Expression of transcription factor CREB1 in human breast cancer and its correlation with prognosis

ALOK CHHABRA, HERMAN FERNANDO, GARETH WATKINS, ROBERT E. MANSEL and WEN G. JIANG

Metastasis and Angiogenesis Research Group, School of Medicine, Cardiff University, Cardiff, UK

Abstract. It has been suggested that the cAMP responsive element-binding protein (CREB) may act as a transcription regulator of aromatase in breast cancer cells. However, there is little knowledge on the expression of CREB1 in human breast cancer and its clinical significance. The current study investigated the expression pattern of CREB1 in human breast cancer at the mRNA and protein level and correlated it with the clinical outcome. CREB1 staining was primarily seen in the nucleus of both normal and tumour cells. At the mRNA level, we found a significantly higher level of CREB1 in breast tumour tissues (n=120) as compared to non-neoplastic mammary tissues (n=33, p=0.0092). When compared between different histological types CREB1 expression was significantly higher in ductal carcinoma as compared to lobular and other breast carcinoma. Patients with a poor prognosis and with metastasis had a markedly raised level of CREB1 compared to patients who were disease free. In addition, node-positive tumours had higher levels of CREB1 than node-negative tumours (p=0.0018). Finally, 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]. This study demonstrates that the level of CREB1 in breast cancer patients is elevated and is significantly raised in patients with a poor prognosis, metastatic disease and nodal involvement. We conclude that the level of CREB1 expression is aberrant in human breast cancer and is associated with disease progression in breast cancer patients.

Introduction

Breast cancer is a major health problem and is the most frequently occurring cancer in women worldwide. It accounts for approximately 25% of all female malignancies and the proportion is higher in women in developed countries with

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Correspondence to: Dr Alok Chhabra, Department of Surgery, School of Medicine, Cardiff University, Cardiff, CF14 4XN, UK
E-mail: chhabraa1@cardiff.ac.uk

the incidence rising in many parts of the world including Europe and the USA. Approximately 70% of all breast cancers are hormone dependent and oestrogen is the primary hormonal factor that stimulates the growth of hormone-dependent breast cancer. Pasqualini *et al* reported that the concentration of oestrogens in breast cancer tissues is greater than in plasma in postmenopausal women (1). Oestrogen biosynthesis from the oestrogen precursor's dihydroepiandrostenedione and testosterone is catalysed by the enzyme aromatase cytochrome P450 which in turn promotes the proliferation of breast cancer cells.

In humans, aromatase is expressed in various glandular and extra-glandular sites including ovary, breast, placenta, bone and adipose tissue. The expression of aromatase is highly aberrant in human breast cancer. Various studies have demonstrated that expression of both aromatase mRNA and aromatase protein were significantly higher in breast cancer tissues than in normal breast tissue (2-4). Tumour aromatase is thought to stimulate breast cancer growth in both an autocrine and a paracrine manner (5). Aromatase expression is dependent on multiple tissue specific promoters. In breast cancer tissue it is directed mainly by cAMP-stimulated promoters 1.3 (6) and II (7), while in normal breast tissue it is driven by glucocorticoid-stimulated promoter 1.4 (8).

CREB, cAMP responsive element-binding protein, belongs to a subfamily of the leucine zipper with basic domain (bZip) family of transcription factors. CREB refers to proteins that bind to the cAMP responsive element (CRE) on the somatostatin promoter (9). CREB binds the cyclic AMP response element (TGACGTCA) (10) in the presence of cAMP and activates transcription in response to a variety of extracellular signals including neurotransmitters, hormones, membrane depolarization, and growth and neurotrophic factors. There is evidence suggesting that the CREB proteins form selective heterodimers with each other or with other bZip proteins such as the fos and jun family of proteins (11). These heterodimers' formation in general can alter DNA binding specificity and transcriptional activity and hence the gene expression.

cAMP responsive elements (CRE1 and CRE2) are essential for cAMP-induced promoter II activity (12), which requires phosphorylation by cAMP-dependent protein kinase at serine-119 and -133 (13) for interaction with DNA and RNA polymerase II. 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 (12). Despite the extensive study of aromatase, little information is available on the expression and role of CREB in human breast cancer. This study was carried out to investigate the expression pattern of CREB1 in a cohort of breast cancer and normal background tissue. We also correlated the level of expression with the prognosis and clinical outcome of the patients.

Materials and methods

An 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, UK). Molecular-biology-grade agarose and DNA ladder were from Invitrogen.

A first-strand cDNA synthesis kit was purchased from Sigma Chemical Ltd. A universal IHC kit was purchased from Vector Laboratories. Low fluorescent 96-well plates were obtained from Abgene and sealing films were from BioRad (Hemel Hempstead, UK).

Sample collection. Breast cancer tissues (n=120) and background tissue samples (normal breast tissue) (n=33) were collected (with approval from the local ethics 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 are shown in Table I.

Tissue processing, RNA extraction and reverse transcription-polymerase chain reaction. Frozen sections of breast tissue specimens were cut using a cryostat at a thickness of 5-10 μm and stored at -20˚C (14). 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 a UV spectrophotometer (Wolfe Laboratories, York, UK). Reverse transcription was carried out from 1 μg total RNA using an RT kit with an anchored oligo-dT primer according to the manufacturer's instructions. The polymerase chain reaction (PCR) was performed with cDNA, master mix and the respective primers [5'ggggactatga ggagatgat'3 and 5' actgaacctgaccgtacagtggaggtcttgatgtgaat'3] using the following reaction conditions: 5 min at 95˚C, 20 sec at 94˚C, 25 sec at 56˚C and 50 sec at 72˚C for 36 cycles followed by a final extension phase of 7 min at 72˚C. ß-actin was used as a housekeeping gene and was amplified simult-aneously using the following primers 5'gctgatttgatggagttgga3' and 5'tcagctacttgttcttgagtgaa3'. The PCR products were then separated on a 0.8% agarose gel, stained using ethidium bromide and visualized under UV light.

Quantitative analysis of CREB1 transcripts. The transcript level of the CREB1 from the above-prepared cDNA was determined using a real-time quantitative PCR, based on the Amplifluor™ uniprimer technology (Intergen Company,

Table I. Clinical information for breast tumour tissues analysed.

Characteristic	n
Tissue type	
Background	33
Tumour	120
Grade	
1	23
2	41
3	56
Nottingham prognostic indicator	
1	66
2	38
3	16
Tumour/nodal status	
1	69
2	40
3	7
4	4
Histology	
Ductal	88
Lobular	14
Other	18
Outcome	
Disease free	81
Metastasis	7
Local recurrence	5
Died of breast cancer	20
Died of unrelated cause	7

Oxford, UK) modified from a previously reported method (14). Specific pairs of PCR primers were designed by the authors using the Beacon Designer software (version 2, Palo Alto, CA, 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 (15) (Intergen), was added. Cytokeratin-19 (CK19) was used for comparison of cellularity during the analysis and primers for CK19 were 5'-caggtccgaggttactgac-3' and 5'-actgaacctgaccgtacacactttctgcc agtgtgtcttc-3' (16).

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 50 ng 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 120 min,
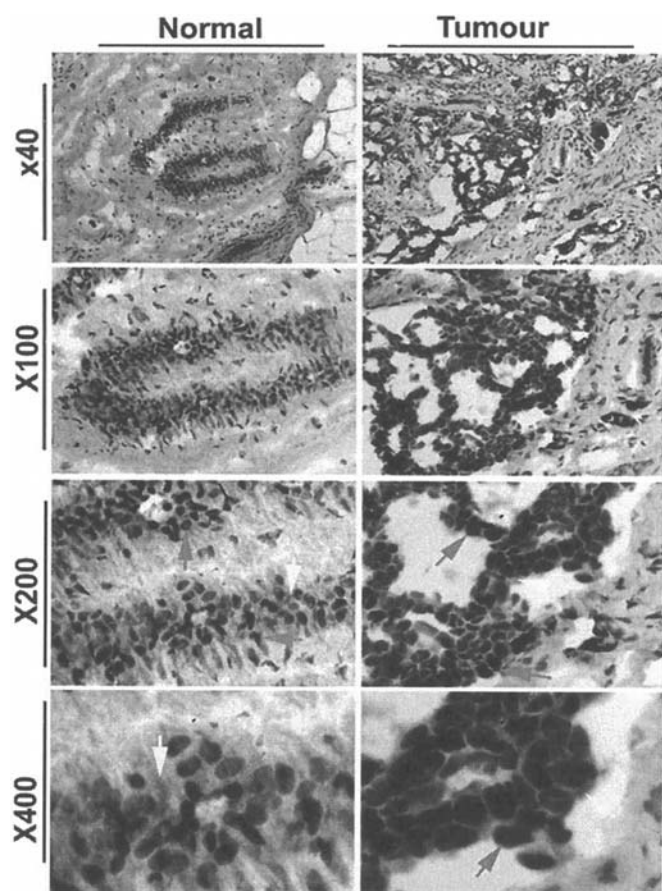
Figure 1. Immunohistochemical staining of normal mammary tissue and breast cancer tissue for CREB1.

50 cycles of 94˚C for 150 sec, 55˚C for 400 sec and 72˚C for 200 sec (14). 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.

Immunohistochemical staining of the CREB1 proteins. Immunohistochemical staining was carried out using frozen sections of breast tumour and normal mammary tissue cut at a thickness of 6 μm using a cryostat. The sections were mounted on Super Frost Plus microscopic slides and air-dried for 20-30 min. The fixed tissue sections were treated in 50% methanol and 50% acetone for 15 min. The sections were then air dried for 10 min and stored at -20˚C (wrapped in foil) for further use or immediately stained. The air dried sections were placed in PBS (Optimax wash buffer) for 5 min to rehydrate. The sections were treated with the primary antibodies at specific dilution and incubated for 1 h. 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 min 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 min to detect the bound antibody. DAB is a substrate for peroxidase and in the presence of H_2O_2 a brown

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 min and treated in Mayer's haematoxylin for 1 min followed by a further wash with water for 10 min (nuclei become blue). The slides were then treated with methanol (3 times) and clearing in 2 changes of 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. Cytoplasmic staining of the respective proteins was quantified using Optimas 6.0 software as we described previously (17).

Statistical analysis was carried out using Mann-Whitney U test, the Kruskal-Wallis test and survival analysis using Kaplan-Meier survival curves and Univariate analysis (SPSS11)

Results

Distribution pattern of CREB1 in mammary epithelial cells and breast cancer cells. We examined the presence of CREB1 in mammary tissue using immunohistochemical staining. CREB1 staining was primarily seen in the nucleus of both normal and tumour cells. The staining pattern of CREB1 in tumour tissue and in normal mammary tissue was then compared. As shown in Fig. 1 the nucleus staining in normal mammary tissue was weaker as compared with the tumour tissue.

Expression of CREB1 in normal mammary and breast cancer tissues. We analysed the expression pattern of CREB1 in both normal mammary and tumour tissues. As shown in Fig. 2A breast tumour tissues had a higher level of CREB1 as compared to normal breast tissues (12.5±1.82 and 3.7±1.35, p=0.0092). When compared between different histological types CREB1 expression was significantly higher in ductal carcinoma (15.4±2.2) as compared to lobular (6.7±0.96) and other breast carcinoma (10±1.26) (Fig. 2B).

Expression of CREB1 and its correlation with nodal involvement and tumour differentiation. As shown in Fig. 3A, grade-2 (19.3±3.6, p=0.075) and grade-3 (7.9±2.5, p=0.1131) carcinomas had higher levels of CREB1 expression as compared to grade-1 (3.4±3.7) carcinoma but they were not statistically significant.

As shown in Fig. 3B, there was a significantly higher level of the CREB1 transcript in node-positive tumours as compared to node-negative tumours (6.2±1.8 in node-negative tumours and 26.5±3 in node-positive tumours, p=0.0018). The same trend was seen when the CREB1 transcript was normalized by CK19 (Fig. 3B insert, p=0.0004).

Tumours from patients with aggressive disease had higher levels of the CREB1 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 was moderate (NPI-2) and >5.4 indicated a poor prognosis (NPI-3). As shown in Fig. 4A, there was a significantly higher level of CREB1 transcript in patients with a moderate (30.8±4.1, p=0.0036) and poor prognosis (13.2±2.2, p=0.0061) as compared to patients with a good prognosis
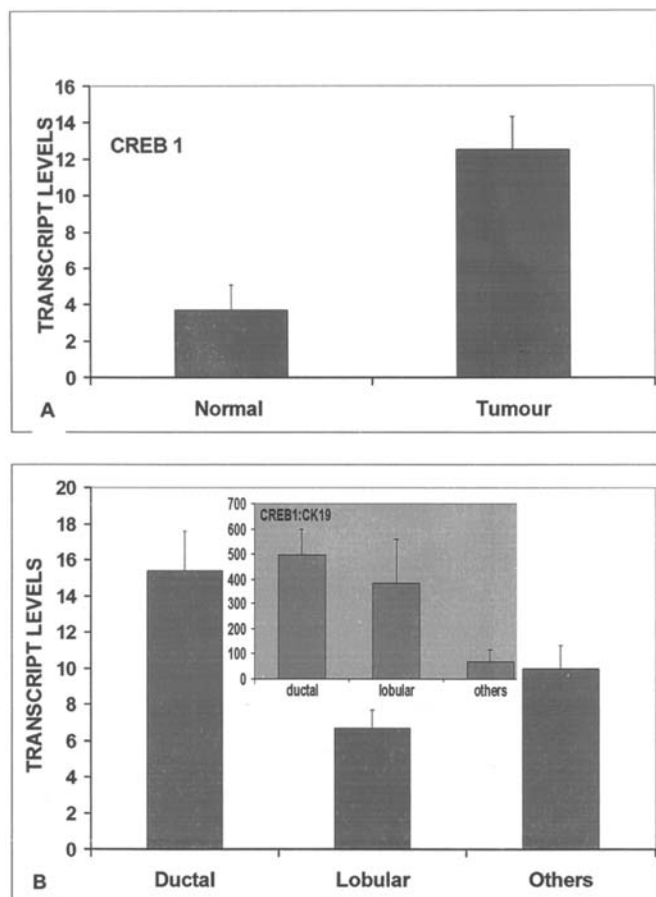
Figure 2. (A) Quantitative real-time PCR demonstrating high expression of CREB1 in tumour samples as compared to normal mammary tissue. (B) Levels of CREB1 transcript levels and its relationship with histological type. Insert: transcript normalised by CK19 (shown as CREB1:CK19 ratio).
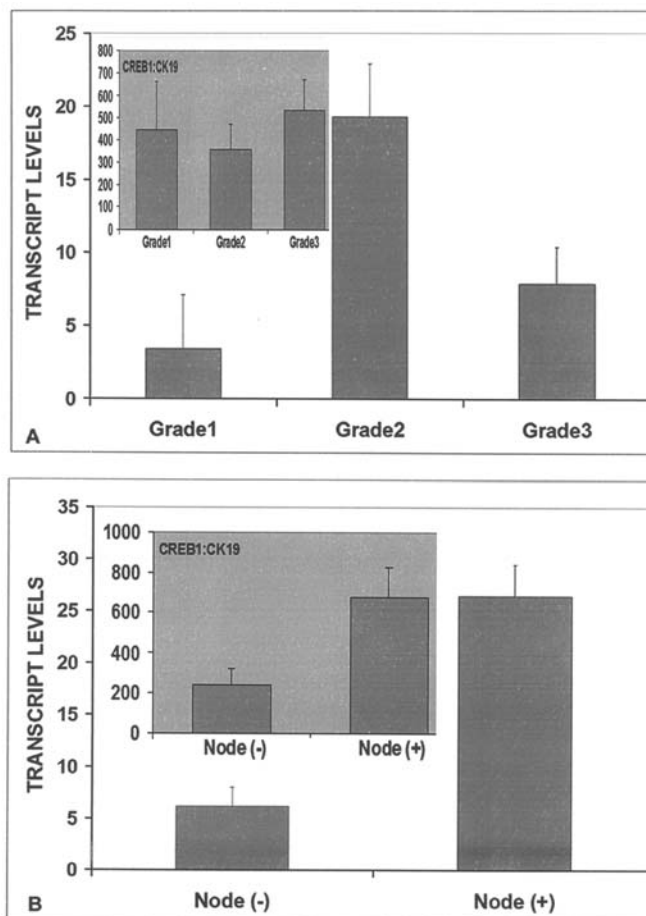

Figure 3. (A) Level of CREB1 and its relationship with tumour grade. Insert: transcript normalised by CK19 (shown as CREB-1:CK19 ratio). (B) RT-PCR analysis of breast cancer tissue samples, showing higher transcript levels of CREB1 in node-positive tumours as compared to node-negative tumours. Insert: transcript normalised by CK19 (shown as CREB1:CK19 ratio).

(6.2±1.8). The same trend was seen when the CREB1 transcript was normalized by CK19 (Fig. 4A insert).

High levels of the CREB1 transcript were linked to both the overall and disease-free survival of the patients. Following a median 120-month follow up, patients were divided into the following groups: those who remained disease free, those who developed metastasis or local recurrence and those who died of breast cancer-related disease (excluding non-cancer-related deaths). As shown in Fig. 4B, the CREB1 level was high in patients who developed metastasis (41±4.6) and who died of breast cancer-related causes (24.2±5.2) as compared to patients who remained disease free (8.9±2.3) but it was not statistically significant. Interestingly, when normalised by CK19 a significant difference was seen in tumours from patients who died of breast cancer (p=0.0003). We combined the three groups (with metastasis, recurrence and mortality), to form a poor prognosis group (Fig. 5A) and compared this group with those who remained disease free. It was demonstrated that the CREB1 level was higher in patients with a poor prognosis (26.5±3) but was statistically not significant. When normalised with CK19 a highly significant difference was seen in patients with a poor prognosis (p=0.0035).

We have divided patients into groups with high levels of CREB1, by using the NPI as a general guide. When the level

of CREB1 transcript in a tumour was higher than the tumour with NPI-2 (3.4-5.4, moderate prognosis group), the tumour was arbitrarily regarded as having a high level. When long-term survival was analysed using the 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 (Fig. 5B).

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 (18), memory (19) and in solid tumours, both benign and malignant (20,21). 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 such as ERRα-1 and CREB1 are present as compared to negative regulatory proteins such as EAR-2, COUP-TFI, Snail and Slug (8). Coxon *et al* have shown a link between an oncogenic chromosomal trans-location and CREB activation in malignant salivary gland
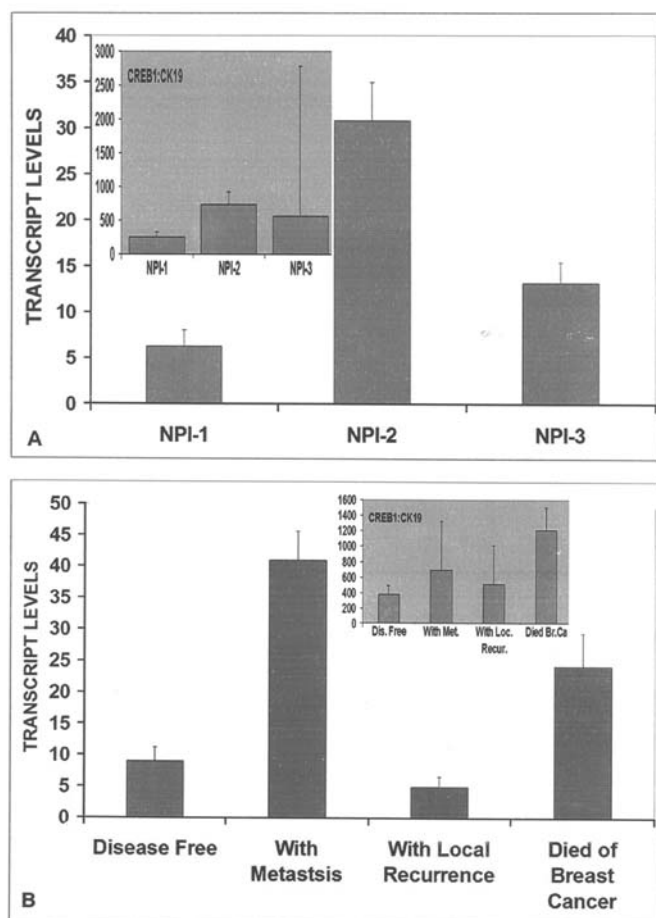
Figure 4. (A) CREB1 and predicted clinical outcome, using the Nottingham Prognostic Index (NPI) as an indicator, in the good prognosis group were patients with NPI<3.4; moderate, with NPI 3.4-5.4; and poor, with NPI>5.4. Insert: transcript normalised by CK19 (shown as CREB1:CK19 ratio). (B) CREB1 transcript levels and clinical outcome (median follow-up 120 months). Insert: transcript normalised by CK19 (shown as CREB1:CK19 ratio).
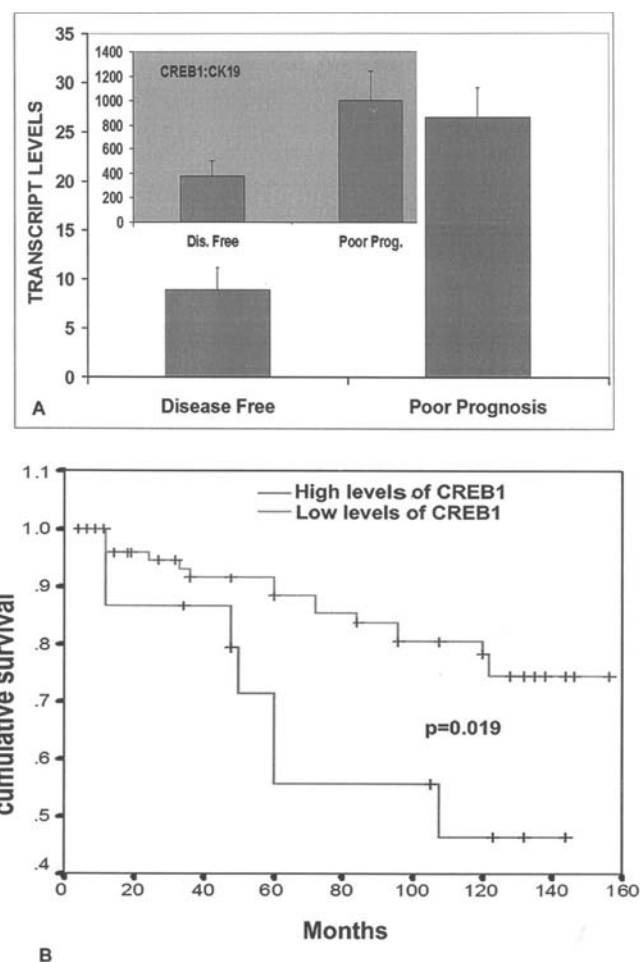

Figure 5. (A) Comparison between patients who remained disease free and who had disease progression (recurrence, metastasis and mortality). Insert: transcript normalised by CK19 (shown as CREB1:CK19 ratio). (B) Kaplan-Meier survival analysis showing correlation between CREB1 transcript levels and the 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.

tumours (22). Sofi *et al* reported that the expression of CREB in tumour bearing breast adipose tissue is higher than in normal breast adipose tissue (12). CREB has an antimitogenic role in solid tumours such as in glioblastoma proliferation (20) and decreased expression is seen in thyroid carcinoma (21).

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

The current study reported for the first time that CREB1 is 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 the CREB1 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.

The role of CREB in relation to aromatase expression is still unclear. Overexpression of aromatase in adipose tissue surrounding breast tumour could arise through an increase in both CREB expression and CREB transcriptional activity. Sofi *et al* reported an increased aromatase expression due to an increase in CREB-induced promoter II activity in tumour bearing breast adipose tissue (12). Further studies needs to be performed to ascertain whether increased expression of CREB leads to aberrant aromatase expression and whether there is a simultaneous decrease in the expression of negative regulatory proteins such as Snail and Slug 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 (23). Inhibition of CREB activity could inhibit aromatase expression and hence decrease oestrogen production in breast tissue. Therefore overexpression 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 an anti-CREB therapy based on inhibiting aromatase expression as a treatment strategy for breast cancer patients.

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