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The mRNA Expression of *DAP1* in Human Breast Cancer: Correlation with Clinicopathological Parameters

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Abstract. *This pilot study is the first to focus on the potential role of death-associated protein 1 (DAP1) in human breast cancer. Materials and Methods: A total of 153 samples were studied. DAP1 transcription levels were determined using quantitative polymerase chain reaction (qPCR). Transcript levels within breast cancer specimens were compared to those of normal background tissues and correlated with clinicopathological data accumulated over a 10-year follow-up period. Results: The expression of DAP1 mRNA was demonstrated to decrease with increasing Nottingham Prognostic Index (NPI2 vs. NPI3, p=0.0026), and TNM stage (TNM1 vs. 4, p=0.0039). Lower DAP1 expression levels were significantly associated with local recurrence (p=0.02) and distant metastasis (p=0.001). Conclusion: This study demonstrates an inverse association between DAP1 mRNA levels and tumour stage and clinical outcome in breast cancer; thus, providing evidence that DAP1 plays a pro-apoptotic role in human breast cancer. The relationship between oncogenesis and the autophagy pathway merits further investigation.*

The role of apoptosis genes in oncogenesis has been the subject of much research in the past. The earliest and most well–documented gene is the so-called 'guardian of the genome', tumour protein 53 (*p53*) (1). Since then, a number of other genes have been studied for their role in programmed cell death, including, but not limited to, B-cell lymphoma 2 protein (*Bcl2*), the Bcl2-associated X protein (*BAX*) (2), and those encoding caspases 3, 6, 8 and 9 (3). Silencing of these genes has been postulated as a possible route to oncogenesis, and this has been demonstrated in various studies (4, 5).

The death-associated proteins (DAPs) are a relatively novel group of proteins found to play a role in programmed cell death. Four proteins were initially identified; DAP kinase, and DAP1, -3 and -5 (6).

DAP1 was recently shown to be a substrate for the mammalian target of rapamycin (mTOR). mTOR is a key regulator of autophagy, a catabolic process by which cells adjust to nutrient–deficient environments (7).

The role of DAP1 in cancer has been overlooked in recent literature. In this pilot study, we investigated the relationship between the expression of DAP1 and breast cancer. We hope this will open a potentially significant avenue of research to further our understanding of the relationship between autophagy and oncogenesis.

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Materials and Methods

Samples. Institutional guidelines including ethical approval and informed consent were adhered to. Immediately after surgical excision, a tumour sample was obtained from the tumour area, while another was obtained from the associated non-cancerous tissue (ANCT) within 2 cm of the tumour, without affecting the assessment of tumour margins. Breast cancer tissues (n=127) and normal background tissues (n=33) were collected and stored at –140˚C in liquid nitrogen until the commencement of this study.

All the patients were treated according to local guidelines, following discussions in multidisciplinary meetings. Patients undergoing breast-conserving surgery also underwent radiotherapy. Hormone-sensitive patients were given tamoxifen. Hormone-insensitive cases, high-grade cancer, and node-positive cases were treated with adjuvant therapy. Clinicopathological data (Table I) were collected from the patient charts, and were correlated in an encrypted database.

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 (8).

Tissue processing, RNA extraction and cDNA synthesis. Approximately 10 mg of cancerous tissue were homogenised. A larger amount of ANCT (20-50 mg) was used, as its high fat content made it difficult to obtain sufficient RNA for analysis. The concentration of RNA was determined using a UV spectrophotometer (Wolf Laboratories, York, UK) to ensure adequate amounts of RNA for analysis. Reverse transcription was carried out using a reverse transcription kit (AbGene) with an anchored olig (dT) primer using 1 mg of total RNA in a 96-well plate to produce cDNA. The quality of cDNA was verified using β-actin primers (primers 5’-ATGATATCGCCGCGCTCGTC-3’ and 5’-CGCTCGGTGAGGATCTTCA-3’) (9).

Quantitative analysis. Transcripts of cDNA library were determined using real-time quantitative PCR based on Amplifluor technology. The PCR primers were designed using Beacon Designer software (Premier Biosoft International Ltd., Pal Alto, CA, USA), but an additional sequence, known as the Z sequence (5’-ACTGAACCTGACCGTACA-3’), which is complementary to the universal Z probe (Intergen Inc., Oxford, UK) was added to the primer. The primers used are detailed in Table II.

The reaction was carried out 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 each transcript were generated from a standard plasmid which contained the specific DNA sequence that was simultaneously amplified within the samples.

With every run of the PCR, a negative and positive control was employed, using a known cDNA sequence (8).

Statistical analysis. Data analysis was performed using the Minitab 14.1 statistical software package (Minitab Ltd., Coventry, UK.) using a custom-written macro (Stat 06e-mtb). Independent variables were compared using the Mann-Whitney *U*-test, while paired variables were compared using the two-sample *t*-test. Transcript levels within breast cancer specimens were compared to those of the ANCT and correlated with clinicopathological data collected over a 10-year follow-up period.

p-Values less than 0.05 were considered significant, whereas *p*-values between 0.05 and 0.10 were considered marginally significant.

For purposes of the Kaplan–Meier survival analysis, the samples were divided arbitrarily into high and low transcription groups, with the value for the moderate prognostic group as defined by NPI serving as the dividing line. Survival analyses were performed using SPSS version 12.0.1 (SPSS Inc., Chicago, IL, USA).

Results

In the course of our study, we recorded a strong inverse correlation between the mean copy number of *DAP1* and survival (Table III). The mean copy number in patients with no disease recurrence was significantly higher than that in patients with local recurrence (15.3 *vs*. 4.01, *p*=0.02), and distant metastasis (15.3 *vs*. 1.76, *p*=0.001). However, this trend was not seen in comparison of patients with no disease recurrence and mortalities (15.3 *vs*. 16.5, *p*=0.91).

The expression of *DAP1* decreased in relation to TNM staging (a mean copy number of 17.3 for TNM1 *vs*. 1.84 for TNM4, *p*=0.0039).

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

Parameter	Category	Number
Node status	Positive	65
	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
	With local recurrence	5
	Alive with metastasis	7
	Died of breast cancer	20

There was an inverse correlation between *DAP1* and NPI staging with a statistically significant decrease in mean *DAP1* copy number between NPI2 and NPI3 (18.4 *vs*. 4.64 , *p*=0.026).

No significant correlation was found between *DAP1* expression and tumour grade.

Discussion

The *DAP1* gene is localized in chromosome 5 band p15, and encodes a small 15 kDa protein. Human DAP1 is proline-rich, and normally exists as a phospho-protein *in vivo* (10). The *DAP1* gene was initially identified in HeLa cells subjected to the continuous presence of apoptosis–inducing interferon gamma. Other genes thus identified encoded DAP3, DAP5 and DAP kinase. These genes are thought to have a role in apoptosis (6). A possible role in advanced cancer for *DAP3* has been a recent subject of research (11).

DAP1 lacks any identifiable functional motifs, and its function was poorly understood until recently. Recent studies suggested it may have a role in autophagy. This is a catabolic process seen in cells in amino acid-deficient conditions. It is thought to help cells survive such conditions. Autophagy shares many of the characteristics of apoptosis (12).

Autophagy is regulated by the mammalian target of rapamycin (mTOR). In nutrient–rich conditions, mTOR phosphorylates its various known substrates. The Unc-51–like kinase 1 (ULK1) complex, when thus

Table II. *Primers used in RT-PCR analysis.*

DAP F1	ATGGACAAGCATCCTTCC
*DAP1*ZR	ACTGAACCTGACCGTACACTCTGTCAGGGAAATACCAA
Beta-actin, forward	ATGATATCGCCGCGCTCGTC
Beta-actin, reverse	CGCTCGGTGAGGATCTTCA

Table III. *Mean (DAP1) mRNA expression levels (copy number) in a cohort of 127 breast cancer patients; a comparison between subgroups with different tumour grade, Nottingham Prognostic Index (NPI), and TNM stage.*

Patients' and tumour characteristics	Mean DAP1 (SD)	*p*-Value
Tumour grade		
1 *vs*. 2	24.9 (50) *vs*. 13.8 (27.5)	0.38
1 *vs*. 3	24.9 (50) *vs*. 11.2 (28.5)	0.27
2 *vs*. 3	13.8 (27.5) *vs*. 11.2 (28.5)	0.67
NPI		
1 *vs*. 2	12.1 (33.2) *vs*. 18.4 (31.1)	0.36
1 *vs*. 3	12.1 (33.2) *vs*. 4.64 (9.74)	0.14
2 *vs*. 3	18.4 (31.1) *vs*. 4.64 (9.74)	0.026
TNM		
1 *vs*. 2	17.3 (37.8) *vs*. 13.2 (28.6)	0.54
1 *vs*. 3	17.3 (37.8) *vs*. 5.5 (10.5)	0.068
1 *vs*. 4	17.3 (37.8) *vs*. 1.84 (3.47)	0.0039
2 *vs*. 3	13.2 (28.6) *vs*. 5.5 (10.5)	0.22
2 *vs*. 4	13.2 (28.6) *vs*. 1.84 (3.47)	0.031
3 *vs*. 4	5.5 (10.5) *vs*. 1.84 (3.47)	0.43
Survival		
DF *vs*. LR	15.3 (34.5) *vs*. 4.01 (6.36)	0.02
DF *vs*. DR	15.3 (34.5) *vs*. 1.76 (1.86)	0.001
DF *vs*. D	15.3 (34.5) *vs*. 16.5 (37.1)	0.91

SD: Standard deviation, DF: disease-free survival, LR: local disease recurrence, DR: distant disease recurrence, D: death from breast cancer.

phosphorylated, is inactive. In nutrient–poor conditions, the activity of mTOR is attenuated, and the now active ULK1 complex mediates the process of autophagy (13).

Recent studies have shown that *DAP1* is also a substrate for mTOR, and is inactive when phosphorylated. In contrast to ULK1, when activated, it has been found to suppress the process of autophagy. It is thought to serve as a 'brake', preventing autophagy from progressing to cell death (7).

This is the first study in the literature to examine the direct relationship between *DAP1* mRNA expression and human breast cancer. Our findings demonstrate that this gene may have a tumour suppressor function. This raises the possibility of a significant overlap between the autophagy pathway and the apoptosis cascade, and a possible role in oncogenesis. This raises the prospect of identification of further therapeutic targets for the treatment of advanced cancer, and would merit further investigation.

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