

SELECTIVE ACTIVATION OF MEMBERS OF THE SIGNAL TRANSDUCERS AND ACTIVATORS OF TRANSCRIPTION FAMILY IN PROSTATE CARCINOMA

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ABSTRACT

Purpose: Cytokines, hormones and growth factors use signal transducers and activators of transcription (STAT) signaling pathways to control various biological responses, including development, differentiation, cell proliferation and survival. Constitutive activation of STATs has been found in a wide variety of human tumors. In this study we examined the activity of STATs in primary human prostate tissues.

Materials and Methods: STAT activity was determined in 104 human primary prostate tissues, including 42 tumors, 42 matched normal prostates adjacent to tumors and 20 normal prostates from donors without cancer by electromobility shift assay.

Results: Significant levels of activated Stat4 and Stat6 were detected in primary prostate tissues. However, little or no expression of active Stat1, Stat2 or Stat5 was detected in primary prostate tissues. Significantly higher levels of constitutive Stat6 activity were found in prostate carcinomas compared with levels in normal tissue adjacent to tumors and normal prostates from donors without prostate cancer. There was no significant difference in Stat6 activity in normal prostate tissues adjacent to tumors and normal prostates from donors without prostate cancer. The levels of Stat4 activity varied but failed to yield statistically significant differences among tumors, matched normal prostates adjacent to tumors and normal prostates from donors without cancer.

Conclusions: We have previously shown that Stat3 is activated in prostate cancer. The results of the current study demonstrate that in addition to Stat3, Stat6 is selectively activated in prostate cancer.

KEY WORDS: prostate; transcription factors; signal transduction; prostatic neoplasms; tumor markers, biological

Signal transducers and activators of transcription (STAT) are latent transcription factors that are activated by phosphorylation. Activated STATs dissociate from the receptor and form homodimers and heterodimers. These dimers translocate to the nucleus and bind to cognate DNA response elements, thus, activating target gene transcription.^{1,2} STAT proteins comprise a family of several known transcription factors, including Stat1 to Stat6.^{1,2} Select STATs can be activated by a number of different cytokines and growth factors. It is known that many cytokines, hormones and growth factors use STAT signaling pathways to regulate various biological responses, including development, differentiation, cell proliferation and survival.^{3,4} Constitutive activation of STATs has been found in a wide variety of human tumors, including leukemias-lymphomas, breast cancer, head and neck cancer, and prostate cancer,^{5–9} suggesting that activated STATs may participate in the development and progression of many types of human cancer.

Stat3 has been shown to mediate interleukin (IL)-6 induced signals in prostate cancer cells.^{10–12} Activated Stat3 induces the androgen independent activation of androgen receptor.¹³ Constitutively active Stat3 has been previously demonstrated in prostate cancer cells.⁸ We previously reported that cells derived from rat and human prostate can-

cers have constitutively activated Stat3 and Stat3 activation is associated with malignant potential.⁸ In addition, blockade of activated Stat3 by ectopic expression of dominant negative Stat3 in human prostate cancer cells that express constitutively activated Stat3 significantly suppresses their growth in vitro and in vivo.⁸ More recent studies showed Stat3 activation in primary human prostate cancer.^{9,14} These results suggest that activation of Stat3 signaling is critical for the growth of prostate cancer cells.

In addition to Stat3, other members of the STAT family proteins have also been found to be activated in human cancers including breast, lymphomas, leukemias, and lungs.⁴ In the current study we examined the levels of activity of the STATs in human primary prostate cancer tissues. Our results demonstrate that in addition to Stat3, Stat6 is selectively activated in prostate cancer.

MATERIALS AND METHODS

Patients and tissue samples. Tissues were obtained from patients undergoing prostatectomy at our institution. Prostatectomy specimens were obtained from radical prostatectomy performed for prostate cancer or donor cystoprostatectomy performed to obtain prostate tissues from tissue donors. Specimen consisted almost purely of cancer. Evaluation of tumor content was done before these samples were used for Stat3 analysis. Tumor content was evaluated by frozen section. Specimens were used for STAT analysis only if tumor constituted 80% or more of the tissue specimen. Inflammation

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tion was not a prominent feature in the samples analyzed. There were occasional inflammatory cells, predominantly lymphocytes. However, neither donor, tumor nor adjacent normal samples had prominent inflammation. We evaluated 84 prostatectomy specimens, including 42 primary prostate tumors and 42 matched normal prostates adjacent to tumors. In addition, samples from 20 normal prostates from organ donors without prostate pathology served as controls. The table 1 lists the characteristics of the tissue samples. The same tissue samples were previously used for Stat3 determination.¹⁴

Electromobility shift and supershift assays. Whole cell extracts were prepared by homogenizing tissues in high salt buffer composed of 20 mM. HEPES, pH 7.9, 20 mM. NaF, 1 mM. Na₃P₂O₇, 1 mM. Na₃VO₄, 1 mM. ethylenediaminetetraacetic acid 1 mM. egtazic acid 1 mM. dithiothreitol, 0.5 mM. phenylmethylsulfonyl fluoride, 420 mM. NaCl, 20% glycerol, 1 μg./ml. leupeptin and 1 μg./ml. aprotinin, followed by snap-freezing in ethanol-dry ice for 5 minutes and thawing on ice for 10 minutes. Freeze and thaw procedures were repeated again for a total of 2 times. The supernatant was then centrifuged and harvested. Protein concentrations were determined using a Coomassie plus protein assay kit (Pierce, Rockford, Illinois) according to the manufacturer protocol. Whole cell extracts (20 μg.) were incubated in a final volume of 20 μl. of 10 mM. HEPES, pH 7.9, 80 mM. NaCl, 10% glycerol, 1 mM. dithiothreitol, 1 mM. ethylenediaminetetraacetic acid and 100 μg./ml. poly(dI-dC) by electromobility shift assay for 20 minutes at room temperature with appropriate radiolabeled double strand STAT consensus binding motif (table 2). For supershift analyses cell extracts were pre-incubated with appropriate antibodies specifically against Stat4 and Stat6 (Santa Cruz Biotechnologies, Santa Cruz, California). Protein-DNA complexes were resolved on 4.5% nondenaturing polyacrylamide gel containing 2.5% glycerol in 0.25 × tris-borate edetic acid buffer at room temperature and the results were autoradiographed.

Western blot analysis. Proteins from whole cell extracts were resolved in 6% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins were then transferred to nitrocellulose membrane. After blocking overnight at 4C in 5% milk in phosphate buffered saline-0.1% Tween 20 membranes were incubated for 1 hour at room temperature with antibody specifically against phosphorylated Stat3 (Signal Technology, Beverly, Massachusetts), diluted 1:1,000 in 1% milk in phosphate buffered saline-Tween. After secondary antibody incubation immunoreactive proteins were visualized with an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom).

Statistical analysis. The significance of the differences in STAT activity detected in tumors, matched normal tissues adjacent to tumors and normal prostates from donors without prostate cancer was evaluated by the t test. Correlation of the levels of STAT activity with Gleason scores and serum prostate specific antigen (PSA) levels was evaluated with regression analysis with p <0.05 considered significant. The StatView statistical program (SAS Institute, Cary, North Carolina) was used for the statistical analysis.

TABLE 1. Characteristics of specimens

Characteristic	Donor	Matched Normal	Matched Tumor
No. pts.	20	42	42
Mean age (range)	41 (20-59)	63 (51-73)	63 (51-73)
Gleason score:	Not applicable		
4		1	1
5-6		17	17
7		7	7
8-9		17	17
Mean ng./ml. pretreatment PSA (range)	Not applicable	7.1 (2.7-16)	7.1 (2.7-16)

TABLE 2. STAT protein oligonucleotide binding motifs

STAT Protein	Sequence
Stat1	GATCTTCAGTTTCATATTACTCTAAATCCAGGATC
Stat2	GATCTTTACAAACAGCAGGAAATA-GAACTTAAGAGAAATACAGATC
Stat4	GATCCTAGAGCCTGATTTCCCGAAATGATGAGCTAGGATC
Stat5	GATCAGATTTCTAGGAATTCAAATCGATC
Stat6	GATCGTATTTCAGAAAAGGAACGATC

RESULTS

To determine the levels of STAT-DNA binding activity 84 specimens from 42 matched primary prostate specimens were evaluated. Samples analyzed consisted of 1 each from an area of prostatic adenocarcinoma and "normal adjacent to tumors" from patients with different Gleason grades of disease. In addition, samples from 20 normal prostates from organ donors without prostate pathology were assessed for activation of each member of the STAT family by electromobility shift assay.

Stat6 was significantly activated in prostate carcinoma. Figure 1, A shows a representative electromobility shift assay analysis of Stat6 activity. DU145 human prostate cancer cells served as the positive control. Quantitation of the amount of Stat6 DNA binding activity in the protein-DNA band shift was measured using PhosphorImager software (Molecular Dynamics, Sunnyvale, California). A positive control lane with DU145 cell protein incubated with the Stat6 probe was routinely run on each gel. PhosphorImager values per sample was normalized to the level in the DU145 cells run on each gel. Supershift analysis using anti-Stat6 antibody pre-incubated with cell extracts verified that the complex contained Stat6 (fig. 1, B).

The levels of Stat6 protein expression were also determined by Western blot analysis using antibody specific against phosphorylated Stat6. Figure 1, A shows phosphorylated Stat6 expression in representative samples. Generally the levels of phosphorylated Stat6 protein expression corresponded to the levels of Stat6 DNA binding activity detected by electromobility shift assay.

The average relative Stat6 activity was 0.72 in the 42 examined primary prostate tumor, 0.47 in the 42 matched normal prostates adjacent to tumors and 0.32 in the 20 normal prostates from donors (fig. 2). Stat6 activity in tumors was significantly higher than activated Stat6 in matched normal tissues adjacent to tumors (p <0.05) and in normal prostates from donors (p <0.01). However, there was no statistically significant difference in Stat6 activity in adja-

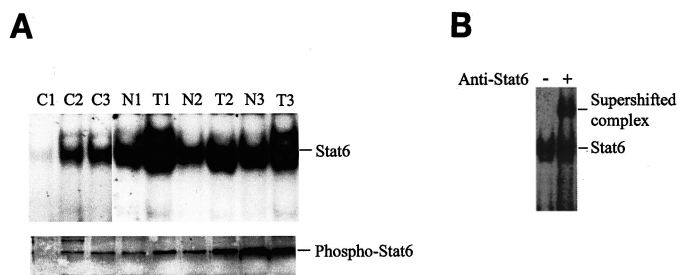


FIG. 1. Stat6 expression in human prostate tissues. A, for Stat6 DNA binding activity 20 μg. whole cell extracts were subjected to electromobility shift assay using ³²P labeled oligonucleotide probe containing consensus binding motif for Stat6. Lanes C1 to C3, normal prostate tissues from 3 organ donors. Lanes N1 to N3, matched normal prostates adjacent to tumors. Lanes T1 to T3, matched prostate carcinomas. For phospho-Stat6 expression 50 μg. proteins from whole cell extracts were subjected to Western blot analysis using antibody specifically against phosphorylated Stat3. B, for supershift assay cell extracts were pre-incubated with antibody specifically against Stat6 antibody. -, negative. +, positive.

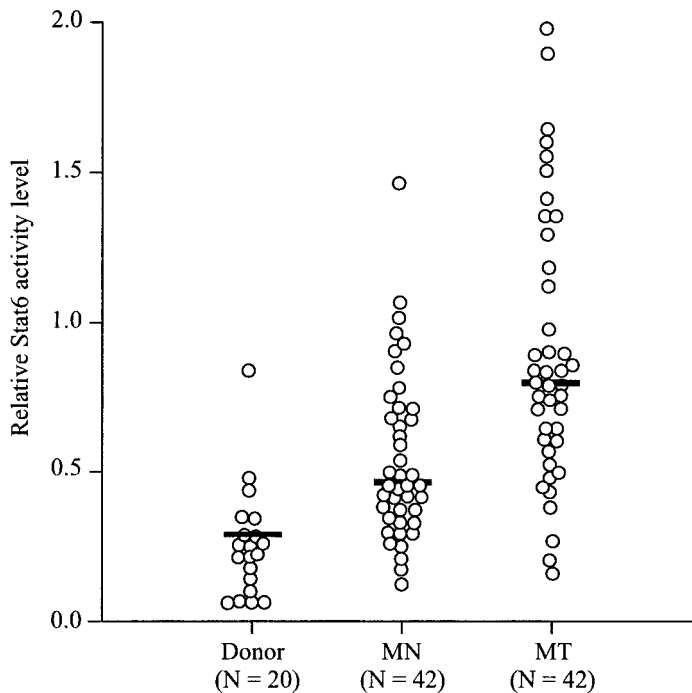


FIG. 2. Quantitation of Stat6 DNA binding activity in normal prostate tissues from 20 organ donors, 42 matched normal tissues adjacent to tumors (MN) and 42 matched prostate carcinomas (MT). Amount of retarded probe was quantitated using PhosphorImager and values were normalized to level in DU145 cells run per gel. Horizontal line indicates mean value.

cent normal tissues and normal donor prostates. Comparing Stat6 activity levels in matched normal prostates adjacent to tumors and tumor tissues revealed higher levels of Stat6 activity in 28 of the overall 42 paired tumor tissue specimens (67%), lower levels in 9 (21%) and comparable levels in 5 (12%) (table 3). Statistical analysis of Stat6 activity and clinicopathological parameters, such as PSA and Gleason grade, demonstrated that the levels of Stat6 activity in tumors did not correlate with pretreatment PSA or Gleason grade (table 3).

Stat4 activation in prostate cancer. The same specimens used for Stat6 analysis were used for Stat4 determinations. Figure 3 shows representative electromobility shift assay analysis of Stat4 activity. DU145 human prostate cancer cells served as the positive control and relative Stat4 activity was calculated to determine relative Stat6 activity, as described. Supershift analysis using anti-Stat4 antibody preincubated with cell extracts verified that the complex contained Stat4 (data not shown).

Average relative Stat4 activity was 0.22 in the 42 primary prostate tumors examined, 0.21 in the 42 matched normal prostates adjacent to tumors and 0.18 in the 20 normal prostates from donors (fig. 4). There was no significant difference in Stat4 activity levels in tumors, matched normal prostates adjacent to tumors and normal prostates from donors. Comparison of Stat4 activity in matched normal prostates adjacent to tumors and tumor tissues revealed higher levels of Stat4 activity in 18 of the overall 42 paired tumor tissue

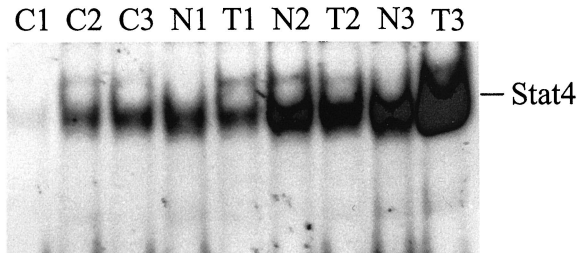


FIG. 3. To determine Stat4 DNA binding activity in representative human prostate tissues 20 μ g. whole cell extracts were subjected to electromobility shift assay using 32 P labeled oligonucleotide probe containing consensus binding motif for Stat4. Lanes C1 to C3, normal prostate tissues from 3 organ donors. Lanes N1 to N3, matched normal prostates adjacent to tumors. Lanes T1 to T3, matched prostate carcinomas.

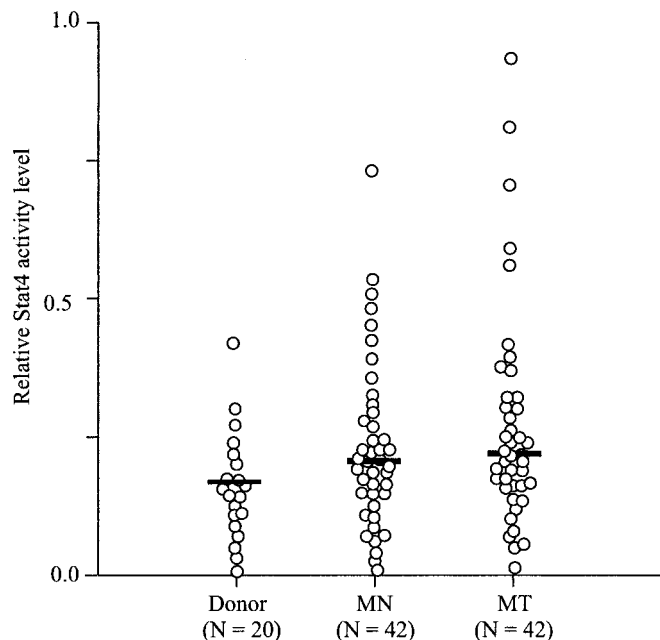


FIG. 4. Cumulative results of Stat4 DNA-binding activity in normal prostate tissues from 20 organ donors, 42 matched normal tissues adjacent to tumor (MN) and 42 matched prostate carcinomas (MT). Amount of retarded probe was quantitated using PhosphorImager and values were normalized to level in DU145 cells run per gel. Horizontal line indicates mean value.

specimens (43%), lower levels in 13 (31%) and comparable levels in 11 (26%).

Stat1, Stat2 and Stat5 activity in prostate cancer. The same specimens used for Stat4 and Stat6 analysis were also analyzed for Stat1, Stat2 and Stat5 activity by electromobility shift assay. Little or no active Stat1, Stat2 or Stat5 was found in these specimens (data not shown).

DISCUSSION

Constitutive activation of STAT proteins are found during abnormal proliferation, especially malignant transformation, as well as during cell apoptosis. Thus, the abnormalities of

TABLE 3. Stat6 activity, Gleason grade and PSA

Stat6 Activity	No. Samples (%)	Mean Pretreatment PSA \pm SEM (ng/ml.)	Mean Gleason Score \pm SEM
Matched tumors greater than matched normal prostates adjacent to tumors	28 (67)	7.0 \pm 3.8	6.4 \pm 1.3
Matched tumors less than matched normal prostates adjacent to tumors	9 (21)	7.4 \pm 2.3	6.6 \pm 0.9
Matched tumors equal to matched normal prostates adjacent to tumors	5 (12)	7.2 \pm 4.4	6.7 \pm 0.8

STAT protein activation may be associated with cancer. In this study we analyzed the levels of activity of the members of the STAT family in human prostate cancer tissues. Our results demonstrate that in addition to Stat3, Stat6 is selectively activated in prostate cancer. Stat6 activity levels in the tumor were significantly higher than in matched normal prostates adjacent to tumors or in normal prostates from noncancer controls. There was no difference in Stat4 activity in tumors, matched normal prostates adjacent to tumors and normal prostates from donors. The levels of active Stat1, Stat2 and Stat5 were negligible in human prostate cancer.

The activity levels of Stat6 did not correlate with Gleason score or tumor stage, which suggests that each has little prognostic value for prostate cancer progression. However, Stat6 activity levels were significantly higher in tumors than in matched normal prostates adjacent to tumors or normal donor prostates. In addition, 28 of the overall 42 paired specimens of tumor tissues (67%) had higher Stat6 activity than matched normal prostates adjacent to tumors. These results suggest that the levels of Stat6 activity may have potential diagnostic value for detecting prostate cancer. It may be interesting to determine whether Stat6 activity levels may be used with current clinicopathological markers, such as PSA and Gleason grade, for more accurately assessing tumor specimens.

Donor samples included men 20 to 59 years old, including 10, 4, 3 and 3 in the 20 to 30, 30 to 40, 40 to 50 and 50 to 59-year-old age groups, respectively. Results showed no significant age related change in STAT activation.

The development of a prostate cancer from a normal prostatic glandular cell requires multiple transformation events. To our knowledge genetic alterations associated with the initial development of histological prostate cancer are incompletely understood. Candidates for regulating prostate cell proliferation include growth factors and their receptors, steroids, adhesion molecules, signal transduction molecules and transcription factors. STAT protein are latent transcription factors that are activated by endogenous protein tyrosine kinase activity of oncoproteins such as v-src as well as by constitutive activity of cytokine receptors or receptor associated Janus kinases. Stat6 and Stat4 are activated through tyrosine phosphorylation by IL-4 and IL-12, respectively.¹⁵ IL-4 is a member of the Th2 or anti-inflammatory cytokine family that can affect the proliferation, gene expression and differentiation of lymphocytes.¹⁶ Binding of IL-4 to its receptor IL-4R phosphorylates membrane associated kinases Jak1 and Jak3, which subsequently activate Stat6.¹⁵ Recent studies demonstrated that levels of IL-4 are significantly elevated in hormone refractory prostate cancer compared with values in hormone sensitive prostate cancer and IL-4 levels directly correlate with elevated PSA.¹⁷ These data support our results that levels of activated Stat6, a major transducer of IL-4-mediated signaling, is significantly higher in tumors than in normal prostate tissues.

CONCLUSIONS

The current study shows that in addition to Stat3, Stat6 is selectively activated in prostate cancer. However, little or no

expression of active Stat1, Stat2 or Stat5 was detected in primary prostate tissues. Although the levels of IL-4 and Stat6 activity in tumor are significantly higher in cancer than those detected in normal prostate, to our knowledge the potential role of IL-4-Stat6 in prostate cancer development and progression is currently unknown. Our results of elevated levels of Stat6 activity in tumors combined with the data of Wise et al¹⁷ that IL-4 levels are significantly elevated in hormone refractory prostate cancer suggest a potential role for IL-4-Stat6 in the development and progression of androgen independent prostate cancer.

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