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RESEARCH REPORT

mRNA expression of DOK1-6 in human breast cancer

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Abstract

AIM: To examine the expression of downstream of tyrosine kinase (DOK)1-6 genes in normal and breast cancer tissue and correlated this with several clinicopathological and prognostic factors.

METHODS: DOK1-6 mRNA extraction and reverse transcription were performed on fresh frozen breast cancer tissue samples (n = 112) and normal background breast tissue (n = 31). Tissues were collected between 1991 and 1996 at two centres and all patients underwent mastectomy and ipsilateral axillary node dissection. All tissues were randomly numbered and the details were only made known after all analyses were completed. Transcript levels of expression were determined using real-time polymerase chain reaction and analyzed against TNM stage, tumour grade and clinical outcome over a 10-year follow-up period.

RESULTS: DOK-2 and DOK-6 expression decreased with increasing TNM stage. DOK-6 expression decreased with increasing Nottingham Prognostic Index (NPI) [NPI-1 *vs* NPI-3 (mean copy number 15.4 *vs* 0.22, 95%CI: 2.7-27.6, P = 0.018) and NPI-2 *vs* NPI-3 (mean copy number 7.6 *vs* 0.22, 95%CI: 0.1-14.6, P = 0.048)]. After a median follow up period of 10 years, higher levels of DOK-2 expression were found among patients who remained disease-free compared to those who developed local or distant recurrence (mean copy number 3.94 *vs* 0.0000096, 95%CI: 1.0-6.85, P = 0.0091), and distant recurrence (mean copy number 3.94 *vs* 0.0025, 95%CI: 1.0-6.84, P = 0.0092). Patients who remained disease-free had higher levels of DOK-6 expression compared to those who died from breast cancer.

CONCLUSION: Decreasing expression levels of DOK-2 and DOK-6 with increased breast tumour progression supports the notion that DOK-2 and DOK-6 behave as tumour suppressors in human breast cancer.

Key words: Adaptor protein; Breast cancer; Downstream of tyrosine kinase-2; Downstream of tyrosine kinase-6; Mitogen-activated protein kinase; Tyrosine kinase, Tumour suppressor

Core tip: Several members of the downstream of tyrosine kinase (DOK) protein family are identified as modulators of cell proliferation/growth pathways. In addition deregulation of specific DOK members has been associated with specific cancers. This study identifies DOK-2 and DOK-6 as potential tumor suppressors in breast cancer.

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INTRODUCTION

Downstream of tyrosine kinase (DOK) family of adaptor proteins consists of 7 members (DOK1-7) that share a structural topology characterized by an NH2-terminal

pleckstrin homology domain, a central phosphotyrosine-binding domain, followed by SH_2 target motifs in the carboxyl-terminal moiety[1-9]. They act as common substrates for multiple protein-tyrosine kinases (PTK's), including receptor tyrosine kinases (RTK's) and non-RTK's from which they modulate signalling pathways involved in various natural cellular processes including proliferation, apoptosis, growth and migration[3,5-8,10-17]. Additionally, PTK signalling has been implicated in the development and progression of various cancers, including breast cancer[18-22].

Principally based on patterns of expression, two sub-groups exist within the DOK family. DOK-1, DOK-2 and DOK-3 comprise one sub-group primarily expressed in haematopoietic tissues[7,9,11,23-28], whereas, DOK-4, DOK-5 and DOK-6 are expressed in non-haematopoietic tissues, predominantly within the nervous system[2-4,29-33]. Separately, expression of DOK-7 is concentrated to skeletal muscle and the heart[34,35]. DOK-1(p62) was initially identified as a substrate for the Bcr-Abl tyrosine kinase oncoprotein in chronic myelogenous leukaemia (CML)[7,9]. Consequently, DOK-1 knockdown in murine bone marrow-derived mast cells resulted in increased cell proliferation following mitogenic stimulus[23]. It was discovered that DOK-1 opposes leukemogenesis by Bcr-Abl through inhibition of the mitogen-activated protein kinase (MAPK) pathway[7,9,10,12,23].

DOK-1, DOK-2 and DOK-3 have all been found to negatively regulate immunoreceptor signalling[6,7]. It was initially posited, DOK-1 and DOK-2 both inhibit the Ras/ERK pathway though interaction with RasGAP[6,7]. Conversely, DOK-3 is understood to inhibit c-Jun N-terminal kinase activation by interacting with SHIP-1 and Grb2[5,36]. However, evidence exists that DOK-1 and DOK-2 can inhibit the MAPK pathway independent of RasGAP *via* epidermal growth factor receptor (EGFR) signalling[18,37,38]. DOK-2 can facilitate the successive recruitment of c-Src and its inhibitor Csk to EGFR, inhibiting MAPK[38]. Individual and concomitant up-regulation of EGFR and c-Src is associated with breast cancer progression. Tyrosine-kinase inhibitors targeted against EGFR are currently in use for breast cancer treatment whilst several against c-Src are being trialled[20,39-41]. Since DOK-1 association with CML, down-regulation of the DOK-1, DOK-2 and DOK-3 sub-group has been linked with several other haematopoietic cancers, including histiocytic sarcoma and Burkitt's lymphoma[6,7,9,24-26]. Investigation into non-haematopoietic tissues identified lung adenocarcinoma development in DOK-1, DOK-2 and DOK-3 knockout mice and down regulation of DOK-2 expression in human lung cancer[1]. Additional non-haematopoietic cancers have since been associated with the DOK-1, DOK-2 and DOK-3 sub-group[42,43]. In particular, DOK-2 has recently been proposed as a potential marker of poor prognosis in patients with gastric cancer after curative resection[43].

The other sub-group of the DOK family, consisting of DOK-4, DOK-5 and DOK-6, are positive regulators of the MAPK pathway and promote neurite outgrowth[3,4,29,32,44]. DOK-4 and DOK-6 are phosphorylated by the tyrosine kinase encoded by the proto-oncogene RET (rearranged by transfection)[3,31,32,44]. Over expression of RET is primarily observed in oestrogen-receptor-positive (ER+) breast cancers where, in models, it is seen to cause increased cell migration and proliferation. In addition, studies have observed RET expression to correlate with poor prognosis of metastasis-free and overall survival in breast cancer patients[18,21,45,46]. Conflictingly, DOK-4 has also been found to inhibit Ret-mediated activation of Elk-1, a member of the ETS oncogene family. Elk-1 is a transcription activator associated with cell survival in breast cancer and has previously been implicated as a potential therapeutic target[18,21,45,46]. Aberrant expression of DOK-4 has been witnessed in non-small cell lung cancer and clear cell renal cell carcinoma[29,30]. Increased expression of DOK-5 is observed in malignant pheochromocytoma and has been suggested to promote cell survival *via* tropomyosin-receptor-kinase (Trk)C receptor signalling, of which DOK-6 is also a substrate[33,47]. Jin *et al*[48] identified TrkC as a critical regulator of breast cancer cell growth and metastasis. It has been established that DOK-7 plays a relatively unrelated role to the rest of the DOK-family in which it promotes acetylcholine receptor clustering on post-synaptic membranes at neuromuscular junctions *via* activating muscle-specific kinase[34,35].

In view of DOK proteins adaptor role in tyrosine kinase signalling pathways regulating cell growth and proliferation and their altered levels of expression in other cancers, we have examined the expression profile of DOK1-6 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.

MATERIALS AND METHODS

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 ℃ until use. Tissues were collected between 1991 and 1996 at two centres (St Georges Hospital in London and University Department of Surgery at Cardiff University School of Medicine). All patients underwent mastectomy and ipsilateral axillary node dissection. All tissues were randomly numbered and the details were only made known after all analyses were completed. Follow-up data were recorded in a custom database. Patients were routinely followed up after surgery (June 2004 was the final comprehensive follow up for the cohort). 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 patients were treated according to local algorithms

Table 1 Clinical and pathological data

Parameter number	Category	*n*
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	1	68
	2	38
	3	16
Clinical outcome	Disease-free	90
	With local recurrence	5
	Alive with metastasis	7
	Died of breast cancer	16

Missing values reflect discarded/un-interpretable values. NPI: Nottingham Prognostic Index.

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)[49].

Materials

RNA extraction kits and reverse transcription kits were obtained from Sigma-Aldrich Ltd (Poole, Dorset, England, United Kingdom). The polymerase chain reaction (PCR) primers were designed using Beacon Designer (Palo Alto, CA, United States) and synthesized by Sigma-Aldrich. Custom made hot-start Master mix for quantitative PCR was obtained from Abgene (Surrey, England, United Kingdom)[49-51].

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 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)[49].

Quantitative analysis

The level of DOK1-6 transcripts from the above prepared DNA were determined using real-time quantitative PCR based on the Amplifluor technology, modified from a method reported previously[49,52]. The PCR primers were designed using Beacon Designer software, but to the reverse primer an additional sequence, known as the *Z* sequence (5'-ACTGAACCTGACCGTACA-3') which is complementary to the universal *Z* probe (Intergen Inc., Oxford, United Kingdom) 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, United Kingdom), which is equipped with an optic unit that allows real-time detection of 96 reactions, under the following conditions: 94 ℃ for 12 min and 50 cycles of 94 ℃ for 15 s, 55 ℃ for 40 s, and 72 ℃ for 20 s. 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 reference gene *CK19*, which was already quantified in these specimens, to correct for varying amounts of epithelial tissue between samples[53]. 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.

Table 2 *DOKs* and *CK19* primers

Gene	Forward	Reverse
DOK-1	TGGCCCTACACTCTGTTG	ACTGAACCTGACCGTACAG GAAGGTGAAGGTTCCAG
DOK-2	ACTGGCCCTACAGGTTTC	ACTGAACCTGACCGTACAC TCAAAGTTGCCCTCTCC
DOK-3	AGAAGGGGAAGTGTGAGG	ACTGAACCTGACCGTACAT CCTTGATAGGGGTCTCC
DOK-4	GCCTCAACGACATCAGTC	ACTGAACCTGACCGTACAC CATACACGTCCAGGTTG
DOK-5	CGTGGTTCACTTTTGAGG	ACTGAACCTGACCGTACAG CAGCAGAGTGGACTTTC
DOK-6	AGAACAGCGTTGGTGAAA	ACTGAACCTGACCGTACAA GCTGGGAAATGTCTGTG
CK19	CAGGTCCGAGGTTACTGAC	ACTGAACCTGACCGTACAC ACTTTCTGCCAGTGTGTCTTC

DOK: Downstream of tyrosine kinase; CK19: Cytokeratin 19.

Statistical analysis

The two-sample *t* test was 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, United Kingdom) using a custom written macro (Stat 2005.mtw).

Table 3 Downstream of tyrosine kinases (1-3) mRNA expression levels (mean ± SD)

Patient and tumour characteristics	DOK-1	*P*	DOK-2	*P*	DOK-3	*P*
NPI						
1 *vs* 2	2250 ± 4652 *vs* 3988 ± 7824	0.48	4.37 ± 10.32 *vs* 4.14 ± 12.26	0.950	6802 ± 18036 *vs* 37024 ± 127523	0.150
1 *vs* 3	2250 ± 4652 *vs* 942 ± 1395	0.19	4.37 ± 10.32 *vs* 1.57 ± 4.55	0.270	6802 ± 18036 *vs* 9808 ± 19782	0.600
2 *vs* 3	3988 ± 7824 *vs* 942 ± 1395	0.21	4.14 ± 12.26 *vs* 1.57 ± 4.55	0.420	37024 ± 127523 *vs* 9808 ± 19782	0.210
Tumour grade						
1 *vs* 2	1346 ± 2587 *vs* 3886 ± 6155	0.18	0.651 ± 1.837 *vs* 4.63 ± 12.49	0.190	1259 ± 2797 *vs* 37803 ± 126737	0.084
1 *vs* 3	1346 ± 2587 *vs* 2100 ± 5520	0.58	0.651 ± 1.837 *vs* 4.23 ± 10.15	0.092	1259 ± 2797 *vs* 8954 ± 23276	0.022
2 *vs* 3	3886 ± 6155 *vs* 2100 ± 5520	0.37	4.63 ± 12.49 *vs* 4.23 ± 10.15	0.910	37803 ± 126737 *vs* 8954 ± 23276	0.170
TNM						
1 *vs* 2	2507 ± 4800 *vs* 3553 ± 7084	0.62	6.62 ± 13.46 *vs* 1.195 ± 3.782	0.052	26175 ± 101693 *vs* 4508 ± 9938	0.100
1 *vs* 3	2507 ± 4800 *vs* 0.782 ± 1.38	0.01	6.62 ± 13.46 *vs* 0.00562 ± 0.00942	0.015	26175 ± 101693 *vs* 16879 ± 39061	0.640
1 *vs* 4	2507 ± 4800 *vs* 626 ± 1252	0.11	6.62 ± 13.46 *vs* 0.00001 ± 0.00001	0.015	26175 ± 101693 *vs* 83.2 ± 80.9	0.050
2 *vs* 3	3553 ± 7084 *vs* 0.782 ± 1.38	0.07	1.195 ± 3.782 *vs* 0.00562 ± 0.00942	0.190	4508 ± 9938 *vs* 16879 ± 39061	0.440
2 *vs* 4	3553 ± 7084 *vs* 626 ± 1252	0.15	1.195 ± 3.782 *vs* 0.00001 ± 0.00001	0.190	4508 ± 9938 *vs* 83.2 ± 80.9	0.014
3 *vs* 4	0.782 ± 1.38 *vs* 626 ± 1252	0.39	0.00562 ± 0.00942 *vs* 0.00001 ± 0.00001	0.320	16879 ± 39061 *vs* 83.2 ± 80.9	0.300
Survival						
DF *vs* LR	1839 ± 4089 *vs* 1957 ± 2149	0.95	3.94 ± 8.96 *vs* 0.00001 ± 0.00001	0.009	18203 ± 86910 *vs* 16027 ± 40813	0.910
DF *vs* DR	1839 ± 4089 *vs* 12051 ± 17043	0.55	3.94 ± 8.96 *vs* 0.00246 ± 0.00349	0.009	18203 ± 86910 *vs* 11083 ± 22940	0.620
DF *vs* DR	1839 ± 4089 *vs* 3023 ± 6758	0.72	3.94 ± 8.96 *vs* 5.93 ± 17.43	0.750	18203 ± 86910 *vs* 21264 ± 60618	0.880

DOK: Downstream of tyrosine kinase; NPI: Nottingham Prognostic Index; DF: Disease Free; LR: Local Recurrence; DR: Distant Recurrence.

RESULTS

DOKs1 - 6 mRNA expression by quantitative PCR

The DOKs1-6 expression profiles were determined both in absolute terms and normalised against CK19.

DOK-1: DOK-1 (Table 3) was found to be expressed in both normal/benign breast tissue and breast cancer specimens. No significant difference was found between DOK-1 expression in breast cancer specimens and its expression in normal background tissue. The expression of DOK-1 mRNA did not significantly differ with increasing Nottingham Prognostic Index (NPI) or between normal background breast tissue and tumour tissues of patients with different NPI levels. No significant difference in the transcript levels observed with different tumour grades or TNM classes. After a median follow up of 10 years, we found DOK-1 mRNA expression levels did not differ among women who remained disease free compared to those who developed recurrence (local or distant) and compared to those who died from breast cancer.

DOK-2: DOK-2 (Table 3) was found to be expressed in both normal/benign breast tissue and breast cancer specimens. No significant difference was found between DOK-2 expression in breast cancer specimens and its expression in normal background tissue. No significant difference in the transcript levels observed with different tumour grades and the decrease in DOK-2 with increasing NPI was not significant. The expression of DOK-2 mRNA was demonstrated to significantly decrease with increasing TNM stage; TNM-1 *vs* TNM-3 (mean copy number 6.6 *vs* 0.0056, 95%CI: 1.4-11.8, $P = 0.015$), and TNM-1 *vs* TNM-4 (mean copy number 6.6 *vs* 0.0000072, 95%CI: 1.4-11.8, $P = 0.015$). After a median follow up of 10 years DOK-2 expression was significantly higher among those who remained disease free compared to those who developed local (mean copy number 3.94 *vs* 0.0000096, 95%CI: 1.0-6.85, $P = 0.0091$), and distant recurrence (mean copy number 3.94 *vs* 0.0025, 95%CI: 1.0-6.84, $P = 0.0092$).

DOK-3: There was neither significant difference in DOK-3 (Table 3) mRNA expression levels between cancer tissue and normal background tissue nor there was a significant difference in the transcript levels with different tumour grades or NPI.

DOK-3 mRNA expression was found to significantly decrease with increasing tumour TNM stage: TNM-1 *vs* TNM-4 (mean copy number 27175 *vs* 83.2, 95%CI: 41-52143, $P = 0.05$); and TNM-2 *vs* TNM-4 (mean copy number 4508 *vs* 83.2, 95%CI: 956-7894, $P = 0.014$).

DOK-4: DOK-4 (Table 4) was found to be expressed in both normal/benign breast tissue and breast cancer specimens. No significant difference was found between DOK-4 expression in normal background tissue and its expression in breast cancer tissue. The expression of DOK-4 mRNA did not significantly differ with increasing NPI or between normal background breast tissue, tumour tissues of patients with different NPI levels and tumour grade. After a median follow up of 10 years, we found DOK-4 mRNA expression levels to be higher among women who remained disease free compared to those who developed local recurrence (mean copy number 328 *vs* 2.22, 95%CI: 19-631.4, $P = 0.038$). No significant difference in expression levels were found between patients who remained disease free and those that developed distant recurrence or died from breast cancer.

DOK-5: There was neither significant difference in

Table 4 Downstream of tyrosine kinases (4-6) mRNA expression levels (mean ± SD)

Patient and tumour characteristics	DOK-4	*P*	DOK-5	*P*	DOK-6	*P*
NPI						
1 *vs* 2	415 ± 1444 *vs* 94.7 ± 275	0.170	31504 ± 163256 *vs* 2466 ± 7194	0.23	15.4 ± 39.98 *vs* 7.55 ± 18.37	0.270
1 *vs* 3	415 ± 1444 *vs* 103.4 ± 182.6	0.180	31504 ± 163256 *vs* 88648 ± 287508	0.52	15.4 ± 39.98 *vs* 0.218 ± 0.468	0.018
2 *vs* 3	94.7 ± 275 *vs* 103.4 ± 182.6	0.910	2466 ± 7194 *vs* 88648 ± 287508	0.32	7.55 ± 18.37 *vs* 0.218 ± 0.468	0.048
Tumour grade						
1 *vs* 2	589 ± 1860 *vs* 188 ± 819	0.410	68655 ± 261421 *vs* 5756 ± 14725	0.32	12.96 ± 28.62 *vs* 12.41 ± 39.55	0.960
1 *vs* 3	589 ± 1860 *vs* 147.5 ± 623.8	0.350	68655 ± 261421 *vs* 28639 ± 155311	0.55	12.96 ± 28.62 *vs* 6.22 ± 21.56	0.430
2 *vs* 3	188 ± 819 *vs* 147.5 ± 623.8	0.830	5756 ± 14725 *vs* 28639 ± 155311	0.35	12.41 ± 39.55 *vs* 6.22 ± 21.56	0.440
TNM						
1 *vs* 2	229 ± 829 *vs* 403 ± 1568	0.610	27103 ± 152075 *vs* 5096 ± 19916	0.30	10.26 ± 33.54 *vs* 13.68 ± 32.53	0.680
1 *vs* 3	229 ± 829 *vs* 83.9 ± 222	0.330	27103 ± 152075 *vs* 200882 ± 446722	0.44	10.26 ± 33.54 *vs* 2.07 ± 4.62	0.130
1 *vs* 4	229 ± 829 *vs* 49.4 ± 62.1	0.160	27103 ± 152075 *vs* 0.198 ± 0.184	0.20	10.26 ± 33.54 *vs* 0.0734 ± 0.1464	0.048
2 *vs* 3	403 ± 1568 *vs* 83.9 ± 222	0.340	5096 ± 19916 *vs* 200882 ± 446722	0.38	13.68 ± 32.53 *vs* 2.07 ± 4.62	0.098
2 *vs* 4	403 ± 1568 *vs* 49.4 ± 62.1	0.280	5096 ± 19916 *vs* 0.198 ± 0.184	0.11	13.68 ± 32.53 *vs* 0.0734 ± 0.1464	0.047
3 *vs* 4	83.9 ± 222 *vs* 49.4 ± 62.1	0.720	200882 ± 446722 *vs* 0.198 ± 0.184	0.37	2.07 ± 4.62 *vs* 0.0734 ± 0.1464	0.340
Survival						
DF *vs* LR	328 ± 1205 *vs* 2.22 ± 2.5	0.038	23013 ± 139049 *vs* 10477 ± 22630	0.53	11.38 ± 33.96 *vs* 9.24 ± 18.47	0.840
DF *vs* DR	328 ± 1205 *vs* 40.5 ± 77.9	0.074	23013 ± 139049 *vs* 1022 ± 2240	0.21	11.38 ± 33.96 *vs* 20.7 ± 35.8	0.700
DF *vs* DR	328 ± 1205 *vs* 84.1 ± 185	0.140	23013 ± 139049 *vs* 81855 ± 276356	0.47	11.38 ± 33.96 *vs* 0.209 ± 0.45	0.013

DOK: Downstream of tyrosine kinase; NPI: Nottingham Prognostic Index; DF: Disease Free; LR: Local Recurrence; DR: Distant Recurrence.

DOK-5 (Table 4) mRNA expression levels between cancer tissue and normal background tissue nor there was a significant difference in the transcript levels with different tumour grades or TNM classes. The expression of DOK-5 mRNA did not significantly differ with increasing NPI and after a median follow up of 10 years, no significant difference in expression was observed between survival statuses.

DOK-6: DOK-6 (Table 4) was found to be expressed in both normal/benign breast tissue and breast cancer specimens. No significant difference was found between DOK-6 expression in normal background tissue and its expression in breast cancer tissue. The expression of DOK-6 mRNA was found to decrease with increasing NPI; NPI-1 *vs* NPI-3 (mean copy number 15.4 *vs* 0.22, 95%CI: 2.7-27.6, $P = 0.018$) and NPI-2 *vs* NPI-3 (mean copy number 7.6 *vs* 0.22, 95%CI: 0.1-14.6, $P = 0.048$), but there was no significant decrease with higher tumour grade. The expression of DOK-6 mRNA was demonstrated to significantly decrease with increasing TNM stage; TNM-1 *vs* TNM-4 (mean copy number 10.3 *vs* 0.073, 95%CI: 0.1-20.3, $P = 0.048$), and TNM-2 *vs* TNM-4 (mean copy number 13.7 *vs* 0.073, 95%CI: 0.2-27.04, $P = 0.047$). After a median follow up of 10 years, patients who died from breast cancer had significantly lower DOK-6 expression compared those whom remained disease free (mean copy number 11.4 *vs* 0.21, 95%CI: 2.5-19.9, $P = 0.013$).

DISCUSSION

Here we analyze the mRNA expression profiles of the DOK1-6 family members in breast cancer specimens and identify decreased expression of individual members with increasing clinical and pathological stage.

The results show a decreased DOK-2 expression with increasing TNM stage. Down regulation of DOK-2 has previously been documented in several human epithelial cancers including lung, colorectal and gastric cancer[1,24,42,43]. Additionally, we have found higher DOK-2 levels to correlate a significantly lower chance of both local and distant recurrence within the 10-year period following surgical resection. Similarly, a recent study also found higher DOK-2 expression to be a good indicator of non-recurrence in gastric cancer patients following resection[43]. The prediction of recurrence and metastasis after curative resection can determine the need for intensive follow-up and adjuvant therapy.

In addition to this the *DOK2* gene is localized to chromosome 8p21.3, one of the most frequently deleted regions in human lung cancer and a region hypothesized to contain multiple tumour suppressor genes[54,55]. DOK-2 has previously been thought to associate with cancer progression *via* its regulatory role of the MAPK pathway. Initially, DOK-2 was found to inhibit the MAPK pathway through interaction with the Ras inhibitor RasGAP[12,23]. Further studies identified a RasGAP-independent route of MAPK inhibition with DOK-2 shuttling c-Src to the EGFR where, after transient activation of c-Src, a phosphorylated DOK-2 recruits the negative regulator of c-Src, Csk. Both EGFR and c-Src are up-regulated in a large percentage of human breast tumours supporting a potential tumour suppressor role of DOK-2 in breast cancer[38].

Furthermore, our results display decreased DOK-6 expression with increased TNM stage as well as NPI, suggesting DOK-6 could hold prognostic value. Interestingly, significantly higher levels of DOK-6 expression were found in specimens of patients that remained dis-

ease free compared to those that died of breast cancer within the following 10-year period. Together these results highlight DOK-6 as potentially possessing tumour suppressor function. In comparison to other family members, less research has been undertaken into DOK-6 function and to our knowledge it has not previously been directly associated with any human cancer, *in vivo*[3,32]. DOK-6 has been suggested to play a role in neurite outgrowth *via* both Ret and TrkC signalling[3,32]. However, it has been indicated that DOK-6 is involved in Ret signalling with less influence when compared with DOK-1 and DOK-4[32]. The exact function of DOK-6 in TrkC signalling has of yet to be established, although knockdown of DOK-6 has been observed to decrease neurite outgrowth in cortical neurons upon NT-3 stimulation[33]. TrkC has recently been proposed to act as a critical regulator of breast cancer cell growth and metastasis. Interaction between TrkC and c-Src was found to activate MAPK cascade *in vivo* in human breast cancer tissues[21,38,48,56,57]. It is worth noting that despite DOK-5 also regulating MAPK activity *via* TrkC interaction, unlike DOK-6, we observe no difference in its expression[47].

DOK-2 and DOK-6 are members of different subgroups within the DOK adaptor protein family and although each is a substrate of multiple tyrosine kinases, current literature places their natural functions and interactions relatively distinct from one another. Our results, however, indicate that they both function within breast tumour cells and loss of their expression is associated with tumour progression. The two DOK proteins may regulate independent pathways or could potentially function together in an as yet undefined pathway.

Goel *et al*[58] have recently published qualitative data of a lucid differential pattern of DOK-1 expression in 8 breast cancer cell lines compared to a non-tumourigenic breast epithelial-derived cell line. We believe our study to be the first that has quantitatively measured DOK-1 expression in human breast cancer specimens. No significant difference was observed between normal/background tissue and breast cancer tissue. Using histologically normal appearing samples as the sole control tissue is probably less appropriate. The use of donor tissues from reduction mammoplasty specimens (ideally obtained under similar conditions as the tumour tissue) will serve as a better control. Donor control, in addition to normal adjacent to tumours, precancerous lesions, and tumour samples will provide the best sample set for resolution of genetic alterations that are relevant to the disease process by minimizing the potential implications of field cancerisation. As there was no normal donor tissue used in this study, it was not clear whether the absence of a difference in DOK family expression between tumour and matched normal background tissue in our cohort was due to the effect of field cancerisation.

To our knowledge, this study is the first to analyze the mRNA expression of the DOK family members 1-6 in breast cancer specimens. Our data support the notion that DOK-2 behave as a tumour suppressor. For the first time we also provide novel data predicting a role of DOK-6 as a potential tumour suppressor in breast cancer. Nevertheless, our understanding of the molecular mechanisms involved, across all DOK members, in their adaptor function to multiple tyrosine kinases requires further study. Our data could be used in further validation studies in order to define clear mechanisms attributing to the development and progression of breast cancer and develop new prognostic markers and novel therapeutic strategies.

COMMENTS

Background

Identification of molecular biomarkers has shown great promise in the identification of metastatic risk and survival rate in breast cancer as well as requirement for adjuvant therapies. The downstream of tyrosine kinase (DOK) family of adaptor proteins have previously been associated with the progression of other cancers including other human epithelial cancers.

Research frontiers

Several members of the DOK protein family are identified as modulators of cell proliferation/growth pathways. In addition deregulation of specific DOK members have been associated with specific cancers. This study identifies DOK-2 and DOK-6 as a potential tumor suppression in breast cancer.

Innovations and breakthroughs

The identification of molecular biomarkers and their subsequent use to determine risk of recurrence and prognosis in breast cancer has begun to make real clinical impact. This is the first study correlate expression of several DOK family members with clinical and pathological parameters of breast cancer and identify DOK-2 and DOK-6 as having potential tumour suppressor function as well as hold prognostic value.

Applications

Further understanding and identification of members of pathways through which breast cancer can progress is essential in the development of effective therapeutic strategies. In addition, the potential prognostic value of DOK-2 and DOK-6 may determine need for post-curative therapies.

Peer review

This work tackles a novel and interesting issue: the expression profile of DOK proteins in breast cancer. The importance of this study lies in that by first time it is suggested a role of DOK proteins as tumour suppressors in breast cancer.

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