoplasm. The results from this study showed that the nuclear architecture of breast cancer cell nuclei is often altered and this could be important for diagnosis and prognosis, as well as correct genome organisation and control of gene expression. Tables 4.4 - 4.11 display the percentages of these characteristics in each of the cell lines.

There are clear differences in nuclear lamins presence and distribution between these cell lines and we noticed that the most severely affected cells were T-47D and BT-474 when stained with lamin B1 and lamin B2 and fixed with methanol: acetone (1:1). It is found that lamin B1 and B2 proteins were found in large foci inside the nucleoplasm (Figure 4.2, Panel

I: G, H and Q, R). The pattern of anti-lamins and lamin binding proteins were different between cell lines. They had strong rim staining in some cells which was absent in others. Some cells showed positive staining throughout the nucleoplasm (homogenous) and some cells were negative.

Lamin B1 Distribution:

The distribution of lamin B1 in normal cells in interphase nuclei is localised at the nuclear periphery as a rim structure with a few small foci in some cells. These cancer cells showed different distributions of this protein. It is found that 87% of MCF-10A cells displayed rim of lamin B1 distribution and 13% of cells had a homogeneous stain within their nuclei after cells fixation with methanol: acetone and 83% of cells had rim and spots after fixation with paraformaldehyde 4% whereas 17% of these cells had many spots. T-47D cells showed lamin B1 distribution as many spots in 100% when fixed with paraformaldehyde and 10% of cells after fixation with methanol: acetone, 1:1. It was found that there were large foci in these cells (25%) when fixed with methanol: acetone as well as rim and spots (65%). GI-101 cells had nuclei with many spots when fixed with paraformadehyde (90%) as well as cells with large foci when fixed with methanol: acetone (55% of cells displayed large spots) as well as many spots (15%). BT-474 cells had lamin B1 in rim and spots within the nucleoplasm with a high percentage 72% when fixed with methanol: acetone and 33% when fixed with paraformadehyde whereas the same cells have displayed the protein around the nuclear membrane (28%) and within the nucleoplasm as many spots (67%) when fixed with methanol: acetone and paraformaldehyde, respectively. Moreover, Sk-Br-3 cells showed an abnormal distribution of the protein with 70% of the cells having many spots when fixed with methanol: acetone and paraformaldehyde although some of nuclei showed abnormal homogeneous in both fixations. 20% of these nuclei were negative after fixation with

methanol: acetone. The large aggregates of lamin B1 observed were widely distributed throughout the nucleoplasm. This is indeed an aberrant distribution of lamin B1 and may well lead to affected genome organisation and gene expression as seen in Chapters 2 and 3. Due to their unusual shape lamin B1 may have been associated with other nuclear structures such as PML bodies or nucleoli.

Table 4.2: The Percentages of Lamin B1 Distribution:

	MCF-10A		T-47D		GI-101		BT-474		Sk-Br-3	
Type of Fixation	**MA**	**P**	**MA**	**P**	**MA**	**P**	**MA**	**P**	**MA**	**P**
Rim Only	87	0	0	0	30	0	0	0	0	0
Rim & Spots	0	83	65	0	0	0	72	33	0	0
Large Spots	0	0	25	0	55	10	0	0	0	0
Homogeneous	13	0	0	0	0	0	0	0	10	30
Many Spots	0	17	10	100	15	90	28	67	70	70
Negative	0	0	0	0	0	0	0	0	20	0

MA=Methanol: Acetone, 1:1 P=Paraformaldehyde 4%

Lamin B2 Distribution:

All cells were immunostained with anti-lamin B2 antibodies. The distribution of lamin B2 in normal cells during interphase nuclei is localised at the nuclear periphery as a rim structure with a few small foci in some cells. The breast cancer cells displayed different patterns of this protein. The experiment showed that when fixed with methanol: acetone MCF-10A cells had 47% of nuclei had a rim and spots, many spots of lamin B2 distribution (20%) ,17% of cells had large spots and 16% were homogeneous.When these cells were fixed with paraformaldehyde 80% of cells had both rim and spots whereas 20% of cells had the protein as rim only when fixed with (. T-47D cells showed lamin B2 distribution as rim and spots in a high percentage of cells (85%) and 15% of cells had many spots only when fixed with

methanol: acetone. Whereas all cell nuclei showed abnormal homogeneous staining (100%) when fixed with paraformaldehyde. It is also observed that GI-101 cells had rim and spots in 80-85% when fixed with both fixations whereas 15-20% had the protein normally at rim only when fixed with both fixations. In addition, BT-474 cells showed nuclei with rim and spots in 80% when fixed with both fixations and 20% of cells had the protein as normally at rim only when fixed with paraformaldehyde and the same percentage (20%) of cells displayed the protein as many spots when fixed with methanol: acetone. BT-474 cells showed the distribution of the protein as many spots within the nucleoplasm in 20% of cells when fixed with methanol: acetone and 80% of cells showed at rim and spots when fixed with paraformadehyde whereas the same cells had the protein around the nuclear membrane and within the nucleoplasm as rim and spots (80%) when fixed with methanol: acetone. Moreover, Sk-Br-3 cells showed an abnormal distribution of the protein in 76% of cells showing many spots and 24% as rim and spots when fixed with paraformadehyde whereas 80% of cells showed rim and spots and 20% of cells had the protein normally as rim only when fixed with methanol: acetone.

Table 4.3: The Percentage of Lamin B2 Distribution:

	MCF-10A		**T-47D**		**GI-101**		**BT-474**		**Sk-Br-3**	
Type of Fixation	**MA**	**P**	**MA**	**P**	**MA**	**P**	**MA**	**P**	**MA**	**P**
Rim Only	0	20	0	0	15	20	0	20	20	0
Rim & Spots	47	80	85	0	85	80	80	80	80	24
Large Spots	17	0	0	0	0	0	0	0	0	0
Homogeneous	16	0	0	100	0	0	0	0	0	0
Many Spots	20	0	15	0	0	0	20	0	0	76
Negative	0	0	0	0	0	0	0	0	0	0

Lamin A Distribution:

The cells were immunostained with anti-lamin A antibodies and fixed with both type of fixations. The normal distribution of lamin A is localised at the nuclear periphery with some small foci inside (Bridger *et al.*, 1993). The control cells MCF-10A cells displayed a more or less normal distribution of lamin A with all cells having a rim stain with internal spots. However, the distribution of lamin A in T-47D cells showed abnormal large spots (75%) and many spots (25%) when fixed with methanol: acetone with an abnormal homogeneous distribution in 24% of cells and many spots in 76% of cells when fixed with paraformaldehyde. The same percentage was scored in GI-101 with the protein as many spots (75%) and rim only in 25% of cells when fixed with methanol: acetone whereas the same cells had the protein in 26% of cells as many spots and 74% had it as a rim and spots when fixed with paraformaldehyde. BT-474 cells displayed the protein in 75% as many spots and 25% of cells as homogeneous when fixed with methanol: acetone whereas the cells had it in 63% as a rim and spots, 22% as many spots, and 5% of cells as homogenous when fixed with paraformadehyde. 10% of BT-474 cells had the protein distribution as a rim only when fixed with paraformadehyde. The same experiment assessed the protein in Sk-Br-3 cells, and found that half of cells displayed it as abnormal homogeneous and many spots when fixed with methanol: acetone whereas 63% displayed it as abnormal many spots, 7% were homogeneous, and 29% expressed it as rim and spots when fixed with paraformadehyde. Thus lamin A has an aberrant distribution in the breast cancer cells which fits with the data for lamin A expression in Wazir *et al.*, in prep).

Table 4.4: The Percentage of Lamin A Distribution:

	MCF-10A		T-47D		GI-101		BT-474		Sk-Br-3	
Type of Fixation	**MA**	**P**	**MA**	**P**	**MA**	**P**	**MA**	**P**	**MA**	**P**
Rim Only	0	0	0	0	25	0	0	10	0	0
Rim & Spots	100	100	0	0	0	74	0	63	0	29
Large Spots	0	0	75	0	0	0	0	0	0	0
Homogeneous	0	0	0	24	0	0	25	5	50	7
Many Spots	0	0	25	76	75	26	75	22	50	63
Negative	0	0	0	0	0	0	0	0	0	0

Lamin C and A/C Distribution:

The distribution of lamins A and C are as for lamin A with a rim staining around the nucleus and some internal foci. The normal distribution of lamin A/C in cells and interphase nuclei is localised at the nuclear periphery as a rim structure as a main component of the nuclear lamina in differentiated cells. None of the cells in this study (from three separate experiments) showed any normal rim or rim and spots staining. Since the lamin A antibody did reveal normal staining in MCF-10A cells it may be that this antibody is not working optimally. This might also be true for the lamin C antibody which was made for the laboratory and uncharacterised.

Table 4.5: The Percentage of Lamin A/C Distribution:

	MCF-10A		T-47D		GI-101		BT-474		Sk-Br-3	
Type of Fixation	**MA**	**P**	**MA**	**P**	**MA**	**P**	**MA**	**P**	**MA**	**P**
Rim Only	0	0	0	0	0	0	0	0	0	0
Rim & Spots	0	0	0	0	0	0	0	0	0	0
Large Spots	0	20	0	0	0	0	4	0	0	35
Homogeneous	60	0	65	30	0	25	10	75	40	65
Many Spots	40	80	0	70	100	75	86	25	60	0
Negative	0	0	35	0	0	0	0	0	0	0

Table 4.6: The Percentage of Lamin C Distribution:

	MCF-10A		T-47D		GI-101		BT-474		Sk-Br-3	
Type of Fixation	**MA**	**P**	**MA**	**P**	**MA**	**P**	**MA**	**P**	**MA**	**P**
Rim Only	0	0	0	0	0	0	0	0	0	0
Rim & Spots	0	0	0	0	0	0	0	0	0	0
Large Spots	40	25	0	0	10	27	15	10	0	6
Homogeneous	40	75	20	72	15	68	50	80	40	30
Many Spots	20	0	70	28	75	5	35	10	60	64
Negative	0	0	10	0	0	0	0	0	0	0

LAP2α Distribution:

The distribution of LAP2α in the nuclei of normal cells is homogeneously distributed. This distribution was seen for both fixations in the MCF-10A control cells. However, the cancer cell lines showed aberrant LAP2α distribution with LAP2α being found at the rim in T-47D (40%), BT-474 (22%) and a few Sk-Br-3 cells (5%). Other patterns seen in the cancer cell lines and not in the control were large spots/aggregates and many small spots. This may be indicative of the chromatin being affected in these cells since LAP2α is a chromatin binding

protein. The rim staining is unusual and could be because there are proteins missing at the nuclear envelope especially in T-47D and BT-474.

Table 4.7: The Percentage of LAP2α Distribution:

	MCF-10A		T-47D		GI-101		BT-474		Sk-Br-3	
Type of Fixation	**M**	**P**	**M**	**P**	**M**	**P**	**M**	**P**	**M**	**P**
Rim Only	0	0	30	0	0	0	0	0	0	0
Rim & Spots	0	0	10	0	0	0	0	22	0	5
Big Spots	0	0	0	10	40	30	20	10	0	10
Homogeneous	100	100	60	0	38	60	39	58	20	42
Many Spots	0	0	0	90	22	10	36	10	80	43
Negative	0	0	0	0	0	0	5	0	0	0

Emerin Distribution:

Emerin is normally distributed at the nuclear rim in cells and can be seen in internal foci as well. MCF-10A cells which had a homogenous in 75% of cells and 25% of cells were negative when fixed with methanol: acetone whereas all cells distributed it as rim and spots in 100% of cells when fixed with paraformaldehyde. The distribution of the protein in T-47D cells showed 50% of cells had rim and spots, large spots in 30% of cells, and 20% as a homogeneous when fixed with paraformaldehyde. GI-101, BT-474 and SkBr3 cells all have quite a number of negative cells which will have an impact on the cells, especially with control of gene expression.

Table 4.8: The Percentage of Emerin Distribution:

	MCF-10A		T-47D		GI-101		BT-474		Sk-Br-3	
Type of Fixation	**M**	**P**	**M**	**P**	**M**	**P**	**M**	**P**	**M**	**P**
Rim Only	0	0	ND	0	0	0	15	0	30	5
Rim & Spots	0	100	ND	50	73	30	50	100	55	65
Large Spots	0	0	ND	30	0	20	5	0	0	5
Homogeneous	75	0	ND	20	0	0	0	0	0	0
Many Spots	0	0	ND	0	0	0	0	0	0	0
Negative	25	0	ND	0	27	50	30	0	15	25

***ND=No Data**

MAN1 Distribution:

MAN1 should be distributed at the rim in normal cells However, none of the cancer cells with either fixation show MAN1 at the nuclear rim. There is a high proportion of nuclei displaying the many spots pattern for both fixations even in the control cells. Thus, in these cells which are epithelial may be MAN1 distribution is different. Some of the cells especially T-47D are displaying large spots of MAN1 which is not a normal distribution. There are also negative cells in the Sk-Br-3.

Table 4.9: The Percentage of MAN1 Distribution:

	MCF-10A		T-47D		GI-101		BT-474		Sk-Br-3	
Type of Fixation	**MA**	**P**	**MA**	**P**	**MA**	**P**	**MA**	**P**	**MA**	**P**
Rim Only	0	0	0	0	0	0	0	0	0	0
Rim & Spots	0	0	0	0	0	0	0	0	0	0
Large Spots	0	0	50	10	10	0	0	3	0	0
Homogeneous	60	0	0	22	20	30	30	0	0	8
Many Spots	40	100	50	63	70	70	60	97	100	72
Negative	0	0	0	5	0	0	10	0	0	20

4.3.3 Distribution of Other Nuclear Structural Proteins by Indirect Immunofluorescence (Methanol: Acetone, 1:1):

Due to the distributions of some of the nuclear envelope antigens, especially the B-type lamins, in the nuclear interior as large foci it was pertinent to determine if these structures were colocalised or complexed with structures normally found as foci in the nuclear interior. This is why the cell lines BT474 and T47D were chosen to be further assessed since they had the most aberrant B-type lamin staining. Thus indirect immunofluorescence with rabbit anti-PML antibody, mouse anti-nucleolin antibody, and rabbit anti-Ki67 antibody was performed on cell lines MCF-10A, T47-D, and BT-474 (Figure 4.3 Panel A (A-E) and Figure 4.4 Panel A (A-D)). Furthermore, due to proteins being not located at the nuclear periphery in these cells we decided to see if antibodies bind to other nuclear envelope proteins i.e. mouse anti-LAP2β and rabbit anti-lamin B receptor were also in the nuclear interior and colocalised with the B-type lamins. The presence of these proteins was also very interesting. Nucleolin and LAPβ were present and correctly localised in almost all cells for nucleolin and in all cells for

LAP2β. Interestingly, BT-474 had a large number of cells where there were no PML bodies present and LBR was very much affected by lacking in BT-474 and T47D cells.

From at least 200 nuclei, the presence of PML, nucleolin, LBR, LAP2β, and Ki67 proteins in BT-474, T-47D, and MCF-10A cell lines was scored. These cells were fixed with methanol: acetone, 1:1 and these proteins showed clear differences in their nuclear distribution (Table 4.12). It is found that PML proteins, protein of stress, were distributed 100% in T-47D cells, 69.5% in BT-474 cells, and 100% in MCF-10A. Moreover, nucleolin, nucleolus protein, was found approximately equal in these three cell lines and between 93-98% of cells showed positive staining. Ki67 proteins, found in the nucleolus of proliferated cells as proliferation marker in breast cancer, were found in high percentages in T-47D cancerous cells (92%) and MCF-10A (75%) normal cells whereas it was 44% in BT-474 cancerous cells. Furthermore, LAP2β proteins were present in a high percentage and equally in these three cell lines (100%). Lamin B receptor (LBR) was present in MCF-10A cells (95%), but reduced in T-47D cells (64%), and low in BT-474 cells (29%). Interestingly, the percentage of LBR positive cells in T-47D and BT-474 was similar to the data from Fischer *et al.*, in 1998, where they found the lack of LBR at the NM in papillary thyroid carcinoma might be linked to *LBR* gene mutation or alterations in the expression of LBR (Fischer *et al.*, 1998) (Figure 4.3 Panel A (A-E); Figure 4.4 Panel A (A-D); Table 4.12).

I. Distribution of Other Nuclear Structure Proteins (Methanol: Acetone, 1:1):

Panel (A):

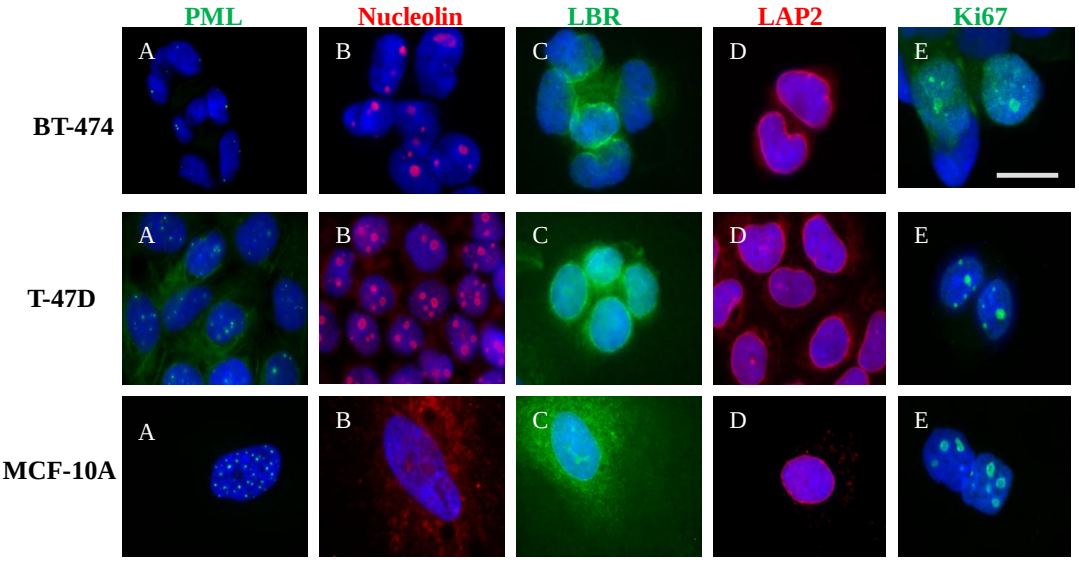


Figure 4.3: Different Localisations of PML, Nucleolin, LBR, LAP2 and KI67 Proteins: These images demonstrate antibody distributions within the nucleoplasm determined by indirect immunofluorescence (IIF) and fixed with methanol: acetone, 1:1. The images demonstrate the localisation of these proteins in BT474, T-47D, and MCF-10A cell lines (A-E) when immunostained with anti-PML (A), anti-nucleolin (B), anti-LBR (C), anti-LAP2 (D), and anti-Ki67 (E) antibodies. The DNA stained with DAPI (Blue). Scale Bar = 10µM.

Table 4.10: The table shows the specific percentages of nuclear proteins which are within these cell lines as shown below. From at least 200 positive nuclei, this table compares the presence and distribution of nuclear proteins in T-47D, BT-474, and MCF-10A cell lines when fixed with methanol: acetone, 1:1. These proteins showed clear differences in their nuclear distribution.

	PML	Nucleolin	Ki67	LAP2	LBR
T-47D Cell Lines	100 %	98.5 %	92 %	100 %	64 %
BT-474 Cell Lines	69.5 %	93 %	44 %	100 %	29 %
MCF-10A Cell Lines	100 %	97.5 %	75 %	100 %	95 %

II. Co-Localisation of Lamins B with Ki67 and Lamin B Receptor with Nucleolin and LAP2 Proteins (Methanol: Acetone, 1:1):

Panel (B):

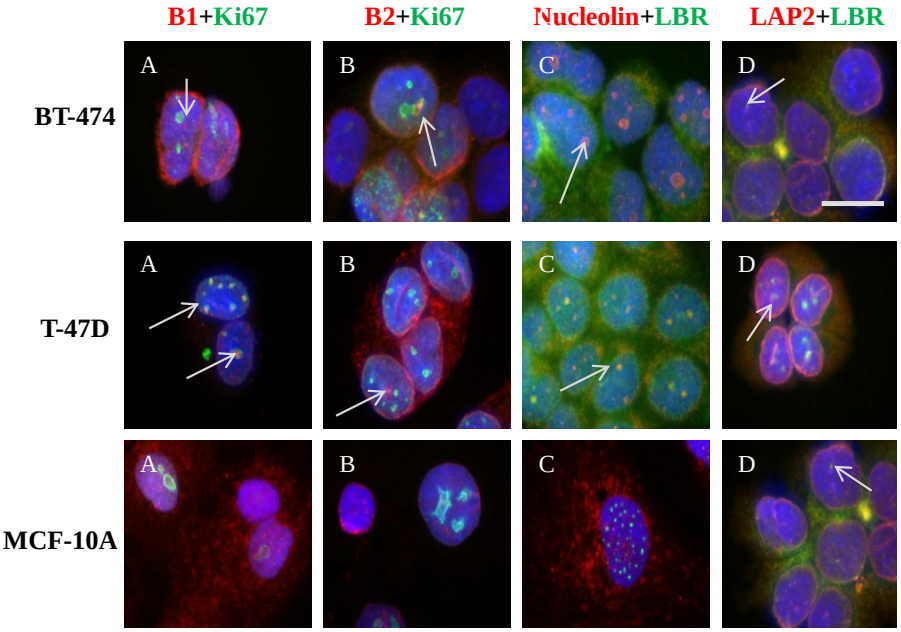


Figure 4.4: Different Co-Localisations of Lamin B with Ki67 and Co-Localisation of LBR with Nucleolin and LAP2: These images demonstrate antibody distributions within the nucleoplasm determined by indirect immunofluorescence (IIF) and fixed with methanol: acetone, 1:1. The images demonstrate the co-localisation of these proteins in BT474, T-47D, and MCF-10A cell lines (A-D) when immunostained with anti-lamins B, anti-nucleolin , anti-LBR, anti-LAP2, and anti-Ki67 antibodies. Ki67 protein was associated or co-localised with lamin B in large spots within the nucleoplasm as well as LBR co-localised with nucleolin and LAP2 in cancer cells. These spots were very interesting within the nucleoplasm. Therefore, dual colour experiments were performed to see these co-localisatioins. The DNA stained with DAPI (Blue). Scale Bar = 10µM.

Since there was very interesting spots from lamin B1, lamin B2, it was important to see if there are co-localisations with these proteins. Therefore, dual colour experiment was performed. The co-localisation assessed lamin B1 with Ki67 antibodies, lamin B2 with Ki67 antibodies, nucleolin with LBR antibodies, and LAP2β with LBR antibodies (Figure 4.4 Panel B (A-D)) as well as lamin B with LBR and lamin B with PML bodies (Figure 4.8, Panel A (I-IV)). In this experiment, it was found that Ki67 protein associated with lamin B in larger foci. LBR colocalised with LAP2β at the nuclear periphery but was also found to colocalise with nucleolin in the interior of the nucleus. In addition to this, large foci were observed for the B-type lamins and LBR which is an important finding because lamin B and LBR has never been seen like this in normal cells and should be at the periphery like a rim in normal situation. These data revealed foci of lamin B with LBR and lamin B with PML bodies deep within the nucleoplasm (Figures 4.7 and 4.9).

4.3.4 Distribution of Other Nuclear Structure Proteins (Paraformaldehyde 4%):

Indirect immunofluorescence staining was performed with rabbit anti-PML antibody, mouse anti-nucleolin antibody, mouse anti-LAP2 antibody, rabbit anti-lamin B receptor antibody, and rabbit anti-KI67 antibody on different cell lines (MCF-10A, T47-D, and BT-474) paraformaldehyde 4% (Figure 4.5, Panel A (A-E)). This experiment was used to assess their localisation and co-localisation of these proteins and their distributions throughout the nucleoplasm. Therefore, dual colour experiment was performed in order to see is there is co-localisation with these proteins. The co-ocalisation assessed between lamin B1 with Ki67 antibodies, lamin B2 with Ki67 antibodies, nucleolin with LBR antibodies, LAP2 with LBR antibodies (Figures 4.6, Panel B (A-D)). In this experiment, it is found that Ki67 protein was associated with lamin B in larger foci as well as foci were found in association of LBR with nucleolin and LBR with LAP2 antibodies. In addition to this, the data revealed very

interesting spots of lamin B with LBR and lamins B with PML bodies within the nucleoplasm when assessed by dual colour experiments as shown below in Figures 4.7 and 4.9.

From at least 200 nuclei, the percentage were counted from different nuclear structures to assess and compare presence and distributions of PML, nucleolin, LBR, LAP2, and Ki67 proteins in BT-474, T-47D, and MCF-10A cell lines. These cells were fixed with paraformaldehyde 4% and these proteins showed clear differences in their nuclear distribution (Table 4.13). It is found that PML proteins were distributed approximately 100% in MCF-10A and T-47D cells, 61% in BT-474 cells, similarly to methanol: acetone fixed cells. Moreover, nucleolin was found in both cancerous cell lines (both T-47D and BT-474) and 50% in normal cells (MCF-10A). This is not a normal situation since nucleolin is always found at nucleoli in normal cells. Ki67 proteins were found in high percentages in T-47D cancerous cells (95.5%) and approximately half of MCF-10A normal cells were expressed it (55%) whereas Ki67 was low in BT-474 cancerous cells (33%). Furthermore, LAP2β proteins were distributed in high percentage in T-47D cells (100%) and MCF-10A normal cells (90%) whereas the same protein was present in very low numbers in BT-474 cells using this fixation as compared to methanol: acetone fixation. LBR was seen in half of MCF-10A cells (52.5%) but in very low numbers in T-47D and BT-474 cells (1%).

I. Distribution of Other Nuclear Structure Proteins(Paraformaldehyde 4%):

Panel (A):

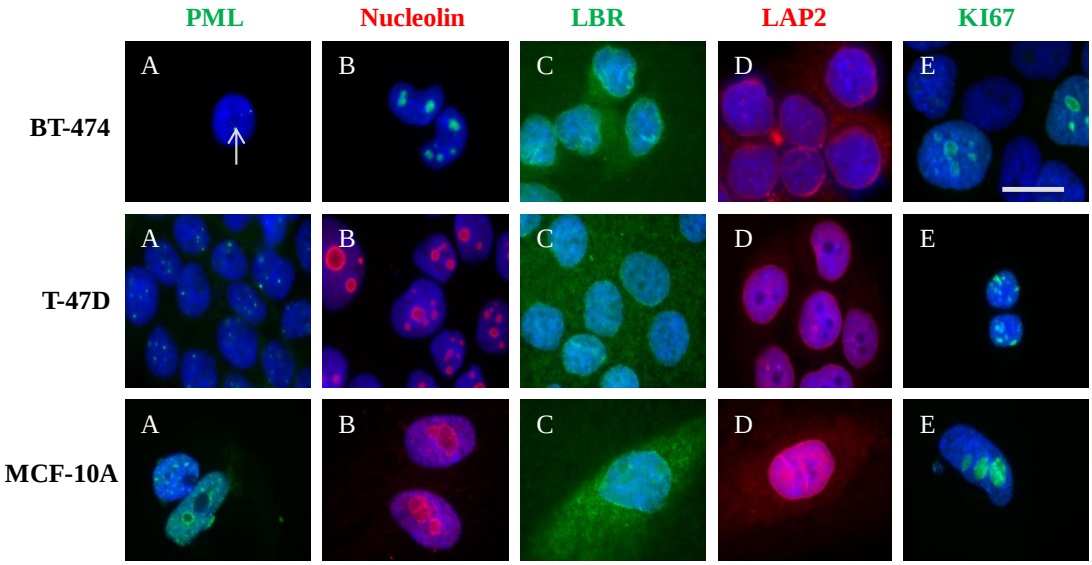


Figure 4.5: Different Localisations of LBR, Nucleolin, PML, LAP2 and KI67 Proteins: These images demonstrate antibody distributions within the nucleoplasm determined by indirect immunofluorescence (IIF) and fixed with paraformaldehyde 4%. The images demonstrate the localisation of these proteins in BT474, T-47D, and MCF-10A cell lines (A-E) when immunostained with anti-PML (A), anti-nucleolin (B), anti-LBR (C), anti-LAP2 (D), and anti-Ki67 (E) antibodies. The DNA stained with DAPI (Blue). Scale Bar = 10µM.

Table 4.11: The table shows the specific percentages of nuclear proteins which are within these cell lines as shown below. From at least 200 positive nuclei, this table compares the presence and distribution of expressed nuclear proteins when fixed with paraformaldehyde 4%. These proteins showed clear differences in their nuclear distribution.

	PML	Nucleolin	Ki67	LAP2	LBR
T-47D Cell Line	98.5 %	100 %	95.5 %	100 %	1 %
BT-474 Cell Line	61 %	97 %	33 %	18.5 %	26.6 %
MCF-10A Cell Line	100 %	50 %	55 %	90 %	52.5 %

II. Co-Localisation of Lamins B with Ki67 and Lamin B Receptor with Nucleolin and LAP2 Proteins (Paraformaldehyde 4%):

Panel (B):

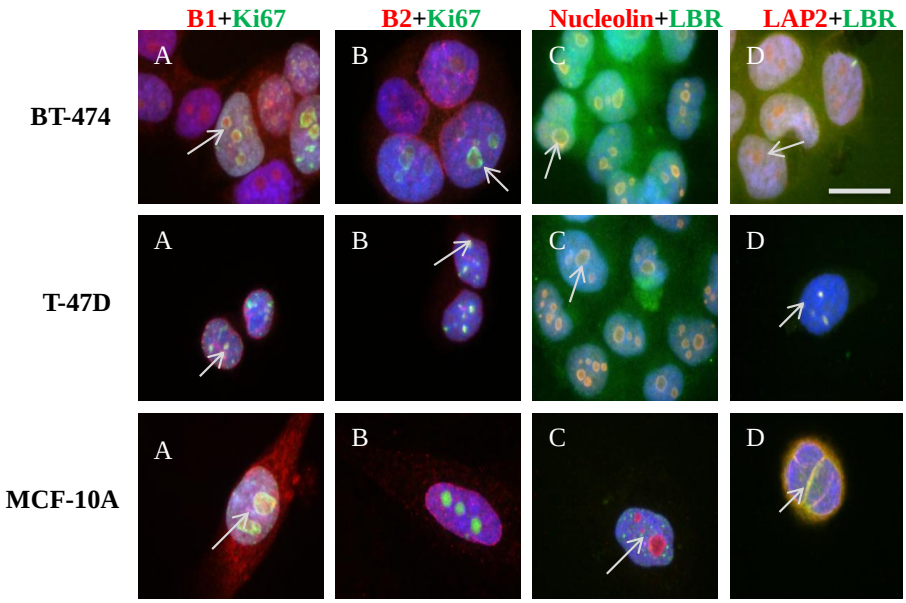


Figure 4.6: Different Co-Localisations of Lamin B with Ki67 and Co-Localisation of LBR with Nucleolin and LAP2: These images demonstrate antibody distributions within the nucleoplasm determined by indirect immunofluorescence (IIF) and fixed with paraformaldehyde 4%. The images demonstrate the co-localisation of these proteins in BT474, T-47D, and MCF-10A cell lines (A-D) when immunostained with anti-lamins B, anti-nucleolin , anti-LBR, anti-LAP2, and anti-Ki67 antibodies. Ki67 protein was associated or co-localised with lamin B in large spots within the nucleoplasm as well as LBR co-localised with nucleolin and LAP2 in cancer cells. These spots were very interesting within the nucleoplasm. Therefore, dual colour experiments were performed to see these co-localisatioins. The DNA stained with DAPI (Blue). Scale Bar = 10µM.

4.3.5 Distribution of Lamin B with PML and LBR Proteins Co-localisation by Indirect Immunofluorescence:

Since B-type lamins were found localised inside the nucleoplasm as large foci it was pertinent to see if they were colocalised with other internal structures such as PML bodies. Further since LBR was also seen to be in internal foci with lamin B being its binding protein – the colocalisation of these proteins needed to be investigated (Figures 4.7 and 4.9).

4.3.5.1 Cells Fixed with Methanol: Acetone, 1:1:

Panel (A):

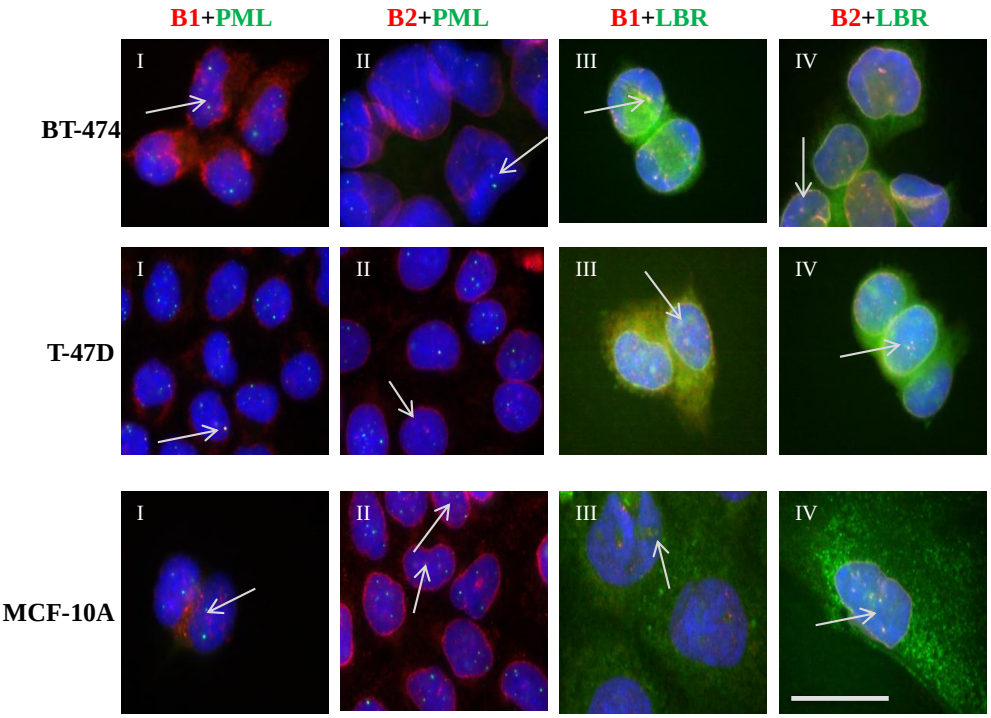


Figure 4.7: Panel (A): Co-staining of Lamin B1 and Lamin B2 with PML Bodies and LBR: These images demonstrate co-staining of proteins distributions within the nucleoplasm and they were processed and determined by indirect immunofluorescence (IIF) method after fixed with methanol: acetone (1:1). Lamins B was found as internal foci within the nucleoplasm. Therefore, dual colour experiments were performed. The images demonstrate the co-localisation of these proteins in BT474, T-47D, and MCF-10A cell lines. Panel A (I-IV): demonstrates the images of BT-474 cells when immunostained with both anti-lamins B with anti-PML and lamins B with anti-LBR antibodies (I-IV). Assessment of co-localisation between these proteins was also performed in T-47D and MCF-10A cells (I-IV). The DNA was stained with DAPI (Blue). Scale Bar = 10µM.

4.3.5.2 Co-Localisation of Lamins B, LBR, and PML (Methanol: Acetone, 1:1):

Figure 4.8 (Panel B) displays the percentages of PML structures that were localised with lamins B1 and B2 as well as the LBR with lamins B1 and B2. These percentages were calculated from different number of cell nuclei ranging from 200-700 nuclei. The indirect immunofluorescence was performed and the cells fixed with methanol: acetone, 1:1.

Panel (B):

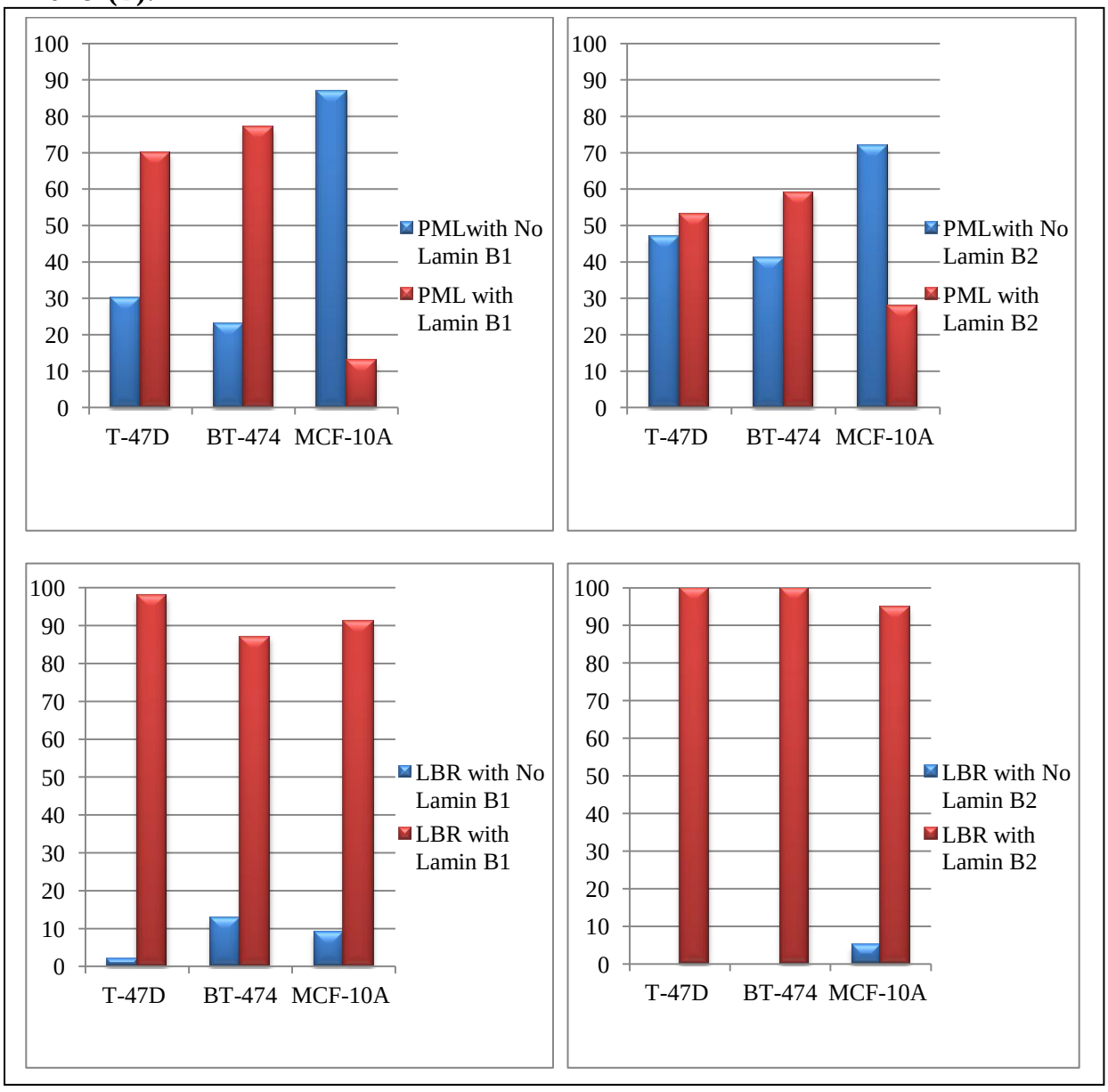


Figure 4.8: Panel (B) Co-Localisation of Lamin B, LBR, and PML: This figure compares the presence and distribution of these nuclear proteins when fixed with methanol: acetone, 1:1. These proteins showed clear differences in their nuclear distribution and localisation as well as co-localisation between lamin B, LBR, and PML. The co-localisation of lamin B with PML in cancer cells is more than the normal cells.

4.3.5.3 Cells Fixed with Paraformaldehyde 4%:

Panel (A):

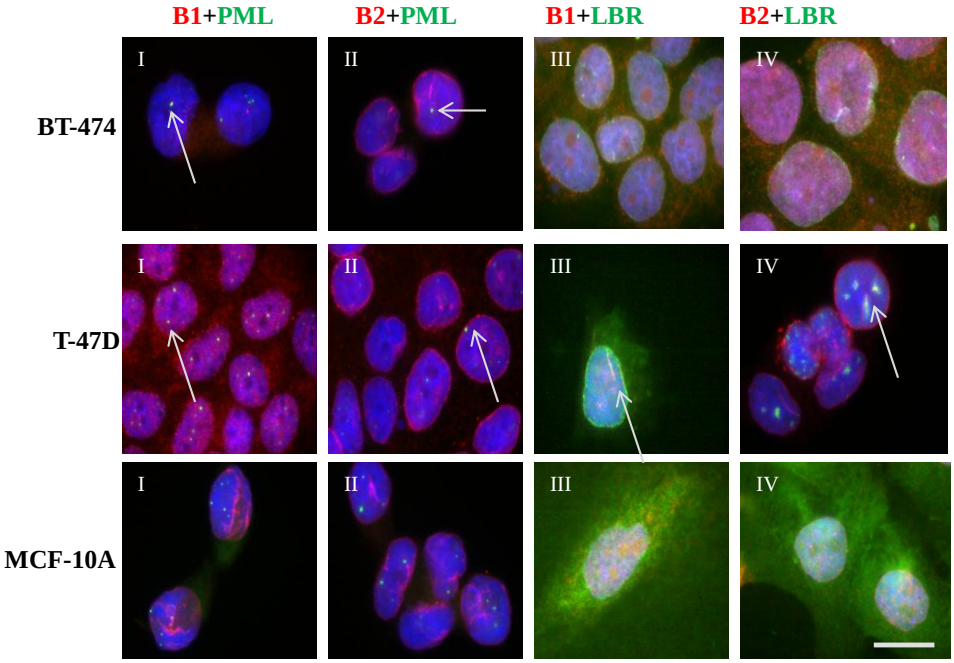


Figure 4.9: Panel (A): Co-staining of Lamins B with LBR and PML: These images demonstrate distributions of antibodies within the nucleoplasm and they were processed and determined by indirect immunofluorescence (IIF) method after fixation with paraformaldehyde (4%). The aim of this experiment is to test the co-localisation of lamins B and PML as well as lamins B and LBR. Lamins B was found as internal foci within the nucleoplasm. The images demonstrate the co-localisation of these proteins in BT474, T-47D, and MCF-10A cell lines. Panel (A): demonstrates the images of BT-474 cells when immunostained with both anti-lamins B with anti-PML and lamins B with anti-LBR antibodies (I-IV). The same assessment of co-localisation between these proteins was performed in T-47D and MCF-10A cells (I-IV). The DNA stained with DAPI (Blue). Scale Bar = 10µM.

4.3.5.4 Co-Localisation of Lamin B, LBR, and PML (Paraformaldehyde 4%):

Figure 4.10 (Panel B) displays the percentages of PML structures that were localised with lamins B1 and B2 as well as the LBR with lamins B1 and B2. These percentages were calculated from different number of cell nuclei ranging from 200-700 nuclei. The indirect immunofluorescence was performed and the cells fixed with paraformaldehyde 4%.

Panel (B):

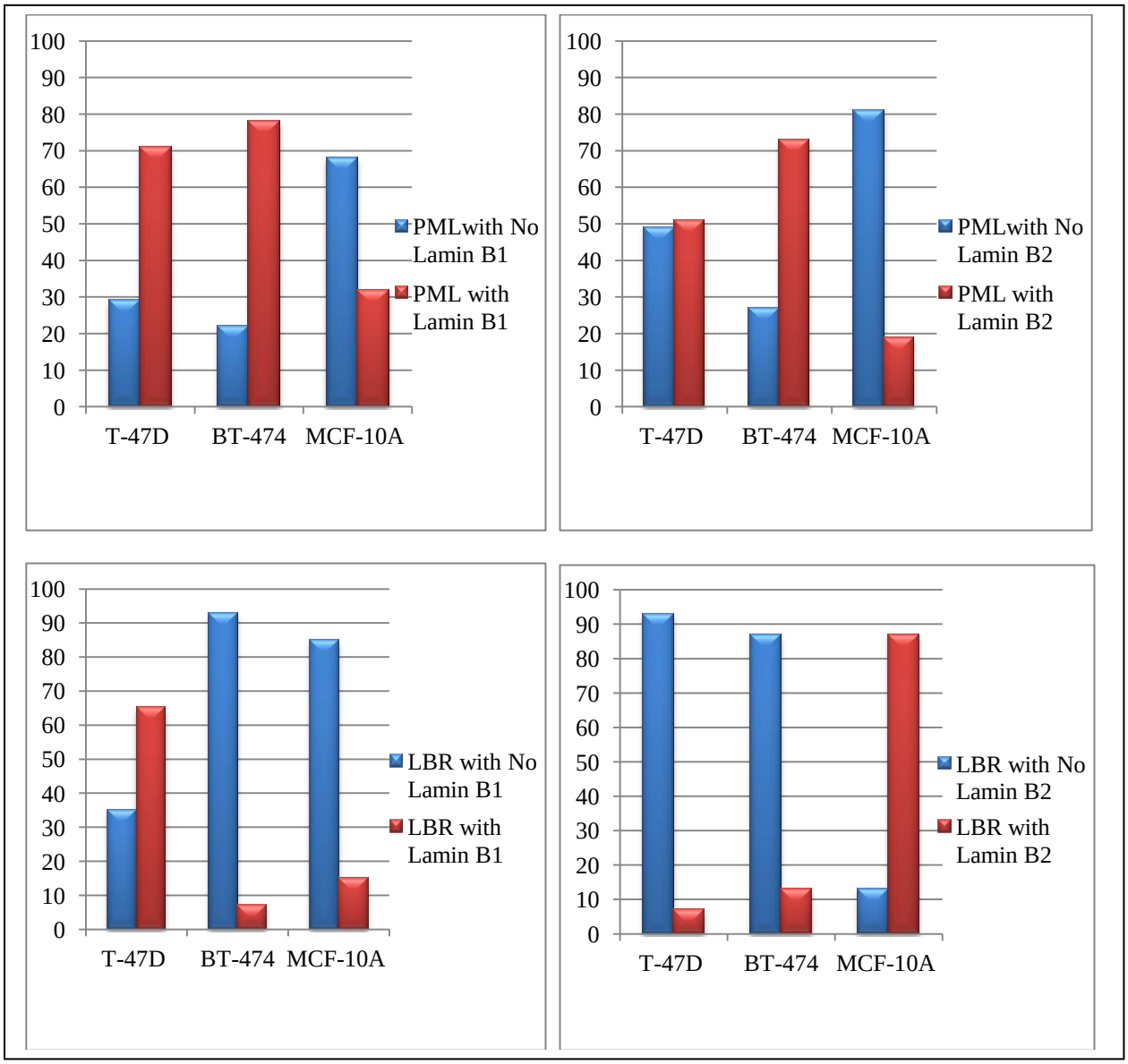


Figure 4.10: Panel (B) Co-Localisation of Lamin B, LBR, and PML: The figure compares the presence and distribution of these nuclear proteins after fixation with paraformaldehyde, 4%. These proteins showed clear differences in their nuclear distribution and localisation as well as co-localisation of lamin B, LBR, and PML. The co-localisation of lamin B with PML in cancer cells is more than the normal cells.

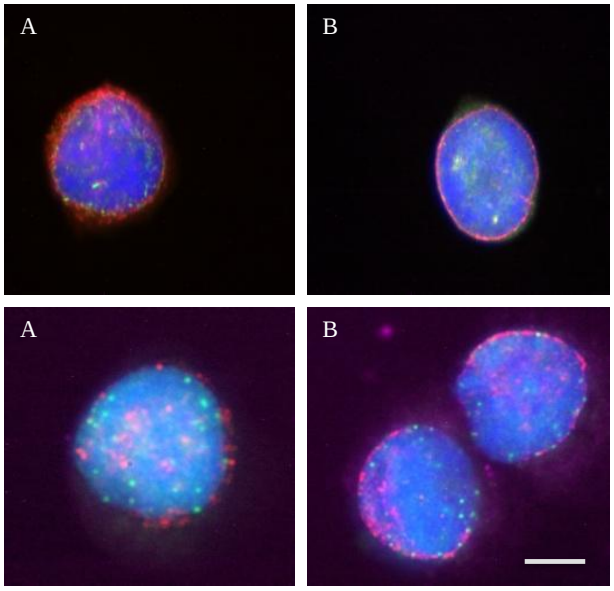
These data show that internal lamin foci of both B-type lamins are indeed colocalised with PML bodies in the cancer cell lines which is very unusual and has not been reported before. The colocalisation fraction is very much less for the control cells. There are also issues with B-type lamins and their colocalisation with LBR which should be 100%.

4.3.6 Four Colour Images by Indirect Immunofluorescence in BT-474 and T-474 Cell Lines: Panels (I and II) Demonstrate Cells after Fixation with Methanol: Acetone (1:1) and Paraformadehyde 4%, Respectively:

In order to assess the colocalisation of a number of antigens in the same nuclei, four colour indirect immunofluorescent experiments were designed. The aim of this experiment was used to confirm our results as compared to the previous experiment results and since these cells have big foci within the nucleoplasm, it is interesting to assess the co-localisation of lamin B1, lamin B2, MAN1, and LAP2α. The images, demonstrate lamins and lamin binding proteins distributions, were processed and determined by indirect immunofluorescence method when fixed with methanol: acetone, 1:1 and paraformaldehyde 4% (Figure 4.11 Panels I and II (A and B)) using anti-lamin B (Conjugated Cy3, Red, A and B), anti-MAN1 (Conjugated Cy5, Pink, A and B), and anti-LAP2α (FITC, Green, A and B) antibodies in both BT-474 and T-47D cells.

Four Colour Images by Indirect Immunofluorescence in BT-474 and T-474 Cell Lines:

Panel (I): Methanol: Acetone, (1:1):



Panel (II): Paraformadehyde 4%:

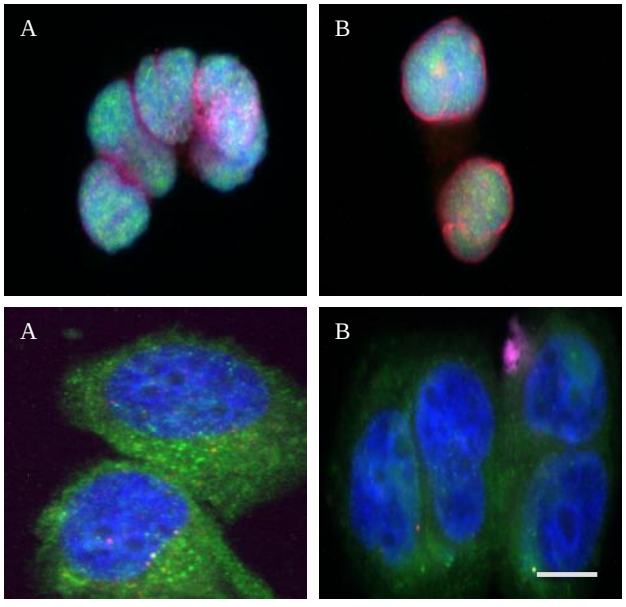


Figure 4.11: Panels (I) and (II): Different Distribution of Nuclear Lamins and Lamin Binding Proteins. Panel (I): These images, demonstrate lamin B1, lamin B2, MAN1, and LAP2α proteins distributions, were processed and determined by indirect immunofluorescence method when fixed with methanol: acetone, 1:1 using anti-lamin B (Red), anti-MAN1 (Pink), and anti-LAP2α (Green) antibodies in both BT-474 and T-47D cells. This figure showed merged colours of anti-lamin B (Red), anti-MAN1 (Pink), and anti-LAP2α (Green) in T-47D and BT-474 cell lines. In both cells we found lamin B as a rim and large foci (A and B) whereas MAN1 is missed or very faint in BT-474 cells and found as foci in T-47D cells. LAP2α was homogeneous and as foci in both cell lines. The four colours were secondary antibodies; anti-lamin B1 (A) and anti-lamin B2 (B) conjugated with Cy3 (red), anti-MAN1 conjugated with Cy5 (pink), anti- LAP2α conjugated with FITC (green), and DAPI (blue) for DNA staining. **Panel (II):** It is similar to panel (I) description and the difference is that the cells were fixed with paraformaldehyde 4%. In BT-474 cells, lamin B1 and MAN1 found as a faint rim and spots (A) whereas lamin B2 was well expressed (B) and LAP2α was homogeneous. In T-47D cells, lamin B and MAN1 were missed (A and B) whereas LAP2α was found as a homogenous and foci (A). Scale Bar = 10µM.

These images indicate that there is a significant difference in the presence, distribution and organisation of nuclear lamins and lamin binding proteins as well as to some important nuclear proteins such as PML, nucleolin, LAP2, and LBR in these cell lines (T-47D and BT-474).

4.3.7 qRT-PCR for Lamin B and Lamin B Receptor Gene Expression:

4.3.7.1 qRT-PCR Analysis for Lamin B and Lamin B Receptor Genes from Different Cells:

The data in this chapter showed that there were problems in the cancer cell lines with lamin B and LBR presence. It was important to determine if these two genes were being expressed in the cell lines. This was assessed by q-RT-PCR. The graphs in Figure 4.12 show the percentage of relative quantification of gene expression by fold changes in expression for each gene using qRT-PCR. The levels of expression of all genes studied here are calculated from $2^{-\Delta\Delta C}{}_t$ or relative quantification (RQ) using RQ Manager and Microsoft Excel software.

The equations are:

$\Delta Ct = Ct\ (target) - Ct\ (housekeeping\ gene)$

$\Delta\Delta Ct = \Delta Ct\ (sample) - \Delta Ct\ (normal\ or\ calibrator)$

$RQ = 2^{-\Delta\Delta C_t}$

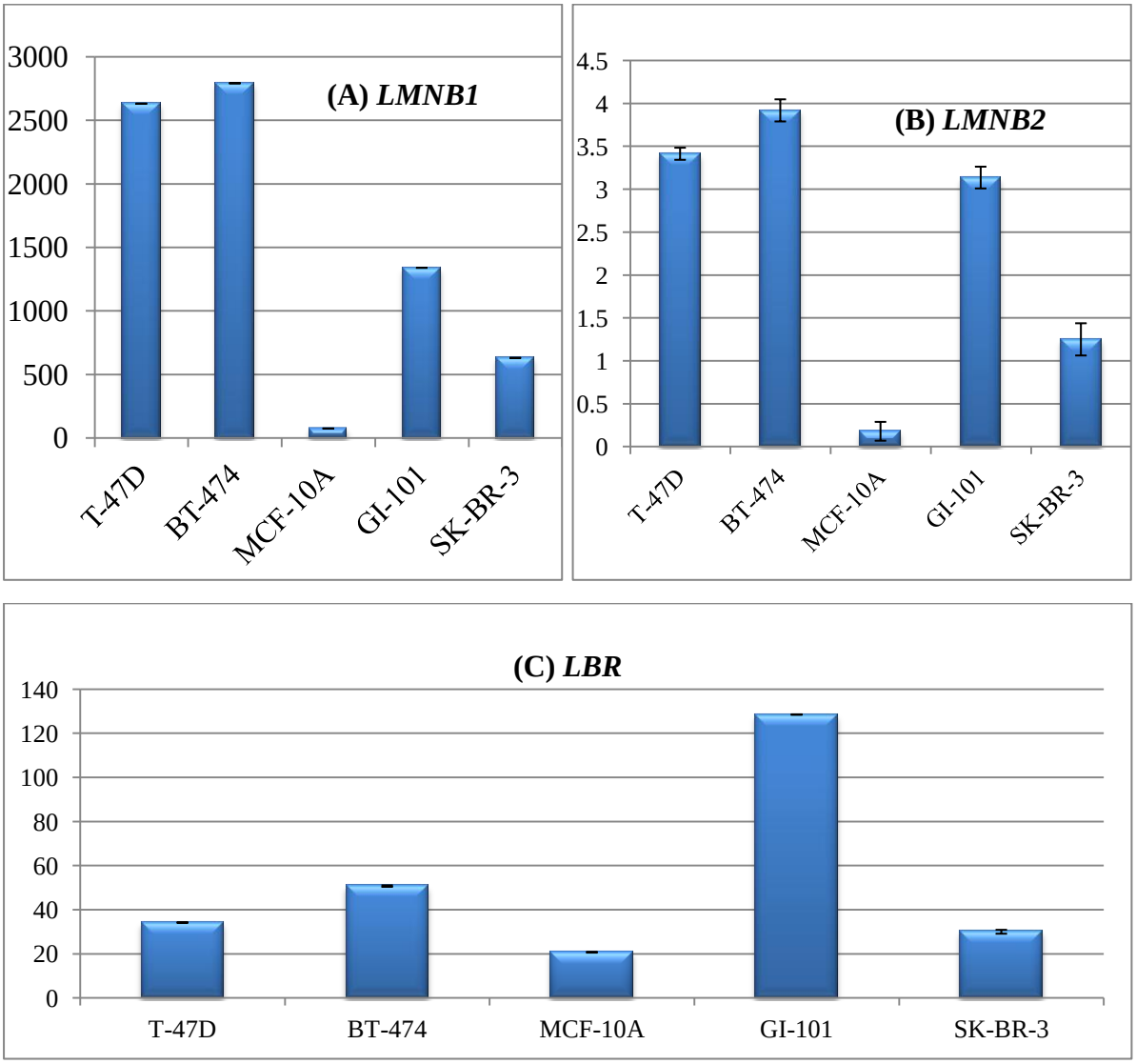


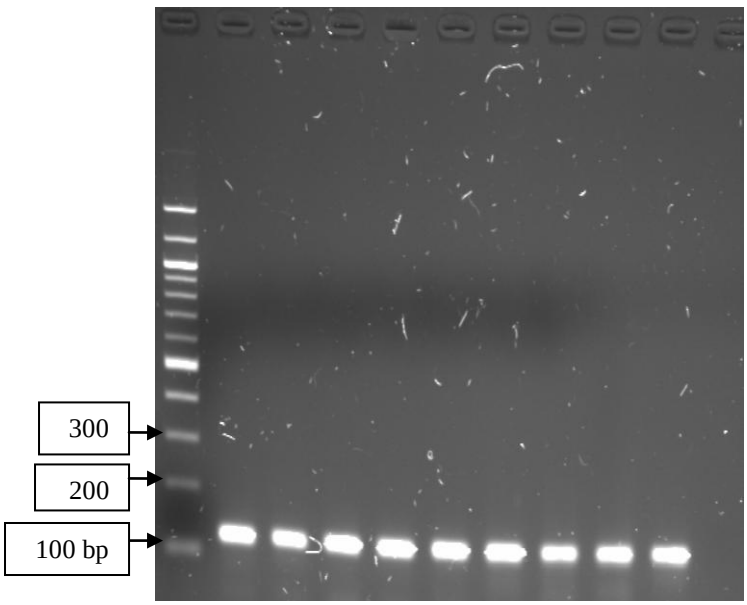
Figure 4.12: Expression Levels of *LMNB1*, *LMNB2*, *LBR*, and *β-Actin* Genes mRNA of Breast Cell Lines using qRT-PCR: These histograms show the percentage of relative quantification (RQ %) of different genes in present of β-actin as a housekeeping gene with no supplement set as 1 (100%) (A-C). The fold changes in different gene expression were assessed between cell lines (MCF-10A, T-47D, BT-474, GI-101, and Sk-Br-3) using RQ Manager and Microsoft Excel Software. Different mRNA levels showed a two-fold increase or decrease change in the gene expression. The expression over 100% is up-regulated and below this percentage is down-regulated. Error bars represent the Standard Error of the Mean (SEM) of three replicates.

The expression data from the qPCR shows that the B-type lamins are expressed well in T-47D and BT-474, maybe even over-expressed as compared to MCF-10A. What is noticeable is that LBR is much less than the other cells. This could cause an issue with the B-type lamins being correctly localised in the cell nuclei.

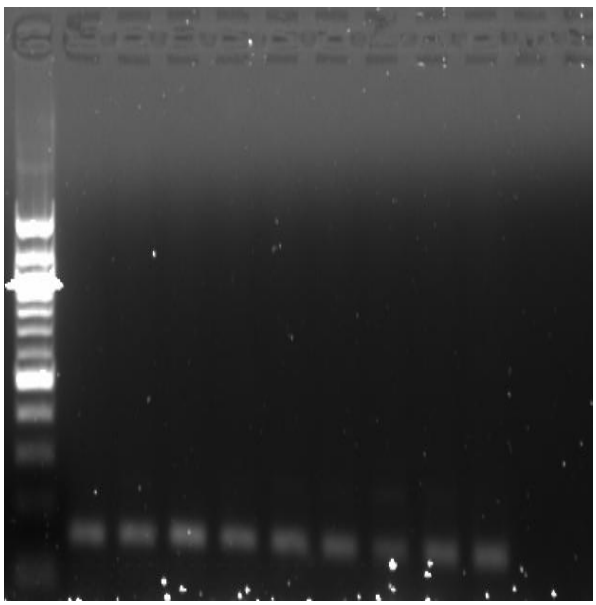
4.3.7.2 Agarose Gels from qPCR Products:

The figures below show the detection of specific gene qPCR products and their molecular sizes (Figure 4.13 and Appendices) by agarose gel electrophoresis. These products are shown after 40 cycles of amplification to evaluate the quality and integrity of these primers.

(1) *LMNB1* (125 bp):



(2) *LMNB2* (153 bp):



(3) *LBR* (128 bp): **(4) *β-Actin* (216 bp):**

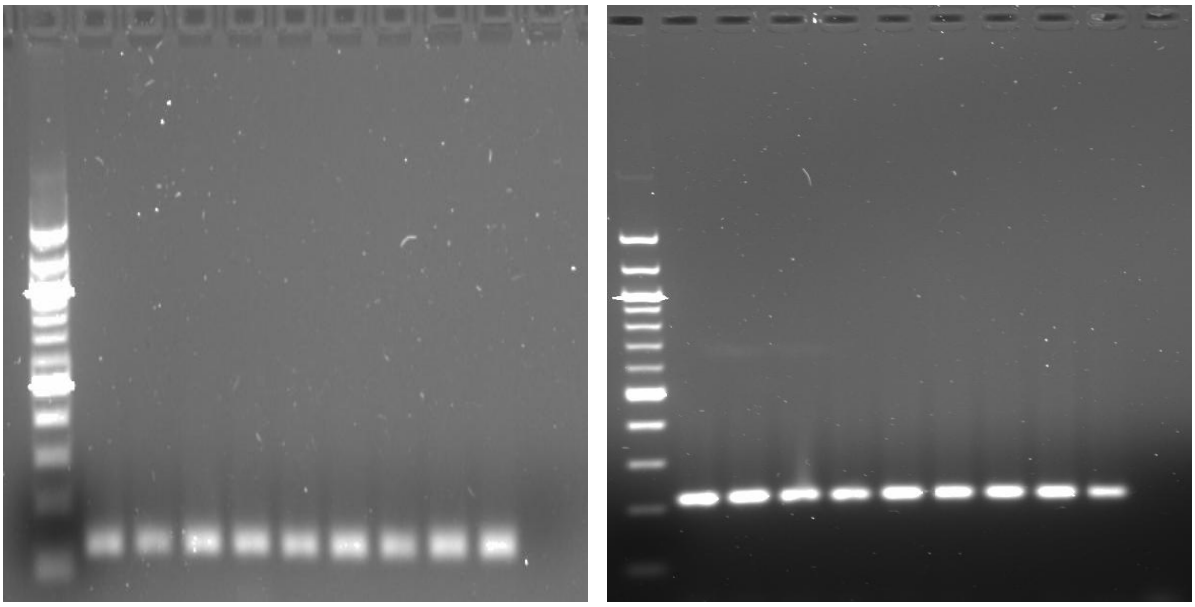


Figure 4.13: Representative Images of Gels Showing the Identification and Specificity of 3 Selected Genes of Interest in Breast Normal and Cancerous Cell Lines, Either Treated or Untreated Cells: cDNA was synthesised from RNA samples obtained from different cell lines. qRT-PCR experiments were performed using specific primers for each of the 3 target genes as well as to the β-actin as a reference gene. For amplification of each gene, qPCR was also performed in triplicate for positive and negative control at the same time on 96-well plate. To confirm and ensure this amplification has worked properly, the amplicons from each experiment were run on 2% agarose gel. The table above showed the size of the band for each product. Moreover, the dissociation curve can also confirm this accuracy, efficiency, specificity, and validity of the cDNA (Data not shown). ***The Key:** M= Marker (100 bp Ladder, BioLabs), 1=T-47D, 2=BT-474, 3=GI-101, 4=Sk-Br-3, 5=MCF-10A, 6=T-47D (12uM/48hours), 7=MCF-10A (12uM/48hours, lovastatin), 8=MCF-10A (24uM/48hours, lovastatin), 9=BT-474 (12uM/48hours, lovastatin), and 10=-ve Control (H_2O). Note: Lanes 6-9 include cells treated and untreated with lovastatin.

4.4 Discussion:

The architecture of cell nuclei is often altered in cancerous cells. The types of cancers are associated with characteristic changes and these may provide important diagnostic features. The normal breast (MCF-10A) and cancer cell lines (T47-D, GI-101, Sk-Br-3, and BT-474) display different distribution of lamins, lamin binding proteins, integral nuclear membrane proteins, and other nuclear proteins such as PML, nucleolin and Ki67 (Figures 4.1 - 4.3, Figure 4.5, and Tables 4.4 - 4.13). The study in this chapter was to assess the distribution and presence of nuclear proteins that if aberrant could lead to the mis-position or disorganisation of the genome. We have found that a number of nuclear envelope proteins and chromatin binding proteins are altered in our cancer cells and even in the MCF-10A control cell line.

For over 140 years, different studies showed that much debate about changes in the cell nucleus structure in cancer diagnosis. One of these changes was observed in 1860 in patient with cancer of the pharynx and observed that there was a clear change in nuclear size and shape variation (Zink *et al.*, 2004). This was followed by changes in cytoplasmic and nuclear structure features of the cells as well as these include changes in nucleolar size and shape, and numbers of nucleoli. These alterations also can be characteristic of cancer type and stage which is a very useful for cancer diagnosis and might also be related to the altered functions of cancer cells (Zink *et al.*, 2004). In this study, T-47D, GI-101, BT-474, and Sk-Br-3 breast cancer cell lines were showed changes and alterations in nuclear proteins distribution when compared with the MCF-10A normal but immortalised cell line.

The structural components of the nucleus are strongly linked to the genome. The correlation between the genome and nuclear structure is that the nuclear envelope proteins have chromatin binding sites and therefore are involved in anchoring interphase chromosomes to

the nuclear periphery. These include lamins, LBR, LAPs 1 and 2, emerin, MAN1, and BAF (Zuleger *et al.*, 2011). In humans the acrocentric chromosomes are embedded in nucleoli and other chromosomes that do not contain rRNA or tRNA are also associated with nucleoli (Bridger *et al.*, 1998b) for example chromosome 17 (van Konigsbruggen *et al.*, 2010) and chromosome 19 (Nemeth *et al.*, 2010). The loss of this organisation or aberrant distribution in nuclear structure will lead to dis-organisation of the genome and the loss of both nuclear structure and genome organisation that are associated with cancer and laminopathies (Young *et al.*, 2012). However, there seem to be no issues with nucleoli in these cancer cells as shown by Ki67 and nucleolin staining. The main issues are with nuclear envelope proteins such as lamin A, emerin and B-type lamins between these five breast cell lines. From the literature, mutation in *LMNA* gene showed a loss of chromatin integrity which is associated with deformation of the nuclear envelope and lamina (Sullivan *et al.*, 1999). Gene expression and chromosome positioning are abnormal in lamin B1 silenced cells (Malhas *et al.*, 2007), while disruption of B-type lamins inhibits DNA replication and RNA pol II transcription (Dechat *et al.*, 2008). Interestingly, lamin B is considered as a binding site for the human genome since lamins have 1,300 lamin associated domains (LADs) which were found to be in gene poor regions at the nuclear periphery of the genome (Guelen *et al.*, 2008). Therefore, these changes in nuclear lamins, integral membrane proteins, and other nuclear structure proteins will affect chromatin organisation and gene expression. Thus, it is important to understand this organisation, regulation and distribution are required and linked to nuclear lamins for efficient normal nuclear functions.

The lamina is thought to be a principal determinant of the nuclear shape (Goldman *et al.*, 2002). The nuclei of most normal cells have a regular shape but an irregular nuclear shape is

observed in cancerous cells. These changes are characteristics of different cancer types. For examples, small-cells lung carcinoma lack the proteins of lamin A whereas lamin B expression varied between normal and cancer cells and therefore this may contribute to the structural changes observed in the lamina (Broers *et al.*, 1993) (Chapter 1, Table 1.1).

These results show that the nuclear lamins and their binding proteins change during breast cancer. Moreover, these changes in distribution might be of the loss of *LMNA* gene that is associated with deformation of the nuclear envelope and lamina. This can lead to changes in nuclear morphology such as herniations and blebs structures. These morphological changes could lead to lack of nuclear pore complexes that prohibit local export of mRNA, thereby inhibiting splicing and transcription. Thus, this will lead to the loss of peripheral heterochromatin and mis-localisation of chromosomes as well as to the loss of lamins A/C expression (Agrelo *et al.*, 2005; Shimi *et al.*, 2008). Therefore, lamins A is important to maintain the stability and regulate transcription. Furthermore, the expression of *LMNA* gene is reduced or absent in cells that are highly proliferating cells (Barboro *et al.*, 2003) but not in other cells. This will support the idea that the genome organisation is tissue specific (Chapter 1, Table 1.1). Moreover, the deregulation of the distribution of these lamins might be due to changes in nuclear matrix proteins that anchor the genome, telomeres, nuclear bodies, matrix attachment regions and transcriptional machinery. This will cause the morphological modifications during the process of carcinogenesis due to disruption in this binding. The depolymerisation of one element in the nucleoskeleton structure could collapse the others (Hozák *et al*., 1995). This is the reason why lamins influence the position of the chromosomes in the interphase nucleus. Thus, this may affect the epigenetic regulation of chromatin. The arrangements in the nucleus are maintained by attachments to nuclear matrix proteins (NMPs) (Fey *et al.*, 1986). Lamins attachments will support their assembly into

functional macromolecules involved in nuclear processes (Nickerson, 2001). The nucleoli and nuclear bodies (PML) also participate in these changes in lamins distribution and expression during breast cancer. These changes include changes in their location, structure, and function.

Previous studies showed that the inactivation of lamin B1 or truncated lamin A inhibit DNA replication and RNA polymerase II transcription, respectively. The effects of lamins on chromatin structure, function, and organisation is through the direct interaction of lamins to histones or lamins binding proteins (Dechat *et al.*, 2008; Shimi *et al.*, 2008). A lack of lamin staining was found to occur in the nuclear periphery in our cells. This could be due to: mutation or loss in *LMN* genes, decreased lamins synthesis, alteration of lamins structure after post-translation modifications, degradation of lamins during apoptosis as a first target by caspases, deficient in lamins processing pathways or defects in enzymes that are involved in prenylation, microtubules disassembly during cell division, and/or epitope masking that binds to specific antibodies at the nuclear periphery. However, lamins staining was observed in the nucleoplasm of cancer cells as foci. Their presence within the nucleus could be due to problems with farnesylation so that they cannot get to the nuclear membrane or that is not enough LBR to enable the lamin B to anchor to the nuclear membrane. It was found that lamin B was associated with PML bodies in this work. The consequence of this to a cancer cell is not easy to ascertain but it could very definitely affect the functionality of PML bodies. Lamin B being in the wrong place could also affect DNA replication, transcription sites or splicing.

Since PML bodies are transcriptional regulators, they are involved in major nuclear functions. In mice, these nuclear bodies are lost and their proteins are dispersed throughout the

nucleaoplasm. These proteins display effects on cell growth, tumourgenesis, and the development of blood cells. Regarding the diffusion of PML bodies and their colocalisation throughout the nucleoplasm, chromatin dynamics have considered in understanding nuclear functions (Chubb and Bickmore, 2003) and PML bodies may also be required for nuclear functions. These nuclear bodies are capable of assembly and disassembly in response to physiological stimuli and have mobility properties in the nucleoplasm. Moreover, their presence surrounding chromatin, either the assembly or disassembly, may be influenced by the local chromatin organisation (Boisvert *et al.*, 2000).

Nuclear structure and genome organisation are essential and important for correct gene expression and normal function of cells (Malhas *et al.*, 2007). Understanding the nuclear behaviour of breast cancer cells will aid in the goal to eliminate cancer as a major health problem. Lamins can be used to distinguish normal tissue from tumour cells. In 2011, Coffinier *et al.* demonstrated that lamin B1 deficiency is sufficient to cause severe neurodevelopmental and nuclear shape abnormalities. In contrast, Yang *et al.*, in 2011, generated mice lacking *both* lamin B1 and lamin B2 in skin keratinocytes and found that skin histology and keratinocyte proliferation was entirely normal. Thus, losing the expression of the B-type lamins are significantly different in the brain and skin. In this study, nuclear lamin B, which is involved in chromosome positioning within cells, is aberrant in T-47D, GI-101, and BT-474 cell lines displaying large foci within the nucleoplasm. However, the functional assessment of lamin B would be difficult and the reason that the function of lamin B2 is not precisely defined particularly when compared with lamin B1. The range of potential roles of lamin B2 includes supporting the inner side of the nuclear envelope, stabilising the nucleus and chromatin, and regulating gene expression.

Chromatin and DNA binding sites are found in LBR (Worman *et al.*, 1990). Since lamins B bind to chromatin, therefore, there is preferential binding between lamins B, chromatin, and LBR within the nucleoplasmic domain. Since lamin B attachment site to LBR, may also function in the attachment of DNA to the inner nuclear membrane. It is also possible that the DNA binding activity of LBR is responsible for targeting mitotic membrane vesicles to decondensing chromosomes at the end of mitosis. These vesicles are targeted to chromatin before nuclear lamins or nuclear pore complex proteins and may initiate nuclear envelope reassembly *in vivo* (Foisner and Gerace, 1993). Thus issues with B-type lamins and LBR could affect chromatin and chromosome binding at the nuclear envelope leading to genome misorganisation and gene repositioning.

Lamin B1 silenced or missed showed clear change of both lamin A/C and lamin B2 structure suggesting that lamin B1 is a critical and provides scaffold required for the proper structural integrity and organising of the nuclear lamins, lamin A/C and B2 (Shimi *et al.*, 2008). Interestingly, the gene poor chromosomal regions are associated with lamin B1 in the lamina at the periphery whereas lamins A are preferentially associated with gene rich regions in the nuclear interior (Goldman *et al.*, 2002; Dechat *et al.*, 2010). Clearly, there is a relationship between lamins, lamin associated proteins, histone and RNA Pol II transcription and their roles in chromatin organisation and gene regulation.

Lamins interact and connect to chromatin through other proteins (lamin binding proteins). This interaction could participate in heterochromatin formation at the nuclear periphery. However, in cancer cells, nuclei can become irregular and nucleoli can be enlarged (Zink *et al.*, 2004). Clearly, any alteration of nuclear shape is associated with alteration in

heterochromatin organisation. This alteration will affect chromatin organisation and gene expression. The variable regulation of gene expression may be due to differences in specific gene regulation at the nuclear periphery of lamina (Shimi *et al.*, 2008).

In order to explain the distribution of nuclear lamina in all these breast cancer cell lines. The possible explanation for the absent of nuclear lamins due to decreased, altered or degradation of nuclear lamin protein synthesis. The steps of processing and modifications are important and critical for lamins localisation into the lamina that means blocking the farnesylation, will inhibit lamins post-translation processing. Although lamin C does not undergo any post-translational modifications and have no CAAX box motif at the C-terminal, it is incorporated to the nuclear periphery in normal cells (Vergnes *et al.*, 2004; Malhas *et al.*, 2007). Therefore, the mechanism of lamins C to be mature and incorporated to the nuclear periphery is unclear. Interestingly, inhibition of lamins farnesylation does not inhibit their incorporation to the lamina. For example, lamins C that lack 98 amino acids in their C-terminal and only have 6 amino acids lacking a CAAX box become incorporated into the nuclear lamina (Dechat *et al.*, 2008). That means CAAX only is not enough for incorporation to the nuclear periphery and other parts are also necessary for full incorporation. Mutations in *LMNA* gene will affect its charge and this will lead to instability of interaction between the α-helix at the rod domain preventing lamins dimerisation thus, inhibit the lamin binding proteins. Moreover, deficient in lamin B1 or the processing pathway could be of the Ras converting enzyme 1 endoprotease deficient or loss of ICMT in the carboxmethyl transferease which is important for the final carboxymethylation step and for Ras functions. This will lead to defective in CAAX processing to be mature and active (Malhas *et al.*, 2007). However, changes in lamina proteins or other nuclear proteins, involved in the process of cancer transformation, remain unclear.

From the data in this chapter, most of the nuclear functions involve not only the nuclear lamina, but also other components of the nuclear structure. This will increase our knowledge to understand the abnormal lamina affects other nuclear structures and functions. To assess the differences in the expression and distribution of other nuclear proteins such as PML, nucleolin, and Ki67 which are included in this chapter between two different cancerous (T-47D and BT-474) cell lines and one normal (MCF-10A) cell line are linked their expression to cancer pathology. The various percentages of nucleolin distribution between normal (50%) and cancer (more than 93%) cells may affect translation and post-translation of proteins and this may lead to nuclear abnormalities and cell cycle deregulation. Moreover, these proteins may affect other proteins that are attached to the nuclear matrix and then perturbed leading to low amount of proteins involved in nuclear processes and functions.

The nuclei that showed negative expression of nucleolin may have dysfunctional nucleoli due to defects in ribosome biogenesis. These defects alter transcription, translation, post-translation modification, apoptosis, and oncogenic pathways. This protein is involved in maintenance of normal nuclear function and structure by maintaining the nucleoplasmic proteins such as nuclear motors and this suggest its interaction with chromosomes therefore, nucleolin can participate in chromosomal organisation and reorganisation (nucleolar organising regions, NORs) (Gautier *et al.*, 1992; Cong *et al.*, 2012).

The various percentages of Ki67 protein distribution between normal and cancer cells depend on the cell cycle phase and cell type. It is seen in early G1 phase when the nuclear compartments broken down during mitosis that includes nuclear envelope and nucleolus reassemble. In addition, Ki67, has been implicated in the regulation of cell cycle, cell proliferation, rRNA synthesis and protection of chromosomes during mitosis, maintenance of

nucleolar structure (Kill, 1996), and can interact with heterochromatin that suppress the transcription and distribution of this protein (Goldman *et al.*, 2004). Furthermore, to these proteins is PML bodies that contain many proteins and have been implicated in multiple regulatory pathways, from the control of cell proliferation to DNA repair or apoptosis and can colocalise with the p53 locus to facilitate cell cycle regulation.

Therefore, the proper distribution of all these proteins within nuclei and nucleolei may result in proper cell functions. Therefore, the presence and distribution of lamins and these proteins may be disturbed resulting in cancer with abnormal nuclear shape, cell cycle up-regulation, and hyperproliferation (Zimber *et al.*, 2004).

4.5 Conclusion:

There is a fundamental correlation between the nuclear structure and genome which is the nuclear structure participates in genome organisation. This organisation and the strong relationship can be influenced by chromatin localisation for correct nuclear functions.

The findings in this chapter have been corroborated in a study by our collaborators, which was suggested by these data in this chapter whereby they show lamin A, B-type lamins and LBR lacking to various extents in actual breast cancers by q-RT-PCR. The expression of all these proteins is negatively correlated with prognosis and positively with survival (Wazir *et al.*, man in prep).

Chapter 5: Investigating the Role of the Drug Lovastatin on Restoration of Nuclear Structure and Genome Organisation in Breast Cancer Cell Lines

Manuscript in Preparation: M. Hassan Ahmed; Harvey A: Bridger JM.

5.1 Introduction:

The regulation, activation, and intracellular targeting of biological molecules are often mediated through the attachment of specific chemical groups. The post-translational modification of specific proteins by phosphorylation, acetylation, fatty acylation, and methylation has been studied extensively, and a variety of biological functions have been

studied are linked to these processes (Karve and Cheema, 2011). Protein prenylation is a process which, until recently, was poorly understood. Prenylation is a post-translation modification of proteins. Some prenylated proteins that have been identified are $p21^{ras}$, Ras related GTP binding protein, lamin B and prelamin A. This post-translational modification plays a critical role in the association of $p21^{ras}$ and lamins to the cell membrane and nuclear envelope, respectively in normal situations (Maltese, 1990). The mevalonate pathway that leads to the production of farnesyl can be targeted through different drugs such as farnesyltransferase inhibitors, statins and bisphosphonates. Lovastatin is a statin that impacts on this pathway.

5.1.1 The Effects of Lovastatin in Cancer:

Statins are used to decrease or reduce the cholesterol levels in the serum as well as inhibition of mevalonate biosynthesis pathways by inhibiting the enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase. Mevalonate is a precursor of dolichol (regulates the cell cycle and DNA synthesis), geranyl-pyrophosphate (GPP) and farnesyl-pyrophosphate (FPP) (cause isoprenylation of the intra-cellular G-proteins Ras and Rho, that regulate the signal transduction of the transcription of genes involved in cell proliferation, differentiation, and apoptosis). The natural inhibitor to HMG-CoA reductase is statin (Hindler *et al.*, 2006; Viccica *et al.*, 2007). Statins have the ability to block the synthesis of mevalonate and isoprenoids and block tumour cell growth for example, lovastatin has been shown to stabilise the cell cycle kinase inhibitors p21 and p27 and to arrest breast cancer cell lines in G1 phase (Rao *et al.*, 1998). Inhibition of GTP binding proteins Ras, is dependent on proper isoprenylation for localisation and function, plays an important role in mediating the biological effects of statins (Liao, 2002) (Chapter 1).

The HMG-CoA reductase inhibitors, or statins, are used to decrease the incidence and risk of cardiovascular diseases, including death, myocardial infarction, stroke, artrial fibrillation, and renal dysfunction, due to their effects on serum cholesterol levels. Statin may also play a potential role in the prevention and treatment of cancer as well as several anti-neoplastic properties, including decreased tumour growth (Hindler *et al.*, 2006), inhibition of angiogenesis (Frick *et al.*, 2003), induction of apoptosis by up-regulating *Bax* and *Bim* and down regulating *Bcl-2* as well as the activation of caspase proteases involved in programmed cell death and repression of metastasis because of its efficacy in the prevention and treatment of cancer (Hindler *et al.*, 2006).

5.1.2 Mutation Can Affect Lamins Assembly to the Nuclear Lamina:

Prelamin A, lamin A and B precursors, is the substrate of ZMPSTE24 in mammals. This enzyme is involved in post-translational proteolytic cleavage of carboxy terminal residues of farnesylated prelamin A to form mature lamin A (Leung *et al.*, 2001; Young *et al.*, 2005). Chapter 1 reviewed in detail lamin biosynthesis, their assembly to the nuclear lamina, and their role in diseases. The role of *ZMPSTE24* gene mutations in human disease has been also revealed. The lack of ZMPSTE24 protein or its activity in cell lines that derived from *ZMPSTE24* mutated patients resulted in misshapen nuclei containing abnormal distribution to lamin A/C associated with accumulation of unprocessed prelamin A (Navarro *et al.*, 2004; Navarro *et al.*, 2005) whereas, the *ZMPSTE24* mutations found in B type mandibuloacral dysplasia (MADB) encoded a protein with considerable reduced residual activity, the mutations found in Restrictive Dermopathy (RD) led to null ZMPSTE24 activity suggesting that a positive correlation between enzyme activity, accumulated prelamin A levels and the severity of the phenotype (Smigiel *et al.*, 2010). In *ZMPSTE24* knockout mice, severe growth and skeletal abnormalities were reported as well as premature death, these characteristics were due to total loss of mature lamin A and an accumulation of prelamin A (Pendás *et al.*,

2002 and Smigiel *et al.*, 2010). Most interestingly, several studies have demonstrated that blocking prelamin A farnesylation using farnesyltransferase inhibitors (FTI) could lead to prelamin A accumulation and prevent lamin A and lamin B maturation. Homozygous *ZMPSTE24* mutation will lead to loss of enzymatic activity, then progeroid and muscle diseases. Therefore, the cleavage cannot occur and the protein remains farnesylated and carboxymethylated and the process is not completed. That means lamins are still on the nuclear envelope and nucleoplasm and not incorporated to the nuclear lamina.

In Progeria where lamin A remains farnesylated there is evidence that statins i.e. Pravastatin can be used to prevent the farnesylation (http://www.ema.europa.eu/docs/en_GB/document_library/Orphan_designation/2010/06/WC500094023.pdf.

The data in this chapters shows that there is a problem with B-type lamins associating with the nuclear envelope and possibly a lack of A-type and B-type lamins in cells. A paper by Swamy *et al.*, 2002 has shown that lamin B expression can be increased by treatment with a statin called Lovastatin. The hypothesis for this chapter is that gene expression is affected in these cells because the genome is mislocalised and this in turn is due to lamin B not being localised properly to the nuclear envelope because it is missing or mislocalised (perhaps by the reduction of LBR) in specific cancer cells. Thus, it was decided to treat T-47D and BT-474 and the control cell line MCF-10A with Lovastatin to determine if nuclear structure and genome organisation could be improved leading to gene expression changes.

This chapter displays the expression of *EGFR*, *PTEN*, *CCND1*, *AKT1*, *HSP90AA1*, *HER2*, *LMNB1*, *LMNB2*, and *LBR* genes in breast cancer cells using qRT-PCR. It also demonstrates

the biological role of statin in the function of specific lamin proteins (lamin A, lamin B1, and lamin B2) and their presence and expression as shown by indirect immunofluorescence (IIF) and the positioning of *AKT1*, *HSP90AA1*, and *ERBB2*/*HER2* genes using 2D-FISH after treatment.

5.2 Material and Methods:

5.2.1 Cell Culture:

As per chapter 2, section 2.2.1

The human breast cancer (T-47D and BT-474) and normal (MCF-10A) cell lines were prepared for indirect immunofluorescence

5.2.1.1 Treatment with Lovastatin:

The cells were treated with lovastatin (Sigma-Aldrich) which was dissolved in absolute ethanol at final concentrations of 0 μM and 12 μM, 24 μM, and 29 μM (lovastatin) for 24 and 48 hours, (Appendices).

5.2.2 Indirect Immunofluoresence:

5.2.2.1 Cell preparation:

As per chapter 4, section 4.2.2.1.

5.2.2.2 Cell Fixation:

As per chapter 4, section 4.2.2.2

5.2.2.3 Primary and Secondary Antibodies Immuno-reactivity:

As per chapter 4, section 4.2.2.3

The primary antibodies used were; goat anti-lamin A (diluted 1:100, Santa Cruz Biotechnology), mouse anti-lamin B1 (diluted 1:50, Abcam), and mouse anti-lamin B2 antibody (diluted 1:500, Abcam). The diluted secondary antibodies were donkey anti-mouse

conjugated to cyanine (Cy3) (diluted 1:100, Jackson Laboratories) and Cy5 conjugated donkey anti-goat (diluted 1:100, Jackson Laboratories).

5.2.2.4 Mounting on Slides:

As per chapter 4, section 4.2.2.4

5.2.2.5 Evaluation and Detection using Fluorescence Microscopy:

As per chapter 4, section 4.2.2.5

5.2.3 2-Dimensional Fluorescence *In Situ* Hybridization (2D-FISH):

As per chapter 2, section 2.2.2 to 2.2.3.10

5.2.4 qRT-PCR

5.2.4.1 RNA Extraction:

As per chapter 3, section 3.2.4 and 3.2.5

5.2.5 cDNA Synthesis:

As per chapter 3, section 3.2.6

Table 5.1: RNA Standardisation Calculation:

The RNA concentrations from each sample were used as shown in table 5.1. Large samples can use up to 500 ng RNA for cDNA synthesis; smaller concentrations can use 100 ng for cDNA synthesis:

Cell Lines With and	Conc. of	Standard	RNA Needed	H_2O

Without Lovastatin Treatment	RNA (ng/ µl)	Amount (ng)	(µl)	Needed (µl)
	X	Y	Y / X	10 – (Y / X)
(6) T-47D (12µM/48 Hours)	423.9	500	1.17	8.83
(7) BT-474 (12µM/48 Hours)	362.7	500	1.37	8.63
(8) MCF-10 A (12µM/48 Hours)	84.1	500	5.94	4.06
(9) MCF-10A (24µM/48 Hours)	72.0	500	6.94	3.06

5.2.6 Reverse Transcriptase - Polymerase Chain Reaction (RT-PCR):

As per chapter 3, section 3.2.8

5.2.7 Western Blots:

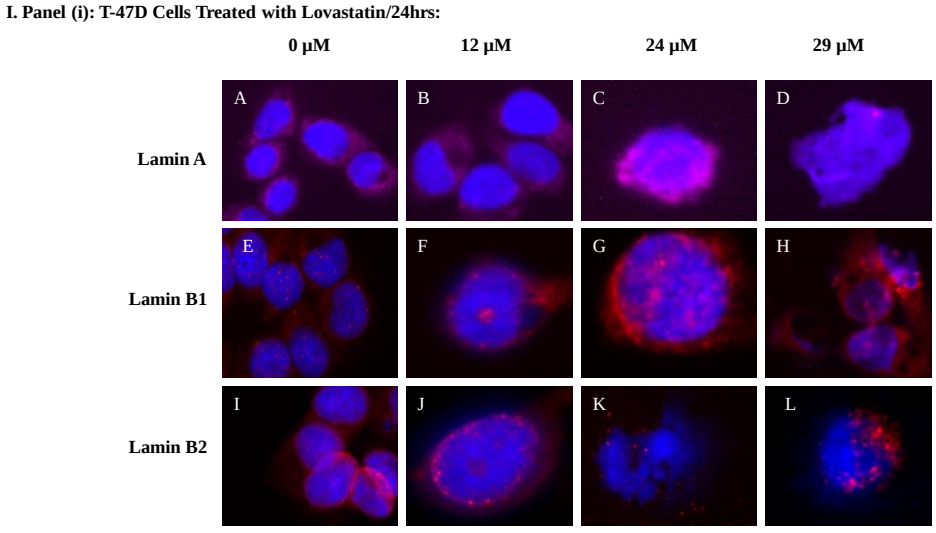
As per chapter 4, section 4.2.7

5.3 Results:

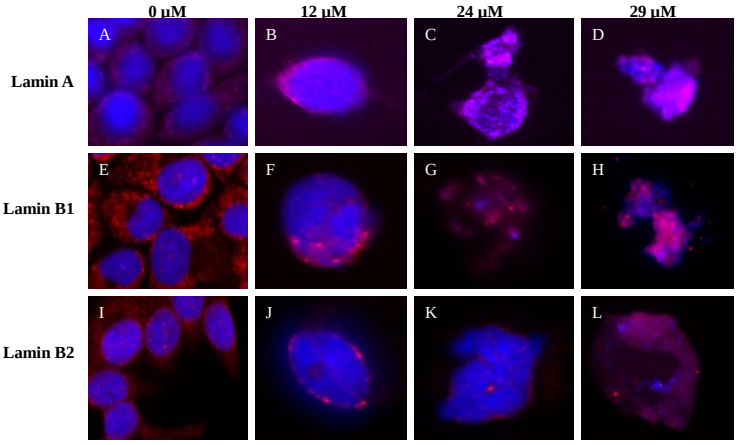
5.3.1 Restoration of Lamins A and B with Lovastatin in MCF-10A, T-47D, and BT-474 Cells:

The cells were treated with different lovastatin concentrations (0 µM, 12 µM, 24 µM, and 29 µM) for 24 and 48 hours. Some cells were killed but a large number of cells survived. Then, the cells were harvested and fixed with methanol: acetone (1:1) for indirect immunofluorescence. The immunoreactivity of lamins A, B1, and B2 within MCF-10A, T-

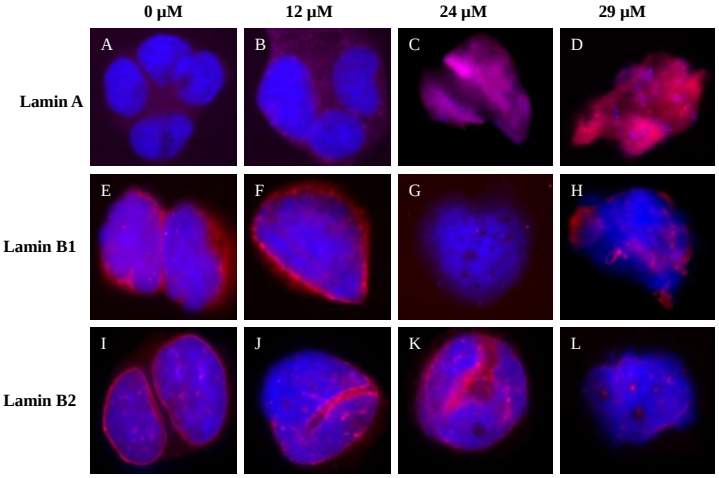
47D, and BT-474 cells was assessed. MCF-10A cells showed clear change in shape from its normal shape as shown in chapter 4 (Results Section) to cells with a more rounded shape (Table 5.2). T-47D cells were less influenced by the drug than BT-474 cells. T47D is the cell line that is used in drug resistance (http://icbp.lbl.gov/breastcancer/list_data.php?id=1, July 2009 and Chapter 1). The drug concentration was shown to be harmful to both T-47D and BT-474 cells at 24 µM and 29 µM concentrations as well as in MCF-10A cells at 29 µM concentration, since these cells showed apoptotic features. The morphological features of apoptosis were observed in these cells were chromatin condensation, cell shrinkage, DNA fragmentation, and membrane blebbing. Thus 12 µM concentration in cells was the best concentration of the drug to use. This concentration has also been used by Corcos and Le Jossic-Corcos in 2013. The presence and distribution of lamins A, B1, and B2 within MCF-10A, T-47D, and BT-474 cells was assessed (Figure 5.1).



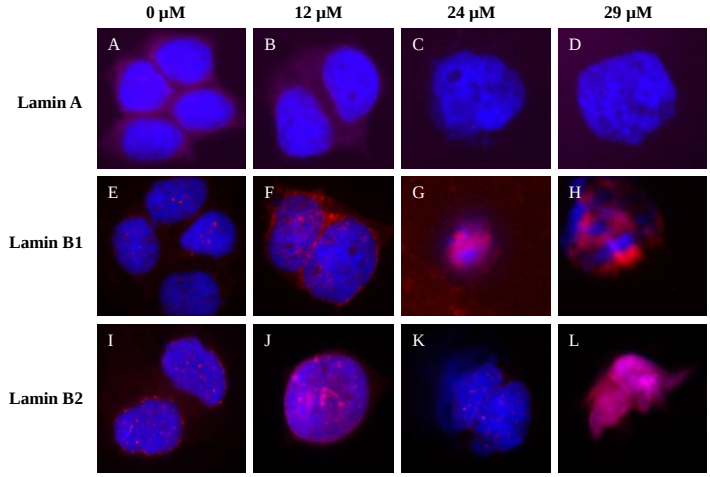
Panel (ii): T-47D Cells Treated with Lovastatin/48hrs:



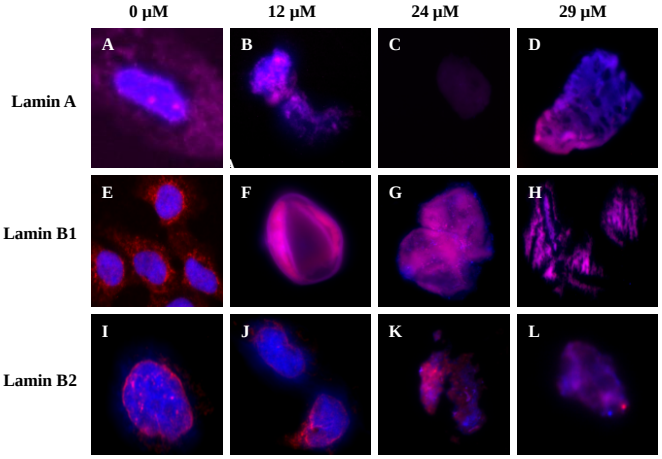
II. Panel (i): BT-474 Cells Treated with Lovastatin/24hrs:



Panel (ii): BT-474 Cells Treated with Lovastatin/48hrs:



III Panel (i): MCF-10A Cells Treated with Lovastatin/24hrs:



Panel (ii): MCF-10A Cells Treated with Lovastatin/48hrs:

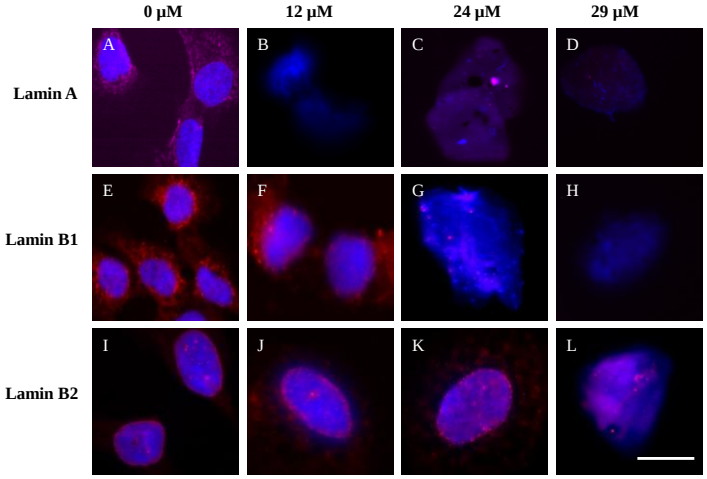


Figure 5.1: The Proteins Presence and Expression of Lamin Proteins by Indirect Immunofluorescence in T-47D, BT-474, and MCF-10A Cell Lines before and after

Treatment with Lovastatin for 24 and 48 Hours at Different Concentrations: These images demonstrate the effects of lovastatin treatment on different cell lines that affect lamins distributions. Cells were processed and determined by indirect immunofluorescence method when fixed with methanol: acetone (1:1) using anti-lamins and anti-lamin binding proteins and the DNA stained with DAPI (Blue). **I. Panels (i and ii):** T-47D cells treated with Lovastatin/24 and 48hrs (0 µM, 12 µM, 24 µM, and 29 µM): demonstrates the images of T-47D cells when immunostained with goat anti-lamin A, mouse anti-lamin B1, and mouse anti-lamin B2 antibodies (A-L). The secondary antibodies at final concentration using Cy5 conjugated donkey anti-goat (pink) and Cy3 conjugated donkey anti-mouse (red) antibodies were employed at final concentration as shown in Chapter 4, Methods Section and Appendices. The same experiment was applied to **II. Panels (i and ii):** BT-474 cells treated with Lovastatin/24 and 48hrs (A-L). **III. Panels (i and ii):** MCF-10A cells treated with Lovastatin/24 and 48hrs (A-L),. Cells were mounted using Vectashield mounting medium containing DAPI to facilitate its visualisation and detection. The DNA stained with DAPI (Blue), Scale Bar = 10µM.

5.3.2 Cellular Morphology:

The cells were treated with lovastatin showed changes in nuclear structure when fixed with methanol: acetone (1: 1) and immunostained with lamin A, lamin B1, and lamin B2 antibodies. The cells were treated with lovastatin at different concentrations 0 µM and 12 µM (MCF-10A, T-47D and BT-474 cells) for 48 hours. The most affected cells by the drug were MCF-10A which showed abnormal morphology in around 80% of cells followed by BT-474 (57%) and then T-47D (29%) cells.

Table 5.2: Cell Morphology after Lovastatin Treatment at 12 µM Concentration for 48 Hours: The table displays the percentages of cellular morphology influenced by lovastatin at concentration 12 µM for 48 hours. The cells were harvested and counted by haemocytometer. The data showed that the more severely affected cells were MCF-10A as showed high number of abnormal nuclei (80%) followed by BT-474 (57%) and T-47D (28%) which is less affected and more resistant to the drug. MCF-10A cells changed their normal shape to

rounded float shape. This was evaluated by light microscope that used to determine the number and shape of these cells.

	Cells with Normal Morphology	**Cells with Abnormal Morphology**
(1) T47-D	71.6%	28.4%
(2) BT-474	43.3%	56.7%
(3) MCF-10A	20.2%	79.8%

5.3.3 Gene Loci position after Treatment with Lovastatin:

Fluorescence *in situ* hybridisation (FISH) was performed to assess the position of human genes *AKT1*, *HSP90AA1*, and *ERBB2*/*HER2* that are located on human chromosomes 14 and 17 territories. Cells were treated with: 0 µM and 12 µM lovastatin for 48 hours. In treated T47-D and BT-474 cell lines, the genes occupy altered localisations in interphase nuclei when compared to MCF-10A treated cells (Figure 5.1I, II, and III Panels i and ii). The position of the genes in treated cells was located at the periphery in MCF-10A and T-47D and in the interior in BT-474. The effect of this drug on the nuclear localisation of these genes in T47-D, BT-474, and MCF-10A cell lines was determined. Treatment with lovastatin relocates the positions of the genes *AKT1*, *HSP90AA1*, *ERBB2*/*HER2* (Table 5.3). To determine the movements and localisations of *AKT1*, *HSP90AA1*, and *ERBB2*/*HER2* genes in T47-D, BT-474, and MCF-10A cell lines, 2D-FISH was performed to determine the gene positions. It was found that *AKT1* was changed its location in MCF-10A from interior to periphery and in BT-474 cells from periphery to interior but there was no change in position in T-47D cells. Moreover, *HSP90AA1* did not change its location in any of the cells after treatment whereas,

ERBB2/*HER2* was changed after treatment in MCF-10A cells from interior to periphery and in BT-474 cells from periphery to interior but no change was found in T-47D cells. These data suggest that lovastatin treatment allows cells to reposition genes in response to the drug (Figures 5.2 and 5.3).

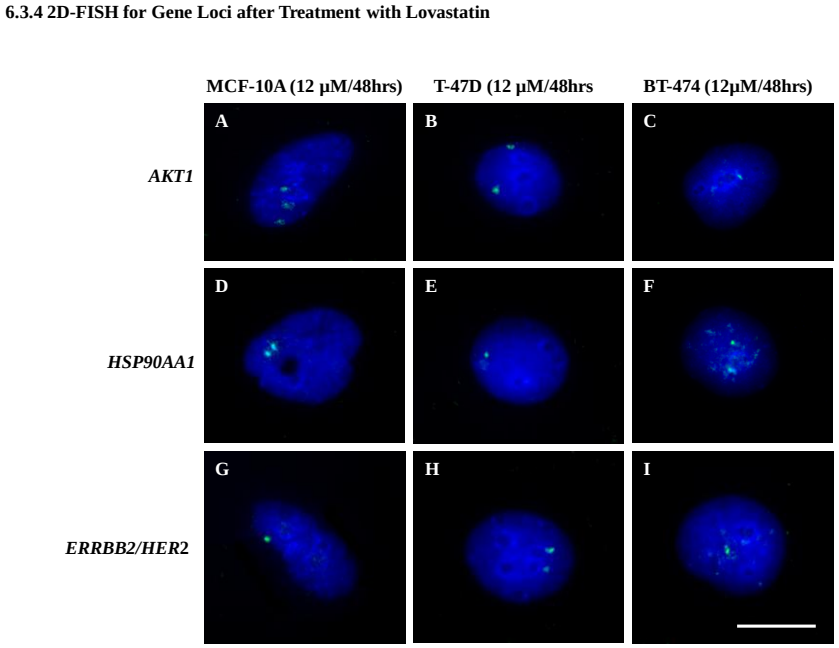


Figure 5.2: 2D-FISH for Gene Loci After Treatment with Lovastatin: Using 2D-FISH to analyse the positioning of specific genes *AKT1*, *HSP90AA1*, and *ERBB2*/*HER2* in normal breast MCF-10A and cancer T-47D and BT-474 treated cell lines. DNA has been counterstained using DAPI (Blue) whereas; gene signals can be seen in Red (labled Cy3-streptavidin) and then pseudocoloured green to facilitate their positioning analysis. Magnification = X100; Scale Bar = 10µM.

5.3.4 Analyses of Gene Loci after Treatment with Lovastatin:

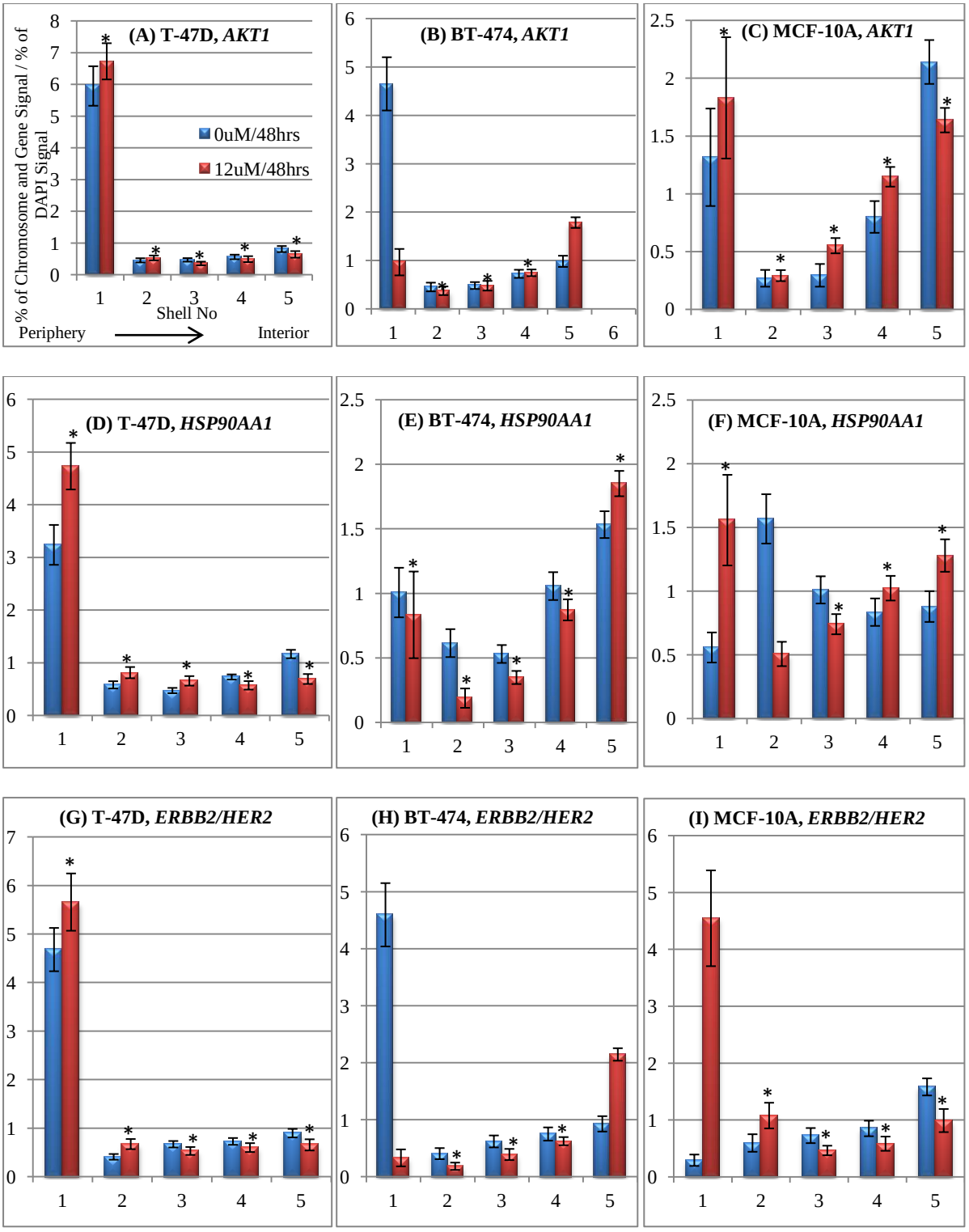


Figure 5.3: Image Analyses of Gene Positioning Before and After Treatment with Lovastatin using 2D-FISH and Erosion Analysis: The histograms display positions of human genes *AKT1* (A, D, and G), *HSP90AA1* (B, E, and H), and *ERBB2*/*HER2* (C, F, and I) that are located and resided on human chromosomes 14 and 17 territories in T47-D and BT-474 breast cancer and MCF-10A normal breast cell lines that occupy altered localisations

during interphase nuclei after treatment with lovastatin. These genes have not changed their position in T-47D cells as well as *HSP90AA1* in BT-474 cells. *AKT1* and *ERBB2*/*HER2* genes moved from the nuclear periphery to the nuclear interior in BT-474 cells whereas the same genes moved from the nuclear interior to the nuclear periphery in MCF-10A cells. No change was observed in *HSP90AA1* gene in MCF-10A. Their positions were assessed by 2D-FISH and analysed by erosion analysis. Error bars indicate standard error of the mean (SEM). Y Axis = % of Chromosome and Gene Signal / % of DAPI Signal whereas X Axis = the position from periphery to interior (1 to 5).

5.3.5 Statistical Analysis After Treatment with Lovastatin using Student's *t*-test:

The Student's *t*-test with Excel Software Programme was used to compare statistically the positions of genes (*AKT1*, *HSP90AA1*, and *ERBB2*/*HER2*) in the treated cell lines as compared to no treatment. The probability-value (*p*-value) in the test was known; $p<0.5$ (Green) was considered significant and any less than 0.05 (Red) that means there is a significant difference in position and indicated with star and more than 0.5 (Black) that means no significant difference in position.

5.3.5.1 Statistical Analyses for Gene Position:

(1) *AKT1*:

	MCF-10A	T-47D	BT-474
Area 1			
Area 2			
Area 3			
Area 4			
Area 5			

(2) *HSP90AA1*:

	MCF-10A	T-47D	BT-474
Area 1			
Area 2			

Area 3			
Area 4			
Area 5			

(3) *ERBB2/HER2*:

	MCF-10A	**T-47D**	**BT-474**
Area 1			
Area 2			
Area 3			
Area 4			
Area 5			

The Key:
Black = Indicates no significant difference between the positions with the *p*-value >0.5
Green = Indicates significant difference between the positions with the *p*-value <0.5
Red = Indicates significant difference between the positions with the *p*-value <0.05

5.3.6 Gene Expression by qRT-PCR Analysis:

The graphs below show the percentages of relative quantification (RQ) of gene expression by fold changes in expression for each gene in treated cell lines using qRT-PCR. The levels of expression of all genes studied here are calculated from $2^{-\Delta\Delta C}{}_t$ or relative quantification using RQ Manager and Microsoft Excel softwares (Figure 5.4). From the literature, the relocation of chromosomes and genes to different sites within the nucleus can affect the gene expression and this is more noticeably in MCF-10A and BT-474 cell lines.

It is observed that an increase in the expression of *LBR* gene in treated BT-474 and T-47D cells whereas it down regulated in MCF-10A treated cells. *LMNB1* and *LMNB2* genes were up-regulated in treated MCF-10A (12 µM/48hrs) and *HSP90AA1* gene was down regulated in T-47D cells and slightly up-regulated in BT-474 and MCF-10A cells. In addition, *HER2* gene

was up-regulated in treated BT-474 cells and *CCND1* gene expression was also up-regulated in treated MCF-10A and T-47D cells. Moreover, *EGFR* gene was up-regulated in treated MCF-10A cells and BT-474 whereas, *PTEN* was up-regulated in all treated cells. *AKT1* was up-regulated in BT474 after treatment and slightly in MCF-10A with no change in T-47D.

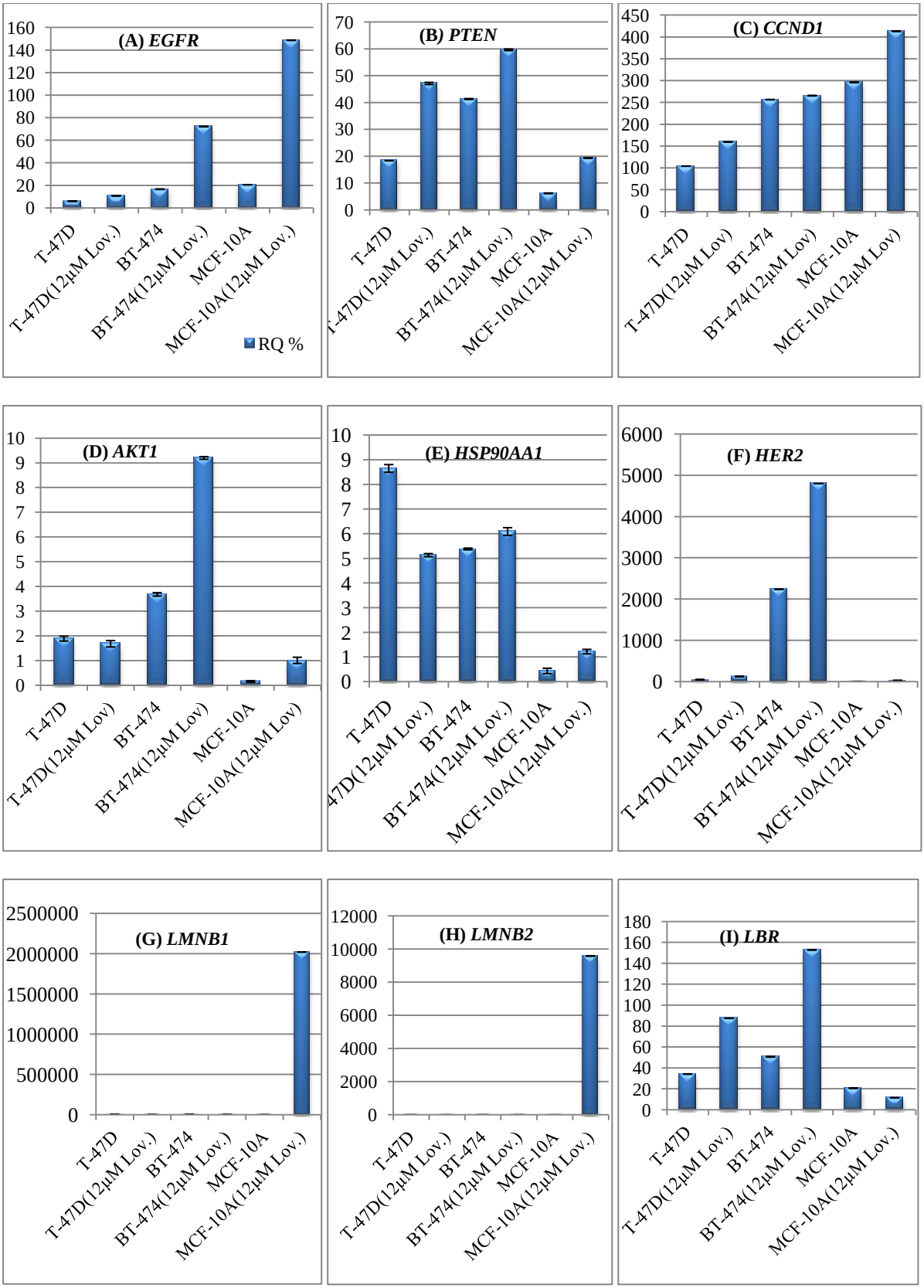


Figure 5.4: Expression Level of *EGFR*, *PTEN*, *CCND1*, *AKT1*, *HSP90AA1*, *HER2*, *LMNB1*, *LMNB2*, *LBR*, and *β-Actin* Genes mRNA After Treatment with Lovastatin in Breast Cell Lines using Quantitative Real Time-PCR (qRT-PCR): These histograms

show the percentage of relative quantification (RQ %) of expression of different genes in present of β-actin as a housekeeping, an endogenous gene with no supplement set as 1. The fold changes in these different gene expressions were assessed in treated T-47D, BT-474, and MCF-10A cell lines using RQ Manager and Microsoft Excel software. Error bars indicate standard error of the mean (SEM) of three replicates.

Changes in gene position were correlated with any changes in gene expression in Table 5.3. For the T-47D cells notably, there were no major alterations in gene positions with the three genes that were positioned. *AKT1* and *ERBB2*/*HER2* genes changed their positions in treated BT-474 cells from the nuclear periphery to the nuclear interior with 12 μM/48hrs and the expression was up-regulated in all the cell lines tested; fitting the hypothesis that gene movement to the interior is correlated with gene up-regulation. *HSP90AA1* gene position was shifted more to the periphery in MCF-10A cells and T-47D cells with an up-regulation and a down-regulation in gene expression, respectively. In BT-474 cells this gene moved slightly towards the interior and became slightly up-regulated. After treatment, *AKT1*, *HSP90AA1*, and *ERBB2*/*HER2* genes did not change their positions in treated T-47D cells from the periphery and *AKT1* and *ERBB2*/*HER2* genes expression did not change in the same cells. *HSP90AA1* gene expression was down-regulated in T-47D cells after treatment. *AKT1* and *ERBB2*/*HER2* genes in treated MCF-10A cells also changed their position from interior to periphery and *AKT1* was up-regulated whereas *ERBB2*/*HER2* gene had no change in expression in the same cell line. *HSP90AA1* gene in MCF-10A cells did not change its position from periphery but down regulated in expression.

Table 5.3: Image Analyses for Gene Positioning and Expression After Treatment with Lovastatin: The table displays positions of human genes *AKT1*, *HSP90AA1*, and *ERBB2*/*HER2* that are located on human chromosomes 14 and 17 territories in T-47D, BT-

474, and MCF-10A cells occupy altered localisations in interphase nuclei after treatment with lovastatin at 12 µM concentrations when incubated for 48 hours.

Gene		MCF-10A (12µM/48hrs)	T-47D (12µM/48hrs)	BT-474 (12µM/48hrs)
AKT1	**Gene Position**	Interior → Periphery	Periphery	Periphery → Interior
	Gene Expression	Up-regulated ↑	No Change	Up-regulated ↑
HSP90AA1	**Gene Position**	More Peripheral	More Peripheral	Slightly More Interior
	Gene Expression	Up-regulated ↑	Down-regulated ↓	Up-regulated Slightly ↑
ERBB2/HER2	**Gene Position**	Interior → Periphery	More Peripheral	Periphery → Interior
	Gene Expression	No Change	No Change	Up-regulated ↑

After treatment and some changes to gene position and expression it was important to determine what had happened to the B-type lamins and LBR. Table 5.4 displays quantitative changes seen with respect to alterations in the fraction of cells displaying more normal distributions of these proteins. Notably, there was improvement and an increase in the presence of lamin B protein within treated MCF-10A cells (12µM/48hrs) and LBR within treated BT-474 and T-47D cells (12µM/48hrs). *LMNB* gene expression was up-regulated in

treated MCF-10A cells whereas treated BT-474 and T-47D cells showed up-regulation in *LBR* gene expression levels. The table below displays these findings before and after treatment with lovastatin.

Table 5.4: Proteins Presence Before and After Treatment and Genes Expression After Treatment with Lovastatin: The table displays the presence of lamin B1, lamin B2, and lamin B receptor (LBR) proteins when fixed with methanol: acetone (1:1) and the expression of their genes (*LMNB1*, *LMNB2*, and *LBR*) in T47-D, BT-474, and MCF-10A cells.

			MCF-10A	**T-47D**	**BT-474**
		Before	Faint Rim (in	Rim + Large	Rim + Large

Lamin B1	**Protein Presence**	**Treatment**	87% of cells)	Brighter Spots (in 65% of cells)	Spots (in 72% of cells)
		After Treatment	Rim only (in 97% of cells)	Rim + Brighter Spots (in 93% of cells)	Rim + Brighter Spots (in 70% of cells
	Gene Expression		Up-regulated ↑	No change	No change
Lamin B2	**Protein Presence**	**Before Treatment**	Faint Rim and Spots (in 47% of cells)	Rim + Large Brighter Spots (in 85% of cells)	Rim + Large Spots (in 80% of cells)
		After Treatment	Rim (in 100% of cells)	Rim only (in 78 % of cells)	Rim + Brighter Spots + (in 74% of cells
	Gene Expression		Up-regulated ↑	No change	No change
Lamin B Receptor (LBR)	**Protein Presence**	**Before Treatment**	Faint (in 95% of cells)	Faint (in 64% of cells)	Faint (in 29% of cells)
		After Treatment	NA	NA	NA
	Gene Expression		No Change	Up-regulated ↑	Up-regulated ↑

***NA = Not Available**

5.4 Discussion:

The question that the spatial organisation of the genome in the nucleus can modulate gene expression is still unanswered. In mammals, regions with low gene-density, and that are late-replicating, locate toward the periphery of the nucleus and inactive genes also locate close to the nuclear periphery and their movement away from the periphery correlates with their transcriptional activation (Szczerbal *et al.*, 2009). However, the expression of other genes in mammalian cells appears to be unaffected by their location to the nuclear periphery (Finlan *et al.*, 2008). Therefore, to what degree this level of nuclear organisation directly affects gene function and position?

In this study, the position of *AKT1*, *HSP90AA1*, and *ERBB2/HER2* genes was assessed and it was found that these genes changed their positioning within BT-474 and MCF-10A after treatment with lovastatin. In BT-474 breast cancer cell lines, the position of *AKT1* was relocated from the periphery to nuclear interior in with up-regulation of expression. In addition to this, the position of *AKT1* was changed or altered from interior to periphery in normal breast MCF-10A cell line with down-regulation of expression. In T-47D breast cancer cell line, the position of *AKT1* remains unchanged at the periphery with no change in its expression. In BT-474 and T-47D breast cancer cell lines, the position of *HSP90AA1* remains unchanged at the interior and peripheral sites, respectively, in both concentrations of 0 µM and 12 µM with increase in expression in BT-474 cells and decrease in expression in T-47D cells. In addition to this, the position of *HSP90AA1* remains unchanged at the periphery in concentrations of 0 µM and 12 µM, for 48 hours in normal breast MCF-10A cell line with increase in its expression within the same cells. In BT-474 breast cancer cell line, the position of *ERBB2*/*HER2* was changed from periphery in concentration of 0 µM for 48 hours to

interior in concentration of 12 μM for the same time. In addition to this, the position of *ERBB2*/*HER2* was altered from interior in concentration of 0 μM for 48 hours to periphery in concentration of 12 μM for the same time in normal breast MCF-10A cell line. In T-47D breast cell line, the position of *ERBB2*/*HER2* remains unchanged at the peripheral in both concentrations of 0 μM and 12 μM for the same time (Figures 5.4 and 5.5, Table 5.3).

In these cell lines it has been shown some correlations with genes being relocated after treatment and either down-regulating their expression with a move to the periphery and up-regulating their expression with a move to the interior. However, the opposite is also true with genes that have moved to the nuclear periphery being up-regulated and genes moving to the interior being down-regulated. Therefore, these findings regarding gene position and expression, it is untrue to consider that the periphery is the site for gene repression in cancer cells. What is important to realise from these studies is that changes to gene behaviour have been elicited by a drug treatment leading to changes in gene expression. Indeed in T-47D there are desirable changes with respect to health and treatment with *AKT1* and *HSP90AA1* being down-regulated and *PTEN* being up-regulated.

The treatment with lovastatin has also increased LBR expression which in cells is correlated with a stronger nuclear B-type lamins, suggesting that the lamin B that was mislocalised as aggregates has been able to locate to the nuclear envelope. In BT-474 this has changed gene positioning back to a more normal situation as in MCF-10A.

The mammalian nuclear lamina protein lamin B1 is post-translationally modified by farnesylation, endoproteolysis, and carboxymethylation processes at a carboxyl-terminal CAAX motif. In 2003, Maske *et al.* demonstrated that the CAAX endoprotease Rce1 is

required for lamin B1 endoproteolysis, demonstrated that proteolysed but non-methylated lamin B1 is required for fully processed lamin B1 during interphase nuclei and showed the role of methylation in the organisation and incorporation of lamin B1 into the nuclear lamina. Deficiency in the endoproteolysis or methylation of lamin B1 results in loss of integrity and deformity of the nuclear lamina. These data showed that the organisation of the nuclear envelope and lamina is dependent on a mechanism that involves the methylation of lamin B1. Since B-type lamins are expressed but not localised properly investigation into their processing in these breast cancer cells would help determine why they are not localised at the membrane if it is not due to the LBR.

However, lamin A and lamin B both are involved in cell proliferation, cell cycle regulation, genome organisation and other nuclear functions such as replication, repair, transcription, expression, and apoptosis. Mutations in *LMNA* and *LMNB* genes may cause disrupting of these pathways. Alteration in these proteins presence, organisation and their distribution in these cell lines after treatment as demonstrated in (Figure 5.2I, II, and III Panels i and ii) influence the changes in gene expression. Alteration in gene expression and the need for specific genes to be expressed lead to the changes in organisation and gene locations. The results demonstrating that the position of *AKT1* and *ERBB2*/*HER2* genes remains unaltered in T-47D cells as well as *HSP90AA1* gene in MCF-10A, T-47D, and BT-474 cells, the reason is that could be of genes that are found in sets or clusters or may be on loops away from their chromosomes, stage of the cancer, solidity of the tumour, dose of the drug, and time for incubation and thus cannot be reorganised (Figure 5.4 and Table 5.3). Moreover, the number of cells showed similar morphology to the cells nuclei before treatment (0 µM/48 hours), while other nuclei showed morphological features of apoptosis (cell death) in response to the drug. The morphological features of apoptosis consist of chromatin condensation, shrinking

of nuclear envelope, and membrane blebbing, DNA fragmentation which can be clearly observed by fluorescence microscopy (Figure 5.2I, II, and III Panels i (Images H, K) and ii (Images G, H)). In addition, there are a number of cells within the treated cultures that are behaving normally and without changing that can still respond to the drug (Figure 5.2I, II, and III Panels i (Images B, F, and J) and ii (Images B, F, and J)). Furthermore, there is still some functioning of lamin A and lamin B within these nuclei. In addition, the repositioning or reorganisation of gene loci could be affected by nuclear motor such as actin and myosin dependent and that mutation in *LMNA* gene may result in perturbed interaction of lamin A with nuclear motor or SUN proteins and other specific inner nuclear membrane proteins (emerin and LAP2α) suggesting that the role of lamin A in chromosomal organisation and chromosomal movement. Similar to this, mutation in *LMNB* gene may result in perturbed interaction of lamin B with its specific receptor (LBR), chromatin, LAP2 β, MAN1, BAF, R-Smad, E2F, and β-catenin proteins and thus, this will lead to changes and alterations in gene repositioning, gene expressions, and dysfunctional characters due to changes in structures.

Although the wide use of lovastatin *in vivo* and *in vitro* as well as animal model is highly successful, limitations of using this treatment should be considered. To our knowledge that lovastatin treatment plays a crucial role in post-translational modifications of proteins such as lamins and RAS that are required to undergo farnesylation and thus may result in various effects. Because lamin A undergoes further proteolytic processing of the CAAX modifications (Kilic *et al.*, 1999), stable interactions of carboxymethyl moiety in the nuclear envelope are probably limited to B-type lamins. Lovastatin treatment may not be able to restore tissues that are already damaged. Similar to this was noticed in MCF-10A cells as it was more influenced by the drug than in other cancerous cells (BT-474 then T-47D cells) (Table 5.2). The improvement of that seen in cell nuclei after lovastatin treatment requires

further study in breast cancer and other cancers. Furthermore, lovastatin could cause changes in the expression and distribution of lamin B1 within treated cells. With this in mind further studies should still go forward to determine if lovastatin or other similar drugs could potentially be used in combination with other treatments to help restore normal nuclear structure and genome organisation to cancerous cells.

The cancer chemotherapeutic agent, lovastatin, has antiproliferative effect through the inhibition of ICMT (by increasing metabolites that directly inhibits the enzyme) and, thus, carboxylmethylation inhibition of prenylated proteins. Because all of the CAAX processing enzymes are potential chemotherapeutic targets, an understanding of the effects of inhibition on nuclear structure, gene expression, and gene activation or repression are essential in the development of therapeutic molecules. Treatment with lovastatin that are used in cancers to induce the apoptosis, improve and increase LBR expression and the nuclear lamina distribution.

5.5 Conclusion:

The effect of lovastatin, as a modern signal transduction inhibitor, was clearly seen in breast cancer cells (T-47D and BT-474) by increasing and up-regulating the expression of *LBR* gene. *LMNB1* and *LMNB2* genes in MCF-10A cells were also increased and up-regulated. This up-regulation allowed these proteins to be more abundant and located in the right place at the nuclear periphery. I also showed that *AKT1* and *HER2* genes changed their position and their expression as well as some other genes that are involved in breast cancer progression. The drug can restore and correct the aberrant nuclear structure and genome organisation in breast cancer cells maybe because lamin B is required for correct gene position and expression and also involved in normal nuclear function. Therefore, lovastatin may provide greater role in tumour specificity and clinical effectiveness than existing systemic chemotherapy and is a promised drug for cancer treatment and for correct gene expression, normal nuclear structures and functions.

Chapter 6: General Discussion and Future prospective

6.1 General Discussion and Future Prospective:

Properly functioning processes, correctly positioned structures, and a well organised genome are required in cell nuclei for regulated gene expression and genome stability. The nuclear lamina is a fundamental nuclear structure composed of lamins proteins that is important in DNA replication, DNA repair and gene positioning at the nuclear envelope. Genes can also be in a different nuclear compartment to their chromosomes, as they can be out on loops away from the chromosome territories. Changes in nuclear positions could severely affect gene expression and the normal function of cells. Here, it is hypothesised that the dysregulation of B-type lamins has contributed to tumour development by relocalisation of genes involved in breast cancer affecting their regulation and expression. In this study, breast cancer cell lines (T-47D and BT-474) were treated with lovastatin which has restored LBR expression and the localisation of lamin B, altering gene positioning and changing expression of genes involved in breast cancer progression such as *AKT1* and *HER2*. Since the drug (lovastatin) affects the prenylation as a post-translation modification process, it is found that B-type lamins and its receptor expression and distribution were improved and increased in the most affected cells (MCF-10A, T-47D, and BT-474). Further, this drug induced cell death in a fraction of cells (Chapter 5).

One of the major roles for lamins is in genome organisation, which can result in correct and regulation of genome organisation, expression, and function (Foster and Bridger, 2005). The lamina is juxtaposed to the inner nuclear membrane and functions in tethering chromatin to the nuclear envelope through both the alpha-helical rod and the carboxyl-terminal end of lamins and in maintaining nuclear shape. A and B type lamins, the main components of the nuclear lamina proteins, contatin a DNA binding domain and have affinity for choromosomes, chromatin and/or DNA (Höger *et al.*, 1991; Glass *et al.*, 1993; Stierlé *et al.*,

2003). The interactions of these lamins with chromatin suggest that alterations in lamin−DNA interactions may play a role in the pathophysiology of some lamin-linked diseases and cancers, as well as in ageing. Studies have assessed chromatin reorganisation as a main contributor to cancer, laminopathies, and ageing in humans. Changes in nuclear architecture are a hallmark of cancers in human cells, laminopathesis and ageing. These changes seem to be driven by DNA damage, gene aberrant expression and localisation which results in alterations and/or diseases that affect gene expression, and may cause cancer (Oberdoerffer and Sinclair, 2007). Not that many studies have looked at nuclear structure in cancer especially breast cancer and as far as we know no studies have correlated gene repositioning in cancer with aberrant nuclear architecture.

Generally, chromosome positioning within cell nuclei is non-random and positioned according to their gene density and activity. From genome organisation theory, the most gene poor chromosomes found at the nuclear periphery and gene rich chromosomes found at the nuclei interior. Different studies showed that the non-random positioning of the genome is involved in controlling and regulating gene expression, but alterations in the levels of transcription and expression have been observed when specific loci change position in nuclei (Misteli, 2004). Furthermore, these genes may be found on loops towards the areas of the nucleus that more active and specified for transcription (Foster and Bridger, 2005). In this study, the experiments have assessed the positioning of specific chromosomes and genes within specific breast cell lines (MCF-10A, T-47D, BT-474, Sk-Br-3, and GI-101) and any aberrant impact of nuclear structure. This assessment was achieved by analysing the presence and distribution of nuclear lamin proteins, genes and chromosome localisation and gene expression. It found that the nuclear architecture of breast cancer cells is often altered. The most severely affected cells (T-47D and BT-474) which have aberrant distribution of nuclear

lamins B1, lamins B2 and lamin B receptor (LBR). B-type lamins were found as large foci within the nucleoplasm and not at the nuclear periphery with little LBR to localise the lamin B. The same cell lines showed that the genes were positioned more peripherally in these two cancer cell lines than in normal cells (MCF-10A). They were also not in the same nuclear compartment as their chromosomes they can be out on loops away from the chromosome. From the literature, B-type lamins are involved in chromosome positioning and correct gene expression (Malhas *et al.*, 2007). This is the first time that B-type lamins, A-type lamins and LBR have been shown to be affected in breast cancer, as well as all the other aberrations in NE proteins and chromatin binding proteins in breast cancer cells. These results were communicated to Prof Mokbel to screen his large array of breast cancer RNA samples finding a correlation with the lack of lamins A, B and LBR (Wazir *et al.*, in prep).

Since lamins and lamin binding proteins disorganisation have the ability of the nuclear membrane to anchor chromatin within the interphase, it is important to consider the possible implications and dysfunction on the nucleus by failing to anchor matrix attachment regions (MARs) and transcription factories correctly. Moreover, the nuclear envelope or nuclear membrane provides the basis for normal function of transcription factories (Iborra *et al.*, 1996). Any incorrect genes positioning, may change the normal local environment and therefore, altering the type of transcription factories and transcription status. Thus, gene expression could be mis-regulated and could lead to cancer. The mis-regulation of transcribed genes and lamin proteins may affect the transcription, translation, and other proper nuclear processes and functions. In 2002, Spann and his co-workers reported that lamin A and B mutation inhibits the activity of RNA polymerase. Furthermore, deletion in lamin B1 levels disrupts RNA polymerase II synthesis and affects interphase chromosome position and gene expression (Malhas *et al.*, 2007; Tang *et al.*, 2008).

These findings suggest that the nuclear membrane contributes to the dysfunctional of lamins and their binding proteins in different ways, by inefficient recruitment of proteins involved in gene and chromosome localisations as well as DNA repair, transcription, cell cycle, gene expression, and apoptosis. However, a few number of published papers regarding the hypothesis that the nuclear lamin subtypes play a role in genome organisation in health and disease cells.

Therefore, nuclear structure and genome organisation are essential and important for correct gene expression and normal function of cells. Understanding the nuclear structure and genome behaviour of breast cancer cells will aid in the goal to eliminate cancer as a major health problem. The mechanistic basis by which alterations in nuclear structure and function lead to cancer is an exciting new area of study.

6.2 Summary of this Project:

To summarise this work, Figure 6.1 describes the findings of this study.

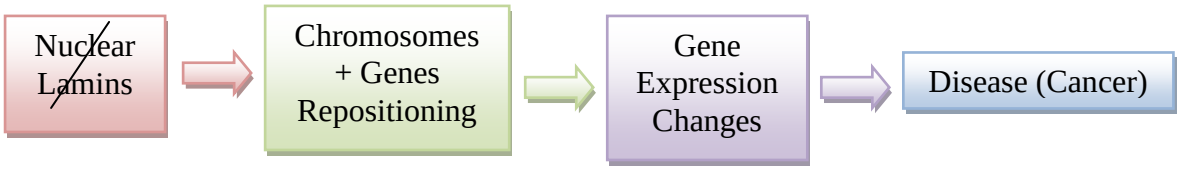


Figure 6.1 Summary of This Project: Loss or aberrant lamins expression will lead to changes in chromosomes and genes positioning. This alteration may change the gene expression and then lead to cancer.

The work in this thesis has a number of novel findings by describing the relocation of genes, and chromosomes in breast cancer cells correlated with changes in gene expression of these genes. The presence and distribution of nuclear lamins and other nuclear structural proteins

are affected in the cell lines. With a drug treatment a number of these aberrations can be corrected or changed. Thus, there is potential to restore nuclear architecture to cancer cells and re-regulate gene expression.

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Appendices

Appendices

❖ Indirect Immunofluorescence:

Methanol : Acetone, 1:1 (v/v):

25 ml Methanol in 25 ml Acetone

4% Paraformaldehyde:

4g paraformaldehyde in 100 ml 1XPBS (heated in 50ºC till to be dissolved)

10X PBS:

160g NaCl, 4g KCl, 22.9g Na_2HPO_4, and 4g KH_2PO_4 adjust this to 2 litres ddH_2O then autoclave it.

1XPBS (v/v):

100 ml 10XPBS in 900 ml ddH_2O

1% Triton-X100:

1 ml Triton-X100 in 99 ml 1XPBS

10% Fetal Calf Serum (FCS):

10 ml Fetal Calf Serum (FCS) in 90 ml 1XPBS (Phosphate Buffer Saline)

1% New Born Calf Serum (NCS, v/v):

1 ml New Calf Serum (NCS) in 99 ml 1XPBS

❖ **Summary of Antibodies Used in Indirect Immunofluorescence Method:**

Primary Antibodies (v/v)			**Secondary Antibodies (v/v)**	
Protein	**Species**	**Dilution (µl)**	**Species**	**Dilution (µl)**
LAP2 α	Rabbit	1:250	Swine (FITC)	1:30
Lamin Cy	Rabbit	1:50		
Lamin Cw	Rabbit	1:50		
LBR	Rabbit	1:500		
PML	Rabbit	1:100		
KI67	Rabbit	1:1500		
Lamin A/C	Mouse	1:100	(1) Donkey (Red) (2) Cy3 Conjugated Donkey	(1) 1:70 (2) 1:100
Lamin B1	Mouse	1:50		
Lamin B2	Mouse	1:100		
Pre-lamin A	Mouse	1:100		
Cleaved Pre-lamin A	Mouse	1:100		
Nucleolin	Mouse	1:200		
LAP2	Mouse	1:100		
MAN1	Goat	1:100	(1) Donkey (FITC) (2) Cy5 Conjugated Donkey (Pink)	(1) 1:50 (2) 1:100
Emerin	Goat	1:100		
Lamin A	Goat	1:100		

❖ Isolation of Genes from Bacterial Artificial Chromosomes (BACs):

Luria-Bertani (LB) Agar Plate:

1 % NaCl (w/v), 1 % Tryptone (w/v), 0.5 % Yeast Extract (w/v), 1.5 % Agar Technical (w/v), and 12.5 % µg/ml Chloramphenicol (w/v).

LB Broth:

1 % NaCl (w/v), 1 % Bactotryptone (w/v), 0.5 % Yeast Extract (w/v), and 12.5 % µg/ml Chloramphenicol (w/v).

P1 Solution:

15 mM Tris (pH 8), 100 µg/ml RNase A, and 10 mM EDTA

P2 Solution:

0.2 M NaOH and 1 % SDS

P3 Solution:

3 M CH_2COOK

10X TBE:

108 g Tris, 55 g Boric Acid, and 9.3 g EDTA in 1 Litre ddH_2O

1X TBE:

100ml 10X TBE in 900ml Sterilised water

❖ Preparation for Running DOP-PCR:

❖ Master Mix for Running DOP-PCR (v/v):

	Without Biotin (µl)	With Biotin (µl)
10x DOP PCR Buffer	10	10
dACGTP (2µM)	10	10
dTTP (2µM)	10	4
DOP-PCR Primer (20 µM)	10	10
Sterilised Water	57	35
DNA Template	2	10
Biotin-16-dUTP	-	20
Taq-KAPA HIFI	1	1
Total	**100**	**100**

❖ DOP-PCR Programme:

Phase	Step (Cycle)	Temperature (°C)	Time (Minutes)
Initial Denaturation	1	94	3
Denaturation	30	94	1
Annealing		62	1
Extension		68	1.5
Final Extension	1	68	8
For Collection		4	∞

❖ Preparation DOP-PCR Gel:

1 % Agarose in 1X TBE and 2 µl Ethidioum Bromide (10 mg/ml)

❖ 2 Dimensional- Fluorescence *In Situ* Hybridisation (2D-FISH):

3M Na Acetate pH 5.0:

0.5g Na Acetate in 2 ml ddH_2O

Adjust pH to 5.0

Methanonl : Acetic Acid, 3:1 (v/v):

30 ml Methanol in 10 ml Acetic Acid

0.075M KCl:

0.7g KCl in 125 ml ddH_2O

70% Ethanol:

70 ml Ethanol in 30 ml ddH_2O

90% Ethanol:

90 ml Ethanol in 10 ml ddH_2O

100% Ethanol:

100 ml Absolute Ethanol

70% Formamide with 2X SSC pH 7.0 (v/v):

70 ml Formamide in 30 ml 2XSSC (10 ml of 2XSSC + 20 ml ddH_2O), then adjust pH to 7.0

Buffer A:

50% Formamide 2XSSC, pH 7.0:

150 ml Formamide in 30 ml 20XSSC +120 ml ddH_2O, then adjust pH to 7.0

Buffer B:

0.1XSSC pH 7.0 (v/v):

1.5 ml 20XSSC in 298.5 ml ddH_2O, then adjust pH to 7.0

20XSSC (w/v):

180.3g NaCl and 88.2g Na Citrate in 1 L ddH_2O, pH7.0 and autoclave it.

4XSSC:

20 ml 20XSSC in 80 ml ddH_2O

4% Bovine Serum Albumin (BSA):

4g BSA in 10 ml 4XSSC

1% BSA:

0.1g BSA in 10 ml 4XSSC

FITC-Streptavidin (1:200 dilution, v/v):

1µl Cy3 in 199µl 1% BSA

Anti-Dig Antibody (1:100 dilution, v/v):

1µl Anti-Dig in 99µl 1% BSA

4XSSC in 0.05% Tween-20 (v/v):

320 ml ddH_2O in 80 ml 20XSSC + 0.2 ml Tween-20

❖ Polymerase Chain Reaction (PCR):

❖ RNA Analysis (Nano – drop):

(1) Untreated Cell Lines:

Cell Lines	Sample Number	Concentration (ng/µl)	260/280	260/230	Base Line (nm)
T-47D	(1a)	170.7	2.04	1.39	340
	(1b)	197.4	2.05	1.33	340
	(2a)	420.2	2.05	1.80	340
	(2b)	372.5	2.06	1.37	340
MCF-10A	(1a)	26.4	2.25	0.72	340
	(1b)	42.0	2.11	1.08	340
	(2a)	50.0	2.09	1.42	340
	(2b)	37.4	2.18	0.37	340
	(3a)	56.3	2.12	0.95	340
	(3b)	59.3	2.12	0.54	340
BT-474	(1a)	234.1	2.05	1.22	340
	(1b)	284.8	2.06	1.52	340
	(2a)	261.5	2.1	1.74	340
	(2b)	226.9	2.11	1.27	340
GI-101	(1a)	380.8	2.07	1.20	340
	(1b)	389.8	2.09	0.77	340
	(2a)	263.3	2.11	1.63	340
	(2b)	252.7	2.06	1.93	340

Sk-Br-3	(1a)	718.1	2.10	1.99	340
	(1b)	647.8	2.06	1.86	340
	(2a)	409.9	2.02	2.36	340
	(2b)	399.7	2.06	1.78	340

(2) Cell Lines Treated with Lovastatin:

Cell Lines	**Sample Number**	**Concentration (ng/µl)**	**260/280**	**260/230**	**Base Line (nm)**
T-47D (12µM/48hours)	(1a)	384.9	2.11	1.46	340
	(1b)	423.9	2.10	1.57	340
MCF-10A (12µM/48hours)	(1a)	84.1	2.11	1.46	340
	(1b)	63.0	2.18	0.64	340
MCF-10A (24µM/48hours)	(1a)	72.0	2.14	1.73	340
	(1b)	69.5	2.10	0.35	340
BT-474 (12µM/48hours)	(1a)	362.7	2.06	2.00	340
	(1b)	320.2	2.08	1.32	340

- **Master Mix 1:**

1 µl Random Primers (Oligonucleotide) + 1 µl dNTPs mix (10 mM) + 10 µl RNA + H_2O (to make up standardised RNA amount in ng)

- **Master Mix 2:**

4 µl First Strand Buffer (250 mM Tris-HCl, pH 8.3, 375 mM KCl, 15 mM $MgCl_2$) + 1 µl Molecular Grade H_2O + 2 µl 0.1M DDT (Dithiothreitol), A Reducing Agent to Prevent DNA Forming Dimmers in Solution + 1 µl Superscript II Reverse Transcriptase.

❖ **RNA Standardisation Calculation (Table Below):**

Large samples can use up to 500 ng RNA for cDNA synthesis; smaller concentrations can use 100 ng for cDNA synthesis:

Cell Lines With and Without Lovastatin Treatment	**Concentration of RNA (ng/ µl)**	**Standard Amount (ng)**	**RNA Needed (µl)**	**H_2O Needed (µl)**
	X	**Y**	**Y / X**	**10 – (Y / X)**
(10) T-47D	420.2	500	1.18	8.82
(11) BT-474	284.8	500	1.75	8.25
(12) GI-101	389.8	500	1.28	8.72
(13) Sk-Br-3	718.1	500	0.69	9.31
(14) MCF-10A	59.3	500	8.43	1.57
(15) T-47D (12µM/48 Hours)	423.9	500	1.17	8.83
(16) BT-474 (12µM/48 Hours)	362.7	500	1.37	8.63
(17) MCF-10 A (12µM/48 Hours)	84.1	500	5.94	4.06
(18) MCF-10A (24µM/48 Hours)	72.0	500	6.94	3.06

❖ **PCR Master Mix as Follows:**

Component from KAPA HiFi	**Total Volume in a 25 µl Reaction**
PCR Grade Water	16.25ul
5X KAPA HiFi Fidelity Buffer (contains 2.0 mM Mg^{2+} at 1X)	5ul
dNTP Mix (10 mM each dNTP)	0.75ul
Forward Primer (100nM)	0.75ul
Reverse Primer (100nM)	0.75ul
cDNA Template	1.0ul (end)
KAPA HiFi DNA Polymerase (1 U/µl)	0.5ul

❖ **The Programme Parameters for PCR Machine:**

Phase	**Step (Cycle)**	**Temperature (°C)**	**Time (Minutes)**
Initial Denaturation		94	3
Denaturation	35	94	1
Annealing		55	1
Extension		72	1.5
Final Extension		72	10
For Collection		4	∞

❖ **Primers Description:**

Primer Name	Primer Sequence	Product Length	%GC	Size (bases)	Tm (°C)
EGFR (F)	CAGCGCTACCTTGTCATTCA	195	50.00	20	60.01
EGFR (R)	TGCACTCAGAGAGCTCAGGA		55.00	20	60.01
PTEN (F)	CATAACGATGGCTGTGGTTG	106	50.00	20	59.99
PTEN (R)	CCCCCACTTTAGTGCACAG		55.00	20	60.03
CCND1 (F)	GAGGAAGAGGAGGAGGAGGA	237	60.00	20	59.88
CCND1 (R)	GAGATGGAAGGGGGAAAGAG		55.00	20	60.01
AKT1 (F)	AGAAGCAGGAGGAGGAGGAG	139	60.00	20	60.09
AKT1 (R)	CCCAGCAGCTTCAGGTACTC		60.00	20	60.01
HSP90AA1 (F)	AGACCCAGTCTTGTGGATGG	120	55.00	20	59.96
HSP90AA1 (R)	ACTCCCCTTTCCCCCTAAAT		50.00	20	60.01
HER2 (F)	ACAGTGGCATCTGTGAGCTG	148	55.00	20	60.06
HER2 (R)	CCCACGTCCGTAGAAAGGTA		55.00	20	59.99
LMNB1 (F)	AGGATCAGATTGCCCAGTTG	125	50.00	20	60.07
LMNB1 (R)	GCGAAACTCCAAGTCCTCAG		55.00	20	59.99
LMNB2 (F)	AAGGACCTGGAGTCCCTGTT	153	55.00	20	59.97
LMNB2 (R)	CTTCTCCAGCTGCTTTTTGG		50.00	20	60.13
LBR (F)	CCGTGAATTAAACCCTCGAA	128	45.00	20	59.93
LBR (R)	CGCGGTCCTGTATTTTCATT		45.00	20	59.96
Beta-Actin (F)	AAGAGAGGCATCCTCACCCT	216			
Beta-Actin (R)	TACATGGCTGGGGTGTTGAA				

*** F=Forward, R=Reverse, and Tm=Temperature**

❖ **Images of 100 bp Ladder Used for PCR Studies:**

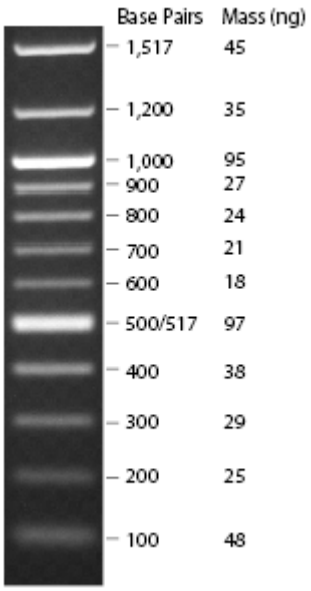

Figure: 100 bp Ladder (Visualized by Ethidium Bromide Staining on a 2% TAE agarose gel).

❖ Drug Treatment:

Lovastatin Drug (10µg/ml) in Ethanol:

1M = 404.54g in 1L

1M = 0.40454g in 1ml

1M = 1000µM = 404µg in 1ml

24µM = 10µg in 1ml

To make it 1000X Stock

List of Publications and Papers in Preparation

List of Publications and Papers in Preparation:

1. Bourne G, Moir C, Bikkul U, **Ahmed MH**, Kill IR, Eskiw CH, Tosi S, and Bridger JM (2013), **Interphase Chromosome Behavior in Normal and Diseased Cells (Book Chapter), Human Interphase Chromosomes: Biomedical Aspects**, *Springer Science*: 9-33.
2. **Ahmed MH** and Bridger JM (2013), **The Nuclear Lamina in Cancer, Cancer Biology and the Nuclear Envelope (Book Chapter)**, (to be edited by Dr. Eric Schirmer and Dr. Jose de las Heras, Published by Springer).
3. Wazir U, **Ahmed MH**, Harvey AJ, Bridger JM, Sharma AK, and Mokbel K (in prep.), **The Clinicopathological Significance of Lamin A, Lamin B and Lamin B Receptor mRNA Expression in Human Breast Cancer**.
4. **Ahmed MH**, Karteris E, Harvey AJ, and Bridger JM (Manuscript in Preparation), **Chromosome Territories, Gene Position, and Gene Expression in Human Breast Cancer Cell Nuclei**.
5. **Ahmed MH**, Harvey AJ, and Bridger JM (Manuscript in Preparation), **Aberrant Distribution and Presence of Nuclear Envelope Proteins in Breast Cancer Cell Lines**.
6. **Ahmed MH**, Harvey AJ, and Bridger JM (Manuscript in Preparation), **Investigating the Role of the Drug Lovastatin on Restoration of Nuclear Structure and Genome Organisation in Breast Cancer Cell Lines**.