An Evaluation of Focal Adhesion Kinase in Breast Cancer by Tissue Microarrays

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Abstract. *Background: Many studies have shown that focal adhesion kinase (FAK) is a positive regulator of tumor progression and invasion. However, there is still very limited information about the role of FAK in breast cancer. Tissue microarrays (TMA) can analyze thousands of tissue samples in a parallel fashion with minimal damage to the origin block. This study was designed with the application of TMA to analyze the FAK status in breast cancer. Patients and Methods: Archival tissue specimens from 98 patients with primary invasive breast cancer were selected and FAK expression was analyzed by immunohistochemical staining with TMA. The data of primary tumor staging, age, estrogen receptor status, lymph node status, histological grading and TNM staging were also collected. Results: There were four patients (4.0%) with grade 1 expression in FAK, 41 patients (41.8%) with grade 2 expression in FAK and 53 patients (54.2%) with grade 3 expression in FAK. There was no significant relationship between FAK expression and age, estrogen receptor status, histological grading, primary tumor staging, lymph node status and TNM stage. By multivariate analysis, the TNM stage was found to be significantly related to the overall five-year survival rate ($p<0.00001$). Conclusion: Immunohistochemical staining with TMA is a convenient and feasible method. Unfortunately, our preliminary results fail to show meaningful prognostic value of FAK in breast cancer. A larger prospective study is warranted for further evaluation.*

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Focal adhesion kinase (FAK) was originally described by Schaller *et al*. (1) and Guan and Shalloway (2) in 1992 as a member of the protein tyrosine kinase (PTK) family and especially of the non-receptor RTKs subfamily (3). The 125-kDa FAK protein is encoded by the *FAK* gene located on human chromosome 8q24. Structurally, FAK consists of an amino-terminal regulatory FERM domain, a central catalytic kinase domain, and a carboxy-terminal focal adhesion-targeting domain (4, 5). Increasing evidence shows that crucial steps in cancer progression such as cell adhesion, migration, and cell- cycle progression are regulated by the composition and organization of the microenvironment (6). The adhesion of cancer cells to components of the microenvironment and the forces transmitted to the cells *via* the actinomyosin network and the signaling complexes organized within focal adhesions allow for cancer cells to sense the local topography of the extracellular matrix and respond efficiently to proximal growth and migration-promoting cues (6). FAK has been claimed to be an important factor of cell adhesion, growth, proliferation, survival, angiogenesis and migration, all of which are often disrupted in cancer cells (7, 8). Many studies have shown FAK to be a positive-regulator of tumor progression and invasion. FAK is noted to be up-regulated in a wide variety of malignancies including colonic, thyroid, prostatic, oral, neck and ovarian cancer (9, 10). These findings have led to the development of FAK inhibitors for the treatment of cancer. In fact, some FAK inhibitors have been included in clinical trials for cancer treatment (9-12).

Breast cancer is a collection of molecularly- and clinically-distinct neoplastic diseases (13-15). The potential for clinical application of the prognostic signature of malignant neoplasms

has been widely explored (16, 17). Recent research has shown that information regarding gene expression in breast cancer, obtained by different methods, could be beneficial in the designing of an optimal treatment plan and may also provide with prognostic information (16, 17).

The application of tissue microarrays (TMA) provides the chance to analyze thousands of tissue samples in a parallel fashion with minimal damage to the origin block (18, 19).

This study was designed with the application of TMA to analyze the FAK status in breast cancer with the hope of elucidating the possible relationship between FAK expression and breast cancer.

Materials and Methods

Patients. This study comprised of 98 female patient with breast cancer whose archival tissue specimens were selected from the pathology files of Kaohsiung Chang Gung Memorial Hospital between January 1994 and December 1998. Clinical records and follow-up information were available in all cases. All the patients underwent modified radical mastectomy due to invasive breast cancer, defined as carcinoma with invasion to or beyond the basement membrane, regardless of histological classification (ductal or lobular) (20). 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 the lymph node status. The data of primary tumor staging, age, estrogen receptor status (21-26), lymph node status, histological grading and TNM staging were also collected.

Tissue microarray assembling. Representative areas of both tumorous and non-tumorous 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 representativity of the selected cores, three areas, each for both tumorous and non-tumorous 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 which was then placed consecutively on the recipient block of approximately 3 cm × 2 cm with a precision instrument (Beecher Instruments, Silver Spring, MD, USA), as described elsewhere (27).

Immunohistochemical analysis. A rabbit polyclonal antibody against FAK (phospho Y397) (ab4803) was purchased from Abcam (Cambridge, MA, USA). Three-micrometer sections of tissue were sliced and incubated overnight in a 37˚C oven. After de-parafinization and antigen retrieval, the sections were incubated with the FAK antibody (1:100 dilution) for 1 h followed by incubation with horseradish peroxidase/Fab polymer conjugate (PicTure™-Plus kit ; Zymed, South San Francisco, CA, USA) for 30 min. Finally, the signals were developed by incubation with peroxidase substrate diaminobenzidine for 5 min.

Immunoreactivity for FAK was scored by a four-tier grading system: 0, no staining in tumor cells; 1+, weak staining in tumor cells; 2+, an intermediate staining intensity between 1+ and 3+ in tumor cells; and 3+, strong staining in tumor cells (Figure 1).

Patients and follow-up. 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 67.7+28.4 months (range=5 to 95 months). Follow-up was usually performed every three months for the first two years and then every six months for the next three years. After five 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 patients) were performed. Radionuclide bone scan, abdominal sonography or other image studies were performeds if specific symptoms, signs or elevated serum alkaline phosphatase level were noted. Data regarding patient survival, clinical status, and clinicopathological factors were obtained from medical records, contact with the patients at the outpatients clinics or by telephone, or from both.

Statistical analyses. Comparisons between groups were performed using x^2 test. For survival analyses, the end-point was overall survival. Survival differences were compared using the log-rank test. To assess the relative influence of the potential prognostic variables on survival, all clinicopathological and genetic variables were entered into the final Cox's proportional hazards model for multivariate analysis. Statistical analyses were conducted using the SPSS software (version 13.0 SPSS, Chicago, IL UAS). Statistical significance was set at $p<0.05$. All p-values are estimated from two -sided tests.

Results

There were four patients (4.0%) with 1+ expression of FAK, 41 patients (41.8%) with 2+ expression and 53 patients (54.2%) with 3+ expression (Table I). By using the x^2 test, comparisons between groups were performed (Table I). There was no significant relationship between FAK expression and age ($p=0.445$), estrogen receptor status ($p=0.225$), histological grading ($p=0.786$), primary tumor staging ($p=0.361$), lymph node status ($p=0.636$) or TNM staging ($p=0.426$). For survival analyses, the end-point was overall survival. The overall five-year survival rates for different categories are listed in Table II. By multivariate analysis, FAK expression did not have any meaningful prognostic value and was not significantly related to the overall five-year survival rate ($p=0.484$; Table III).

Discussion

Constitutive tyrosine kinase activity contributes to cancer cell proliferation and to other aspects of malignant transformation, with a number of possible mechanisms for elevated kinase activity, increased gene dosage or altered protein expression and stability (28, 29). It has been reported that FAK is altered at the genetic level in tumors and in cell lines derived from a variety of human tumors (30).

There is circumstantial evidence linking aberrant FAK expression with malignant disease, with elevated FAK protein levels reported in a long list of human epithelial cancer types (4). The capability of tumor cells to invade surrounding tissue during the initiation of metastatic disease is of crucial significance in cancer progression. Recent studies support a role for FAK in promoting the invasive process (31, 32). Inhibition of FAK activity or blocking of FAK expression has been noted to inhibit the invasive motility of human carcinoma cells (33). Invading tumor cells require a means of degrading the surrounding tissues to facilitate their dissemination during the invasive process. On this respect, there is evidence that

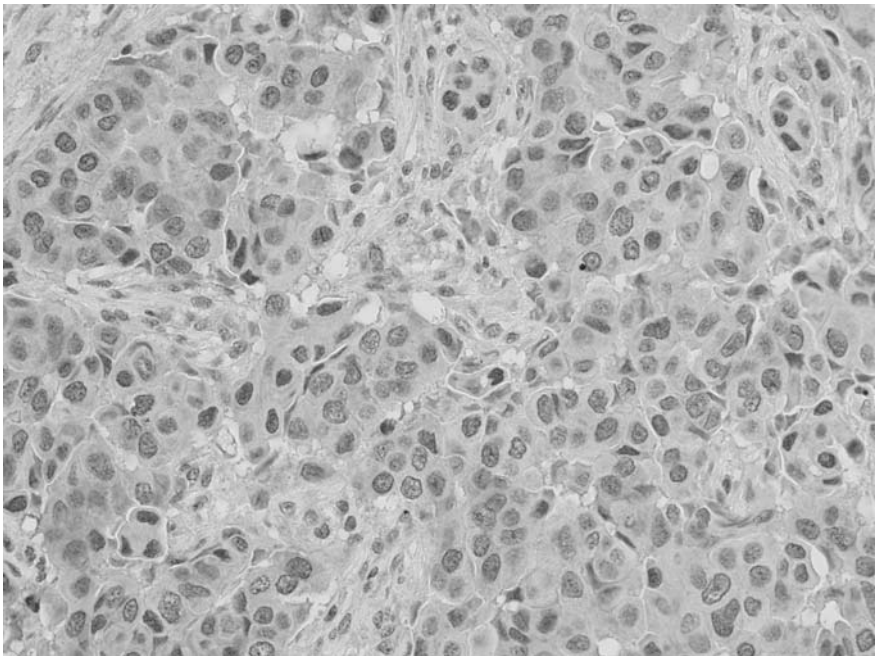

Figure 1. *A representative case of breast cancer scored as 3+ for Focal adhesion kinase expression. Diffuse and strong immunostaining for FAK is noted in both the nuclear and cytoplasmic areas of the tumor cells. Original magnification, ×200.*

Table I. *Focal adhesion kinase expresssion in relation to clinicopathological variables.*

	FAK score						
	1+		2+		3+		*p*-Value
Age, years							0.445
<50	3	75.0%	23	56.1%	25	47.2%	
≥50	1	25.0%	18	43.9%	28	52.8%	
ER status							0.255
Negative	4	100.0%	24	58.5%	34	64.2%	
Positive	0	0.0%	17	41.5%	19	35.8%	
Histological grading							0.786
1	0	0.0%	5	12.2%	8	15.1%	
2	2	50.0%	24	58.5%	32	60.4%	
3	2	50.0%	12	29.3%	13	24.5%	
Primary tumor staging							0.361
T1	0	0.0%	6	14.6%	11	20.8%	
T2	3	75.0%	23	56.1%	25	47.2%	
T3	1	25.0%	10	24.4%	8	15.1%	
T4	0	0.0%	2	4.9%	9	17.0%	
N Status							0.636
N0	2	50.0%	17	41.5%	28	52.8%	
N1	1	25.0%	9	22.0%	8	15.1%	
N2	1	25.0%	10	24.4%	7	13.2%	
N3	0	0.0%	5	12.2%	10	18.9%	
TNM Stage							0.426
I	0	0.0%	2	4.9%	9	17.0%	
II	3	75.0%	20	48.8%	24	45.3%	
III	1	25.0%	18	43.9%	17	32.1%	
IV	0	0.0%	1	2.4%	3	5.7%	

ER: estrogen receptor.

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

Variable	Category	5-year survival rate (%)	*p*-Value
Age, years	<50	76.3	0.148
	≥50	60.9	
TNM Stage	I	100.0	<0.00001
	II	91.3	
	III	38.9	
	IV	0	
ER status	Negative	62.3	0.033
	Positive	80.1	
Histological grading	1	61.5	0.549
	2	72.0	
	3	66.7	
FAK	1	50.0	0.474
	2	73.2	
	3	67.3	

FAK: Focal adhesion kinase; ER: estrogen receptor.

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

Variable	*p*-Value	OR	95% CI
Age (age >=50 *vs*. <50 years)	0.773	1.1	0.6-3.2
TNM stage (I, II, III, IV)	0.000	10.4	5.8-32.5
ER status (postive *vs*. negative)	0.050	0.4	0.2-1.1
Histologic grading (I, 2, 3)	0.110	1.6	0.9-2.9
FAK (1, 2, 3)	0.483	1.3	0.4-1.4

OR: Odds ratio; CI: confidence interval; FAK: Focal adhesion kinase.

FAK may control proteolytic pathways that facilitate degradation of the surrounding matrix (28, 35, 36).

Kononen *et al*. (37) originally developed a TMA technology by which hundreds of individual tissue specimens can be arrayed to a single tumor array block. This method allows for consecutive sections to be cut from the block to provide the material for the simultaneous *in situ* detection of DNA, RNA, or protein targets in a very high number of tissue samples (38). The most important advantages of TMA are increased capacity, negligible damage to the original tissue block, the precise localization of sample tissue specimens and the ability for automated construction and analysis of arrays (37). In contrast to immunohistochemical analyses on large sections, TMA allows for a high level of standardization for immunohistochemical staining because all tumor samples are pretreated and stained under exactly the same conditions. In contrast to the reading of large sections, which is always an attempt to integrate the observations in multiple different regions of a tissue section, the morphological classification and interpretation of immunoreactivity in TMA are based on the findings within one small, highly-defined tissue area in TMAS. The criteria for diagnostic decisions are, therefore, much easier to establish between the individual samples on the array and to compare among different observers (19, 38, 39).

The critique of whether minute tissue samples of potentially heterogeneous tumor are representative enough to allow for meaningful analysis has been raised. Torhort *et al*. showed that by using TMA, the prognostic value of estrogen receptor, progesterone receptor and p53 in a series of 553 cases of breast cancer could be fully-reproduced (40). Rubin *et al*. also noted that in prostate cancer, which is well-known for its heterogeneity, TMA was still proven to be a reliable method for its evaluation of the prognostic value of biomarkers (41). Lars *et al*. (42) recently showed that immunohistochemistry with TMA is valid and provides results equivalent to conventional immunochemistry with respect to expression patterns and clinicopathological characterizations (42).

In our study, a TMA technique was used and we found there was no significant relationship between FAK expression and other basic clinicopathological parameters. Furthermore, no survival difference was noted among the three groups with different FAK expression (Table II, p=0.474). By multivariate analysis, FAK expression did not show meaningful prognostic value and was not significantly related to the overall five-year survival rate (p=0.484, Table III). Our preliminary results fail to show any meaningful prognostic value of FAK expression in breast cancer. This is probably due to the retrospective nature of this study and the small number of patients in this study. We believe a prospective study with larger number of patient is warranted for further evaluation.

In conclusion, immunohistochemical staining with TMA was convenient and feasible for the analysis of FAK expression in breast cancer, yet FAK expression did not show a significant correlation with the overall survival rate in this preliminary study. A larger prospective study is warranted for further evaluation.

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