Insulin-like Growth Factor-binding Protein-3 in Breast Cancer: Analysis with Tissue Microarray

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Abstract. *Background: IGFBP-3 has been reported to be growth-stimulatory. The creation of tissue microarray (TMA) allows for the rapid immunohistochemical analysis of thousands of tissue samples in a parallel fashion. This study was designed with the application of TMA to analyze the IGFBP-3 status in breast cancer with the hope to elucidate the possible relationship between IGFBP-3 expression and breast cancer biology. Materials and Methods: Archival tissue specimens from 97 patients with primary invasive breast cancer were selected. The IGFBP-3 expression was analyzed by 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 40 patients (41%) with 0-1 expression of IGFBP-3, 52 patients (54%) with 2 expression of IGFBP-3 and 5 patients (5%) with 3 expression in IGFBP-3. By multivariate analysis, the IGFBP-3 expression turned out to be significantly related to the overall five-year survival rate. Conclusion: The preliminary results about IGFBP-3 expression in breast cancer are intriguing and require further evaluation.*

Insulin-like growth factors are multifunctional peptides that appear to be important in regulation of growth of normal cells and resistance to apoptosis in neoplastic tissue (1, 2). Contrary to most other growth factors, IGF peptides appear in large concentrations in the circulation and show systemic, hormonal and local paracrine effect on cell behavior (3). In the circulation, IGF-I binds mostly to the main IGF binding protein. Insulin-like growth factor-binding protein-3 (IGFBP-3) (3). IGFBP-3, a 45-kDa glycoprotein abundant in the circulation and extracellular environment, is a key regulator of the peptide hormones IGF-I and IGF-II (1). Through its high affinity for these growth factors, IGFBP-3 competes for ligand binding with the receptor mainly responsible for mediating the actions of IGF-I and -II, the type I IGF receptor (IGFR1) (3), and thereby blocks mitogenic and anti-apoptotic signaling initiated by its activation. A crucial role for IGFBP-3 in modulating the proliferative effects of IGFs in many cell types is well known, and both exogenous and endogenous IGFBP-3 have been shown to block IGF action in breast cancer cells *in vitro* (4-7). On the contrary, IGFBP-3 has been reported to be growth-stimulatory *in vitro*. IGFBP-3 may strengthen IGF-stimulated DNA synthesis in MCF-7 breast cancer cells (8). Recently, IGFBP-3 has been found to have the capability of increasing proliferation of LNCaP prostate cancer cells in the absence of serum or IGFs (9). IGFBP-3 is secreted primarily by the liver (10), furthermore, secretion by breast cancer cells (11) resulting in local effect has also been reported.

The creation of tissue microarray (TMA) allows for the rapid immunohistochemical analysis of thousands of tissue samples in a parallel fashion with minimal damage to the origin blocks (12, 13).

This study was designed with the application of TMA to analyze the IGFBP-3 status in breast cancer with the hope to elucidate the possible relationship between IGFBP-3 expression and breast cancer.

Materials and Methods

Specimen selection and data collection. Archival tissue specimens from 97 patients with primary invasive breast cancer were selected from the pathology files of Chang Gung Memorial Hospital at Kaohsiung between January 1994 and December 1998. All the patients underwent modified radical mastectomy due to invasive

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breast cancer, defined as carcinoma with invasion to or beyond the basement membrane regardless of histological classification (ductal or lobular) (14). The data of primary tumor staging, age, estrogen receptor status (15-20), lymph node status, histological grading and TNM staging were also collected. The hematoxylin-eosinstained slides of the paraffined-embedded tumor specimens were reviewed by our pathologists to confirm the accuracy of the histological diagnoses and lymph node status.

Tissue microarray assembling. The 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 assure 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 into the recipient blocks with a precision instrument (Beecher Instruments, Silver Spring, MD, USA) as described elsewhere (21).

Immunohistochemical analysis. The rabbit polyclonal antibody against human insulin-like growth factor-binding protein-3 (IGFBP3) (sc-9028) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and was diluted 1:50 in phosphate buffered saline (PBS). Five-micrometer sections were cut from the recipient blocks of the tissue microarray, incubated overnight in a 37˚C 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 deprive the endogenous peroxidase activity and microwaved in 10 mM citrate buffer pH6.0 to unmask the epitopes. After antigen retrieval, the sections were incubated with diluted IGFBP3 antibody for 1 hour 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 minutes. After washing, the sections were incubated with peroxidase substrate diaminobenzidine for 5 minutes and counterstained with hematoxylin.

Grading for IGFBP3. Immunoreactivity of IGFBP3 was classified into a four-grade scale for evaluation: 0, absence of staining in tumor cells; 1+, weak cytoplasmic and/or nuclear staining in tumor cells; 2+, an intermediate staining intensity between 1+ and 3+ in tumor cells; and 3+, strong cytoplasmic and nuclear staining in tumor cells (Figure 1).

Patients and follow-up. All of the patients were women from 26 to 76 years old, with a mean age of 48.2±10.5 years. The mean follow-up was 69.7±25.8 months (range, 6 to 95 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 clinicopathological factors were obtained from medical records, contact with the patients at the outpatients clinics or by telephone, or both.

Table I. *Univariate analysis for overall five-year survival rate*.

Variable	Category	Case No.	5-y survival rate (%)	*p*-value
Age (Y)	<50	54	82	0.261
	≥50	43	70	
T stage	1	21	95	<0.001
	2	55	84	
	3	12	67	
	4	9	0	
N stage	0	46	94	<0.001
	1	19	90	
	2	20	50	
	3	12	33	
M stage	Negative	94	79	<0.001
	Positive	3	0	
TNM stage	1	14	100	<0.001
	2	47	92	
	3	33	52	
	4	3	0	
Histological grading	1	12	69	0.627
	2	58	79	
	3	26	73	
IGFBP3 expression	0-1	40	75	0.069
	2	52	81	
	3	5	40	
IGFBP3 expression	0-2	92	78	0.023
	3	5	40	

Statistics. All analyses were done using the Statistical Package for the Social Sciences, release 13.0 (SPSS, Inc. Chicago, IL USA). Differences of clinicopathological features among groups by immunostaining were assessed with the X^2 method and Fisher's exact test, whichever was appropriate. Overall survival was calculated using univariate analysis by the Kaplan-Meier method. Differences were tested using the log-rank test. To control for confounding factors, the Cox proportional hazard model was used. Survival plots were constructed using Kaplan-Meier method. All tests were two sided. Statistical significance was set at $p<0.05$.

Results

There were 40 patients (41%) with 0-1 expression in IGFBP-3, 52 patients (54%) with 2 expression in IGFBP-3 and 5 patients (5%) with 3 expression in IGFBP-3 .The end point was overall survival. The results of univariable analysis for overall five-year survival are listed in Table I. When the IGFBP-3 expression was categorized into 0-1, 2 and 3, the *p* value for the five-year survival rate difference was 0.069. Nevertheless, when the patients were categorized into those with strong expression of IGFBP-3 (3) and those with none to intermediate expression of IGFBP-3 (0, 1 and 2), there was a significant five-year survival rate difference (40% *vs*. 78%, Figure 2, $p=0.023$). Furthermore, by multivariate analysis, the IGFBP-3 expression still turned out to be significantly related to the overall five-year survival rate (Table II).

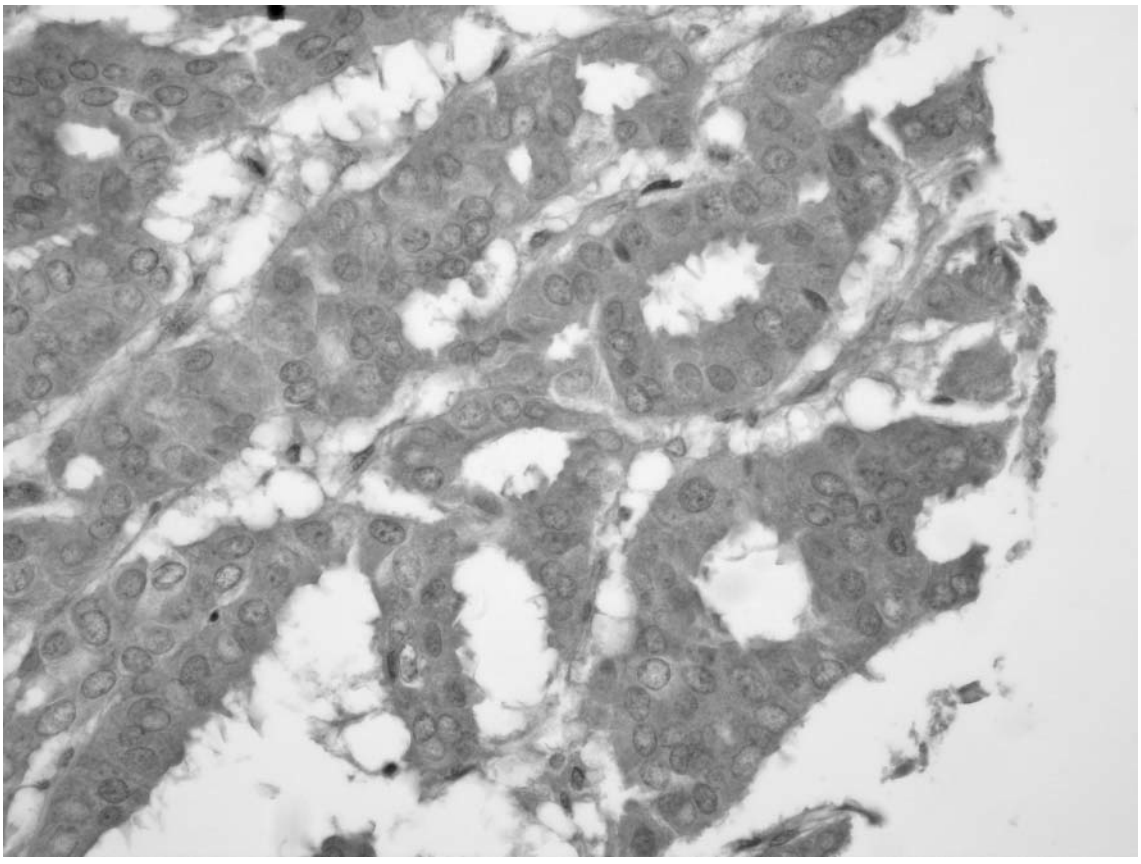

Figure 1. *Immunostaining with the IGFBP3 antibody on the tissue microarray slides of the breast cancers. The representative 3+ case reveals strong cytoplasmic and nuclear immunoreactivity in the tumor cells. Original magnification, ×400.*

Discussion

There is increasing evidence that a family of peptide hormones including insulin and insulin-like growth factors I and II (IGF-I, IGFII) may have a crucial role in the development and prognosis of breast cancer (22). Although the clinical relevance of IGF-I in breast cancer is still not fully elucidated, the support for an association between IGF-I and breast cancer is growing. Hankinson *et al*. (23) reported a modest adverse effect of IGF-I in premenopausal women less than 50 years of age. Rasmussen *et al*. (24) reported that IGF-I may be secreted locally by either tumor or stromal cells. Therefore, it is assumed that autocrine or paracrine rather than endocrine effects of IGF-I may lead to its prognostic effect. Thus circulating or locally produced IGF binding proteins (IGFBP's) were postulated to influence biological activity of IGF-I with resultant effects on breast cancer risk and prognosis. At least seven IGFBP's were identified (25). IGFBP-3, the largest of them, was reported to bind the majority of IGF-I in the circulation. In *in vitro* studies, IGFBP-3 was found to exert either enhancing or inhibitory effects on IGF-I action, depending on the circumstances (3). IGFBP-3 is secreted primarily by the liver (10), although secretion by breast cancer cells (11) resulting in local effect has also been reported. Furthermore, both IGFBP-3 m-RNA expression and cytosolic IGFBP-3 expression in primary breast cancer tissue have been reported to be associated with poor prognostic factors (estrogen and progesterone receptor negativity, aneuploidy, high S-phase fraction (26, 27).

Kononen *et al*. (28) recently described an array-based high-throughput technique that facilitates analysis of very large numbers of tumors at once, either at the DNA, RNA, or

Table II. *Multivariate analysis for overall five-year survival rate.*

Variable	Category	OR	95% CI	*p*-value
Age	≥50 *vs*. <50	1.5	0.6-3.7	0.343
IGFBP3 expression	3 *vs*. 0-2	2.0	1.0-3.9	0.036
Histologic grading	1, 2, 3	1.8	0.9-3.5	0.102
ER status	positive *vs*. negative	0.4	0.2-1.2	0.112
Staging	1, 2, 3, 4	9.4	4.0-22.2	0.000

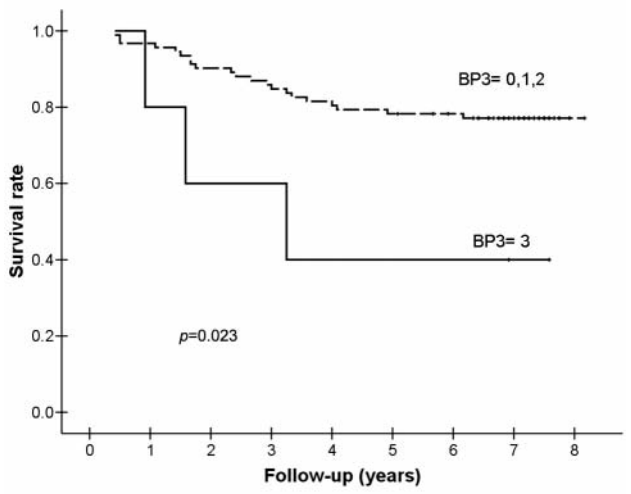

Figure 2. *There is significant five-year survival rate difference (78% vs. 40% , p=0.023) between none to intermediate expression and strong expression of IGFBP-3 groups.*

protein level. As many as 1000 cylindrical tissue biopsy specimens from individual tumor can be arrayed in a single tissue microarray (TMA) block. The power of the TMA technique is the capability of performing a series of analyses on thousands of specimens in a parallel fashion with minimal damage to the origin blocks (28-30). In contrast to immunohistochemical analyses on large section, TMA allows a high level of standardizations for immunohistochemical staining because all tumor samples are pretreated and stained under exactly the same conditions. Contrary from the reading of large sections which always is an attempt to integrate the observations in multiple different regions of a tissue section, the morphological classification and interpretation of immunoreactivity are based on the findings within one small, highly defined tissue area in TMA. The criteria for diagnostic decisions are therefore much easier to establish between the individual samples on the array and to compare among different observers (28-30).

Nevertheless, critique of TMA arises as to whether these small specimens (diameter 0.6 mm) are really representative of their donor tumors. It has been reported that some alternations are not detected if the analysis of heterogenous tumors is restricted to samples measuring 0.6 mm (31). However, Moch *et al*. (29) pointed out that the TMA approach has been designed to examine tumor populations and not to survey individual tumors. They have analyzed the impact of tissue heterogeneity on TMA data and compared results obtained from TMA with results from large sections in multiple different studies, and found that the results did show heterogeneity within tumors but suggested that this heterogeneity did not influence the identification of prognostic parameters. The reliability of tissue microarrays in detecting protein expression and gene amplification in breast cancer has been confirmed (32-35). The presented study analyzed IGFPB-3 expression in breast cancer by immunohistochemical staining with TMA and the results were obtained smoothly. By multivariate analysis, the IGFBP-3 expression turned out to be significantly related to the overall five-year survival rate (Table II). To the best of the authors knowledge, this is probably the first report with long term follow-up about IGFBP-3 expression in breast cancer analyzed by using TMA.

In conclusion, immunohistochemical staining with tissue microarray was convenient and feasible for the analysis of IGFBP-3 expression status in breast cancer. Furthermore, by multivariate analysis, the IGFBP-3 expression turned out to be significantly related to the overall five-year survival rate. These preliminary results about IGFBP-3 expression in breast cancer are intriguing and deserve further evaluation.

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