Tissue Microarray Analysis of FAS, Bcl-2, Bcl-x, ER, PgR, Hsp60, p53 and Her2-neu in Breast Carcinoma

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Abstract. *Background: The aim of this study was to detect immunohistochemical markers in breast carcinoma by means of tissue microarray analysis (TMA) and to associate their expressions with clinicopathological features and prognosis. Fatty acid synthase, bcl-2, bcl-x, p53, estrogen and progesterone receptors, heat shock protein 60 and Her2-neu (c-erbB-2) were evaluated in a group of 149 breast carcinoma patients with a 5-year follow-up period. Materials and Methods: TMA blocks were made by using duplicate 0.6-mm diameter tissue cores from each paraffin block. Results: Statistical analysis revealed that tumor stage (p=0.003) and node status (p=0.001) were the only two prognostic markers of disease-free survival. Moreover, FAS and bcl-x showed an independent effect on recurrence (p=0.005). The node status was the only marker of overall survival (p=0.05). Conclusion: Our data confirmed recent reports associating the stage of disease, FAS and Bcl-x expressions with recurrence and outcome. These data demonstrated that TMA is an effective substitute for conventional histochemical-immunohistochemical techniques.*

Breast carcinoma remains the most commonly occurring cancer in women (1). The ability to predict the outcome of a breast carcinoma is important in planning therapy, however it is a heterogeneous disease and, unfortunately, the ability to predict its biological behaviour has not kept pace with improvements in detection. Classic associations between the clinical course of the disease and histo-pathological features have failed to differentiate aggressive from indolent lesions (2), whereas data regarding the correlation of prognosis with single-, newly-isolated oncogenes or enzymes expressed by neoplastic breast tissue is controversial. The evaluation of multiple markers in large numbers of cases would help stratify patients into different follow-up groups and in decision-making for appropriate management. However, standard immunohistochemistry may lead to technical inconsistencies, including lack of a simultaneous scoring system, inability to simultaneously use different antibodies or antigen retrieval techniques, inability to simultaneously evaluate the variation in sensitivity and specificity of tumor tissues to different antibody reagents, all of which contribute to discrepancies. Moreover, in studies involving a large number of cases, the separate examination of each archival tumor block would be laborious and time-consuming, technically challenging and expensive. In contrast, is the use of tissue microarrays (TMA), each of which can be constructed from as many as 1000 donor tumors (3). The aim of this study was to correlate the survival results with patient age, menopausal status, histological tumor type and grade, tumor size, estrogen receptor, progesterone receptor, heat shock protein 60 (Hps60), Her2-neu (c-erbB-2) protein, p53 protein, bcl-2, bcl-x protein and fatty acid synthase (FAS) immunohistochemical expressions using tissue microarray analysis in a group of 149 breast carcinoma patients with a 5-year follow-up period.

Materials and Methods

Case selection. One hundred and forty-nine patients, surgically treated for breast carcinoma at the University of Rome "La Sapienza", Italy, between January 1996 and January 2000, were studied. Clinical information was obtained from the medical records. The clinical data included the patient's name, the family and patient's breast cancer history, type of surgery, postoperative treatment, date and site of the eventual recurrence, and the patient's current status (alive or deceased). Disease-free months of survival were calculated from the date of diagnosis to the date of

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Key Words: FAS, bcl-2, bcl-x, p53, Hsp60, Her2-neu, breast carcinoma, prognosis, TMA.

the first recurrence. Ethical approval was appropriately acquired. The histopathological data included tumor size, histological subtype (4) and grade (5), evidence of necrosis, and stage of the disease according to the TNM classification (6). Control specimens were obtained from patients submitted to mastoplasty. Representative blocks of the tumor were chosen for the immunohistochemical stainings.

Tumor collection and tissue microarray construction. The tissue sections were cut from the specimens and afterwards fixed in buffered formalin and paraffin embedded at the Department of Experimental Medicine and Pathology, University of a Rome "La Sapienza", Italy, in accordance with institutional guidelines for the use of discarded human tissue. For statistical purposes, the breast carcinomas were divided into three main groups: infiltrating duct, lobular and medullary carcinomas. The histological grading was evaluated on hematoxylin and eosin-stained sections, according to the criteria described by Bloom and Richardson (5). Areas of well-fixed invasive carcinoma were marked on both the slide and matching paraffin tissue block for construction of tissue microarrays (7). A total of 149 formalin-fixed, paraffin-embedded breast carcinoma samples were obtained. The tumor samples were arrayed according to Kononen *et al.* (3). Briefly, a hematoxylin and eosin-stained section was prepared from each selected tissue block. The tissue cores (cylinder) from designated zones of each "donor" block were punched; relatively small areas of tissue (down to 0.6 mm in diameter) were obtained from the "donor" blocks. The tissue cores and sample were deposited into a recipient block using a tissue-arraying instrument (Beecher Instruments Sun Prairie, WI, USA). After construction of the array blocks, sections from these numerous paraffin blocks were cut down on microtome and were placed on to standard glass slides; multiple consecutive 4-micron sections were cut until all of the tissue cores were present on a single slide (Figure 1). Once constructed, the tissue microarrays were used for immunohistochemical staining (Figure 2).

Patient follow-up techniques. All patients were followed-up postoperatively for a minimum of 5 years until January 2005. The disease-free survival (DFS) and overall survival (OS) were calculated as the period from surgery until the date of the first recurrence or death. Recurrence of disease was defined as the first evidence of new disease manifestation(s) at or near the area of the original cancer or at distant site(s). Any new disease involvement was assessed by clinical, radiological and, whenever feasible, histological examination of the site of first relapse.

Immunohistochemical assay. The indirect avidin-biotin complex (ABC) immunoperoxidase assay was performed on dewaxed and rehydrated cores of formalin-fixed, paraffin-embedded tissues obtained from the Department of Experimental Medicine and Pathology of the University of Rome ("La Sapienza") using a commercially available Dako ABC kit (Dako, Carpinteria, CA, USA). Sections were incubated with non-immune rabbit serum at 1:100 dilution in Tris-buffered saline (pH 7.6 at 37˚C) for 30 minutes and, after intervening washes in Tris-buffered saline, incubated with each primary antibody at concentrations ranging from 50 to 3000 μg/ml for 60 minutes, at 25˚C in a moist chamber. To block endogenous peroxidase activity, the sections were subsequently incubated with 3% hydrogen peroxidase in methanol for 30 minutes. Following incubation with biotynlated rabbit anti-mouse antibody, for 1 hour at 25˚C, the immunoenzymatic reaction was developed using 3'3-diamonobenzidine (DAB) as a chromogen substrate. After extensive washing, nuclear counterstaining was achieved using Meyer's hematoxylin. The primary antibodies used are listed in Table I. Some were obtained from Dako (bcl-2, bcl-x, p53, Her2-neu, estrogen and progesterone receptors), Hsp60 was from Neomarkers (Lab Vision Corporation, Fremont, CA, USA), while FAS was a gift from FASgen Inc. (Baltimore, MD, USA).

Table I. *Antibodies used in this study.*

Antiserum (clone) against	Source	Pattern of reactivity
FAS	F. Kuhajda	cytoplasmic
Bcl-2	Dako	nuclear
Bcl-x	Dako	nuclear
p53	Dako	nuclear
Hsp 60	Neomarkers	cytoplasm
Her2-neu	Dako	membraneous
Estrogen receptor	Dako	nuclear
Progesterone receptor	Dako	nuclear

Scoring of immunoreactivity. The immunohistochemical expressions of FAS, bcl-2, bcl-x, p53, Hsp60, Her2-neu, estrogen and progesterone receptors on each single core were simply graded as positive or negative. A reaction was considered positive when at least 10% of the entire core revealed stained cells. The intensity of the staining was not considered.

Statistical analysis. Cut-off end-points were decided on by the positive and negative immunohistochemical expressions observed. The FAS, Hsp60, bcl-2, bcl-x, estrogen and progesterone receptors, p53 protein and Her2-neu protein immunostainings were associated with the clinicopathological features and prognosis, as determined by the Chi-square test or the Fisher's exact test where appropriate. The primary statistical outcomes were the DFS and the OS from the date of surgery. Univariate and multivariate analyses were performed using the Cox regression model. All the analyses were conducted using the SYSTAT® software statistic package (SPSS Inc., Chicago, IL, USA)

Results

Histotype. One hundred and twenty-eight samples were of infiltrating duct carcinoma, seven were infiltrating lobular carcinoma and 14 were medullary carcinoma.

Histological grade. Twenty-one cases were well-differentiated (G1), while 79 cases were moderately-differentiated (G2) and 49 cases were poorly-differentiated (G3) carcinomas. Sixty cases were stage I, 72 cases were stage II and 17 cases were stage III.

Immunohistochemical expression. One hundred and eighteen (79%) carcinomas showed a positive overall score for FAS immunostaining, while twelve (8%) were considered negative. Nineteen cases were not valuable because the tissue cores were missing on the slide (12%). Bcl-2 expression was positive in 88 patients (59%), negative in 34 patients (22%) and 27 tissue cores were missing (18%). Bcl-x expression was positive in 110 patients (74%), negative in 23 patients (15%) and 26 tissue cores were missing (11%). Seventy-nine (53%) carcinomas showed a positive overall score for Hsp60 immunostaining, 63 (42%) were considered negative and seven tissue cores were missing (4%). p53 expression was positive in 62 patients (41%), negative in 63 patients (42 %) and 24 tissue cores were missing (17%). Estrogen-positive receptors were expressed in 72 patients (48%), negative in 63 patients (42%) and 15 tissue cores were missing (9%). Progesterone-positive receptors were expressed in 64 patients (42%), negative in 49 patients (32%) and 35 tissue cores were missing (24%). Her2-neu expression was positive in 81 patients (54%), negative in 43 patients (28%) and 25 tissue cores were missing (16%).

Statistical analysis. The analysis revealed that the stage of the disease ($p=0.003$) and the node status ($p=0.0001$) were the only markers of disease-free survival (DFS) by univariate analysis, while FAS and bcl-x showed an independent effect on recurrence ($p=0.005$). The node status was the only marker of overall survival (OS) by multivariate analysis ($p=0.05$), while patients younger than 50 years and with well-differentiated carcinomas (G 1) were associated with survival after a 5-year follow-up period. Other clinicopathological markers had no statistical significance.

Discussion

Breast carcinomas are heterogeneous diseases with uncertain prognosis. The clinicomorphological features evaluated in the past have been demonstrated to be unable to define prognosis, while biological markers evaluated separately have revealed controversial results. The clustering of biological markers could be useful to assess associations between the clinicomorphological expression and disease progression. Detection of these markers, independently, can be time-consuming, expensive and may be biased because of technical problems. To avoid these biases, here the tissue microarray analysis (TMA) technique was applied to the study of a group of markers in patients with breast carcinomas in order to reveal statistical associations between the marker expressions, clinicopathological features and prognosis. Most tissues with high cellular turnover appear to utilize circulating lipids for the synthesis of new structural lipids, but hyperplastic as well as neoplastic tissues seem to require alternative sources for energy storage. A minor metabolic pathway for the accumulation of energy involves the biosynthesis of fatty acids. In mammals and birds, the *de novo* synthesis of fatty acids is consolidated into a single protein that is the product of a single gene. This multifunctional enzyme, the key enzyme in fatty acid biosynthesis, is FAS, being the major enzyme involved in the anabolic conversion of dietary carbohydrates to fatty acids. FAS synthesizes long-chain fatty acids by using acetyl-CoA as a primer, malonyl-CoA as a two-carbon donor, and NADPH as a reductant of the intermediates, mainly synthesizing palmitate (80%), myristate (10%) and stearate (10%). The FAS expression in normal tissues is regulated by several hormonal signals and is related to dietary fat intake and metabolism, while FAS expression in tumor tissues occurs at very high rates; in fact, it has been shown that the FAS expression parallels the increased malignant potential during neoplastic progression. It seems that FAS overexpression confers a selective growth advantage to neoplastic cells (9-11).

Our data revealed, by univariate analysis, that FAS had an independent effect on recurrence. This is not surprising since FAS overexpression has been demonstrated, using conventional immunohistochemistry, in many human carcinomas with aggressive features and poor outcome, such as carcinomas of the ovary (12), prostate (13), vulva (14), colon (15), bladder (16), esophagus (17) and endometrium (18), some pediatric tumors (19), mesotheliomas (20), melanomas (21) and soft tissue sarcomas (22). Previous studies established that FAS may clinically predict the recurrence of breast cancer when combined with the progesterone receptor status (23). p53, a tumor suppressor gene, is a transcription factor present in minute levels in normal cells. Although the number of genes activated by p53 is rather large, the outcome of p53 activation is either cell cycle arrest in G1, in G2 or apoptosis. The arrest of cell growth by p53 allows the activation of the DNA repair system of the cell. p53 is mutated in 30% of breast cancer, particularly in advanced and aggressive forms (24). Univariate and multivariate analyses showed no associations between p53 expression, OS and DFS by the TMA technique, confirming that there is conflicting data concerning this marker for some human malignancies. The bcl-2 family of proteins are important regulators of apoptosis, are widely expressed in human cancer cells and are induced in response to diverse survival signals; they are expressed at significant levels in cell lines derived from ovarian, colon and breast carcinomas (25, 26). Bcl-2 and bcl-x inhibit cell death and it was observed that bcl-x had an independent effect on prognosis, emphasizing its role as an anti-apoptotic agent. Heat shock proteins (Hsp) are thought to play important roles in the cell cycle and in various processes of carcinogenesis. Heat shock is an important apoptosis-inducing stresser which is known to cause the

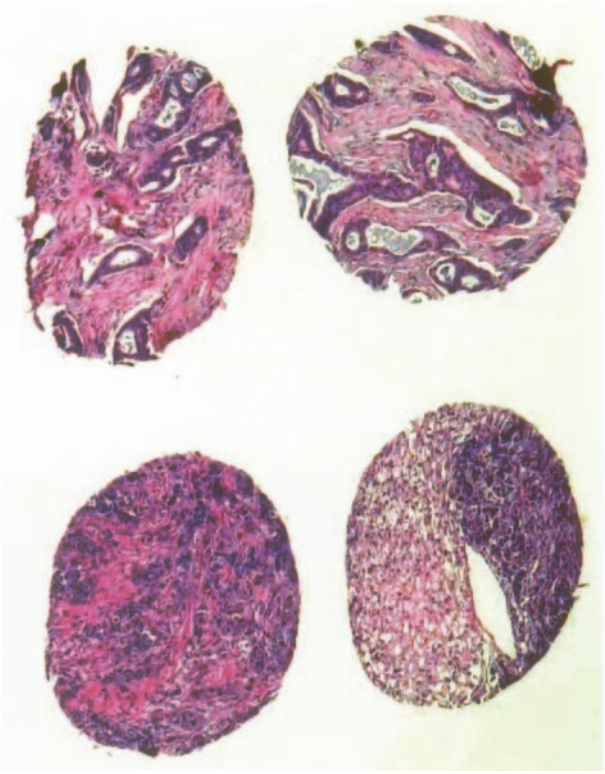

Figure 1. *Tissue microarray analysis. Four cores of breast carcinoma patients. (H-Ex20)*

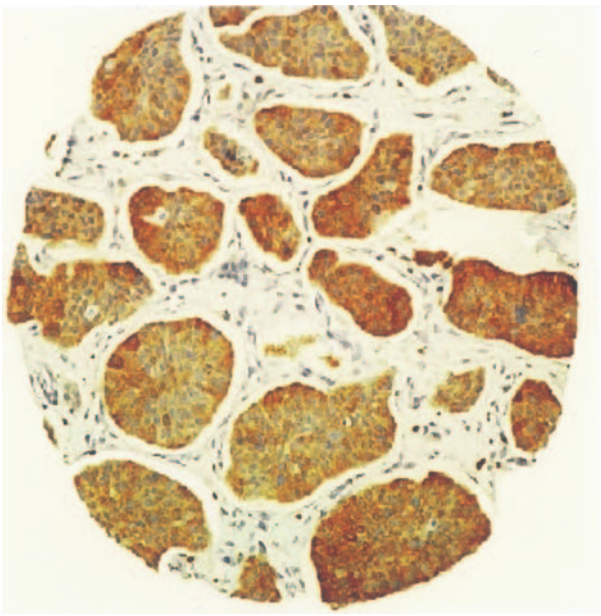

Figure 2. *Tissue microarray analysis. Single core with immunohistochemical cytoplasmic staining of human breast carcinoma by anti-fatty acid synthase (FAS). (DABx40)*

synthesis of proteins called heat shock proteins. Hps60, particularly, is an abundant protein found primarily in the mithochondria, though 15% to 20% of this protein is found in the cytosol. In humans, Hsp is overexpressed in cancer cells of the ovary, endometrium, breast, prostate and digestive tract. In some cases, overexpression of this protein may favor metastatic disease (27-29). However, no association was found between Hsp60 and prognosis in our population. The Her2-neu gene encodes an integral type I glycoprotein of 185 kDa with extracellular, transmembrane and intracellular domains. Her2-neu is a valuable prognostic marker in primary breast carcinoma, the gene being amplified and overexpressed in 20 to 25% of breast tumors. Tumors showing Her2-neu amplification have predominantly a loss of estrogen receptor expression (ER–) and are of ductal invasive type (30). Our data revealed no association between Her2-neu expression and prognosis in breast carcinomas.

Conclusion

Our study revealed that tumor stage and node status are the most powerful markers in order to predict DFS in breast carcinoma patients, while positive nodes have a major impact on the OS. Moreover, anti-apoptotic markers such as bcl-x and endogenous fatty acid synthesizing enzymes, such as FAS, may be indicators of recurrence. We suggest that TMA can easily substitute for the conventional histological-immunohistochemical technique, without significant statistical bias. TMA is a powerful technique with many advantages: it can help to expand the use of archival paraffin blocks by facilitating the construction of multiple copies of blocks sparing tissue loss; it can expand the capacity of the tissue samples, since more studies can be performed using limited samples; finally, it is time-saving and limits the costs of antibodies and reagents.

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Received March 1, 2006
Revised May 19, 2006
Accepted May 23, 2006