Molecular insight of the cAMP Responsive Element Binding Protein (CREB) in Human Breast Cancer

ALOK CHHABRA

A dissertation submitted to the School of Medicine, Cardiff University in candidature for the degree of
Doctor of Medicine (MD)

Metastasis and Angiogenesis Research Group
Department of Surgery
School of Medicine, Cardiff University
Cardiff, Wales

June 2012

DECLARATION

This work has not previously been accepted in substance for any degree and is not concurrently submitted in candidature for any degree.

Signed …….. (Candidate) Date …….....

STATEMENT 1

This thesis is being submitted in partial fulfilment of the requirements for the degree of MD.

Signed …….. (Candidate) Date …….....

STATEMENT 2

This thesis is the result of my own independent work / investigation, except where otherwise stated.
Other sources are acknowledged by explicit references.

Signed …….. (Candidate) Date …….....

STATEMENT 3

I hereby give consent for my thesis, if accepted, to be available for photocopying and for inter-library loan, and for the title and summary to be made available to outside organisations.

Signed …….. (Candidate) Date ….....

STATEMENT 4: PREVIOUSLY APPROVED BAR ON ACCESS

I hereby give consent for my thesis, if accepted, to be available for photocopying and for inter-library loans **after expiry of a bar on access previously approved by the Graduate Development Committee.**

Signed…….. (Candidate) Date …….....

ACKNOWLEDGEMENTS

I would like to express my sincere thanks and appreciation to my supervisors Professor Wen G Jiang and Professor Robert E Mansel for giving me the opportunity, continued guidance, encouragement and support throughout the period of this study and in the preparation and proofing of this thesis.

I would like to offer my thanks to the late Dr Gaynor Davies, Dr Tracey Martin, Gareth Watkins, Dave Grimshaw, Andrew J Sanders and Lin Ye for their constant support and encouragement throughout my studies. I am thankful to them for all their advice, helpful suggestions and discussions that helped me to solve my day to day problems in the Laboratory. I am extremely grateful to them for being a fantastic colleague and creating a brilliant working atmosphere and for making my stay most memorable and enjoyable. I feel grateful and privileged to have had the opportunity to meet and work with all those in the department during my research.

I am extremely thankful to Miss Helen Sweetland and Mr David Scott- Coombes for continuous support and guidance that helped me complete my thesis.

Finally I would like to thank my family for their constant support and encouragement they have shown throughout this study.

The work in this thesis was supported by a generous grant from E J Demery Bequest Fund.

PUBLICATIONS AND PRESENTATIONS

"Expression of transcription factor CREB-1 in human breast cancer and its correlation with prognosis"

Alok Chhabra, H. Fernando, Gareth Watkins, Robert E. Mansel, Wen G. Jiang

Oncol Rep. 2007 Oct; 18(4):953-8.

"Pattern of expression of Activating Transcription Factor [ATF] in human breast cancer and the prognostic significance"

Alok Chhabra, Gareth Watkins, Robert E. Mansel, Wen G. Jiang

Breast Cancer Research and Treatment (Supplement), 2006, 5125: 254

"Expression of Transcription factor, CREB-1 in human breast cancer and its correlation with prognosis"

Alok Chhabra, Gareth Watkins, Robert E. Mansel, Wen G. Jiang

British Journal of Surgery, 2006, Volume 93, Issue S3

"Pattern of expression of Activating Transcription Factor [ATF] in human breast cancer and the prognostic significance"- Poster Presentation

29th Annual San Antonio Breast Cancer Symposium, Texas Dec 2006

"Expression of Transcription factor, CREB-1 in human breast cancer and its correlation with prognosis"- Oral Presentation

Society of Academic and Research Surgery 2006, Edinburgh [Shortlisted for **President Prize**]

"Molecular insight of the CREB [cAMP Responsive Element Binding Protein] in human breast cancer"- Oral Presentation

Metastasis and Angiogenesis Research Group Meeting, Cardiff 2005

"Expression of Activating Transcription Factors in human breast cancer and association with the clinicopathological characteristics"

Under Preparation

SUMMARY

CREB, cAMP responsive element binding protein is a positive regulatory protein transcriptional factor, for genes including aromatase, an enzyme that converts androgens to oestrogens, c-fos, tyrosine hydroxylase and neuropeptides like somatostatin and enkephalin. The expression of aromatase is highly aberrant in human breast cancer and has been implicated in the disease progression. Aromatase expression in breast cancer tissue is directed mainly by promoters 1.3 and II. CRE1 and CRE2 are essential for cAMP induced promoter II activity. CRE binding protein (CREB) bound to this element (CRE) and that this interaction was enhanced in the presence of cAMP. Despite the extensive work on aromatase, little information is available on the expression and role of CREB in human breast cancer.

The aim of this study was to investigate the molecular impact of CREB family of proteins on the aggressive nature of breast cancer cells and to investigate the expression pattern in breast cancer tissues in relation to tumour histopathological grade, stage, nodal status and the clinical outcome of the patients.

In this study we examined the expression of CREB1 and ATFs (Activating transcription factors) in breast cancer cell lines using RT-PCR, which allowed us to design the strategy of *in vitro* experiments. Ribozyme knockdown technology was used to target the expression of CREB1 in a breast cancer cell line MDA-MB-231. Knockdown of CREB1 using ribozyme transgenes resulted in decrease in *in vitro* cell growth and invasiveness in breast cancer cells. The results presented here demonstrate that the level of CREB-1 and ATFs in breast cancer patients was elevated. The study results presented here revealed a significant link between CREB and mortality, in that high levels are associated with shorter disease free survival and interestingly we found significantly low levels of ATFs in patients with poor prognosis, metastatic disease and nodal involvement. We conclude that the level of CREB-1 and ATFs are aberrantly expressed in human breast cancer which may be associated with disease progression in breast cancer patients and has significant bearing to the clinical outcome of the patients.

Over-expression of aromatase in adipose tissue surrounding breast tumour could arise through increase in both CREB expression and CREB transcriptional activity. Inhibition of CREB activity could inhibit aromatase expression and hence decrease oestrogen production in breast tissue. An understanding of the molecular mechanisms of expression of CREB, together with aromatase between non-cancerous and cancerous breast tissue at both transcriptional and translational levels may help in the design of a therapy based on suppressing aromatase expression in breast cancer tissues.

CONTENTS

TABLE OF CONTENTS

List of Figures

List of Tables

Abbreviations

Aa	Amino acid
Ab	Antibody
ABC	Avidin biotin complex
Ag	Antigen
bp	Base pair
BSA	Bovine serum albumin
BSS	Balanced Salt Solution
DAB	Diaminobenzidine tetrahydrochloride
cDNA	Complementary Deoxyribonucleic acid
dH2O	Distilled water
DEPC	Diethyl pyrocarbonate
DMEM	Dulbeccos's Modified Eagles Medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
E. coli	Escherichia coli
EDTA	Ethylene Diaminetetraacetic acid
FCS	Foetal calf serum
G418	Geneticin
hr	Hour
Kb	Kilo-base

LB	Luria-Bertani
m	Metre
M	Molar
mA	Milli-amp.
mg	Milligram
min.	Minute
ml	Milli litre
mM	Milli molar
mRNA	Messanger Ribonucleic acid
NaCl	Sodium chloride
NaOH	Sodium Hydroxide
ng	Nano- gram
PAGE	Polyacrylamide gel electrophoresis
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolutions per minute
RT	Reverse Transcription
SD	Standard Deviation
sec	Second
TBE	Tris/ Borate/ EDTA electrophoresis buffer
U	Unit
V	Volt
WT	Wild type

CHAPTER 1

GENERAL INTRODUCTION

1.1 Breast Cancer

1.1.1 Epidemiology

Breast Cancer is a major health issue and is the most frequently occurring cancer in women worldwide. It accounts for about 25 per cent of all female malignancies and the proportion is higher in women in developed countries with incidence rising in many parts of the world including Europe and USA. Over one million new cases occurs each year worldwide (Parkin *et al.,* 2001). There is substantial geographical variation in age-adjusted incidence rates with the highest rates reported in Northern America and Europe and the lowest in the Far East (Figure 1.1) which is mostly attributed to the introduction of screening programme in these regions. As shown in the graph the survival rate in developed countries is much higher as compared to developing countries and hence the differences in mortality rates worldwide being much less marked than for incidence (Figure 1.2).

Breast cancer is the most common female cancer in UK accounting for 31% of all cases in women (Cancer Research UK 2011). The life time risk of developing breast cancer in the UK is 1 in 8, with an estimated 48,000 new cases diagnosed in 2008 (Cancer Research UK 2011). Incidence rates of breast cancer in UK have continued their upward trend as well, increasing by 80 per cent between 1975 and 2008. (Figure 1.2) (Cancer Research UK 2011). Breast cancer is the second most common cause of death from cancer accounting for 16% of all cases in women and around 12,000 women died from breast cancer in United Kingdom in 2008. Fortunately, the incidence of breast cancer appears to have reached a plateau and mortality rates are improving mainly due to more effective treatments and earlier detection of cancer by screening.

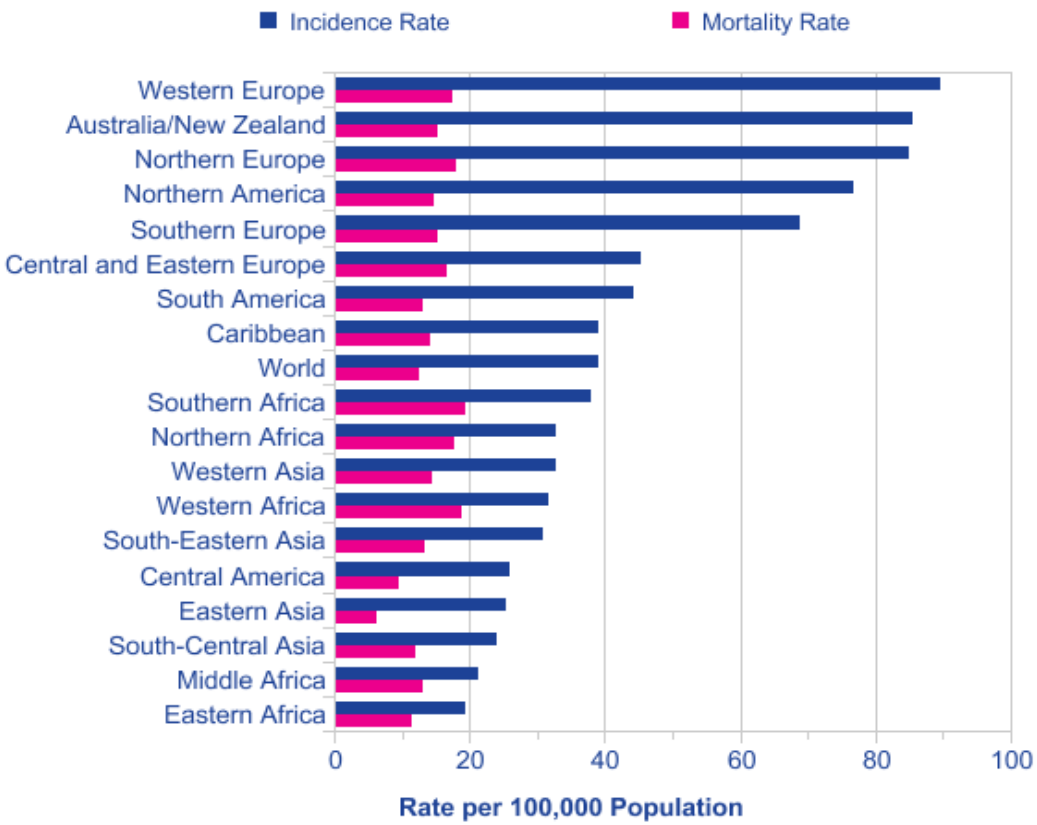


Figure 1.1: Age standardised incidence and Mortality rates of female breast cancer in selected world regions, 2008
(Adapted from Cancer Research UK, 2011)

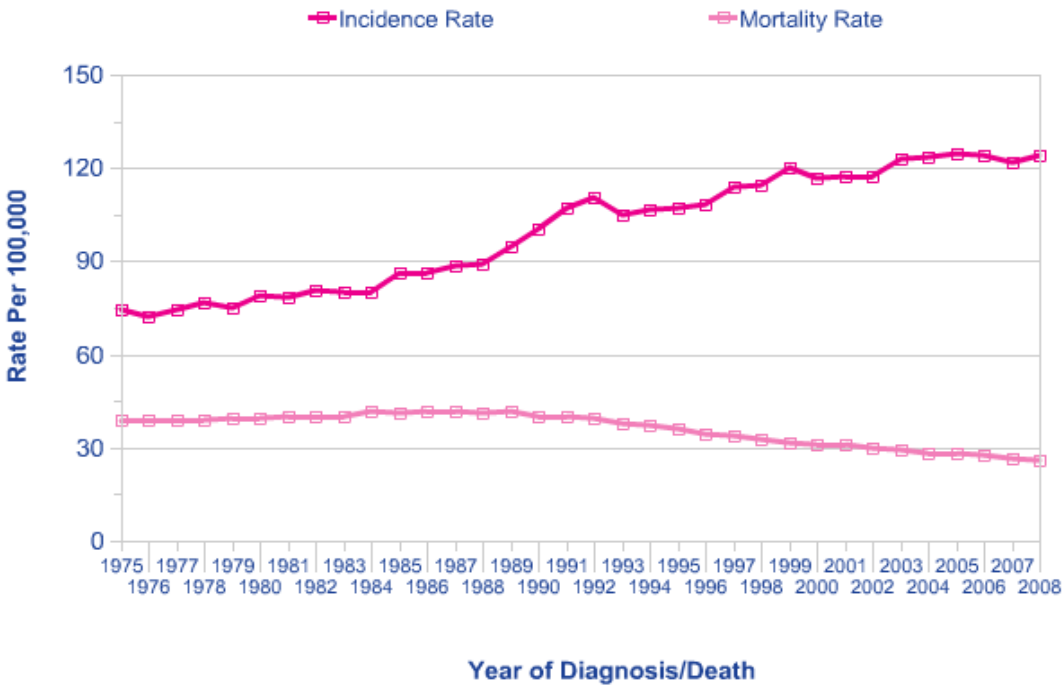


Figure 1.2: Age standardised incidence and mortality rates of female breast cancer- United Kingdom (1975-2008)
(Adapted from Cancer Research UK, 2011)

1.1.2 Aetiology of Breast Cancer: Environmental and Genetic Factors

Female breast cancer is a complex, multifactorial disease, the aetiology of which involves a strong association between environmental, genetic and behavioural factors. Several risk factors are well established and are shown in Table 1.1. The strongest associated risk factor that affects most women is increasing age. Breast cancer is a rare disease in women under 35 years old but its incidence double every 5 years until the age of 50 with the peak in the 50 to 64 age group (Figure 1.3 & Figure 1.4) (Cancer Research UK, 2011) (Kelsey et al., 1988). Eighty per cent of cases occur in post-menopausal women. This cancer accounts for almost one in three of all cancer cases in women, and the life time risk for breast cancer in women is one in eight. The estimated risk of developing breast cancer for different age group is shown in Table 1.2.

The most important influence is hormonal factors, especially the determinants of menstrual life and childbirth history. Women who began having periods early (before 12 years of age) or who attain menopause after the age of 55 have a slightly increased risk of breast cancer. Women who had no children, or who had their first child after age 30, have a slightly higher risk of breast cancer (Kelsey *et al.,* 1988). Being pregnant more than once and at an early age reduces the risk of breast cancer. Some studies have shown that breast-feeding slightly lowers breast cancer risk, especially if the breast-feeding lasts 1½ to 2 years. The use of oral contraceptives at a young age and the use of hormone replacement therapy are also known to increase the breast cancer risk.

Table 1.1 Risk factors for the development of breast cancer

Risk Factors	High risk	Low risk	Relative risk
Age at 1st full term pregnancy	>30	<20	2-4
Age at menarche	Early	Late	1-1.9
Age at menopause	Late	Early	1-1.9
Family history of premenopausal bilateral breast cancer	Yes	No	>4
Past history of breast cancer	Yes	No	>4
Any 1st degree relative with breast cancer	Yes	No	2-4
Biopsy showing atypical hyperplasia	Yes	No	>4

Adapted from (Kelsey *et al.,* 1988)

Table 1.2 Estimated risks for different age group

Age Group (in years)	Estimated Risk
20-29	1 in 1,900
30-39	1 in 233
40-49	1 in 69
50-59	1 in 38
60-69	1 in 27
70-79	1 in 11
≥ 80	1 in 10

Adapted from Breast Cancer.org, 2012

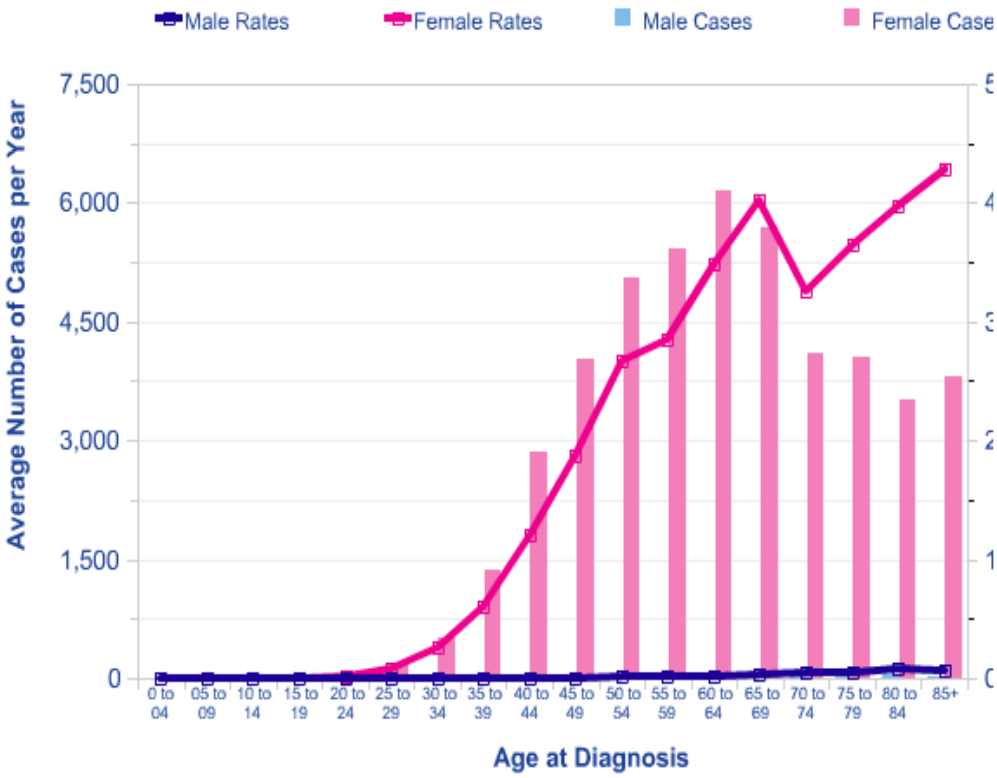


Figure 1.3: Numbers of new cases and age specific incidence rates, by sex, breast cancer, UK
(Adapted from Cancer Research UK, 2011)

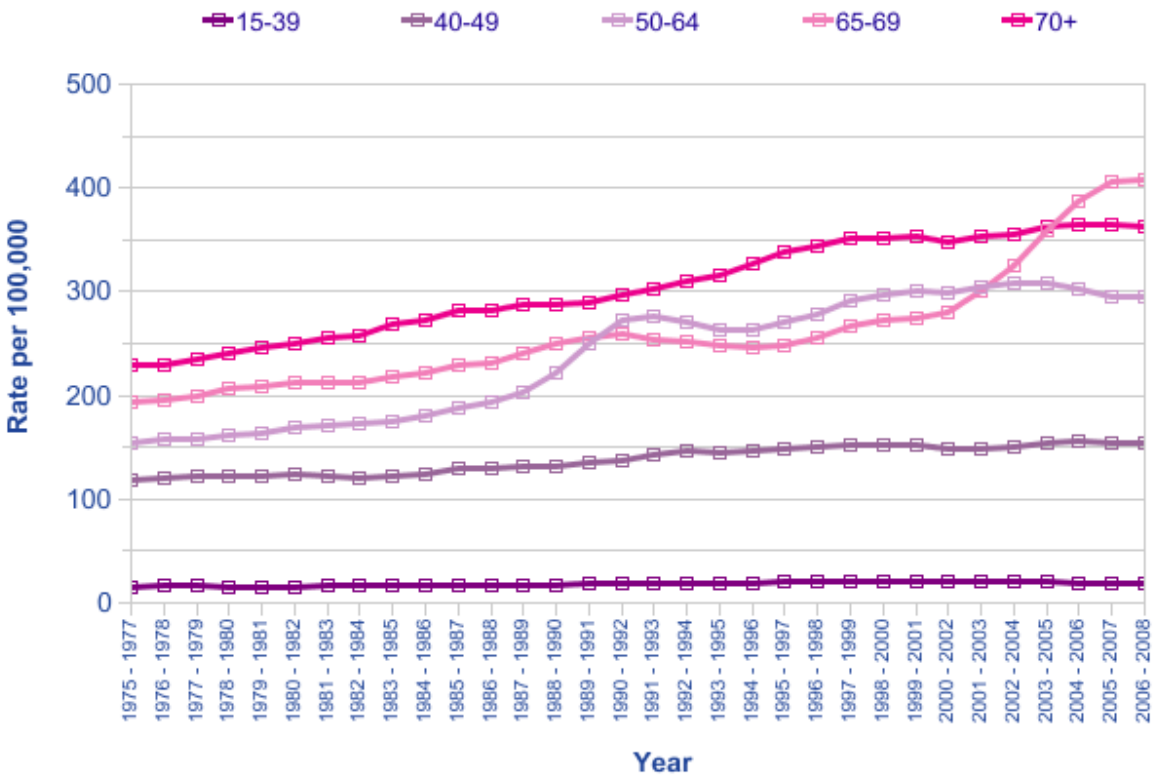


Figure 1.4: Age specific incidence rate of female breast cancer in United Kingdom, 1975-2008
(Adapted from Cancer Research UK, 2011)

The risk of breast cancer appears to be higher in women from more affluent background and white women as compared to African-American women. Breast cancer risk increases in women who migrated from low risk to high risk countries. Asian, Hispanic, and American Indian women have a lower risk of getting breast cancer.

Various studies have shown that about 5% to 10% of breast cancer cases are hereditary as a result of gene mutations. The most common gene mutations are those of the BRCA1 and BRCA2 genes which lie on chromosome 17q and chromosome 13q respectively. Normally, these genes help to prevent cancer by making proteins that keep cells from growing abnormally. Over 200 described mutations of these genes exist, each carrying different risks of breast cancer. Overall individuals with these gene mutations have up to 85% chance of getting breast cancer during their lifetime. These genes have been found in approximately 85 per cent of families with four or more cases of breast cancer diagnosed under the age of 60. Mutation in other genes like p53, Rb and Myc may raise breast cancer risk as well.

Breast cancer risk is higher among women whose first degree blood relatives have this disease. The relatives can be either from the maternal or paternal side of the family. Other factors associated with increased risk are a previous history of breast cancer or benign disease with atypical histology, exposure of breast tissue (chest area) to ionizing radiation (particularly before 20 years of age), an increased body size (obesity) as the conversion of adrenal androstenedione to oestrone by the aromatase enzyme is related to body weight and alcohol consumption.

1.1.3 Pathology

The breast is divided into 15-20 lobes composed of lobules embedded in fibro fatty tissue. Each lobe is drained by a single collecting duct, formed by coalescence of the ducts of the component lobules which in turn converge to open on the summit of the nipple. The smallest ducts, closest to the lobules are termed as the terminal ducts and along with the lobule form terminal duct –lobular units. The entire breast duct- lobular units is lined by two cell types, an inner luminal epithelial cell which differentiates into the milk secreting cell and an incomplete outer layer of myoepithelial cells. Basement membrane surrounds the epithelial cell layers. Breast cancer arises from the epithelium of the duct system extending from the nipple end of major lactiferous ducts to the terminal duct unit in the breast lobule. Breast cancer is morphologically described as either invasive or in situ (non invasive) carcinoma. A classification of breast carcinoma modified from that of the world health organisation is shown in Table 1.3.

Invasive ductal carcinoma (also known as Not Otherwise Specified-NOS) is the most common type and constitutes about 70% of the breast cancer. Lobular carcinoma is the second most common and accounts for 10% of breast cancers. A small percentage of breast cancers belong to rarer histological variants, usually towards the well-differentiated end of the spectrum and hence carrying a better prognosis. These include medullary carcinoma with a marked lymphocytic infiltration, colloid or mucinous carcinoma, whose cells produce abundant mucin, and tubular carcinoma.

Inflammatory carcinoma is a fortunately rare, highly aggressive cancer that presents as a painful, swollen breast with cutaneous oedema. This is due to blockage of the subdermal lymphatic with carcinoma cells.

Another variant is the *In situ* carcinoma in which the malignant cells are confined within the myoepithelial layer and basement membrane and hence do not have the metastatic potential. This was previously a rare, usually asymptomatic finding in breast biopsy specimens, but is becoming increasingly common with the advent of mammographic screening. It now accounts for over 20% of cancers detected by screening in the UK. *In situ* carcinoma may be ductal (DCIS) or lobular (LCIS) carcinoma in situ, the latter often multifocal and bilateral. Both are precursor of invasive cancer, which will go on to develop in at least 20% of cases. DCIS may be classified by the Van Nuys system, which combines the patient's age, type of DCIS (high, intermediate and low grade based on nuclear atypia) and presence of microcalcification, extent of resection margin and size of disease. Patients with a high *score* benefit from radiotherapy after excision, whereas those of low grade, who are completely excised, need no further treatment.

Table 1.3 WHO classification of Breast carcinoma

Tumour type	
In situ carcinoma	8 %
In situ ductal carcinoma	
In situ lobular carcinoma	
In situ carcinoma with microinvasion	
Infiltrating carcinoma	
Infiltrating ductal carcinoma	70 %
Infiltrating lobular carcinoma	10 %
Carcinoma with particular clinical manifestations	
Pagets disease of the nipple	1 % - 4 %
Inflammatory carcinoma	1 % - 5 %
Others	3 % - 10 %
Medullary carcinoma	
Mucoid / colloid carcinoma	
Tubular carcinoma	
Adenoid cystic carcinoma	

1.1.3.1 Tumour Grade

Grade refers to how similar the tumour is to the surrounding normal breast tissue. Different grading systems have been proposed in the past but the generally accepted system is the modified Bloom and Richardson Grading System of breast cancer [based on mitotic index, tubule formation and nuclear pleomorphism and correlates with outcome] which is detailed in Table 1.4.

1.1.4 Diagnosis and Staging

1.1.4.1 Diagnosis

Breast cancer can arise from any portion of the breast, including the axillary tail. It most commonly arises in the upper outer quadrant. Breast cancer usually present as:

- Breast lump
- Bloody nipple discharge
- Skin changes like tethering, peau d'orange appearance, indrawing of the nipple, nipple eczema and rarely frank ulceration.
- Mammographic screening abnormalities

Breast cancer is diagnosed by triple assessment which includes clinical evaluation, imaging (ultrasound <35 years old or mammography >35 years old) and cytological or histological examination.

Table1.4: Modified Bloom and Richardson Grading System of breast cancer

Tubule Formation (% of tumour exhibiting gland formation)	
>75% of tumour	1
10- 75% of tumour	2
<10% of tumour	3
Pleomorphism (Variation in size and shape of nuclei)	
Mild	1
Moderate	2
Marked	3
Mitotic activity	
<6 mitosis/ 10hpf	1
7- 15 mitosis/ 10hpf	2
>15 mitosis/ 10hpf	3
Total Score is derived from adding scores for the 3 parameters. The score varies from 3- 9.	
Grade 1 (well differentiated)	3-5
Grade 2 (moderately differentiated)	6-7
Grade 3 (poorly differentiated)	8-9

1.1.4.2 Staging

Following diagnosis the stage of the disease is determined using various staging system and the most commonly used staging system is the Tumour size-Node- Metastasis (TNM) classification.

T: Tumour Size

The letter T followed by a number from 0 to 4 describes the tumour's size and whether it has spread to the skin or chest wall under the breast. Higher T numbers indicate a larger tumour and/or more extensive spread to tissues surrounding the breast.

- TX: Tumour cannot be assessed
- T0: No evidence of a tumour
- Tis: Insitu carcinoma-Ductal carcinoma in situ or Lobular carcinoma in situ
- T1: Tumour < 2 cm in diameter
- T2: Tumour between 2 and 5 cm in diameter
- T3: Tumour >5 cm in diameter
- T4: Tumour of any size, fixed to the chest wall

N: Palpable Nodes

The letter N followed by a number from 0 to 3 indicates whether the cancer has spread to lymph nodes.

- NX: Lymph nodes cannot be assessed
- N0: No lymph node involvement
- N1: Ipsilateral axillary lymph nodes involvement, mobile
- N2: Spread to ipsilateral lymph nodes and fixed
- N3: Spread to the ipsilateral internal mammary lymph nodes or the ipsilateral supraclavicular lymph nodes

M: Metastasis

The letter M followed by a 0 or 1 indicates whether or not the cancer has metastasized (spread) to distant organs (i.e., the lungs or bones).

- MX: Metastasis cannot be assessed
- M0: No distant metastasis
- M1: Distant metastasis

Staging of Breast Cancer

The American Joint Committee on Cancer (AJCC) staging of a breast cancer describes its size and the extent to which it has spread. The staging system ranges from Stage 0 to Stage IV as shown in table 1.5.

Table 1.5: Stages of Breast Cancer

Stage	Tumour Size	Lymph Node Involvement	Metastasis
0	Tis	N0	M0
I	T1	N0	M0
II A	T0, T1	N1	M0
	T2	N0	M0
II B	T2	N1	M0
	T3	N0	M0
III A	T0, T1, T2	N2	M0
	T3	N1, N2	M0
III B	T4	Any N	M0
	Any T	N3	M0
IV	Any T	Any N	M1

1.1.4.3 Nottingham Prognostic Index (NPI)

Like all types of cancer, breast cancer is an unpredictable condition and no one can be absolutely certain of the outcome of treatment. The important prognostic factors are lymph node involvement, tumour size and tumour grade. The Nottingham prognostic index (NPI) (Galea *et al.,* 1992) is a predictive index of the clinical outcome of breast cancer which has been devised combining the following factors: tumour size, node involvement and modified Scarff Bloom Richardson (SBR) grading system. The NPI can be calculated as:

NPI = 0.2 x tumour size (cm) + lymph node stage + histological tumour grade

The lymph node stage is 1 (if there are no nodes affected), 2 (if up to 3 nodes are affected) or 3 (if more than 3 nodes are affected). Similarly the tumour grade is scored as 1 (for grade I), 2 (for a grade II) or 3 (for a grade III).

Applying the formula gives scores which fall into three bands:

- a score of less than 3.4 - suggests a good outcome with a 80% 15 year survival
- a score of between 3.4 to 5.4 - suggests an intermediate level with a 42% 15 year survival
- A score of more than 5.4 - suggests a poor outcome with a 13% 15 year survivial.

Beside this there are other important independent prognostic variables like lymphovascular invasion and oestrogen and progesterone receptor status which are useful in determining the clinical outcome of the breast cancer. The presence or absence of lymphovascular invasion is considered to be useful in predicting local recurrences.

1.1.5 Treatment of Breast Cancer

Treatment of breast cancer largely depends upon clinical stage of the disease at the time of presentation. Surgery remains the cornerstone of breast cancer management and has been the primary treatment for breast cancer patient for more than 100 years. Radical surgery for breast cancer has been the 'gold standard' in the past. Over the last two decades, various trials including the National Surgical Adjuvant Breast and Bowel Project –NSABP-06 (Fisher *et al.,* 1992) have demonstrated no difference in the disease free survival between early breast cancer patients who had conservative breast surgery and those who had mastectomy. Wide local excision of the tumour followed by radiotherapy is now well accepted practice for early breast cancer. Breast cancer commonly spreads to the regional axillary lymph nodes and occasionally the tumour cells may spread to the internal mammary lymph nodes. Since the majority of tumour spread is to the axilla, Axillary Clearance and the minimally invasive procedure Sentinel Lymph Node Biopsy (SLNB) constitutes a part of standard treatment for breast cancer. Systemic therapy which includes chemotherapy or hormonal therapy is given if there are associated poor prognostic factors such as lymph node metastasis and high tumour grade as there is increase chance of local or metastatic recurrence. On the other hand in metastatic breast cancer surgery play a very limited role and is usually treated by either radiotherapy or systemic chemotherapy for symptomatic relief.

1.1.5.1 Endocrine therapy

Reproductive and certain other sensitive tissues like mammary adipose tissue possess high affinity protein receptors for oestrogen and progesterone. Specific receptors for both hormones may be present in tumour tissue of mammary origin and play key roles in both normal breast development and breast cancer progression. These receptor proteins are activated when occupied by their specific hormone ligand leading to the induction of cellular genes, including those that may encode critical enzymes and secrete peptide growth factors.

Oestrogen receptors (ER) are intracellular DNA-binding transcription protein expressed in different tissue types including endometrium, breast cancer cells and ovarian stroma cells. There are two different forms of ER, referred as ERα and ERβ which are encoded by seperate genes ESR1 and ESR2 on the 6q25.1 and 14q23.2 chromosomes respectively. Hormone-activated estrogen receptors are coexpressed in many cell types and may form homodimers or heterodimers with each other (Li et al., 2004). ER interacts with transcriptional coactivators and corepressors responsible for tissue selective agonistic or antagonistic effect of the ligands like oestrogen and tamoxifen (Deroo et al., 2006). ER binds to the oestrogen and this complex subsequently binds to the specific sequences of DNA which in turn recruits other proteins responsible for the proliferation of mammary cells. ER form complexes with G proteins and tyrosine kinases like EGFR and IGF-1 (Levin ER, 2005; Zivadinovic et al., 2005) and the signals are sent to the nucleus through the mitogen activated protein kinase (MAPK/ERK) pathway and phosphoinositide 3-kinase (Pl3K/AKT) pathway (Kato et al.,1995).

HER2 (Human Epidermal Growth Factor Receptor 2) protein also known as ErbB-2 is a member of the epidermal growth factor receptor (EGFR/ErbB) familyof proto-oncogene. HER2 is encoded by ERBB2 proto-oncogene located at the long arm of human chromosome 17. It has been shown that over-expression of HER2 play an important role in the pathogenesis and progression of breast cancer and recently has evolved as an important biomarker and therapy target in patients with breast cancer (Saini et al., 2011; Eccles SA., 2011; Moasser MM., 2007).

The clinical importance of amplification of HER2 in breast cancer was first recognised in 1987 (Slamon et al., 1987). Amplification of the HER-2 was a significant predictor of overall survival and time to relapse in patients with breast cancer and is associated with increased recurrence and poor prognosis (Slamon et al., 1987; Barnes DM., 1993). Over-expression of HER2 occurs in approximately 15% to 20% of breast cancer and is measured using immunohistochemistry or fluorescence in situ hybridisation technique (Belengeanu et al., 2010). HER2 activates various signaling pathways like mitogen activated protein kinase (MAPK), phosphoinositide 3- kinase (P13K/ AKT) and protein kinase C (PKC) pathways (Roy et al., 2009) leading to proliferation and uncontrolled cell growth (Nagy et al., 1999).

Endocrine therapy for breast cancer involves selective oestogen receptor modulators like tamoxifen and aromatase inhibitors such as anastozole and letrozole. ER status is used to determine sensitivity of breast cancer to tamoxifen and aromatase inhibitors. (Clemons et al., 2002).

Surgically induced menopause in the form of bilateral oophrectomy became the first effective means to control advanced breast cancer. Over the last two decades hormonal manipulation for the treatment of breast cancer has been simplified by the introduction of tamoxifen and aromatase inhibitors. This is assessed by measuring oestrogen and progesterone receptor status by immunohistochemistry of the resected tumour and reported using a standardised technique such as the Allred scoring system. This assay may be done on either frozen or paraffin embedded tumour sections. In general, if greater than 10% of tumour cells stain positive for the nuclear receptor, the assay is reported as positive and a response to hormonal therapy is likely. The majority of human breast cancer contain hormonal receptor either oestrogen or progesterone or both. Approximately 70% of primary breast cancers express ER (Oestrogen Receptor), and over half of this also expresses PgR (Progesterone Receptor). The presence of oestrogen receptor predicts the clinical response to all types of endocrine therapies including the hormonal therapy. Treatment aimed at inhibiting the action of ER represents a highly successful example of targeted therapy for clinical breast cancer (Campos et al., 2003).

ER positive tumours have a 5-10% better chance of survival than ER negative tumours. In addition patients with ER positive tumours respond well to anti-oestrogen treatment with better clinical outcome as compared to ER negative tumours (Dowsett et al., 2006). Patients with both ER and PgR positive have better prognosis than those with ER positive but PgR negative tumours (Bardou et al., 2003). It has been demonstrated that patients with ER+/HER2+ compared with ER-/HER2+ breast cancers may benefit more from drugs that inhibit the PI3K/AKT molecular pathway (Loi et al., 2009). The expression of HER2 is regulated by signaling through estrogen receptors. It has been shown that oestradiol and tamoxifen acting through the estrogen receptor down-regulate the

expression of HER2. (Hurtado et al., 2008). Low ER or PgR or high HER-2 expression is associated with a high risk of recurrence with either anastrozole or tamoxifen

Tamoxifen is an oestrogen receptor modulator and acts as a weak agonist / antagonist. It acts as a competitive antagonist of oestrogen activity in the breast tissue but not in other oestrogen sensitive tissues. Tamoxifen reduces the risk of local recurrence by 25% and mortality by 17% in oestrogen/ progesterone receptor- positive breast cancer patients. Aromatase inhibitors are agents that block the conversion of androstenedione to oestrone by inhibiting the aromatase enzyme. Aromatase inhibitors like anastrozole and Letrozole are now well established proven adjuvant endocrine therapy in the treatment of breast cancer. Forbes et al., 2008 reported significant improvement in disease-free survival and risk of disease recurrence in ER positive breast cancer patients treated with anastrozole.

1.1.6 Prognosis

The prognosis of breast cancer has been improving over last three decades and more women are being successfully treated than ever before. As Shown in figure1.5 the estimated relative five-year survival rate for women diagnosed with breast cancer in England in 2001-2006 was 82%, compared with only 52% for women diagnosed in early 1970s (Cancer Research UK, 2011).The estimated relative twenty year survival rate for women with breast cancer has gone from 44% in the early 1990s to 64% in 2003 (Cancer Research UK, 2011). As figure1.6 shows, breast cancer survival rates are higher in women diagnosed in their 50s and 60s than for either younger or older women. The maximum improvement in five-year survival have been for women aged 50-69 years (Cancer Research UK, 2011).

Prognostic indicators serve as a guide for clinical decision and estimates of outcome. The prognosis depends on the invasiveness and the metastatic potential of the breast cancer. In an attempt to define which tumours will behave aggressively, a host of prognostic factors has been described. These include tumour size, lymph node status, histological grade of the tumour, hormone receptor status, measures of tumour proliferation such as S-Phase fraction and thymidine labelling index, growth factor analysis and oncogene or oncogene product measurements. Breast cancer spreads via the lymphatic system and primarily spreads to axillary lymph nodes. The best indicators of likely prognosis in breast cancer are the histological grade, tumour size and the lymph node status. The regional lymph node status remains the single most important prognostic factor (Fisher *et al.,* 1983) and a positive correlation has been noted between the number of lymph nodes involved and poor prognosis (Carter *et al.,* 1989). Patients with ≥ 5 positive nodes have a 5 –year survival of less than 20% (Fisher *et al.,* 1983). Various other studies have shown that the prognosis for node-negative patients is better than those with node positive patients (Kim et al., 2011; Carter *et al.,* 1989; Quiet *et al.,* 1995; Saimura *et al.,* 1999).

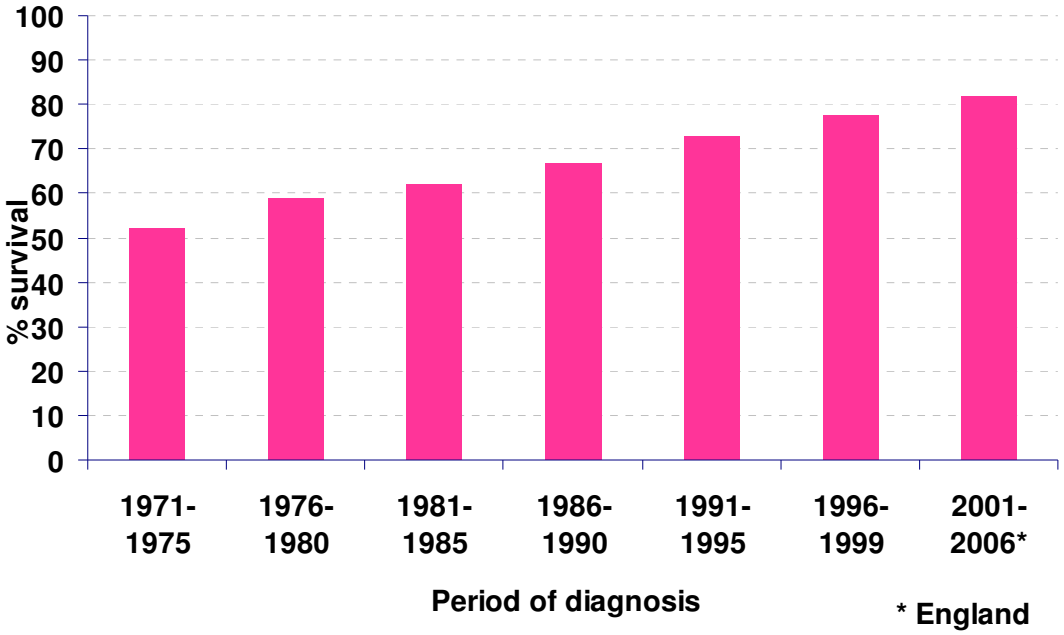


Figure 1.5: Age-standardised five-year relative survival rate, female breast cancer, England and Wales, 1971-2006
(Adapted from Cancer Research UK, 2011)

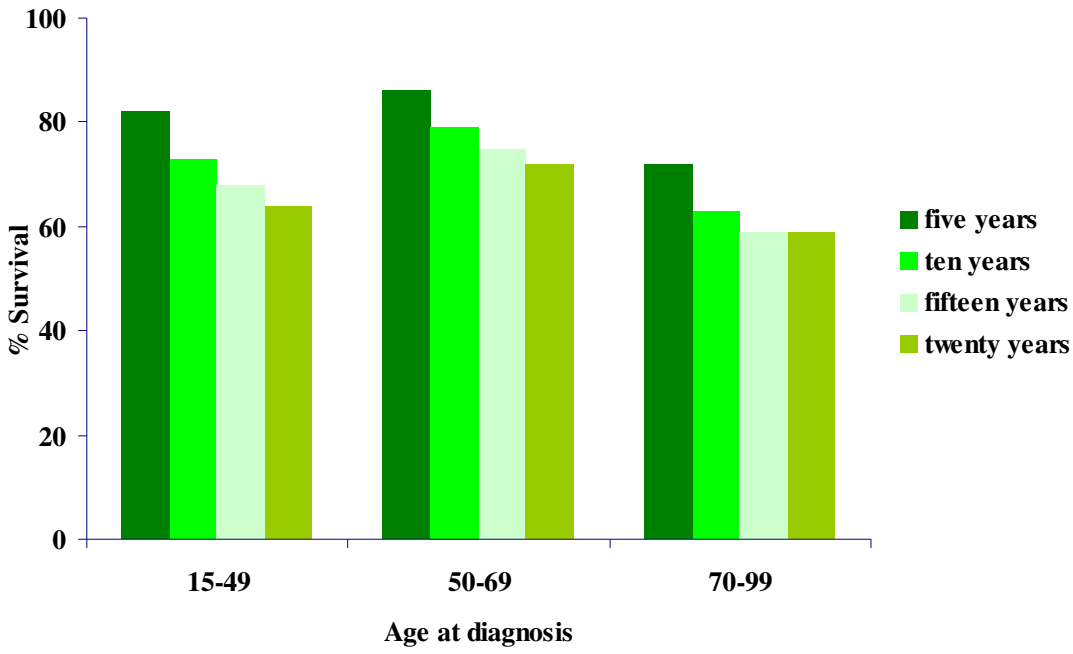


Figure 1.6: Relative five, ten, fifteen and twenty year survival (%) breast cancer, by age at diagnosis, England and Wales, 2001-2003
(Adapted from Cancer Research UK, 2011)

1.2 Transcription factor:

Transcription is defined as the synthesis of RNA from a DNA template. It consists of three steps:

Initiation- In this step RNA polymerase binds to double stranded DNA (promoter). Promoter is defined as the DNA sequence containing binding sites for RNA polymerase and the transcription factors necessary for normal transcription.

Elongation- In this step single stranded DNA is formed by the addition of nucleotides to the 3'end of the polynucleotide chain.

Termination- In this step the process of transcription terminates the recognition of transcription termination sequence and the RNA polymerase is released.

The process of transcription requires other associated gene regulatory proteins (transcription factors) along with RNA polymerase. A transcription factor is a protein that binds DNA at a specific promoter or enhancer region or site, where it regulates transcription. These proteins are primarily involved in the initiation stage of RNA transcription and play an important role in determining where the DNA chain becomes unzipped, creating a single strand to which RNA can be attached. These proteins can be selectively activated or deactivated by other proteins and binds to DNA at a sequence specific site and regulates transcription. Different DNA binding motifs found in transcription factors are as follows:

- **Leucine zippers DNA binding motif:** helps in associating the transcription factors with each other. Examples of this group includes CREB family of transcription factors and C/EBP.

- **Zinc fingers:** are protein motifs act as a structural platform for DNA binding by the coordination of one or more zinc ions. Example of Zinc fingers includes SP1 TFIIIA and oestrogen receptors
- **bHLH (Basic-helix-loop- helix):** binds DNA with two alpha helices containing basic amino acid residues. Example includes Myc protein, E12/E47 and myogenic regulatory factors.
- **HTH (Helix- turn- helix):** binds the major groove of the DNA. Example includes Myb and ETS protein.

There are three classes of transcription factors as described below:

- **General transcription factors:** These are ubiquitous factors and are involved in the formation of a preinitiation complex by interacting with the promoter region surrounding the transcription start site. Examples of general transcription factors are RNA polymerase II holoenzyme (RNAPII), TFIIA, TFIIB, TFIID, TFIIE, TFIIF and TFIIH.
- **Upstream transcription factors:** These are unregulated proteins which bind to initiation site to activate or inhibit transcription. These factors increase the efficiency of transcription initiation. Examples of upstream transcription factors are SP-1, AP-1, ATF/CREB, c-myc and C/EBP
- **Inducible transcription factors:** These factors act in the same manner as an upstream factor but require activation or inhibition before binding to transcription site. Examples of inducible transcription factors are c-Jun, c-Fos, JunD, JunB and FosB

1.2.1 Functions of transcription factors

- Binds to RNA polymerase
- Binds to another transcription factor
- Binds to cis- acting DNA sequences

1.3 Transcription factors and breast cancer

Transcription factors helps in various physiological processes including DNA replication, cell growth and cellular differentiation. Hence the defect in transcriptional factors either inherited or acquired may contribute to various types of malignancy including breast cancer. Transcription factors when genomically altered have the potential oncogenic role in various malignancy including lymphomas, leukaemia's and breast cancer.

Transcription factors represent prime targets for disruption in the process of tumourigenesis as they possess specific structural features that encode transactivating or repressing functions as well as dimerization, nuclear localization and DNA binding functions. These transcription factors are cell cycle associated and are expressed ubiquitously in various malignant tumours rather than lineage restricted expression (Rabbitts *et al.,* 1994). Some tumours like lymphomas and childhood tumours can have lineage- restricted transcription factors abnormality leading to dysregulated cell growth (Look *et al.,* 1997). It has been suggested that the ubiquitously expressed transcription factor can achieve lineage restricted gene control and upon disruption or activation can lead to abnormal cell growth. Various proto-oncogenes and anti-oncogenes are known to encode proteins which act as transcriptional regulators. These proteins which include

oncoproteins like Myc and Myb and tumour suppressor proteins like Rb and p53 are cell cycle associated and are ubiquitously expressed.

Breast cancer results from acquired or inherited gene defects leading to genomic instability and hence the tumour growth due to increased proliferation of transformed mammary cells (Jackson *et al.,* 1998; Loeb *et al.,* 1998). Various oncogenes and tumour suppressor genes leading to abnormal transcription factor expression in breast tumours are Myc, Rb, and p53.

Various defective gene controlling mechanisms resulting in the expression of abnormal transcriptional factors in breast tumourigenesis are as follows:

- Growth dysregulation by defective transcription factor expression
- Post- translational activation of transcription factors mediating breast tumourigenesis
- Breast tumour co regulators and cooperating transcription factors
- Transcriptional silencing in breast tumours

1.3.1 Growth dysregulation by defective transcription factor expression

Defects in ER, Myc, Rb and p53 contribute to a common growth dysregulatory mechanism that drives the uncontrolled cellular proliferation underlying breast tumours. The growth dysregulating consequences of overexpressed oestrogen receptor (ER) represent the most common transcriptional defect found in breast cancer and occur in more than 50% of all newly diagnosed breast cancer (Tripathy *et al.,* 1992). It is an important molecular target for the treatment of this malignancy.

The overexpression and transcriptional upregulation of Myc occurs in 30% of primary breast cancers (Escot *et al.,* 1986) and underexpression of p53 due to various genomic point mutations occurs in 20-40% of breast cancer (Hartmann *et al.,* 1997). Lania et al. have shown the relationship between these transcriptional factors and the regulation of cell cycle progression between G1 and S phases leading to loss of G1 –S cell cycle control (Lania *et al.,* 1999).

1.3.2 Post translational activation of transcription factors mediating breast tumourigenesis

Expression of transcription factors is dependent on both transcriptional and post translational control. Tumourigenesis can be mediated by disturbances in the post translational modification of the transcriptional factors, as the control of the genes they regulate may be influenced by the post translational activating mechanisms. These activating mechanisms may be ligand activated as characteristically occurs with members of the oestrogen receptor (ER) family or by intracellular signalling pathways that produce covalent modifications in the structure of the transcription factors or by Mitogen-activated protein kinase (MAPK) (Kato *et al.,* 1995; Katzenellenbogen *et al.,* 1997; Kato *et al*., 2001).

1.3.3 Breast tumour coregulators and cooperating transcription factors

Most transcription factors, including the members of the steroid receptor superfamily, work to regulate gene expression in conjunction with co-regulatory factors known as coactivators or corepressors. Amplification and overexpression of the coregulators has

been found in a significant proportion of primary breast tumours (Horwitz *et al.,* 1996; Smith *et al*., 1997).

1.3.4 Transcriptional silencing in breast tumours

Gene repression by DNA hypermethylation with the formation of M5C in gene regulatory region may represent part of a more fundamental imbalance in genomic methylation associated with breast tumours (Baylin *et al.,* 1998). This hypermethylation mechanism has been shown to lead to transcriptional repression of tumour suppressor genes encoding various transcription factors like Rb, VHL and E- cadherin.

1.4 CREB/ ATF Family

As described earlier transcription factors are divided into various families such as basic-helix- loop- helix (bHLH), leucine zipper with basic domain (bZip), zinc- finger, homeobox (HOX) and Ets factors. cAMP responsive element binding (CREB)/ Activating transcription factor (ATF) protein belongs to a subfamily of the bzip family of transcription factors. The cAMP transcription factors belong to a multigene family with several isoforms that may function as transcriptional activators or repressors (Hai *et al.,* 1989). The common motif shared by them is a basic domain – leucine zipper (bZip) (Hai *et al.,* 1989) at the carboxyl- terminal end that promotes dimmer formation. This family of transcription factors is a component of intricate intracellular signaling pathway that is important for regulating biological functions ranging from spermatogenesis to circadian rhythms and memory (Silva *et al.,* 1998).

Activating transcription factor (ATF) was first isolated and named by Lee et al. in 1987 (referred to proteins that bind to adenovirus early promoters E2, E3 and E4)(Lee *et al.,* 1987). cAMP responsive element binding protein (CREB) was named in 1987 to refer to proteins that bind to the cAMP responsive element (CRE) on the somatostatin promoter (Montminy *et al.,* 1987). The CREB/ ATF family of transcription factor was identified by their ability to bind to the consensus CRE/ ATF site "TGACGTCA" (Montminy *et al.,*. 1987). Hai *et al*. identified cDNAs encoding 8 different human CREB/ ATF binding proteins (Hai *et al.,* 1989). At present there are over 20 different CREB/ ATF cDNAs which have been identified and described. All members of this family share significant sequence similarity within a leucine zipper DNA- binding motif and an adjacent basic region. The members are grouped into subfamilies based on sequence homology outside the bzip domain (amino acid similarity).

CREB 1 is mapped to human chromosome 2q32.3-q34. The nomenclature for this family of proteins is confusing. Over the year's cDNA encoding homologous or identical proteins encoding bZip DNA binding domain were isolated by different laboratories and given different names. In various literatures alternative names were used to identify the same protein (Table 1.6) as well as the same name was used to refer to different proteins. As for instance CREB2 has been used to refer to three different proteins: CRE-BP1 (ATF2) (Flint *et al.,* 1991), ATF4 (Karpinski *et al.,* 1992), and an alternatively spliced CREB (Yoshimura *et al.,* 1990). Hence to ensure the identity of a given protein, the best way is to inspect its amino acid sequence. These proteins are therefore grouped into subgroups according to amino acid similarity.

Table 1.6: The mammalian CREB/ ATF family of transcription factors
(Hai *et al*., 2001)

Subgroup	Members	Alternative names
CREB	• CREB-1 • CREM • ATF-1	ATF-47 TREB-36,TCRATF-1, ATF-43
CRE-BP-1	• CRE-BP-1 • ATFa • CRE-BPa	ATF-2, HB-16, TREB, TCR-ATF-2, mXBP
ATF-3	• ATF-3 • JDP-2	LRF-1, LRG-21, CRG-5, TI-241
ATF-4	• ATF-4 • ATFx	CREB-2, TAXREB-67, mATF4, C/ATF, mTR-67. hATF-5
ATF-6	• ATF-6 • CREB-RP	ATF-6α G-13, ATF-6β
B-ATF	• B-ATF • JDP-1	

1.4.1 Cross talk between CREB/ ATF, AP1 and C/EBP Proteins

Besides CREB/ ATF, other proteins also contain the bZip DNA binding domain; they include the AP1 and C/EBP families of proteins (Cao *et al.,* 1991; Yeh *et al.,* 1995). This family of proteins can cross bind to each others consensus sites (Table 1.7) and can form heterodimers with members of another family. These proteins can form selective heterodimers with ATF2 (Hai *et al.,* 1989), cJun (Hai *et al.,* 1991; Hsu *et al.,* 1992) Jun B (Hsu *et al.,* 1992) and JunD (Chu *et al.*, 1994; Fawcett *et al.*, 1999). These heterodimers can act as activators or repressors depending on the promoter. Besides this, members of one family can regulate transcription in manner characteristics of the other family like Jun protein regulate the proencephalin promoter in a cAMP –dependent manner (Kobierski *et al.,* 1991) and C/EBP protein regulates certain promoters via a CRE site instead of a C/EBP site (Park *et al.,* 1993). Examples of crosstalk between CREB/ ATF, AP1 and C/EBP are shown in the table1.7.

Table1.7: Examples of crosstalk between CREB/ ATF, AP1 and C/EBP

Cross family binding
CREB/ ATF proteins bind to the AP1 consensus (Hai *et al.,* 1991; Masquilier *et al.,* 1992)
AP1 proteins bind to the CRE/ ATF consensus (Ryseck *et al.,* 1991)
C/EBP proteins bind to the CRE/ ATF consensus (Bakker *et al.,* 1991)
Cross family Heterodimer formation
CREB/ ATF and AP1 (Benbrook *et al.,* 1990; Chatton *et al*., 1994)
AP1 and C/EBP (Hsu *et al.,* 1994)

1.4.2 Mode of action of CREB

CREB is a 43 kDa basic /leucine zipper (bZip) transcription factor that regulates gene expression through the activation of cAMP dependent or independent signal transduction pathways. The leucine zipper is needed for dimerization whereas the adjacent basic domain mediates the contact with DNA (De Cesare *et al.,* 2000). These transcription factors acts through cAMP (Montminy *et al*., 1997) which is an important intracellular second messenger for a number of hormones and mediates the transcriptional induction of many genes. It binds to cAMP responsive element (CRE) 5'-TGACGTCA-3' (Deutsch *et al.,* 1988) and activates transcription in response to a variety of extracellular signals including neurotransmitters, hormones, membrane depolarization, and growth and neurotrophic factors. The unique aspect of the genes encoding CREB and other members of this family is that they can originate different isoforms by mechanisms of alternative exon splicing, alternative promoter usage and autoregulation of promoters (De Cesare *et al.,* 2000). Some isoforms act as activator of transcription such as phosphorylated CREB whereas others such as the inducible cAMP Early repressor (ICER) act as repressors. Activation of CREB requires phosphorylation by cAMP- dependent protein kinase at serine-119 and serine-133 for interaction with DNA and RNA polymerase II (Gonzalez *et al., 1989*; Lee *et al.,* 1995; Wong *et al.,* 1995; Shaywitz *et al.,* 1999; Johannessen *et al.,* 2007). Phosphorylation occurs via p44 / 42 MAP kinase and p90RSK and also via p38 MAP kinase and MSK 1. Although CREB binds to DNA independent of its phosphorylation state, only the phosphorylated form is competent as a transcription factor. P- CREB in turn increases the expression of the CREB gene itself as well as of the repressor ICER upon binding to CRE sequences in the promoter region of these genes

(De Cesare *et al.,* 2000). There are evidence suggesting that the CREB/ ATF proteins binds to cAMP response element (CRE) consensus sequence in target genes as a homodimer or heterodimer with other members of the family or with other bZip proteins like fos and jun family of proteins (AP-1) (Chinenov *et al.,* 2001). These alter DNA binding specificity and transcriptional activity and hence the gene expression. These bZip proteins can also bind to each other's consensus site and regulate transcription similar to other family proteins.

1.5 CREB/ ATF and Malignancy

The cAMP response element – binding protein (CREB) overexpression is associated with increased risk of relapse of acute myeloid leukemia. CREB promotes abnormal proliferation, cell cycle progression and survival of myeloid cells through upregulation of specific target genes. Activation of CREB is downstream of mitogen activated protein kinase kinase (MAKK) and pp90 ribosomal S6 kinase (RSK) – dependent pathways in myeloid cells (Lee *et al.,* 1995; Kwon *et al.,* 2000). CREB also induces proliferation of T cells in response to a variety of stimuli (Kuo *et al.,* 1999). CREB is expressed in the normal adrenal cortex and in adrenal adenomas but no CREB transcript is detectable in adrenal carcinoma and is reduced in thyroid carcinoma (Reincke *et al.,* 1997).

CREB mediates cellular responses to various mitogens and stressors. Various stress signals, growth factors and kinases promote phosphorylation –mediated activation of the CREB, involved in glial cell fate determination (Bayatti *et al.,* 2001). CREB has an antimitogenic role in astrocytes and suppresses the proliferation of glioblastoma through an intrinsic transcriptional regulation mechanism of stress induced acetylcholinestrase

variant, AChE-R. CREB promotes proliferation and survival of neurons and glial cells in the injured brain (Ong *et al.,* 2000) and mediates cell viability during early embryonic development (Bleckmann *et al.,* 2002).

Various growth factors, stress signals and kinases including PKA, PKC, calcium / calmodulin kinase 2 and MAPK activated protein, promote CREB activation by Ser 133 phosphorylation resulting in diverse cellular outcomes including cell proliferation (Goren *et al.,* 2001) (Table 1.8). PKC – mediated CREB activation induced proliferation of early oligodendrocytes (Afshari *et al.,* 2001). PKA – dependent CREB activation promotes astroglia differentiation (Bayatti *et al.,* 2001) and is required for Shwann cell proliferation (Lee *et al.,* 1999).

Table1.8 Extra cellular regulations of CREB/ ATF proteins

Member	Mode of regulation	Regulators
CREB, CREM, ATF1	Post translational	cAMP/PKA (Liu *et al.,* 1993) calcium ionophores (Montminy *et al.,* 1997)
CRE-BP1, ATF2	Post translational RNA level	TPA stress kinases (Gupta *et al.,* 1995)
ATF3	Post translational RNA level	HTLV-1 Tax (Low *et a*l., 1994) PKA (Chu *et al.,* 1994) Hepatitis virus X protein (Barnabas *et al.,* 1997)

1.6 CREB/ ATF in other conditions

This family of transcription factors is a part of intricate intracellular signaling pathway that is required in the regulation of various biological and cellular functions including cell survival, spermatogenesis, glucose homeostasis, circadian rhythm, memory and neuronal plasticity (Barton *et al.,* 1996; Silva *et al.,* 1998; De Cesare *et al., 1*999; Bonni *et al.,* 1999; Travnickova-Bendova *et al.,* 2002). Its activity is dependent on serine -133 phosphorylation, which is catalysed by various groups of serine/ threonine kinases including calcium/ calmoudlin-dependent protein kinases, mitogen – activated protein kinases(MAPK'S), protein kinase A, protein kinase G and PP90 ribosomal S6 kinase (Gonzalez *et al.,* 1989; Sheng *et al.,* 1991; Tan *et al.,* 1996; Xing *et al.,* 1996; Du *et al.,* 1998). It is also activated in response to a broad spectrum of stimulant in different cell types (De Cesare *et al.,* 1999; Shaywitz *et al.,* 1999). Known target genes are induced in response to CREB activation in other systems. Cyclin A 1 has been reported to be a CREB target gene and regulates G1to S transition through activation of CDK2 (Woo and Poon *et al.,* 2003). CREB directly binds the CRE in the cyclin A1 upon activation of CREB (Deutsch *et al.,* 1988; Mayr *et al.,* 2001; Kothapalli *et al.,* 2003). CREB overexpression leads to upregulation of a CREB target gene, cyclin A1, and hence promoting aberrant cellular proliferation. CREB/ ATF protein is involved in both the positive and negative regulation of smooth muscle cell growth and motility. ATF-1 mediates protease activated receptor-1 induced DNA synthesis in vascular smooth muscle cells (Ghosh *et al.,* 2002). CREB protein mediates thrombin (Tokunou *et al.,* 2001) and angiotensin 2 (Funakoshi *et al.,* 2002)induced proliferation of vascular smooth muscle cells. CREB mediates Alpha tumour necrosis factor (α TNF) induced vascular smooth

muscle migration. CREB mediates Cycloxygenase induced vascular smooth muscle cells motility (Reddy *et al.,* 2002) whereas its activation by Ser – 133 phosphorylation leads to decrease expression of multiple cell cycle regulatory genes and hence reduced proliferation (Klemm *et al.,* 2001). These differential roles of CREB-1 in the smooth muscle cells growth and motility may reflect its coupling to various signaling pathways targeting either the stimulation or suppression of growth and motility and therefore can function either as an inducer or as a suppressor of gene expression.

CREB plays a key role in the development of different neuronal cells and is activated by a variety of signaling molecule. CREB enhances both proliferation and survival of neuronal cells (Riccio *et al.,* 1999 ; Monti *et al.,* 2002; Lonze *et al.,* 2002). CREB 1 interacts with CRE-11 in the SMN promoter and its overexpression upregulates SMN promoter activity. CREB has been shown to be a critical regulator of proliferation in normal neuronal development and plays an important role in learning and memory (Shaywitz *et al.,* 1999; Mayr *et al.,* 2001). CREB knock out mice show diminished learning ability.

1.7 ATF 3

ATF 3 has been implicated in various physiological and pathological processes including stress response, HBV-mediated processes and tumourigenecity. ATF3 was first isolated a decade ago, and since then several studies had shown that it can be induced by various stress signals. ATF3 is regulated by multiple signaling pathways in signal and cell type-dependent manners. ATF 3 interacts with the HTLV-1 Tax protein leading to increase transcriptional repression activity (Barnabas *et al.,* 1997). ATF3 interacts with the hepatitis B virus X protein which is implicated in HBV mediated hepatocarcinogenesis

and apoptosis (Table 1.8). Besides this ATF3 play a role in metastatic processes in melanoma and has a tumourigenecity- inhibiting effect on the HT29 colon cancer cells (Ishiguro *et al.,* 2000).

ATF3 is the immediate early gene which is induced by various extracellular signals. In animal models ATF3 is induced in the heart by myocardial ischaemia and in the liver by hepatic ischaemia and partial hepatectomy. In cultured cells ATF3 is induced by a variety of signals such as ultraviolet rays and ionizing radiation and cytokines. Allen and Jennings demonstrated that ATF3 is induced during pancreatic stress such as partial pancreatectomy and ischemia – reperfusion of the pancreas(Allen-Jennings *et al.,* 2001). In most systems ATF3 mRNA increases shortly after the exposure of cells to the signals. Several genes have been implicated to be the targets like gadd153/chop10 (Wolfgang *et al.,* 1997; Fawcett *et al.,* 1999), E- selectin (Nawa *et al.,* 2000) and phosphoenolpyruvate carboxykinase (Allen-Jennings *et al.,* 2002).

ATF3 gene is induced in a variety of cell types by many stress signals suggesting that ATF3 is a key regulator in cellular stress responses. Although the induction of ATF3 gene is neither tissue nor stimulus – specific, it is shown that this also induce cellular damage (Chen *et al.,* 1996; Tsujino *et al.,* 2000). It has been suggested that ATF3 appears to be part of the cellular response that leads to poor outcomes. Allen and Jennings showed that in transgenic mice expressing ATF3 in liver results in liver dysfunction and reduced expression of gluconeogenic genes (Allen-Jennings *et al.,* 2001). Okamoto demonstrated that in transgenic mice expressing ATF3 in the heart have conduction abnormality and contractile dysfunction (Okamoto *et al.,* 2001).

1.8 CREB/ ATF in Breast Cancer

1.8.1 Aromatase expression in breast cancer tissue

Oestrogen is a steroidal hormone which is involved in various reproductive and metabolic processes. Pathologically, oestrogen is the primary hormonal factor that stimulates growth of hormone-dependent breast cancer. Approximately 75% of all breast cancers are hormone dependent of which 50% - 60% of premenopausal and 70% - 75% of postmenopausal patients has *oestrogen*-dependent breast cancer. The effects of *oestrogen* on breast cancer cells are predominantly mediated via interaction with the *oestrogen* receptor (ER) which inturn induces the expression of peptide growth factors that are responsible for the proliferation of cancer cells.

Oestrogen biosynthesis from C_{19} steroids is catalysed by the enzyme aromatase cytochrome P450. Aromatase catalyzes three consecutive hydroxylation reactions converting androstenedione and testosterone to oestrone and oestradiol respectively. Since aromatase is the enzyme necessary for the synthesis of *oestrogen*s, and *oestrogen* plays a major role in the development of breast cancer, aberrant expression of aromatase in breast cancer cells and surrounding stromal cells may have a significant influence in breast tumour pathogenesis.

Aromatase is expressed in a tissue-specific manner. This enzyme is mainly expressed in the ovaries and placenta of premenopausal women. In postmenopausal women, adipose tissue and skin are the major sources of *oestrogen* production. However, the aromatase activity in these tissues is significantly lower than that in the ovaries of premenopausal women, and the level of circulating *oestrogen* is much lower in postmenopausal women as compared to premenopausal women.

The expression of aromatase in breast cancer cells is up regulated as compared to that of surrounding non cancerous tissue (James *et al.,* 1987). Aromatase is expressed at a higher level both at mRNA and protein level in the breast cancer cells as compared to normal breast tissue (Esteban *et al.,* 1992; Santen R J., 1994). The expression of aromatase in breast cancer tissue has been demonstrated by enzyme activity measurement (Miller *et al.,* 1987), immunocytochemistry (Sasano *et al.,* 1994; Lu *et al.,* 1996) and RT-PCR analysis (Zhou *et al.,* 1996; Harada *et al.,* 1997). Cell culture (Santner *et al.,* 1993) and nude mouse experiments (Yue *et al.,* 1994) using aromatase transfected MCF-7 cells has shown that aromatase expressed in breast cancer cells play a significant role in the growth of the breast cancer. Aromatase can stimulate breast tumour growth in both an autocrine and a paracrine manner (Sun *et al.,* 1997)

The human aromatase gene was mapped to chromosome 15, band q21 by in situ hybridization studies (Chen *et al.,* 1988) and confirmed by human genome analysis (Sebastian *et al*., 2001). Human aromatase expression is regulated by a complex mechanism, in that seven promoters (I.1, I.2, I.3, I.4, I.5, I.6, and PII) have been identified and found to be utilized in a tissue-selective manner. It has been shown that various exon I-containing RNA messages are present at different levels in different aromatase expressing tissues. It is thought that aromatase expression is driven by the promoters situated upstream from these exon Is, providing tissue-specific control of aromatase expression. Increased expression of aromatase in breast tumour is due to changes in the transcriptional control of aromatase expression. It is known that in normal mammary adipose tissue aromatase is expressed through the use of promoter 1.4 (Mahendroo *et al.,* 1993; Zhao *et al.,* 1995) and in tumour tissue through the use promoter II (Zhou *et al.,* 1996) and promoter I.3 (Agarwal *et al.,* 1996; Zhou *et al.,* 1996). It has been suggested

that there is a switch in the regulatory mechanism of aromatase expression from normal breast tissue to cancer tissue. In normal mammary tissue, the function of promoters I.3 and II is suppressed through the binding of EAR-2, COUP-TFI, and RARγ to S1 (Yang *et al.,* 1998 ; Yang *et al.,* 2002), and Snail/Slug proteins to CREaro (Figure 1.8).

In cancer tissue, it has been shown that when the expression level of EAR-2, EARγ, Snail, and Slug decreases, the expression of aromatase is up-regulated through the binding of CREB1 to CREaro and that of ERRα to S1 (Figure 1.8). ERRα is expressed at higher level in the breast cancer tissue and is thought to be a positive regulatory factor that up-regulates promoter I.3 and II (Yang *et al.,* 1998). Through the binding to ERα, E2 up-regulates the expression of MTA3 which suppresses the activity of Snail/Slug (Fujita *et al.,* 2003). These two zinc-finger proteins have been shown to down-regulate the activity of promoter 1.3. By suppressing the expression of Snail/Slug through MTA3, E2/ERα indirectly enhances the activity of promoter 1.3. On the other hand, E2/ERα can up-regulate PKC-δ/MAP kinase and promoter I.1 of the aromatase gene through cross-talk with ErbB1/ErbB2 (Figure 1.9). Furthermore Gotzmann demonstrated that ERα's and Snail's function can be modulated by the PI3 kinase/Akt pathway (Gotzmann *et al.,* 2002). Therefore, the ligands of ERα and ERRα, e.g., phytoestrogens, can regulate aromatase expression. This similar positive and negative regulatory mechanism to explain the interaction between SF-1 and COUP-TFI with S1 was demonstrated in uterine endometrial cells (Zeitoun *et al.,* 1999). A silencer element (S1) situated between promoters I.3 and II acts as a negative regulatory element which down-regulates the action of these promoters (Zhou *et al.,* 1998). S1 behaves as an enhancer when ERRα-1 binds and as a repressor when COUP-TF, EAR-2 or RARγ binds (Yang *et al.,* 1998 ; Yang *et al.,* 2002).

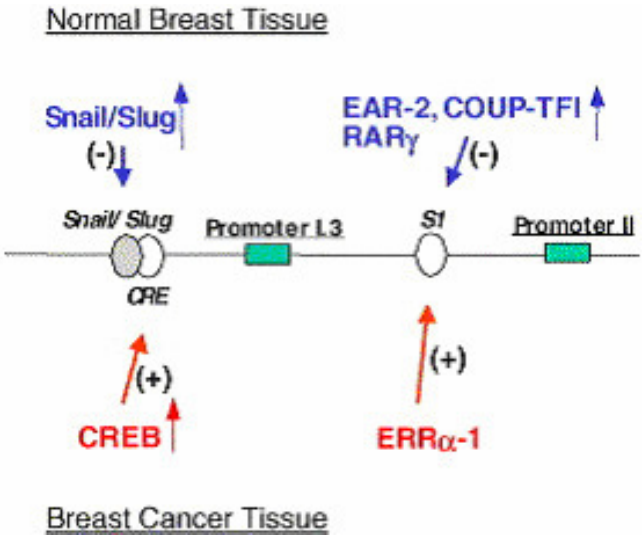


Figure 1.7: Proposed mechanism of the regulation of aromatase activity in normal breast and cancer tissue in relation to CREB and their promoters
(Adapted from Chen et al., 2001)

Snail acts as a repressor that down-regulates the expression of aromatase in normal breast tissue by suppressing the function of promoter I.3. A reduction of the expression of Snail/ Slug protein in breast cancer tissue further suggests a cancer-protective role for this protein in normal breast tissue (Chen *et al.,* 2002). In normal breast epithelial cells and stromal fibroblasts, Snail and Slug are expressed and prevents activators from binding to CREaro. This results in a suppression of promoter I.3 activity. On the other hand, in cancer tissue, human aromatase promoter usage switches to promoter I.3 (Zhou *et al.,* 1996), which in response to cAMP activation of CRE binding proteins such as CREB1 decreases the Snail and Slug expression (Figure 1.10).This reveal that cAMP plays a critical role in upregulating the activity of promoters I.3 and II, leading to an increase of the expression of aromatase/oestrogen biosynthesis in breast cancer tissue. Several factors may be involved in increasing the level of cAMP in breast cancer tissue. For example, Zhao et al. suggested that prostaglandin PGE2 synthesized in breast cancer cells induces cAMP response (Zhao *et al.,* 1996). It has also been demonstrated that COX2 inhibitor reduces the aromatase expression by suppressing prostaglandin formation. Furthermore, *oestrogen* is capable of increasing cAMP production in breast cancer cells by stimulating adenylate cyclase (Aronica *et al.,* 1994). All these observations suggest a paracrine loop between cAMP synthesis in breast cancer tissue and *oestrogen* synthesis by aromatase enzyme. Hence suppression of in situ *oestrogen* biosynthesis can be achieved by the prevention of aromatase expression in breast tumours or by the inhibition of aromatase activity.

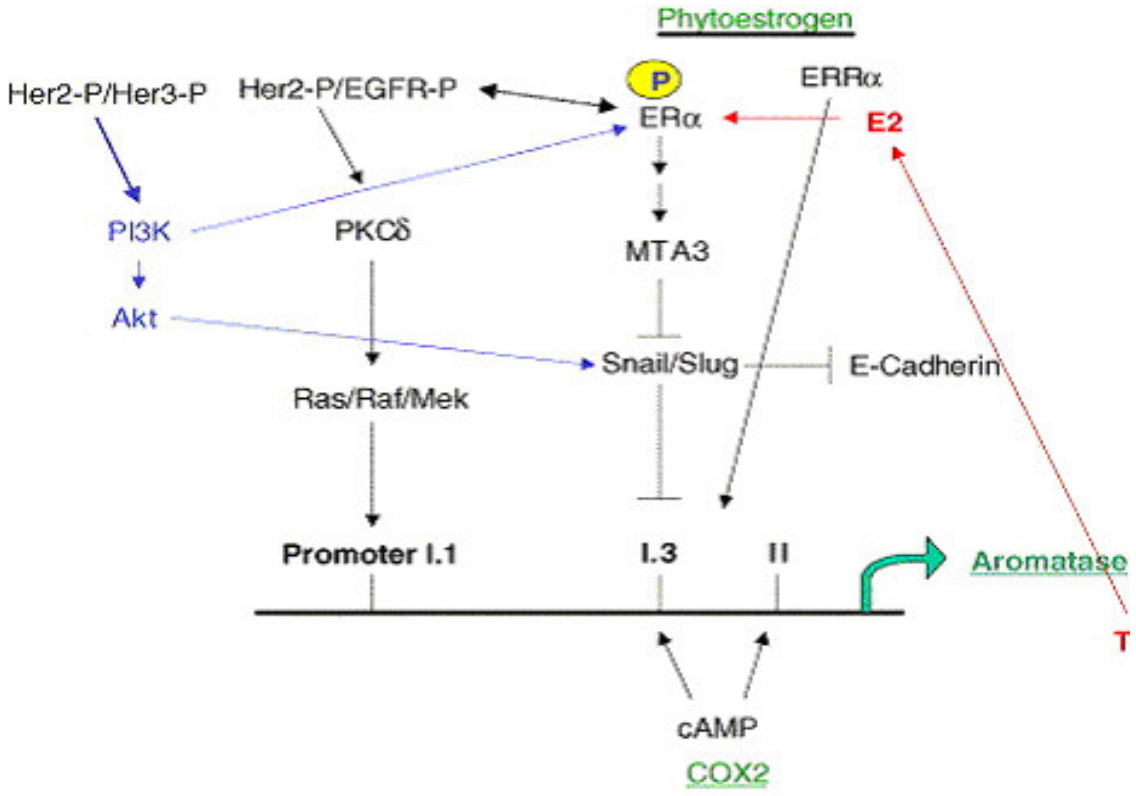


Figure 1.8: Schematic Diagram showing a complex mechanism to modulate aromatase expression in human breast cancer tissue
(Adapted from Chen et al., 2005)

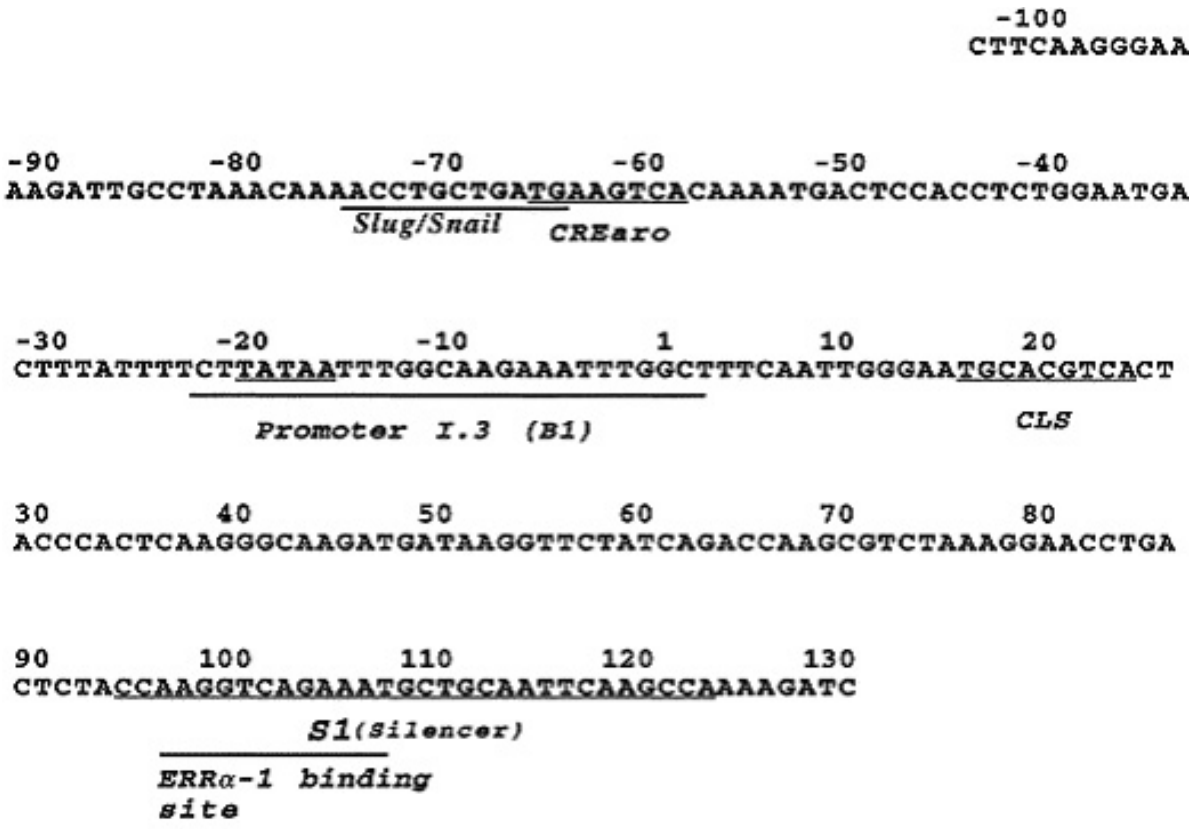


Figure 1.9: The nucleotide sequence of the −104/+130 bp region containing promoter I.3 of the human aromatase gene

(Adapted from Zhou et al., 1999).

1.8.2 Role of CREB in aromatase expression in breast tissue

It has been reported that cAMP Response Element-Binding Proteins (CREB) may act as a positive transcription regulator of aromatase via cAMP (CRE1 and CRE2) induced promoter II activity in breast tumour tissue and hence increased expression of oestrogen in breast cancer cells (Sofi *et al.,* 2003).

To date, there is very little study which has shown any direct link between CREB expression and breast cancer, CREB may be involved in tumour growth and metastasis through increased expression of aromatase.

1.9 Aims of this study

The aims of this study were threefold:

1. To investigate the molecular impact of CREB family of proteins on the aggressiveness of breast cancer cells and to investigate the expression pattern in cohort of breast cancer and normal background tissue in relation to tumour histopathological grade, stage, nodal status and the clinical outcome of the patients

2. To develop ribozyme transgenes to knockdown the expression of CREB in breast cancer cell lines and its effect on the biological functions of the breast cancer cells

3. To investigate the role of CREB on *in vitro* growth and invasiveness of breast cancer cells following knockdown of CREB.

CHAPTER 2

MATERIALS AND METHODS

2.1 General Materials

The following cell lines have been used in this study:

Cell Lines	Origin	Characteristics
MDA-MB- 231	Human Breast cancer cell line	Ethnicity: Caucasian Gender: Female Age: 51 years Isolation date: 1973 Morphology: epithelial cell type Derived from metastatic site: Pleural effusion Oestrogen receptor: Negative Antigen Expression: epidermal growth factor (EGF), transforming growth factor alpha (TGF alpha),
MCF 7	Human Breast cancer cell line	Ethnicity: Caucasian Gender: Female Age: 69 years Isolation date: 1970 Morphology: epithelial cell type Derived from metastatic site: Pleural effusion Oestrogen receptor: Positive Her2/ ERBB2: Negative
MDA-MB- 453	Human Breast cancer cell line	Ethnicity: Caucasian Gender: Female Age: 48 years Isolation date: 1976 Morphology: epithelial cell type Derived from metastatic site: Pericardial effusion Oestrogen receptor: Negative Her2/ ERBB2: Negative Antigen Expression: fibroblast growth factor (FGF)
MRC 5	Immortalised human Fibroblast cell line	Ethnicity: Caucasian Gender: Male Age: 14 weeks old foetus Isolation date: 1966 Morphology: fibroblast cell type Derived from Human foetal lung
HECV	Transformed human umbilical vein endothelial cell line	Endothelial cell type derived from neonatal umbilical vein

The above cells were obtained from the European collection of animal cell culture (ECACC), except HECV which was purchased from Interlab, Milan, Italy.

2.1.1 Preparation of Reagents, Buffers and Standard solutions

All the standard reagents and chemicals which were routinely used throughout this study, unless otherwise stated, were purchased from Sigma-Aldrich Inc., Poole, Dorset, UK.

Tris- Boric acid- EDTA (TBE) Electrophoresis Buffer

A stock solution of TBE was prepared as a 5 × concentrate. The solution was made by dissolving 540g Tris-Cl (Melford Laboratories Ltd., Suffolk, UK), 275g Boric acid (Duchefa Biochemie, Haarlem, The Netherlands) and 46.5g EDTA (Duchefa Biochemie, Haarlem, The Netherlands) in 10 litre dH_2O. A working solution was prepared by dissolving 200ml of stock solution in 800ml of distilled water.

DEPC water

A stock solution was prepared by dissolving 250µl of Diethyl Pyrocarbonate (DEPC) (Sigma-Aldrich Inc., Poole, Dorset, UK) in 4750µl distilled water and autoclaved before use.

Preparation of Ethidium Bromide (EtBr) Staining Solution (Strength: 10mg/ml)

The Working solution was prepared by dissolving 100mg Ethidium Bromide (Sigma-Aldrich Inc., Poole, Dorset, UK) in 10ml of distilled water. The solution is mixed well to dissolve powder and wrapped in aluminium foil.

Preparation of Complete Cell Culture Medium

This solution was prepared by mixing 50ml of heat inactivated Foetal calf serum (FCS) (PAA Laboratories, Austria) in 500ml DMEM/F12 (PAA Laboratories, Austria) with 2mM L- glutamine. To this 250µl of Benzyl penicillin (100 units/ml, Britannia Pharmaceuticals Ltd., UK) and 200µl of Streptomycin (100µg/ml, Gibo BRC, Paisley, Scotland) was added and the solution was then stored up to a month at 4ºC.

Preparation of G418 Selection Media

- 250µl of G418 (200mg/ml)
- 500ml of complete cell culture medium
- Wrap in aluminium foil to protect from light and stored at 4ºC.

Balanced Salt Solution (BSS)

A 5 litre stock solution of 1XBSS (137mM NaCl, 2.6mM KCl, 1.7Mm Na_2HPO_4 (BDH Chemicals Ltd., Poole, UK) and 8mM KH_2PO_4) was prepared by dissolving 40g NaCl, 1g KCl (Fisons Scientific Equipment, Loughborough, UK), 5.72g Na_2HPO_4 and 1g KH_2PO_4 (BDH Chemicals Ltd., Poole, UK) in dH_2O. The pH was adjusted to 7.4 with 1M NaOH before use.

Preparation of 0.05M EDTA

A 5 litre stock solution at 1×BSS contained 137mM NaCl, 2.6Mm KCl, 1.7mM Na_2HPO_4 and 8mM KH_2PO_4 and 0.05M EDTA (Duchefa Biochemie, Haarlem, The Netherlands). These were dissolved in 5 litres distilled H_2O and pH adjusted to 7.4 before autoclaving.

Trypsin (25mg / ml)

A stock solution of trypsin was prepared by dissolving 500mg trypsin in 20ml 0.05M EDTA. This solution was then filtered through a 0.2µm ministart filter (Sartorius, Epsom, UK) and stored at -20ºC. The working solution was prepared by further dissolving 250µl of trypsin solution in 10ml of 0.05 EDTA.

Penicillin (120mg/ml)

The stock solution was prepared by dissolving 600mg benzyl penicillin sodium (Britannia Pharmaceuticals Ltd., UK) in 5ml sterile injection water (Braun, Germany).

Tris Buffered Saline (TBS)

TBS Buffer was prepared as a 10× concentrate. A stock solution was prepared by dissolving 24.22g Tris-Cl (Melford Laboratories Ltd., Suffolk, UK) and 80.06 NaCl in 1 litre of distilled water. This solution contained 200mM Tris and 1.37M of NaCl.

Preparation of Antibodies

Antibodies obtained from the manufacturers (detailed in respective chapters) are diluted in 0.1% Bovine serum albumin (BSA) in BSS buffer and stored in 100µl aliquots at -20ºC.

Preparation of Inhibitor Buffer

We dissolve 2.76g of sodium nitrate, 630g of sodium fluoride, 5.58g EDTA, 10g $Na_2H_2PO_4$ and 3×10^5 Units of Aprotonin. The solution is made up to 1 litre with distilled water, prepared as a 3× concentrate and stored at 4ºC.

Preparation of Cell Lysis Buffer

The Stock Solution was prepared by dissolving 0.24g Tris-Cl, 0.435g NaCl, 0.25g Sodium Deoxycholate, 0.135g Sodium Orthovanadate , 0.01g Sodium Azide, 1ml of Triton x-100 in 50ml of distilled water. The solution is prepared as a 2× concentrate and stored at 4ºC.

A working solution is prepared by diluting 10ml of the stock solution in 6.6ml of inhibitor buffer, 200µl of 0.1mM PMSF (phenylmethylsulfonyl fluoride), 80µL of 10mM $CaCl_2$, 3ml of 10%Triton X-100 made up to 20ml with distilled water.

Transfer Buffer (for Western Blot)

A stock solution of 5 litres was prepared by dissolving 15.15g Tris, 72g Glycine and 1 litre of methanol (Fisher Scientific, Leicestershire, UK) in dH_2O.

Preparation of Amino Black

A working solution is prepared by dissolving 10mg Napthalene black (Edward Gurr Ltd., Suffolk, UK) in 10ml Acetic acid (Fisher Scientific, Leicestershire, UK) and 25ml Ethanol (Fisher Scientific, Leicestershire, UK). This solution was made up to 100ml with distilled water and stored at room temperature.

Preparation of Fluorescamine for protein quantisation

The working solution is prepared fresh by dissolving 3mg of Fluorescamine in 10ml of acetone.

Preparations of Amino Black destain

This solution is prepared by mixing 25ml ethanol and 10ml acetic acid and made up to 100ml with distilled water.

Preparation of Protein Loading Dye

This solution is prepared as a 10× concentrate. We dissolve 0.125g Bromophenol Blue (Sigma-Aldrich Inc., Poole, Dorset, UK) and 20g sucrose in 50ml of distilled water and freeze it in microcentrifuge tubes until required.

Preparation of Ponceau S

A working solution is prepared by mixing 20ml of Ponceau S (Sigma-Aldrich Inc., Poole, Dorset, UK) concentrate in 180ml of distilled water and stored at room temperature.

Preparation of 0.1% Comassie Blue

The working Solution is prepared by dissolving 1g Comassie, 400ml Methanol and 100ml Acetic acid in distilled water. The total volume is made up to 1litre. It is then filtered and stored at room temperature.

2.2 General Methods

2.2.1 Cell Culture and Storage

Cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM- Sigma: pH 7.3) containing 2mM L-glutamine, 15mM HEPES and 4.5mM $NaHCO_3$ (a pH buffer) supplemented with 10% heat inactivated (55°C for 30 minutes) foetal calf serum (PAA Laboratories, Austria), 50units/ml Benzylpenicillin (Britannia Pharmaceuticals Ltd) and 50µg/ml Streptomycin (Gibco BRC, Paisley). Foetal calf serum contains the necessary serum growth factors. All cell lines were grown in either 25cm² or 75cm² tissue culture flasks (Greiner Labortechnik, Germany) in monolayers depending on the application. Culture flasks were loosely capped, to allow gaseous exchange and then placed horizontally in a humidified incubator (98% humidification achieved with water tray in the incubator) (Sanyo Electric, Japan) at 37ºC with 5% CO2 (act as a buffer with the $NaHCO_3$) in air. The culture flasks were then left until sub-confluent (2-3 days) for experimental work or until fully confluent (7 days) for subculture.

2.2.2 Trypsinization and Counting of cell lines

- All procedures were carried out under sterile condition using class II hoods and autoclaved instruments to prevent any potential contamination.
- Tissue culture flask was taken out from incubator and the medium aspirated.
- Following this the flasks were rinsed with 5ml of EDTA in Hanks Balanced Salt Solution (HBSS containing 137mM NaCl, 8mM Na2HPO4, 3mM KCl and 1.5mM KH2PO4) buffer to remove traces of serum which would inhibit the enzymatic action of trypsin.

- After this 1-2 ml of trypsin / EDTA solution (Trypsin0.01% (w/v) and EDTA 0.05% (w/v) in HBSS buffer) was added to the flask and was incubated for 5 minutes at 37ºC to allow cell detachment. Following this 5ml of DMEM was added to the flask to neutralised trypsin and transferred to the universal container.
- The cells were centrifuged at 1500g for 5 minutes and medium aspirated.
- Following this the pellets were re-suspended in 5mls of DMEM solution and cells were re-cultured in tissue flasks, counted for immediate experimental work or stored by freezing in liquid nitrogen.
- Cell counts were performed using Neubauer haemocytometer counting chamber with an inverted microscope (Reichert, Austria) at ×10 magnification.

2.2.3 Storage of cell lines

The cell lines were stored in liquid nitrogen by re-suspending at a cell density of 1×10^6 cells/ml in complete medium (DMEM) containing 10% dimethylsulphoxide (DMSO; Fisons, UK). 1ml of cell suspension was transferred into pre-chilled cryopreserve vial and then frozen at -80ºC for 1 day before storage in liquid nitrogen (-196ºC).

2.2.4 Resuscitation of cell lines

To thaw cells, the cryo-vial was removed from liquid nitrogen and was allowed to thaw rapidly in a 37ºC water bath. The cell suspension was transferred to a universal container containing 2ml of pre-warmed DMEM and incubated at 37ºC for 10 minutes. The cell suspension was then centrifuged at 1600 rpm for 5 minutes and the supernatant discarded. The cell pellet was re-suspended in 5ml of DMEM and washed twice to remove any

possible trace of DMSO. The cell pellet was then re-suspended in 5ml of DMEM and transferred to a 25cm² tissue culture flasks. The cells were incubated at 37ºC with 98% humidification and 5% CO2 in air.

2.3 Isolation of total RNA from cells using the guanidine thiocyanate method

Reagents Required:

- RNA isolation reagent
- Chloroform
- Isopropanol
- 75% Ethanol
- DEPC treated water

Ribonucleic acid (RNA) is present within the nucleus, cytoplasm and mitochondria of all living eukaryotic cells. There are three main subtypes of RNA which are classified as ribosomal RNA (rRNA), transfer RNA (tRNA) and messenger RNA (mRNA). mRNA is of particular importance as it carries the genetic information for subsequent protein synthesis. The presence of specific mRNA therefore gives a good indication of which proteins are being produced by the cell at any one time.

RNA is susceptible to degradation by RNase and hence special precautions are taken to minimise this during isolation. All methods involving RNA isolation rely on the use of strong denaturants to inhibit the action of endogenous RNase. Guanidine, thiocyanate and chlorine are amongst the most effective protein denaturants and inhibitors of ribonucleases.

RNA isolation using the guanidine thiocyanate method was first described by Chomczynski and Sachhi in 1987. This procedure involves combining of acid guanidine thiocyanate, phenol and chloroform in a single step RNA extraction. RNA is prone to degradation by RNase and hence this method relies on use of strong denaturants like guanidine and thiocyanate to inhibit the action of endogenous RNase. The reaction is set under acidic conditions, so that the DNA is selectively partitioned into the organic phase whilst the RNA remains in the aqueous phase. Cells of approximately 80- 90% confluence were harvested from the tissue culture flask using a cell scraper and lysed in 1ml of RNA isolation reagent (Advanced Biotechnologies Ltd, Surrey, UK) by repetitive pipetting. The resultant homogenates were transferred to microcentrifuge tubes (Fisher Scientific, UK) and placed on ice for 5 minutes to allow the complete dissociation of nucleoprotein complexes. 200µl of chloroform per ml of RNA isolation reagent was added to the homogenate followed by vigorous shaking for 10-15 seconds. The samples were then incubated on ice for 10 minutes and centrifuged (Biofuge13, Heraeus, Sepatech) at 15000rpm for 10 minutes at 4ºC. Following centrifugation the homogenate gets separated into two distinct phases: the lower organic phase of DNA and protein and the upper aqueous phase of RNA. The aqueous phase containing RNA (40- 50% of total volume of homogenate) was transferred to a fresh tube and 500µl of isopropanolol (Sigma-Aldrich Inc., Poole, Dorset, UK) was added to precipitate RNA. Samples were then placed on ice for 10 minutes and centrifuged at 12000 rpm for 10 minutes at 4ºC. Following this the supernatant was removed and the resultant RNA pellets were mixed with 1ml of 75% ethanol by vortexing. The RNA pellets were precipitated by centrifugation at 12000 rpm for 5 minutes at 4ºC, briefly dried at 55ºC for 5 minutes in order to evaporate the remaining ethanol and then treated with 50µl DEPC (Diethyl

pyrocarbonate, Sigma) water by vortexing for 1 minute. RNA concentration was determined by measuring its absorbance at wavelength of A260nm/A280nm (WPA UV 1101, Biotech Photometer). A260nm/A280nm ratio gives an estimate of the purity of the RNA. Pure RNA solutions have an optical density ratio of 2.0. Optical density values less than 1.5 indicates ethanol or protein contamination. The RNA sample then was stored at -80ºC for future use.

2.4 Reverse Transcription- Polymerase Chain Reaction (RT-PCR)

Reverse transcription leads to synthesis of complementary cDNA from RNA. It is followed by polymerase chain reaction (PCR) and the two processes together are known as RT- PCR. RT-PCR is a sensitive and highly versatile technique that has greatly enhanced the study of genes and how they are controlled. RT- PCR is a more sensitive technique for the analysis of mRNA as compared to other techniques like Northern blots and RNA dot blots. This technique requires a small amount of RNA and is more sensitive and rapid in detecting genetic sequences as compared to other procedures. In this study RT- PCR was performed using a Dura Script RT-PCR Kit (Sigma-Aldrich Inc., Poole, Dorset, UK)) according to manufacturer's instructions. The following components were mixed together in a polypropylene tube (Appendoff):

Reagent	Volume	Concentration
RNA template	Depends on RNA concentration	×
PCR water	Depends on RNA concentration	×
Deoxynucleotide	1µl	500µM each dNTP
Anchored oligo dT	1µl	0.5µg/µl
Reverse Transcriptase	1µl	1 unit/µl
RNase inhibitor	1µl	1 unit/µl
PCR buffer	2µl	5×concentrate
Total Volume	20µl	×

The reaction mixture was incubated at 47ºC for 1hr to commence cDNA synthesis followed by incubation at 75ºC for 10 minutes to inactivate RNase. The cDNA was diluted to 1:2 in PCR water (distilled, deionised and UV- treated H2O) and the samples were either stored at -20ºC until required or used for PCR amplification.

2.5 Polymerase chain reaction (PCR)

Polymerase Chain Reaction, invented by Karey Mullis in 1983 is a simple technique of amplifying DNA to produce a desire amount from a single target DNA molecule.

Principle:

- It relies on the principle of a chain reaction in which each target molecule is subject to further amplification.
- It requires two short DNA sequences (oligonucleotides- Forward and Reverse primers) which binds by base pairing and are complementary to a defined sequence on each of the two strands of the DNA. These primers are aligned with the 3' ends directed towards each other.

- It involves the use of heat-stable DNA polymerase (Taq polymerase, a thermostable enzyme from Thermus aquaticus bacteria which is native to hot springs). The use of this enzyme means that fresh polymerase does not need to be added after each denaturation step as the bacteria survives temperature over 95ºC.
- It involves cycles of denaturation (96ºC) of template, annealing (50ºC) of primers and extension (72ºC) by DNA polymerase (Figure: 2.1) for amplification. The whole reaction is performed using a PCR thermal cycler (Gene Amp 9700, Perkin Elmer) which is a programmable machine that can rapidly change temperature and can incorporate upto 96 reactions simultaneously.

Amplification of cDNA templates previously prepared was performed using PCR. The following method was used for cDNA amplification in this study. All reactions were performed to a total volume of 20µl, with 10µl of Mastermix^{TM} (0.625 U of *Taq* DNA polymerase from *Thermus aquaticus*, 75mM Tris- HCL (pH 8.8 at 25°C), 20Mm $(NH_4)_2SO_4$, 1.5mM $MgCl_2$, 0.01% (v/v) Tween20, 0.2 mM from each of the following: dATP, dCTP, dGTP, and dTTP (ABgene , Surrey, UK), 1µl of cDNA template, 1µl of each forward and reverse primer and 7µl of PCR water in a thin walled PCR tube. PCR reactions were performed using a PCR thermal cycler as mentioned above. PCR conditions were variable depending on the application and consisted of 35-40 cycles. The PCR product is then analyzed on an agarose gel and is detected with ethidium bromide staining.

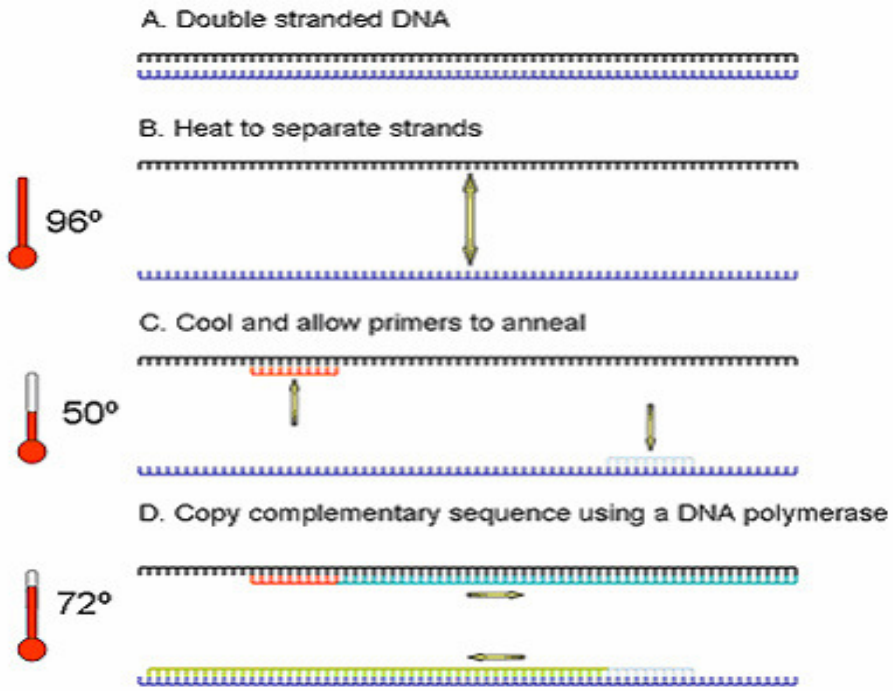


Figure 2.1: Different steps in PCR

2.6 Agarose gel electrophoresis

The resultant DNA PCR products were separated using horizontal gel electrophoresis apparatus (Gibco BRL, Life Technologies) at either 0.8% or 2% depending on the PCR product. A low percentage agarose gel (0.8%) is used to separate PCR products with a high number of base pairs (>500bp) and 2% agarose gel to separate DNA fragments with low number of base pair (<500bp).

Procedure:

20mls of agarose gel solution which was prepared by dissolving 0.8g or 2g of agarose (depending on the size of PCR product) in 100ml of TBE (Tris Borate Electrophoresis) buffer (0.45M Tris-HCl, 0.44M Borate, 12.5mM EDTA). The solution was then boiled at 100C until the agarose had completely dissolved (when the solution had become clear). The agarose gel solution of appropriate concentration was poured into a universal container and cooled down before casting it into the mould in the electrophoresis apparatus. Following this a comb was inserted into the liquid gel to create wells. The gel was then allowed to set at room temperature for about 30 minutes. Once the gel had set, TBE buffer was added into the electrophoresis apparatus upto a level of about 5mm above the surface of the gel and the comb was removed from the gel, leaving a row of wells. A 1kb pair ladder (Pharmacia Biotech, USA), prepared according to the manufacturer's instructions in a loading buffer (Glycerol, Bromophenol blue), and PCR products were loaded into separate wells of the gel (5µl per well). The gel was then run at a constant voltage of 100 volts (Gibco BRL Power pack model 250EX, Life Technologies) and was continued for 35-55 minutes or until the samples had migrated about two thirds down the agarose gel (depending on the PCR product size). The DNA PCR product was then

stained using ethidium bromide solution for 5 min with continuous agitation to provide uniform staining. Ethidium bromide is a dye that binds to double stranded DNA by interpolation between the base pairs and fluoresces when exposed to the UV light. This was sometime followed by destaining in tap water for 30-60 minutes if necessary for removal of background staining on the agarose gel from the fluorescent dye. PCR DNA products were then visualised using an Ultra Violet Transilluminator (UVP, Cambridge, UK) and photographed using a thermal printer or scanned for storing images electronically.

2.7 Real time quantitative Polymerase Chain Reaction (QPCR)

Over the last two decades PCR has become an essential part of most laboratories involved in biomedical research. The development of real-time QPCR added a new dimension to PCR and a significant improvement in the quantification of nucleic acid samples. Real-time quantitative polymerase chain reaction (QPCR) is a new technology, developed in 1992 which allows quantification of DNA, cDNA, or RNA templates as the products accumulates with each cycle of amplification. It provides a broad dynamic range for detecting specific gene sequences with excellent sensitivity and specificity. It is based on the detection of a fluorescent reporter molecule that increases as PCR product accumulates with each cycle of amplification (the level of fluorescence detected is directly proportional to the PCR product yield). The level of fluorescence is continuously monitored on a computer screen and hence the term 'real-time'. Fluorescent reporter molecules include dyes that bind double-stranded DNA (i.e. SYBR® Green I) or sequence–specific probes (i.e. Molecular Beacons®, Amplifluor® probes, Scorpions® probes or TaqMan® Probes).

There are two basic types of QPCR which includes SYBR® Green I based quantitative PCR and probe based quantitative PCR. The first method uses SYBR Green I fluorescent DNA binding dye, which binds to all double–stranded DNA and detection, is monitored by measuring the increase in fluorescence throughout the cycle. This dye binds non-specifically and hence can binds to the contaminated products that are amplified during PCR as well. The second method which is a newer improved version of QPCR uses sequence–specific probes. These are oligonucleotide sequences which contains a fluroscopically- labelled base known as fluorophore and a quencher. Prior to PCR, the fluorophore and the quencher lie next to each other, so that there is no fluorescence. However during the annealing stage the fluorophore and quencher get separated as the oligonucleotide probe becomes incorporated into the PCR product and results in an increase in fluorescence. These probes are specific for the desired PCR product and hence any contaminants in the reaction which are amplified will not fluoresce.

The iCycler iQ^{TM} system (Bio-Rad, Camberley, UK) was used throughout this project and consists of a 96-well thermal cycler module connected to a laser and charge-coupled device (CCD) optics system. It consists of two key components, namely an excitation system and a detection system. The excitation system consist of a cooler fan, a tungsten halogen lamp, an infrared absorbing heat filter, a filter wheel that is fitted with optical filters, and a dual mirror arrangement that allows simultaneous illumination of all the reaction wells. The laser light is directed through the filter and a pre- selected colour optical filter to excite the fluorescent molecules that have become unquenched in the PCR solution in each reaction well. Emissions are then sent to the CCD camera (detection system). The primary components of the detection system include an emission filter

wheel, an image intensifier and a cooled detection camera. Fluorescent light emitted from each well passes through both the emission filter and intensifier, which increases the fluorescence prior to detection by the camera where they are analysed by the software's algorithms. Collected data are subsequently sent to the computer. Emissions are measured constantly. During the initial cycles of QPCR there is very little change in fluorescence from the initial level as the products accumulate exponentially. After further cycles, the number of products rapidly increases and reaches the exponential phase of PCR and then plateau again as the substrates are progressively consumed.

There are four types of probe systems available which are as follows: Molecular Beacons®, Amplifluor® probes, Scorpions® probes or TaqMan® Probes).

2.7.1 TaqMan® probe based assay

The TaqMan® assay is a sensitive method to determine the presence or absence of specific target sequences. TaqMan® probes are used to detect the presence and quantify the amount of specific target sequences by employing the 5'→3' exonuclease activity of Taq polymerase. These Probes are designed to anneal to the target sequence between the forward and reverse primers. The probe consists of oligonucleotide sequence which is labelled at the 5' end with a reporter fluorochrome (6-carboxyfluorescein [6-FAM]) and a quencher fluorochrome (6-carboxy-tetramethyl-rhodamine) at the 3' end. These probes

have a higher T_m (Melting Temperature) than the primers, and during the extension phase must be 100% hybridized for success of the assay. The close proximity of the quencher to the reporter fluorochrome prevents its natural fluorescence. However, as the PCR progresses the probe binds to the PCR products and Taq polymerase cleaves the reporter

fluorochrome and the quencher from the probe, releasing the sequence specific fluorescent signal (reporter fluorochrome). The amount of fluorescence released during the amplification cycle is proportional to the amount of PCR product generated in each cycle.

2.7.2 Amplifluor® probe based assay

Nazarenko first described the Amplifluor based Q-PCR in 1997 (Nazarenko et al., 1997). The principle is based on energy transfer from an excited fluorophore to the acceptor moiety which results in quenching of the fluorescence. This quenching is accomplished by linking the fluorophore and the acceptor to an oligonucleotide primer. This system requires three oligonucleotides which are as follows: A target specific forward primer, a target specific reverse Z primer which incorporates a unique Z sequence at the 5' end of the primer and a universal primer labelled with fluorescein (FAM) which also contains a Z sequence attached at the 3'end. Amplifluors® combine primer and probe in one molecule. The Amplifluor® Probe and the forward primer are used in a 10-fold excess over the reverse primer to ensure high yield of the fluorescent product. As the reaction commence, the forward primer attaches to the target cDNA and begins extending. The reverse primer, which is specific to the target DNA, also extends in the opposite direction. Since the latter has a Z sequence attached, when the forward primer attaches to this newly synthesised sequence, it also synthesises an oligonucleotide containing a complementary Z sequence. After few cycles, the reverse primer/Z sequence is depleted, leaving only the forward primer and the probe. At this point, the Z sequence of the probe attaches to the newly synthesised Z sequence. When the forward primer extends to the probe and beyond, it results in a conformational change of the probe, separating the quencher and

fluorophore. This results in an increase in fluorescence. The fluorescent signal produced during each PCR cycle directly correlates to the amount of amplified DNA generated.

2.7.3 Molecular Beacons probe assay

Molecular Beacons probes belong to a special class of dual-labelled probes having self-complementary ends that form a stem-loop structure (hairpin) in their native state. It contains a reporter dye (fluorophore) at the 5'free end of the hairpin and a quencher at the 3' free end. In their native state, the reporter dye and quencher lie close to each other, preventing fluorescence. Once the reaction proceeds the oligonucleotide anneals to the developing PCR product sequence and the probe undergoes a spontaneous conformational change that results in separation of the fluorophore and quencher from each other, leading to the production of fluorescence. As PCR progresses, the number of amplicons (PCR products) increase and the number of probes that bind to the products also increase, hence an increase in fluorescence intensity.

2.7.4 Scorpions probes based assay

Scorpions probes based assay is a valuable tool for Rapid, Real-time qPCR and Gene Quantification. Scorpions probes are bi- functional molecules which incorporate two distinct structures: 1) a target-specific DNA probing sequence and 2) a target-specific PCR primer. These probes are based on a uni- molecular mechanism unlike TaqMan® probes and Molecular Beacons which are based on a bi-molecular probing mechanism and hence does not require enzymatic cleavage of the probe during PCR cycling. This structural arrangement provides a number of important advantages over other PCR probe formats:

• Simplified Assay Design

• Enhanced Sensitivity and Specificity

• Measurably Faster Reactions

Furthermore, they combine primer and probe in one molecule, with the primer at the 3' end and the probe contained within a hairpin-loop structure at the 5' end. The molecules also contain a fluorophore. Scorpions probes, unlike other probes, are designed in such a way that they emit light only when bound to their complementary target sequence during PCR amplification. In the quiescent state, the fluorophore and quencher lie next to each other, hence no fluorescence. As the reaction progresses, the scorpion primers is extended on the target DNA sequence. During the annealing phase, the scorpions molecule undergoes a conformational change, so that the probe containing the fluorophore bends over to anneal with the corresponding sequence on the target DNA. Since the oligonucleotide /quencher is now free, fluorescence is produced.

2.8 Western Blotting

2.8.1 Preparation of protein from cell lysates

To extract proteins, cell lines were grown as monolayers in 25cm2 or 80cm2 culture flasks until 90% confluent is achieved. The cells were extracted using a disposable cell scraper. The cells were then transferred, using a pipette to a universal container and centrifuged at 1600 rpm for 5 min. The supernatant was discarded and cell pellet resuspended in 200 µl of cell lysis buffer (10mM NA3VO4, 0.5% SDS, 0.5% Triton X-100m, 2mM CaCl2, 100µg/ml (1mM) phenylmethylsulfonyl fluoride, 1mg/ml (10U/ml)

aprotinin, 1mg/ml leupeptin and 0.1% IGEPAL CA-630) and the resultant lysates transferred into 1ml microcentrifuge tubes. The samples were then rotated on a rotating wheel for 40 min to ensure appropriate lysis and extract protein from the cell lysate. The tubes were centrifuged (Biofuge 13 Heraeus, Sepatech) at 13,000 rpm for 10 minutes to remove cellular debris and collect the protein. The supernatant was transferred to a clean Appendoff tubes and stored at -20ºC until ready to use for SDS-PAGE.

The protein concentrations were adjusted to a working range of 1-2mg/ml by the addition of sample buffer (10% glycerol; 5% 2-mercaptoethanol; 3% SDS; 80mM Tris-HCl (pH 6.8); 0.012% bromophenol blue) for crude cell lysate analysis. The crude cell lysate samples were then denatured under reducing conditions by boiling at 100°C for 5 minutes. The samples were, stored at -20°C

2.8.2 Determination of cellular protein concentration

Equal amounts of protein from each sample were used to determine the total cellular protein concentration and to detect the differences in protein levels in cells following various treatments.

The method employed to measure cell lysate protein concentrations was modified from the method described by Bohlen (Bohlen *et al.,* 1973) using the reagent 4-phenylspiro [furan-2(3H), 1'-phthalan]-3, 3'- dione (fluorescamine). Fluorescamine reacts with protein amino groups to yield a highly fluorescent product at an alkaline pH and this product can be detected using a fluorescent plate reader as described below:

40µl of protein from each lysate sample and 40µl of bovine serum albumin [100mg/ml, diluted in a cell lysis buffer to give a working concentration range between 0.50-

0.79mg/ml] was mixed in a 96 well microtitre plate. 60µl of sodium phosphate buffer was then added to each well and the pH adjusted to 8.8 using 1M Sodium Hydroxide. In addition, 25µl of fluorescamine (Sigma) solution (working concentration of 0.3mg/ml achieved by dissolving in acetone) was added to each well. The plate was then placed into a fluorescence plate reader (Denly, UK) and the fluorescence measured at an excitation wavelength of 540nm and emission wavelength of 590nm. A standard protein curve was then constructed from the bovine serum albumin and the protein concentrations from the cell samples were determined accordingly.

2.8.3 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

In this method equal volumes of samples and the protein standard (Ladder) were loaded into the wells of the polyacrylamide gel. A high voltage current (Bio Rad power pac 300) was then applied (30mA at 180V) which causes the protein samples to migrate towards positively charged anode. The SDS binds to protein and the resultant protein –SDS complex is then separated on the basis of their molecular mass. The protein size was then determined by comparing the gel mobility of a particular band with that of the ladder. Following electrophoresis bands were then visualised using Coomassie Blue or the proteins are electroblotted onto the nitrocellulose membrane.

2.8.4 SDS- PAGE Gel Preparation

SDS- PAGE was carried out by assembling the gel (Mini Protean 11 gel system) apparatus according to the manufacturer's instructions (Bio- Rad Laboratories, Richmond Ca, USA). Polyacrylamide gel is prepared by polymerising acrylamide with a cross-linking agent (methylene- bis- acrylamide) in the presence of a catalyst (TEMED

N,N,N,N-tetramehylethylenediamine) and the initiator (ammonium persulphate). The pore size is determined by the relative proportion of the acrylamide to the cross-linking agent and the rate at which the gel set is controlled by varying the concentrations of persulphate and TEMED within the acrylamide mixture. The ingredients used in preparing 8% or 10% resolving and stacking gel are shown below.

A brief description of the procedure is shown below: The resolving gel prepared was transferred to the gel system until the gel is 0.5 cm from the lower end of the gel plate. Following this 0.1% SDS solution was added to form a layer on the top of the gel solution. This is to ensure that the gel set with the smooth surface. The gel was then allowed to set at the room temperature for about 40 minutes. Once the gel is set the SDS solution is removed and the gel washed with distilled water. The stacking gel was then added to the gel system till it reaches the top end of the gel plate. A well forming comb was then inserted between the glass plates till it reaches the top of the resolving gel and allowed to set at room temperature. The well forming comb was removed and the gel cassette assembled in the electrophoresis tank as per manufacturers guide (Bio Rad Laboratories, USA). The tank was then filled with the running buffer. The protein samples (mixed with the loading buffer) and the standard protein were loaded into the wells. The tank was then connected to the power pack at a constant current of 15mA Electrophoresis was then continued till the bromophenol blue in the loading buffer reach the bottom of the gel. Following this the gel was removed and washed in the transfer buffer to remove the electrophoresis buffer, detergents and salts.

Preparation of SDS-PAGE Gel

Resolving gel composition (10mls)	Strengths (mls)	Strengths (mls)
	10%	8%
Distilled Water	4.0	4.6
30%Acrylamide/ 0.8% Bisacrylamide	3.3	2.7
1.5M Tris (pH = 8.8)	2.5	2.5
10% SDS	0.1	0.1
10% APS	0.1	0.1
TEMED	0.004	0.006

Stacking gel composition (2mls)	Strengths (mls)
	5%
Distilled Water	1.4
30%Acrylamide/ 0.8% Bisacrylamide	0.33
1.0M Tris (pH = 6.8)	0.25
10% SDS	0.02
10%APS	0.02
TEMED	0.002

2.8.5 Electroblotting

After gel electrophoresis proteins were transferred to nitrocellulose membrane where the proteins are detected by specific monoclonal or polyclonal antibody against them. A nitrocellulose membrane (Amersham International Plc) and filter paper (Whatman, Kent, UK) were cut to the dimension of the gel (6 x 9cm) and immersed in transfer buffer for 10-15 minutes. Two wet filter paper were placed over the cathode and nitrocellulose membrane along with the gel was placed on top of this which was then covered with two further pieces of pre soaked filter paper forming a sandwich. The surface of this sandwich was flattened out to avoid the formation of any air bubbles that might interfere with protein transfer. The positive electrode (anode) was placed on top of this and a constant current 5 Volts, 500mA and 8 Watts was applied for 40 minutes to facilitate protein transfer from gel to the nitrocellulose membrane.

Following this, the nitrocellulose membrane was removed and immersed in ponceau S solution for one minute at room temperature. Ponceau S stains proteins on nitrocellulose membranes and excess staining was removed by rinsing the membranes in distilled water until the bands were visible. The position of the molecular weight markers were then indicated on the membranes using a pencil. Following Ponceau S staining the nitrocellulose membranes were transferred to 50ml polypropylene tubes and incubated in 15ml of blocking buffer (10% milk solutions in TBS) for 40 – 60 minutes to block non-specific protein binding of the antibody. The blocking buffer was then removed and 5ml of 3 % milk solution in TBS and 0.1% Tween 20 containing the relevant primary antibodies in the appropriate concentrations was added. This was then incubated with agitation for 60 minutes at room temperature which facilitates an even distribution of

antibody on the membrane. Excess primary antibody was removed by washing the membranes three times, for 10 minutes per wash, using 10ml of wash buffer. The membranes were then incubated with 5ml of secondary horseradish peroxidase - conjugated antibody (prepared in a similar manner to that of the primary antibodies) at room temperature on a rotating wheel for 60 minutes. Unbound secondary antibody was removed by washing the membranes in the similar manner described for the primary antibody. The membranes were then washed twice in 10ml of 0.2% TTBS buffer (Tween 20 Tris Buffered Solution) for 15 minutes each. Finally the membranes were washed with 10ml of TSB buffer only to remove any residual detergent and transferred to weighing boats until ready for chemiluminescent detection.

2.8.6 Detection of protein using chemiluminescence

In this Horse Radish Peroxidase (HRP), in the presence of hydrogen peroxide (H_2O_2) oxidises the chemiluminescent substrate luminal, with concomitant production of light which is detected by exposing the nitrocellulose membrane to a photographic film. The intensity of light emission is increased 1000 fold in the presence of phenol (chemical enhancer). 8ml of KPL chemiluminescent reagent A and 8ml of KPL chemiluminescent reagent B were mixed together in a universal container and poured into a weighing boat. The nitrocellulose membrane was then placed over the tissue paper to drain the excess buffer. Following this the membrane was immersed in the chemiluminescent solution for 60 seconds and excess solution removed as before by using tissue paper. The chemiluminescent treated membrane was then wrapped in Saranwrap™ and placed in an autoradiography film cassette. In the dark room, using safe lighting condition a piece of autoradiography film (Kodak XOMAT-AR) was placed over the nitrocellulose membrane

and the cassette closed. The membrane was then exposed to X-ray film for 60 seconds for the best possible resolution of the antibody signal and the film developed using an automated film developer (X-ograph Imaging Systems, Malmesbury, Wiltshire, UK). After developing the film, the membrane was removed from the cassette and stained in Amido black stain for 15 seconds. The Amido black stain provides a permanent record of the protein positions on the membrane. The membranes were then placed in an Amido black de-stain solution until the protein bands are seen on the membrane.

2.9 Immunohistochemical staining

Immunohistochemical staining was carried out using frozen sections of breast tumour and normal mammary tissue cut at a thickness of 6µm using a cryostat. The fixed tissue sections were mounted on Super Frost Plus microscopic slides and were treated in 50% methanol and 50% acetone for 15 minutes. The sections were then air dried for 10 minutes and stored at -20ºC (wrapped in foil) for further use or immediately stained. The air dried sections were placed in PBS (Optimax wash buffer) for 5 minute to rehydrate. The sections were then treated with the primary antibodies at specific dilution and incubated for 1 hour. The slides were subjected to 4 washes with PBS and then treated in universal multi- link biotinylated secondary antibody at a specific concentration. The slides were incubated for 30 minutes followed by 4 washes with PBS and then placed in avidin biotin complex (ABC- Vector Labs). Diaaminobenzidine tetrahydrochloride (3,3'-diaminobenzidine) – DAB (Sigma) was added for 5 minutes to detect the bound antibody [DAB (3,3´-diaminobenzidine) is a substrate for peroxidase and in the presence of H_2O_2 brown coloured precipitate is formed indicating the presence of a specific protein when used in conjunction with specific antibodies].The slides were washed with water for 5

minutes and treated in Mayer's haematoxyline for 1 minute followed by further wash with water for 10 minutes (nuclei become blue). The slides were then treated with methanol (3 times) and clearing in 2 changes xyline before mounting under a cover slip. Negative controls (an irrelevant antibody instead of the primary antibody or PBS buffer) were used in this study. The concentrations of antibodies were different to each staining, details of which is explained separately in the relevant chapters.

2.10 Knockdown of gene transcripts using Hammerhead Ribozyme Transgenes

The hammerhead ribozyme transgenes was discovered by Forster et al in 1987 as a self cleaving domain in the RNA genome of different plant viroids (Forster and Symons *et al.,* 1987). It was then demonstrated that the Ribozyme transgenes can act as a catalyst for cleaving a variety of RNA targets by incorporating into short synthetic oligonucleotides (Haseloff and Gerlach et al., 1988). The ribozyme transgenes possess a typical secondary structure consisting of three helical stems with a catalytic core junction comprising of several invariant nucleotides (Figure 2.2).

To knockdown a specific gene using a hammerhead ribozyme transgene, we designed primers according to secondary structure of the gene predicted by using Zuker's RNA mFold software (Zuker et al., 2003). A suitable ribozyme target site was selected from within the secondary structure of the specific gene and the ribozyme designed to specifically bind and cleave the sequence surrounding this target site of the mRNA transcript using a touchdown PCR technique. The touchdown PCR conditions are shown below:

- Step 1: Initial denaturating period – 94ºC for 5 minutes
- Step 2: Denaturating step- 94ºC for 10 seconds

- Step 3: Annealing steps- 70ºC for 15 seconds, 65ºC for 15 seconds, 60ºC for 15 seconds, 57ºC for 15 seconds, 54ºC for 15 seconds and 50ºC for 15 seconds
- Step 4: Extension step- 72ºC for 20 seconds
- Step 5: Final extension period of 72ºC for 7 minutes

Step 2-4 were repeated over 48 cycles, comprising of 8 cycles for each different annealing temperature. Following this the ribozyme transgenes were inserted into the pEF6 plasmid in the TOPO cloning reaction as described later. After 4 weeks selection with a specific antibiotic, a stable cell line containing transgene was produced and verified using RT-PCR and Western Blotting. This technique is used extensively and reported in our laboratory previously (Jiang *et al.,* 2001, 2003b). Using this technique a hammerhead ribozyme transgene was constructed for the CREB 1 which is explained in detail in the respective chapter.

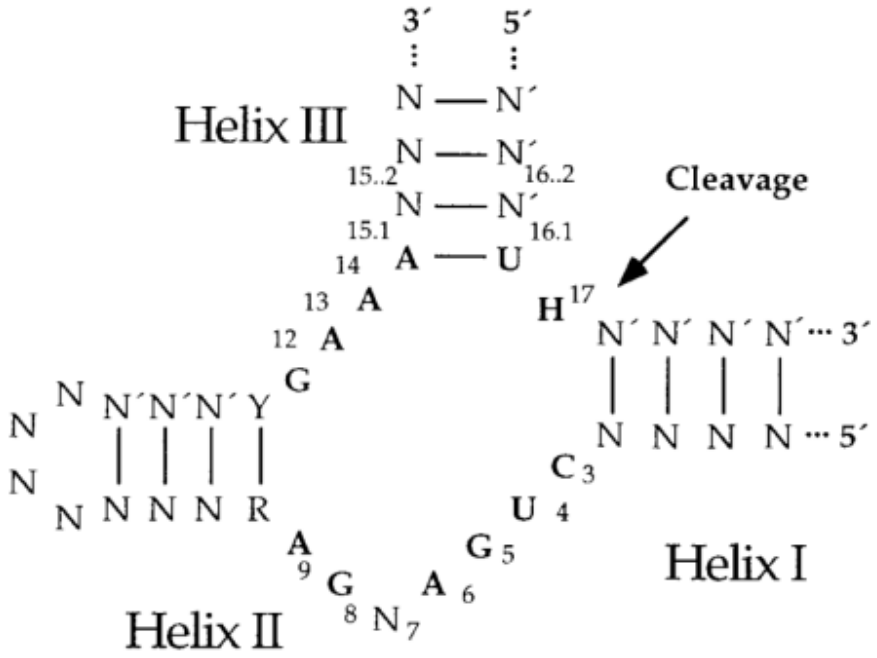


Figure 2.2: Two-dimensional structure of the hammerhead ribozyme

N, any nucleotide; N′, nucleotide complementary to N; H, any nucleotide but G; Y, pyrimidine nucleotide; R, purine nucleotide complementary to Y
(Adapted from Narendra K. Vaish et al., 1998)

2.11 Cloning and expression of PCR products

Cloning is defined as the method by which PCR products are inserted into plasmid vectors for constitutive or induced expression in mammalian cell line. It involves the ligation of the PCR insert into the reading frame of the plasmid vector and requires the proper vector insert molar ratio (1:1-1:3) depending on vector. This technique enables a large number of copies of the PCR products to be synthesised.

The design of primers is crucial for correct expression of PCR products in a vector. The PCR products should reveal a single discrete band to be used directly in the cloning reaction. Alternatively, if multiple bands are produced, the PCR reaction may require modification to optimise conditions, before subjecting to the cloning reaction. Through out this study cloning was performed using TOPO TA Cloning® kit (Invitrogen, Paisley, UK) in accordance with the manufacturer's instructions.

2.11.1 TOPO TA Cloning

TOPO TA Cloning® TA Cloning System (TOPO Cloning) is extensively used and provides an extremely efficient one step cloning technique for the direct insertion of Taq polymerase- amplified PCR products into a plasmid vector. TOPO Cloning ligates the PCR product into the pEF6/V5-His-TOPO® plasmid vector with topoisomerase I. The vector has been engineered to be a linearized plasmid with 3' deoxythymidine (T) overhangs that is activated by being covalently bonded to topoisomerase I (Activated TOPO cloning vector). The 3'A overhangs of the PCR product complement the 3'T overhangs of the vector and allow for fast ligation with the topoisomerase I. Once the PCR product is cloned into pEF6/V5-His-TOPO and transformants analyzed for the

correct orientation, the plasmid may be transfected into the mammalian cell line of choice.

2.11.2 TOPO® Cloning Reaction

The table below showed the ingredients required to set up TOPO® Cloning reaction to produce the recombinant vector for eventual transformation into chemically competent TOP10 One Shot® E. coli (Figure 2.3).

Reagent	**Volume** (6 µl)
Fresh PCR product	2 µl
Salt Solution	1 µl
Water	2 µl
TOPO® vector	1 µl

The red colour of the TOPO® vector solution is used to visualize the solution. Inclusion of salt (final concentration of 200 mM NaCl, 10 mM MgCl2) allows for longer incubation times as it prevents topoisomerase I from rebinding and potentially nicking the DNA after ligating the PCR product and dissociating from the DNA. This results in more stable molecules leading to higher transformation efficiency.

The solution was mixed gently and incubated for 5 minutes at room temperature (22°C - 23°C). Depending on the size of the PCR products the length of the TOPO® Cloning reaction can be varied from 30 seconds to 30 minutes. During incubation the PCR product ligates with the free end of the linear vector to form a circular plasmid. The reaction was then placed on ice and immediately proceeds to Transforming Competent Cells to avoid decrease in transformation efficiency.

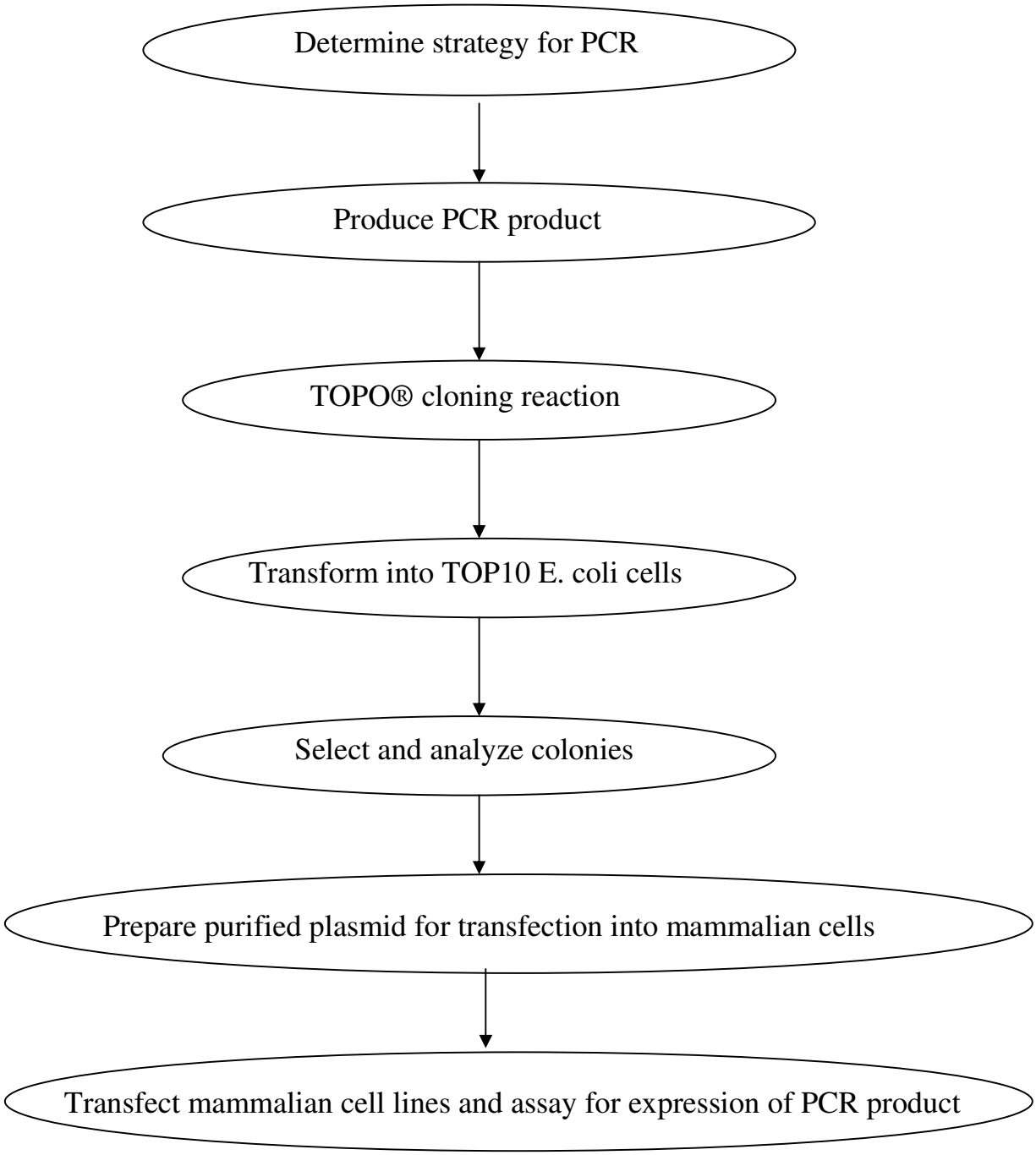


Figure 2.3: Flow Chart Showing the TOPO TA Cloning Method
(Invitrogen web catalogue)

2.11.3 Transformation into Competent Cells (E. coli)

After the TOPO® cloning reaction, we then transform the TOPO® construct into the **One Shot® TOP10** Chemically Competent *E. coli*.

Transforming One Shot® TOP10 Competent Cells (chemical transformation)

Materials required for chemical transformation is outlined below

- TOPO® Cloning reaction solution
- S.O.C. medium
- LB plates containing 50 µg/ml ampicillin
- 40 mg/ml X-gal in dimethylformamide (DMF)
- Water bath
- 37°C shaking and non-shaking incubator

For each transformation, we used one vial of competent cells and two LB plates.

- Equilibrate a water bath to 42°C
- Warm the vial of S.O.C. medium to room temperature.
- Warm LB plates containing 50-100 µg/ml ampicillin at 37°C for 30 minutes
- Spread 40 µl of 40 mg/ml X-gal on each LB plate and incubate at 37°C until ready for use.
- Thaw on ice 1 vial of One Shot® cells for each transformation.

2 µl of the TOPO® Cloning reaction solution was gently mixed into a vial of One Shot® Chemically Competent E. coli and incubated on ice for 30 minutes. The cells were then heat-shocked for 30 seconds in a 42°C water bath and the vial immediately transferred back on ice. Following this 250 µl of pre-warmed S.O.C. medium to room temperature was added to the vial and incubated at 37°C for 1 hour on an orbital shaker (200rpm).

After this 50 µl of transformed solution was then spread on a pre-warmed LB agar selective plate containing 50µg/ml ampicillin and incubated overnight at 37°C.

2.12 LB (Luria-Bertani) medium and agar plates (1 litre)

LB medium was prepared by dissolving 10g tryptone, 5g yeast extract, 10g NaCI in 950 ml deionized water. We then adjusted the pH to 7.0 with NaOH, and the solution was made up to 1 litre. 15g of agar was then added to the solution and autoclaved. The solution was allowed to cool, during which time the desired antibiotic (Ampicillin 50µg/ ml) was added. The solution was poured into Petri dish plates (Bibby Sterlin Ltd., Staffd, UK) and allowed to set before being stored inverted at 4°C in a refrigerator. Ampicillin was added to the solution to select out the positive colonies containing the plasmid with the PCR sequence. The pEF6/V5-His-TOPO® plasmid contains an ampicillin resistance sequence which confers resistance to E. coli containing this plasmid to the ampicillin. Normally E. coli are sensitive to ampicillin and hence do not grow in the LB medium. 50µl of the transformed solution was then evenly spread out in the LB agar plate and incubated overnight at 37ºC.

2.13 Selection and Analysis of positive colonies

Following overnight incubation, the LB agar plates were inspected for bacterial colonies containing the plasmid vector. We than analysed the colonies to identify the correct insertion and orientation of the target sequence in the plasmid to ensure that the resulting product is viable prior to amplification. 10-15 colonies were randomly selected and the plates marked and numbered over the selected positive colonies. After this we screened these bacterial colonies for the plasmid vector incorporating the PCR insert using RT-

PCR. This is achieved through the use of the forward primer for the plasmid and the reverse primer for the insert. This ensures that the amplified products are that of the insert and the plasmid in the correct orientation prior to amplification.

2.14 Amplification, extraction and purification of plasmid DNA

Individual colonies producing the correct sized PCR product were identified and picked from the LB agar plate. This was then inoculated into 20ml of LB medium containing 100µg/ml ampicillin in a polypropylene tube and incubated overnight at 37°C in a rotatory shaker (220rpm). Following this the resultant culture was added into 50ml of LB medium (with the appropriate antibiotic) and transferred into the conical flask. This was then incubated overnight at 37°C in a rotatory shaker. After this the solution was transferred to the universal container and centrifuged at 3000g for 15 minutes at 4ºC. The supernatant was discarded and the remaining pellet containing the E. coli were used for plasmid extraction.

Plasmid was extracted and purified using the QIAGEN Mini Purification Kit (Qiagen, Crawley, UK) in accordance with the manufacturer's protocol.

The pellet was resuspended in 0.3ml of resuspension buffer which contains RNase inhibitors that inactivates any RNA contaminants. 0.3ml of cell lysis buffer was then added to the solution and mixed gently to avoid shearing of genomic DNA. The solution was incubated at room temperature for 5 minutes. 0.3ml of neutralization buffer was added to the solution and then incubated on ice for 5 minutes. The mixture was centrifuged at 13000rpm for 10minutes at 4ºC and the supernatant transferred to the pre-equilibrated QIAGEN- tip (filter cartridge). This was then allowed to enter the resin by gravity flow and the plasmid DNA blinds to the ion-exchange resin within the filter

cartridge. The QIAGEN – tip was then washed 3 times with 1ml of medium salt buffer (QC wash buffer) to remove contaminants like RNA, proteins, dyes, and low molecular weight impurities from the plasmid preparation. The plasmid DNA was eluted from the QIAGEN –tip resin using 0.8ml of high salt buffer (QF buffer), and was collected in a 2ml microfuge tube. The Plasmid DNA was then precipitated with 0.56ml of isopropanol and immediately centrifuged at 10000 rpm for 15 minutes. The pellet was then washed 2 times with 1ml of 70% ethanol, centrifuged and air dried for 5 minutes. After this the pellet was resuspended in DEPC water. The yield of DNA was then determined through DNA quantisation using UV spectrophotometry at a wavelength of A_{260nm} and Gel electrophoresis performed using small amount of plasmid DNA to check both plasmid purity and size. The plasmid solution was stored at -20ºC.

2.15 Electroporation (Transfection) of mammalian cells

The purified plasmid DNA obtained as described above was incorporated into cultured mammalian cells utilising electroporation. The cells were harvested by trypsinisation of 80-90% confluent cell culture as described above. The cell pellet obtained following centrifugation was mixed in 1 ml of complete medium and then placed on ice. 500µl of the cell suspension was transferred into electroporation cuvette and stored on ice. This technique was performed using the Easy jet Plus system (Flowgen, Staffordshire, UK). The parameters were set at 310 volts (to destabilise the cell wall integrity and hence allowing the plasmid DNA to cross the cell membrane to be integrated into the native cellular DNA) and a capacitance value of 1050µF. 10 µg of plasmid DNA was added to cell suspension in the electroporation cuvette, mixed gently and kept at room temperature for 2-5 minutes. The cuvette was then loaded into the electroporator and a current of 310

volts was passed through the cuvette. The electroporated cells were immediately transferred into culture flask containing 5ml of pre-warmed culture medium and incubated at 37ºC with a humidified atmosphere of 5% CO_2.

Following this, isolation of a stable cell line expressing the gene of interest was achieved by harvesting cells in the culture medium containing 100µg/ml of G418. The electroporated cells were then allowed to grow till the cells are semi-confluent. The selective culture medium was changed every 2-3 days for 3-4 weeks to remove the dead cells and to allow colonies of resistant cells containing the plasmid and the inserted DNA to grow until sufficient numbers of cells were available for future experiments. Cells were routinely tested for the presence of the plasmid and insert, using RT-PCR.

2.16 *In vitro* Functional Assays

2.16.1 *In vitro* cell growth assay

This is a simple assay for obtaining quantitative information of relative density of cells adhering to multi-well cluster dishes. The dye Crystal violet used in this assay stains DNA. Upon solubalization, the amount of dye taken up by the monolayer can be quantified in a spectrophotometer. The method is described below which has been previously reported by Jiang et al., 2005.

Cells were detached from the culture flask and cell density per ml determined. Cells were then seeded into a 96 well plate equally (2500cells/well in 200l of normal medium) (Nunclon, Fisher Scientific, UK). Three sets of plates were incubated for 1, 3 and 5 days. Following this the medium was removed on the day of plating and the cells were fixed in 10% formaldehyde for 30 minutes. This was followed by staining in 0.5% (w/v) crystal violet for 5 minutes. The plates were then washed and stain extracted with 10% acetic

acid. The cell density was then determined by measuring the absorbance at a wavelength of 540nm by using a plate reading spectrophotometer (EL*800, Bio-Tek, Wolf Laboratories, UK). Cell Growth was determined as percentage increase and calculated by comparing the colorimetric absorbance obtained for each incubation period as follows:

Percentage increase = (Day 3 or Day 5 absorbance) - Day 1absorbance/ Day 1 absorbance.

2.16.2 *In vitro* invasion assay

The invasiveness of the breast cancer cells used in this study was determined using an *In vitro* Matrigel invasion assay model. This method was previously reported by Albini et al., 1987 and modified in our Laboratory (Jiang et al., 1995). This assay measures the cells ability to invade through an artificial basement membrane. The technique works upon the principle that a culture plate is equipped with an insert which has one end sealed with a polycarbonate membrane. The rationale behind using the polycarbonate membrane is that this membrane has a pore size of 8µm in diameter which allows cells to migrate through it. The surface of this membrane is coated with an extracellular matrix protein solution, matrigel (rich in basement membrane) (Becton-Dickinson Biosciences, Oxford, UK) to form a thin layer of gel matrix. Since this technique determines the capacity of tumour cells to penetrate through the gel matrix and porous membrane, it presents an indication of invasive capacity. The technique used during this study is described below. Twenty four well pre-chilled Cell culture inserts (Becton-Dickinson Biosciences, Oxford, UK) containing 8µm pores were coated in 50µg of Matrigel. To prevent irreversible gelling of Metrigel the following procedure was carried out at 4ºC. A stock solution and a working solution of Matrigel was prepared. 100µl (50µg) of working Matrigel solution was added to these cell culture inserts to form a thin even coating of Matrigel. This was

then allowed to set and incubated at 45ºC in a drying oven. The inserts were then placed into twenty four well plates and the Matrigel layers were rehydrated by incubating at room temperature for 60 minutes using 300µl of sterile water. Following rehydration, the water was aspirated from each insert. Cell suspensions (15000 cells in 200µl of normal medium) were then added to each insert and incubated for upto 72 hours at 37ºC. Following this the Matrigel layer together with the non-invasive cells were removed from the inside of the insert using a cotton swab. The cells which had invaded into the Matrigel and migrated through the porous membrane to the underside of the insert were then fixed in 4% formaldehyde for 10 minutes at room temperature. Following this the cells were washed using distilled water and stained in 0.5 % crystal violet for 10 minutes at room temperature. Excess stain was removed by washing the cells with distilled water. The cells were then air dried and the numbers of invading cells (random fields) were counted using a light microscope.

2.17 Statistical analysis

Statistical analysis was performed using the Minitab 14 Statistical Software Package. The statistical comparisons were made using either a non-parametric Mann- Whitney test if the data was not normalised or a Students two tailed t- test if the data was found to be normalised and have equal variances. Graphs were constructed using Microsoft Excel Software Package.

CHAPTER 3

EXPRESSION OF CREB 1 IN HUMAN BREAST CANCER AND THE ASSOCIATION WITH CLINICOPATHOLOGICAL CHARACTERISTICS

3.1 Introduction

CREB belongs to a subfamily of the leucine zipper with basic domain (bZip) family of cellular transcription factors. CREB binds to the DNA sequence called the cyclic AMP response element (TGACGTCA) (Deutsch PJ et al., 1988)in the presence of cAMP to increase or decrease gene transcription in response to extracellular signals. CREB mediates cellular responses to various mitogens and stressors. Various stress signals, growth factors and kinases promote phosphorylation –mediated activation of the CREB. cAMP responsive elements (CRE1 & CRE2) are essential for cAMP induced promoter II activity (Sofi *et al.,* 2003). It requires phosphorylation by cAMP- dependent protein kinase at serine-119 and serine-133 (Johannessen *et al.,* 2004) for interaction with DNA and RNA polymerase II.

It has been previously shown that CREB may act as a positive or negative transcription regulator in various human benign and malignant conditions (Luciani *et al.,* 2003; Persani *et al.,* 2000; Shankar *et al.,* 2004; Perry *et al.,* 2004; Dronadula *et al.,* 2006; Melnikova *et al.,* 2008; Deng *et al.,* 2008 ; Catalano *et al.,* 2009; Makhov *et al.,* 2009; San-Marina *et al.,* 2008) CREB is also involved in the regulation of various biological and cellular functions including cell survival, spermatogenesis, glucose homeostasis, circadian rhythm, memory and neuronal plasticity (Barton *et al.,* 1996; Silva *et al.,* 1998; De Cesare *et al.,* 1999; Bonni *et al.,* 1999 ; Travnickova-Bendova *et al*., 2002). It has been reported that CREB may act as a positive transcription regulator of aromatase and hence increased expression and oestrogen synthesis in breast cancer cells (Sofi *et al.,* 2003).

However, despite the extensive work on aromatase, little information is available on the expression and role of CREB in human breast cancer. There is no available data about the association of CREB with tumour or patients clinicopathological characteristics in human

breast cancer. This study was carried out to investigate the expression pattern of CREB family of proteins in cohort of breast cancer and normal background tissue in relation to tumour histopathological grade, stage and nodal status. We also correlate the level of expression with the prognosis and clinical outcome of the patients.

3.2 Materials and Methods

RNA-extraction kit, RT (Reverse Transcriptase) kit and Mastermix for routine PCR and quantitative PCR were obtained from AbGene (Surrey, UK). PCR primers were designed using Beacon Designer (Palo Alto, CA, USA) and synthesized by Invitrogen (Paisley, Scotland, UK). Molecular-biology-grade agarose and DNA ladder were from Invitrogen.
A first strand cDNA synthesis kit was purchased from Sigma Chemical Ltd. Low fluorescent 96 plates were obtained from Abgene and sealing films were from BIO- Rad (Hemel Hempstead, England, UK).

3.2.1 Sample Collection

Breast cancer tissues (n=120) and background tissue samples (normal breast tissue) (n=33) were collected (with ethical approval from local committee) immediately after surgery and stored in liquid nitrogen before processing. Patients were routinely followed after surgery and the median follow up period was 120 months for this study. The histopathological details, tumour grading (modified Bloom and Richardson's grading system), tumour staging (TNM) and the prognostic index for the patients is shown in table 3.1.

Table 3.1 - Clinical Information for breast tumour tissues analysed (n=120)

Tissue Type	Histological Grade (G)	NPI and Node Status	TNM	Histology	Outcome
Backgound normal breast tissue n = 33					
Tumour n = 120	G1 n = 23 G2 n = 41 G3 n = 56	NPI 1 n = 66 NPI 2 n = 38 NPI 3 n = 16 Node +ve n = 55 Node -ve n = 65	TNM 1 n =69 TNM 2 n =40 TNM 3 n =7 TNM 4 n =4	Ductal n =88 Lobular n =14 Other n =18	Disease Free n =81 Metastasis n =7 Local recurrence n =5 Died of breast cancer n =20 Died of unrelated cause n =7

- Histological Grade (Modified Richardson Bloom Classification) : G1 – Well Differentiated, G2 – Moderately Differentiated and G3 – Poorly Differentiated
- NPI (Nottingham Prognostic Indicator): NPI = 0.2 x tumour size (cm) + lymph node stage + histological tumour grade (Galea MH et al., 1992). NPI 1 (Good prognosis) ≤ 3.4 NPI 2 (Moderate prognosis)- Between 3.4 to 5.4 and NPI 3 (Poor prognosis)- ≥ 5.4
- TNM: T- Tumour, N- Nodal and M- Metastasis

3.2.2 Tissue processing, RNA extraction and reverse transcription – polymerase chain reaction

The frozen sections of breast tissue specimen were cut using a cryostat at a thickness of 5–10 µm and stored at -20°C. Approximately 15–20 sections from each breast tissue sample were homogenized using a hand-held homogenizer in ice-cold RNA extraction buffer. The purity and the concentration of RNA were determined using agarose gel electrophoresis and UV spectrophotometer (Wolf Laboratories, York, UK). Reverse transcription was carried out from 1µg total RNA using a RT kit with an anchored oligo-dT primer according to manufacturer's instructions. The polymerase chain reaction (PCR) was performed with cDNA, master mix and the respective primers (Table 3.2) using the following reaction conditions: 5 min at 95°C, 20 seconds at 94°C, 25 seconds at 56°C and 50 seconds at 72°C for 36 cycles followed by final extension phase of 7 minutes at 72°C. ß-actin was used as a housekeeping gene and was amplified simultaneously using the primers as shown in the table. The PCR products were then separated on a 0.8% agarose gel, stained using ethidium bromide and visualized under UV lights.

3.2.3 Quantitative analysis of CREB 1 Transcripts

The transcript level of the CREB1 from the above-prepared cDNA was determined using a real-time quantitative PCR, based on the AmplifluorTM uniprimer technology (Intergen Company, Oxford, UK) modified from a previously reported method (Jiang et al., 2004). Specific pair of PCR primers were designed using the Beacon Designer software (version 2, Palo Alto, California, USA) and to one of the primers (routinely the antisense primer

in our laboratory), an additional sequence, known as the Z sequence (5'-actgaacctgaccgtaca-3'), which is complementary to the universal Z probe (IA Nazarenko et al., 1997) (Intergen, Oxford, UK) was added. Cytokeratin-19 (CK19) was used for comparison of cellularity during the analysis and primer for CK19 is shown in table 3.2. The reaction was carried out using the following: Hot-start Q-master mix (Abgene), 10 pmol of specific forward primer, 10 pmol of reverse primer which has the Z sequence, 100 pmol of 6-carboxyfluorescein (FAM)-tagged probe (Intergen), and cDNA from approximately 50ng RNA (calculated from the starting RNA in the reverse transcriptase reaction). The reaction was carried out using IcyclerIQ™ (BioRad) which is equipped with an optic unit that allows real-time detection of 96 reactions, using the following conditions: 94°C for 5 minutes followed by 50 cycles at 94°C for 10 seconds, 55°C for 15 seconds and 72°C for 20 seconds (Jiang *et al.,* 2003a). The levels of the transcripts were generated from an internal standard that was simultaneously amplified with the samples, and are shown here in two ways: levels of transcripts based on equal amounts of RNA, and as a target/CK19 ratio.

Table 3.2: Primers sequences used in the quantitative RT- PCR

Gene	Primer Name	Primer Sequence (5'-3')	Optimal Annealing Temperature	Product Size (bps) Accession no:
CREB 1	CREB 1 F1	ggggactatgaggagatgat	55°C	816 NM_005171
	CREB 1 ZR	actgaacctgaccgtacagtggaggtcttgatgtgaat		
ß-actin	ß-actin F	atgatatcgccgcgctcg	55°C	580 NM_001101 81-660
	ß-actinR	cgctcggtgaggatcttca		
	ß-actin ZF	ggacctgactgactacctca		117 NM_001101 622-720
	ß-actin ZR	actgaacctgaccgtacaagcttctccttaatgtcacg		
CK19	CK19 F	caggtccgaggttactgac		
	CK19 ZR	actgaacctgaccgtacacactttctgccagtgtgtcttc		

3.2.4 Immunohistochemical staining of the CREB-1 proteins

Immunohistochemical staining was carried out using frozen sections of breast tumour and normal mammary tissue cut at a thickness of 6μm using a cryostat. The sections were mounted on Super Frost Plus microscopic slides and air-dried for 20-30 minutes. The fixed tissue sections were treated in 50% methanol and 50% acetone for 15 minutes. The sections were then air dried for 10 minutes and stored at -20ºC (wrapped in foil) for further use or immediately stained. The air dried sections were placed in PBS (Optimax wash buffer) for 5 minute to rehydrate. The sections were treated with the primary antibodies (Santa Cruz Biotechnology, Santa Cruz, California, USA) at specific dilution and incubated for 1 hour. The slides were subjected to 4 washes with PBS and then treated in universal multi- link biotinylated secondary antibody (Sigma, Poole, Dorset, UK) at a specific concentration. The slides were incubated for 30 minutes followed by 4 washes with PBS and then placed in avidin biotin complex (ABC- Vector Labs). Diaminobenzidine tetrahydrochloride (3,3-diaminobenzidine) – DAB (Sigma) was added for 5 minutes to detect the bound antibody. [DAB (3,3´-diaminobenzidine) is a substrate for peroxidase and in the presence of H_2O_2 a brown coloured precipitate is formed indicating the presence of a specific protein when used in conjunction with specific antibodies].The slides were washed with water for 5 minutes and treated in Mayer's haematoxyline for 1 minute followed by further wash with water for 10 minutes (nuclei become blue). The slides were then treated with methanol (3 times) and clearing in 2 changes xyline before mounting under a cover slip. Negative controls (an irrelevant antibody instead of the primary antibody or PBS buffer) were used in this study. The

concentrations of antibodies were different to each staining. Staining of the respective proteins was quantified using Optimas 6.0 software as described below.

3.2.5 Quantification of CREB expression in breast cancer tissues

Quantification of expression of these proteins and immunohistochemical grading were carried out as described by Paleri et al. (Paleri *et al*., 2001). The staining was assessed on random pattern fields and grading performed on a four point scale. The grading was scored as 0 = negative, 1 = weakly positive, 2 = positive and 3 = strongly positive. This scoring was carried out by two independent observers and any disparity in the findings was resolved by re-evaluation of the slides.

Statistical analysis was carried out as described in section 2.17

3.3 Results

We screened three breast cancer lines (MDA-MB-231, MCF 7 and MDA- MB- 453) to assess the expression of CREB 1, using RT-PCR. The mRNA level of CREB 1 in these cell lines are illustrated in Figure 3.1. CREB 1 was expressed at a relatively higher level in MDA-MB- 231 and MCF 7 cell lines and was detectable in MDA- MB- 453 cell line. Human fibroblast cell line MRC5 and endothelial cell line HECV had very low levels of CREB1.

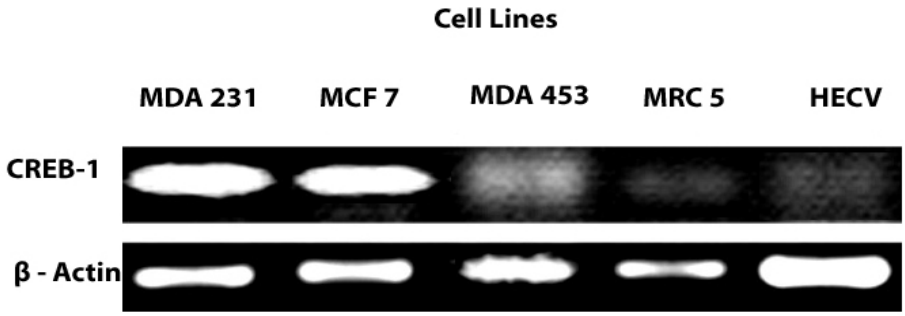


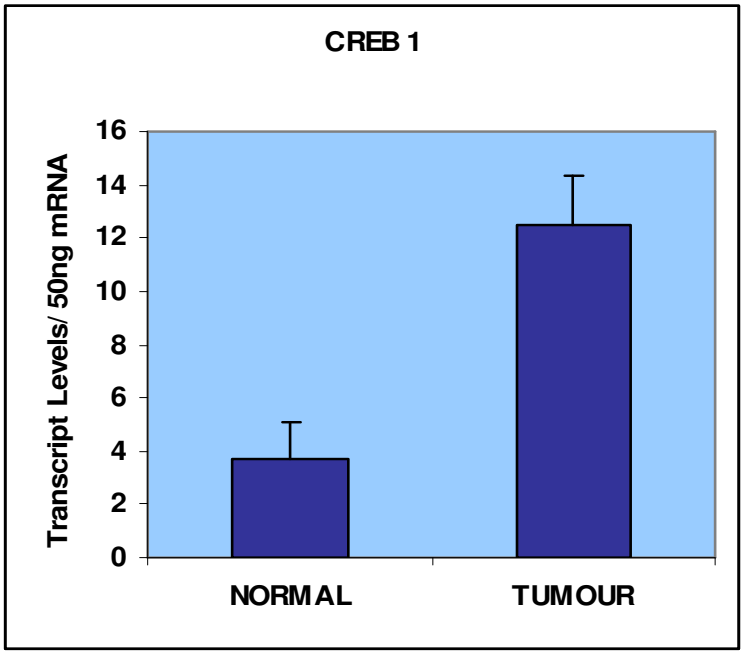
Figure 3.1: The expression of CREB 1 in breast cell lines, fibroblast and endothelial cell lines was assessed using RT- PCR.
40 cycles were performed for these PCR reactions. ß-actin was used a housekeeping control.

3.3.1 Expression of CREB 1 in normal mammary and breast cancer tissues

We analysed the expression pattern of CREB-1 in both normal mammary and tumour tissues using the real time quantitative RT-PCR. As shown in Figure 3.2 breast tumour tissues had higher level of CREB-1 as compared to normal breast tissues (p=0.0092) (Table 3.3).

Table 3.3: Means and standard deviation of the levels of expression of CREB 1 in normal and breast cancer tissues.

	CREB1		CREB 1: CK19 ratio	
Normal	3.7 ± 1.35		720 ± 232	
Cancer	12.5 ± 1.82	p=0.0092	519 ± 105	p=0.43



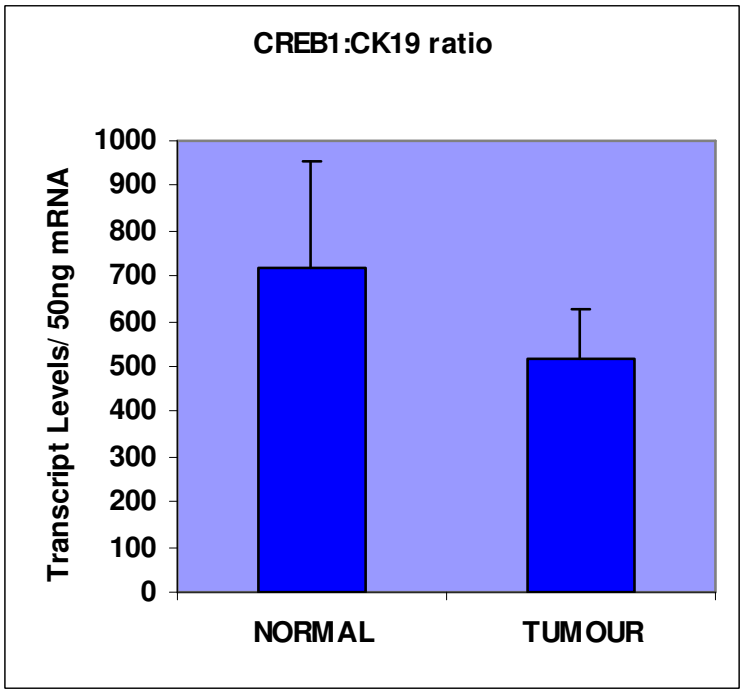


Figure3.2a: Quantitative real time PCR demonstrating high expression of CREB 1, in tumour samples as compared to normal mammary tissue.
Figure3.2b: Transcript normalised by CK19 (shown as CREB-1: CK19 ratio).

3.3.2 Expression of CREB 1 and its correlation with nodal involvement

As shown in Figure 3.3a, there was a significantly higher level of the CREB-1 transcript in node positive tumours as compared to node negative tumours (Table 3.4) The same trend was seen when CREB-1 transcript was normalized by CK19 (Figure 3.3b & Table 3.4).

Table 3.4: Means and standard deviation of the levels of expression of CREB 1 in relation to nodal status.

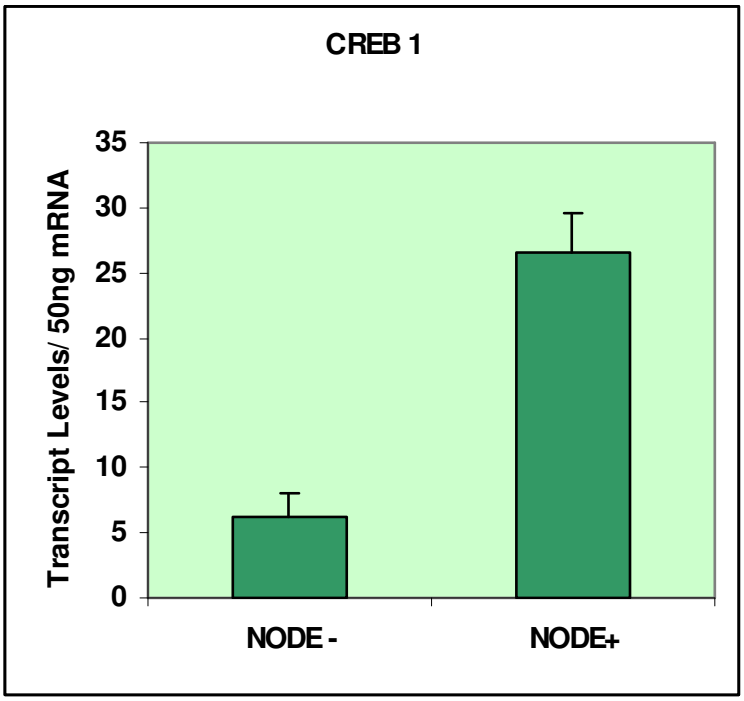
	CREB1		CREB1:CK19 ratio	
Node -ve	6.2 ± 1.8		238 ± 85	
Node +ve	26.5 ± 3	p=0.0018	680 ± 149	p=0.0004

3.3.3 Expression of CREB family proteins and its relation to tumour grade

As shown in Figure 3.4a, Grade-2 carcinoma (19.3 ± 3.6, p=0.075) and grade-3 (7.9 ± 2.5, p=0.1131) had higher level of CREB-1 expression as compared to grade-1 (3.4 ± 3.7) carcinoma but were not statistically significant. The same trend was seen with Grade 3 tumours when CREB-1 transcript was normalized by CK19 but interestingly Grade 1 carcinoma had higher level of CREB-1 expression as compared to grade-2 (Figure 3.4b & Table 3.5).

Table 3.5: Means and standard deviation of the levels of expression of CREB 1 in relation to tumour grade.

	CREB1		CREB1:CK19 ratio	
Grade 1	3.4 ± 3.7		448 ± 214	
Grade 2	19.3 ± 3.6	p=0.075	357 ± 112	p=0.73
Grade 3	7.9 ± 2.5	p=.1131	535 ± 135	p=0.76



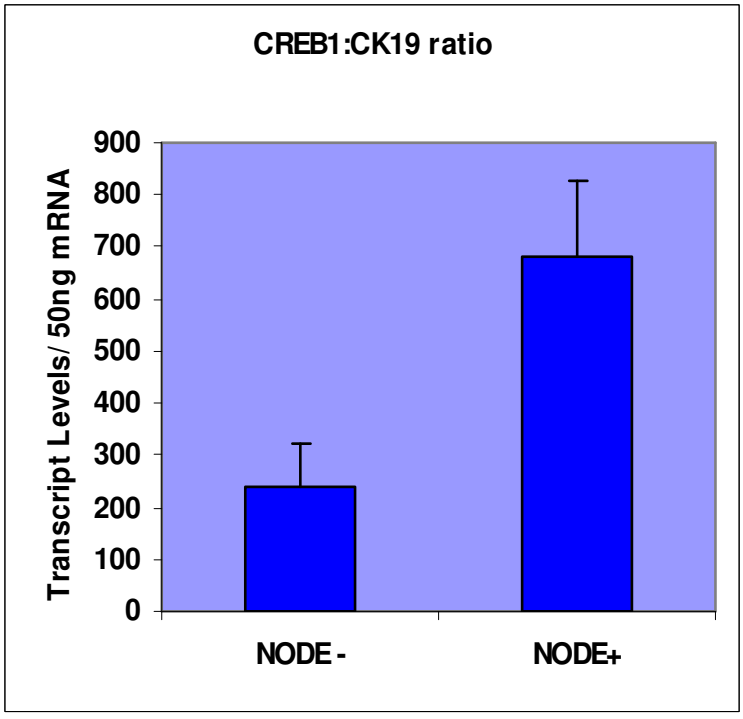
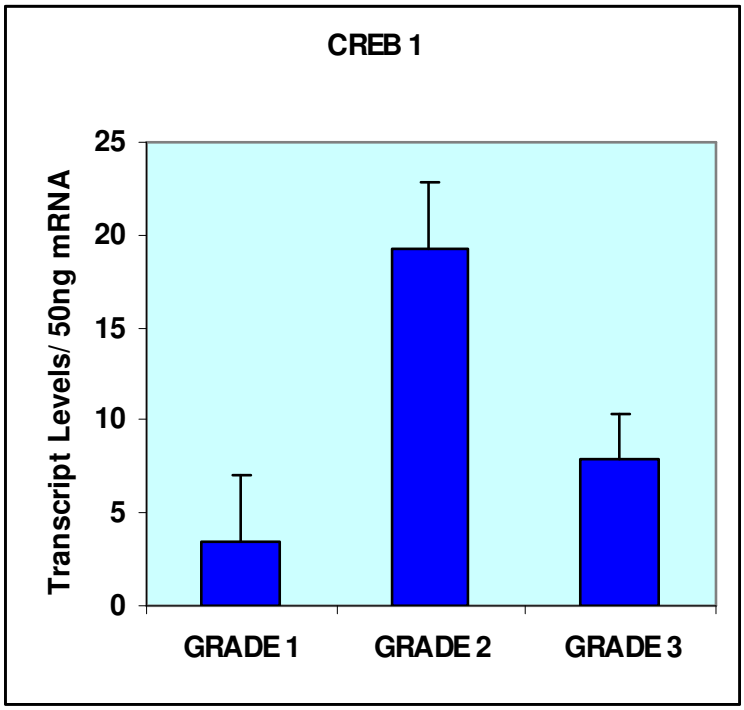


Figure3.3a: RT –PCR analysis of breast cancer tissue samples, showing higher transcript levels of CREB 1 in node positive tumours as compare to node negative tumours.
Figure3.3b: Transcript normalised by CK19 (shown as CREB-1: CK19 ratio).



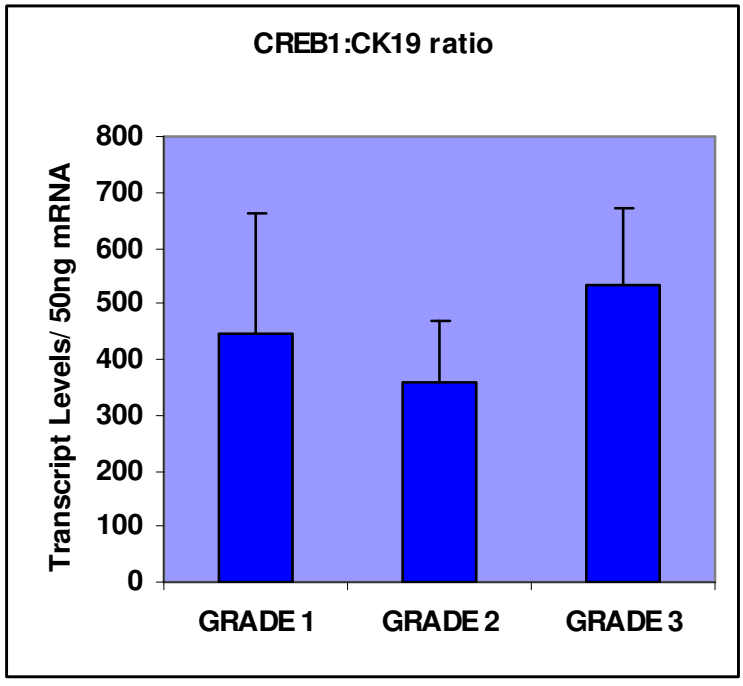


Figure3.4a: Levels of CREB1 in relationship with tumour grade.
Figure3.4b: Transcript normalised by CK19 (shown as CREB-1: CK19 ratio).

3.3.4 Tumours from patients with aggressive disease had higher levels of the CREB-1 transcript

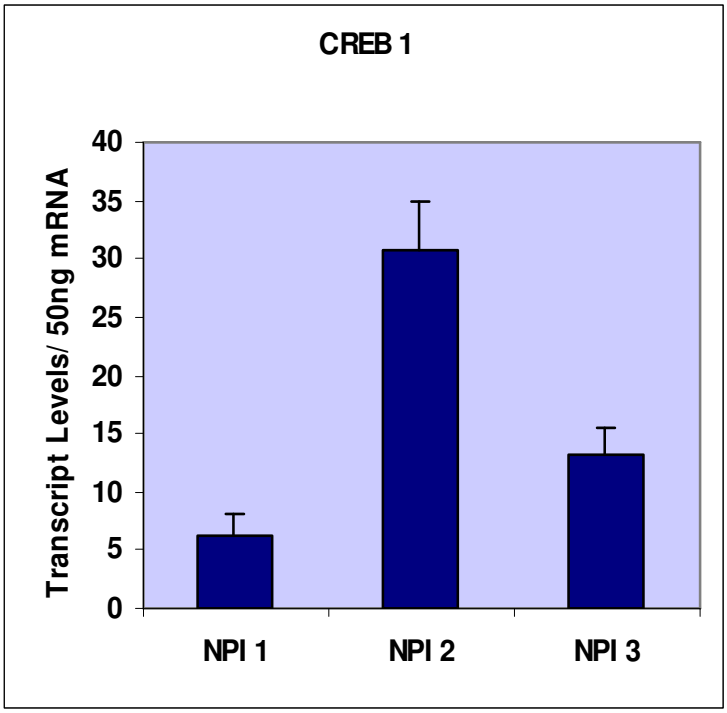
Nottingham prognostic index was used as a tool to determine the prognosis of the patients. NPI< 3.4 was regarded as having a good prognosis (NPI-1), 3.3-5.4 moderate (NPI-2) and >5.4 as poor prognosis (NPI-3). As shown in Figure 3.5a, there was significantly higher level of CREB-1 transcript in patients with moderate (30.8 ± 4.1, p=0.0036) and poor prognosis (13.2 ± 2.2, p=0.0061) as compared to patients with good prognosis (6.2 ± 1.8) (Table 3.6). The same trend was seen when CREB-1 transcript was normalized by CK19 (Figure 3.5b). Based on TNM staging the expression of CREB 1 is markedly raised in TNM 3 tumours but was statistically not significant (Table 3.7). Interestingly the expression of CREB 1in stage 4 tumour was decreased as compared to stage 1 tumour (Figure 3.6a) but when normalised by CK19 the expression of CREB 1 increases dramatically as compared to stage 1 & 2 tumours (Figure 3.6b).

Table 3.6: Means and standard deviation of the levels of expression of CREB 1 in relation to patient prognosis using the Nottingham Prognostic Index (NPI)

	CREB1		CREB 1: CK19 ratio	
NPI 1	6.2 ± 1.8		238 ± 85	
NPI 2	30.8 ± 4.1	p=0.0036	730 ± 190	p=0.0050
NPI 3	13.2 ± 2.2	p=0.0061	553 ± 215	p=0.0018

Table 3.7: Means and standard deviation of the levels of expression of CREB 1 in relation to tumour stage using the TNM classification.

	CREB1		CREB1:CK19 ratio	
TNM 1	15 ± 1.7		389 ± 108	
TNM 2	6.4 ± 4.1	p=0.79	490 ± 146	p=0.58
TNM 3	23.5 ± 4	p=0.0928	594 ± 441	p=0.76
TNM 4	7.9 ± 5.1	p=0.699	2040 ± 1659	p=0.39



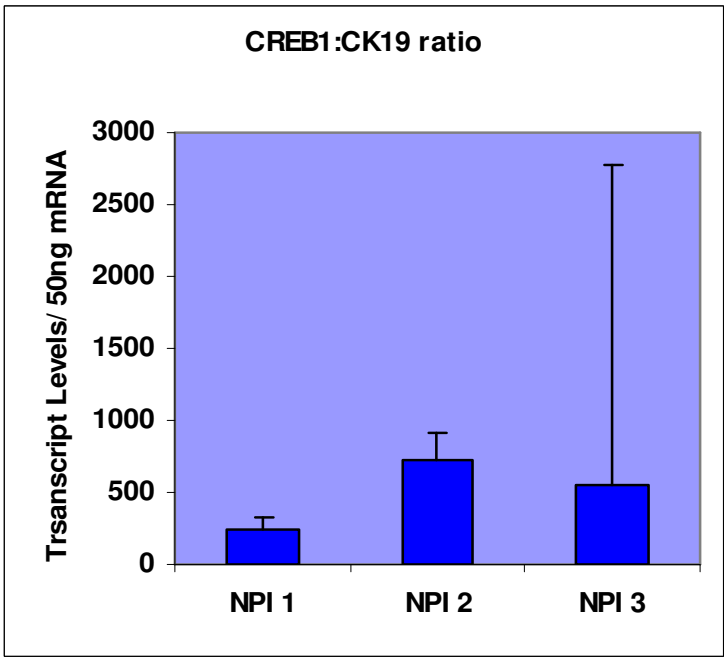
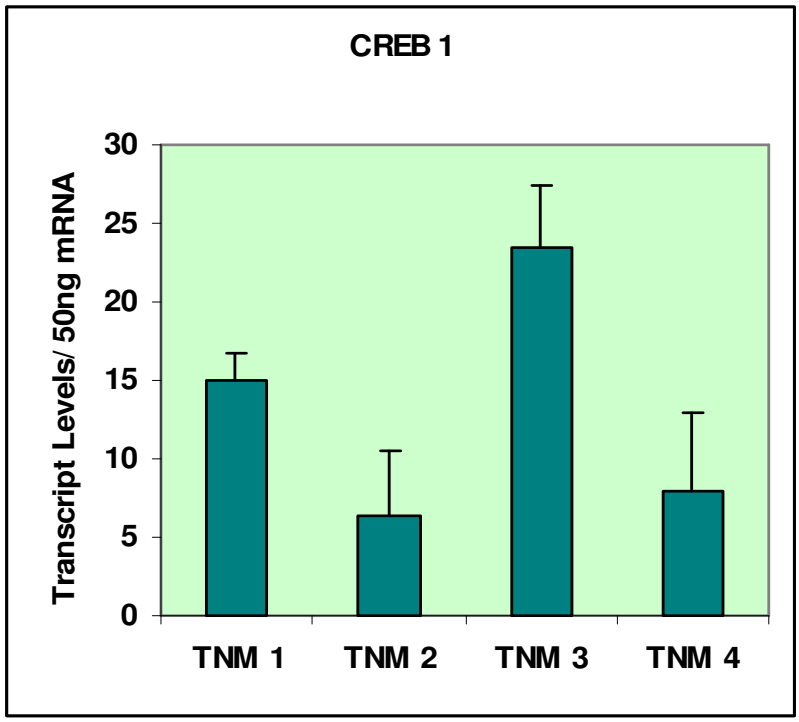


Figure3.5a: CREB-1 and predicted clinical outcome, using the Nottingham Prognostic Index (NPI) as an indicator
Figure3.5b: Transcript normalised by CK19 (shown as CREB-1: CK19 ratio).

(NPI-1 - good prognosis group, NPI-2 - moderate prognosis group and NPI-3 - poor prognosis group)



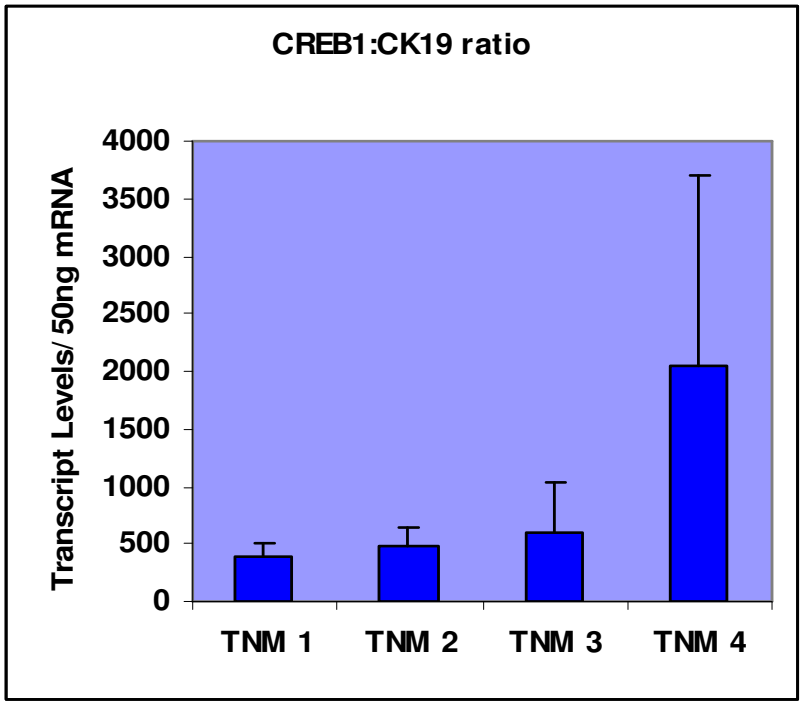


Figure3.6a: Levels of CREB1 in relationship with TNM Staging.
Figure3.6b: Transcript normalised by CK19 (shown as CREB-1: CK19 ratio).

3.3.5 High levels of the CREB family protein transcript was linked to both the overall and disease-free survival of the patients

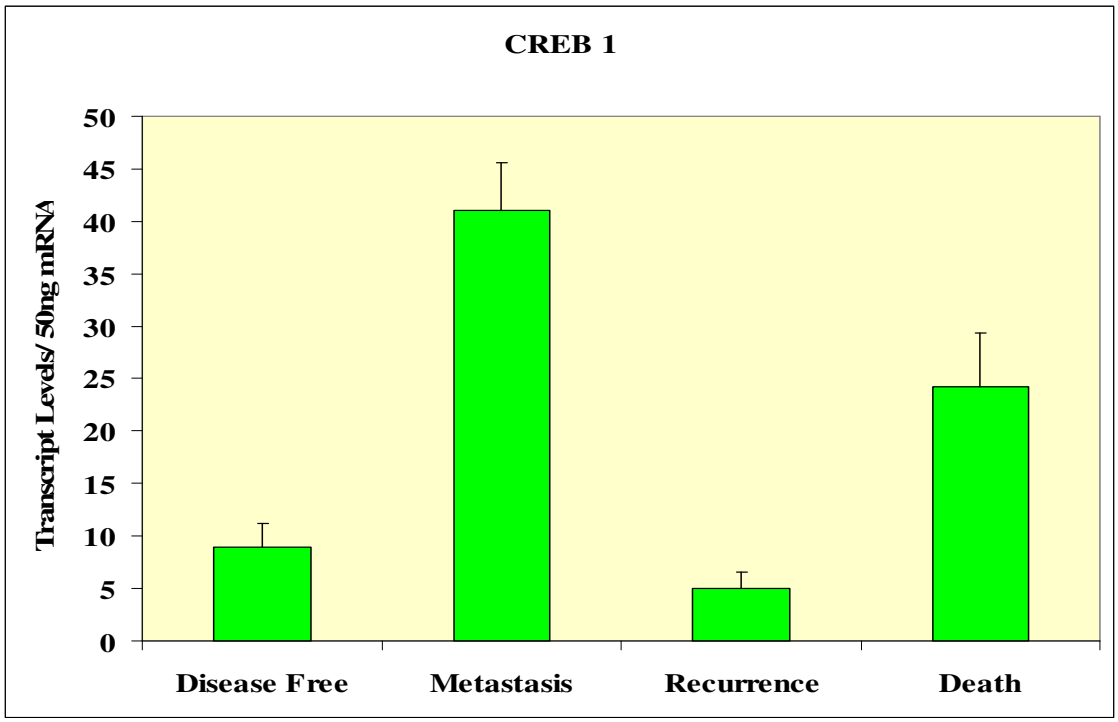
Following a median 120 months follow up, patients were divided into the following groups: those who remained disease free, who developed metastasis or local recurrence and those who died of breast cancer related disease (excluding non – cancer related deaths). As shown in figure 3.7a, CREB 1 level was high in patients who developed metastasis (41 ± 4.6) and who died of breast cancer (24.2 ± 5.2) related cause as compared to patients who remained disease free (8.9 ± 2.3) but were statistically not significant. Interestingly, when normalised by CK19 a significant difference was seen in tumours from patients who died of breast cancer (p=0.0003; Figure 3.7b & Table 3.8). We combined the three groups (with metastasis, recurrence and mortality), to form a poor prognostic group (referred to as poor prognosis in figure 3.8a) and compared this group with those who remained disease free. It was demonstrated that CREB 1 level was higher in patients with poor prognosis (26.5 ± 3) but was statistically not significant. When normalised with CK19 a highly significant difference was seen in patients with poor prognosis (p=0.0035). (Figure 3.8b & Table 3.9) We have divided patients into groups with high levels of CREB 1, by using the NPI as a general guide. When the level of CREB 1 transcript is higher than the mean of NPI-2 (3.4-5.4, moderate prognostic group), the tumour is arbitrarily regarded as having a high level. When long term survival was analysed using Kaplan-Meier survival method, patients with high levels of CREB1 had a significantly shorter disease free survival {95.3 (68.4-122.3) months} compared with those with lower levels {133.9 (123.5-144.2) months}, p=0.0193 (figure 3. 9).

Table 3.8: Means and standard deviation of the levels of expression of CREB 1 in relation to clinical outcome
(median follow- up 120 months).

	CREB1		CREB1:CK19 ratio	
Disease Free Survival	8.9 ± 2.3		375 ± 120	
Metastasis	41 ± 4.6	p=0.089	692 ± 640	p=0.65
Local Recurrence	5 ± 1.6	p=0.288	516 ± 502	p=0.89
Death (Breast Cancer)	24.2 ± 5.2	p=0.326	1215 ± 295	p=0.0003

Table 3.9: Means and standard deviation of the levels of expression of CREB 1 in patients who remained disease free and who had disease progression

	CREB1		CREB1:CK19 ratio	
Disease Free Survival	8.9 ± 2.3		375 ± 120	
Poor Prognosis	26.5 ± 3	p=0.2998	999 ± 239	p=0.0035



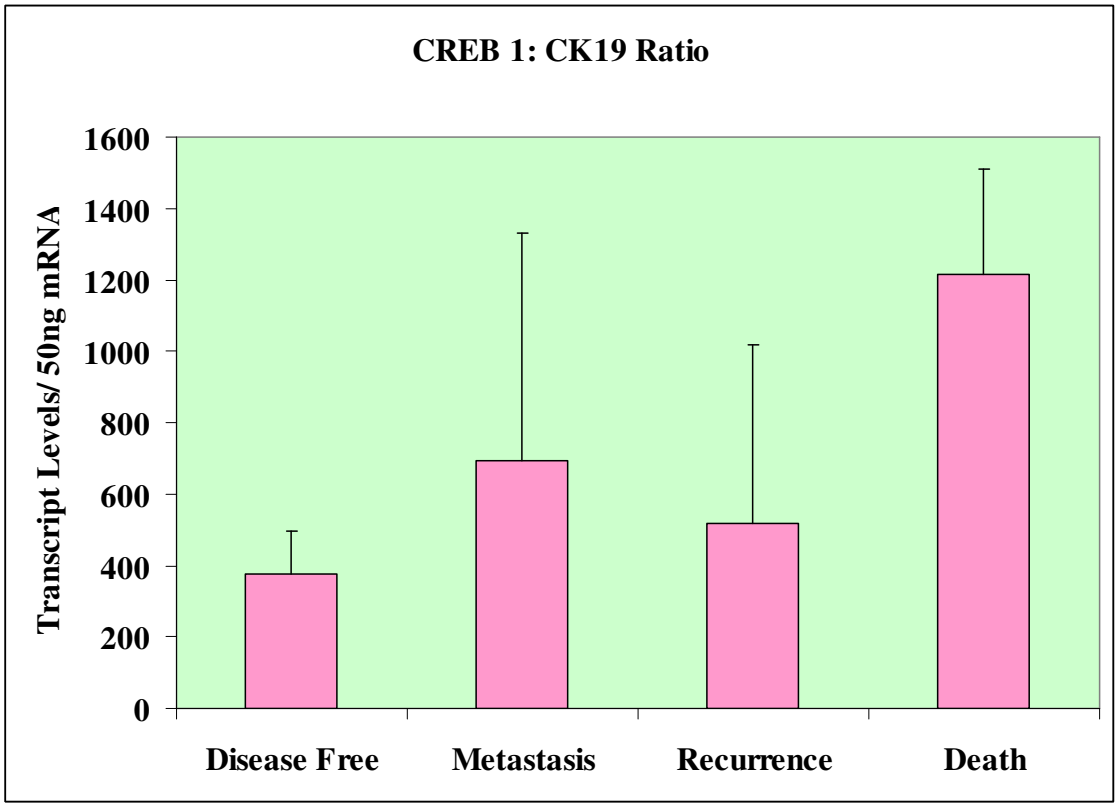
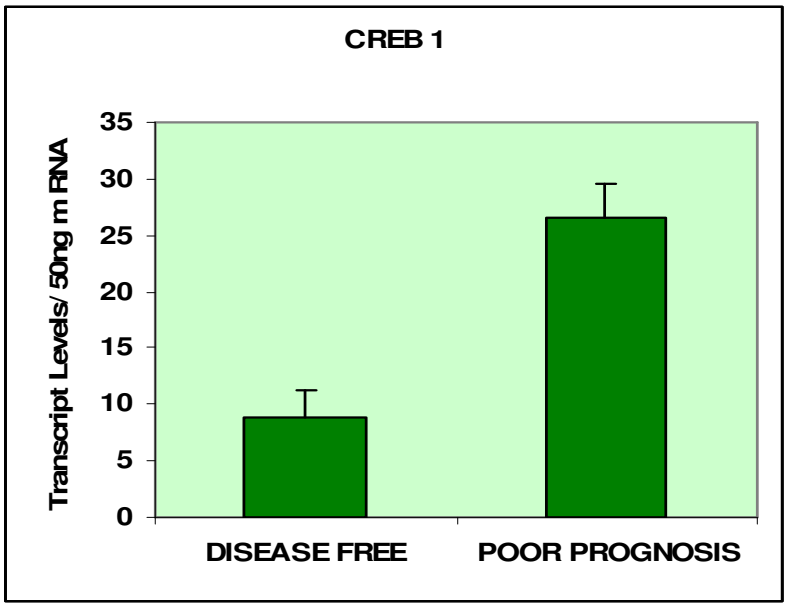


Figure3.7a: CREB-1 transcript levels and clinical outcome (median follow- up 120 months)

Figure3.7b: Transcript normalised by CK19 (shown as CREB-1: CK19 ratio).



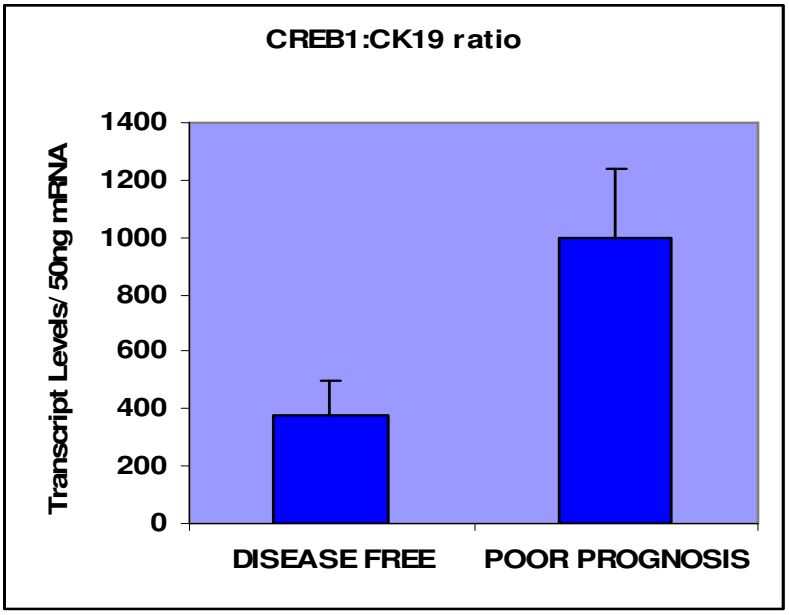


Figure3.8a: Comparison between patients who remained disease free and who had disease progression (recurrence, metastasis and mortality).
Figure3.8b: Transcript normalised by CK19 (shown as CREB-1: CK19 ratio).

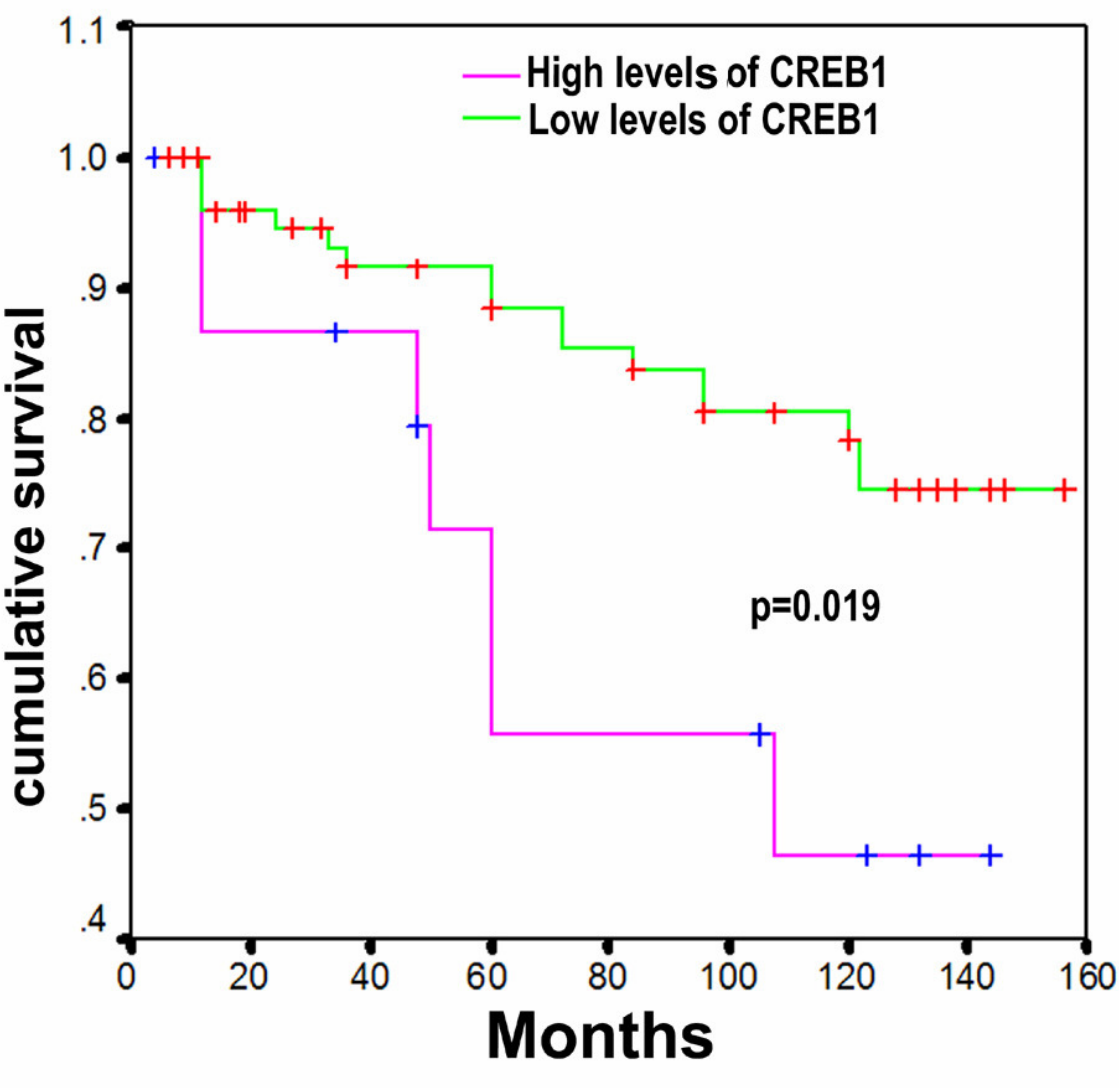


Figure3.9: Kaplan- Meier survival analysis showing correlation between CREB-1 transcript levels and the disease free survival.

Patients with high levels of CREB1 had a significantly shorter disease free survival (95.3 (68.4-122.3, 95%CI) months) compared with those with lower levels (133.9 (123.5-144.2) months), p=0.0193. Patients divided into groups with high and low levels of CREB 1 by using NPI as a general guide as explained earlier.

3.3.6 Distribution of CREB 1 protein in breast cancer and normal mammary tissue

We examined the presence of CREB-1 in mammary tissue using immunohistochemical staining. CREB-1 staining was primarily seen in nucleus of both normal and tumour cells. The staining pattern of CREB-1 in tumour tissue and in normal (background) mammary tissue was then compared. As shown in Figure 3.10 CREB-1 is mainly localised in the nucleus both in normal mammary tissue and the tumour tissue. Semi – quantitative analysis of the 8 paired (normal and tumour tissue) samples showed that CREB 1 staining in normal mammary tissue was weaker as compared with the tumour tissue (Figure 3.10 & Table 3.11). Using a Chi-square test, there was a significant difference between background normal and cancer tissues (Chi square value=16, p=0.001). By grouping negative and weakly positive, positive and strongly positive (Table-3.11) and using Fisher's Exact test, there was a significant difference between background normal and cancer tissues ($p<0.0001$).

3.3.7 Immunostaining of CREB 1 in relation to tumour prognosis (Nottingham prognostic Index – NPI), TNM stage and tumour grade

The level of CREB 1 expression is higher in the grade 3 tumours as compared to grade 1 tumours. Of the 6 G3 samples (background =3 and tumour =3), 5 samples staining was scored as 3+ for CREB 1 whereas out of the 6 G1 samples (background =3 and tumour =3), G1 tumours were scored negative (0) or weakly positive (+1) (Table 3.12, Figure 3.11). Similar trend was seen in NPI 3 and TNM 4 tumours as compared to NPI 1 and TNM 1 tumours respectively (Table 3.12, Figure 3.12 & 3.13).

Table 3.10 Pathological characteristics of Breast Cancer

GRADE	TNM	NPI
GRADE 1 n = 3	TNM 1 n = 2	NPI 1 n = 2
GRADE 2 n = 2	TNM 2 n = 1	NPI 2 n = 3
GRADE 3 n = 3	TNM 3 n = 2	NPI 3 n = 3
	TNM 4 n = 3	

Table 3.11 Immunohistochemistry staining scores of CREB 1 in normal and cancer tissue specimen

CREB1	negative or weakly positive		positive or strongly positive	
	0	+1	+2	+3
Normal	2	6	0	0
Cancer	0	0	3	5

Table 3.12 Immunohistochemistry staining scores of CREB 1 in relation to tumour grade, TNM stage and Nottingham Prognostic Index (NPI)

CREB1	Background				Cancer			
IHC	0	+1	+2	+3	0	+1	+2	+3
GRADE 1 n = 3	3	0	0	0	1	2	0	0
GRADE 2 n = 2	0	1	1	0	0	1	1	0
GRADE 3 n = 3	0	0	1	2	0	0	0	3
TNM 1 n = 2	2	0	0	0	1	1	0	0
TNM 2 n = 1	1	0	0	0	0	1	0	0
TNM 3 n = 2	0	0	1	1	0	0	0	2
TNM 4 n = 3	0	0	0	3	0	0	1	2
NPI 1 n = 2	1	1	0	0	0	2	0	0
NPI 2 n = 3	0	0	1	2	0	0	1	2
NPI 3 n = 3	0	0	2	1	0	0	0	3

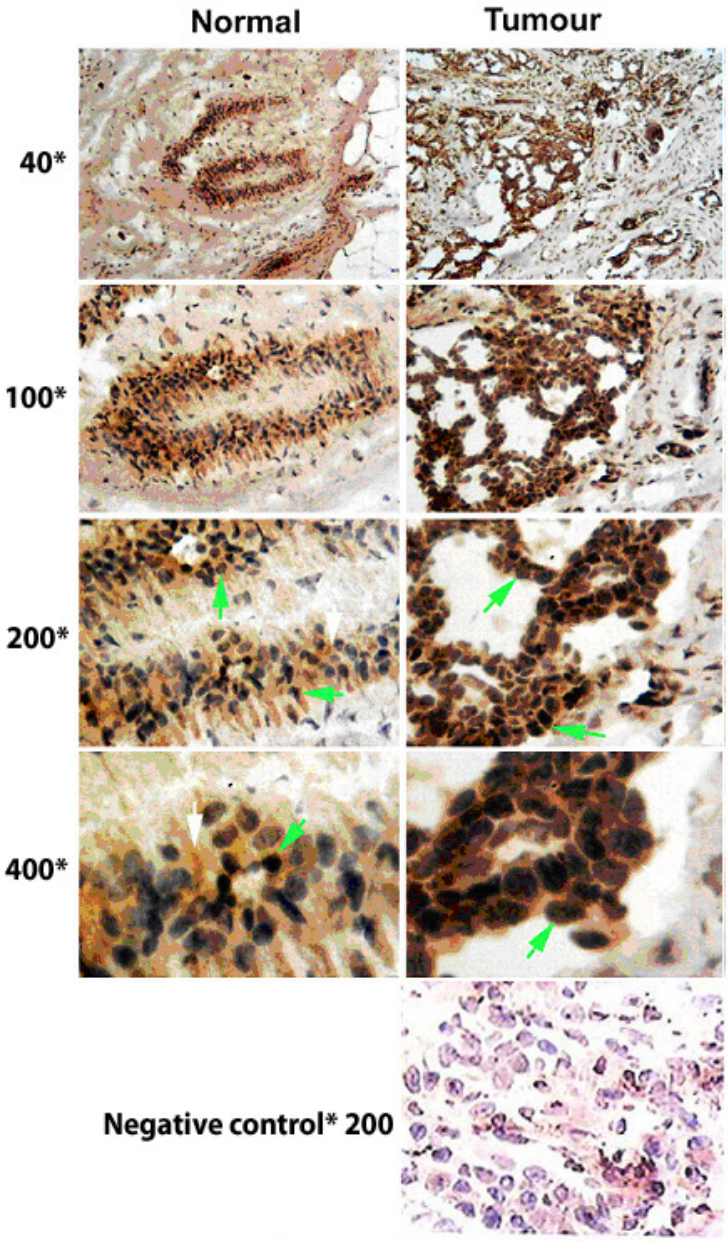


Figure3.10: Immunohistochemical staining of CREB-1 in normal mammary and tumour tissue

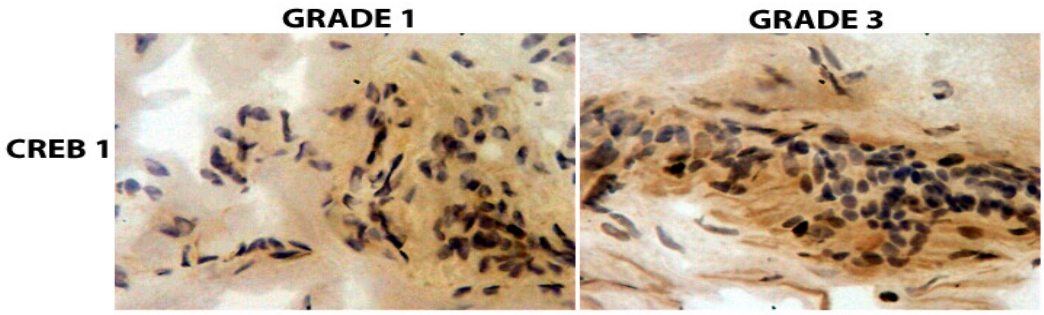


Figure3.11: CREB 1 immunostaining in breast cancer tissue.

Grade 3 tumour displayed strong immunoreactivity for CREB 1 as compared to Grade 1 tumour which showed weak immunostaining.

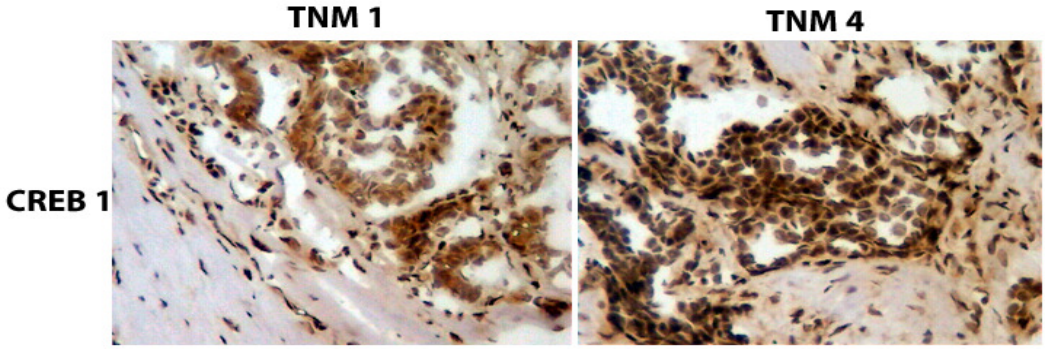


Figure 3.12: CREB 1 immunostaining in breast cancer tissue.

TNM 4 tumour displayed strong immunoreactivity for CREB 1 as compared to TNM 1 tumour which showed weak immunostaining.

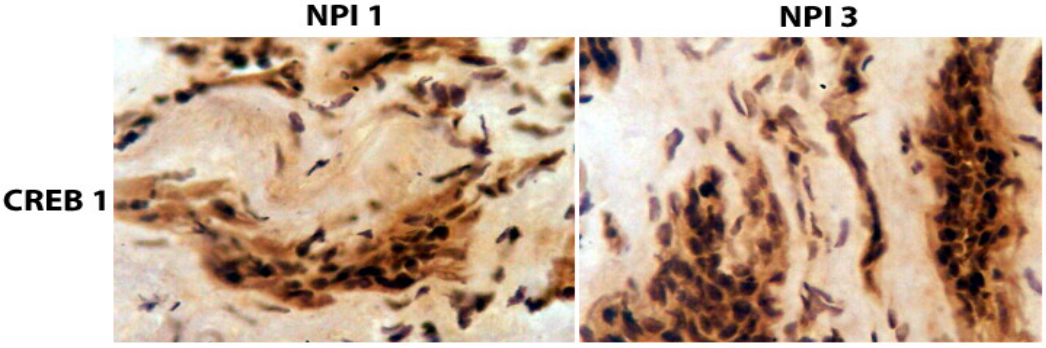


Figure 3.13: CREB 1 immunostaining in breast cancer tissue.

NPI 3 tumour displayed strong immunoreactivity for CREB 1 as compared to NPI 1 tumour which showed weak immunostaining

3.4 Discussion

Aberrant transcriptional activity and expression of CREB in cancer cells and clinical tumours has long been recognised. This molecule has been extensively studied in relation to stress response (Sabban *et al.,* 2006), memory (Wagatsuma *et al.,* 2006) and in solid tumours, both benign and malignant (Luciani *et al.,* 2003; Perry *et al.,* 2004). This protein behaves as both negative and positive regulatory proteins in different solid tumours. Chen et.al reported that in cancer tissue increased levels of positive regulatory proteins like ERRalpha -1 and CREB-1 is present as compared to negative regulatory proteins like EAR-2, COUP-TFI, Snail and Slug proteins (Chen *et al.,* 2005). Coxon et al. have shown a link between an oncogenic chromosomal translocation and CREB activation in malignant salivary gland tumours (Coxon *et al.,* 2005). Sofi et al reported that the expression of CREB in tumour bearing breast adipose tissue is higher than the normal breast adipose tissue (Sofi *et al.,* 2003). CREB has an antimitogenic role in solid tumours like glioblastoma proliferation (Perry *et al.,* 2004) and decreased expression is seen in thyroid carcinoma (Luciani *et al.,* 2003).

This study has found that CREB 1 is expressed in both tumour tissues and the normal breast adipose tissues with higher levels in tumour tissues. CREB 1 was shown to be predominantly a nucleus protein which was highly stained in breast cancer cells nucleus as compared with normal epithelial cells.

The current study reported for the first time that CREB 1 is aberrantly expressed in human breast cancer at mRNA levels and has significant bearing to the clinical outcome of the patients. We assessed CREB-1 level in breast cancer tissue samples against the

known prognostic factors and found significantly raised levels of CREB1 in patients with poor prognosis, metastatic disease and nodal involvement. The study has revealed a significant link between CREB 1 and mortality, in that high levels are associated with shorter disease free survival.

The immunohistochemical based analysis has shown similar findings to that of quantitative transcript analyses. However, due to the limited availability of the tissue materials for histological analysis, IHC assay was only conducted on a small number of samples, which preclude the possibility to carry out an appropriate statistical analysis, namely in Grade and NPI subgroups. Based on the favourable IHC results, it will be highly useful to conduct a full range IHC analysis on a suitable cohort of breast tumour tissues in future.

The role of CREB in relation to aromatase expression is still unclear. Over-expression of aromatase in adipose tissue surrounding breast tumour could arise through increase in both CREB expression and CREB transcriptional activity. Sofi *et al*. reported that there is increase aromatase expression due to increase in CREB induced promoter II activity in tumour bearing breast adipose tissue(Sofi *et al.,* 2003). Further studies needs to be done whether increased expression of CREB leads to aberrant aromatase expression and whether there is simultaneous decrease in the expression of the negative regulatory proteins like Snail and Slug proteins in breast cancer tissue.

CHAPTER 4

EXPRESSION OF ATF 1, ATF 2 AND ATF 3 IN HUMAN BREAST CANCER AND THE ASSOCIATION WITH CLINICOPATHOLOGICAL CHARACTERISTICS

4.1 Introduction

Transcription factors play a vital role in the process of tumourigenesis as they possess specific structural features that encode transactivating or repressing functions. Breast cancer results from acquired or inherited gene defects resulting in the expression of abnormal transcriptional factors which in turn leads to increased proliferation of transformed mammary cells (Jackson *et al.,* 1998; Loeb *et al.,* 1998).

Activating transcription factor (ATF) protein belongs to a subfamily of the bzip family of transcription factors that may act as transcriptional activators or repressors (Hai *et al.,* 1989). All members of this family share significant sequence similarity within a leucine zipper DNA- binding motif and an adjacent basic region.

ATF protein is involved in both the positive and negative regulation of smooth muscle cell growth and motility. ATF-1 mediates protease activated receptor- 1 induced DNA synthesis in vascular smooth muscle cells (Ghosh *et al.,* 2002).

ATF 3 has been implicated in various processes including stress response, HBV-mediated processes and tumourigenecity. ATF3 interacts with the hepatitis B virus X protein which is implicated in HBV mediated hepatocarcinogenesis and apoptosis. ATF3 has been shown to play an important role in metastatic processes in melanoma and has a tumourigenecity- inhibiting effect on the HT29 colon cancer cells (Ishiguro *et al.,* 2000). Allen and Jennings demonstrated that ATF3 is induced during pancreatic stress such as partial pancreatectomy and ischemia –reperfusion of the pancreas (Allen and Jennings *et al.,* 2001).

ATF3 has also been demonstrated to activate transcription when coexpressed with other proteins such as HTLV-1 Tax (Barnabas *et al.,* 1997; Low *et al.,* 1994), Ras (Nilsson *et*

al., 1997), and PKA (Chu *et al.,* 1994). ATF3 increases following exposure of cells to the signals and targets genes like gadd153/chop10 (Wolfgang *et al.,* 1997; Fawcett *et al.,* 1999), E- selectin (Nawa *et al.,* 2000) and phosphoenolpyruvate carboxykinase (Allen-Jennings *et al.,* 2002)

Till date there is no available data about the association of ATFs with tumour or patients clinicopathological characteristics in human breast cancer. This study was carried out to investigate the expression pattern of ATFs family of proteins in cohort of breast cancer and normal background tissue in relation to tumour histopathological grade, stage and nodal status. We also correlate the level of expression with the prognosis and clinical outcome of the patients.

4.2 Material and Methods

RNA-extraction kit, RT (Reverse Transcriptase) kit and Mastermix for routine PCR and quantitative PCR were obtained from AbGene (Surrey, UK). PCR primers (Table: 4.1) were designed using Beacon Designer (Palo Alto, CA, USA) and synthesized by Invitrogen (Paisley, Scotland, UK). Molecular-biology-grade agarose and DNA ladder were from Invitrogen (see section 3.2)

4.2.1 Sample Collection

Breast cancer tissues (n=120) and background tissue samples (normal breast tissue) (n=33) were collected (with ethical approval from local committee) immediately after surgery and stored in liquid nitrogen before processing. Patients were routinely followed after surgery and the median follow up period was 120 months for this study. The histopathological details, tumour grading (modified Bloom and Richardson's grading

system), tumour staging (TNM) and the prognostic index for the patients is shown in chapter 3 (For detail see section 3.2.1 and Table 3.1)

4.2.2 Tissue processing, RNA extraction and reverse transcription – polymerase chain reaction

For detail see section 3.2.2

4.2.3 Quantitative analysis of ATF Transcripts

The transcript level of the ATF from the above-prepared cDNA was determined using a real-time quantitative PCR, based on the Amplifluor™ uniprimer technology (Intergen Company, Oxford, UK). (For detail see section 3.2.3) The reaction was carried out as explained previously, using the following conditions: 94°C for 5 minutes followed by 50 cycles at 94°C for 10 seconds, 55°C for 15 seconds and 72°C for 20 seconds. The levels of the transcripts were generated from an internal standard that was simultaneously amplified with the samples, and are shown as levels of transcripts based on equal amounts of RNA.

4.2.4 Immunohistochemical staining of the ATF proteins

Immunohistochemical staining was carried out using frozen sections of breast tumour and normal mammary tissue as explained in section 3.2.4. However, given that ATF proteins are nucleus in their cellular location, modifications were carried out in order to visualise the nucleus distribution of the protein. At the end of DAB colour development, the slides were dried and mounted. The first evaluations were carried out by examining the brown staining in nucleus of the cells. Subsequently, all the slides were placed in Xylene for overnight in order to remove the coverslips. After removal of the cover slips, the slides were series treated using fresh xylene (X2), pure ethanol (X2), 90% Ethanol (X2), 70%

Ethanol (X2) and 50% Ethanol (X2), before finally merged in water and stained with hematoxyline to counter stain the nucleus. Images are presented here as both counter stained and non-conterstained.

Statistical analysis was carried out as described in section 2.17

Table 4.1: Primers sequences used in the quantitative RT- PCR

Gene	Primer Name	Primer Sequence (5'-3')	Optimal Annealing Temperature	Product Size (bps) Accession no:
ATF1	ATF1F1	ttctcatattgctcaacaggt	55°C	816 NM_005171
	ATF1 ZR	actgaacctgaccgtacactttctgtgaggagcctatg		
ATF2	ATF2F1	cttgccatgagaaaaagaat	55°C	2117 NM_001880
	ATF2ZR	actgaacctgaccgtacagaaagggtttgtcatcactc		
ATF3	ATF3F1	gcgacgagaaagaaataaga	55°C	1914 NM_001674
	ATF3ZR	actgaacctgaccgtacatcaatctgagccttcagttc		
ß-actin	ß-actin F	atgatatcgccgcgctcg	55°C	580 NM_001101 81-660
	ß-actinR	cgctcggtgaggatcttca		
	ß-actin ZF	ggacctgactgactacctca		117 NM_001101 622-720
	ß-actin ZR	actgaacctgaccgtacaagcttctccttaatgtcacg		

4.3 Results

We screened three breast cancer lines (MDA-MB 231, MCF 7 and MDA- MB 453) to assess the expression of ATF 1, ATF 2 and ATF 3, using RT-PCR. The mRNA level of ATF 1, ATF 2 and ATF 3 in these cell lines are illustrated in Figure 4.1. ATF 1 was expressed at a relatively higher level in all the three breast cancer cell line. ATF 2 and ATF 3 were expressed at a higher level in MDA- MB 453 cell line as compared to MDA-MB 231 and MCF 7 cell lines (Figure 4.1)

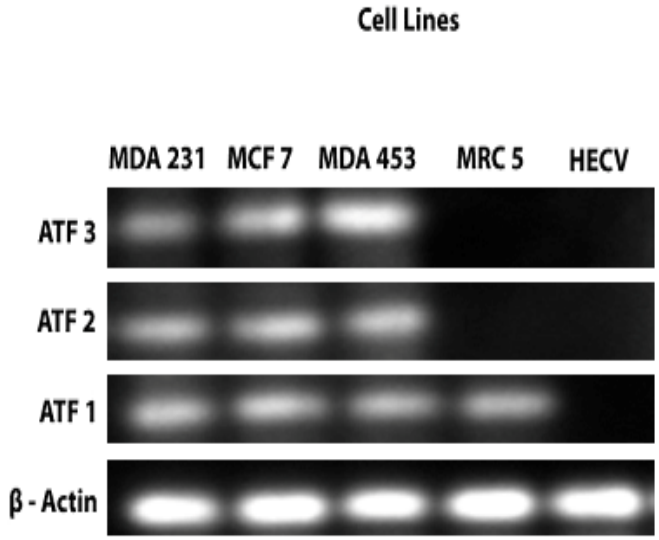


Figure 4.1: The expressions of ATF 1, ATF 2 and ATF 3 in human breast cancer, fibroblast and endothelial cell lines were assessed using RT- PCR.

40 cycles were performed for these PCR reactions.

4.3.1 Expression of ATFs in normal mammary and breast cancer tissues

We first analysed the overall expression levels of ATF 1, ATF 2 and ATF 3 in normal mammary and tumour tissues using the real time quantitative RT-PCR. As shown in figure the expression of ATF 1 transcript is significantly higher in breast tumour tissues as compared to normal mammary tissue (Figure 4.2 & Table 4.2). The expression of ATF 2 and ATF 3 is higher in tumour tissue but no statistical significant difference was seen as compared to normal breast tissue (Figure 4.2 & Table 4.2).

4.3.2 Expression of ATFs and its correlation with nodal involvement and tumour grade

We then analysed the expression pattern of ATF 1, ATF 2 and ATF 3 in various tumour characteristics and interestingly we demonstrated that the level of expression of ATF 1, ATF 2 and ATF 3 transcript is higher in node negative tumours as compared to node positive tumours (Figure 4.3 & Table 4.3). The expression of ATF 1, ATF 2 and ATF 3 is higher in grade 1 tumour as compared to grade 3 tumour (Figure 4.4 and Table 4.4) but no statistical significant difference was seen.

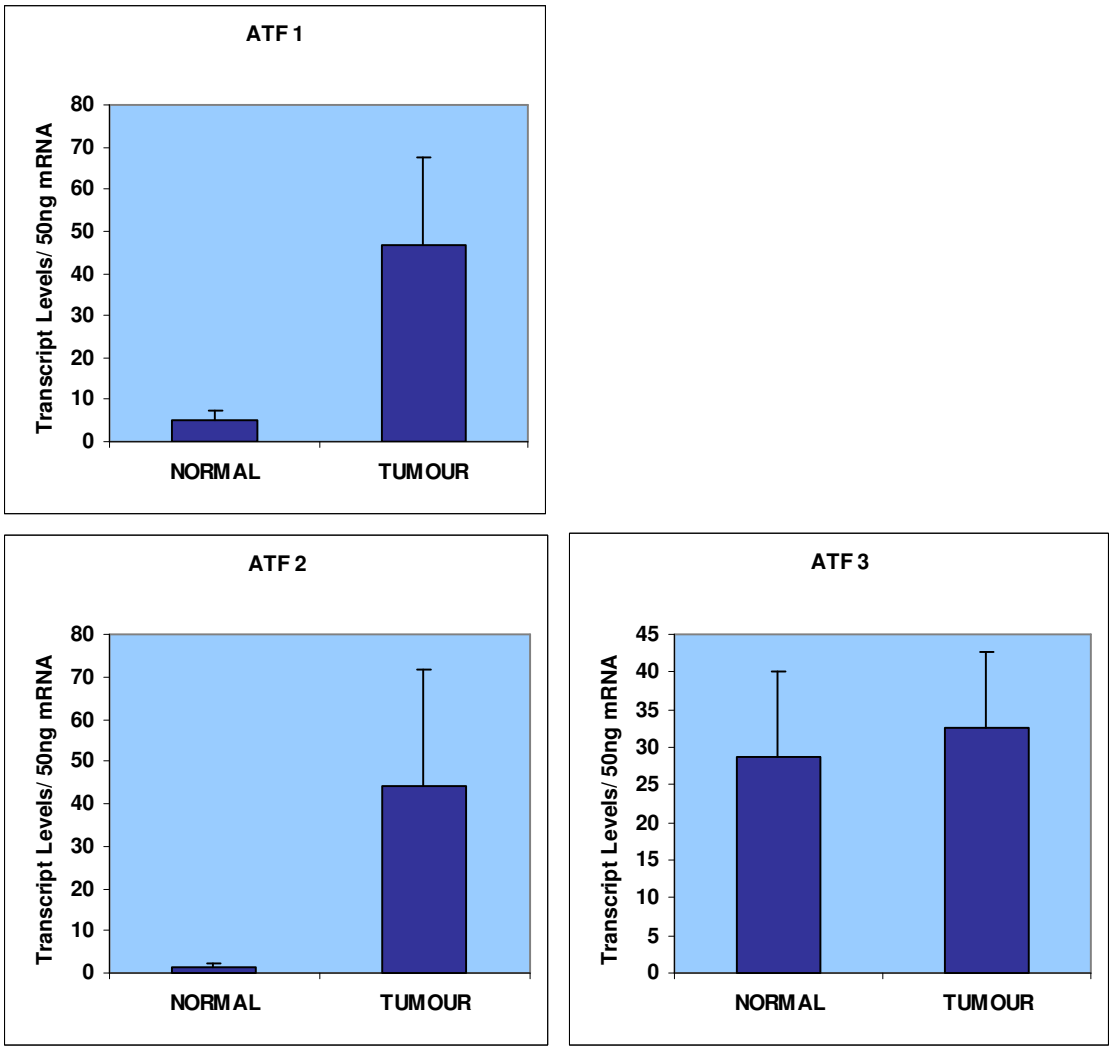


Figure 4.2: Quantitative real time PCR demonstrating high expression of ATF 1, ATF 2 and ATF 3 in tumour samples as compared to normal mammary tissue.

Table 4.2: Means and standard deviation of the levels of expression of ATF 1, ATF 2 and ATF 3 in normal and breast cancer tissues.

	ATF1		ATF 2		ATF 3	
Normal	5 ± 2.3		1.5 ± 0.96		28.7 ± 11.5	
Cancer	46.7 ± 20.7	p=0.048	44.3 ± 27.6	p=0.12	32.6 ± 10.1	p=0.8

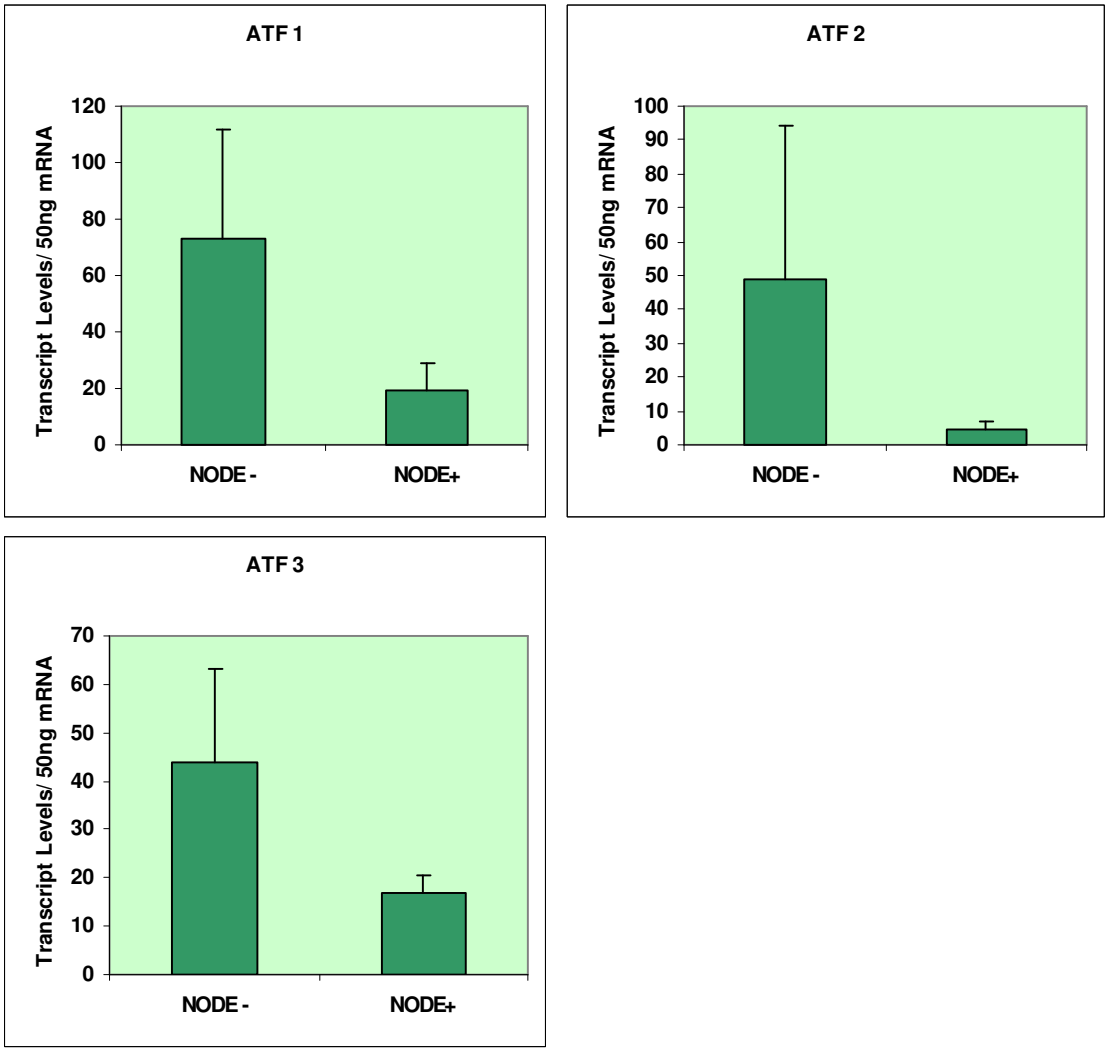


Figure 4.3: RT –PCR analysis of breast cancer tissue samples, showing low transcript levels of ATFs in node positive tumours as compare to node negative tumours.

Table 4.3: Means and standard deviation of the levels of expression of ATF 1, ATF 2 and ATF 3 in relation to nodal status.

	ATF1		ATF 2		ATF 3	
Node -ve	73 ± 39		49 ± 45		44 ± 19	
Node +ve	19.1 ± 9.8	p=0.18	4.6 ± 2.3	p=0.33	17 ± 3.6	p=0.16

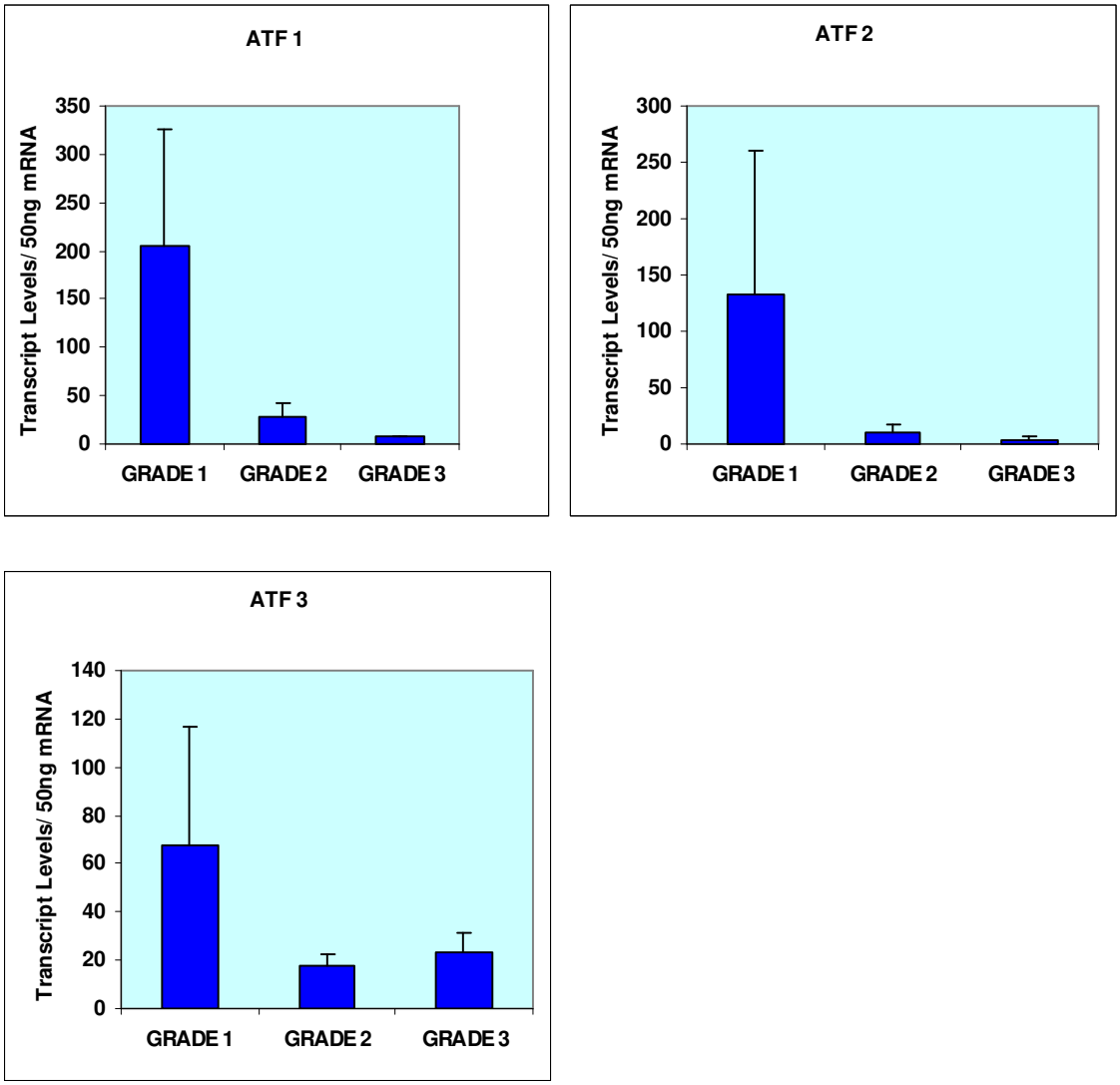


Figure 4.4: Levels of ATF 1, ATF 2 and ATF 3 in relationship with tumour grade.

Table 4.4: Means and standard deviation of the levels of expression of ATF 1, ATF 2 and ATF 3 in relation to tumour grade.

	ATF1		ATF 2		ATF 3	
Grade 1	205 ± 120		133 ± 128		67.9 ± 48.8	
Grade 2	28.3 ± 14.4	p=0.16	10.6 ± 7.2	p=0.35	17.7 ± 4.5	p=0.32
Grade 3	7.1 ± 1.6	p=0.12	4.3 ± 2.4	p=0.33	23.1 ± 8.6	p=0.38

4.3.3 Expression of ATFs and its relation to tumour prognosis

Nottingham prognostic index was used as a tool to determine the prognosis of the patients. NPI< 3.4 was regarded as having a good prognosis (NPI-1), 3.4-5.4 moderate (NPI-2) and >5.4 as poor prognosis (NPI-3). As shown in figure, there was significantly higher level of ATFs transcript in patients with good prognosis as compared to patients with moderate and poor prognosis tumour (Figure 4.5 and Table 4.5). The expression of ATF1 and ATF3 is markedly raised in TNM 1 and TNM 2 tumours as compared to TNM 4 tumours. Interestingly the expression of ATF 2 in stage 4 tumour is high as compared to stage 1 tumour (Figure 4.6, Table 4.6)

4.3.4 High levels of the ATFs family protein transcript was linked to both the overall and disease-free survival of the patients

Following a median 120 months follow up, patients were divided into the following groups: those who remained disease free, who developed metastasis or local recurrence and those who died of breast cancer related disease (excluding non – cancer related deaths). As shown in figure, Patients with metastatic disease and with local recurrence had low levels of the three ATFs, when compared with patients who remained disease free. Most interestingly, patients who died of breast cancer had a markedly reduced levels of ATF-1 and ATF-3 as compared with the patient who remained disease free (Figure 4.7 and Table 4.7).

We combined the three groups (with metastasis, recurrence and mortality), to form a poor prognostic group (referred to as poor prognosis in figure 4.8) and compared this group with those who remained disease free. It was demonstrated that patients with predicted good prognosis had higher levels of ATF transcripts (73.1±38.7 ATF1, 49.4±45.4 ATF2 and 44.0±18.6 ATF-3), than those with poor prognosis (6.1±2.6, 8.9±7.7 and 19.3±8.5 for ATF-1, -2 and -3 respectively) (Figure 4.8 and Table 4.8).

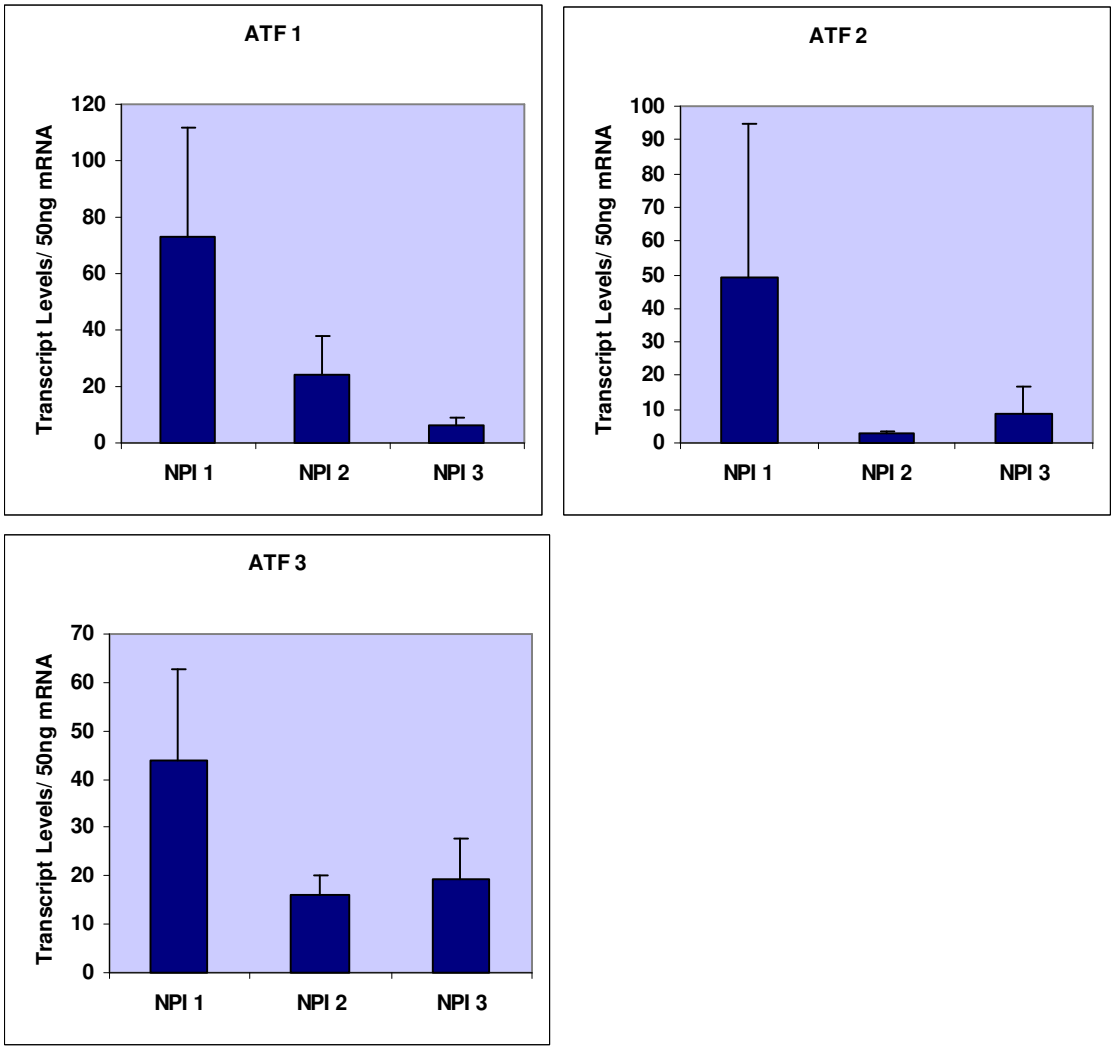


Figure 4.5: ATFs and predicted clinical outcome, using the Nottingham Prognostic Index (NPI) as an indicator

(NPI <3.4 - good prognosis group, NPI 3.4-5.4 - moderate prognosis group and NPI > 5.4 - poor prognosis group)

Table 4.5: Means and standard deviation of the levels of expression of ATF 1, ATF 2 and ATF 3 in relation to patient prognosis using the Nottingham Prognostic Index (NPI)

	ATF1		ATF 2		ATF 3	
NPI 1	73.1 ± 38.7		49.4 ± 45.4		44 ± 18.6	
NPI 2	24.3 ± 13.7	p=0.24	2.8 ± 0.9	p=0.31	16.1 ± 3.9	p=0.15
NPI 3	6.1 ± 2.6	p=0.089	8.9 ± 7.7	p=0.38	19.3 ± 8.5	p=0.23

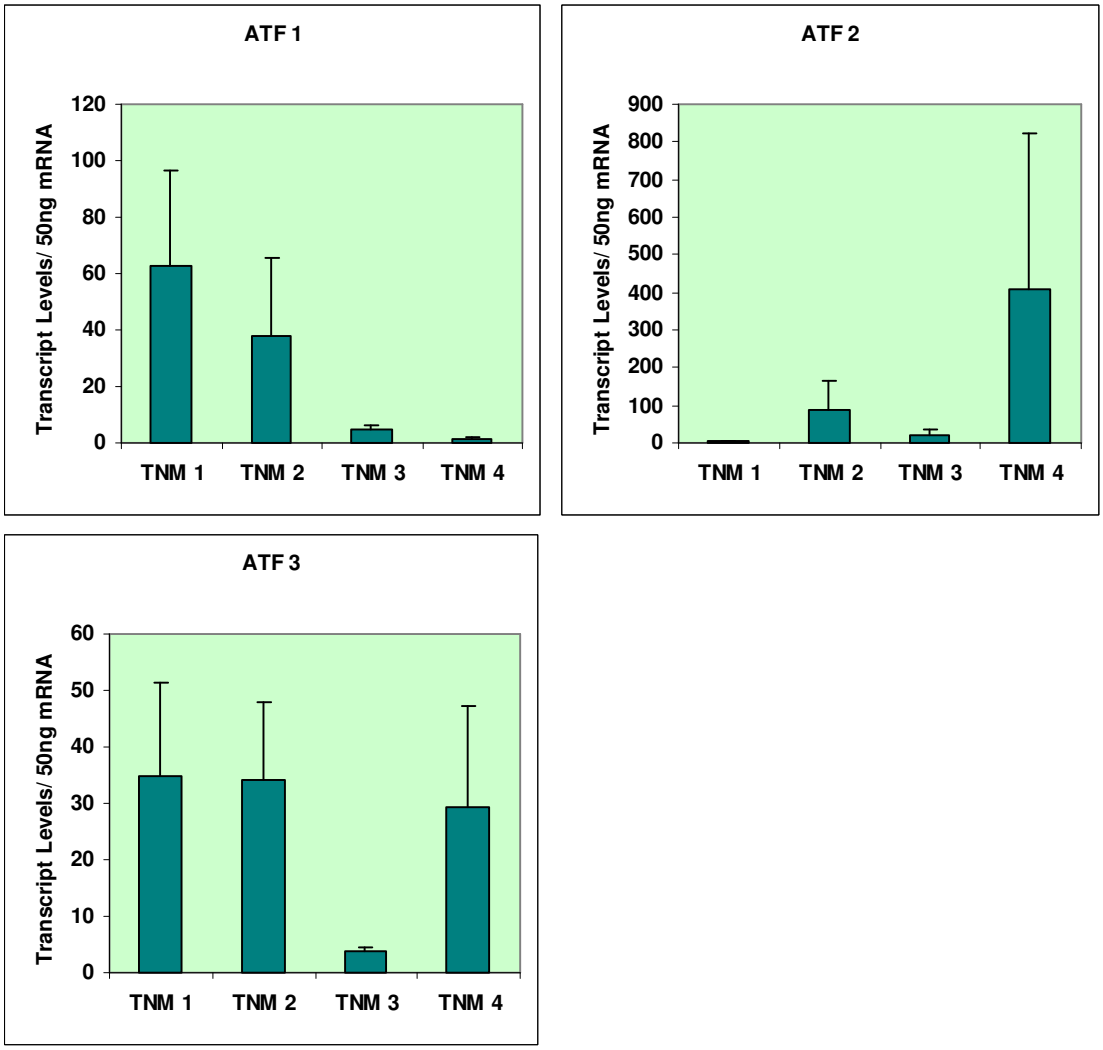


Figure 4.6: Levels of ATF 1, ATF 2 and ATF 3 in relationship with TNM staging.

Table 4.6: Means and standard deviation of the levels of expression of ATF 1, ATF 2 and ATF 3 in relation to tumour stage using the TNM classification.

	ATF1		ATF 2		ATF 3	
TNM 1	62.5 ± 34.3		2.9 ± 0.9		35 ± 16.3	
TNM 2	37.9 ± 27.8	p=0.58	87.5 ± 76.3	p=0.28	34 ± 14	p=0.96
TNM 3	4.8 ± 1.5	p=0.098	21.8 ± 16	p=0.28	3.7 ± 0.8	p=0.05
TNM 4	1.5 ± 0.76	p=0.08	411 ± 410	p=0.39	29.2 ± 18.1	p=0.75

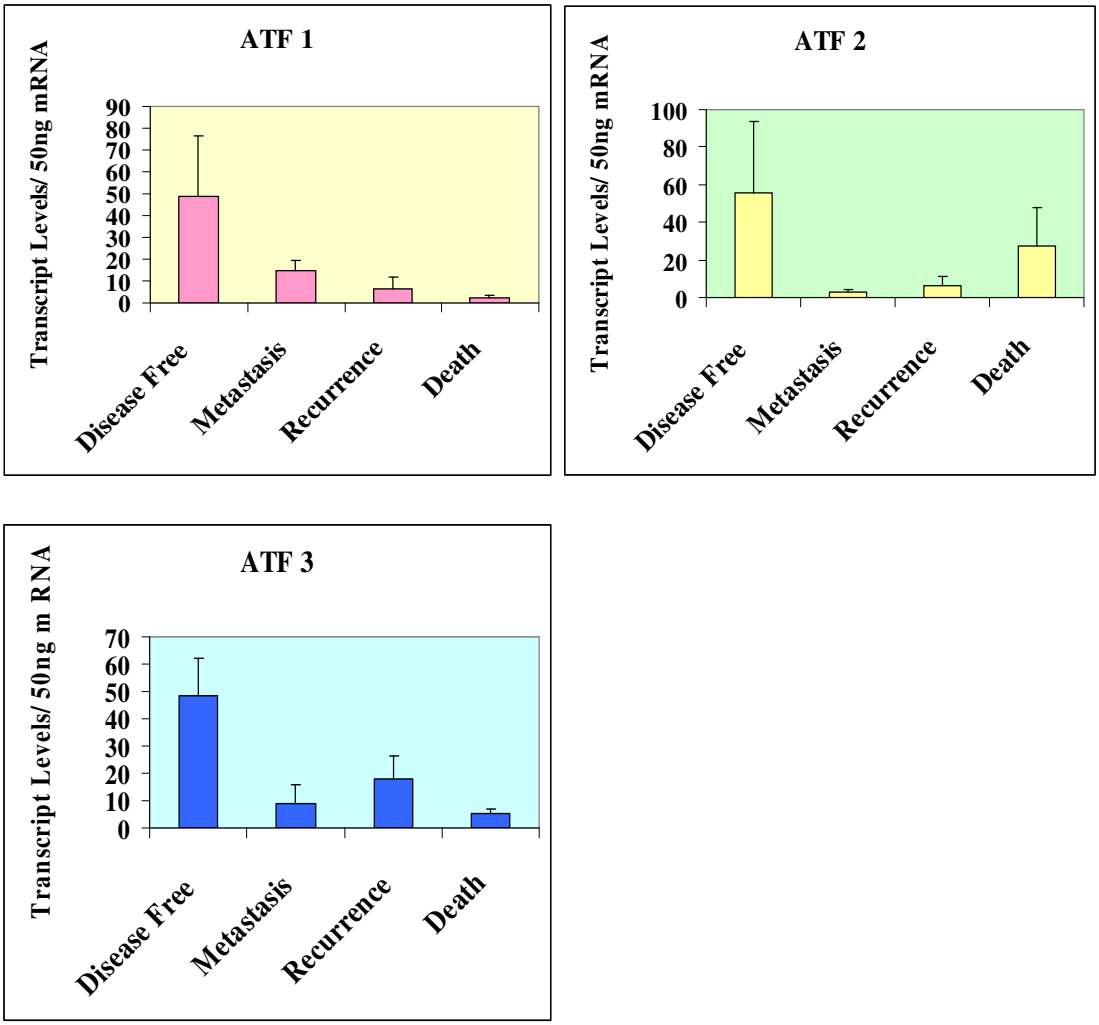


Figure 4.7: ATFs transcript levels and clinical outcome

Table 4.7: Means and standard deviation of the levels of expression of ATF 1, ATF 2 and ATF 3 in relation to clinical outcome

(Median follow- up 120 months)

	ATF1		ATF 2		ATF 3	
Disease Free Survival	49.1 ± 27.4		55.9 ± 38.1		48.2 ± 13.9	
Metastasis	15 ± 4.4	p=0.22	2.6 ± 1.3	p=0.17	9.1 ± 6.5	p=0.039
Local Recurrence	6.7 ± 5.1	p=0.13	6.5 ± 4.9	p=0.2	17.8 ± 8.5	p=0.15
Death (Breast Cancer)	2.6 ± 0.71	p=0.094	27.6 ± 20.6	p=0.52	5.3 ± 1.3	p=0.11

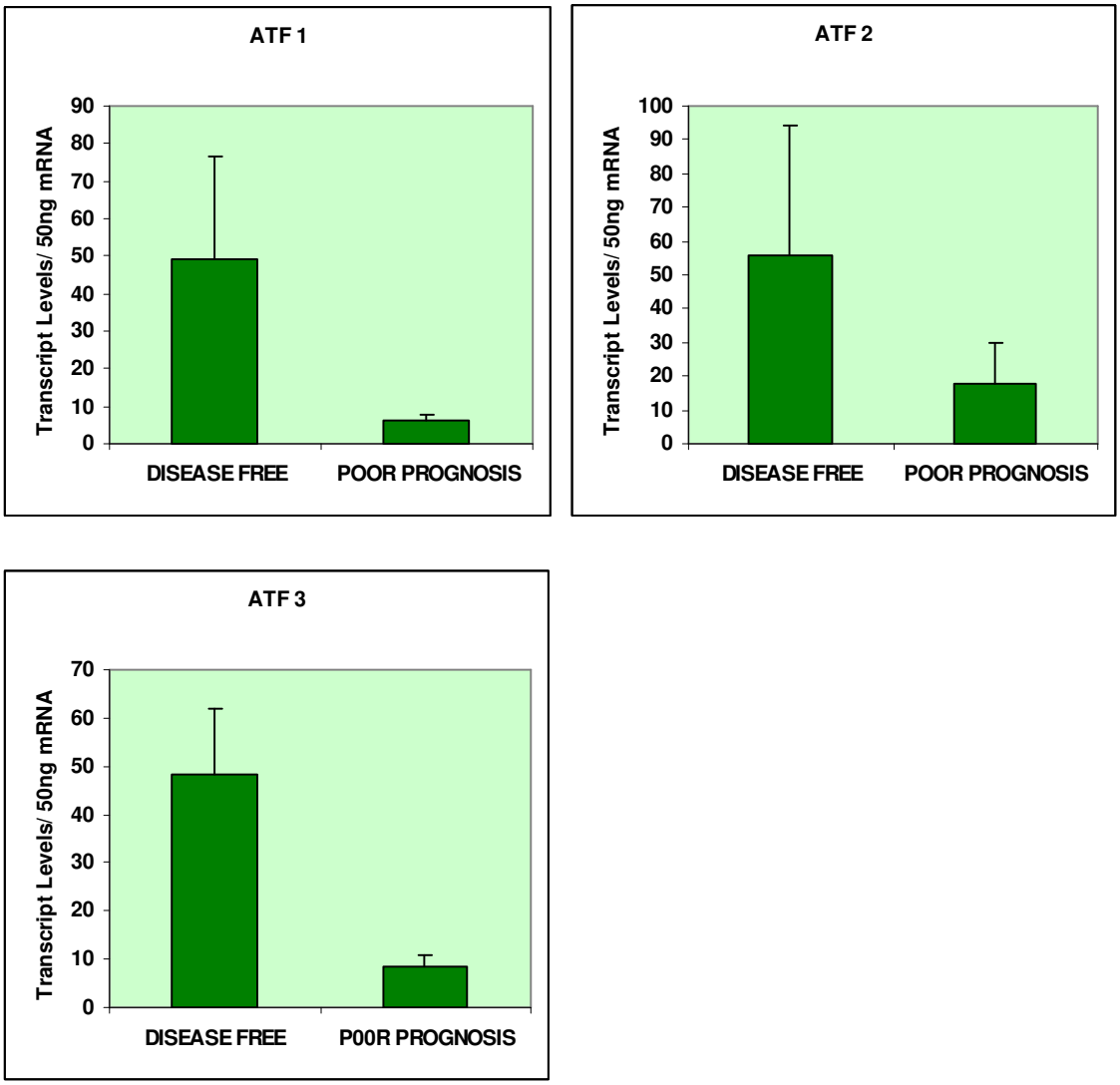


Figure 4.8: Comparison between patients who remained disease free and who had disease progression (recurrence, metastasis and mortality).

Table 4.8: Means and standard deviation of the levels of expression of ATF 1, ATF 2 and ATF 3 in relation to Disease Free Survival and Poor Prognosis
(median follow- up 120 months)

	ATF1		ATF 2		ATF 3	
Disease Free Survival	49.1 ± 27.4		55.9 ± 38.1		48.2 ± 13.9	
Poor Prognosis	6.2 ± 1.6	p=0.12	17.8 ± 12	p=0.34	8.5 ± 2.3	p=0.021

4.3.5 Immunohistochemical staining of the ATFs protein in breast cancer and normal mammary tissue

The immunohistochemical staining of ATF1, ATF2 and ATF3 protein was performed in breast cancer and normal mammary tissue. Here, we took an extra step, by first staining the tissue sections with anti-ATF antibodies (Santa Cruz Biotechnology, Santa Cruz, California, USA) and evaluated the nucleus staining without carrying out nucleus counter staining. The sections were subsequently restained using hematoxyline after removing the cover slips, thus allowing for further matched analysis. The staining pattern of ATF in tumour tissue and in normal mammary tissue was then compared. Semi –quantitative analysis of the 8 paired (normal and tumour tissue) samples showed that ATF1 (Table 4.10 & Figure 4.9), ATF2 (Table 4.12 & Figure 4.13), and ATF3 (Table 4.14 & Figure 4.17), protein staining in normal mammary and tumour tissues were showing differing patterns.

4.3.6 Immunohistochemical staining of ATFs in relation to tumour prognosis (Nottingham prognostic Index – NPI), TNM stage and tumour grade

The level of ATF1, ATF2 and ATF3 protein expression is higher in the grade 1 tumours as compared to grade 3 tumours. Of the 6 G1 samples (background =3 and tumour =3), 5 samples each of ATF1, ATF2 and ATF3 protein staining was scored as 3+ whereas out of the 6 G3 samples (background =3 and tumour =3), G3 tumours were either scored negative (0) or weakly positive (+1) (Table 4.11, 4.13 & 4.15, Figure 4.10, 4.14 & 4.18). Similar trend was seen for ATF1, ATF2 and ATF3 protein in relation to tumour prognosis (Nottingham prognostic Index – NPI) with the levels higher in the NPI 1 tumours as compared to NPI 3 tumours (Table 4.11, 4.13 & 4.15, Figure 4.11, 4.15 &

4.19). Interestingly the expression of ATF2 protein level is higher in the TNM 4 tumours as compared to TNM 1 tumours (Table 4.13 Figure 4.16). However ATF1 and ATF3 protein expression was higher in the TNM 1 tumours as compared to TNM 4 tumours (Table 4.13; Figure 4.12 & 4.20)

Table 4.9 Pathological characteristics of Breast Cancer

GRADE	TNM	NPI
GRADE 1 n = 3	TNM 1 n = 2	NPI 1 n = 2
GRADE 2 n = 2	TNM 2 n = 1	NPI 2 n = 3
GRADE 3 n = 3	TNM 3 n = 2	NPI 3 n = 3
	TNM 4 n = 3	

Table 4.10 Immunohistochemistry staining scores of ATF1 in normal and cancer tissue specimen

ATF1	0	+1	+2	+3
Normal	5	2	1	0
Cancer	0	1	3	4

Table 4.11 Immunohistochemistry staining scores of ATF1 in relation to tumour grade, TNM stage and Nottingham Prognostic Index (NPI)

ATF1	Background				Cancer			
IHC	0	+1	+2	+3	0	+1	+2	+3
GRADE 1 n = 3	0	0	1	2	0	0	0	3
GRADE 2 n = 2	0	1	1	0	0	0	0	2
GRADE 3 n = 3	1	2	0	0	0	2	1	0
TNM 1 n = 2	0	0	1	1	0	0	0	2
TNM 2 n = 1	0	0	1	0	0	0	0	1
TNM 3 n = 2	1	1	0	0	0	1	1	0
TNM 4 n = 3	2	0	1	0	0	2	1	0
NPI 1 n = 2	0	0	2	0	0	0	0	2
NPI 2 n = 3	0	1	0	2	0	0	1	2
NPI 3 n = 3	1	1	1	0	1	2	0	0

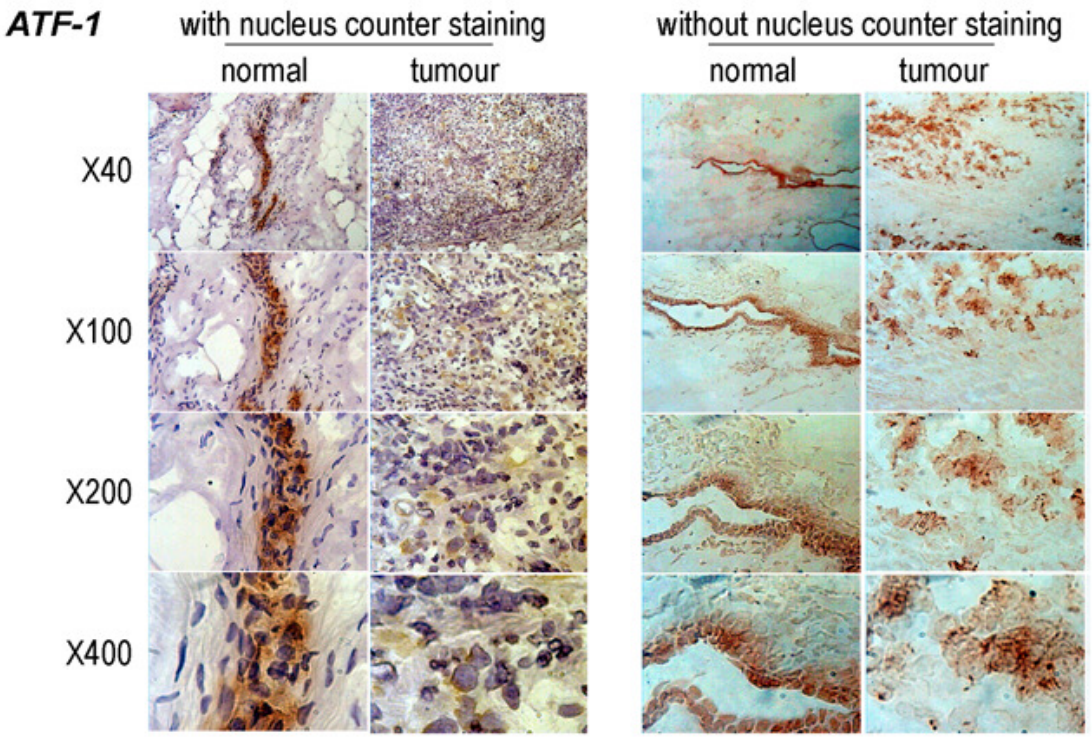


Figure 4.9: Immunohistochemical staining of ATF 1 in normal mammary and tumour tissue

Shown are images from non-counter stained (right panel) and counterstained sections (left panel).

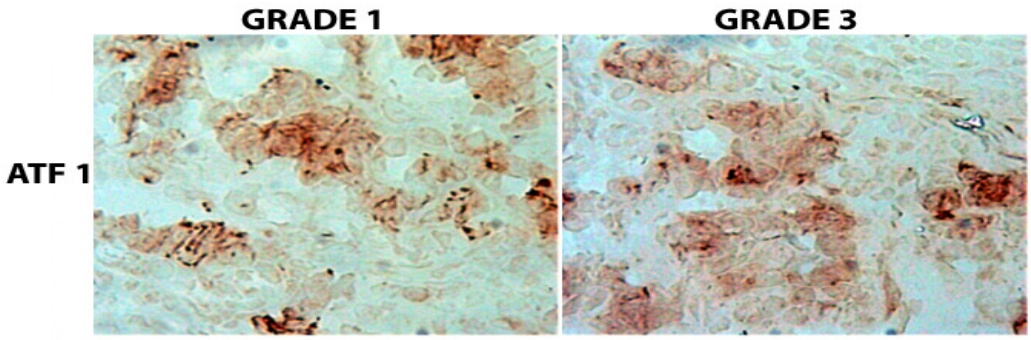


Figure 4.10: ATF 1 immunostaining in breast cancer tissue

Grade 1 tumour displayed strong immunoreactivity for ATF 1 as compared to Grade 3 tumour which showed weak immunostaining.

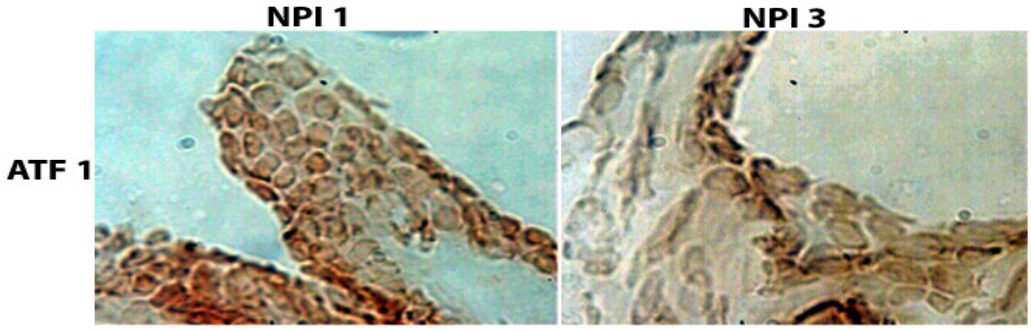


Figure 4.11: ATF 1 immunostaining in breast cancer tissue

NPI 1 tumour displayed strong immunoreactivity for ATF 1 as compared to NPI 3 tumour which showed weak immunostaining

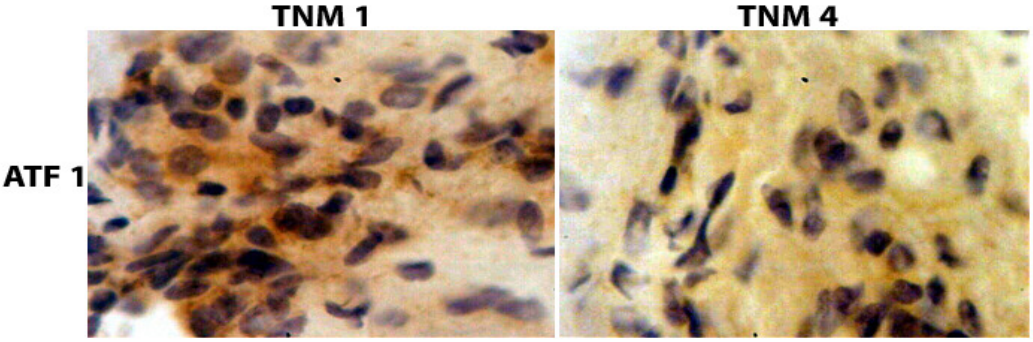


Figure 4.12: ATF 1 immunostaining in breast cancer tissue

TNM 1 tumour displayed strong immunoreactivity for ATF 1 as compared to TNM 4 tumour which showed weak immunostaining.

Table 4.12 Immunohistochemistry staining scores of ATF2 in normal and cancer tissue specimen

ATF2	0	+1	+2	+3
Normal	6	2	0	0
Cancer	0	2	2	4

Table 4.13 Immunohistochemistry staining scores of ATF2 in relation to tumour grade, TNM stage and Nottingham Prognostic Index (NPI)

ATF2	Background				Cancer			
IHC	0	+1	+2	+3	0	+1	+2	+3
GRADE 1 n = 3	0	0	1	2	0	0	0	3
GRADE 2 n = 2	1	0	1	0	0	0	1	1
GRADE 3 n = 3	1	2	0	0	2	1	0	0
TNM 1 n = 2	2	0	0	0	1	1	0	0
TNM 2 n = 1	1	0	0	0	0	1	0	0
TNM 3 n = 2	0	1	1	0	0	0	2	0
TNM 4 n = 3	0	1	1	1	0	0	1	2
NPI 1 n = 2	0	0	0	2	0	0	1	1
NPI 2 n = 3	0	1	2	0	0	0	1	2
NPI 3 n = 3	1	2	0	0	2	0	1	0

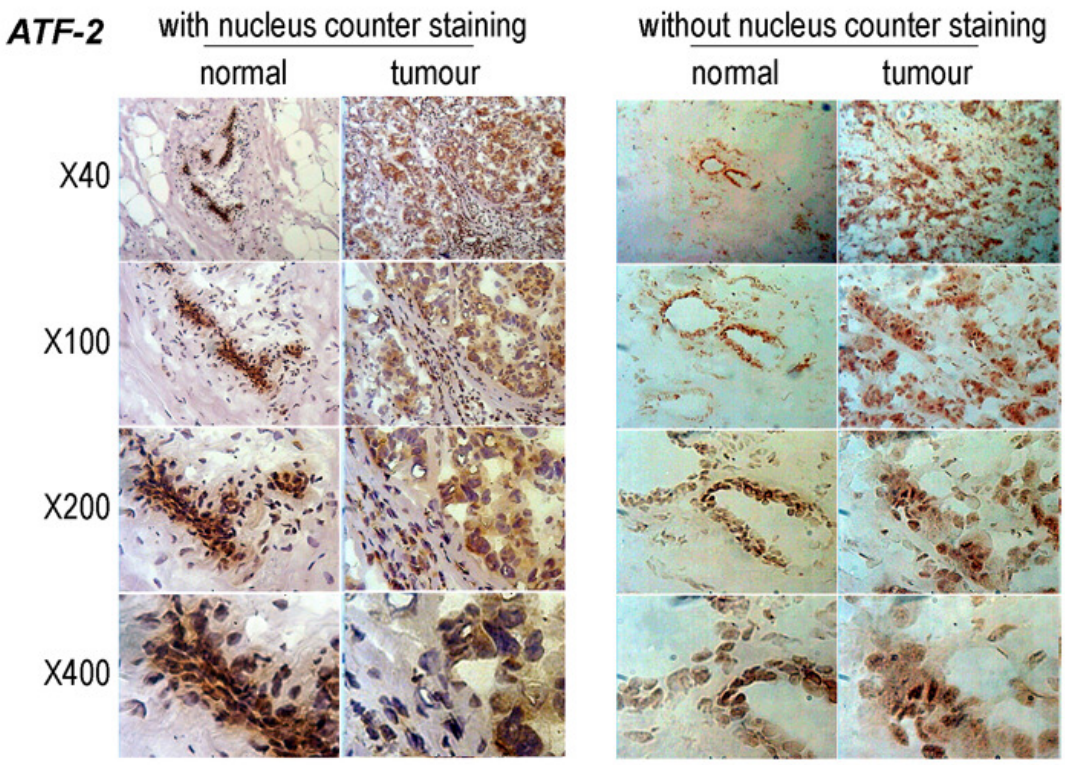


Figure 4.13: Immunohistochemical staining of ATF 2 in normal mammary and tumour tissue

Shown are images from non-counter stained (right panel) and counterstained sections (left panel).

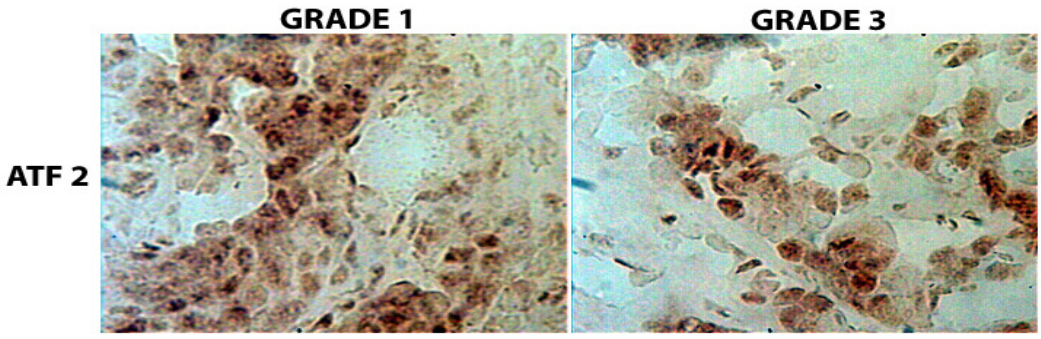


Figure 4.14: ATF 2 immunostaining in breast cancer tissue

Grade 1 tumour displayed strong immunoreactivity for ATF 2 as compared to Grade 3 tumour which showed weak immunostaining.

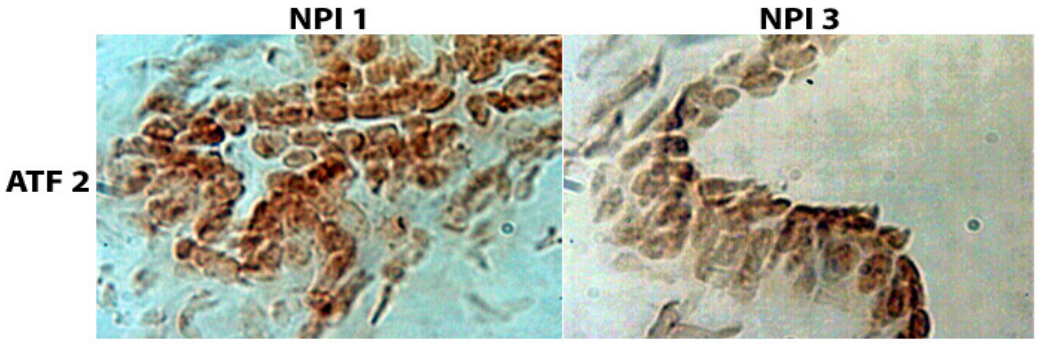


Figure 4.15: ATF 2 immunostaining in breast cancer tissue

NPI 1 tumour displayed strong immunoreactivity for ATF 2 as compared to NPI 3 tumour which showed weak immunostaining.

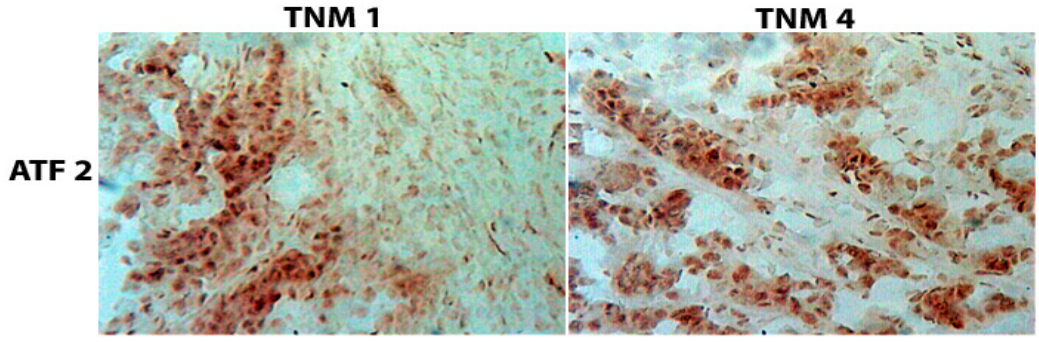


Figure 4.16: ATF 2 immunostaining in breast cancer tissue

TNM 1 tumour displayed strong immunoreactivity for ATF 2 as compared to TNM 4 tumour which showed weak immunostaining.

Table 4.14 Immunohistochemistry staining scores of ATF 3 in normal and cancer tissue specimen

ATF 3	0	+1	+2	+3
Normal	6	2	0	0
Cancer	0	2	3	3

Table 4.15 Immunohistochemistry staining scores of ATF 3 in relation to tumour grade, TNM stage and Nottingham Prognostic Index (NPI)

ATF 3	Background				Cancer			
IHC	0	+1	+2	+3	0	+1	+2	+3
GRADE 1 n = 3	0	0	0	3	0	0	1	2
GRADE 2 n = 2	0	1	1	0	1	0	1	0
GRADE 3 n = 3	2	1	0	0	0	2	1	0
TNM 1 n = 2	0	0	1	1	0	0	0	2
TNM 2 n = 1	0	0	0	1	0	0	0	1
TNM 3 n = 2	0	2	0	0	1	1	0	0
TNM 4 n = 3	2	1	0	0	0	3	0	0
NPI 1 n = 2	0	0	1	1	0	0	0	2
NPI 2 n = 3	2	0	1	0	1	0	0	2
NPI 3 n = 3	2	0	0	1	0	2	0	1

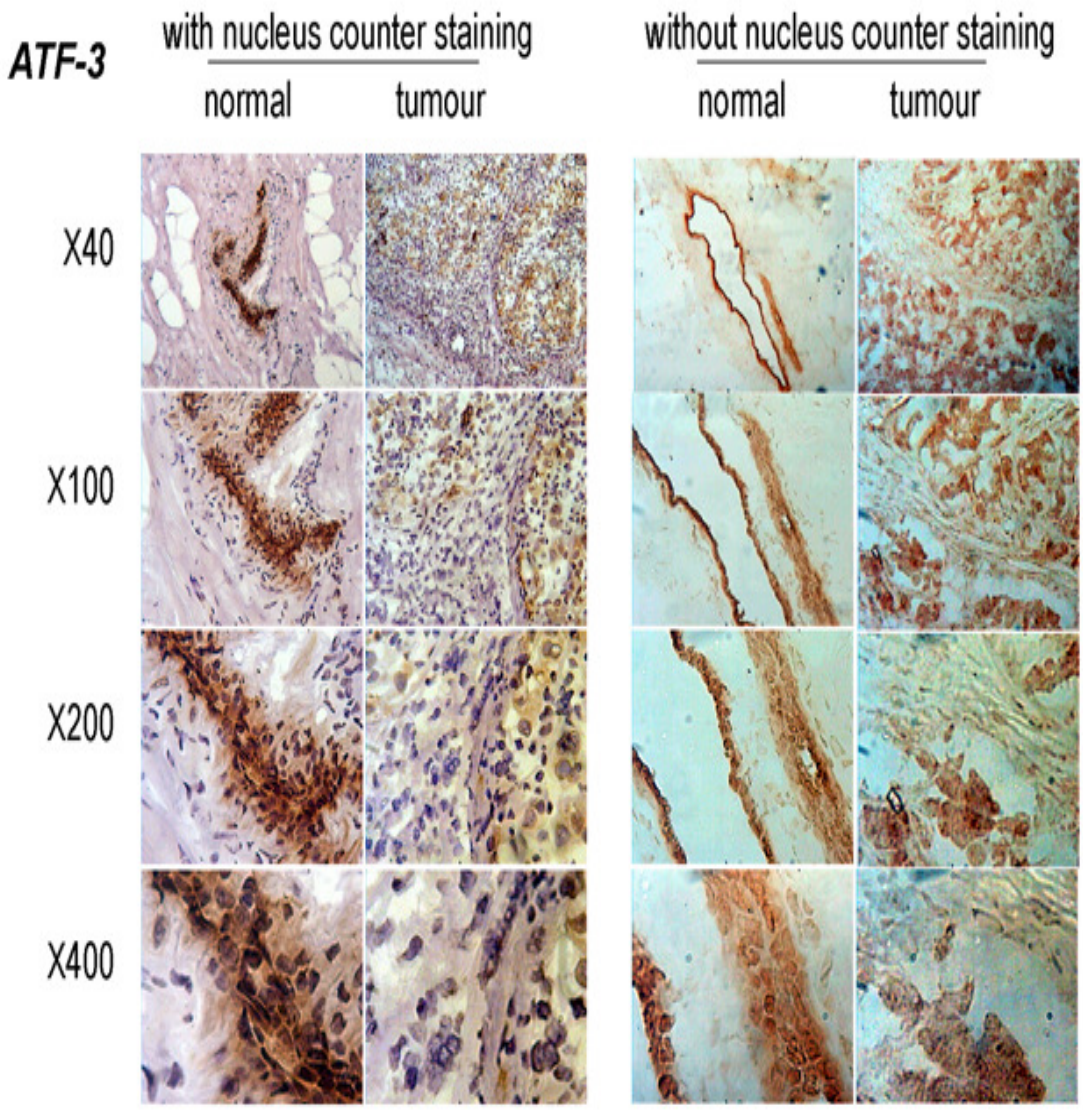


Figure 4.17: Immunohistochemical staining of ATF 3 in normal mammary and tumour tissue

Shown are images from non-counter stained (right panel) and counterstained sections (left panel).

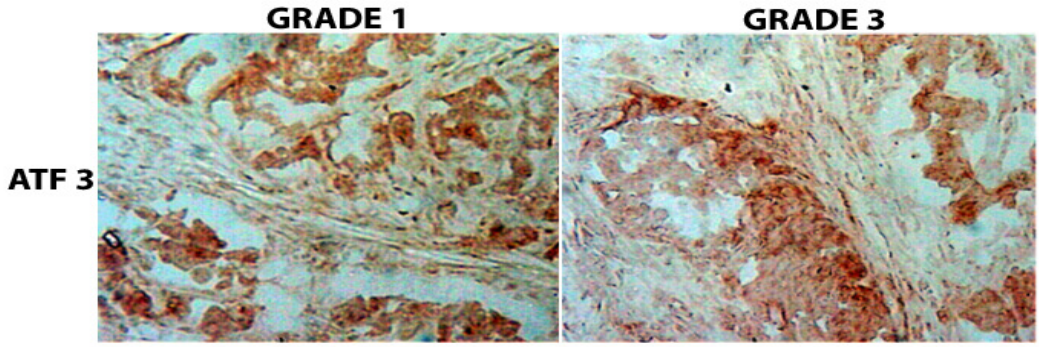


Figure 4.18: ATF 3 immunostaining in breast cancer tissue

Grade1 tumour displayed strong immunoreactivity for ATF 3 as compared to Grade 3 tumour which showed weak immunostaining.

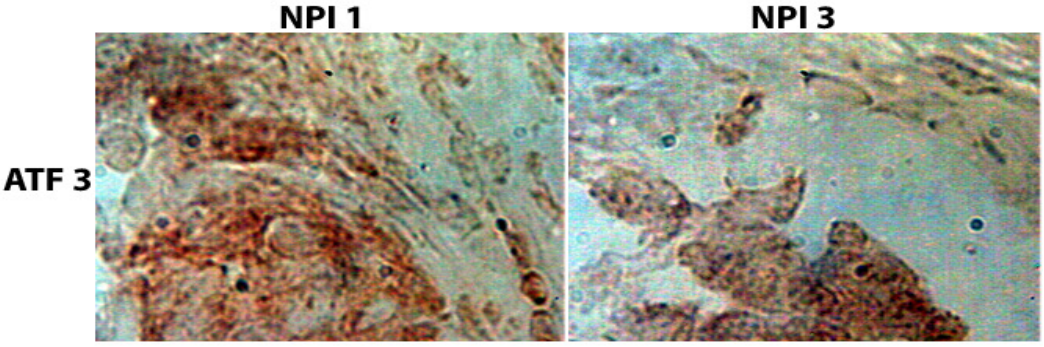


Figure 4.19: ATF 3 immunostaining in breast cancer tissue

NPI 1 tumour displayed strong immunoreactivity for ATF 3 as compared to NPI 3 tumour which showed weak immunostaining.

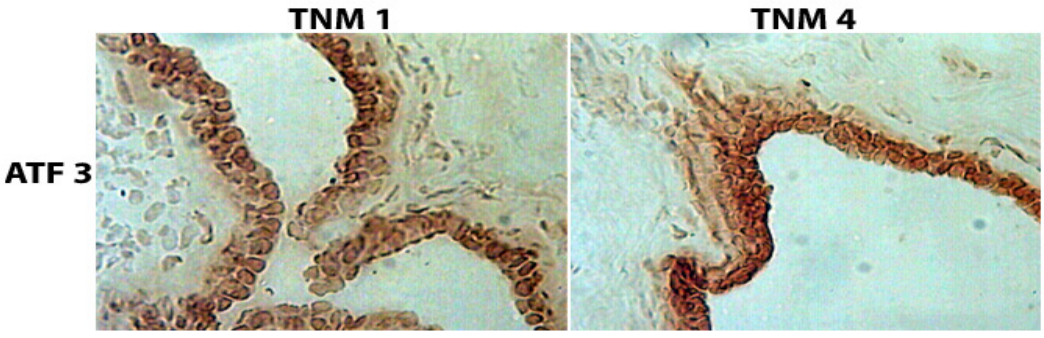


Figure 4.20: ATF 3 immunostaining in breast cancer tissue

TNM 1 tumour displayed strong immunoreactivity for ATF 3 as compared to TNM 4 tumour which showed weak immunostaining.

4.4 Discussion

The ATF family of transcription factors are involved in various cellular functions, including cell stress responses, cell survival, and cell growth. The pattern of expression of ATFs in benign and malignant solid tumours has showed that this protein behaves as both negative and positive regulatory proteins. The ATF consist of an N terminal DNA-binding domain and a C-terminal B-ZIP (basic leucine zipper) domain which binds to other B-ZIP transcription factors to form homodimers and heterodimers to either activate or repress gene transcription and therefore have potential to regulate the transcription of many target genes involved in breast cancer pathology and suppression (Vinson et al., 2002; Hai *et al.,* 1991; Hsu *et al.,* 1992).

This study has demonstrated that ATF is expressed in both tumour tissues and the normal breast adipose tissues with higher levels in tumour tissues. ATF was shown to be predominantly a nucleus protein which was highly stained in breast cancer cells nucleus compared with normal epithelial cells.

The current study reported for the first time that ATF is aberrantly expressed in human breast cancer at mRNA levels and has significant bearing to the clinical outcome of the patients. We assessed ATFs level in breast cancer tissue samples against the known prognostic factors and found significantly low levels of ATFs in patients with poor prognosis, metastatic disease and nodal involvement. The study has revealed a significant link between ATFs and mortality, in that low levels are associated with shorter disease free survival.

Similar to that of CREB1 IHC study, the number of tissues available for the immunohistological analyses were limited, which had refrained us from carrying out full statistical analysis on the semi-quantitative data. However, a future IHC study using a large sample cohort will be invaluable.

This study demonstrate that the level of ATF in breast cancer patients was found to be elevated and is significantly raised in patients with good prognosis. Breast cancer with increased expression of ATF has good prognosis which could be due to anti proliferative, apoptotic or anti invasive property of this protein as described in various studies in other human cancer cell lines. We conclude that the level of ATF expression is aberrant in human breast cancer and may be associated with good prognosis in breast cancer patients.

CHAPTER 5

KNOCKDOWN OF CREB 1 BY HAMMERHEAD RIBOZYME TRANSGENES INHIBIT CELL GROWTH AND INVASION IN BREAST CANCER CELLS

5.1 Introduction

Oestrogen synthesis is catalysed by the enzyme aromatase cytochrome P450 and plays an important role in the development of breast cancer. Hence aberrant expression of aromatase in breast cancer cells and surrounding stromal cells may have a significant influence in breast tumour pathogenesis.

Aromatase expression upregulation in breast tumours is attributed to changes in the transcriptional control of aromatase expression. It has been shown that aromatase is expressed at a higher level both at mRNA and protein level in the breast cancer cells as compared to normal breast tissue (Esteban *et al.,* 1992; Santen *et al.,* 1994). It is known that in normal mammary adipose tissue aromatase is expressed through the use of promoter 1.4 (Mahendroo *et al.,* 1993; Zhao *et al.,* 1995) and in tumour tissue promoter II (Zhou et al., 1996) and promoter I.3 (Agarwal *et al.,* 1996; Zhou *et al.,* 1996) directs aromatase expression..

In normal mammary tissues, Snail and Slug are expressed and prevents activators from binding to CREaro. This results in a suppression of promoter I.3 activity. In cancer tissue, human aromatase promoter usage switches to promoter I.3, activated by CRE binding proteins like CREB1 and also due to decrease in Snail and Slug expression. This shows that cAMP plays a critical role in upregulating the activity of promoters I.3 and II, leading to an increase in the expression of aromatase and hence oestrogen in breast cancer tissue.

Suppression of *oestrogen* synthesis can be achieved by the prevention of aromatase expression or by the inhibition of aromatase activity in breast tumour tissues. It has been demonstrated that there is a switch in the aromatase promoter usage from a

glucocorticoid-stimulated promoter I.4 in normal tissue to cAMP-stimulated promoters I.3 and II in cancerous tissue.

Although there is no study which have shown any direct link between CREB expression and breast cancer, CREB may be involved in tumour growth and metastasis through increased expression of aromatase. It has been reported that cAMP Response Element-Binding Proteins (CREB) may act as a positive transcription regulator of aromatase and hence increased expression and oestrogen synthesis in breast cancer cells (Sofi *et al.,* 2003).

5.2 Materials and Methods

5.2.1 Materials

Polyclonal goat anti CREB 1 IgG and monoclonal mouse anti Actin were obtained from Santa Cruz Biotechnology (Santa Cruz, USA). Peroxidase conjugated anti- goat and anti-mouse IgG for western blotting were purchased from Sigma (Poole, Dorset UK). MDA-MB-231 cells were grown and maintained in DMEM medium with 10% foetal calf serum and antibiotics.

5.2.2 Construction of ribozyme transgenes targeting at CREB 1

A hammerhead ribozyme for CREB 1 was designed based on their secondary structure (Figure 5.1) using Zuker's RNA mFold programme (Zuker *et al.,* 2003). Based on the cleavage site of target gene and the sequence of hammerhead ribozyme, the primers were designed (Table 5.1). Hammerhead ribozyme and antisense were pre-arranged so that correctly oriented ribozymes were amplified.

The respective ribozymes were synthesised using touchdown PCR. The ribozymes thus synthesised carried an overhang at the 3' end of the product. The ribozyme was then cloned into pEF6/V5- His- TOPO plasmid vector (Invitrogen, Paisley, UK) as described earlier in chapter 2. Following this the vector was then transformed in E.Coli using electroporation. The transformed E.Coli was then incubated for 60 minutes in SOC medium inorder to allow the bacteria to develop resistance to the antibiotic. This was then plated and incubated overnight at 37ºC. Following this the clones were analysed using direction specific PCR to verify the presence of the ribozymes in the clone. The colony with correctly oriented ribozyme insert was identified using two different PCR reactions.

PCR reactions were carried out using T7F coupled with RBBMR primer and RBTPF respectively (Table 5.1). Colonies with positive T7F/RBBMR and negative T7F/ RBTPF were regarded as the colonies with correctly oriented ribozymes insert. These colonies were then selected and cultured in LB medium containing ampicillin. Following this the plasmid was extracted and verified using DNA electrophoresis.

Table 5.1: Primers for synthesis of CREB 1 ribozymes

Ribozyme	Name of Primer	Sequence of Primers
	T7F	5'-TAATACGACTCACTATAGGG
	RBBMR	5'-TTCGTCCTCACGGACTCATCAG
	RBTPF	5'-CTGATGAGTCCGTGAGGACGAA
CREB 1 ribozyme-1	CREB RIB1F	5'-CTGCAGAGTTCAGGGAAGAACATGGAGACTGATGAGTCCGTGAGGA
	CREB RIB1R	5'-ACTAGTCCAGACCAAGATAGAACCTGTTTCGTCCTCACGGACT
CREB 1 ribozyme-2	CREB RIB2F	5'-CTGCAGGGATGGGCCCATGCTGATCTGCTGATGAGTCCGTGAGGA
	CREB RIB2R	5'-ACTAGTCCCAGCCCCCTCACTTGGACCTGTTTCGTCCTCACGGACT

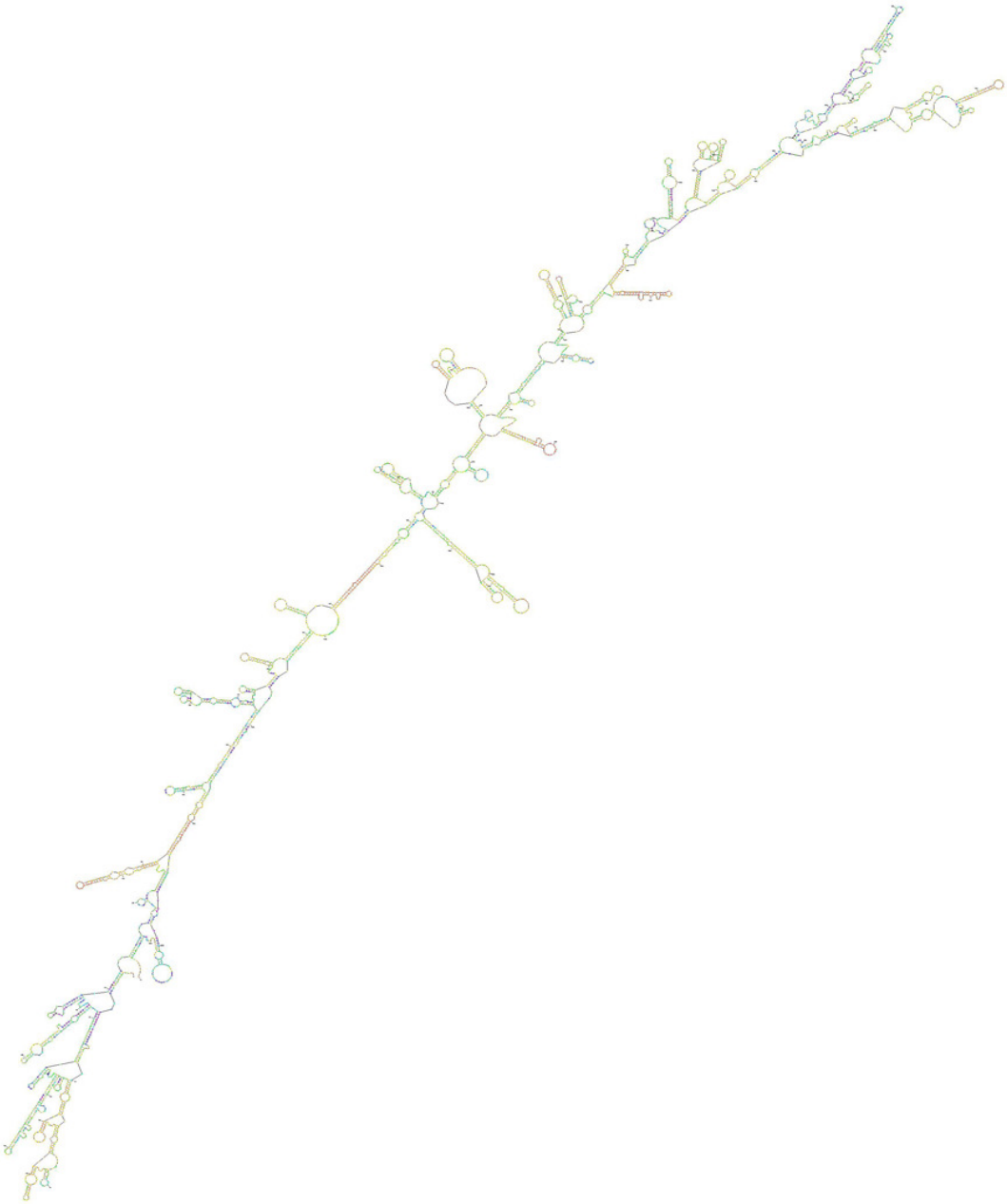


Figure 5.1: The predicted secondary structure of the CREB 1 mRNA transcript.

Computed using mFOLD method at: http://mfold.rna.albany.edu.Folding ID 709981.

5.2.3 Transfection of Breast Cancer cells and establishment of the stable transfectants

Ribozyme transgene and empty plasmid vectors were transfected using the electroporator (Easyjet Plus, Equibio Ltd., UK) into MDA-MB-231 breast cancer cell lines. The cells were then immediately transferred into the 25 cm3 culture flask containing 5ml of prewarmed culture medium and incubated. Selection of the transfected cells was then carried out once the cells reached 50% -70% confluent using the selection medium containing the antibiotic. Following this the presence of the ribozyme and its effect on the expression of the respective target gene transcript was verified using RT-PCR. Verified transfectant were then cultured in the maintenance medium containing antibiotic (2 weeks for G418 and 1 week for blasticidin) to obtain sufficient cells for further experimental studies.

5.2.4 Synthesis of complementary DNA and RT–PCR for CREB 1

cDNA was synthesised by reverse transcription using a Dura Script RT-PCR Kit (Sigma) according to manufacturer's instructions as described in chapter 2.

The reaction conditions were as follows: 5 min at 95°C, 20 seconds at 94°C, 25 seconds at 56°C and 50 seconds at 72°C for 36 cycles followed by final extension phase of 7 minutes at 72°C. The PCR products were then separated on a 0.8% agarose gel, stained using ethidium bromide and visualized under UV lights.

5.2.5 Western blot analysis of CREB 1

Cells were lysed and after extraction protein concentrations were measured as described in chapter 2 using a Bio-Rad DC protein Assay Kit (Bio-Rad laboratories, UK). Briefly the protein samples were mixed with a sample buffer containing SDS, glycerol and a loading dye. Following electrophoresis proteins were blotted onto nitrocellulose membrane. This was followed by probing with specific primary antibody (anti- CREB 1, 1:250) and corresponding peroxidase – conjugated secondary antibodies (1:1000). Protein bands were then visualised using a Supersignal TM West Dura System (Pierce Biotechnology, USA).

5.2.6 *In vitro* Functional Assays

5.2.6.1 *In vitro* cell growth assay

The method used for growth assay was previously been reported by Jiang *et al*. 2005. Cells were detached from the culture flask and cell density per ml determined. Cells were then seeded into a 96 well plate equally (2500cells/well in 200µl of normal medium) (Nunclon, Fisher Scientific, UK). Three sets of plates were incubated for 1, 3 and 5 days. Following this the medium was removed on the day of plating and the cells were fixed in 10% formaldehyde for 30 minutes. This was followed by staining in 0.5% (w/v) crystal violet for 5 minutes. The cell density was then determined by measuring the absorbance at a wavelength of 540nm by using spectrophotometer (Bio-Tek, Wolf Laboratories, UK). Cell Growth was determined as percentage increase and calculated by comparing the colorimetric absorbance obtained for each incubation period as follows:

Percentage increase = (Day 3 or Day 5 absorbance) - Day 1absorbance/ Day 1 absorbance.

Three independent experiments were performed with and without aromatase inhibitors.

5.2.6.2 *In vitro* invasion assay

The invasiveness of the breast cancer cells used in this study was determined using an *In vitro* Matrigel invasion assay model. The technique used during this study is described below.

Twenty four well pre-chilled Cell culture inserts (Becton-Dickinson Biosciences, Oxford, UK) containing 8µm pores were coated in 50µg of Matrigel. 100µl (50µg) of working Matrigel solution was added to these cell culture inserts and was then allowed to set and incubated at 45ºC in a drying oven. The inserts were then placed into twenty four well plates and the Matrigel layers were rehydrated by incubating at room temperature for 60 minutes using 300µl of sterile water. Following rehydration, the water was aspirated from each insert. Cell suspensions (15000 cells in 200µl of normal medium) were then added to each insert and incubated for upto 72 hours at 37ºC. The cells which had invaded into the Matrigel and migrated through the porous membrane to the underside of the insert were then fixed in 4% formaldehyde for 10 minutes at room temperature. Following this the cells were washed using distilled water and stained in 0.5 % crystal violet for 10 minutes at room temperature. The cells were then air dried and the numbers of invading cells (random fields) were counted using a light microscope.

5.3 Results

5.3.1 Effect of ribozyme transgenes on mRNA level of CREB 1 in MDA-MB-231 cells

As shown earlier CREB 1 was highly expressed in the breast cancer cells. We transfected MDA-MB-231 cells with empty plasmids and the ribozyme transgenes which target CREB 1. The expression of CREB 1 mRNA was significantly reduced in the ribozyme transgene transfected cells (MDA-MB-231$^{\Delta CREB\ 1}$ and MDA-MB-231$^{\Delta CREB\ 2}$) as compared to the level of expression seen in wild type (MDA-MB-231^{WT}) cells and MDA-MB-231 empty plasmid (MDA-MB-231$^{pEF/His}$) cells (Figure5.2).

5.3.2 Effect of ribozyme transgenes on the expression of CREB 1 protein in MDA-MB-231 cells

Using western blot technique we found that the expression of CREB 1 protein was significantly reduced in the ribozyme knockdown cells as compared to the wild type (MDA-MB-231^{WT}) cells and MDA-MB-231 empty plasmid (MDA-MB-231$^{pEF/His}$) cells (Figure 5.3).

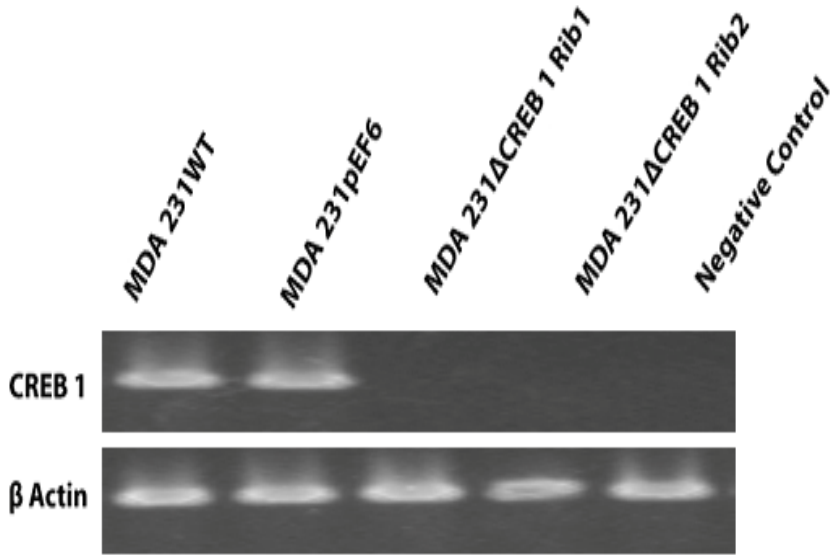


Figure 5.2: RT-PCR for mRNA level of CREB 1 in MDA-MB-231 cells following knock down using ribozyme transgene.

The gene transcripts of CREB 1 was significantly reduced in the MDA-MB-231ΔCREB 1 Rib1 and MDA-MB-231ΔCREB 1 Rib2 as compared to MDA-MB-231WT and MDA-MB-231pEF6. (Shown are representative of three separate PCR reactions).

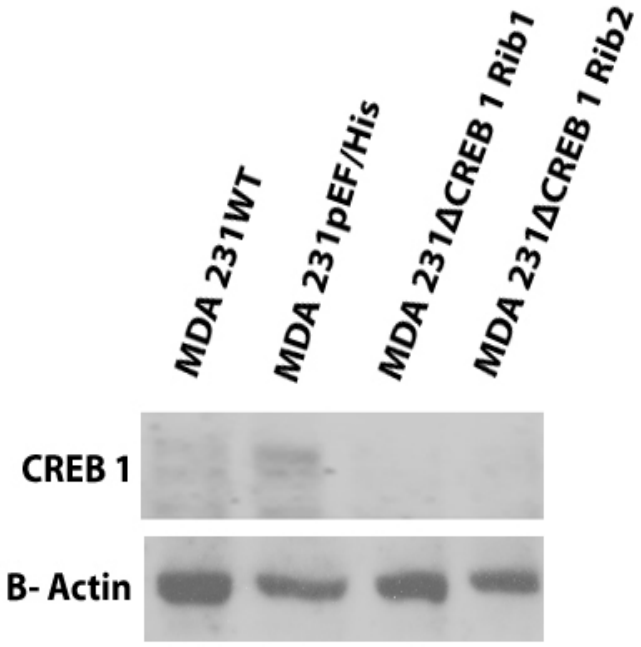


Figure 5.3: Western blot analysis for protein production of CREB 1 in MDA-MB-231 cells following knockdown using ribozyme transgene.

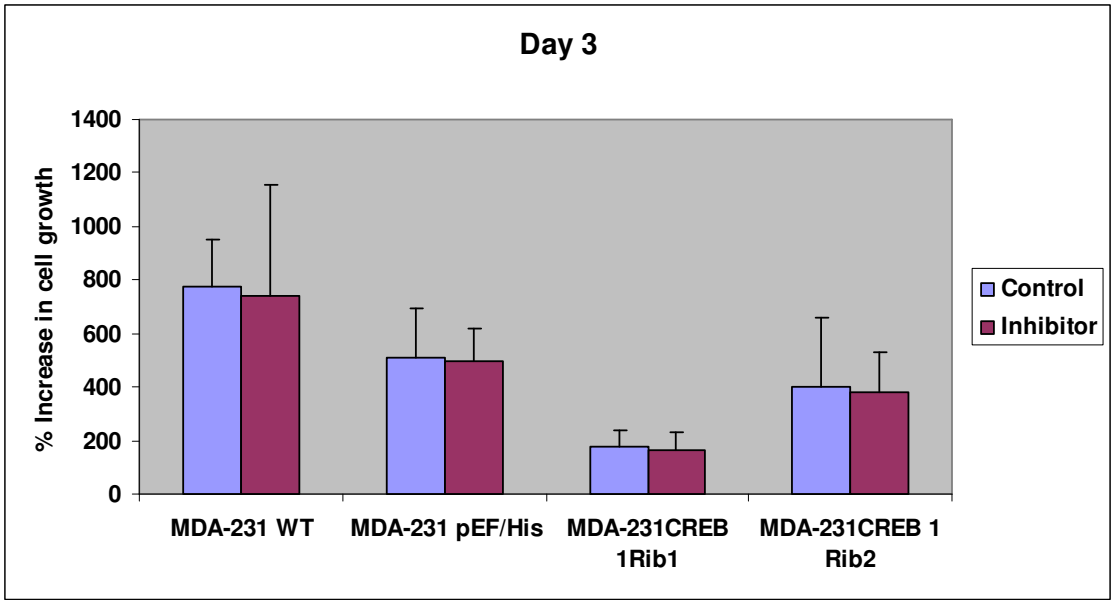
The protein production of CREB1 is eliminated in the MDA-MB-231ΔCREB 1 Rib1 and MDA-MB-231ΔCREB 1 Rib2 as compared to MDA-MB-231WT and MDA-MB-231pEF6. 15µg protein of each sample was used to load for SDG- PAGE. (Shown are representative of three separate experiments).

5.3.3 Effect of CREB 1 knockdown on cell growth in breast cancer

We examined the influence on cell growth at day 3 and day 5 after knockdown of CREB 1 in MDA-MB-231 cells with and without aromatase inhibitors. Cell growth following knockdown of CREB 1 was significantly reduced as compared to WT and pEF/His cells (Table 5.3 & 5.5; Figure 5.4 & 5.5). The cell growth of knockdown cells is further reduced following treatment with aromatase inhibitors (Table 5.2 & 5.4; Figure 5.4 & 5.5).

5.3.4 Effect of CREB 1 knockdown on invasiveness in breast cancer

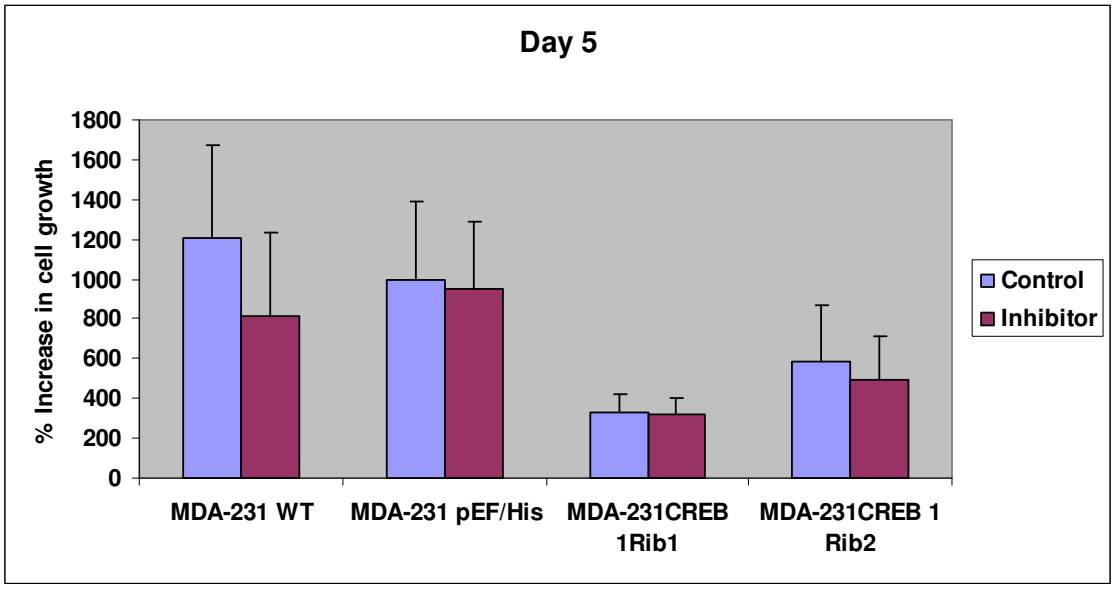
We further studied the effect on invasiveness following the elimination of CREB 1 in MDA-MB-231 cells. There was significant decreased in the invasiveness of the MDA-MB-231$^{\Delta CREB\ 1\ Rib1}$ and MDA-MB-231$^{\Delta CREB\ 1\ Rib2}$ cells as compared to WT (MDA-MB-231^{WT}) and vector control (MDA-MB-231^{pEF6}) cells (Table 5.6- 5.9; Figure 5.6- 5.10). The invasiveness of knockdown cells is further reduced following treatment with aromatase inhibitors.



Day 3	Control (n=6) Mean ± SD	Inhibitor (n=6) Mean ± SD
MDA-MB-231^{WT}	774.49 ± 176.79	742.34 ± 411.17
MDA-MB-231$^{pEF/His}$	506.52 ± 184.26	495.51 ± 124.90
MDA-MB-231$^{\Delta CREB\ Rib\ 1}$	174.01 ± 63.86	164.90 ± 65.54
MDA-MB-231$^{\Delta CREB\ Rib\ 2}$	400.53 ± 258.44	379.81± 151.13

Figure 5.4: Effect on cell growth of MDA-MB-231 cells following knockdown of CREB 1 using *in vitro* cell growth assay with and without aromatase inhibitor (Day 3).

The error bar represents standard deviation, n=6. This showed a significant decrease in the growth of the breast cancer cells following knockdown of CREB 1 as compared to the WT and pEF/His plasmid control cell lines ($P<0.01$ without aromatase inhibitor) and with aromatase inhibitor the data showed statistically significant decrease in the growth as compared to pEF/His plasmid control cell lines ($P<0.01$) but interestingly not with MDA-MB-231^{WT} cell lines



Day 5	Control (n=6) Mean ± SD	Inhibitor (n=6) Mean ± SD
MDA-MB-231 ^{WT}	1205.00 ± 468.56	813.57 ± 415.80
MDA-MB-231 $^{pEF/His}$	996.59 ± 392.80	954.15 ± 337.30
MDA-MB-231 $^{\Delta CREB\ Rib\ 1}$	327.72 ± 90.45	323.51 ± 74.46
MDA-MB-231 $^{\Delta CREB\ Rib2}$	585.27 ± 282.74	490.43 ± 226.11

Figure 5.5: Effect on cell growth of MDA-MB-231 cells following knockdown of CREB 1 using *in vitro* cell growth assay with and without aromatase inhibitor (Day 5).

The error bar represents standard deviation, n=6. This showed a significant decrease in the growth of the breast cancer cells following knockdown of CREB 1 as compared to the WT and pEF/His plasmid control cell lines ($P < 0.05$ with aromatase inhibitor, $P<0.01$ without aromatase inhibitor).

Table 5.2: Raw data for the *in vitro* invasion assays following knockdown of CREB 1 in MDA231 breast cancer cell line treated with General Medium only

Data for the individual repeats are depicted along with mean, standard deviation (StDev), number of repeats (= n), square root of n (SQRT) and standard error for the mean (SEM).

	MDA-MB-231^{WT}	MDA-MB-231^{pEF6}	MDA-MB-231$^{CREB\ 1\ Rib1}$	MDA-MB-231$^{CREB\ 1\ Rib2}$
	46	42	22	24
Medium	41	40	23	19
	40	41	21	23
Mean	42.33	41	22	22
StDev	3.21	1	1	2.65
n	3	3	3	3
SQRT	1.73	1.73	1.73	1.73
SEM	1.9	0.58	0.58	1.5

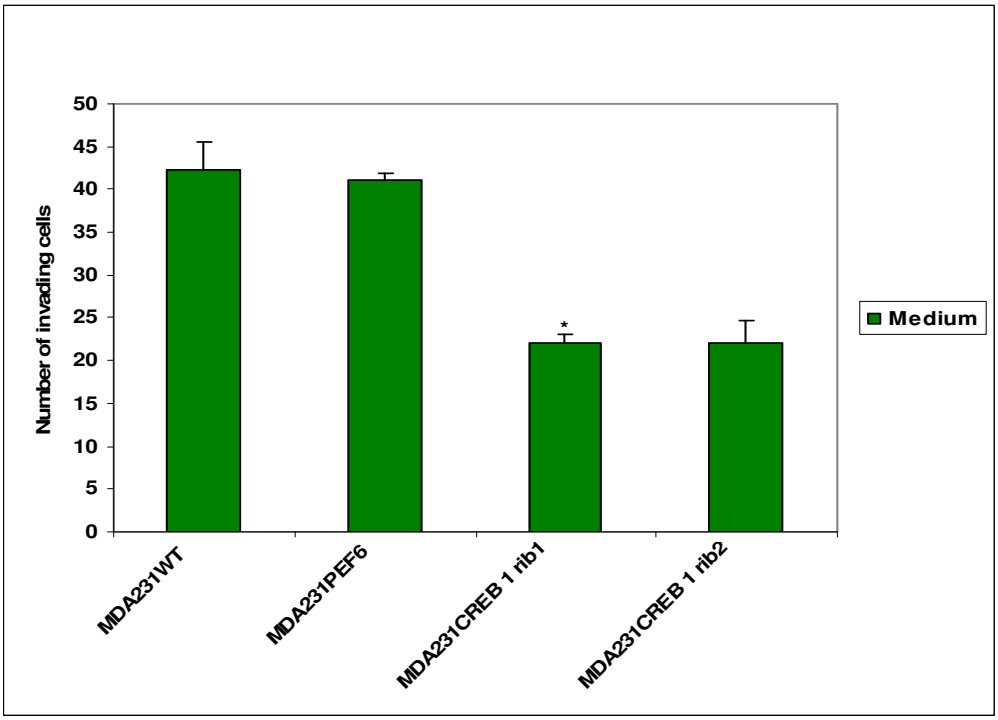


Figure 5.6: Influence on the invasiveness of MDA-MB-231 breast cancer cells treated with general medium, following knockdown of CREB 1 using in vitro invasive assay.

The Error Bars represent standard deviation, n=3. This showed a significant decrease in the invasiveness of the breast cancer cells following knockdown of CREB 1 as compared to the WT and pEF6 plasmid control cell lines (* represent $P \leq 0.01$).

Table 5.3: Raw data for the *in vitro* invasion assays following knockdown of CREB 1 in MDA231 breast cancer cell line treated with General Medium and Aromatase Inhibitor.

Data for the individual repeats are depicted along with mean, standard deviation (StDev), number of repeats (= n), square root of n (SQRT) and standard error for the mean (SEM).

	MDA-MB-231^{WT}	MDA-MB-231^{pEF6}	MDA-MB-231$^{CREB\ 1\ Rib1}$	MDA-MB-231$^{CREB\ 1\ Rib2}$
	40	38	20	21
Medium +Aromatase Inhibitor	45	42	21	19
	41	41	19	22
Mean	42	40.33	20	20.67
StDev	2.65	2.08	1	1.53
n	3	3	3	3
SQRT	1.73	1.73	1.73	1.73
SEM	1.5	1.2	0.58	0.88

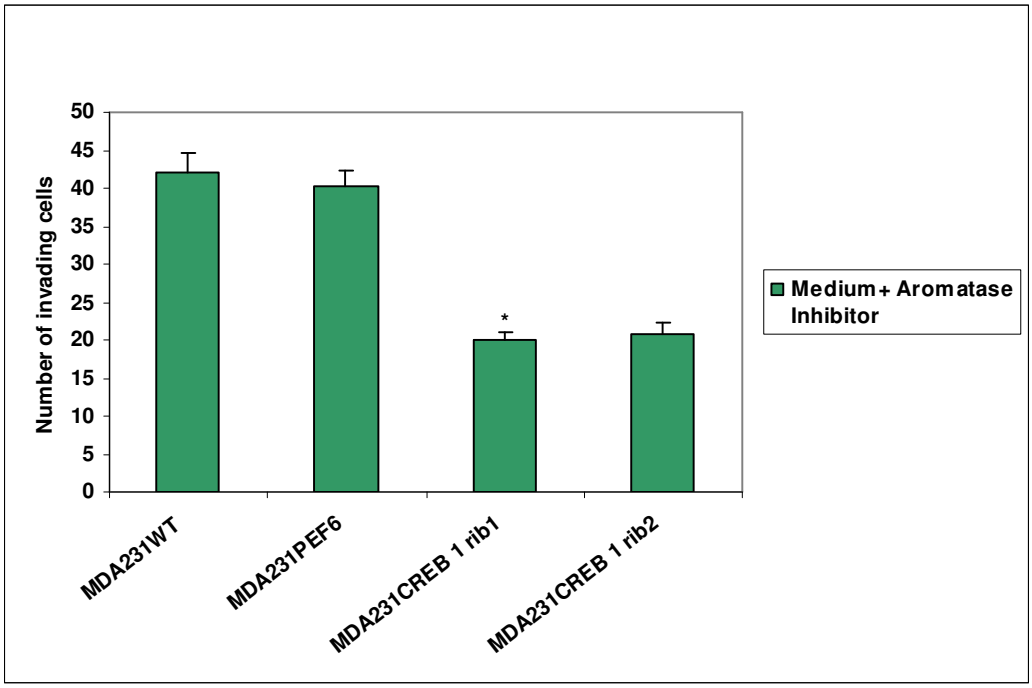


Figure 5.7: Influence on the invasiveness of MDA-MB-231 breast cancer cells treated with general medium and Aromatase Inhibitor, following knockdown of CREB 1 using *in vitro* invasive assay.

The Error Bars represent standard deviation, n=3. This showed a significant decrease in the invasiveness of the breast cancer cells following knockdown of CREB 1 as compared to the WT and pEF6 plasmid control cell lines (* represent $P \leq 0.001$).

Table 5.4: Raw data for the *in vitro* invasion assays following knockdown of CREB 1 in MDA-MB-231 breast cancer cell line treated with HGF.

Data for the individual repeats are depicted along with mean, standard deviation (StDev), number of repeats (= n), square root of n (SQRT) and standard error for the mean (SEM).

	MDA-MB-231^{WT}	MDA-MB-231^{pEF6}	MDA-MB-231$^{CREB\ 1\ Rib1}$	MDA-MB-231$^{CREB\ 1\ Rib2}$
	48	44	26	25
HGF	46	41	23	21
	43	42	23	24
Mean	45.67	42.33	24.33	23.33
StDev	2.52	1.53	1.53	2.08
n	3	3	3	3
SQRT	1.73	1.73	1.73	1.73
SEM	1.5	0.88	0.88	1.2

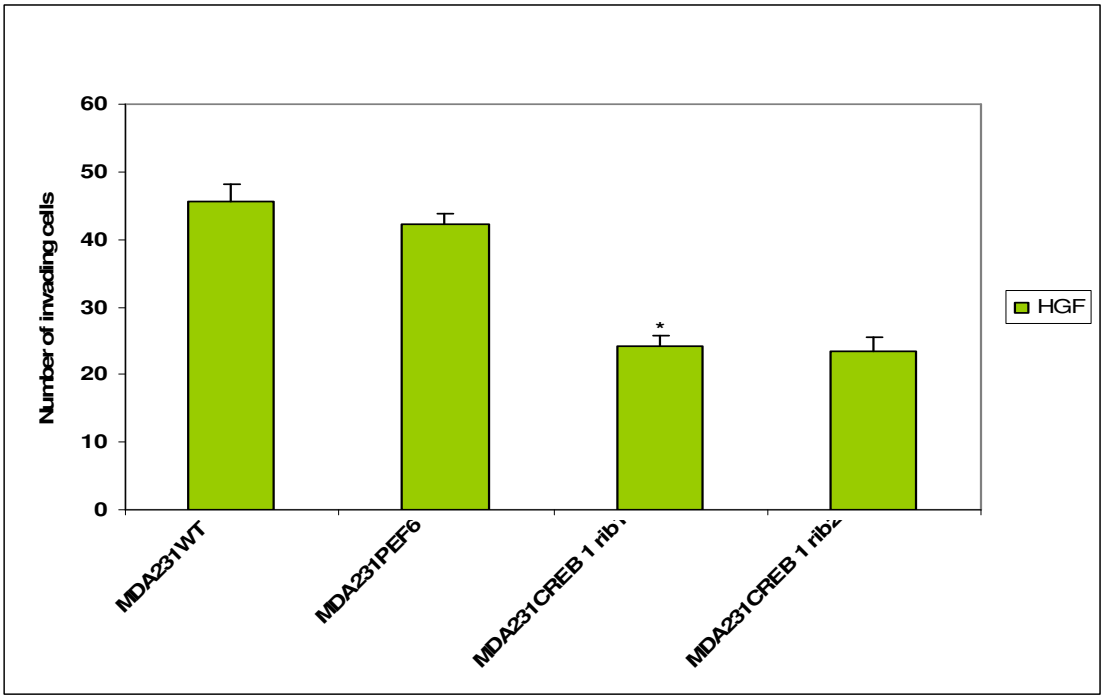


Figure 5.8: Influence on the invasiveness of MDA-MB-231 breast cancer cells treated with HGF, following knockdown of CREB 1 using in vitro invasive assay.

The Error Bars represent standard deviation, n=3. This showed a significant decrease in the invasiveness of the breast cancer cells following knockdown of CREB 1 as compared to the WT and pEF6 plasmid control cell lines (* represent $P \leq 0.01$).

Table: 5.5: Raw data for the *in vitro* invasion assays following knockdown of CREB 1 in MDA-MB-231 breast cancer cell line treated with HGF and Aromatase Inhibitor.

Data for the individual repeats are depicted along with mean, standard deviation (StDev), number of repeats (= n), square root of n (SQRT) and standard error for the mean (SEM).

	MDA-MB-231^{WT}	MDA-MB-231^{pEF6}	MDA-MB-231$^{CREB\ 1\ Rib1}$	MDA-MB-231$^{CREB\ 1\ Rib2}$
	47	39	21	23
HGF +Aromatase Inhibitor	44	45	22	19
	46	42	25	21
Mean	45.67	42	22.67	21
StDev	1.53	3	2.08	2
n	3	3	3	3
SQRT	1.73	1.73	1.73	1.73
SEM	0.88	1.7	1.2	1.2

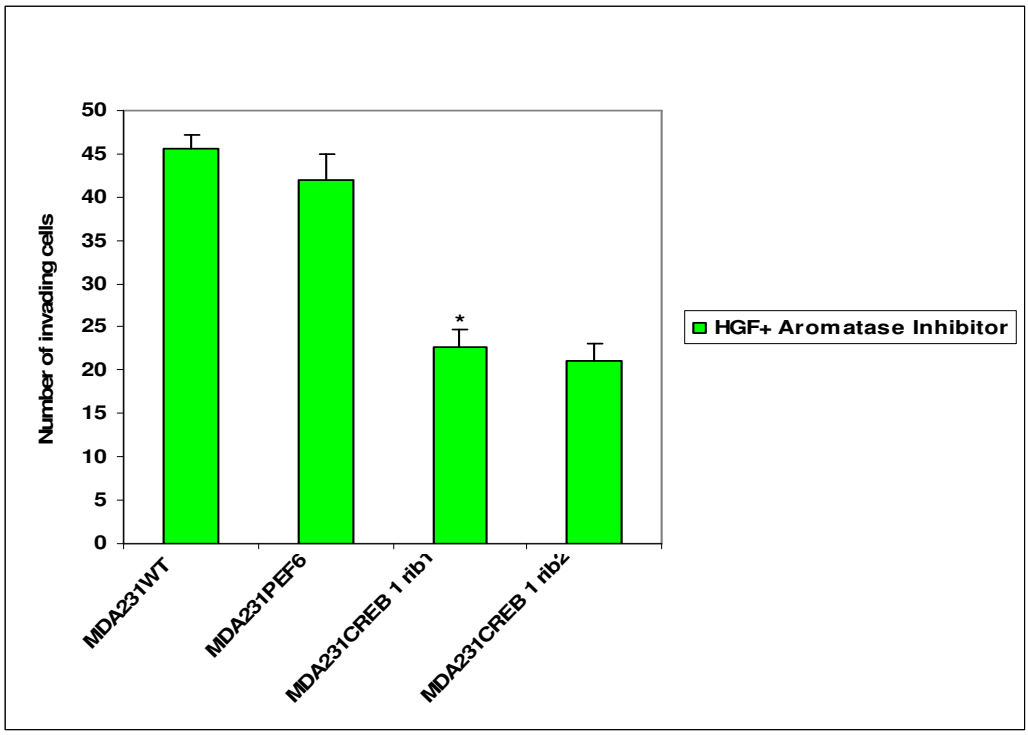
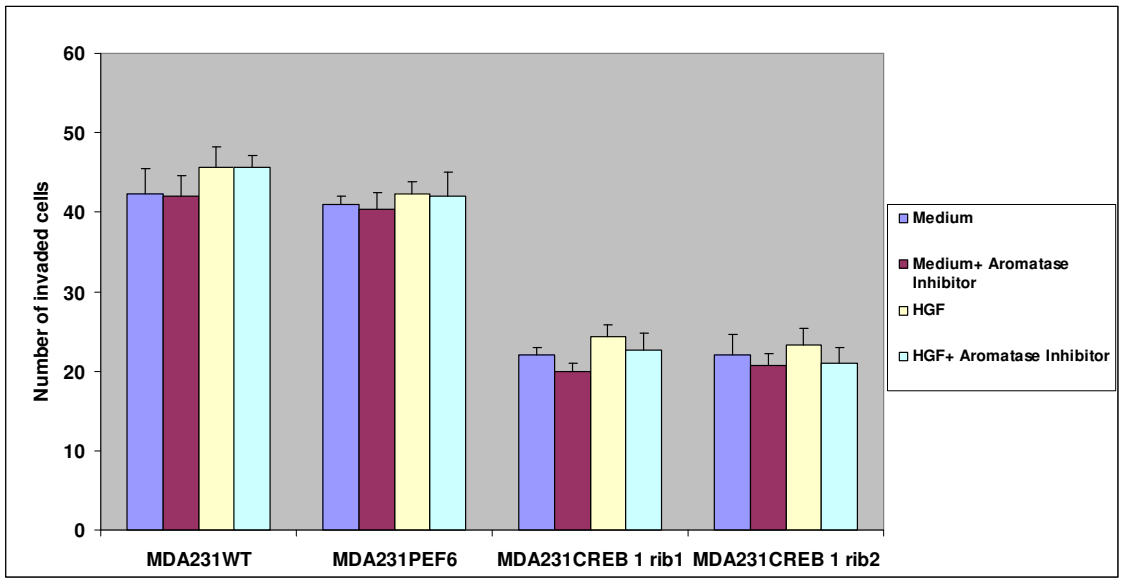
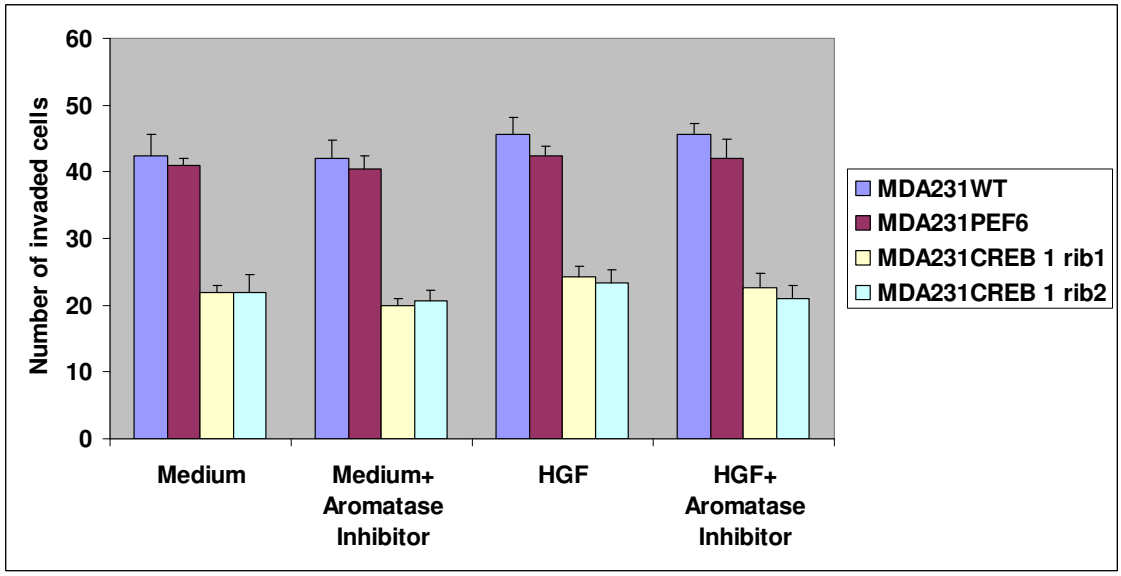


Figure 5.9: Influence on the invasiveness of MDA-MB-231 breast cancer cells treated with HGF and Aromatase Inhibitor, following knockdown of CREB 1 using *in vitro* invasive assay.

The Error Bars represent standard deviation, n=3. This showed a significant decrease in the invasiveness of the breast cancer cells following knockdown of CREB 1 as compared to the WT and pEF6 plasmid control cell lines (* represent $P \leq 0.001$).





	MDA-MB-231^{WT}	MDA-MB-231^{pEF6}	MDA-MB-231$^{CREB\ 1\ Rib1}$	MDA-MB-231$^{CREB\ 1\ Rib2}$
medium	42.33 ± 3.21	41± 1	22 ± 1	22 ± 2.65
Medium+ Aromatase Inhibitor	42 ± 2.65	40.33 ± 2.08	20 ± 1	20.67± 1.53
HGF	45.67 ± 2.52	42.33 ± 1.53	24.33± 1.53	23.33 ± 2.08
HGF+ Aromatase Inhibitor	45.67 ± 1.53	42 ± 3	22.67± 2.08	21± 2

Figure 5.10: Effect on invasiveness following knockdown of CREB 1 with and without Aromatase Inhibitor.

The Error Bar represents standard deviation, n=3. This showed a decrease in the invasiveness of the breast cancer cells following knockdown of CREB 1

5.4 Discussion

Oestrogen synthesis is catalysed by the enzyme aromatase cytochrome P450 and is the primary hormonal factor that stimulates growth of hormone-dependent breast cancer. The expression of aromatase in breast cancer cells is up regulated compared to that of surrounding non cancerous tissue and can stimulate breast tumour growth in both an autocrine and a paracrine manner (Sun *et al.,* 1997) It has been repeatedly demonstrated that aromatase is highly expressed and is directly implicated in the pathogenesis of breast cancer (Miller *et al.,* 1987; Sasano *et al.,* 1994; Lu *et al.,* 1996; Zhou *et al.,* 1996; Harada *et al.,* 1997).

Till date there is no study which has shown any direct link between CREB expression and breast cancer. CREB may be involved in tumour growth and metastasis through increased expression of aromatase. Sofi *et al*. in 2003 reported that cAMP Response Element-Binding Proteins (CREB) may act as a positive transcription regulator of aromatase and hence increased expression and oestrogen synthesis in breast cancer cells (Sofi *et al.,* 2003).

In this study we have developed the hammerhead ribozymes transgene targeting the CREB 1 breast cancer cells. These knocked down cells were then utilised to further evaluate the effects on the biological behaviours of breast cancer cells.

In this study, we utilised the hammerhead ribozymes transgene to interfere with the expression of CREB 1 after transcription. The expression of CREB 1 can be successfully down regulated by *in vitro* application of hammerhead ribozymes transgene. The ribozymes described here are highly effective and have significantly reduced the levels of CREB 1 at both protein and mRNA levels.

We examined the influence on cell growth and invasiveness after knockdown of CREB 1 in MDA-MB-231 cells with and without aromatase inhibitors. Loss of CREB 1 is associated with decreased growth and invasion potential in breast cancer cells. These demonstrated the potential positive regulatory effect of CREB 1 on cellular growth in breast cancer. There was no difference on cell growth and invasiveness between MDA-MB-231 cells treated with aromatase inhibitors when compared with cells without aromatase inhibitor. Limited by time, the present study was unable to further examine the impact of CREB1 manipulation on the expression of aromatase and the cells response to aromatase inhibitors especially in oestrogen positive cell lines, which will be an exciting research lead to pursue. It is hope that this will form a future research project.

This study showed that the growth was significantly reduced following knockdown of CREB 1 and may have independent role in the metastatic potential of breast cancer and hence have the therapeutic potential in both oestrogen positive and negative tumours.

CHAPTER 6

DISCUSSION

6.1 Discussion

Transcription factors are gene regulatory proteins and play a vital role in the process of cancer cell proliferation. Transcription factors are involved in various organismal processes like cellular differentiation, DNA replication, cell growth and apoptosis. They represent prime targets for disruption in tumourigenesis, attribute to their inherent modular structure encoding various activating or repressing cellular functions. Breast cancer results from acquired or inherited gene defects leading to genomic instability and hence abnormal expression of transcriptional factors which in turn leads to increased proliferation of transformed mammary cells (Jackson *et al.,* 1998; Loeb *et al.,* 1998).

Aberrant transcriptional activity and expression of CREB family of proteins in cancer cells and clinical tumours has long been recognised. These molecules have been extensively studied in relation to stress response, memory and in solid tumours, both benign and malignant. CREB proteins behave as both negative and positive regulatory proteins in different solid tumours. Chen et al. reported that in cancer tissue increased levels of positive regulatory proteins like ERRalpha1 and CREB1 is present as compared to negative regulatory proteins like EAR-2, COUP-TFI, Snail and Slug proteins (Chen et al., 2005). Coxon *et al.* have shown a link between an oncogenic chromosomal translocation and CREB activation in malignant salivary gland tumours (Coxon *et al., 2005)*. CREB has an antimitogenic role in solid tumours like glioblastoma proliferation and decreased expression is seen in thyroid carcinoma. Beside this ATFs has been implicated in various other malignant processes including HBV mediated hepatocarcinogenesis and melanoma (Ishiguro T et al., 2000).

Studies on any direct link between the expression of these proteins and breast cancer are yet to come. We therefore conducted this study to investigate the expression pattern of CREB family of proteins in cohort of breast cancer and normal background tissue in relation to tumour histopathological grade, stage and nodal status. We also correlate the level of expression with the prognosis and clinical outcome of the patients.

In this study, we examined the expression of these transcription factors and aromatase in breast cancer cell lines using RT-PCR. Interestingly we found increased expression of CREB family of proteins in both MDA-MB-231 and MCF 7 cell lines with minimal aromatase activity in MDA-MB-231 cell line. These results provided us with a basic assessment of the function of these proteins and helped to choose a suitable cell line for further biological functional study. We further examined the expression of these proteins in Breast specimen using immunohistochemical staining. We found that CREB is expressed in both tumour tissues and the normal breast adipose tissues with higher levels in tumour tissues. CREB was shown to be predominantly a nucleus protein which was highly stained in breast cancer cells nucleus compared with normal epithelial cells.

The current study reported for the first time that CREB 1, ATF 1, ATF 2 and ATF 3 proteins are aberrantly expressed in human breast cancer at both protein and mRNA levels and has significant bearing to the clinical outcome of the patients. We assessed CREB-1 level in breast cancer tissue samples against the known prognostic factors and found significantly raised levels of CREB1 in patients with poor prognosis, metastatic disease and nodal involvement. The study has revealed a significant link between CREB and mortality, in that high levels are associated with shorter disease free survival.

We further assessed the ATF's level in breast cancer tissue samples and demonstrated that the level of ATF in breast cancer patients was found to be elevated and is significantly raised in patients with good prognosis as contrast to the expression of CREB1. Breast cancer with increased expression of ATF has good prognosis which could be due to anti proliferate, apoptotic or anti invasive property of this protein as described in various studies in other human cancer cell lines (Ishiguro *et al.,* 2000 ; Allen and Jennings *et al.,* 2001). Oestrogen is a steroidal hormone that stimulates growth of hormone-dependent breast cancer. Oestrogen synthesis from C_{19} steroids is catalysed by the enzyme aromatase cytochrome P450 and hence aberrant expression of aromatase in breast cancer cells and surrounding stromal cells may have a significant influence in breast cancer pathogenesis. Aromatase is expressed at a higher level at mRNA and protein level in the breast cancer cells as compared to normal breast tissue (Esteban *et al.,* 1992). It has been reported previously that there is increase aromatase expression due to increase in CREB induced promoter II and promoter I.3 activity in tumour bearing breast adipose tissue via CRE1 and CRE2 (Sofi *et al.,* 2003). In normal mammary tissue, the function of promoters I.3 and II is suppressed through the binding of EAR-2, COUP-TFI, and RARγ to S1 (Yang *et al., 1*998; Yang *et al.,* 2002), and Snail/Slug proteins to CREaro (Chen *et al.,* 2002). In cancer tissue, the expression levels of EAR-2, COUP-TF1, EARγ, Snail, and Slug decreases, and aromatase expression is then up-regulated through the binding of ERRα to S1 and the binding of CREB1 to CREaro (Yang *et al.,* 1998 ; Yang *et al.,* 2002). Hence cAMP plays a critical role in upregulating the activity of promoters I.3 and II, leading to an increase of the expression of aromatase/oestrogen synthesis in breast cancer cells.

The role of CREB in relation to aromatase expression is still unclear. Over-expression of aromatase in adipose tissue surrounding breast tumour could arise through increase in both CREB expression and CREB transcriptional activity and that CREB may be involved in tumour growth and metastasis through increased expression of aromatase However, despite the extensive work on aromatase and its association with breast cancer, little information is available on the potential role of CREB in human breast cancer.

Following the examination of the expression of CREB in breast cancer cells, we successfully knocked down CREB 1in breast cancer cell line using ribozyme transgene. This is the first time a ribozyme transgene system has been used to reduce the expression of CREB 1 in breast cancer cell line. Down – regulation of the expression of CREB 1 in the MDA -231 cells following knock down by ribozyme transgene resulted in decrease cellular invasiveness and reduces the cellular growth. We further treated the knockdown cells with aromatase inhibitor and HGF. The result demonstrated that aromatase inhibitor treated cells showed further reduction in both cellular invasion and growth but was not significant as compared to untreated CREB 1 knock down cells. The results in this work showed that there is a potential direct link between CREB 1 and breast cancer progression as reported previously in various cancer cell lines like ovarian cancer cells and breast cancer cells using CRE- transcription factor decoy oligonucleotides (Lee *et al.,* 2000).

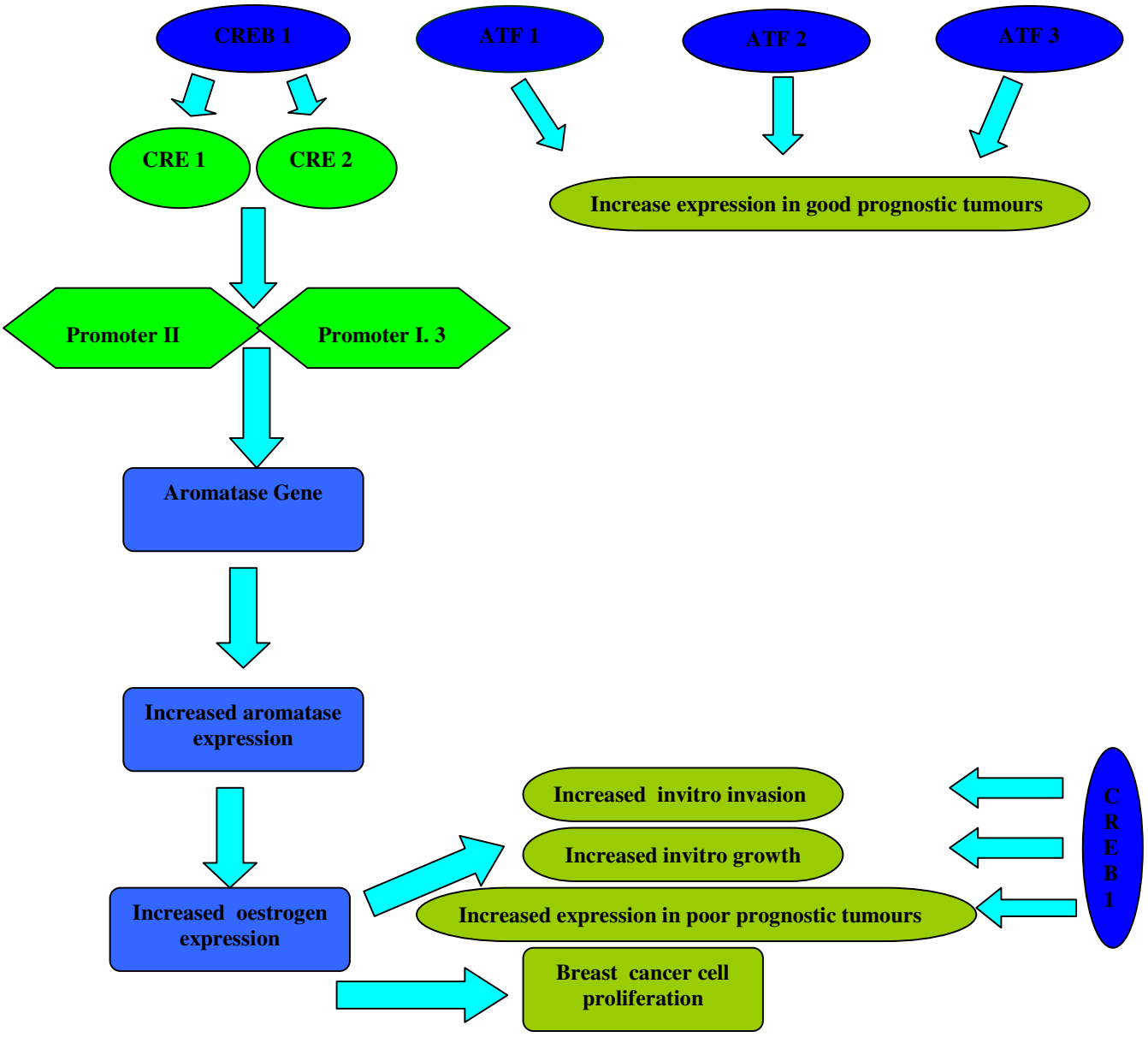


Figure 6.1: Diagrammatic presentation of CREB 1, ATF 1, ATF 2 and ATF 3 showing the expression pattern in breast cancer tissue.

CREB 1 is associated with poor prognostic tumours and is implicated in increase invasion and growth of breast cancer cells. ATF'S are associated with good prognostic tumours with decreased expression in poor prognostic breast tumours.

6.2 Prospects of future study

In this study, we have demonstrated the positive regulatory effect of CREB 1 in breast cancer cells. As explained earlier the possible mechanism whereby CREB 1 elicit changes in the biological function of breast cancer cells still remains unclear and will require further study. Furthermore the potential role of other members of this family including ATF 1, ATF 2 and ATF 3 in breast cancer needs further input.

Limited by time, we were unable to examine the impact of CREB1 manipulation on the expression of aromatase and the CREB-1 knockdown cells response to aromatase inhibitors especially in oestrogen positive cell line, which will be an exciting research lead to pursue.

Further studies needs to be done whether increased expression of CREB 1 leads to aberrant aromatase expression and whether there is simultaneous decrease in the expression of the negative regulatory proteins like Snail and Slug proteins in breast cancer tissue.

Aromatase inhibitors have been extensively used in the treatment of advanced breast cancer, both in the adjuvant and in the neo adjuvant setting and are shown to be superior to the ER antagonist. Inhibition of CREB activity could inhibit aromatase expression and hence decrease oestrogen production in breast tissue. Henceforth over-expression of CREB, together with aromatase in breast cancer tissue at both transcriptional and translational levels may provide a link between CREB pathways and breast

tumourigenesis and in future may help in the design of anti CREB therapy based on inhibiting aromatase expression as therapeutic strategies in breast cancer patients.

CHAPTER 7

BIBLIOGRAPHY

Afshari FS, C. A., Sato-Bigbee C (2001). "Effect of cyclic AMP on the expression of myelin basic protein species and myelin proteolipid protein in committed oligodendrocytes: differential involvement of the transcription factor CREB." J Neurosci Res 66(1):37-45.

Agarwal VR, B. S., Leitch M, Rohrich R, Simpson ER (1996). "Use of alternative promoters to express the aromatase cytochrome P450 (CYP19) gene in breast adipose tissues of cancer-free and breast cancer patients." J Clin Endocrinol Metab 81(11):3843-9.

Albini A, Iwamoto Y, Kleinman HK, Martin GR, Aaronson SA, Kozlowski JM, McEwan RN (1987). “A rapid in vitro assay for quantitating the invasive potential of tumor cells.”Cancer Res. 47(12):3239-45.

Allen-Jennings AE, H. M., Kociba GJ, Hai T (2001). "The roles of ATF3 in glucose homeostasis. A transgenic mouse model with liver dysfunction and defects in endocrine pancreas." J Biol Chem 276(31):29507-14.

Allen-Jennings AE, H. M., Kociba GJ, Hai T (2002). "The roles of ATF3 in liver dysfunction and the regulation of phosphoenolpyruvate carboxykinase gene expression." J Biol Chem 277(22):20020-5.

Aronica SM, K. W., Katzenellenbogen BS (1994). "Estrogen action via the cAMP signaling pathway: stimulation of adenylate cyclase and cAMP-regulated gene transcription." Proc Natl Acad Sci U S A 91(18):8517-21.

Bakker O, P. M. (1991). "CAAT/enhancer binding protein is able to bind to ATF/CRE elements." Nucleic Acids Research 19(6):1213-7.

Bardou VJ, Arpino G, Elledge RM, Osborne CK, Clark GM. (2003) “Progesterone receptor status significantly improves outcome prediction over estrogen receptor status alone for adjuvant endocrine therapy in two large breast cancer databases.” J Clin Oncol. 21(10):1973-9.

Barnabas S, H. T., Andrisani OM (1997). "The hepatitis B virus X protein enhances the DNA binding potential and transcription efficacy of bZip transcription factors." J Biol Chem 272(33):20684-90.

Barnes DM. (1993) c-erbB-2 amplification in mammary carcinoma. J Cell Biochem Suppl. 17G:132-8.

Barton K, M. N., Chanyangam M, Fischer C, Clendenin C, Leiden JM (1996). "Defective thymocyte proliferation and IL-2 production in transgenic mice expressing a dominant-negative form of CREB." Nature 379(6560):81-5.

Bayatti N, E. J. (2001). "Cyclic AMP modulates the response of central nervous system glia to fibroblast growth factor-2 by redirecting signalling pathways." J Neurochem 78(5):972-80.

Baylin SB, H. J., Graff JR, Vertino PM, Issa JP. (1998). "Alterations in DNA methylation: a fundamental aspect of neoplasia." Adv Cancer Res 72:141-96.

Belengeanu A, Mureşan A, Stoicănescu D, Lazăr E. (2010) Amplification of HER-2 gene in breast cancer: immunohistochemical and FISH assessment. Rom J Morphol Embryol. 51(2):321-6.

Benbrook DM, J. N. (1990). "Heterodimer formation between CREB and Jun proteins." Oncogene 5(3):295-302.

Blais JD, F. V., Bi M, Harding HP, Ron D, Koumenis C, Wouters BG, Bell JC (2004). "Activating transcription factor 4 is translationally regulated by hypoxic stress." Mol Cell Biol 24(17):7469-82.

Bleckmann SC, B. J., Rudolph D, Monaghan AP, Schmid W, Schütz G (2002). "Activating transcription factor 1 and CREB are important for cell survival during early mouse development." Mol Cell Biol 22(6):1919-25.

Böhlen P, Stein S, Dairman W, Udenfriend S (1973). “Fluorometric assay of proteins in the nanogram range.”Arch Biochem Biophys. 155(1):213-20

Bonni A, B. A., West AE, Datta SR, Takasu MA, Greenberg ME (1999). "Cell survival promoted by the Ras-MAPK signaling pathway by transcription-dependent and -independent mechanisms." Science 286(5443):1358-62.

Boudreaux JM, Towler DA. (1996). “Synergistic induction of osteocalcin gene expression: identification of a bipartite element conferring fibroblast growth factor 2 and cyclic AMP responsiveness in the rat osteocalcin promoter”. J Biol Chem. 271(13):7508-15.

Cancer Research UK, Breast Cancer Statistics- 2011

Campos SM, W. E. (2003). "Hormonal therapy in postmenopausal women with breast cancer." Oncology 64(4):289-99.

Cao Z, U. R., McKnight SL (1991). "Regulated expression of three C/EBP isoforms during adipose conversion of 3T3-L1 cells." Genes Dev 5(9):1538-52.

Carter CL, A. C., Henson DE (1989). "Relation of tumor size, lymph node status, and survival in 24,740 breast cancer cases." Cancer Res 1;63(1):181-7.

Catalano S, G. C., Rizza P, Gu G, Barone I, Bonofiglio D, Giordano F, Malivindi R, Gaccione D, Lanzino M, De Amicis F, Andò S (2009). "Evidence that leptin through STAT and CREB signaling enhances cyclin D1 expression and promotes human endometrial cancer proliferation." J Cell Physiol 218(3):490-500.

Chatton B, B. J., Goetz J, Gaire M, Lutz Y, Kedinger C (1994). "Jun and Fos heterodimerize with ATFa, a member of the ATF/CREB family and modulate its transcriptional activity" Oncogene 9(2):375-85.

Chen BP, W. C., Hai T (1996). "Analysis of ATF3, a transcription factor induced by physiological stresses and modulated by gadd153/Chop10." Mol Cell Biol 16(3):1157-68.

Chen BP, Liang G, Whelan J, Hai T. (1994). "ATF3 and ATF3 delta Zip. Transcriptional repression versus activation by alternatively spliced isoforms". J Biol Chem. 3; 269(22):15819-26.

Chen S, Zhou D, Yang C, Okubo T, Kinoshita Y, Yu B, Kao YC, Itoh T.(2001). "Modulation of aromatase expression in human breast tissue". J Steroid Biochem Mol Biol. 79(1-5):35-40

Chen S, Ye J, Kijima I, Kinoshita Y, Zhou D. (2005). “Positive and negative transcriptional regulation of aromatase expression in human breast cancer tissue”. J Steroid Biochem Mol Biol. 95(1-5):17-23.

Chen S, I. T., Wu K, Zhou D, Yang C (2002). "Transcriptional regulation of aromatase expression in human breast tissue." J Steroid Biochem Mol Biol 83(1-5)(93-9.).

Chen SA, B. M., Sparkes RS, Zollman S, Klisak I, Mohandas T, Hall PF, Shively JE (1988). "Human aromatase: cDNA cloning, Southern blot analysis, and assignment of the gene to chromosome 15." DNA 7(1):27-38.

Chevray PM, N. D. (1992). "Protein interaction cloning in yeast: identification of mammalian proteins that react with the leucine zipper of Jun." Proc Natl Acad Sci U S A 89(13):5789-93.

Chinenov Y, K. T. (2001). "Close encounters of many kinds: Fos-Jun interactions that mediate transcription regulatory specificity." Oncogene 20(19)(2438-52).

Chomczynski P, Sacchi N (1987). “Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction.” Anal Biochem. 162(1):156-9.

Chu HM, T. Y., Kobierski LA, Balsam LB, Comb MJ (1994). "Activating transcription factor-3 stimulates 3',5'-cyclic adenosine monophosphate-dependent gene expression." Mol Endocrinol 8(1):59-68.

Cibelli G, S. S., Thiel G (1999). "Nuclear targeting of cAMP response element binding protein 2 (CREB2)." Eur J Cell Biol 78(9):642-9.

Clemons M, Danson S, Howell A (2002). "Tamoxifen (Nolvadox): A Review". Cancer Treat. Rev. 28 (4): 165–180

Coxon A, R. E., Park YS, Joshi N, Tsurutani J, Dennis PA, Kirsch IR, Kaye FJ. (2005). "Mect1-Maml2 fusion oncogene linked to the aberrant activation of cyclic AMP/CREB regulated genes." Cancer Res 65(16): 7137-44.

De Cesare D, F. G., Sassone-Corsi P (1999). "Signaling routes to CREM and CREB: plasticity in transcriptional activation." Trends Biochem Sci 24(7):281-5.

De Cesare D, Sassone-Corsi P (2000). "Transcriptional regulation by cyclic AMP-responsive factors." Prog Nucleic Acid Res Mol Biol 64:343-69.

Deng X, L. H., Huang J, Cheng L, Keller ET, Parsons SJ, Hu CD (2008). "Ionizing radiation induces prostate cancer neuroendocrine differentiation through interplay of CREB and ATF2: implications for disease progression." Cancer Res 68(23):9663-70.

Deroo BJ, Korach KS. (2006) “Estrogen receptors and human disease.” J Clin Invest. 116(3):561-70

Deutsch PJ, H. J., Jameson JL, Lin JC, Habener JF (1988). "Structural determinants for transcriptional activation by cAMP-responsive DNA elements." Biol Chem 263(34): p. 18466-72.

Dowsett M, Allred C, Knox J, Quinn E, Salter J, Wale C, Cuzick J, Houghton J, Williams N, Mallon E, Bishop H, Ellis I, Larsimont D, Sasano H, Carder P, Cussac AL, Knox F, Speirs V, Forbes J, Buzdar A. (2008) “Relationship between quantitative estrogen and progesterone receptor expression and human epidermal growth factor receptor 2 (HER-2) status with recurrence in the Arimidex, Tamoxifen, Alone or in Combination trial.” J Clin Oncol. 26(7):1059-65.

Dronadula N, R. F., Blaskova E, Li Q, Rao GN (2006). "Involvement of cAMP-response element binding protein-1 in arachidonic acid-induced vascular smooth muscle cell motility." J Lipid Res 47(4):767-77.

Drysdale BE, Howard DL, Johnson RJ. (1996). “Identification of a lipopolysaccharide inducible transcription factor in murine macrophages”. Mol Immunol. 33(11-12):989-98.

Du K, M. M. (1998). "CREB is a regulatory target for the protein kinase Akt/PKB." J Biol Chem 273(49):32377-9.

Eccles SA. (2011)“The epidermal growth factor receptor/Erb-B/HER family in normal and malignant breast biology.” Int J Dev Biol. 55(7-9):685-96.

Escot C, T. C., Lidereau R, Spyratos F, Champeme MH, Gest J, Callahan R. (1986). "Genetic alteration of the c-myc protooncogene (MYC) in human primary breast carcinomas." Proc Natl Acad Sci U S A 83(13):4834-8.

Esteban JM, W. Z., Haniu M, Hall P, Shively JE, Chen S (1992). " Detection of intratumoral aromatase in breast carcinomas. An immunohistochemical study with clinicopathologic correlation." Am J Pathol 140(2): p. 337-43.

Estes SD, S. D., Anderson GR (1995). "Normal fibroblasts induce the C/EBP beta and ATF-4 bZIP transcription factors in response to anoxia." Exp Cell Res 220(1):47-54.

Estes SD, S. D., Anderson GR (1995). "Normal fibroblasts induce the C/EBP beta and ATF-4 bZIP transcription factors in response to anoxia." Exp Cell Res 220(1):47-54.

Fawcett TW, M. J., Guyton KZ, Hai T, Holbrook NJ (1999). "Biochem JComplexes containing activating transcription factor (ATF)/cAMP-responsive-element-binding protein (CREB) interact with the CCAAT/enhancer-binding protein (C/EBP)-ATF composite site to regulate Gadd153 expression during the stress response." 339 (Pt 1):135-41.

Fisher B, B. M., Wickerham DL, Redmond CK, Fisher ER, Cruz AB, Foster R, Gardner B, Lerner H, Margolese R (1983). "Relation of number of positive axillary nodes to the prognosis of patients with primary breast cancer. An NSABP update." Cancer Biol Ther 52(9):1551-7.

Fisher ER, A. S., Redmond C, Fisher B (1992). "Ipsilateral breast tumor recurrence and survival following lumpectomy and irradiation: pathological findings from NSABP protocol B-06." Semin Surg Oncol 8(3):161-6.

Flint KJ, J. N. (1991). "Differential regulation of three members of the ATF/CREB family of DNA-binding proteins." Oncogene 6(11):2019-26.

Forbes JF, Cuzick J, Buzdar A, Howell A, Tobias JS, Baum M. (2008) “Effect of anastrozole and tamoxifen as adjuvant treatment for early-stage breast cancer: 100-month analysis of the ATAC trial.” Lancet Oncol. 9(1):45-53.

Forster AC, Symons RH (1987). “Self-cleavage of plus and minus RNAs of a virusoid and a structural model for the active sites.” Cell 24;49(2):211-20.

Fujita T, K. Y., Wada O, Tateishi Y, Kitada L, Yamamoto Y, Takashima H, Murayama A, Yano T, Baba T, Kato S, Kawabe Y, Yanagisawa J (2003). "Full activation of oestrogen receptor alpha activation function-1 induces proliferation of breast cancer cells." J Biol Chem 278(29):26704-14.

Funakoshi Y, I. T., Takeda K, Tokuno T, Iino N, Takeshita A (2002). "Critical role of cAMP-response element-binding protein for angiotensin II-induced hypertrophy of vascular smooth muscle cells." J Biol Chem 277(21):18710-7.

Gachon F, P. A., Thebault S, Dick J, Lemasson I, Devaux C, Mesnard JM (1998). "CREB-2, a cellular CRE-dependent transcription repressor, functions in association with Tax as an activator of the human T-cell leukemia virus type 1 promoter." J Virol 72(10):8332-7.

Galea MH, B. R., Elston CE, Ellis IO (1992). "The Nottingham Prognostic Index in primary breast cancer." Breast Cancer Res Treat 22(3):207-19.

Ghosh SK, Gadiparthi L, Zeng ZZ, Bhanoori M, Tellez C, Bar-Eli M, Rao GN. (2002). “ATF-1 mediates protease-activated receptor-1 but not receptor tyrosine kinase-induced DNA synthesis in vascular smooth muscle cells”. J Biol Chem. 14;277(24):21325-31.

Gonzalez GA, M. M. (1989). "Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133." Cell 59(4):675-80.

Goren I, T. E., Goldblum A, Honigman A. (2001). "Two cysteine residues in the DNA-binding domain of CREB control binding to CRE and CREB-mediated gene expression." J Mol Biol 313(4):695-709.

Gotzmann J, H. H., Thallinger C, Wolschek M, Jansen B, Schulte-Hermann R, Beug H, Mikulits W (2002). "Hepatocytes convert to a fibroblastoid phenotype through the cooperation of TGF-beta1 and Ha-Ras: steps towards invasiveness." J Cell Sci 115(Pt 6):1189-202.

Gupta S, C. D., Dérijard B, Davis RJ (1995). "Transcription factor ATF2 regulation by the JNK signal transduction pathway." Science 267(5196):389-93

Hai T, C. T. (1991). "Cross-family dimerization of transcription factors Fos/Jun and ATF/CREB alters DNA binding specificity." Proc Natl Acad Sci U S A 88(9):3720-4.

Hai T, Hartman MG. (2001). "The molecular biology and nomenclature of the activating transcription factor/cAMP responsive element binding family of transcription factors: activating transcription factor proteins and homeostasis". Gene. 25; 273(1):1-11

Hai TW, Liu F, Coukos WJ, Green MR. (1989) "Transcription factor ATF cDNA clones: an extensive family of leucine zipper proteins able to selectively form DNA-binding heterodimers". Genes Dev. 3(12B):2083-90.

Harada N (1997). "Aberrant expression of aromatase in breast cancer tissues." J Steroid Biochem Mol Biol 61(3-6):175-84.

Hartmann A, B. H., Kovach JS, Sommer SS (1997). "The molecular epidemiology of p53 gene mutations in human breast cancer." Trends Genet 13(1):27-33.

Haseloff J. Gerlach WL (1988). “Simple RNA enzymes with new and highly specific endoribonuclease activities.” Nature 18;334(6183):585-91.

Horwitz KB, J. T., Bain DL, Richer JK, Takimoto GS, Tung L. (1996). "Nuclear receptor coactivators and corepressors." Mol Endocrinol 10(10):1167-77.

Hsu W, K. T., Chen PL, Curran T, Chen-Kiang S (1994). " Fos and Jun repress transcription activation by NF-IL6 through association at the basic zipper region." Mol Cell Biol 14(1):268-76.

Hsu JC, Bravo R, Taub R. (1992) “Interactions among LRF-1, JunB, c-Jun, and c-Fos define a regulatory program in the G1 phase of liver regeneration”. Mol Cell Biol. 12(10):4654-65.

Hurtado A, Holmes KA, Geistlinger TR, Hutcheson IR, Nicholson RI, Brown M, Jiang J, Howat WJ, Ali S, Carroll JS. (2008) “ERBB2 regulation by Estrogen Receptor-Pax2 determines tamoxifen response”. Nature 456 (7222): 663–6.

IA Nazarenko, S. B. a. R. H. (1997). "A closed tube format for amplification and detection of DNA based on energy transfer." Nucleic Acids Research Vol 25(12): 2516-2521.

Ishiguro T, N. H., Naito M, Tsuruo T. (2000). "Inhibitory effect of ATF3 antisense oligonucleotide on ectopic growth of HT29 human colon cancer cells." Jpn J Cancer Res 91(8):833-6.

Iyer VR, Eisen MB, Ross DT, Schuler G, Moore T, Lee JC, Trent JM, Staudt LM, Hudson J Jr, Boguski MS, Lashkari D, Shalon D, Botstein D, Brown PO. (1999).”The transcriptional program in the response of human fibroblasts to serum”. Science. 1;283(5398):83-7.

Jackson AL, L. L. (1998). "The mutation rate and cancer." Genetics 148(4):1483-90.

James VH, M. J., Lai LC, Newton CJ, Ghilchik MW, Reed MJ (1987). "Aromatase activity in normal breast and breast tumor tissues: in vivo and in vitro studies." Steroids 50(1-3): p. 269-79.

Jiang WG, Ablin R, Douglas-Jones A, Mansel RE (2003a). “Expression of transglutaminases in human breast cancer and their possible clinical significance.” Oncol Rep. 10(6):2039-44.

Jiang WG, Davies G, Fodstad O (2005). “Com-1/P8 in oestrogen regulated growth of breast cancer cells, the ER-beta connection.”Biochem Biophys Res Commun. 330(1):253-62.

Jiang WG, Grimshaw D, Lane J, Martin TA, Abounader R, Laterra J, Mansel RE (2001). “A hammerhead ribozyme suppresses expression of hepatocyte growth factor/scatter factor receptor c-MET and reduces migration and invasiveness of breast cancer cells.” Clin Cancer Res. 7(8):2555-62.

Jiang WG, Grimshaw D, Martin TA, Davies G, Parr C, Watkins G, Lane J, Abounader R, Laterra J, Mansel RE (2003b). “Reduction of stromal fibroblast-induced mammary tumor growth, by retroviral ribozyme transgenes to hepatocyte growth factor/scatter factor and its receptor, c-MET.” Clin Cancer Res. 9(11):4274-81.

Jiang WG, Hiscox S, Hallett MB, Scott C, Horrobin DF, Puntis MC (1995). “Inhibition of hepatocyte growth factor-induced motility and in vitro invasion of human colon cancer cells by gamma-linolenic acid.”Br J Cancer. 71(4):744-52.

Jiang WG, Watkins G, Fodstad O, Douglas-Jones A, Mokbel K, Mansel RE (2004). “Differential expression of the CCN family members Cyr61, CTGF and Nov in human breast cancer.” Endocr Relat Cancer. 11(4):781-91.

Johannessen M, D. M., Moens U. (2004). "What turns CREB on?" Cell Signal 16(11): 1211-27.

Johannessen M, M. U. (2007). "Multisite phosphorylation of the cAMP response element-binding protein (CREB) by a diversity of protein kinases." Front Biosci 12:1814-32.

Karpinski BA, M. G., Huggenvik J, Uhler MD, Leiden JM. (1992). "Molecular cloning of human CREB-2: an ATF/CREB transcription factor that can negatively regulate transcription from the cAMP response element." Proc Natl Acad Sci U S A 89(11):4820-4.

Kato S, E. H., Masuhiro Y, Kitamoto T, Uchiyama S, Sasaki H, Masushige S, Gotoh Y, Nishida E, Kawashima H, Metzger D, Chambon P. (1995). "Activation of the oestrogen receptor through phosphorylation by mitogen-activated protein kinase." Science 270(5241):1491-4.

KatoS (2001). "Estrogen receptor-mediated cross-talk with growth factor signaling pathways." Breast Cancer Res Treat 8(1):3-9.

Katzenellenbogen BS, M. M., Ekena K, Herman ME, McInerney EM. (1997). "Antiestrogens: mechanisms of action and resistance in breast cancer." Breast Cancer Res Treat 44(1):23-38.

Kelsey JL, B. G. (1988). "Breast cancer epidemiology." Cancer Res. 48(20):5615-23.

Kim JY, Ryu MR, Choi BO, Park WC, Oh SJ, Won JM, Chung SM. (2011) “The prognostic significance of the lymph node ratio in axillary lymph node positive breast cancer.” J Breast Cancer. 14(3):204-12.

King JA, O.-A. S., Stevens T, Al-Mehdi AB, Fodstad O, Jiang WG. (2004). "Activated leukocyte cell adhesion molecule in breast cancer: prognostic indicator." Breast Cancer Res Treat. 6(5): R478-87.

Klemm DJ, W. P., Frid MG, Dempsey EC, Schaack J, Colton LA, Nesterova A, Stenmark KR, Reusch JE (2001). "cAMP response element-binding protein content is a molecular determinant of smooth muscle cell proliferation and migration." J Biol Chem 276(49):46132-41.

Kobierski LA, C. H., Tan Y, Comb MJ (1991). "cAMP-dependent regulation of proenkephalin by JunD and JunB: positive and negative effects of AP-1 proteins." Proc Natl Acad Sci U S A 88(22):10222-6.

Kokame K, K. H., Miyata T (1996). "Homocysteine-respondent genes in vascular endothelial cells identified by differential display analysis GRP78/BiP and novel genes." J Biol Chem 271(47):29659-65.

Kothapalli D, S. S., Smyth EM, Azonobi I, Pure E, Assoian RK (2003). "Prostacylin receptor activation inhibits proliferation of aortic smooth muscle cells by regulating cAMP response element-binding protein- and pocket protein-dependent cyclin a gene expression." Mol Pharmacol 64(2):249-58.

Kuo CT, L. J. (1999). "Transcriptional regulation of T lymphocyte development and function." Annu Rev Immunol 17:149-87.

Kwon EM, R. M., Blenis J, Sakamoto KM. (2000). "Granulocyte-macrophage colony-stimulating factor stimulation results in phosphorylation of cAMP response element-binding protein through activation of pp90RSK." Blood 95(8):2552-8.

Lania L, M. B., Napolitano G (1999). "Transcriptional control by cell-cycle regulators: a review." J Cell Physiol 179(2):134-41.

Lassot I, S. E., Berlioz-Torrent C, Durand H, Groussin L, Hai T, Benarous R, Margottin-Goguet F (2001). "ATF4 degradation relies on a phosphorylation-dependent interaction with the SCF(betaTrCP) ubiquitin ligase." Mol Cell Biol 21(6):2192-202.

Lee HJ, M. R., Sakamoto KM. (1995). "Transcriptional activation of egr-1 by granulocyte-macrophage colony-stimulating factor but not interleukin 3 requires phosphorylation of cAMP response element-binding protein (CREB) on serine 133." J Biol Chem 270(27):15979-83.

Lee KA, H. T., SivaRaman L, Thimmappaya B, Hurst HC, Jones NC, Green MR (1987). "A cellular protein, activating transcription factor, activates transcription of multiple E1A-inducible adenovirus early promoters." Proc Natl Acad Sci U S A 84(23):8355-9.

Lee MM, B. A., DeVries GH (1999). "Phosphorylation of CREB in axon-induced Schwann cell proliferation." J Neurosci Res 55(6):702-12.

Lee YN, Park YG, Choi YH, Cho YS, Cho- Chung YS (2000). "CRE-transcription factor decoy oligonucleotide inhibition of MCF-7 breast cancer cells: cross-talk with p53 signaling pathway." Biochemistry 25;39(16):4863-8.

Levin ER. (2005) “Integration of the extranuclear and nuclear actions of estrogen.” Mol Endocrinol. 19(8):1951-9.

Li X, Huang J, Yi P, Bambara RA, Hilf R, Muyan M. (2004) “Single-Chain Estrogen Receptors (ERs) Reveal that the ERα/β Heterodimer Emulates Functions of the ERα Dimer in Genomic Estrogen Signaling Pathways.” Mol Cell Biol. 24(17): 7681–7694.

Liu F, T. M., Wagner S, Greenberg ME, Green MR (1993). "Activating transcription factor-1 can mediate Ca(2+)- and cAMP-inducible transcriptional activation." J Biol Chem 268(9):6714-20.

Loeb LA (1998). "Cancer cells exhibit a mutator phenotype." Adv Cancer Res 72:25-56.

Loi S, Sotiriou C, Haibe-Kains B, Lallemand F, Conus NM, Piccart MJ, Speed TP, McArthur GA (2009). “Gene expression profiling identifies activated growth factor signaling in poor prognosis (Luminal-B) estrogen receptor positive breast cancer.” BMC Med Genomics 2: 37

Lonze BE, R. A., Cohen S, Ginty DD (2002). "Apoptosis, axonal growth defects, and degeneration of peripheral neurons in mice lacking CREB." Neuron 25;34(3):371-85.

Look AT (1997). "Oncogenic transcription factors in the human acute leukemias." Science 278(5340): 1059-64.

Low KG, C. H., Schwartz PM, Daniels GM, Melner MH, Comb MJ (1994). "Novel interactions between human T-cell leukemia virus type I Tax and activating transcription factor 3 at a cyclic AMP-responsive element." Mol Cell Biol 14(7):4958-74.

Lu Q, N. J., Savinov A, Yue W, Weisz J, Dabbs DJ, Wolz G, Brodie A (1996). "Expression of aromatase protein and messenger ribonucleic acid in tumor epithelial cells and evidence of functional significance of locally produced oestrogen in human breast cancers." Endocrinology 137(7):3061-8.

Luciani P, B. L., Conforti B, Tonacchera M, Agretti P, Elisei R, Vivaldi A, Cioppi F, Biliotti G, Manca G, Vitti P, Serio M, Peri A. (2003). "Expression of cAMP response element-binding protein and sodium iodide symporter in benign non-functioning and malignant thyroid tumours." Eur J Endocrinol 148(5): 579-86.

Mahendroo MS, M. C., Simpson ER (1993). "Tissue-specific and hormonally controlled alternative promoters regulate aromatase cytochrome P450 gene expression in human adipose tissue." J Biol Chem 268(26):19463-70.

Makhov P, G. K., Uzzo RG, Wuestefeld T, Scoll BJ, Kolenko VM (2009). "Transcriptional regulation of the major zinc uptake protein hZip1 in prostate cancer cells."Gene 431(1-2) : 39-46.

Masquilier D, S.-C. P. (1992). "Transcriptional cross-talk: nuclear factors CREM and CREB bind to AP-1 sites and inhibit activation by Jun." J Biol Chem 267(31):22460-6.

Mayr B, M. M. (2001). "Transcriptional regulation by the phosphorylation-dependent factor CREB." Nat Rev Mol Cell Biol 2(8):599-609.

Mayr BM, C. G., Montminy MR (2001). "Distinct effects of cAMP and mitogenic signals on CREB-binding protein recruitment impart specificity to target gene activation via CREB." Proc Natl Acad Sci U S A 98(19):10936-41.

Melnikova VO, B.-E. M. (2008). "Transcriptional control of the melanoma malignant phenotype." Cancer Biol Ther 7(7):997-1003.

Mielnicki LM, H. R., Chevray PM, Pruitt SC (1996). "Mutated Atf4 suppresses c-Ha-ras oncogene transcript levels and cellular transformation in NIH3T3 fibroblasts." Biochem Biophys Res Commun 228(2):586-95.

Miller WR, O. N. J. (1987). "The importance of local synthesis of oestrogen within the breast." Steroids 50(4-6):537-48.

Moasser MM. (2007) “Targeting the function of the HER2 oncogene in human cancer therapeutics.” Oncogene. 26(46):6577-92.

Monti B, M. L., Contestabile A (2002). "NMDA receptor-dependent CREB activation in survival of cerebellar granule cells during in vivo and in vitro development." Eur J Neurosci 16(8):1490-8.

Montminy MR and B. LM. (1987). "Binding of a nuclear protein to the cyclic-AMP response element of the somatostatin gene." Nature 328(6126): 175-8.

MontminyM (1997). "Transcriptional regulation by cyclic AMP." Annu Rev Biochem 66:807-22.

Nagy P, Jenei A, Kirsch AK, Szöllosi J, Damjanovich S, Jovin TM (1999) "Activation-dependent clustering of the erbB2 receptor tyrosine kinase detected by scanning near-field optical microscopy". J. Cell. Sci. 112 (11): 1733–41.

Narendra K. Vaish, Anilkumar R. Kore and Fritz Eckstein (1998). " Recent developments in the hammerhead ribozyme field." Nucl. Acids Res. 26 (23): 5237-42.

Nawa T, N. M., Cai Y, Zhang C, Uchimura I, Narumi S, Numano F, Kitajima S (2000). "Repression of TNF-alpha-induced E-selectin expression by PPAR activators: involvement of transcriptional repressor LRF-1/ATF3." Biochem Biophys Res Commun 275(2):406-11.

Nazarenko IA, Bhatnagar SK, Hohman RJ (1997). “A closed tube format for amplification and detection of DNA based on energy transfer.” Nucleic Acids Res. 25(12):2516-21

Ngan Vo and Richard H. Goodman (2001). "CREB-binding Protein and p300 in Transcriptional Regulation." The Journal of Biological Chemistry 276: 13505-13508.

Nilsson M, Ford J, Bohm S, Toftgård R. (1997). “Characterization of a nuclear factor that binds juxtaposed with ATF3/Jun on a composite response element specifically mediating induced transcription in response to an epidermal growth factor/Ras/Raf signaling pathway”. Cell Growth Differ. 8(8):913-20.

Okamoto Y, C. A., Chen J, Kelley R, Jones K, Weed HG, Gardner KL, Gangi L, Yamaguchi M, Klomkleaw W, Nakayama T, Hamlin RL, Carnes C, Altschuld R, Bauer J, Hai T. (2001). "Transgenic mice with cardiac-specific expression of activating transcription factor 3, a stress-inducible gene, have conduction abnormalities and contractile dysfunction." Am J Pathol 159(2):639-50.

Ong WY, L. H., Lim TM, Lutz B. (2000). "Kainate-induced neuronal injury leads to persistent phosphorylation of cAMP response element-binding protein in glial and endothelial cells in the hippocampus." Exp Brain Res 131(2):178-86.

Paleri V, Pulimood A, Davies GR, Birchall MA. (2001). Interleukins 7 and 12 are expressed in head and neck squamous cancer. Clin Otolaryngol Allied Sci. 26(4):302-6.

Park EA, G. A., Nizielski SE, Hakimi P, Cao Z, Moorman A, Hanson RW (1993). "Relative roles of CCAAT/enhancer-binding protein beta and cAMP regulatory element-binding protein in controlling transcription of the gene for phosphoenolpyruvate carboxykinase (GTP)." J Biol Chem 268(1):613-9.

Parkin DM, B. F., Devesa SS. (2001). "Cancer burden in the year 2000. The global picture." Eur J Cancer Suppl 8:S4-66.

Parkin DM, B. F., Ferlay J, Pisani P. (2001). "Estimating the world cancer burden: Globocan 2000." Int J Cancer 94(2):153-6.

Perry C, S. E., Soreq H (2004). "CREB regulates AChE-R-induced proliferation of human glioblastoma cells." Neoplasia 6(3): p. 279-86.

Persani L, L. A., Alberti L, Romoli R, Mantovani G, Filetti S, Spada A, Conti M (2000). "Induction of specific phosphodiesterase isoforms by constitutive activation of the cAMP pathway in autonomous thyroid adenomas." J Clin Endocrinol Metab 85(8):2872-8.

Quiet CA, F. D., Weichselbaum RR, Hellman S (1995). "Natural history of node-negative breast cancer: a study of 826 patients with long-term follow-up." J Clin Oncol 13(5):1144-51.

RabbittsTH (1994). "Chromosomal translocations in human cancer." Nature 372(6502): 143-9.

Reddy MA, A. S., Kim YS, Lanting L, Rossi J, Kang SW, Nadler JL, Shahed A, Natarajan R (2002). "Interaction of MAPK and 12-lipoxygenase pathways in growth and matrix protein expression in mesangial cells." Am J Physiol Renal Physiol 283(5):F985-94.

Reddy TR, T. H., Li X, Wong-Staal F (1997). "Functional interaction of the HTLV-1 transactivator Tax with activating transcription factor-4 (ATF4)." Oncogene 14(23):2785-92.

Reincke M, Mora P, Beuschlein F, Arlt W, Chrousos GP, Allolio B. (1997). Deletion of the adrenocorticotropin receptor gene in human adrenocortical tumors: implications for tumorigenesis. J Clin Endocrinol Metab. 82(9):3054-8.

Riccio A, A. S., Davenport CM, Blendy JA, Ginty DD (1999). "Mediation by a CREB family transcription factor of NGF-dependent survival of sympathetic neurons." Science 286(5448):2358-61.

Roy V, Perez EA. (2009)"Beyond trastuzumab: small molecule tyrosine kinase inhibitors in HER-2-positive breast cancer". Oncologist. 14(11):1061-9.

Ryseck RP, B. R. (1991). "c-JUN, JUN B, and JUN D differ in their binding affinities to AP-1 and CRE consensus sequences: effect of FOS proteins." Oncogene 6(4):533-42.

Sabban EL, L. X., Serova L, Gueorguiev V, Kvetnansky R. (2006). "Stress Triggered Changes in Gene Expression in Adrenal Medulla: Transcriptional Responses to Acute and Chronic Stress." Cell Mol Neurobiol.

Saimura M, F. T., Tsuda H, Sato H, Miyamoto K, Akashi-Tanaka S, Nanasawa T (1999). "Prognosis of a series of 763 consecutive node-negative invasive breast cancer patients without adjuvant therapy: analysis of clinicopathological prognostic factor." J Surg Oncol 71(2):101-5.

Saini KS, Azim HA Jr, Metzger-Filho O, Loi S, Sotiriou C, de Azambuja E, Piccart M. (2011) “Beyond trastuzumab: new treatment options for HER2-positive breast cancer.” Breast. Suppl 3:S20-7.

San-Marina S, H. Y., Suarez Saiz F, Trus MR, Minden MD (2008). "Lyl1 interacts with CREB1 and alters expression of CREB1 target genes." Biochim Biophys Acta 1783(3):503-17.

Santen RJ, M. J., Hoagland M, Naftolin F, Roa L, Harada N, et al (1994). "Stromal spindle cells contain aromatase in human breast tumors." J Clin Endocrinol Metab 79(2): p. 627-32.

Santner SJ, C. S., Zhou D, Korsunsky Z, Martel J, Santen RJ (1993). "Effect of androstenedione on growth of untransfected and aromatase-transfected MCF-7 cells in culture." J Steroid Biochem Mol Biol 44(4-6):611-6.

Sasano H, N. H., Harada N, Goukon Y, Kimura M (1994). "Immunolocalization of aromatase and other steroidogenic enzymes in human breast disorders." Hum Pathol 25(5):530-5.

Sebastian S, B. S. (2001). "A highly complex organization of the regulatory region of the human CYP19 (aromatase) gene revealed by the Human Genome Project." J Clin Endocrinol Metab 86(10):4600-2.

Shankar DB, S. K. (2004). "The role of cyclic-AMP binding protein (CREB) in leukemia cell proliferation and acute leukemias." Leuk Lymphoma 45(2):265-70.

Shaywitz AJ, G. M. (1999). "CREB: a stimulus-induced transcription factor activated by a diverse array of extracellular signals." Annu Rev Biochem 68:821-61.

Sheng M, T. M., Greenberg ME (1991). "CREB: a Ca(2+)-regulated transcription factor phosphorylated by calmodulin-dependent kinases." Science 252(5011):1427-30.

Silva AJ, K. J., Frankland PW, Kida S (1998). "CREB and memory." Annu Rev Neurosci 21:127-48.

Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. (1987) Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science. 235(4785):177-82.

Smith CL, N. Z., O'Malley BW. (1997). "Coactivator and corepressor regulation of the agonist/antagonist activity of the mixed antiestrogen, 4-hydroxytamoxifen." Mol Endocrinol 11(6):657-66.

Sofi M, Y. M., Papamakarios T, Simpson ER, Clyne CD (2003). "Role of CRE-binding protein (CREB) in aromatase expression in breast adipose tissue." Breast Cancer Res Treat 79(3): p. 399-407.

Sun XZ, Z. D., Chen S (1997). "Autocrine and paracrine actions of breast tumor aromatase. A three-dimensional cell culture study involving aromatase transfected MCF-7 and T-47D cells." J Steroid Biochem Mol Biol 63(1-3): p. 29-36.

Talukder AH, V. R., Mandal M, Kumar R (2000). "Heregulin induces expression, DNA binding activity, and transactivating functions of basic leucine zipper activating transcription factor 4." Cancer Res 60(2):276-81.

Tan Y, R. J., Zhang A, Cariati S, Cohen P, Comb MJ (1996). "FGF and stress regulate CREB and ATF-1 via a pathway involving p38 MAP kinase and MAPKAP kinase-2." EMBO J 15(17):4629-42.

Tokunou T, I. T., Takeda K, Funakoshi Y, Iino N, Takeshita A (2001). "cAMP response element-binding protein mediates thrombin-induced proliferation of vascular smooth muscle cells." Arterioscler Thromb Vasc Biol 21(11):1764-9

.

Travnickova-Bendova Z, C. N., Reppert SM, Sassone-Corsi P (2002). "Bimodal regulation of mPeriod promoters by CREB-dependent signaling and CLOCK/BMAL1 activity." Proc Natl Acad Sci U S A 99(11):7728-33.

Tripathy D, B. C. (1992). "Activated oncogenes and putative tumor suppressor genes involved in human breast cancers." Cancer Treat Res 63:15-60.

Tsujimoto A, N. H., Morita T, Sato T, Shimotohno K (1991). "Isolation of cDNAs for DNA-binding proteins which specifically bind to a tax-responsive enhancer element in the long terminal repeat of human T-cell leukemia virus type I." J Virol 65(3):1420-6.

Tsujino H, K. E., Fukuoka T, Dai Y, Tokunaga A, Miki K, Yonenobu K, Ochi T, Noguchi K (2000). "Activating transcription factor 3 (ATF3) induction by axotomy in sensory and motoneurons: A novel neuronal marker of nerve injury." Mol Cell Neurosci 15(2):170-82.

Vallejo M, R. D., Miller CP, Habener JF (1993). "C/ATF, a member of the activating transcription factor family of DNA-binding proteins, dimerizes with CAAT/enhancer-binding proteins and directs their binding to cAMP response elements." Proc Natl Acad Sci U S A 90(10):4679-83.

Vinson C, Myakishev M, Acharya A, Mir AA, Moll JR, Bonovich M. (2002) "Classification of Human B-ZIP Proteins Based on Dimerization Properties." Mol Cell Biol. 22(18):6321-35

Wagatsuma A, A. S., Sakura M, Hatakeyama D, Aonuma H, Ito E (2006). "De Novo synthesis of CREB in a presynaptic neuron is required for synaptic enhancement involved in memory consolidation." J Neurosci Res 84(5): 954-60.

Weir E, Chen Q, DeFrances MC, Bell A, Taub R, Zarnegar R. (1994)."Rapid induction of mRNAs for liver regeneration factor and insulin-like growth factor binding protein-1 in primary cultures of rat hepatocytes by hepatocyte growth factor and epidermal growth factor". Hepatology. 20(4 Pt 1):955-60.

Wolfgang CD, C. B., Martindale JL, Holbrook NJ, Hai T (1997). "gadd153/Chop10, a potential target gene of the transcriptional repressor ATF3." Mol Cell Biol 17(11):6700-7.

Wong A, S. K. (1995). "Granulocyte-macrophage colony-stimulating factor induces the transcriptional activation of egr-1 through a protein kinase A-independent signaling pathway." J Biol Chem 270(51):30271-3.

Woo RA, Poon RY (2003) Cyclin-dependent kinases and S phase control in mammalian cells. Cell Cycle. 2(4):316-24.

Xing J, G. D., Greenberg ME (1996). "Coupling of the RAS-MAPK pathway to gene activation by RSK2, a growth factor-regulated CREB kinase." Science 273(5277):959-63.

Yang C, Y. B., Zhou D, Chen S (2002). "Regulation of aromatase promoter activity in human breast tissue by nuclear receptors" Oncogene 21(18):2854-63.

Yang C. Z. D., Chen S (1998). "Modulation of aromatase expression in the breast tissue by ERR alpha-1 orphan receptor." Cancer Res 58(24):5695-700.

Yang X, M. K., Bialek P, Jacquot S, Masuoka HC, Schinke T, Li L, Brancorsini S, Sassone-Corsi P, Townes TM, Hanauer A, Karsenty G (2004). "ATF4 is a substrate of RSK2 and an essential regulator of osteoblast biology; implication for Coffin-Lowry Syndrome." Cell 117(3):387-98.

Yeh WC, C. Z., Classon M, McKnight SL (1995). "Cascade regulation of terminal adipocyte differentiation by three members of the C/EBP family of leucine zipper proteins." Genes Dev 15;9(2):168-81.

Yoshimura T, F. J., Yoshida M. (1990). "Multiple cDNA clones encoding nuclear proteins that bind to the tax-dependent enhancer of HTLV-1: all contain a leucine zipper structure and basic amino acid domain." EMBO J 9(8):2537-42.

Yue W, Z. D., Chen S, Brodie A (1994). "A new nude mouse model for postmenopausal breast cancer using MCF-7 cells transfected with the human aromatase gene " Cancer Res 54(19):5092-5.

Zeitoun KM, B. S. (1999). "Aromatase: a key molecule in the pathophysiology of endometriosis and a therapeutic target." Fertil Steril 72(6):961-9.

Zhao Y, A. V., Mendelson CR, Simpson ER (1996). "Estrogen biosynthesis proximal to a breast tumor is stimulated by PGE2 via cyclic AMP, leading to activation of promoter II of the CYP19 (aromatase) gene." Endocrinology 137(12):5739-42.

Zhao Y, M. C., Simpson ER (1995). "Characterization of the sequences of the human CYP19 (aromatase) gene that mediate regulation by glucocorticoids in adipose stromal cells and fetal hepatocytes." Mol Endocrinol 9(3):340-9.

Zhou C, Z. D., Esteban J, Murai J, Siiteri PK, Wilczynski S, et al (1996). "Aromatase gene expression and its exon I usage in human breast tumors. Detection of aromatase messenger RNA by reverse transcription-polymerase chain reaction." J Steroid Biochem Mol Bio 59(2): p. 163-71.

Zhou D, Clarke P, Wang J, Chen S.(1996).“Identification of a promoter that controls aromatase expression in human breast cancer and adipose stromal cells”. J Biol Chem. 271(25):15194-202.

Zhou D, C. S. (1998). "Characterization of a silencer element in the human aromatase gene." Arch Biochem Biophys 353(2):213-20.

Zhou D, C. S. (1999). "Identification and characterization of a cAMP-responsive element in the region upstream from promoter 1.3 of the human aromatase gene." Arch Biochem Biophys 15;371(2): p. 179-90.

Zivadinovic D, Gametchu B, Watson CS. (2005) “Membrane estrogen receptor-alpha levels in MCF-7 breast cancer cells predict cAMP and proliferation responses.” Breast Cancer Res. 7(1):R101-12.

Zuker M (2003). “Mfold web server for nucleic acid folding and hybridization prediction.” Nucleic Acid Res 1; 31 (13):3406-15.