

## Signal Transducer and Activator of Transcription 1 in Breast Cancer: Analysis with Tissue Microarray

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**Abstract.** *Background:* Constitutively activated signal transducer and activator of transcription (STAT) proteins are found in various types of tumors. However, there is still very limited information about the role of STATs in breast cancer. The power of the tissue microarray analysis (TMA) technique is the capability of performing a series of analyses of thousands of specimens in a parallel fashion with minimal damage to the original blocks. This study was designed to use TMA in determining the STAT1 status in breast cancer tissues. *Materials and Methods:* Archival tissue specimens from 102 patients with primary invasive breast cancer were selected and STAT1 expression was analyzed using immunohistochemical staining with tissue microarray. The data of primary tumor staging, age, estrogen receptor status, lymph node status, histological grading and TNM staging were also collected. *Results:* There were 18 patients (17.6%) with 0 expression in STAT1, 29 patients (28.4%) with 1 expression in STAT1, 21 patients (20.6%) with 2 expression in STAT1 and 34 patients (33.4%) with 3 expression in STAT1. There was no significant relationship between STAT1 expression and age ( $p=0.203$ ), estrogen receptor status ( $p=0.221$ ), histological grading ( $p=0.861$ ), primary tumor staging ( $p=0.918$ ), lymph node status ( $p=0.53$ ), or TNM staging ( $p=0.826$ ). There was no survival difference noted among the four groups with different STAT1 expression ( $p=0.859$ ). *Conclusion:* Immuno-histochemical staining with tissue microarray analysis was convenient and feasible for the analysis of STAT1 expression status in breast cancer. STAT1 expression did not show significant correlation with the overall survival rate.

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Signal transducers and activators of transcription (STATs) constitute a family of latent transcription factors whose activation is dependent on tyrosine phosphorylation at a site in their C-termini (1). Tyrosine phosphorylation of STAT proteins induces dimer formation, followed by their translocation to the nucleus, where they bind DNA response elements and thereby regulate gene expression (2). It has been suggested that STAT proteins have primarily evolved to mediate cytokine signaling, particularly in cells of the immune system (3), indeed, gene knockout experiments in mice indicate a pivotal role for STAT proteins in the development and regulation of the immune system (4).

Although originally discovered as effectors of normal cytokine signaling, subsequent studies have demonstrated the participation of STATs in signaling by polypeptide growth factors and oncoproteins. Precise regulation of STAT activation is critical with regard to eliciting appropriate responses to extracellular signals. For example, through constitutive ligand/receptor engagement or oncogenic tyrosine kinase (TK) activity, aberrant STAT signaling may contribute to malignant transformation by promoting cell cycle progression and/or cell survival (5-11). Because STATs directly regulate gene expression, implicit in the constitutive activation of STATs observed during oncogenesis is the acquisition of a permanent alteration in the genetic program. Moreover, better understanding of the mechanisms underlying aberrant STAT signaling during oncogenesis may lead to the development of novel cancer therapies based on interrupting key steps in this pathway (12-17).

Constitutively activated STAT proteins are found in various types of malignancy including leukemia, prostate cancer and neck tumors (18-20). However, there is still limited information about the role of STATs in breast cancer (21-22).

Tissue microarray (TMA) analysis allows for the rapid immunohistochemical analysis of thousands of tissue samples in parallel with minimal damage to the original tissue blocks (23, 24).

This study was designed with the application of TMA to analyze STAT1 status in breast cancer tissue in the hope of elucidating the possible relationship between STAT1 expression and breast cancer.

## Materials and Methods

**Specimen selection and data collection.** Archival tissue specimens from 102 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 breast cancer, defined as carcinoma with invasion to or beyond the basement membrane regardless of histological classification (ductal or lobular) (25). The data of primary tumor staging, age, estrogen receptor status (26-31), lymph node status, histological grading and TNM staging were also collected. The hematoxylin-eosin stained slides of the paraffin-embedded tumor specimens were reviewed by our pathologists to confirm the accuracy of the histological diagnoses and lymph node status.

**Tissue microarray assembly.** Representative areas of both tumorous and non-tumorous tissue for each case were selected and circled to match the blocks for the tissue microarray. The blocks matching the circled slides were then retrieved to prepare the recipient block for the microarray. To ensure the accurate representation of the selected cores, three areas each for both tumorous and non-tumorous tissue per case were determined for assembling the recipient blocks. Each target area on the selected block was punched to form a 0.6-mm-diameter tissue core and placed consecutively on the recipient blocks of approximately 3 cm x 2 cm with a precision instrument (Beecher Instruments, Silver Spring, MD, USA) as described elsewhere (32).

**Immunohistochemical analysis.** The rabbit polyclonal antibody against human signal transducer and activator of transcription 1 (STAT1) (sc-592) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and 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 min to deprive the endogenous peroxidase activity and microwaved in 10 mM citrate buffer at pH 6.0 to unmask the epitopes. After antigen retrieval, the sections were incubated with dilute STAT1 antibody for 1 h followed by a 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 the peroxidase substrate diaminobenzidine for 5 min and counterstained with hematoxylin.

**Grading for STAT1 immunoreactivity.** For evaluating the immunoreactivity of STAT1, staining of samples was classified using a four-grade scale: 0, absence of staining in tumor cells; 1+, weak nuclear and/or cytoplasmic staining in tumor cells; 2+, an intermediate staining intensity between 1+ and 3+ in tumor cells; and 3+, strong nuclear and cytoplasmic staining in tumor cells (Figure 1).

**Patients and follow-up.** All of the patients were women aged from 26 to 76 years, 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 a detailed physical examination were usually performed at follow-up. Annual mammography or breast sonography (for the younger patients) were performed. A 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.

**Statistical analyses.** Comparisons between groups were performed using  $\chi^2$  test. For survival analyses, the end-point was overall survival. Overall survival rates were calculated using the Kaplan-Meier method and the differences were assessed with the log rank test. Statistical analyses were conducted using SPSS software (version 11.0 SPSS, Chicago, IL, USA). A *p*-value of less than 0.05 was considered statistically significant. All *p*-values were estimated from two-sided tests.

## Results

There were 18 patients (17.6%) with 0 STAT1 expression, 29 patients (28.4%) with grade 1 expression, 21 patients (20.6%) with grade 2 expression and 34 patients (33.4%) with grade 3 expression (Table I). Using  $\chi^2$  test, comparisons between groups were performed. There was no significant relationship between STAT1 expression and age, estrogen receptor status, histological grading, primary tumor staging, lymph node status or TNM staging. For survival analyses, the end-point was overall survival. The overall five-year survival rates were 77.8% (grade 0 expression), 72.3% (grade 1 expression), 66.7% (grade 2 expression) and 73.3% (3 grade expression). Survival differences were compared using the log-rank test. There was no survival difference noted among the four groups with different STAT1 expression (*p*=0.859, Figure 2).

## Discussion

Kononen *et al.* (33) recently described an array-based high-throughput technique that facilitates analysis of very large number of tumors at once, either at the DNA, RNA, or protein level. As many as 1,000 cylindrical tissue biopsy specimens from an individual tumor can be arrayed in a single tissue microarray (TMA) block. The power of the TMA technique is the ability to perform a series of analyses of thousands of specimens in a parallel fashion with minimal damage to the origin blocks (23, 24, 33). In contrast to immunohistochemical analyses on large sections, the TMA allows a high level of standardization for immunohistochemical staining because all tumor specimens are pretreated and stained under exactly the same conditions. Being different from the reading of large sections, which always is an attempt to integrate the observations in multiple different regions of

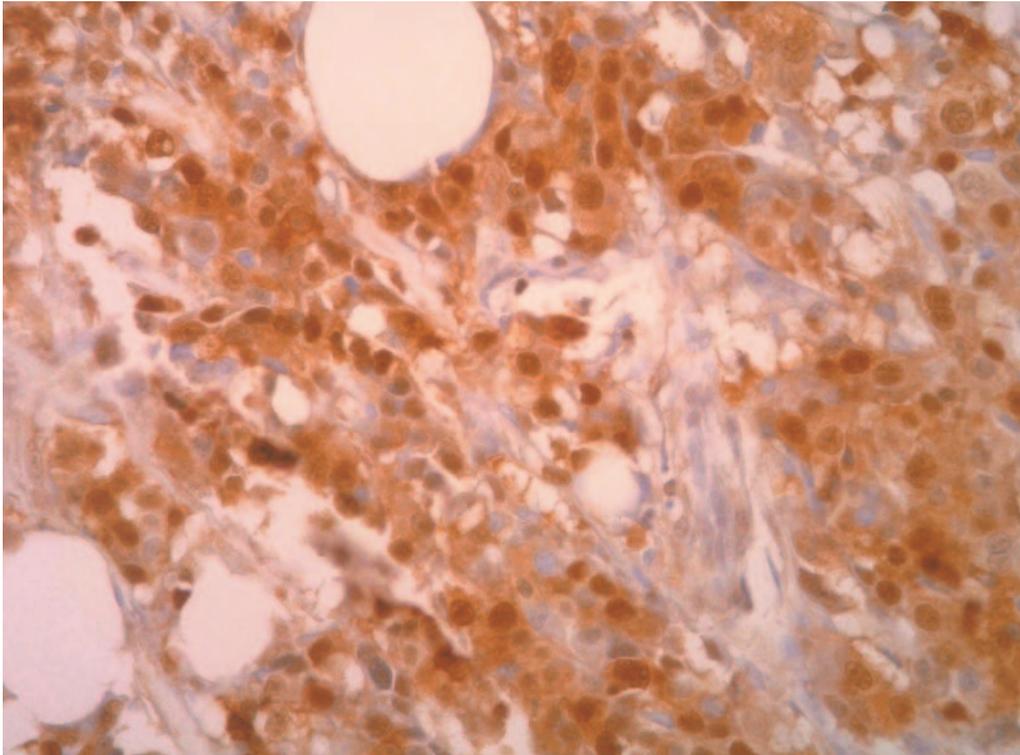


Figure 1. Immunostaining with the STAT1 antibody of the breast cancer tissue microarray slides. The representative 3+ case reveals strong nuclear and cytoplasmic immunoreactivity in the tumor cells. Original magnification, x400.

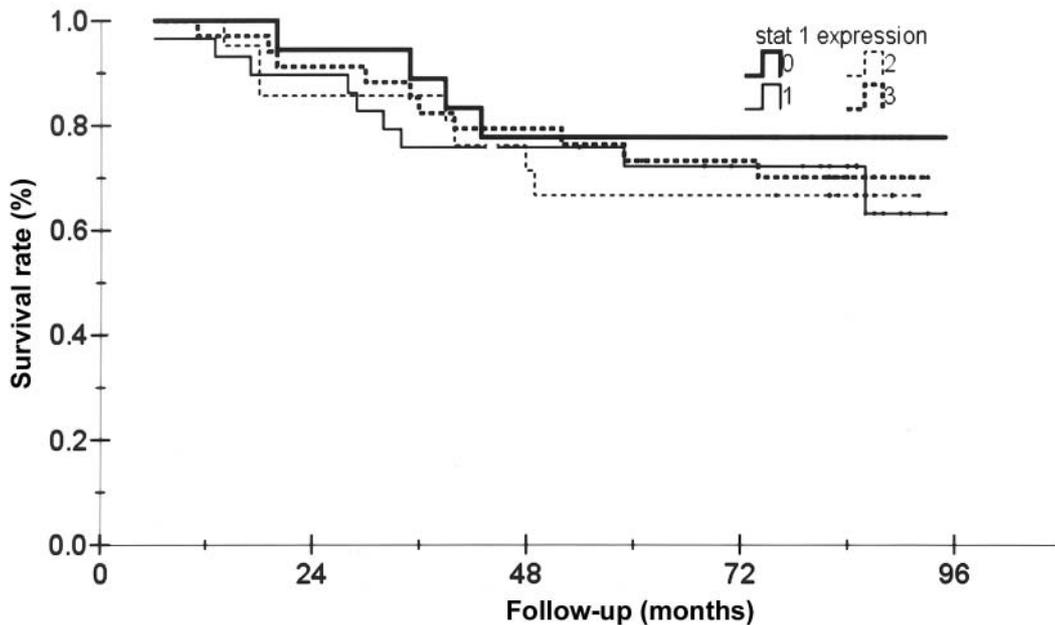


Figure 2. There was no survival difference among the four groups with different STAT1 expression ( $p=0.859$ ).

a tissue section, the morphological classification and interpretation of immunoreactivity 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 (23, 24, 33).

Table I. *STAT1* expression in relation to clinicopathologic variables.

STAT1 expression (no. of pts %)	0	1	2	3	P-value
Age (yrs)					
≤50	7 (11.48%)	19 (31.15%)	12 (19.67%)	23 (37.70%)	0.203
≥50	11 (26.83%)	10 (24.39%)	9 (21.95%)	11 (26.83%)	
Estrogen receptor status					
Negative	13 (20.63%)	21 (33.33%)	12 (19.05%)	17 (26.98%)	0.221
Positive	5 (12.82%)	8 (20.51%)	9 (23.08%)	17 (43.59%)	
Histological grading					
1	2 (15.38%)	5 (38.46%)	0 (0.00%)	6 (46.15%)	0.861
2	13 (22.03%)	10 (16.95%)	15 (25.42%)	21 (35.59%)	
3	3 (10.00%)	14 (46.67%)	6 (20.00%)	7 (23.33%)	
Primary tumor staging					
T1	3 (15.00%)	7 (35.00%)	5 (25.00%)	5 (25.00%)	0.918
T2	11 (20.75%)	14 (26.42%)	9 (16.98%)	19 (35.85%)	
T3	3 (15.00%)	5 (25.00%)	6 (30.00%)	6 (30.00%)	
T4	1 (11.11%)	3 (33.33%)	1 (11.11%)	4 (44.44%)	
Lymph node status					
N0	8 (16.67%)	12 (25.00%)	12 (25.00%)	16 (33.33%)	0.53
N1	3 (15.79%)	7 (36.84%)	1 (5.26%)	8 (42.11%)	
N2	5 (22.73%)	6 (27.27%)	3 (13.64%)	8 (36.36%)	
N3	2 (15.38%)	4 (30.77%)	5 (38.46%)	2 (15.38%)	
TNM staging					
I	1 (7.69%)	5 (38.46%)	4 (30.77%)	3 (23.08%)	0.826
II	9 (19.15%)	13 (27.66%)	8 (17.02%)	17 (36.17%)	
III	8 (19.05%)	11 (26.19%)	9 (21.43%)	14 (33.33%)	

Nevertheless, criticism 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 alterations

are not detected if the analysis of heterogenous tumors is restricted to samples measuring 0.6 mm (34). However, Moch *et al.* (23) pointed out that the TMA approach has been designed to examine tumor populations and not to survey individual tumors. They (23) analyzed the impact of tissue heterogeneity on TMA data by comparing 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 (35, 36). Our study analyzed *STAT1* expression in breast cancer using immunohistochemical staining with TMA and the results were obtained smoothly. To the best of our knowledge, this is the first report with long term follow-up regarding *STAT1* expression in breast cancer analyzed using TMA.

The *STATs* are implicated as important regulators of the development and differentiation of multicellular organism. Recent studies suggest that activated *STAT* signaling was observed in cancer and that dysregulation of these factors may participate in oncogenesis (37-39). Chin *et al.* (40) has concluded that activation of the *STAT1* signal pathway can induce apoptosis through the induction of interleukin-1 beta converting enzyme (*ICE*) gene expression and therefore *STAT1* has been suggested to serve as a tumor suppressor (41,42). However, our studies showed that there was no survival difference among the four groups with different *STAT1* expression ( $p=0.859$ , Figure 1). The real reason for this discrepancy is not fully known. Gooch *et al.* (43) reported that *IFN-gamma*-mediated growth inhibition requires *STAT1* activation and even though *IFN-gamma* inhibited proliferation of monolayer cultured MCF-7 and MDA-MB-231 breast cancer cells, *IFN-gamma* had no effect on MDA-MB-231 colony formation; they finally concluded that *STAT1* activation does not appear to be sufficient for *IFN-gamma*-mediated growth inhibition. Clinically, the breast cancer burden is probably not reflected in the monolayer status and Gooch *et al.*'s findings may provide a rational basis for our results.

**Conclusion**

Immunohistochemical staining with tissue microarray analysis was convenient and feasible for the analysis of *STAT1* expression status in breast cancer, yet its expression did not show significant correlation with the overall survival rate.

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