An Evaluation of Prognostic Value of Death-associated Protein Kinase 1 in Breast Cancer

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Abstract. *Background: The benefit of tissue microarray (TMA) is its capability to analyse large numbers of tissue samples in a uniform fashion. This study was designed to evaluate the capability of TMA for analyzing the status of death-associated protein kinase 1 (DAPK1) in breast cancer patient and to explore its potential in the management of breast cancer. Patients and Methods: Over a 60-month period, tissue specimens of 99 patients with primary invasive breast cancer were selected. Tissue microarray (TMA) was applied to detect the DAPK1 expression. The data for the other clinicopathologic variables, including age, histological grading, estrogen receptor status and TNM staging were also recorded. Results: Tumor in 11 patients (11.1%) scored 1 for DAPK1 expression, 55 patients (55.6%) scored 2 and 33 (33.3%) scored 3. We found no obvious link between DAPK1 expression and age, histologic grading, primary tumor staging, lymph node status, estrogen receptor and TNM stage. TNM staging was found to be significantly linked to the overall five-year survival rate through multivariate analysis. Conclusion: DAPK1 expression did not show any meaningful value in predicting outcome for patients with breast cancer.*

Prognostic signatures have been explored for many malignant neoplasms, but whether these are valid for clinical application is still uncertain (1, 2). Many studies have shown the potential value of gene expression signatures in assessment of the postoperative outcomes in breast cancer and have inspired further studies on breast cancer prognosis (1, 2)

Death-associated protein kinase is a multidomain calcium/calmodulin-regulated and cytoskeletal-associated serine/threonine-kinase mandatory for interferon-gamma, tumor necrosis factor α, and activated Fas-induced apoptotic cell death and detachment from the extracallular matrix, comprising modules such as ankyrin repeats mediating protein-to-protein interactions and a death domain (3). This 160 kDa protein kinase is normally localized in the cytoskeleton in association with actin microfilaments. The death-promoting effects of DAPK depend on its intact catalytic activity, correct intracallular localization, and the presence of the death domain (4). The relation between DAPK and human cancer has already been mentioned. It has been reported that DAPK mRNA and protein expression are frequently lost in various human cancer cell lines, such as B-cell lymphoma and leukemia cell lines, as well as bladder and renal cell carcinoma-derived cell lines, indicating its potential role as a tumor suppressor gene (5, 6).

The benefit of tissue microarray (TMA) is its capability to analyses of large numbers of tissue samples in a uniform fashion (7, 8). This study was designed to evaluate the capability of TMA for analyzing the DAPK1 status in breast cancer patients and to explore its potential in the management of breast cancer.

Patients and Methods

Ninety-nine patients with primary invasive breast cancer were selected from the pathology files of Kaohsiung Chang Gung Memorial Hospital between January 1994 and December 1998. Modified radical mastectomy was performed for these cases, except those patients with stage IV. The hematoxylin-eosin-stained slides of the paraffined-embedded tumor specimens were reviewed by our pathologists to confirm the accuracy of the histological diagnoses and lymph node status.

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The data of the other clinicopathologic variables including age, histological grading, estrogen receptor status (9-11) and TNM staging, were also recorded.

Construction of TMA. Representative areas of both tumor and non-tumor parts for each case were selected and circled to match the blocks for the tissue microarray. Then the blocks matching the circled slides were retrieved to prepare the recipient block for the microarray. To ensure the representation of the selected cores, three areas each for both tumor and non-tumor parts per case were determined for assembling the recipient blocks. Each target area on the selected blocks was punched to form a 0.6-mm-diameter tissue core and placed consecutively on the recipient blocks of approximately 3 cm × 2 cm with a precision instrument (Beecher Instruments, Silver Spring, MD, USA) as described elsewhere (12).

Immunohistochemistry. The rabbit polyclonal antibody against DAPK1 (ab33526) was purchased from Abcam plc. (Cambridge, UK) and was diluted 1:150 in phosphate buffered saline (PBS).

Five-micrometer sections were cut from the recipient blocks of the tissue microarray, incubated overnight at 37˚C in an oven, dewaxed in xylene, and dehydrated in a series of graded alcohols. The sections were then treated with 3% hydrogen peroxide for 10 minutes to neutralize endogenous peroxidase activity and microwaved in 10 mM citrate buffer pH 6.0 to unmask the epitopes. After antigen retrieval, the sections were incubated with diluted DAPK1 antibody for 1 h followed by PBS wash. Horseradish peroxidase/Fab polymer conjugate (PicTure™-Plus kit) (Zymed, South San Francisco, CA, USA) was then applied to the sections for 30 min. After washing, the sections were incubated with peroxidase substrate diaminobenzidine for 5 min and counterstained with hematoxylin.

DAPK1 immunoreactivity grading. The intensity of the immunoreactivity for DAPK1 was classified by a four-tier grading system: 0, absence of staining in tumor cells; 1+, weak or focal (fewer than 10% cells) cytoplasmic staining in tumor cells; 2+, an intermediate staining intensity between 1+ and 3+ in tumor cells; and 3+, strong and diffuse (more than 90% cells) cytoplasmic staining in tumor cells.

Follow-up of patients. All of the patients were women from 29 to 76 years old, with a mean age of 49.4±10.2 years. The mean follow-up was 68.2±27.5 months (range, 5 to 98 months). Follow-up was usually performed every 3 months for the first 2 years and then every 6 months for the next 3 years. After 5 years, follow-up became annual. Chest radiography, serum alkaline phosphatase level, and detailed physical examination were usually performed at follow-up. Annual mammography or breast sonography (for the younger patient) were performed. Radionuclide bone scan, abdominal sonography or other image studies were performed if specific symptoms, signs or elevated serum alkaline phosphatase level were noted. Data regarding patient survival, clinical status, and clinicopathologic factors were obtained from medical records, contact with the patients at the outpatients clinics or by telephone, or from both.

Methodology for statistics. All analyses were carried out using the Statistical Package for the Social Sciences, release 17.0 (SPSS, Inc. Chicago, IL). Differences in clinicopathologic features by immunostaining among groups were assessed with the Chi-squared method and Fisher's exact test, whichever was appropriate. Overall survival was calculated by the Kaplan-Meier method. Differences in survival were tested using the log-rank test. To control for the confounding factors, the Cox proportional hazard model was used. Survival plots were constructed using the Kaplan-Meier method. All tests were two sided. Statistical significance was set at $p<0.05$.

Table I. *Death-associated protein kinase-(DAPK1) expression in relation to clinicopathologic variables*.

	DAPK1 Score						
	1		2		3		*P*-Value
Age, Years							0.475
<50	6	54.5%	26	47.3%	20	60.6%	
≥50	5	45.5%	29	52.7%	13	39.4%	
ER status							0.256
Negative	8	72.7%	37	67.3%	17	51.5%	
Positive	3	27.3%	18	32.7%	16	48.5%	
Histologic grading							0.420
1	3	27.3%	7	12.7%	4	12.1%	
2	4	36.4%	36	65.5%	19	57.6%	
3	4	36.4%	12	21.8%	10	30.3%	
Primary tumor staging							0.849
T1	1	9.1%	12	21.8%	7	21.2%	
T2	6	54.5%	29	52.7%	14	42.4%	
T3	3	27.3%	9	16.4%	7	21.2%	
T4	1	9.1%	5	9.1%	5	15.2%	
LN status							0.472
N0	7	63.6%	22	40.0%	18	54.5%	
N1	1	9.1%	13	23.6%	5	15.2%	
N2	1	9.1%	13	23.6%	4	12.1%	
N3	2	18.2%	7	12.7%	6	18.2%	
TNM stage							0.562
I	1	9.1%	5	9.1%	7	21.2%	
II	7	63.6%	26	47.3%	13	39.4%	
III	3	27.3%	22	40.0%	11	33.3%	
IV	0	.0%	2	3.6%	2	6.1%	

Results

Eleven patients (11.1%) scored for expression of DAPK1, 55 patients (55.6%) scored 2 and 33 (33.3%) scored 3 (Figure 1). By using the Chi-squared method, comparisons between groups were made and we found no obvious link between DAPK1 expression and age ($p=0.475$), histologic grading ($p=0.420$), primary tumor staging ($p=0.849$), lymph node status ($p=0.472$), estrogen receptor status ($p=0.256$) and TNM stage ($p=0.562$, Table I). For survival analyses, the end point was overall survival. The overall 5-year survival rates for different categories are listed in Table II. TNM staging was found to be significantly linked to the overall five-year survival rate through multivariate analysis. Nevertheless, DAPK1 expression did not show a meaningful value in predicting outcome for patients with breast cancer ($p=0.288$, Table III).

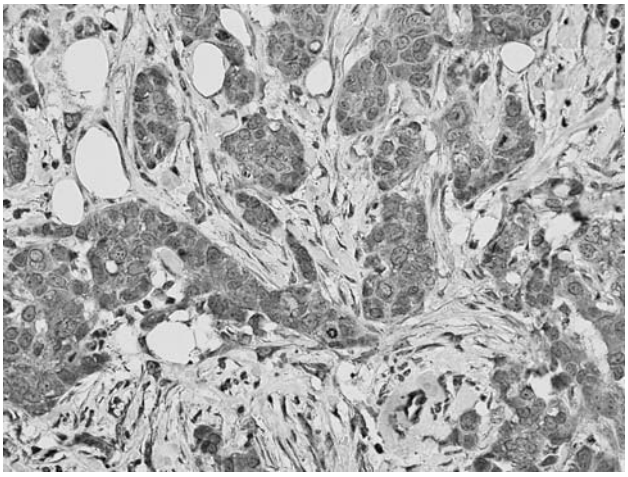

Figure 1. *DAPK1 immunostaining of a representative case of breast tissue graded as 3+. Strong and diffuse nuclear and cytoplasmic staining for DAPK1 are noted in the tumor cells of the breast cancer tissue. Original magnification, ×200.*

Table II. *Overall 5-year survival rate for each category of breast cancer.*

Variable	Category	Survival rate (%)	*P*-Value
Age, Years	<50	78.7	0.097
	≥50	61.7	
TNM stage	I	100.0	<.00001
	II	93.4	
	III	38.9	
	IV	0	
ER status	Negative	64.5	0.046
	Positive	80.7	
Histologic grading	1	64.3	0.549
	2	74.5	
	3	65.4	
DAPK1 score	1	63.6	0.848
	2	70.9	
	3	72.6	

DAPK1 : Death-associated protein kinase 1.

Table III. *Multivariate analysis for overall 5-year survival rate.*

Variable	*P*-Value	OR	95% CI	
Age (age≥50 *vs*. <50 years)	0.470	1.4	0.6	3.2
TNM stage (I, II, III, IV)	0.000	13.7	5.8	32.5
ER status (positive *vs*. negative)	0.076	0.4	0.2	1.1
Histologic grading (I, 2, 3)	0.084	1.6	0.9	2.9
DAPK1 (1, 2, 3)	0.288	0.7	0.4	1.4

OR: Odds rate; CI: confidence interval; DAPK1: death-associated protein kinase 1.

Discussion

Inactivation of tumor suppressor genes and reduced cell apoptosis have been reported to be essential mechanisms for breast tumorigenesis, contributing to deregulated tumor cell proliferation, invasion and metastasis (13). Tumor sensitivity to therapeutic process is usually mediated by initiation of programmed cell death *via* active apoptotic pathways (14). Some genes related to a defined apoptotic cascades, such as p53, have already demonstrated their association with breast tumor prognosis (15).

Loss of DAPK expression has been reported to correlate strongly with negative prognosis of several types of human cancer, such as small cell lung cancer, B-cell malignancies, primary head and neck tumors, colon and bladder cancer, and multiple myeloma (16, 17). Moreover, restoration of DAPK expression to physiological levels in a murine model of highly metastatic lung carcinoma cells strongly suppressed their metastatic ability (18). It seems that the loss of DAPK expression leads to a selective advantage for cancer cells and may play a role in tumor progression. Thus, DAPK expression may be useful in identifying aggressive neoplasms more likely to spread and alter patient long-term outcome.

With TMA, multiple specimens can be simultaneously evaluated under identical laboratory conditions, leading to a dramatic reduction of cost and time when compared with conventional pathologic studies (19, 20). Moreover, this technique is less exhausting for the specimen, allowing for an increased number of analyses per case (19, 20).

The major concern about TMA is to what extent tissue heterogeneity influences the validity and reproducibility of results achieved by TMA. Many studies have evaluated this issue. Torhorst *et al*. (21) demonstrated that by using TMA, the prognostic value of estrogen receptor, progesterone receptor and p53 in a series of 553 cases of breast cancer was fully reproduced. Rubin *et al*. (22) also found that in prostate cancer, which is well known for its heterogeneity, TMA was also proven to be a reliable tool for evaluation of the prognostic value of biomarkers. Recently, Lars *et al*. (20) found that when compared conventional method immunohistochemistry with tissue microarray is valid and provides results equivalent to these with conventional immunohistochemistry with respect to expression patterns and clinicopathological characterization.

In this study, TMA was used to analyze the DAPK1 status in 99 patients, with a mean follow-up of 68.2±27.5 months (range, 5 to 98 months). We found the DAPK1 level in breast cancer patients is not associated with overall survival (Table III).The case number of our series is still limited, further larger group study might provide a more objective answer. To the best of our knowledge, this is probably the first report with long-term follow-up regarding DAPK1 expression in breast cancer patient analyzed by using TMA.

In summary, DAPK1 expression by immunohistochemical staining with TMA did not show prognostic value in patients with breast cancer.

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