Significance of STAT3 activation in breast cancer

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Summary

Signal transducer and activator of transcription 3 (STAT3) is a latent transcription factor whose activation plays immense role in cell proliferation, survival, differentiation and development. Activation of STAT3 relies on phosphorylation at tyrosine residues promoting dimerization and subsequent nuclear translocation and activation of transcription. This study explores the relation between STAT3 and activated STAT3 (p-STAT3) in malignancy of breast. A high expression of STAT3 was found in breast cancer compared to adjacent normal breast tissues. A significant correlation between STAT3 and p-STAT3 was also observed. We also examined possible associations of these biomarkers with various clinicopathological parameters of breast cancer patients. The results revealed that both STAT3 and p-STAT3 are strongly associated with some of the major risk factors of breast cancer. Our findings implicate that targeting STAT3 signalling pathway could be an effective therapeutic approach for breast cancer.

Keywords : STAT3, p-STAT3, breast cancer, clinicopathological features.

Introduction

Breast cancer is the most prevalent cancer among women in India and continues to maim global health. It is a heterogeneous disease arising from multiple genetic changes in oncogenes and tumor suppressor genes with pivotal roles in the homeostatic control of mammary epithelial cell proliferation, differentiation and death. Aberrations in the expression and function of these genes leads to clonal expression with subsequent acquisition of invasive and metastatic phenotypes (Sutherland and Musgrove, 2002). Finding a successful treatment regime for breast cancer is a Sisyphean task and advocates understanding of breast cancer at the molecular level.

Transcription factors are key regulators of cell growth and differentiation and their dysfunction leads to many human diseases, including cancer (Jiang et al., 2006). Transcription factors that are gene regulator proteins are bestowed with sequence specific DNA recognition. They positively or negatively influence the rate and efficiency of transcript initiation at a gene containing the factor's cognate recognition sequence or DNA response element. In order to accomplish intricate cellular processes, STAT family of proteins serves both as cytoplasmic signal transducers and transcriptional activators controlling gene expression (Darnell Jr, 1997; Sriuranpong et al., 2003; Ivashkiv and Hu, 2004).

The Janus kinase (JAK)/ STAT pathway plays pleiotrophic roles in animal development and its signalling effects have attributed largely to direct transcription regulation by STAT of specific target genes that promote tumor cell proliferation or survival (Shi et al., 2006). Delineation of cytokine signalling pathways have defined critical and nonredundant roles of STATs (Darnell JE Jr, 1997). To date, seven STAT family members have been identified in mammals namely STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6, of which only deficiency of STAT3 leads to early embryonic lethality and is also ubiquitously expressed in most tissues (Takeda et al., 1997; Bromberg, 2000; Benekli, 2003). STAT3 is the most pleotropic member and is most strongly implicated in oncogenesis (Levy and Lee, 2002). Cytokine's engagement to its cognate receptors activates STAT3 proteins by phosphorylation. The phosphorylated STAT3 rapidly translocates to the nucleus to induce target gene transcription. Subsequently, they are inactivated by tyrosine dephosphorylation and are deported back to the cytoplasm (Bromberg, 2000; Caldera et al., 2008). Precise regulation of STAT3 activation is critical for eliciting appropriate responses to extracellular signals. However, constitutive activation of STAT3 is often observed in oncogenesis and implicit permanent alteration in genetic program. Aberrant STAT3 signalling leads to malignant transformation by promoting cell cycle progression or cell survival (Bowman et al., 2000). Towards understanding the importance of STAT3 and p-STAT3 in oncogenesis, we investigated the expression levels of both these markers at the mRNA level using Reverse Transcriptase (RT) -PCR and at the protein level using immunohistochemistry and Western blotting in both breast tumor and adjacent normal breast tissues. We also

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analysed the associations between STAT3 and p-STAT3 in breast cancer and how they correlated with the clinicopathological features of breast cancer patients.

Materials and Methods

Study subjects and sample collection

A total of 70 primary breast tumor samples and 20 adjacent normal breast tissues were collected from breast cancer patients undergoing mastectomy at the Surgical Oncology Department of Regional Cancer Centre, Thiruvananthapuram. The clinicopathological features of breast cancer patients included in this study are summarized in table1. Patient's consent was obtained and the study was approved by the Institute Review Board and Ethical Committee.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from breast tumor samples and adjacent normal breast samples using TRI Reagent (Sigma, USA) following manufacturer's protocol and was immediately quantified by spectrophotometry (A_{260}/A_{280} ratio). 2 µg of total RNA was reverse transcribed to cDNA at 42ºC for 1 hour in a 25 µL reaction mix containing 200 U of M-MLV Reverse Transcriptase in 1x reaction buffer with 1µg of Random Primers; 8 U of RNase inhibitor and 2 mM of dNTP mix. PCR was performed using specific primers (Integrated DNA Technologies, USA) for human STAT3 (sense, 5'- TTG CCA GTT GTG GTG ATC-3'; and antisense, 5'- AGA ACC CAG AAG GAG AAG-3') and GAPDH (sense, 5'- ACC ACA GTC CAT GCC ATC AC-3'; and antisense, 5'- TCC ACC ACC CTG TTG CTG TAG-3'). For the amplification of specific genes, 2.5 µL of cDNA was used. The PCR amplification was done in a 20 µL reaction mix containing 0.2 µL Taq DNA Polymerase in 5x reaction buffer containing 1.2 µL dNTP mix and 0.85 µM primers of STAT3. 1 µM of GAPDH primers served as internal control. The thermocycling protocol was as follows: 94ºC for 3 minutes (Initial Denaturation), 35 cycles of 94ºC for 30 seconds (Denaturation), 55ºC for 40 s (Annealing, STAT3) and 59ºC for 40 s (Annealing, GAPDH), 72ºC for 90 s (Extension) followed by a final extension at 72ºC for 7 min. Amplification products were resolved on 1.2% agarose gel containing ethidium bromide.

Immunohistochemical analysis and evaluation

Sections (4 µm thick) of formalin-fixed, paraffin-embedded breast tumor and adjacent normal breast samples were deparaffinised in xylene and rehydrated in graded alcohol. Endogenous peroxidase activity was blocked using 1.5% H_2O_2 for 30 minutes. These sections were then subjected to antigen retrieval by boiling in 10mM citrate buffer (pH 6.0) for 15 minutes. The slides were then cooled and nonspecific binding sites were blocked by incubating with 3% bovine serum albumin (BSA) for 20 min in a humidified chamber at room temperature. The sections were incubated at 4ºC overnight with STAT3, diluted 1:800 (Cell Signaling Technology, USA) and p-STAT3, diluted 1:500 (Cell Signaling Technology, USA) primary antibodies. The bound primary antibody was detected by addition of secondary antibody conjugated with horseradish peroxidase polymer and DAB substrate using Super-sensitive polymer HRP detection system (Biogenex, USA). The sections were then counterstained with haematoxylin. Immunostaining of both nuclear and cytoplasm were evaluated. A nuclear score of 0 was considered negative, samples that stained for nucleus between 1 and 25% were designated as +, 25-60% as ++ and > 60% as +++ while cytoplasmic staining was designated as weak, moderate and strong based on the intensity of staining. Prior to immunohistochemistry (IHC) both the breast tumor and adjacent normal breast tissue sections were subjected to Haematoxylin and Eosin (H&E) staining to confirm tumor and normal samples and were reconfirmed by a Pathologist.

Protein extraction and Western blot analysis

Adjacent normal breast samples and tumor breast tissues were lysed using RIPA (Radio Immuno Precipitation Assay) buffer (50 mM Tris HCl, 150 mm NaCl, 1 mM EDTA, 1% Triton-X 100, 1% sodium deoxy cholate, 0.1% sodium dodecyl sulphate (SDS), 0.1 M PMSF, 1 M sodium orthovanadate and protease inhibitor cocktail obtained from Sigma, USA). After centrifugation at 15,000 xg for 20 min at 4ºC, the protein concentrations in tissue extracts were determined by Bradford assay. For Western blot analysis, tissue extracts were mixed with 2x Laemlli buffer at 1:1 ratio and were boiled for 5 min at 95ºC. 60-80 µg of protein were separated by electrophoresis on a 6-10% SDS-polyacrylamide gel using mini-PROTEAN3 cell (Biorad, Hercules, CA). The separated proteins were electrophoretically transferred on to polyvinylidinene difluoride (PVDF) membrane (Millipore Corp., MA) for 2 h at 80 V and blocked with 5% nonfat milk in Tris-buffered saline solution (10 mM Tris, pH 7.4; 150 mM NaCl) containing 0.05% tween-20 (TBST). After blocking, membranes were incubated at 4ºC overnight with STAT3 and p-STAT3 (Santa Cruz Biotechnology) primary antibodies in TBST containing 5%

BSA. The expression of β-actin was used as normalization control for protein loading. The membranes were then washed with TBST and were incubated with corresponding alkaline-phosphatase conjugated secondary antibodies (Sigma, USA). Subsequently, membranes were washed and immune complexes were detected using 5 Bromo 4-Chloro-3-Indoyl Phosphate (BCIP)/Nitro Blue Tetrazolium (NBT) (Sigma, USA) in alkaline phosphatase buffer. The specific proteins were detected with reference to prestained molecular weight markers (Sigma, USA).

Statistical Analysis

Statistical analyses were performed using SPSS statistical software. Associations between STAT3 and p-STAT3 and their correlations with patient's clinicopathological features were studied using Spearman's correlation. $P < 0.05$ was considered statistically significant.

Results

Expression of STAT3 at the mRNA level in breast tumor tissues and normal breast tissues

70 breast tumor samples and 20 normal breast samples were subjected to RT-PCR to analyze the mRNA expression of STAT3. A high expression of STAT3 mRNA (99%) was observed in breast tumor tissues while only 80% STAT3 mRNA expression was observed in adjacent normal breast tissues (Fig. 1). Concomitantly, the band intensity of STAT3 mRNA in breast tumor samples was higher compared to that of normal breast tissues (Fig. 2). The results, therefore, revealed a robust increase in the expression of STAT3 mRNA in breast tumor samples when compared to the adjacent normal breast tissues.

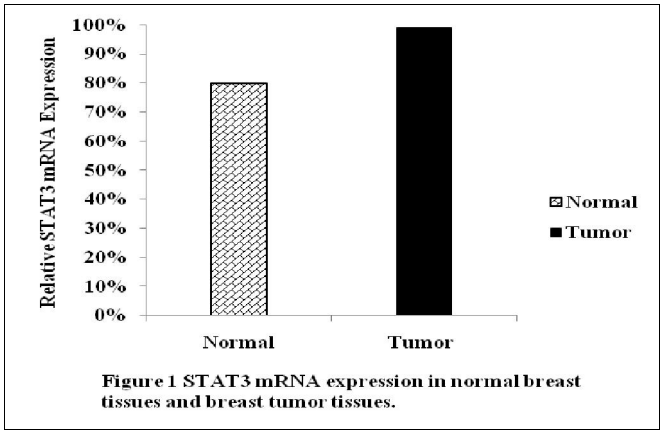

Figure 1 STAT3 mRNA expression in normal breast tissues and breast tumor tissues.

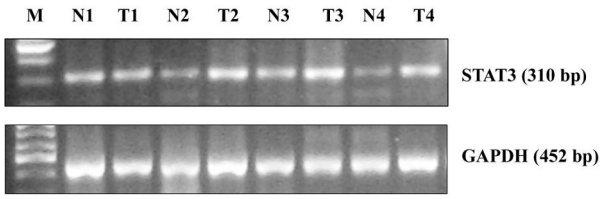

Figure 2 Representative RT-PCR analysis of STAT3 and GAPDH in adjacent normal (N) breast samples and corresponding breast tumor (T) tissues. Lane 1: 100 bp ladder; lanes 2, 4, 6, 8 : STAT3 and GAPDH mRNA expression in normal breast samples; lanes 3, 5, 7, 9: STAT3 and GAPDH mRNA expression in corresponding breast tumor samples.

Immunohistochemical and immunoblot analysis of STAT3 and p-STAT3 in normal and tumor breast tissues

60 breast tumor samples and 13 adjacent normal breast tissues were subjected to both immunohistochemical and immunoblot analysis. The rest of the samples had only adipose and connective tissues. Among the representative breast tumor samples, 97% expressed STAT3 while only 52% expressed p-STAT3. The low expression of p-STAT3 is also attributed to its transient expression and requires snap frozen samples. In this study, both nuclear and cytoplasmic staining was considered for STAT3 and only nuclear staining for p-STAT3. 87% of the tumor samples showed STAT3 nuclear staining and 97% expressed cytoplasmic STAT3 while 85% of breast tumor samples expressed both cytoplasmic and nuclear STAT3. In adjacent normal breast samples, only 31% expressed nuclear p-STAT3. Immunohistochemical nuclear localization of STAT3 was observed in 61% of adjacent normal breast tissues while only 54% of normal breast samples demonstrated cytoplasmic STAT3. Both nuclear and cytoplasmic STAT3 expression was discernible only in 46% of adjacent normal breast samples (Fig. 3, 4). Similarly, immunoblot analysis also revealed a high expression of STAT3 and p-STAT3 in breast tumor samples compared to adjacent normal breast samples (Fig. 5). The results of both immunohistochemical analysis and Western blotting correlated with each other.

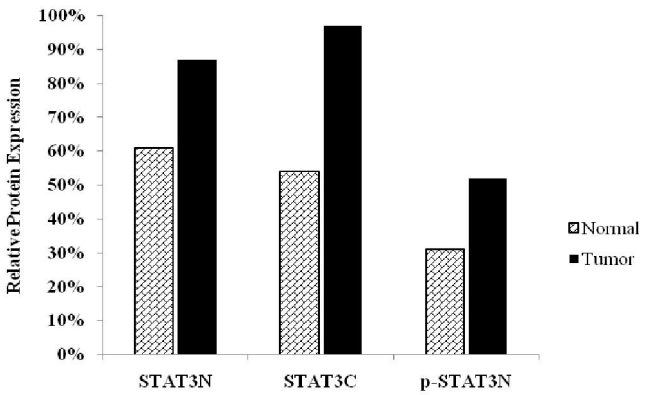

Figure 3 Immunohistochemical localization of cytoplasmic and nuclear expression of STAT3 and p-STAT3 in normal breast tissues and breast tumor tissues.

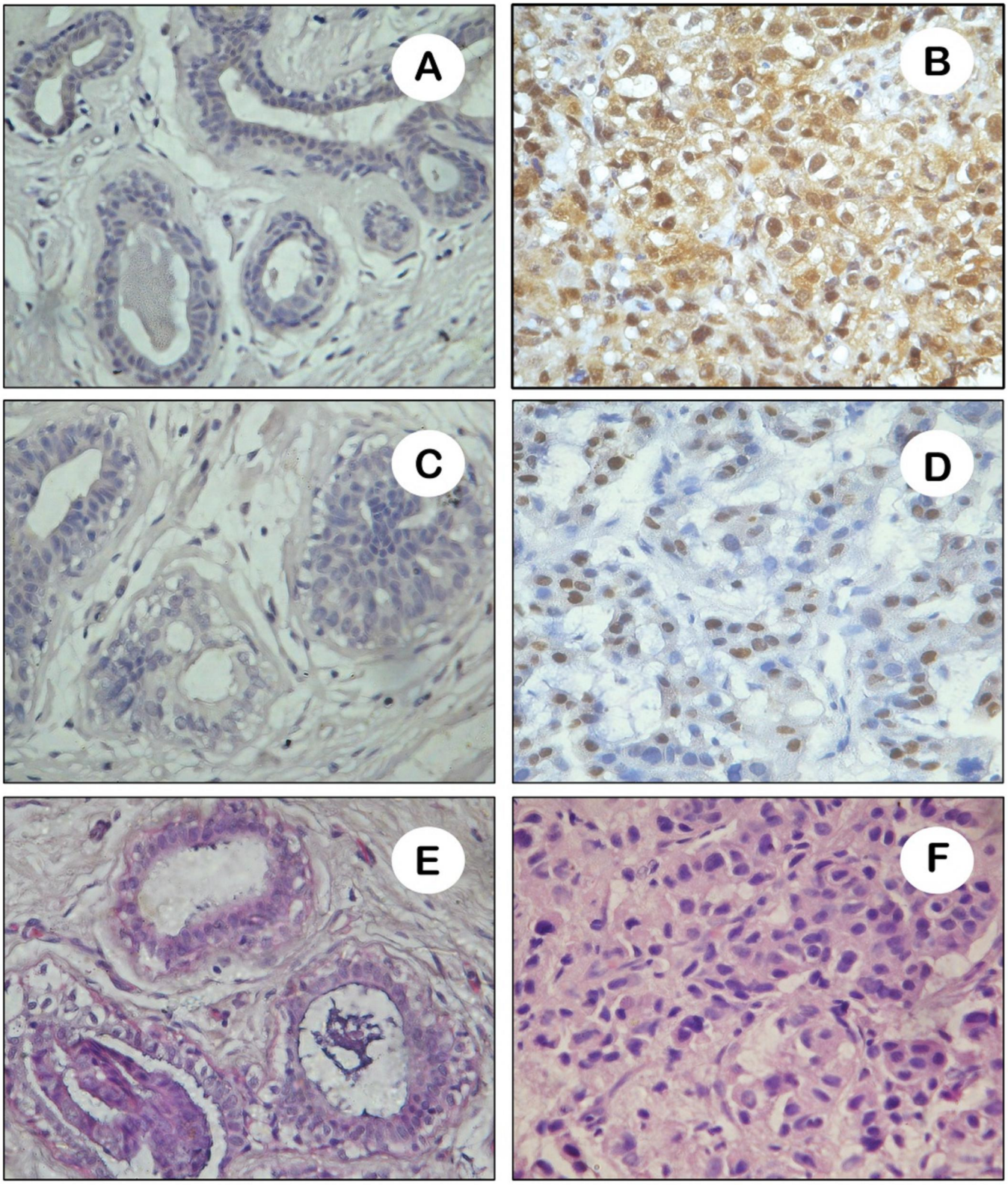

Fig. 4. Immunohistochemical localization of STAT3 (A & B) and p-STAT3 (C & D) in normal breast tissues (A & C) and breast tumor tissues (B & D). H & E staining in representative normal breast tissues (E) and breast tumor tissues (F). Original magnification of figures are 400 x

Table 1: Clinicopathological features of breast cancer

Clinicopathological parameters (n = 70)		**Numbers (%)**
Patient's age (years)	≤ 50	40 (57)
	> 50	30 (43)
Menopausal status	Premenopausal	32 (46)
	Postmenopausal	38 (54)
Parity	Nulliparous	1 (1)
	Mono/multiparous	69 (99)
Family history of cancer	Present	19 (27)
	Absent	51 (73)
Lymph node metastasis	Negative	33 (47)
	1-3 nodes	16 (23)
	4-9 nodes	13 (19)
	≥ 10 nodes	8 (11)
Histology grade	IDC grade I	1 (1)
	IDC grade II	14 (20)
	IDC grade III	55 (79)
Tumor size	pT1 < 2 cm	10 (14)
	pT2 = 2-5 cm	49 (70)
	pT3/4 > 5 cm	11 (16)

IDC – Infiltrating Ductal Carcinoma

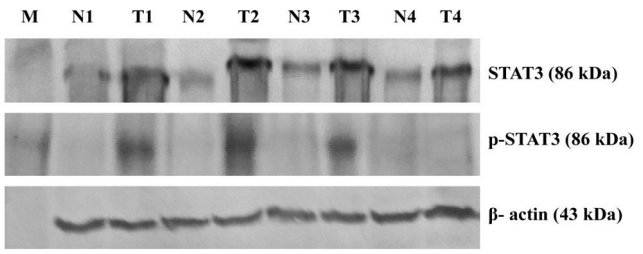

Figure 5 Representative immunoblot analysis of STAT3 and p-STAT3 in adjacent normal (N) breast tissues and corresponding breast tumor (T) tissues. Lane 1: Protein marker; lanes 2, 4, 6, 8: expression of STAT3 and p-STAT3 proteins in normal breast tissues; lanes 3, 5, 7, 9 : expression of STAT3 and p-STAT3 proteins in the corresponding breast tumor samples. β-actin represents equal protein loading control.

Associations between STAT3 and p-STAT3 proteins in breast cancer

This study revealed a significant correlation between STAT3 and p-STAT3 (r = 0.588, P = 0.000). Nuclear expression of STAT3 strongly associated with cytoplasmic expression of STAT3 (r = 0.417, P = 0.001). Further, the cytoplasmic expression of STAT3 significantly correlated with nuclear expression of p-STAT3 (r = 0.396, P = 0.002).

Clinicopathological significance of STAT3 and p-STAT3 in breast cancer

At the protein level, STAT3 significantly associated with breast cancer patient's clinicopathological features such as patient's age (r = 0.221, P = 0,090), menopausal status (r = 0.446, P = 0.000), parity (r = 0.011, P = 0.933), tumor histology grade (r = 0.511, P = 0.000), metastatic lymph nodes (r = 0.738, P = 0.000) and tumor size (r = 0.711, P = 0.000). However, p-STAT3 protein demonstrated strong associations to only patient's age (r = 0.107, P = 0.367), parity (r = 0.024, P = 0.838), metastatic lymph nodes (r = 0.325, P = 0.011) and histology grade (r = 0.008, P = 0.946). At the mRNA level, no significant associations were observed between STAT3 and patient's clinicopathological features. Nevertheless, STAT3 mRNA expression positively correlated with patient's age, parity and tumor histology grade.

Discussion

The JAK/STAT pathway is one of the pleiotropic cascades known to transduce a multitude of signals for

development and homeostasis. It is the principal signalling mechanism for a wide array of cytokines and growth factors (Rawlings et al., 2004). STAT3 is one of the members of STAT family and is known to transcriptionally activate numerous important cellular genes involved in growth regulation, differentiation and cell cycle (Hirano et al., 2000). In this study, we investigated the expression of STAT3 and p-STAT3 in breast cancer tissues and in adjacent normal breast tissues both at the mRNA and protein levels. We used immunohistochemistry and Western blot methods to assess STAT3 expression and its phosphorylation status at the protein level and RT-PCR at the mRNA level. Another objective of this study was to find out whether STAT3 and p-STAT3 correlated with the clinicopathological features of breast cancer patients which are also the major risk factors for developing breast cancer. Our results certainly implies that STAT3 is constitutively activated in breast cancer and its strong association with some of the clinicopathological parameters of breast cancer patients may bestow it with an important role in development of breast cancer.

In the present study, we found an extremely robust increase in the expression level of STAT3 with 99% of the breast cancer tissues expressing STAT3 at the mRNA level and 97% at the protein level. In contrast, the adjacent tumor free normal breast tissues expressed only 80% STAT3 and the positive ones had a relatively low intensity PCR bands compared to those from the breast cancer samples. At the protein level, 97% of the breast cancer tissues expressed STAT3 while only 46% expression of STAT3 was discernible in adjacent normal breast tissues. Further, p-STAT3 protein expression was 52% in breast cancer tissues while only 31% of adjacent normal breast tissues expressed p-STAT3. These results indicate that STAT3 and p-STAT3 is likely to contribute to breast tumorigenesis and may be considered as a relevant biomarker for breast cancer. The constitutive activation of STAT3 is found to be required for the transformed phenotype of tumor-derived breast cancer cell lines (Bromberg, 2002). Inhibition of constitutively activated STAT3 can induce apoptosis and cancer cell growth indicating constitutive STAT3 signalling is required for cancer cell growth and survival (Buettner, 2002). We also investigated the nuclear expression of STAT3 and p-STAT3 proteins and the cytoplasmic expression of STAT3 proteins in both breast tumor and adjacent normal breast tissues. An increased cytoplasmic expression of STAT3 (97%) followed by 87% expression of nuclear STAT3 was found in breast tumor tissues while 85% of the breast tumor samples expressed both cytoplasmic and nuclear localization of STAT3 protein. Whereas in normal breast tissues, 61% of samples demonstrated nuclear localization of STAT3, 54% cytoplasmic expression of STAT3 and 46% of normal breast tissues revealed both cytoplasmic and nuclear expression of STAT3. Immunoblot analysis also showed a high expression of STAT3 and p-STAT3 in breast tumor tissues compared to adjacent normal breast tissues. Our data indicates that studying the expression levels of STAT3 and p-STAT3 in breast cancer may help in understanding better the cancer progression. The expression of STAT3 and its phosphorylated forms was found to increase as a function of severity of the cervical lesions from precancer to cancer stages (Shukla, 2010). Consistent with this, our correlation analysis also revealed a strong positive association between nuclear and cytoplasmic expression of STAT3 with p-STAT3 proteins. The requirement of STAT3 during cell transformation suggests that STAT3 possess a transformation specific function, consistent with its postulated role as an oncogene (Schlessinger and Levy, 2005).

Another potentially important aspect of this work relates to investigating the clinicopathological significance of STAT3 and p-STAT3 in breast cancer. Our data reveals a strong association of STAT3 proteins with almost all the clinicopathological features of breast cancer patients included in this study. Notably, STAT3 proteins significantly correlated with breast cancer patient's age, menopausal status, lymph node metastasis, parity, tumor histology grade and tumor size whereas p-STAT3 demonstrated strong positive associations with breast cancer patient's age, parity, metastatic lymph nodes and histology grade of tumor. Increased levels of p-STAT3 significantly correlated with the existence of nodal metastasis and its stage in colorectal carcinoma (Ma et al., 2004). The expression of p-STAT3 was statistically indicative of a poor prognosis for overall survival and disease-free survival in patients with cervical squamous-cell carcinoma (Takemoto et al., 2009). In head and neck small cell carcinoma (HNSCC),

elevated levels of activated form of STAT3 had a significant association with the clinical stage of HNSCC (Masuda et al., 2002). It was demonstrated that STAT3 DNA binding activity correlated with malignant potential in both human prostate cancer cell lines and a large series of rat Dunning prostate cancer cell lines. The most aggressive cell lines were found to have the highest STAT3 DNA binding activities (Ni et al., 2002). However in this study, no significant associations were found between STAT3 and patient's clinicopathological parameters at the mRNA level. Our data reveals that since STAT3 and p-STAT3 proteins strongly correlate with the clinicopathological features of breast cancer patients, enhanced expression of these two proteins implicate poor prognosis in breast cancer. Our results, therefore, open perspectives for understanding the interplay between STAT3 and p-STAT3 and their importance in breast cancer. We speculate that inhibition or downregulation of STAT3 could be of therapeutic relevance to breast cancer treatment.

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References

Benekli M, Baer MR, Baumann H, Wetzler M (2003) Signal transducer and activator of transcription proteins in leukemias. *Blood* **101**:2940-2954.

Bowman T, Garcia R, Turkson J, Jove R (2000) STATs in Oncogenesis. *Oncogene* **19**:2474-2478.

Bromberg J (2000) Signal transducers and activators of transcription as regulators of growth, apoptosis and breast development. *Breast Cancer Res*. **2**:86-90.

Bromberg J (2002) Stat proteins and oncogenesis. *J Clin Invest*. **109**:1139-1142.

Buettner R, Mora L, Jove R (2002) Activated STAT signalling in human tumours provides a novel molecular targets for therapeutic intervention. *Clin Cancer Res*. **8**:945-954.

Caldera V, Mellai M, Annovazzi L, Valente G, Tessitore L, Schiffer D (2008) Stat3 expression and its correlation with proliferation and apoptosis/autophagy in gliomas. *J Oncol*. **2008**:219-241.

Darnell JE Jr (1997) STATs and gene regulation. *Science* **277**:1630-1635.

Hirano T, Ishihara K, Hibi M (2000) Roles of STAT3 in mediating the cell growth, differentiation and survival signals relayed through the IL-6 family of cytokine receptors. *Oncogene* **19**:2548-2556.

Ivashkiv LB, Hu X (2004) Signaling by STAT3. *Arthritis Res Ther*. **6**:159-168.

Jiang X, Roth L, Lai C, Li X (2006) Profiling activities of transcription factors in breast cancer cell lines. *Assay and Drug Development Technologies* **4**:293-305.

Levy DE, Lee CK (2002) What does stat3 do? *J Clin Invest.* **109**:1143-1148.

Ma XT, Wang S, Ye YJ, Du RY, Cui ZR, Somsouk M (2004) Constitutive activation of Stat3 signaling pathway in human colorectal carcinoma. *World J Gastroenterol*. **10**:1569-1573.

Masuda M, Suzui M, Yasumatu R, Nakashima T, Kuratomi Y, Azuma K, Tomita K, Komiyama S, Weinstein IB (2002) Constitutive activation of signal transducers and activators of transcription 3 correlates with cyclin D1 overexpression and may provide a novel prognostic marker in head and neck squamous cell carcinoma. *Cancer Res*. **62**:3351-3355.

Ni Z, Lou W, Lee SO, Dhir R, DeMiguel F, Grandis JR, Gao AC (2002) Selective activation of members of the signal transducers and activators of transcription family in prostate carcinoma. *J Urol*. **167**:1859-1862.

Rawlings JS, Rosler KM, Harrison DA (2004) The JAK/STAT signalling pathway. *J Cell Sci*. **117**:1281-1283.

Schlessinger K, Levy DE (2005) Malignant transformation but not normal cell growth depends on signal transducer and activator of transcription 3. *Cancer Res*. **65**:5828-5834.

Shi S, Calhoun HC, Xia F, Li J, Le L, Li WX (2006) JAK signalling globally counteracts heterochromatic gene silencing. *Nature Genetics* **38**:1071-1076.

Shukla S, Shishodia G, Mahata S, Hedau S, Pandey A, Bhambhani S, Batra S, Basir SF, Das BV, Bharti AC (2010) Aberrant expression and constitutive activation of STAT3 in cervical carcinogenesis: implications in high- risk human papillomavirus infection. *Mol Cancer* **9**:282-298.

Sriuranpong V, Park JI, Amornphimoltham P, Patel V, Nelkin BD, Gotkind JS (2003) Epidermal growth factor receptor-independent constitutive activation of STAT3 in head and neck squamous cell carcinoma is mediated by the autocrine/paracrine stimulation of the interleukin 6/gp 130 cytokine system. *Cancer Res*. **63**:2948-2956.

Sutherland RL, Musgrove EA (2002) Cyclin D1 and mammary carcinoma: new insights from transgenic mouse models. *Breast Cancer Res*. **4**:14-17.

Takeda K, Noguchi K, Shi W, Tanaka T, Matsumoto M, Yoshida N, Kishimoto T, Akira S (2000) Targeted disruption of the mouse stat3 gene leads to early embryonic lethality. *Proc Natl Acad Sci USA*. **94**:3801-3804.

Takemoto S, Ushijima K, Kawano K, Yamaguchi T, Terada A, Fujiyoshi N, Nishio S, Tsuda N, Ijichi M, Kakuma T, Kage M, Hori D, Kamura T (2009) Expression of activated signal transducer and activator of transcription-3 predicts poor prognosis in cervical squamous-cell carcinoma. *Br J Cancer* **101**:967-972.