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Evidence of a Tumour Suppressive Function of *E2F1* Gene in Human Breast Cancer

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Abstract. *Background: The E2F family of transcription factors are key regulators of genes involved in cell cycle progression, cell fate determination, DNA damage repair and apoptosis. E2F1 is unique in that it contributes both to the control of cellular proliferation and cellular death. Furthermore, unlike other E2Fs, E2F1 responds to various cellular stresses. This study aimed to examine the level of mRNA expression of E2F1 gene in normal and malignant breast tissue and correlate the level of expression to tumour stage. Materials and Methods: One hundred and twenty-seven breast cancer tissue and 33 normal tissues were analyzed. Levels of transcription of E2F1 were determined using real-time quantitative PCR, normalized against CK19. Levels of expression were analyzed against TNM stage, nodal involvement, tumour grade and distant metastasis. Results: The levels of E2F1 mRNA were lower in malignant tissues. They declined further with increasing TNM stage. This became statistically significant when TNM stages 3 and 4 were compared to TNM stages 1 and 2 disease (TNM1 vs. TNM3 p=0.032; TNM1 vs. TNM4 p=0.032; TNM2 vs. TNM3 p=0.019; TNM2 vs. TNM4 p=0.021). The levels of E2F1 also fell with increasing tumour grade, when comparing grade 2 and 3 with grade 1, however, the differences were not statistically significant. Conclusion: These results are highly suggestive of the role of E2F1 as a tumour suppressive gene in human breast cancer.*

E2F refers to a group of genes that codifies a family of transcription factors in mammalian cells. The *E2F* family of transcriptional factors are key regulators of genes involved in cell cycle progression, cell fate determination, DNA damage repair and apoptosis. The family can be classified into eight different groups of transcription factors based on domain conservation and transcriptional activity (1, 2). *E2F1*, *E2F2*, and *E2F3a* are potent transcriptional activators of *E2F*-responsive genes important for cell cycle progression and nucleotide synthesis. Other *E2Fs*, such as *E2F3b*, *E2F4*, *E2F5*, and *E2F6*, generally function as repressors of *E2F* gene expression. The recently discovered *E2F7* and *E2F8* genes form a separate group with antiproliferative function (3-5).

Various studies also demonstrate the unique and complex role of *E2F1* amongst the activators of *E2F* responsive genes, contributing to the control of both cellular proliferation and cell death. *E2F1* appears to be a stronger inducer of apoptosis than either *E2F2* or *E2F3* when expressed in the absence of growth factors (6-11). Unlike the other activators of *E2F* gene expression, *E2F1* responds to various cellular stresses. *E2F1*, but not *E2F2* or *E2F3*, is phosphorylated by ATM/ATR and Chk2 kinases in response to DNA damage, leading to accumulation of *E2F1*, through enhanced protein stability (12, 13).

The Rb/E2F pathway regulates the expression of genes essential for cell proliferation but also triggers apoptosis. The retinoblastoma (Rb) protein was the first tumour suppressor to be identified and it is absent or mutated in at least one-third of all human tumours (14). Rb restrains proliferation, in part, by modulating the activity of *E2F* transcription factors. In quiescent cells (G0 and early G1), Rb is hypophosphorylated, and hence binds with several E2Fs, resulting in the repression of proliferation-associated genes. Mitogenic growth factors induce the sequential activation of the cell cycle-dependent cyclin kinase complexes Cdk4/Cdk6-cyclin D and Cdk2-cyclin E, which phosphorylate Rb and cause it to release E2F. The resultant activation of E2F-responsive genes during late G1, commits the cell to initiate DNA replication and S-phase entry. Furthermore, the unrestrained E2F activity also promotes apoptosis through p53-dependent and -independent mechanisms (15, 16).

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During normal proliferation, PI3K/Akt signalling blocks *E2F1*-induced apoptosis, thus serving to balance proliferation and death (17). Growth factor-activated PI3K signalling directly regulate *E2F1* transcriptional output and apoptosis induction, through repressing a subset of previously unidentified *E2F1* target genes (17). Akt is a serine/threonine kinase that phosphorylates several cellular proteins (*e.g.* p27 KIP1, BAD, capsase-9), either inhibiting their proapoptotic or enhancing an antiapoptotic activity (18-20). Akt phosphorylation also degrades p53 by activating its inhibitor Mdm (21, 22). Furthermore, Akt can indirectly inhibit *E2F1* transcriptional activity by phosphorylating TopBP1, promoting its binding to and repression of *E2F1* (23).

Our study aimed to examine the levels of mRNA expression for *E2F1* gene in normal and malignant breast tissue and correlate the levels of this gene to the clinicopathological parameters of the tumour samples using quantitative PCR (Q-PCR), which has been shown to be a sensitive and specific method of measuring gene copy number.

Materials and Methods

Materials. RNA extraction kits and reverse transcription kits were obtained from Abgene Ltd. (Surrey, UK). PCR primers were designed using Beacon Designer (Palo Alto, CA, USA) and synthesized in house. Custom made hot-start Master mix for quantitative PCR was from Abgene (24, 25).

Sample collection. Breast cancer tissues (n=127) and normal background tissues (n=33) were collected immediately after surgery (Table I) and stored at –80˚C until use. A Consultant Pathologist (A.D-J) who examined H&E-stained frozen sections verified the presence of tumour cells in the collected tissues. Details of histology were obtained from pathology reports.

Tissue processing, RNA extraction and cDNA synthesis. Frozen sections of tissue were cut at a thickness of 5-10 μm and were kept for routine histology. An additional 15-20 sections were mixed and homogenized using a handheld homogeniser, in ice-cold RNA extraction solution. The concentration of RNA was determined using a UV spectrophotometer. Reverse transcription was carried out using a reverse transcription kit with an anchored olig(dT) primer supplied by Abgene, using 1 μg of total RNA in a 96 well plate. The quality of cDNA was verified using β-actin primers (primers 5'-ATGATATCGCCGCGCTCGTC-3' and 5'-CGCTCGG TGAGGATCTTCA-3').

Quantitative analysis of E2F1. The levels of *E2F1* transcripts from the above prepared DNA was determined using real-time quantitative PCR based on the Amplifluor technology, modified from a method reported previously (25).

PCR primers were designed using Beacon Designer software but to the reverse primer an additional sequence, known as the Z sequence (5'-ACT-GAACCTGACCGTACA-3') which is complementary to the universal Z probe (Intergen Inc., Oxford, UK) was added. The primers were designed to amplify only *E2F1*. The product expands one intron. The reaction was carried out using the following: Hot-start Q-master mix (Abgene), 10 pmol of specific forward primer, 1 pmol reverse primer which had the Z sequence, 10 pmol of FAM- tagged probe (Intergen Inc., Oxford, UK) and cDNA from 50 ng of RNA. The reaction was carried out using the IcyclerIQ (BioRad, UK) which is equipped with an optical unit that allows real-time detection of 96 reactions under the following conditions: 94˚C for 12 min and 50 cycles of 94˚C for 15 s, 55˚C for 40 s and 72˚C for 20 s. The levels of the transcript were generated from a standard that was simultaneously amplified with the samples. Levels of *E2F* expressions were then normalized against CK19 expression already measured in these specimens to correct for varying amounts of epithelial tissue among samples.

Table I. *Clinical data*.

Parameter	Category	Number
Node status	Positive	54
	Negative	73
Tumour grade	1	24*
	2	43
	3	58
Tumour type	Ductal	98
	Lobular	14
	Medullary	2
	Mucinous	4
TNM staging	1	70**
	2	40
	3	7
	4	4

*2 Values were discarded as they were not interpretable; **6 Values were discarded as they were not interpretable.

Primer sequences: Forward primers: *E2F1*F1-TGCTCTCCGAG GACACTG, *E2F1*F2-CATTGCCAAGAAGTCCAAGAAC. Reverse primers: *E2F1*ZR2-ACTGAACCTGACCGTACACGGAG GTCCT GGGTCAAC, *E2F1*ZR1-ACTGAACCTGACCGTACATCA CCAT AACCATCTGCTCTG.

CK19 was quantified as previously reported (25) using the primers: 5'-CAGGTCCGAGGTTACTGAC-3' and 5'-CTGAACCTGACCG TACACACTTTCGCCAGTGTGTCTTC-3'.

Statistical analysis. The statistical analysis of the data was carried out using the SPSS software package (SPSS Inc., Chicago, IL, USA; Version 11.5, 2003). Mann-Whitney and two sample *t*-test were performed for analysis of probable associations. The significance levels were considered for results with *p*-value lower than 0.05.

Results

The raw data (Table II) showed that levels of *E2F1* were 6 times higher in normal breast tissues than in breast cancer tissues (median values: 499 *vs*. 82.1 respectively, p=0.0571). Furthermore, it also demonstrated a negative correlation between *E2F1* levels in the tissues and stage of the breast cancer. The *E2F1* levels were normalized against CK19 (an

Table II. *Level of E2F1 mRNA according to tumour characteristics (raw data).*

Parameter	Subgroups	No.	Median	Mean±SD
All samples	E2F1 all tissue	160	101	4067±11865
	Normal tissue	33	499	4158±6434
	Tumour tissue	127	82	3715±12373
Grade	Grade1	24	42	7847±16709
	Grade2	43	153	651±1309
	Grade3	58	64	4821±14865
TNM staging	TNM1	70	168	4557±14003
	TNM2	40	101	1831±4080
	TNM3	7	0.9	14±25.6
	TNM4	4	22	48±66.3
Nodal status	Node-positive	54	88	6371±17441

epithelial maker) to correct for the varying amounts of epithelial tissue in each sample.

E2F1 levels were found to decline with increasing TNM stage and this was found to be statistically significant. When TNM stage 3 and 4 were compared to TNM 1, a statistically significant decline in *E2F1* level was noted (TNM1 *vs*. TNM3 p=0.032; TNM1 *vs*. TNM4 p=0.032). Similarly, *E2F1* levels were lower in TNM 3 and 4 than TNM 2 cancer (TNM2 *vs*. TNM3 p=0.019; TNM2 *vs*. TNM4 p=0.021) at a statistically significant level. Those patients with positive nodal status showed lower levels of *E2F1* than did normal tissues (mean values: 499 *vs*. 88), however, this was not statistically significant.

In contrast, the levels of *E2F1* also fell with increasing tumour grade, however, this did not reach a statistical significance.

Discussion

The study demonstrated a compelling trend for lower level of expression of *E2F1* gene in malignant when compared to normal tissues. Most remarkably, the study established a statistically significant inverse correlation between the expression of *E2F1* genes and increasing TNM stage of breast cancer. Such down-regulation of *E2F1* in malignant tissues suggests a likely tumour suppressive role for *E2F1* in human breast cancer.

The above hypothesis is consistent with the finding in bladder cancer that reduced expression of *E2F1* is associated with increased risk of progression to metastasis and death (26). Similarly, Ho *et al*. (27) demonstrated a lower expression of both *E2F1* and *E2F4* in the majority (70%) of the primary breast carcinomas and in all metastatic nodal breast tissues when compared with those of their corresponding normal tissue. In contrast, however, Seki *et al*. (28) reported the amplification and overexpression of *E2F1* in 63% of gastric cancer cases, suggesting that *E2F1* may act as an oncogene in gastrointestinal carcinoma. These findings indicate the likely tissue-specific nature of the net effect of the different functions of E2Fs. However, breast cancer based studies (29-31), have also shown that low *E2F1* transcript levels are strong determinants of favourable breast cancer outcome instead. Such findings highlight the two diametrically opposite effects of *E2F1*-dependent gene expression program and their likely role as a 'molecular switch' determining the balance between proliferation and cell death.

The deregulation of apoptosis may be important in breast tumourgenesis (32) and there is evidence to suggest that the tumour-suppressive potential of *E2F* is linked to its apoptotic function. Bargou *et al*. (33) demonstrated that inhibition of endogenous *E2F* activity in a normal breast epithelial cell line could confer resistance to apoptosis without any effect on the rate of cell proliferation and DNA synthesis and can also induce tumour growth in severe combined immunodeficiency mice. Furthermore, *E2F1*-deficient mice have a defective apoptotic function and develop various types of tumours (34, 35).

Similarly, the finding that *E2F1* overexpression mediates apoptosis (6, 7, 10, 34, 36) suggests an alternative explanation for the poor outcome in patients with low *E2F1* expression. Shan and Lee (7) overexpressed *E2F1* in Rat-2 fibroblasts, demonstrating that apoptosis occurs in an *E2F1* dose-dependent fashion. Qin *et al*. (6) also demonstrated that induction of *E2F1* expression in transfected Rat-1a fibroblasts resulted in S-phased entry and apoptosis. Fueyo *et al*. (36) showed that *E2F1* overexpression in nude mice harbouring subcutaneously implanted gliomas caused growth inhibition or tumour regression as a result of *E2F1*-mediated apoptosis as suggested by *in vitro* experiments. This *E2F1*-mediated apoptosis was independent of the endogenous *p53, Rb,* or *p16* status; *E2F1* overexpression did not increase *Bax* expression but did result in activation of the capsase cascade (36). Thus, the relative resistance to apoptosis may account for the poor outcome in patients with low *E2F1* reactivity. Other investigators have suggested additional mechanisms that may potentially explain the poor outcomes observed in patients with low *E2F1* reactivity, including changes in *E2F1* phosphorylation (phosphorylation of *E2F1* by cyclin A cdk2) or cyclin A-cdk2 impairing binding of *E2F1* to the E2 promoter (37) and facilitation of Rb binding to *E2F1* (38), and alteration of the DP proteins required for stable interaction of *E2F1* with Rb (39). Furthermore, Crowe *et al*. demonstrated that *E2F1* had an atypical function as a transcriptional repressor of the *hTERT* gene in human cells (40).

While our observations require further validation by larger series, the results support the role of *E2F1* as a tumour suppressor in breast cancer. Hence, *E2F1* gene may have a potential use as a prognostic marker and a therapeutic target. Reports of recombinant adenovirus-mediated gene therapy

with *E2F1* as a powerful inducer of apoptosis in human breast cancer cell lines (41) demonstrated the use of *E2F1* as a potential therapeutic gene. Further studies that clarify the mechanisms involved in *E2F* down-regulation in breast cancer cells are also required.

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