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Ets2 transcription factor in normal and neoplastic human breast tissue

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ABSTRACT

The Ets family of transcription factors regulate the expression of multiple genes involved in tumour formation and progression. The aim of this work was to test the hypothesis that the expression of Ets2 in breast cancers was associated with parameters of tumour progression and metastasis. Using reverse-transcriptase polymerase chain reaction (RT-PCR), Ets2 mRNA was detected in 69% of 181 breast carcinomas, 63% of 43 fibroadenomas and 47% of 43 specimens of normal breast tissue. Levels were significantly higher in carcinomas compared with normal breast tissue ($P = 0.006$). Using Western blotting, Ets2 protein was found to migrate as two bands with molecular masses of 52 kDa (p52) and 54 kDa (p54). Levels of both proteins were significantly higher in the carcinomas compared with both fibroadenomas ($P = 0.0001$) and normal breast tissue ($P = 0.0001$). In the carcinomas, a significant relationship was found between the p52 and p54 form of Ets2 ($r = 0.51$, $P < 0.0001$; Spearman correlation). Also, in the carcinomas, a significant correlation was found between both forms of Ets2 protein and urokinase plasminogen activator (uPA) (for p52, $r = 0.43$, $P = 0.0005$, $n = 68$; for p54, $r = 0.50$, $P = 0.0001$, $n = 68$). As Ets2 binding sites are present on the uPA promoter, Ets2 may be one of the transcription factors regulating uPA expression in human breast cancer.

1. Introduction

The *ets* genes encode a family of transcription factors that regulate a wide variety of biological processes.[1] A highly conserved DNA-binding domain known as the *ets* domain defines the family. The *ets* domain is necessary for the specific recognition of a purine-rich core sequence, GGAA/T, within the promoter/enhancer regions of multiple target genes. Amongst the genes whose promoter/enhancer regions contain Ets binding sites are those involved in both tumourigenesis and metastasis, such as human epidermal growth factor receptor-2 (HER-2/*neu*),[2] p16INK4A,[3] p21WAF,[4] urokinase plasminogen activator (uPA)[5] and specific matrix metalloproteinases (MMPs).[6]

Ets2 is one of the founder members of the Ets family and was initially characterised as a proto-oncogene.[1] It is known to be a downstream target for both the Ras MAP kinase[7] and phosphatidylinositol 3-kinase/Akt[8] pathways. As a transcription factor, Ets2 has been shown to regulate genes involved in the cell cycle,[9] apoptosis,[10] oncogenesis and tumour progression.[11,12]

Multiple studies with cell lines and animal models suggest that Ets2 is causally involved in breast cancer formation and/or progression.[13,14] For example, Sapi and colleagues[15] showed that an Ets2 transdominant mutant abolished anchorage-independent growth and macrophage colony-stimulated factor-stimulated invasion by BT20 breast cancer cells. In the MCF7 breast cancer cell line, overexpression of Ets2 was found to

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inhibit breast cancer associated gene 1 (*BRCA1*) expression but increased expression of both uPA and MMP-3.[16] On the other hand, transfection of the non-tumourigenic but immortalised MCF-12A breast cells with Ets2 resulted in EGF-independent proliferation, growth in soft agar, alterations in adhesiveness, and enhanced invasiveness.[17] Furthermore, mammary tumours that developed in a mouse model possessing only a single copy of the *ets2* wild-type gene were approximately only half the size of those formed in the control group.[18] In another model system, substituting alanine for threonine 72 in Ets2 protein created a hypomorphic allele that restricted mammary tumour formation.[19] This reduction in tumour development correlated with decreased expression of macrophage MMP-3 and MMP-9.[19]

Although these studies implicate Ets2 in breast carcinogenesis and/or progression, little work has been carried out at the mRNA and protein level on this Ets factor in human breast cancers. The aim of this investigation was therefore to test the hypothesis that the presence of Ets2 in breast carcinomas correlated with parameters of tumour progression. In the carcinomas, we showed that Ets2 protein exists as two forms, migrating with molecular masses of 52 and 54 kDa. We also showed that levels of both the p52 and p54 forms of Ets2 correlated significantly with those of uPA, a protease causally linked with cancer progression and one of the most potent biological prognostic factors so far described for breast cancer.[20]

2. Materials and methods

2.1. Human breast tissues

Following histopathological examination, residual tumour tissue was immediately frozen in liquid nitrogen. Normal breast tissue included tissue remote from primary carcinoma ($n = 9$), remote from fibroadenoma ($n = 33$), and tissue from reduction mammoplasty ($n = 1$). Although these specimens cannot strictly be regarded as normal healthy breast tissues, for this article they are referred to as 'normal' breast tissue. As similar Ets2 expression levels were observed in these three types of normal breast tissue, their values were combined. The characteristics of the carcinomas used are summarised in Table 1. In total, 181 primary breast carcinomas were analysed for Ets2 mRNA expression. Of these 181 cases, 111 representative samples were also analysed for Ets2 protein expression. All carcinoma samples were dissected out by a qualified pathologist and confirmed to be malignant using frozen section analysis. A total of 43 fibroadenomas were analysed for Ets2 mRNA expression, 38 of which were also analysed for Ets2 protein expression. In addition, 43 normal breast tissues were analysed for mRNA expression, 12 of which were also analysed for protein expression.

Table 1 – Patient and disease characteristics in primary breast carcinomas analysed for Ets2 expression ($n = 181$)

Factors	Patients	
	n	%
Nodal status		
Negative	81	45
Positive	90	50
Unknown	10	5
Tumour size		
T1 (⩽2 cm)	45	25
T2 (2–5 cm)	94	52
T3–4 (⩾5 cm)	28	15
Unknown	14	8
Histology type		
Ductal (D)	146	81
Lobular (L)	26	14
D & L	9	5.0
Tumour grade		
1	11	6
2	70	39
3	100	55
ER status[a]		
Negative	52	29
Positive	128	71
Unknown	1	1
PR status[b]		
Negative	75	41
Positive	77	43
Unknown	29	16

PR, progesterone receptor; ER, oestroegn receptor.
a Cutoff point = 200 fmol/G of wet weight tissue.
b Cutoff point = 1000 fmol/G of wet weight tissue.

2.2. RNA extraction

Samples were powdered using the Braun Mikrodismembrator (Braun Apparate, Melsungen, Germany) and the powder stored at −80 °C. Total RNA was extracted from 100 to 200 mg of breast tissue using the guanidine isothiocyanate/phenol chloroform method.[21] The integrity of the RNA was visualised by running 10 μl on a 2.5% (w/v) agarose gel with ethidium bromide staining and checking the integrity of the 28 S and 18 S bands. The quality of RNA was determined by reading the absorbance at 260 nm.

2.3. cDNA synthesis

One microgram of total RNA was reversed transcribed to single-stranded complementary DNA (cDNA) in a final volume of 20 μl. The reaction mixture contained 0.4 mM of each deoxynucleotide triphosphates (dNTP), 10 μg/ml of oligo $(dT)_{12–18}$, 10 mM dithiothreitol (DTT), 50 mM Tris–HCl (pH 8.3), 75 mM KCl and 3 mM $MgCl_2$. This reaction mixture was incubated for 5 min at 70 °C to remove secondary DNA structures, centrifuged and cooled on ice, followed by the addition of 4.6 U of human placenta ribonuclease inhibitor (GibcoBRL®) and 200 U of Moloney murine leukaemia virus reverse transcriptase (Promega). Samples were incubated for 1 h at 37 °C and finally heated for 5 min at 65 °C. cDNA was stored aliquoted at −20 °C until required for polymerase chain reaction (PCR) amplification.

2.4. Polymerase chain reaction (PCR)

Semi-quantitation of Ets2 mRNA was performed following normalisation with an internal control sequence, glyceraldehyde-3-phosphate-dehydrogenase (GAPDH). The primers for

Ets2 and GAPDH were designed and numbered according to Genbank notation (accession numbers = NM_005329, NM_002046). Primers specific for the amplification of the two transcripts were chosen using the 'Primer Select' software, and the specificity confirmed by carrying out a detailed BLAST search. Details of all primer pairs are as follows: Ets2: sense, 5′-AAG AAC CCC TGG CTG GCT GTG GG-3′;antisense, 5′-CCG CCT TTG GGG GTA AAT TC-3′ (nucleotides 592–615 and 915–1938, respectively); GAPDH: sense, 5′-CCA CCC ATG GCA AAA TTC CAT GGC A-3′; antisense, 5′-TCT AGA CGG CAG GTC AGG TCC ACC-3′ (nucleotides 227–250 and 801–824, respectively).

The PCR reaction comprised 2 μl of cDNA template obtained from 1 μg of RNA, 100 ng each of upstream and downstream Ets2 primer (Genosys, Pampisford, United Kingdom (UK)), 0.25 mM each of dNTP, 1.5 mM $MgCl_2$ and 1.25 U Taq DNA polymerase (Promega) in a reaction buffer made up to a final volume of 50 μl. Amplified products were obtained in the exponential phase for all sets of primers. All PCR reactions were performed in an automated thermocycler (MJ Research, Watertown, MA, United States of America (USA)). The cycling conditions for each of the primer sets were as follows: Ets2: a denaturing step for 2 min at 94 °C, followed by 1 min at 94 °C, 1 min at 58 °C and 1 min at 72 °C for 33 cycles, followed by 5 min at 72 °C; GAPDH: a denaturing step for 2 min at 94 °C, followed by 1 min at 94 °C, 1 min at 60 °C and 1 min at 72 °C for 30 cycles. With these conditions, amplification products were obtained in the exponential phase for both sets of primers. Following amplification, 20 μl of PCR product from each reaction was run out on a 2.5% (w/v) agarose gel and visualised by ethidium bromide staining under ultraviolet (UV) light. The intensity of the bands was determined by densitometry (EagleEye™, Stratagene, UK), and expressed as a ratio of the GAPDH band intensity. Negative controls included omission of reverse transcriptase and replacement of cDNA by water. The identity of the PCR products was confirmed by direct sequencing (ABI prism 310 technology).

2.5. *Western blot analysis*

Frozen powdered tissue samples were suspended in 50 mM Tris–HCl, pH 7.4, (2 ml per 100 mg of sample) containing Triton X-100 at a final concentration of 1% (v/v). These homogenates were agitated for 20 min at 4 °C and centrifuged at 13000*g* for 20 min at 4 °C. The pellet was discarded and the supernatant containing the protein was transferred to a clean tube. Total protein concentration was determined using the micro-bicinchoninic acid (BCA) protein assay (Pierce, Rochford, IL, USA) according to the manufacturer's instructions. Samples containing 30 μg protein were subjected to 12% (v/v) polyacrylamide gel electrophoresis under reducing conditions. Following electrophoresis, the separated proteins were transferred to a nitrocellulose membrane (Sigma Chemical Company, St. Louis, MO, USA). A cell lysate from Jurkat cells (Upstate Biotechnology, Lake Placid, NY, USA) was used as a positive control. After non-specific sites were blocked with 5% (w/v) powdered milk in 0.05% Triton X-100/Tris buffered saline (TBS-T) for 1 h, blots were incubated overnight with an affinity purified rabbit polyclonal Ets2 (C-20, Santa Cruz Biotechnology, Inc.) antibody at a concentration of 0.4 μg/ml. The blots were then washed three times in TBS-T for 10 min each and incubated with a HRP-conjugated goat anti-rabbit IgG (Sigma–Aldrich Ireland Ltd., Dublin, Ireland) at a concentration of 1 μg/ml in 5% (w/v) powdered milk in 0.05% TBS-T. All samples were also blotted for β-actin (Clone AC-15, Sigma–Aldrich Ireland Ltd., Dublin, Ireland) to normalise protein amounts. Bands were detected by the addition of a chemiluminescent substrate (Luminol System, Santa Cruz Biotechnology, Inc.). Exposing the blots to Fuji X-ray film for 10 s to 20 min captured light emitted during the enzyme-catalysed decomposition reaction. Scanning densitometry was performed on the protein bands using the EagleEye™ Still Video system (EagleEye™, Stratagene, UK) and arbitrary units assigned. Values are expressed relative to β-actin.

2.6. *Enzyme-linked immunosorbent assay (ELISA)*

uPA was assayed by ELISA using kits obtained from American Diagnostica Inc., Stamford, CT, USA. Oestrogen receptors and progesterone receptors were also determined by ELISA (Abbott Diagnostics, North Chicago, IL, USA).

2.7. *Statistical analysis*

The strength of associations between the various parameters measured in this study was tested using non-parametric tests. The Mann–Whitney *U* test was used for categorical data and the Spearman rank correlation were used for continuous variables. Two-sided *P*-values below 0.05 were considered statistically significant.

3. Results

3.1. *Ets2 mRNA in normal breast tissue, fibroadenomas and breast carcinomas*

Representative RT-PCR blots for Ets2 and GAPDH mRNA are shown in Fig. 1(a). GAPDH was used as an internal control and confirmed an equivalent amount of RNA loading for each sample. Table 2 summarises the median values, ranges and frequency of expression of Ets2 mRNA levels in normal breast tissue, fibroadenomas and breast carcinomas. Both the levels and frequency of expression of Ets2 mRNA were significantly higher in breast carcinomas compared with normal breast tissue (for levels, $P = 0.0001$, Mann–Whitney *U* test; for frequency, $P = 0.006$, χ^2 test) (Fig. 1(b)). No significant difference in Ets2 expression was found between breast carcinomas and fibroadenomas. Levels of Ets2 mRNA were however, higher in fibroadenomas vis-á-vis normal breast tissue ($P = 0.03$) (Fig. 1(b)).

3.2. *Ets2 protein in normal breast tissue, fibroadenomas and breast carcinomas*

Fig. 2(a) shows Ets2 protein measured by Western blotting. Two different protein forms were detected, migrating with molecular masses of 52 kDa (p52) and 54 kDa (p54). The distribution of p52 and p54 in normal breast tissue, fibroadenomas and breast carcinomas is summarised in Table 2. Both the p52 and p54 forms of Ets2 protein were expressed at significantly higher levels in carcinomas versus both fibroadenomas (for

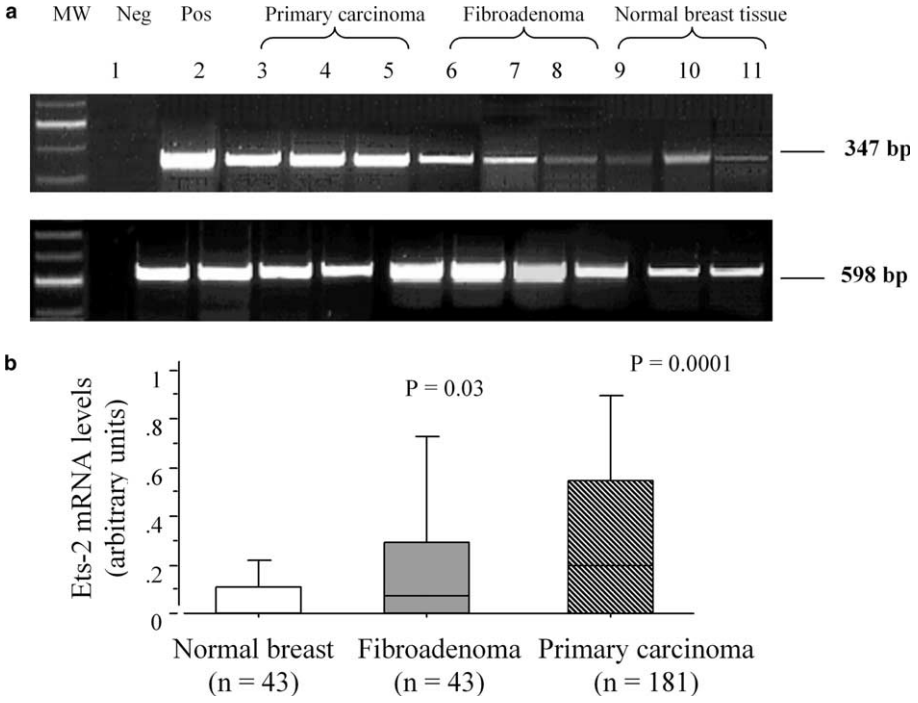

Fig. 1 – (a) Representative reverse-transcriptase polymerase chain reaction (RT-PCR) blots of Ets2 and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) detected in breast carcinomas, fibroadenomas and normal breast tissue. Lane MW, 100 base pair ladder; lane 1, negative control without reverse transcriptase and lane 2, positive control (Jurkat cell cDNA), lanes 3–5, primary breast carcinomas, lanes 6–8, fibroadenomas, lanes 9–11, normal breast tissue. (b) Relative levels of Ets2 mRNA in normal breast tissue, fibroadenomas and primary carcinomas as detected by RT-PCR. mRNA levels are represented as arbitrary units relative to GAPDH. Box boundaries indicate the 25th and 75th percentiles of the observed values; capped bars indicate the 10th and 90th percentiles and the solid line indicates the median. Data were analysed using the non-parametric Mann–Whitney *U* test. Levels were significantly higher in both carcinomas and fibroadenomas compared with normal breast tissue ($P = 0.0001$ and 0.03, respectively).

both p52 and p54, $P = 0.0001$) and normal breast tissue (for both proteins, $P = 0.0001$) (Fig. 2(b and c)). Both forms of Ets2 protein were found in a similar proportion of carcinomas with a moderately strong significant correlation observed between the p52 and p54 forms ($r = 0.51$, $P = < 0.0001$, $n = 83$; Spearman rank correlation) (Fig. 3). No significant correlation was found between either of the protein forms and Ets2 mRNA.

Table 2 – Levels of Ets2 mRNA and protein in normal breast tissue, fibroadenomas and carcinomas

Tissue type	*n*	Median	Range	% positive
Ets2 mRNA				
Normal breast	43	0	0–0.96	47
Fibroadenoma	43	0.07	0–1.5	63
Primary carcinoma	181	0.19	0–3.0	69
Ets2 52 kDa				
Normal breast	12	0	0–0.48	17
Fibroadenoma	38	0.01	0–0.86	43
Primary carcinoma	111	0.85	0–11.0	85
Ets2 54 kDa				
Normal breast	12	0	0–0.42	33
Fibroadenoma	38	0.01	0–2.3	58
Primary carcinoma	111	0.63	0–5.8	89

Values are given in arbitrary units following semi-quantification of both Ets2 mRNA and protein bands by scanning densitometry. mRNA levels are expressed relative to GAPDH and the protein levels are expressed relative to β-actin.

3.3. *Correlation between Ets2 and the established prognostic factors for breast carcinoma*

Neither the p52 or p54 forms of the Ets2 protein correlated significantly with tumour size, nodal status, tumour grade, ER or PR levels in the primary carcinomas. Similarly, no significant relationship was observed between Ets2 mRNA and these prognostic factors.

3.4. *Relationship between Ets2 proteins and uPA*

As mentioned in Section 1, Ets binding sites have been identified in the promoter/enhancer regions of the uPA promoter[5] and indeed Ets2 has been shown to upregulate expression of uPA in a number of different cell lines.[22] We therefore investigated whether Ets2 protein levels correlated with those of uPA in human breast cancers. As shown in Fig. 4, a statistically significant and moderately strong relationship were observed between both forms of the Ets2 protein and uPA (for p52, $r = 0.43$, $P = 0.0005$; for p54, $r = 0.50$, $P = 0.0001$; Spearman rank non-parametric test) (Fig. 4(a and b)).

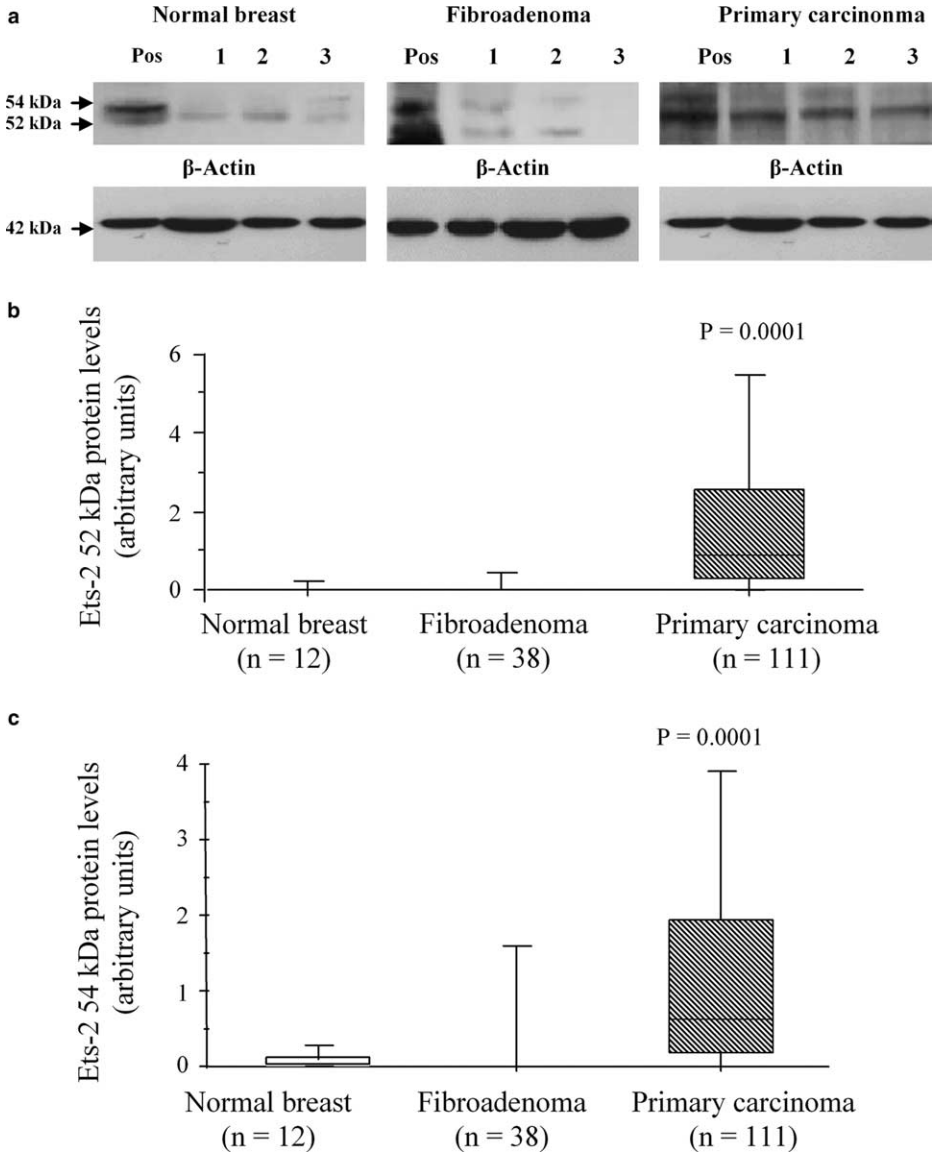

Fig. 2 – (a) Ets2 protein expression in primary carcinomas, fibroadenomas and normal breast tissue. Ets2 protein levels were determined by Western blotting as described in Section 2. Prim., primary breast carcinoma; FA, fibroadenoma; N, normal breast tissue; Pos., positive control (Jurkat cell lysate). Arrows show Ets2 p52 (full-length) and p54 (phosphorylated) proteins detected using an Ets2 specific antibody. β-Actin (42 kDa) was used as an internal positive control for each sample. Gels were allowed to run for 3 h to separate 54 and 52 kDa bands. (b) Relative levels of Ets2 p52 and p54 protein in normal breast tissue, fibroadenomas and primary carcinomas as detected by Western blotting. Protein levels are represented as arbitrary units relative to β-actin. Box boundaries indicate the 25th and 75th percentiles of the observed values, capped bars indicate the 10th and 90th percentiles and the solid line indicates the median. Data were analysed using the non-parametric Mann–Whitney *U* test. (c) Significantly higher levels were found in carcinomas compared with fibroadenomas and normal breast tissues for both p54 and p52 (*P* = 0.001).

4. Discussion

Although a number of studies have been published on Ets2 in breast cancer cell lines[22,23] and mice models,[18] few reports exist on Ets2 in human breast cancer. Here, we show that Ets2 at both mRNA and protein levels were significantly elevated in breast cancer compared with normal breast tissue. Furthermore, both forms of Ets2 protein were significantly higher in breast cancer vis-á-vis fibroadenomas. Using animal models, Galang and colleagues[13] recently reported that although Ets2 mRNA was abundantly expressed in normal mouse mammary gland, levels were not significantly increased in mammary tumours. In that study however, other members of the *ets* family, including PEA3, ER.81, ERM, Pse, Ese2, Ese3, Tel and Elf2, were overexpressed in mammary tumours compared with normal breast tissue.[13] In another report, PEA3, ERM and ER81 were found to be overexpressed in some breast cancers lines compared with normal breast epithelial cells.[24]

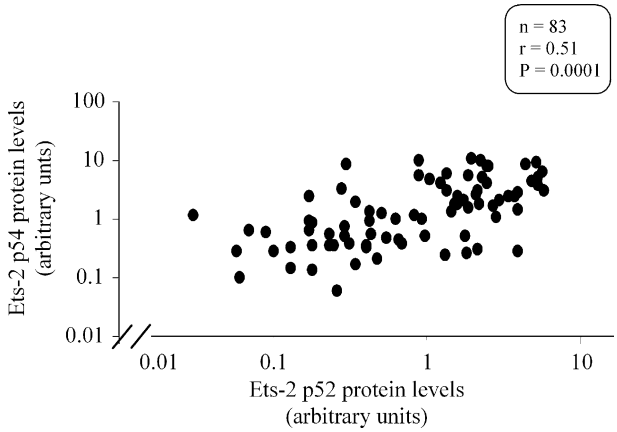

Fig. 3 – Relationship between the p52 and p54 forms of Ets2 protein in breast cancer Ets2 protein measurements were determined using Western blotting. Arbitrary units were assigned to each protein band following scanning densitometry. Values are expressed as a ratio to β-actin. Data were analysed using the non-parametric Spearman rank test. Each dot represents a value for a particular sample.

In our study, Ets2 protein was detected in 85–89% of breast cancers. While this work was in progress, Svensson and colleagues[25] using tissue arrays reported that a specific phosphorylated form of Ets2 protein was present in 69% of breast cancers while Myers and colleagues,[26] using standard immunohistochemistry, found Ets2 in 52% of samples. The reason(s) for these different findings is likely to relate to varying sensitivities of the different assays used, i.e., use of Western blotting on cell-free extracts of frozen tissue in this study compared with immunohistochemistry on formalin-fixed and paraffin-embedded fixed in the two published studies.[25,26]

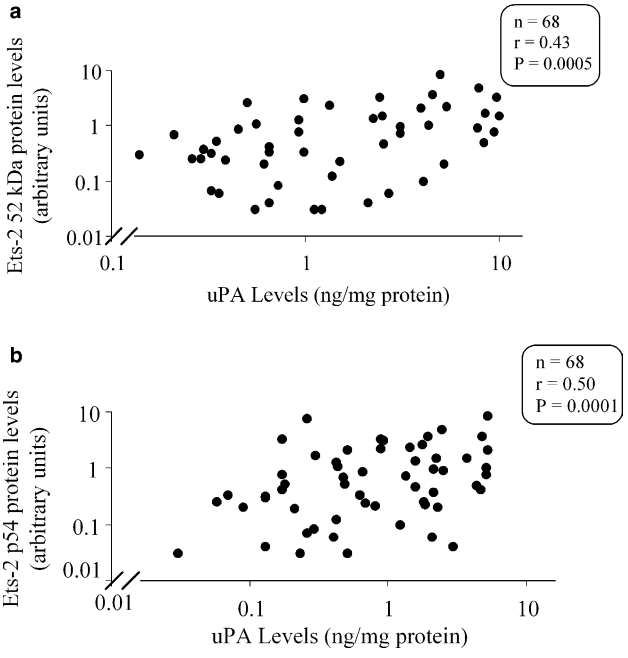

Fig. 4 – Relationship between (a) Ets2 p52 and uPA protein and (b) Ets2 p51 and uPA protein. Ets2 protein measurements were determined using Western blotting. Arbitrary units were assigned to each protein band following scanning densitometry. Values are expressed as a ratio to β-actin. uPA protein was measured using enzyme-linked immunosorbent assay (ELISA). Data were analysed using the non-parametric Spearman rank test. Each dot represents a value for a particular sample.

In this investigation, expression of Ets2 was independent of the established prognostic factors for breast cancer. Svensson and colleagues[25] however, reported an inverse association between the phosphorylated form of Ets2 referred to above and tumour size. The relationship between this phosphorylated form of Ets2 and the p52 and p54 proteins identified in our study is unclear.

Using Western blotting, we found that Ets2 protein exists as two forms in human breast cancer. Other investigators have also reported the existence of multiple forms of Ets2 protein.[27] In unstimulated RAW cells in culture, only the 54 kDa form of Ets2 was identified.[27] However, following stimulation with interferon gamma or lipopolysaccharide (LPS), additional bands at 58 and 109 kDa were observed. Further stimulation with LPS gave rise to bands at 52, 119 and 165 kDa. In our study, the high molecular forms of Ets2 protein found in RAW cells were not detected.

Although Ets2 displayed no significant relationship with traditional prognostic factors for breast cancer, both forms of Ets2 protein correlated significantly with uPA levels. uPA is a serine protease causally involved in invasion and metastasis and is one of the most potent biological prognostic factors so far described for breast cancer.[20] Recently, its prognostic impact in lymph node-negative breast cancer patients was validated in both a prospective randomised trial [28] and a pooled analysis.[29,30] Ets2 binding sites have been located on the *u*PA promoter and, as mentioned in the introduction, Ets2 has been shown to upregulate uPA expression in breast cancer cells in culture.[22] In this study, both forms of the Ets2 protein were found to associate significantly with uPA protein expression in primary carcinomas. Previously, we also found a significant association between Ets1 protein levels and uPA in breast cancer.[31] These findings suggest that both Ets1 and Ets2 play a role in the upregulation of uPA in human breast cancer.

In the breast cancers investigated in this study, levels of Ets2 expression at mRNA were not significantly related to protein levels. The reason(s) for the discordance are unknown but could relate to varying turn-over rates of Ets2 at mRNA and protein levels in the different tumours. It is of interest, that in a recent study on lung adenocarcinoma, only 21/98 (21.4%) of the genes investigated displayed a significant correlation between mRNA and protein levels.[32]

In conclusion, we have shown that Ets2 at both mRNA and protein levels were overexpressed in breast carcinomas compared with normal breast tissue. In breast carcinomas, Ets2 protein was found to exist in two main forms. Both these forms of Ets2 correlated significantly with uPA, suggesting that Ets2 may be, at least, partially responsible for the increased uPA levels found in breast cancer. The correlation between Ets2 and uPA suggests that Ets2 may also be a significant prognostic factor in breast cancer. Indeed, Span and colleagues[33] recently reported that high expression of Ets1 mRNA predicted adverse outcome in patients with breast cancer.

Conflict of interest statement

None declared.

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