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Evidence for Tumour Suppressor Function of DOK7 in Human Breast Cancer

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ABSTRACT

Introduction: Downstream of tyrosine kinase 7 (DOK-7) is a member of the DOK family, which has been associated with the development and progression of various humancancers. Previously, identification of CpG hypermethylation in DOK-7 promoter was identified in breast cancer. Method: DOK-7 mRNA extraction and reverse transcription were performed on fresh frozen breast cancer tissue samples and normal background breast tissue. Transcript levels of expression were analyzed against TNM stage, tumour grade and clinical outcome over a 10-year follow-up period. Results: Levels of DOK-7 expression decreased significantly with increasing TNM stage. Higher DOK-7 expression was correlated with longer disease free and overall survival times. Conclusion: To our knowledge, this is the first study to investigate DOK-7 expression in human breast cancer. We identify a potential DOK-7 tumour suppressor role. DOK-7 as a prognostic biomarker in human breast cancer should be included in future validation studies.

KEYWORDS

Breast Cancer; DOK-7; CpG Hypermethylation; Tumour Suppressor; Prognostic Marker

1. Introduction

Identification of breast cancer biomarkers has shown great promise in not only increasing prognostic ability and molecular understanding at all stages of tumorigenesis but also aid in the decision of specific clinical interventions, potentially leading to the production of individualized therapies [1-6]. Clinical impact has begun to be achieved by several biomarkers most notably oestrogen receptor (ER) and human epidermal growth factor receptor 2 (HER2/neu) [7].

The downstream of tyrosine kinase (DOK) family of adaptor proteins consists of 7 members that share a structural topology characterized by an NH_2-terminal pleckstrin homology (PH) domain, a central phosphotyrosine-binding (PTB) domain, followed by SH_2 target motifs in the carboxyl-terminal [8,9]. Several members of the DOK family are associated with various human cancers; recently DOK-2 has been suggested as a marker for poor prognosis in gastric cancer [10,11]. Whilst two subgroups exist within the DOK family; DOK 1-3 primarily expressed in haematopoietic tissues [12] and DOK 4-6 predominantly within the nervous system [13,14]; DOK-7, expressed in skeletal muscle and the heart, plays a distinct role in other members [15].

Mutations in DOK7 are a common cause of congenital myasthenic syndrome (CMS). DOK-7 promotes transautophosphorylation and activation of muscle-specific kinase (MuSK) through formation of a dimeric structural unit following PTB domain interaction with the phosphorylated juxtamembrane region of MuSK. Activation of MuSK results in downstream induction of acetylcholine receptor (AChR) clustering on the post-synaptic membrane, essential for efficient neuromuscular transmission [15-20]. MuSK activation is also reliant on the motor neuron-derived ligand Agrin and suggested phosphorylation of its intracellular domain by Casein Kinase

2 (CK2) [21,22]. Agrin has previously been linked as a biomarker for colorectal and liver cancer [23] whilst CK2 association with breast cancer is well established through oncogene phosphorylation and over expression correlating with metastatic risk [21,24].

In a recent methylation profiling study of twins discordant for breast cancer, Heyn *et al.*, identified hypermethylation of the DOK-7 gene in primary breast cancer tissues, cell lines and whole blood samples [25]. CpG site hypermethylation was observed within the DOK-7 promoter region. Recently, the importance of epigenetic modifications in cancer development and progression has been well-established [26-28]. In breast cancer, CpG island hypermethylation is associated with down regulation of various tumour suppressor genes, controlling all aspects of cellular function [3,29]. One way down regulation is thought to be that result is via abrogation of transcription factor binding to methylated promoter regions. The Sp1 transcription factor has been demonstrated to activate DOK-7 expression [30]. Sp1 has been found to participate in the expression of several oncogenes and up-regulation of its own expression has been observed in a percentage of breast tumours [31-37].

In view of the association between DOK-7 hypermethylation and breast cancer, we examined the expression profile of DOK-7 in a cohort of archival normal and breast cancer specimens. Transcript levels were evaluated against established pathological and prognostic parameters in addition to clinical outcome.

2. Method

2.1. Patients and Samples

Institutional guidelines, including ethical approval and informed consent were followed. Primary breast cancer tissues (n = 112) and adjacent non-cancerous mammary tissue (n = 31) were collected immediately after surgical excision and stored at −80˚C. An independent specialist pathologist examined haematoxylin and eosin stained frozen sections to verify the presence of tumour cells in the collected samples. Where normal non-neoplastic tissues were used, no tumour cells were found in the sections. 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 clinico-pathological data (Table 1) [38].

2.2. Materials

RNA extraction kits and reverse transcription kits were obtained from Sigma-Aldrich Ltd (Poole, Dorset, England, UK). The PCR primers were designed using Beacon Designer (Palo Alto, CA, USA) and synthesized by Sigma-Aldrich. Custom made hot-start Master Mix for quantitative Polymerase Chain Reaction (PCR) was obtained from Abgene (Surrey, England, UK) [38-40].

2.3. Tissue Processing, RNA Extraction and cDNA Synthesis

Frozen sections of tissue were cut at a thickness of 5 - 10 µm and kept for routine histological analysis. Additional 15 - 20 sections were mixed and homogenized using a hand-held homogenizer in ice-cold RNA extraction solution. The concentration of RNA was determined using UV spectrophotometry. Reverse transcription was carried

Table 1. Clinical and pathological 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
	Tubular	2
	Mucinous	4
	Other	7
TNM staging	1	70
	2	40
	3	7
	4	4
NPI	NPI1	68
	NPI2	38
	NPI3	16
Clinical outcome	Disease-free	90
	With local recurrence	5
	Alive with metastasis	7
	Died of breast cancer	16

Note: missing values reflect discarded/un-interpretable values.

out using a reverse transcription kit with an anchored oligo (dT) primer supplied by Abgene, using 1 μg of total RNA in a 96-well plate. The quality of cDNA was verified using Cytokeratin 19 (CK19) primers (Table 2) [38].

2.4. Quantitative Analysis

The level of DOK-7 transcripts from the above prepared DNA was determined using real-time quantitative PCR based on the Amplifluor technology, modified from a method reported previously [38,41]. The PCR primers were designed using Beacon Designer software, but to the reverse primer an additional sequence known as a Z sequence (5'-ACTGAACCTGACCGTACA-3') which is complementary to the universal Z probe (Intergen Inc., Oxford, UK) was added. The product expands one intron. The primers used are detailed in Table 2. The reaction was carried out using Hotstart Q-master mix (Abgene), 10 pmol of specific forward primer, 1 pmol reverse primer which had the Z sequence, 10 pmol of FAM (fluorogenic reporter dye, carboxyfluorescein) tagged probe (Intergen Inc.), and cDNA from 50 ng of RNA. The reaction was carried out using the IcyclerIQ (Bio-Rad Ltd, Hemel Hempstead, England, UK), which is equipped with an optic 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 sec, 55˚C for 40 sec, and 72˚C for 20 sec. The levels of the transcript were generated from a standard that was simultaneously amplified with the samples. The levels of gene expression were then normalized against the housekeeping gene CK19, which was already quantified in these specimens, to correct for varying amounts of epithelial tissue between samples [42]. The primers used for CK19 are detailed in Table 2. With every PCR run, a negative control without a template and a known cDNA reference sample as a positive control, were included.

2.5. Statistical Analysis

The Mann-Whitney U-test and two-sample t-test were used for statistical analysis of absolute and normalised gene copy number. The transcript levels within the breast cancer specimens were compared to normal background tissues and analyzed against conventional pathological parameters and clinical outcome over a 10 year follow-up period. The statistical analysis was carried out using Minitab version 14.1 (Minitab Ltd. Coventry, England, U.K.) using a custom written macro (Stat 2005. mtw). For purposes of the Kaplan-Meier survival analysis, the samples were divided arbitrarily into two groups, "high transcript level" or "low transcript level", for the DOK-7 gene. The cut-off was guided by the Nottingham Prognostic Index (NPI) value, with which the value of the moderate prognostic group was used as the dividing line at the start of the test. Disease Free Survival (DFS) and Overall Survival (OS) analyses were performed using SPSS version 12.0.1 (SPSS Inc. Chicago, IL, USA). For multivariate analysis using the Cox regression model, PASW Statistics 18 Software (Chicago, IL, USA) was used.

Table 2. DOK-7 and CK19 Primers.

DOK-7	
Forward	gagtaggtggctggtgct
Z Reverse	actgaacctgaccgtacacagatgtcctctagcgtca
CK19	
Forward	caggtccgaggttactgac
Reverse	actgaacctgaccgtacacactttctgccagtgtgtcttc

3. Results

DOK-7 expression profile was determined via quantitative PCR in both absolute terms and normalized against CK19. DOK-7 was found to be expressed in both normal/benign breast tissue and breast cancer specimens. Overall, no difference was found between DOK-7 expression in breast cancer specimens and its expression in normal background tissue.

The expression of DOK-7 mRNA was demonstrated to significantly decrease with increasing TNM class; TNM-1 vs. TNM-4 [mean copy number 21,895 vs. 1239, 95% CI (3787, 37,526), p = 0.02] and TNM-2 vs. TNM-4 [mean copy number 7982 vs. 1239, 95% CI (−58, 13,544), p = 0.05]. DOK-7 expression in TNM-4 specimens was also significantly lower than normal breast samples [mean copy number 1239 vs. 39,810, 95% CI (−75,665, −1478), p = 0.04] (Table 3).

A noticeable trend in decreasing DOK-7 expression with increasing Nottingham Prognostic Index (NPI) was observed; however, this did not reach statistical significance (NPI-3 compared to NPI-1 and NPI-2, p = 0.08 and p = 0.16, respectively). Transcript levels were significantly lower in Grade-1 tumour specimens than Grade-2 [mean copy number 752 vs. 32,796, 95% CI (−58,069, −6019), p = 0.02] although no overall trend existed amongst tumour grades (Table 3).

After a median follow up of 10 years, DOK-7 mRNA expression levels were significantly higher in women that remained disease free compared to those who developed local recurrence [mean copy number 21,675 vs. 2310, 95% CI (3134, 35,596), p = 0.02] or those that died from breast cancer [mean copy number 216,75 vs. 1835, 95% CI (3790, 35,890), p = 0.02]. Furthermore, expression levels of women who developed local recurrence or died from breast cancer were significantly lower than normal breast samples [mean copy number 2310 vs. 39,810, 95%

Table 3. DOK-7 mean mRNA expression level.

Patient and tumour Characteristics	DOK-7 mean (SD)	p
NPI		
NPI 1 vs. 2	23538 (81191) vs. 20632 (67371)	0.85
NPI 1 vs. 3	23538 (81191) vs. 4267 (8882)	0.08
NPI 2 vs. 3	20632 (67371) vs. 4267 (8882)	0.16
Tumour Grade		
Grade 1 vs. 2	752 (1584) vs. 32796 (80234)	0.02
Grade 1 vs. 3	752 (1584) vs. 14742 (72914)	0.17
Grade 2 vs. 3	32796 (80234) vs. 14742 (72914)	0.27
TNM		
TNM 1 vs. 2	21895 (65670) vs. 7982 (20072)	0.13
TNM 1 vs. 3	21895 (65670) vs. 79286 (199804)	0.48
TNM 1 vs. 4	21895 (65670) vs. 1239 (1253)	0.02
TNM 2 vs. 3	7982 (20072) vs. 79286 (199804)	0.38
TNM 2 vs. 4	7982 (20072) vs. 1239 (1253)	0.05
TNM 3 vs. 4	79286 (199804) vs. 1239 (1253)	0.34
Survival		
DF vs. LR	21675 (70979) vs. 2310 (5029)	0.02
DF vs. DR	21675 (70979) vs. 80140 (173108)	0.49
DF vs. D	21675 (70979) vs. 1835 (5438)	0.02

CI (−74,775, −226), p = 0.05] and [mean copy number 1835 vs. 39,810, 95% CI (−75,165, −785), p = 0.05], respectively (Table 3).

There is a trend for specimens with lower levels of DOK-7 expression to associate with shorter disease-free (DFS) and overall survival (OS) times. Survival curves (DFS and OS) for women with tumours expressing "high levels" of DOK-7 differed significantly from those classified as having "low levels". The survival curves show higher levels of DOK-7 were of significant benefit in predicting higher DFS (p = 0.006) and better OS (p = 0.009) (Figures 1 and 2).

4. Discussion

Here we present the mRNA expression profile of DOK-7 in breast cancer specimens and demonstrate decreased expression levels with increasing pathological and prognostic statuses.

We have observed a significant decrease in DOK-7 expression level with increasing TNM stage, raising the potential for a novel tumour suppressor function outside its essential role in neuromuscular synaptogenesis. In addition, DOK-7 expression in TNM-4 stage tumours was significantly lower than that of normal breast tissue. Prior to this study, Heyn *et al.* detailed hyper-methylation of a CpG site within the DOK-7 promoter in twins discordant for breast cancer [25]. CpG promoter hypermethylation is associated with down regulation of gene expression, concurrent with our results. The scale of epigenetic modifications associated with tumour development and progression is beginning to be appreciated with particular efforts placed in the identification of methylation signatures that could serve as prognostic/predictive markers in breast cancer [7,27].

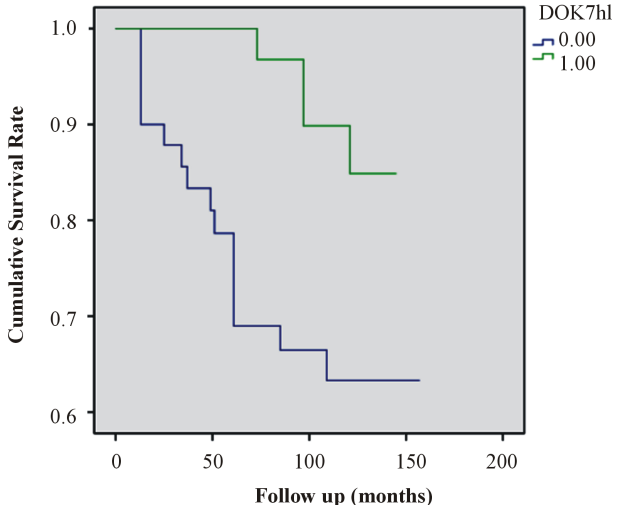

Figure 1. Kaplan Meier Disease Free Survival (DSF) Curves for DOK-7. Survival times are expressed as mean number of months with 95% confidence interval. DFS (p = 0.006).

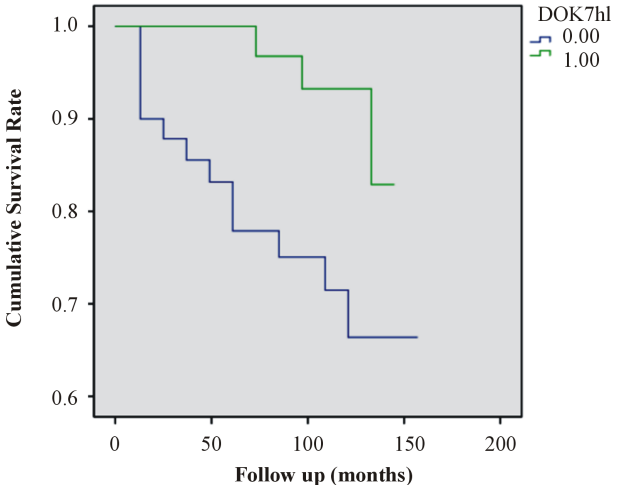

Figure 2. Kaplan Meier Overall Survival (OS) Curves for DOK-7. Survival times are expressed as mean number of months with 95% confidence interval. OS (p = 0.009).

Other members of the DOK family of adaptor proteins have been identified to possess tumour suppressor roles; none more so than DOK-1, which is down regulated in several human cancers as a result of hyper-methylation of its promoter region [43]. Whilst other members of the DOK family modulate proliferative signalling pathways, DOK-7 is currently seen to have a distinct expression pattern and role in MuSK activation to promote AChR clustering [16], making it difficult to posit the nature of any potential DOK-7 tumour suppressor function. However, several proteins employed in the Agrin/MuSK pathway that harbour additional roles, such as Ck2, have been associated with human breast cancer [21,22,44]. Moreover, one mechanism by which methylation can down-regulate expression, is by blocking transcription factors from accessing target-binding sites within the promoter region. Amongst its broad spectrum, Sp1 transcriptionally activates DOK-7 and is up-regulated in a percentage of breast cancers [30,45].

Furthermore, our results demonstrated significantly lower DOK-7 expression levels in women who developed local recurrence or died from breast cancer following a median 10-year follow up period compared to both normal breast tissue and women that remained disease free over the same period of time. Disease free survival (DFS) and overall survival (OS) curves revealed that a higher DOK-7 expression level was a significant predictor of superior DFS and OS, supporting the suitability of DOK-7 as a prognostic biomarker for breast cancer. Biomarker prediction of recurrence after curative resection is useful for determining intensity of clinical surveillance and adjuvant therapies.

Limitations of the present study included the use of background parenchyma from breast cancer patients to provide "normal tissue" for comparison. Ideally, such material should be derived from patients without breast cancer in order to avoid any "field change" that may exist within cancer bearing tissues. Although the follow-up period was substantial, sample size was relatively small and it is possible that a larger cohort may have influenced several results that approached, but failed to reach, statistical significance. Furthermore, the protein expression and epigenetic modifications were not analysed in the present study and should be included in future investigations.

5. Conclusion

To our knowledge, this is the first study to investigate DOK-7 expression in human breast cancer and identify a potential tumour suppressor role. We also present data to support the value of DOK-7 as a prognostic biomarker in breast cancer. Restoring or mimicking the function of DOK-7 could provide a novel therapeutic modality against cancer.

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Abbreviations

NPI: Nottingham Prognostic Index
DF: Disease Free
LR: Local Reoccurrence
DR: Distant Reoccurrence
TNM: TNM Classification of Malignant Tumours