Evidence of a Tumour Suppressor Function for DLEC1 in Human Breast Cancer

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Abstract. *DLEC1 (deleted in lung and oesophageal cancer), located on 3p22.3, is a candidate tumour suppressor gene in lung, esophageal, and renal cancer. The aim of this study was determine whether the mRNA expression levels of DLEC1 were consistent with a tumour suppressive function. Materials and Methods: A total of 153 samples were analysed. The levels of transcription of DLEC1 were determined using quantitative PCR and normalised against (CK19). Transcript levels within breast cancer specimens were compared to normal background tissues. Results: Levels of transcription were higher in tumour samples compared to adjacent non cancerous tissue (ANCT) samples but this was not statistically significant (median 0.167 vs. 0.03; p=0.138). DLEC1 expression levels were significantly lower in samples from patients who developed metastasis, local recurrence, or died of breast cancer when compared to those who were disease free for >10 years (p=0.041). Discussion: These findings are consistent with a possible tumour suppressor function of DLEC1 in breast cancer.*

Breast cancer remains the leading cause of mortality among women in the western world. Complete understanding of the genetic abberations in breast cancer may help early diagnosis, prognostication and management of the disease. This has stimulated research into the identification and characterization of mutations that occur frequently during breast carcinogenesis (1).

Loss of heterozygosity on chromosomes 1, 3p, 6q, 7q, 8p, 11p, 13q, 17p, 17q, 18q, and 22q has been reported in breast carcinomas and other tumours, indicating a role for tumour suppressor genes located in these regions in the development and progression of human cancer (1, 2). There is an increasing body of evidence that the short arm of chromosome 3 (3p) harbours potential tumour suppressor genes in breast cancer. By means of hemizygosity and homozygosity mapping, cytogenetic analysis, and functional studies, distinct regions on 3p (3p25–26, 3p 21–22, 3p14.2, and 3p12) have been shown to be important for the development of several common sporadic cancer types including lung, breast, kidney, ovarian, cervical, and head and neck cancer (2-7). It was previously demonstrated that *SETD2*, located on 3p21.31, was a potential tumour suppressor gene (8).

The *DLEC1* gene that is deleted in lung and oesophageal cancer is located in the 3p22.3 region, which has been identified as one of the common deleted regions in lung cancer (9). The *DLEC1* gene contains 37 exons and spans approximately 59 kb. The predicted *DLEC1* protein contains 1755 amino acids. However, its exact biological function is still unclear because the predicted amino acid sequence of *DLEC1* has no significant homology to any of the known proteins or domains (10, 11). Loss of *DLEC1* expression has been observed in lung, oesophageal, renal, ovarian and nasopharyngeal carcinoma cell lines and primary tumours, and functional analyses strongly suggest that *DLEC1* is a tumour suppressor gene (10, 12). Promoter hypermethylation has been shown to be responsible for silencing of *DLEC1* in ovarian cancer, nasopharyngeal carcinoma and lung cancer (13).

The aim of this study was to examine the mRNA expression of *DLEC1* in breast cancer using quantitative PCR and to determine whether the mRNA expression levels of *DLEC1* were consistent with a tumour suppressive function. This is the first study in the literature to examine the direct relationship between *DLEC1* and breast cancer.

Materials and Methods

Patients and samples. Institutional guidelines, including ethical approval and informed consent, were followed. Breast cancer

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tissues (n=120) and adjacent non cancerous tissues (ANCT) (n=33) were collected immediately after excision during surgery, and stored at –80˚C until use. A consultant pathologist examined hematoxylin- and eosin-stained frozen sections to verify the presence of tumour cells in the collected samples. ANCT was derived from the background breast parenchyma of breast cancer patients within the study group. All tissues were randomly numbered and the details were only made known after all analyses were completed. All patients were treated according to local algorithms of management following a multidisciplinary discussion. Patients treated with breast-conserving surgery received adjuvant radiotherapy. Those with hormone-sensitive malignancy received tamoxifen. Fit patients with node-positive breast cancer or hormone-insensitive large and/or high-grade cancer were offered adjuvant chemotherapy. Medical notes and histology reports were used to extract clinicopathological data (Table I). A customised database was established to record data.

Materials. RNA extraction kits and reverse transcription kits were obtained from Sigma-Aldrich Ltd (Poole, Dorset, England, UK). PCR primers were designed using Beacon Designer (Palo Alto, CA, U.S.A.) and synthesised by Sigma-Aldrich. A custom made hot-start Master-mix for quantitative PCR was obtained from Abgene (Surrey, England, UK) (14, 15).

Tissue processing, RNA extraction and cDNA synthesis. Frozen sections of tissue were cut to a thickness of 5-10 mm and kept for routine histological analysis. Additional 15-20 sections were mixed and homogenised using a hand-held homogeniser in ice-cold RNA extraction solution. The concentration of RNA was determined using UV spectrophotometry. Reverse transcription was carried out using a reverse transcription kit with an anchored olig (dT) primer supplied by Abgene, using 1 mg of total RNA in a 96-well plate.

Quantitative analysis. The level of DLEC1 transcripts from the above prepared DNA were determined using real-time quantitative PCR based on the Amplifluor technology (15), modified from a method reported previously (15). PCR primers were designed using Beacon Designer software (15), but to the reverse primer an additional sequence, known as the Z sequence which is complementary to the universal Z probe (Intergen Inc., Oxford, UK) was added. The product expanded one intron. The primers used for each *DLEC1* are detailed in Figure 1. The reaction was carried out using the following: Hotstart 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.), and cDNA from 50 ng of RNA. The reaction was carried out using the IcyclerIQ (Bio-Rad Ltd, Hemel Heamstead, England, 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 *DLEC1* expression were then normalised against CK19 expression, already measured in these specimens, to correct for varying amounts of epithelial tissue between samples. *CK19* transcripts were quantified as previously reported (16), using the primers detailed in Figure 1. With every PCR run, a negative control without a template was included and a known cDNA reference sample was used as a positive control.

Table I. *Clinical data showing number of patients in each category.*

Parameter	Category	Number of patients
Node status	Node positive	65
	Node negative	55
Tumour grade	1	23
	2	41
	3	56
Tumour type	Ductal	94
	Lobular	14
	Medullary	2
	Tubular	2
	Mucinous	4
	Other	4
TNM staging	1	69
	2	40
	3	7
	4	4
Clinical outcome	Disease free	81
	Alive with metastasis	7
	With local recurrence	5
	Died of breast cancer	20
	Died of unrelated disease	7
ER status	ERα negative	26
	ERα positive	62
	ERβ negative	17
	ERβ positive	71

Statistical analysis. The Mann–Whitney *U*-test was used for statistical analysis. *DLEC1* transcript levels within breast cancer specimens were compared to ANCT and analysed against conventional pathological parameters and clinical outcome over a 10-year follow-up period. In each case, the true copy number was used for statistical analysis and hence samples were not classified as positive or negative. Statistical analysis was carried out using Minitab v.14.1 (Minitab Inc., State College, PA, USA) using a custom written macro (Stat 2005.mtw).

Results

The levels of transcription in tumour samples were first examined and compared to those in ANCT samples. Levels were more than 5 times higher in ANCT samples (median 0.167 *vs*. 0.03), however this was not statistically significant (p=0.1385).

Levels in tumour samples were also examined according to their respective Nottingham Prognostic Index (NPI). This showed a statistically significant difference between levels in NPI1 compared to NPI2 or NPI3 and similarly with NPI2 compared to NPI3 (p=0.43, 0.06 and 0.2, respectively). Tumour samples showed no significant increase in levels when comparing grade 1 to grade 2 or grade 3 and grade 2 to grade 3 (p=0.08, 0.09 and 0.73, respectively). Similarly, levels did not correlate with TNM staging. No statistically significant differences were

DLEC1

DLEC1F1: aaacagtgggctgctaga
DLEC1Zr1: actgaacctgaccgtacatactcctggcagtgaagaaa

CK19

CK19F1: caggtccgaggttactgac
CK19Zr1: actgaacctgaccgtacacactttctgc cagtgtgtcttc

Figure 1. *Primers for DLEC1 and CK19.*

observed between TNM1 and TNM2, TNM3 or TNM4 (p=0.12, 0.06 and 0.84, respectively).

DLEC1 expression levels were significantly lower in samples from patients with progressive disease who developed metastasis, local recurrence or died of breast cancer when compared to those who were disease free for >10 years (p=0.041).

Levels of transcription were also significantly higher in samples from patients with progressive disease and node positive patients when compared to ANCT samples (p=0.022 and 0.044, respectively). No correlation with ERα and ERβ receptor status was found (p=0.08 and 0.7, respectively). Kaplan-Meier analysis of overall survival of breast cancer patients depending on the expression levels of *DLEC1* showed a statistically significant difference (p=0.05) (Figure 2).

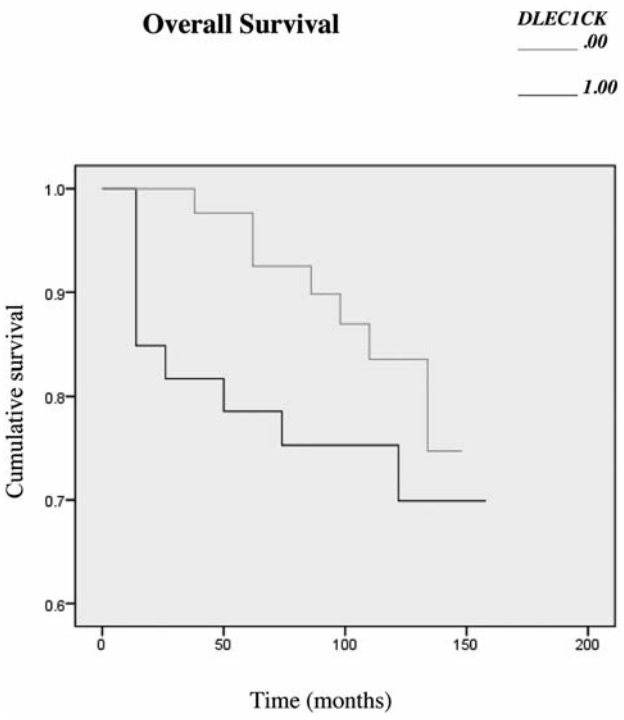

Figure 2. *Kaplan-Meier overall survival analysis depending on the expression of DLEC1. .00= low expression levels; 1.00=high expression levels; p=0.05.*

Discussion

The tumour-suppressing properties of *DLEC1* were supported by studies showing aberrant expression of *DLEC1* in several types of human cancer, including lung, oesophageal, and renal tumours (11). *DLEC1* encodes a novel protein which has no significant homology to known proteins or domains and the function of which remains unknown.

Previous research demonstrated the loss of *DLEC1* expression in ovarian cancer and the suppression of ovarian cancer cell growth by *DLEC1* re-expression. The loss of *DLEC1* expression in ovarian cancer is related to promoter hypermethylation and histone hypoacetylation, but not to loss of chromosome 3p22.3 (10). Seng *et al*. (13) showed that the *DLEC1* promoter is methylated in lung cancer and *DLEC1* methylation was associated with shorter overall survival and nodal involvement in the whole cohort (13). It has been recently shown that *DLEC1* is also subject to long-range epigenetic regulation in colon cancer. Multiple genes in this region can be silenced simultaneously through promoter hypermethylation and histone methylation in colorectal cancer (17). It has been previously demonstrated that *SETD2*, also located on the short arm of chromosome 3, is a potential tumour suppressor gene (8). *SETD2* is a histone trimethyltransferase protein that is involved in histone modification which could explain a link between *DLEC1* and *SETD2*.

The findings of the present study are consistent with a possible tumour suppressor function of *DLEC1* in breast cancer. The strength of these findings lies in the use of robust RT-PCR methodology to analyse *DLEC1* mRNA expression in a cohort of breast cancer patients with a long follow-up. However, this report has some inherent limitations including the lack of data regarding *DLEC1* protein expression. This is nevertheless the first study to investigate *DLEC1* expression in human breast cancer and identify a possible tumour suppressor function.

Further research is required to confirm the role of *DLEC1* gene in the pathogenesis of breast cancer including immunohistochemistry studies, *in vitro* experiments, and the preparation of animal models with suppressed *DLEC1* gene. If the current observations are confirmed by other studies, *DLEC1* may prove to be a valuable prognostic marker and its artificial expression could represent a novel therapeutic strategy.

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