Activation of Signal Transducer and Activator of Transcription-5 in Prostate Cancer Predicts Early Recurrence

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Abstract

Purpose: We have shown previously that the signal transducer and activator of transcription-5 (Stat5) is a critical survival factor in human prostate cancer cells. In addition, we recently showed that Stat5 is activated at a high level, particularly in high-grade human prostate cancers. Here, we investigated whether activation of Stat5 in prostate cancer was linked to clinical outcome with disease recurrence as end point.

Experimental Design: Immunohistochemistry was used to detect active, nuclear Stat5 in 357 paraffin-embedded prostate cancer specimens on a tissue microarray with clinical follow-up data. Stat5 activation status in prostate cancer specimens was analyzed by univariate and multivariate survival analysis to determine whether activation of Stat5 predicts earlier prostate cancer recurrence. Separate sets of statistical analysis were done for all patients regardless of Gleason grade and for patients with prostate cancer of intermediate Gleason grades (3 and 4).

Results and Conclusions: Stat5 activation in prostate cancer was associated with early disease recurrence ($P = 0.0399$). Importantly, active Stat5 also predicted shorter progression-free survival in intermediate Gleason grade prostate cancers ($P = 0.0409$). Stat5 activation remained an independent prognostic marker after adjusting for Gleason grade, pT stage, perineural invasion, or seminal vesicle infiltration in all patients ($P = 0.0565$) and in Gleason grade 3 or 4 patients ($P = 0.0582$). The results of this work also confirmed our previous finding of association of Stat5 activation with a high histologic grade of prostate cancer ($R = 0.11$, $P = 0.033$). In summary, our study shows that active Stat5 distinguished prostate cancer patients whose disease is likely to progress earlier; therefore, active Stat5 may be a useful marker for selection of more individualized treatment. The results of this study need to be validated in a large prospective cohort.

We have shown previously that the signal transducer and activator of transcription-5 (Stat5) is a key survival factor for human prostate cancer cells (1). This finding was later confirmed in mouse prostate cancer by data emerging from another laboratory (2). Recently, we also showed that activation of Stat5 in human prostate cancer strongly associates with high histologic grade (3). Stat5 is the key signaling protein activated by prolactin (Prl) in normal and malignant prostate (3, 4). Prl, in turn, we have shown being a mitogen (5, 6) and survival factor (7) for both rodent and human prostate epithelium. Transgenic mice overexpressing *Prl* gene develop massive hyperplasia of dorsolateral prostate (8), and correspondingly, Prl-null mice have smaller prostates than their wild-type counterparts (9). Furthermore, Prl itself is produced by normal and malignant prostate cells (10, 11), and our recent data showed that production of Prl protein is significantly increased in high-grade human prostate cancer (3), indicating that Prl is involved in progression of prostate cancer as a local autocrine/paracrine Stat-activating growth factor.

Stat5 becomes activated by phosphorylation on a conserved tyrosine residue in its COOH-terminal end (12) by a protein tyrosine kinase, typically of the Janus-activated kinase family (12). Phosphorylated Stat5 dimerizes and translocates to the nucleus where it binds to promoter regions of its target genes to regulate transcription (12). Here, we evaluated levels of active Stat5 in 357 human prostate cancer specimens (13) using *in situ* detection of tyrosine-phosphorylated and nuclear Stat5 in paraffin-embedded tissue (14, 15) and correlated the data with clinical outcome. We show that activation of Stat5 is associated with early recurrence of prostate cancer and thus may serve as a prognostic biomarker for intermediate Gleason grade prostate cancers.

Materials and Methods

Prostate cancer material. The material represented paraffin-embedded prostate cancer tissue specimens on a tissue array from a total of 548 patients who were treated for clinically localized prostate cancer

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by radical prostatectomy or transurethral resection at one of two Kaiser hospitals in Portland, OR between 1971 and 1996. The material on a tissue microarray is a subset of prostate cancers described previously by Zhang et al. (16). The tissue and microarray construction has been previously described by Zellweger et al. (13). The use of the deidentified archival tissue specimens in research was approved by the Committee for the Protection of Human Subjects of Kaiser Permanente, Portland, OR and by the Georgetown University institutional review board. Medical records for the entire cohort had been abstracted at one time, 1999 to 2001, to assure uniform criteria for diagnosis, progression, and staging. Immunohistochemical analysis of levels of active Stat5 was uninformative for 191 tumor samples because of missing or unrepresentative samples in the array sections studied. Demographic and clinical characteristics of the prostate cancer specimens are presented in Table 1. Gleason grades rather than Gleason scores were assigned to individual specimens in the tissue microarray because of the small size of each specimen. Moreover, the small-size specimens on the tissue array mimic biopsies of prostate cancer that the initial treatment decisions are based upon. There was no statistically significant difference in progression-free survival, pT stage, or age at diagnosis in patients whose prostate cancer specimens were uninformative for active Stat5 compared with those with positive or negative Stat5 level. Progression was defined clinically (bone scan, chest X-ray, and digital rectal examination) and by increase in prostate-specific antigen (PSA) serum concentrations as described before (13). Moreover, death related to prostate cancer was regarded as progression.

Immunohistochemistry of active Stat5. Mouse monoclonal antibody AX1, which recognizes activated Stat5, was provided by Advantex BioReagents (Conroe, TX). Its specificity to tyrosine-phosphorylated Stat5 in immunohistochemistry and immunoblotting has been validated by peptide competition, inducible phosphorylation studies, site-directed mutagenesis, and Stat5 knockout model analyses (14). Sections of paraffin-embedded, formalin-fixed tissues from human prostate cancer were deparaffinized in xylene for 2 × 15 minutes followed by rehydration in graded ethanol. Slides containing deparaffinized tissue sections were microwave treated in a pressure cooker with antigen retrieval solution AXAR1 (Advantex BioReagents). For detection of total Stat5, parallel tissue sections were microwave treated in citrate solution (BioGenex Laboratories, San Ramon, CA). Endogenous peroxidase activity was blocked by incubating slides in 0.3% hydrogen peroxide for 10 minutes at room temperature, and nonspecific binding of immunoglobulin was minimized by preincubation in normal goat serum for 2 hours at room temperature. The primary antibodies recognizing phosphorylated (Y694/Y699) Stat5 (monoclonal antibody; Advantex BioReagents) and total Stat5 (monoclonal antibody; Santa Cruz Biotechnology, Santa Monica, CA) were diluted in 1% bovine serum albumin in PBS at concentrations of 0.6 and 2 μg/mL, respectively. Antigen-antibody complexes were detected using biotinylated goat anti-mouse secondary antibody (Biogenex Laboratories) followed by streptavidin-horseradish peroxidase complex, using 3,3′-diaminobenzidine as chromogen and Mayer's hematoxylin as counterstain. Lactating human breast tissue (14) was used as a positive control tissue for total and activated Stat5 immunohistochemistry. E-cadherin and P53 immunostainings were done and scored by Zellweger et al. as reported previously (13).

Scoring of levels of active Stat5. Individual prostate tumor samples were scored (MTN and HL) for active and nuclear Stat5 levels on a scale from 0 to 1, where 0 was undetectable and 1 represented positive immunostaining. Score 1 for phosphorylated and nuclear Stat5 was used in the statistical analysis to define positive Stat5 activation status.

Statistical methods. Curves for overall survival, tumor-specific survival, and progression-free survival from the date of surgery were calculated using the method of Kaplan and Meier (17) stratified by selected histopathologic characteristics (active Stat5, Gleason grade, pT

Table 1. Characteristics of the prostate cancers in the tissue microarray

Continuous variables	No. patients	%
Age at diagnosis, y		
Median (min, max)	65.0 (45.0, 88.0)	
Mean (SE)	64.61 (0.3)	
No. patients	548	
PFS follow-up, y		
Median (min, max)	5.76 (0.04, 27.27)	
Mean	6.48	
PFS follow-up, y (Gleason 3 or 4)		
Median (min, max)	5.77 (0.04, 27.27)	
Mean	6.51	
TSS follow-up, y		
Median (min, max)	5.95 (0.04, 28.36)	
Mean	6.77	
OS follow-up, y		
Median (min, max)	6.01 (0.93, 28.36)	
Mean	6.80	
Gleason grade		
2	26	4.7
3	333	60.8
4	171	31.2
5	18	3.3
pT stage		
T1	35	6.4
T2	401	73.1
T3	87	15.9
T4	21	3.8
Unknown	4	0.7
Perineural invasion		
Yes	232	42.3
No	316	57.7
Seminal vesicle infiltration		
Yes	40	7.3
No	508	92.7
Stat5 activation status		
Negative	141	25.7
Positive	216	39.4
Unknown	191	34.9
Hormone therapy		
No	449	81.9
Yes	99	18.1
Radiation therapy		
No	63	11.5
Yes	485	88.5
E-cadherin		
Negative	204	37.2
Positive	333	60.8
Unknown	11	2.0
P53		
Negative	426	77.7
Positive	99	18.1
Unknown	23	4.2

NOTE: Hormone therapy was typically Leuprolide given monthly (one to three doses).
Abbreviations: PFS, progression-free survival; TSS, tumor specific survival; OS, overall survival.

stage, perineural invasion, seminal vesicle infiltration, and age at diagnosis). Univariate analyses were done to determine the patient characteristics related to progression-free survival, overall survival, and tumor-specific survival by testing the statistical significance of the differences between curves using a generalized Wilcoxon test (18) because this test does not require an assumption of proportional hazards. Multivariate Cox regression models (19) were separately fit to progression-free survival data from date of surgery for all the patients and those with Gleason grades 3 and 4 only to determine the combination of patient characteristics best related to outcomes. The models were fit in stepwise fashion beginning with the factors that were the most interesting (Gleason grade, pT stage, perineural invasion, seminal vesicle infiltration, and active Stat5) and interactions between the factors (active Stat5 and Gleason grade interaction, active Stat5, and seminal vesicle interaction). A backward model selection method was applied in which nonsignificant covariates were eliminated step-by-step so that the final Cox regression models included only statistically significant covariates. In the fitting of the Cox models, interrelationships among the covariates were taken into account so that if one factor (e.g., Gleason grade) was statistically significant, another factor might have lost significance because it correlated with Gleason grade. Association of Stat5 activation with cell surface E-cadherin or with positive immunostaining for p53 was analyzed by two-sided χ^2 test.

Results and Discussion

Stat5 is an independent marker for early recurrence in prostate cancer. To assess the ability of activated Stat5 to predict progression of prostate cancer, activation of Stat5 was analyzed in paraffin-embedded prostate cancer specimens (Table 1) by a highly sensitive *in situ* detection method, which is based on immunohistochemical detection of nuclear localized phosphorylated Stat5 (refs. 1, 3, 4, 14, 15; Fig. 1A, *top*). The specificity of anti-phosphoStat5 mouse monoclonal antibody AX-1 to tyrosine-phosphorylated Stat5 in immunohistochemistry and immunoblotting has been validated by peptide competition, inducible phosphorylation studies, site-directed mutagenesis, and Stat5 knockout model analyses (14). This method was complemented with immunohistochemical detection of nuclear Stat5 protein (Fig. 1A, *bottom*). We have shown previously both expressions of Stat5 protein and mRNA in fresh human prostate cancer specimens (3). Prostate cancers of positive (*top* and *bottom left*) or negative (*top* and *bottom right*) Stat5 activation status are presented in Fig. 1.

Stat5 activation status in prostate cancer specimens (Table 1) was analyzed separately by univariate and multivariate survival analysis. By univariate analysis, positive Stat5 activation status was associated with significantly earlier prostate cancer recurrence (P = 0.0399; Table 2). Kaplan-Meier estimates indicated a progression-free 15-year survival rate of 65% in prostate cancer patients with negative Stat5 activation status compared with ~44% in patients with Stat5-positive tumors (Fig. 1B). This corresponds to an ~21% benefit in progression-free survival at 15 years associated with negative Stat5 activation status of prostate cancer. Consistent with previous observations, Gleason grade, pT stage, perineural invasion, and seminal vesicle infiltration were also highly significant predictive

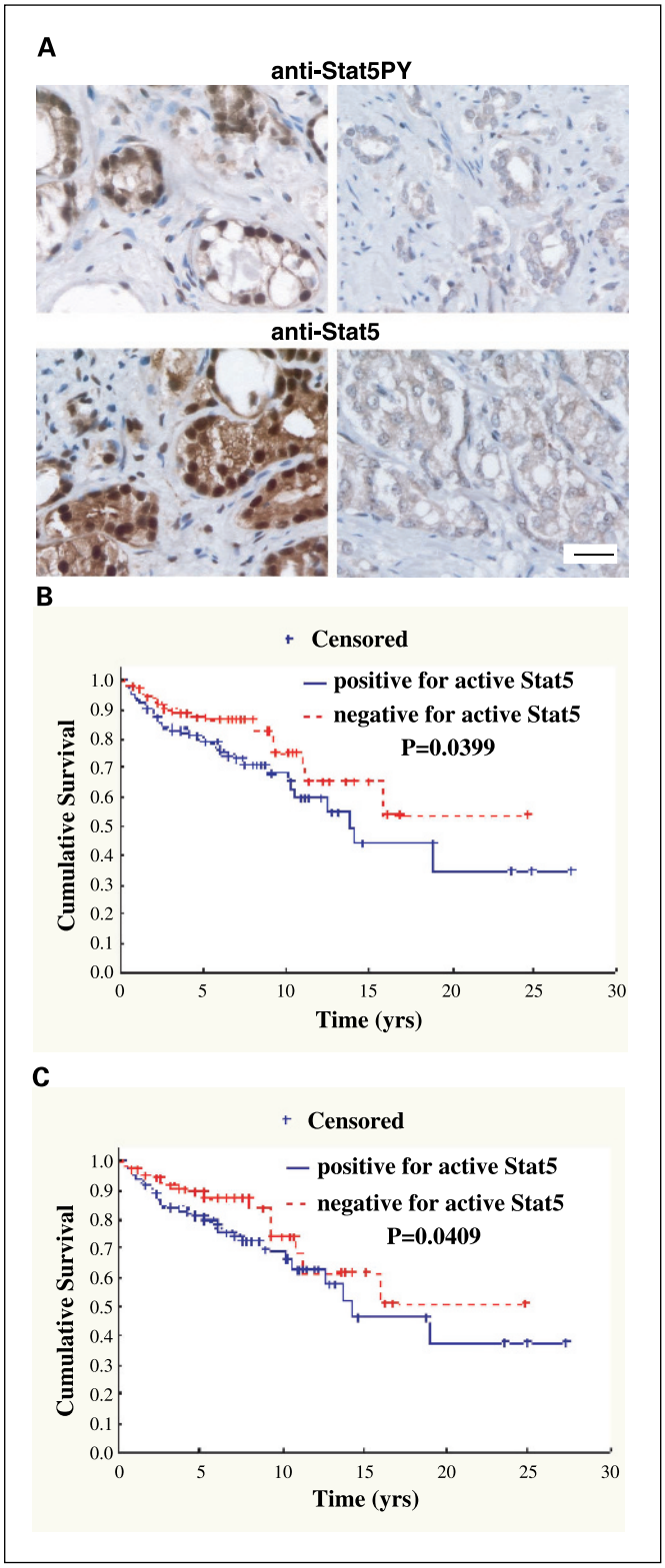

Fig. 1. *A,* immunohistochemical detection of active Stat5 in prostate cancer tissue. Paraffin-embedded tissue sections of human prostate specimens were immunostained with a monoclonal activation state – specific anti-pTyrStat5 antibody (*top*) and with an anti-Stat5 antibody (*bottom*). 3,3′-Diaminobenzidine was used as a chromogen and Mayer's hematoxylin as counterstain. Biotin-streptavidin amplified peroxidase antiperoxidase immunodetection shows intense positive reactions for active Stat5 in the nuclei of epithelial cells of prostate cancer acini (*bottom*). Right, prostate cancer specimens that were negative for active, nuclear Stat5. *B* and *C,* activation of Stat5 in prostate cancer predicts shorter progression-free survival. Actuarial curves for progression-free survival according to negative (*top curve*) or positive (*bottom curve*) Stat5 activation status in all patients (n = 357) regardless of Gleason grade (*A*) and in Gleason grade 3 and 4 patients (n = 325; *B*). Censored cases (+) and number of patients are indicated.

Table 2. Univariate and multivariate analysis of progression-free survival comparing active Stat5 with other prognostic factors in prostate cancer

	Univariate analysis		Regression coefficient (SE)	Multivariate analysis	
	No. patients*	*P*		*P*	Hazard ratio (95% confidence interval)
Entire material† progression-free survival					
Stat5 activation status	357	0.0399	0.4884 (0.256)	0.0565	1.630 (0.99 to 2.69)
Negative‡	141				
Positive	216				
Gleason grade	548	<0.0001	0.7031 (0.234)	0.0026	2.020 (1.28 to 3.194)
2 or 3‡	359				
4 or 5	189				
pT stage	544	<0.0001	0.6897 (0.262)	0.0085	1.993 (1.19 to 3.33)
T1-T2‡	436				
T3-T4	108				
Perineural invasion	548	0.0285			
No‡	316				
Yes	232				
Seminal vesicle infiltration	548	0.001	0.6534 (0.329)	0.0469	1.922 (1.01 to 3.66)
No‡	508				
Yes	40				
Age at diagnosis	548	0.30			
45-64‡	265				
65-69	146				
≥70	137				
Gleason grade 3 or 4 patients only§, progression-free survival					
Stat5 activation status	325	0.0409	0.5174 (0.273)	0.0582	1.678 (0.98 to 2.87)
Negative‡	121				
Positive	204				
Gleason grade	504	<0.0001	0.4726 (0.246)	0.0543	1.604 (0.99 to 2.60)
3‡	333				
4	171				
pT stage	500	<0.0001			
T1-T2‡	402				
T3-T4	98				
Perineural invasion	504	0.0366			
No‡	285				
Yes	219				
Seminal vesicle infiltration	504	<0.001	1.1698 (0.307)	0.0001	3.221 (1.77 to 5.88)
No‡	466				
Yes	38				
Age at diagnosis	504	0.28			
45-64‡	248				
65-69	137				
≥70	119				

NOTE: Statistical analyses were done by Cox proportional hazard regression analysis to evaluate the prognostic power in a multivariate manner. Prognostic variables evaluated include Stat5 activation (negative versus positive), Gleason grade, T stage, perineural invasion (negative versus positive), and seminal vesicle infiltration (negative versus positive).

*Number of cases available for analysis of each variable from a total of 548 cases with progression-free survival data.

†Entire material: median age, 65 years; median follow-up, 5.76 years.

‡Favorable.

§Gleason 3 or 4 patients only: median age, 65 years; median follow-up, 5.77 years.

markers of short progression-free survival (Table 2). Multivariate analysis, using Cox proportional hazard regression method (19) to take all prognostic markers into account, showed that Stat5 activation remained an independent prognostic factor for early disease recurrence (hazard ratio, 1.6; $P = 0.0565$; Table 2). In addition, Gleason grade, pT stage, and seminal vesicle infiltration were independent markers of early disease recurrence for prostate cancer (Table 2).

Activation of Stat5 is associated with shorter progression-free survival in intermediate Gleason grade prostate cancers. Currently, the treatment decisions for prostate cancer are predominantly based on the clinical stage, serum PSA value, and the histologic grade of the tumor obtained by needle biopsy at the time of diagnosis. The Gleason grading system in biopsy or prostatectomy specimens is a measure of biological aggressiveness (20). In addition, serum PSA values highly correlated to tumor volume and also correlates with stage (20). Therefore, both Gleason grade and serum PSA value provide significant prognostic information as individual variables when their values are at the very high or at the very low of the spectrum. However, most patients have intermediate PSA levels and intermediate Gleason grade (20); therefore, the treatment of intermediate Gleason grade (3 and 4) prostate cancers would greatly benefit from identification of additional markers that predict the clinical course of the disease so that the appropriate treatment strategy for an individual patient can be selected.

Because active Stat5 would provide a simple immunohistochemical marker for paraffin-embedded tissue sections, we next specifically analyzed the ability of Stat5 activation status to predict progression-free survival of intermediate Gleason grade prostate cancers in this material (n = 325). In Gleason grade 3 and 4 prostate cancers, univariate analysis identified Stat5 activation as a prognostic factor that predict early disease recurrence (P = 0.0409; Table 2). Importantly, active Stat5 continued to be a significant and independent marker to predict early prostate cancer recurrence after adjusting for Gleason grade, pT stage, and perineural invasion or seminal vesicle infiltration (hazard ratio, 1.7; P = 0.0582; Table 2) in prostate cancers of intermediate histologic grades. Specifically, the data indicated that a patient with Gleason grade 3 or 4 prostate cancer and whose prostate tumor stains positively for active Stat5 is 1.7 times more likely to experience disease progression when compared with a patient with negative status for Stat5 activation. By Kaplan-Meier estimates in Gleason grade 3 or 4 prostate cancers, this corresponds to a 15-year progression-free survival rate of 46% with positive Stat5 activation status compared with ~62% in patients with Stat5-negative tumors (Fig. 1C). Thus, there was an ~16% benefit in progression-free survival at 15 years associated with negative Stat5 activation status among Gleason grade 3 or 4 prostate cancers. In our material, activation of Stat5 was not significantly associated with shorter overall survival or tumor-specific survival. This is likely to be due to a relatively low number of deaths caused by prostate cancer in our material.

Activation of Stat5 is involved in progression of prostate cancer. The present study identifies active Stat5 as an independent marker of poor clinical outcome of prostate cancer based on significantly shorter progression-free survival associated with activation of Stat5 in the tumor. Importantly, active Stat5 predicted early disease recurrence specifically in intermediate Gleason grade (3 and 4) prostate cancers. The most immediate use of active Stat5 in prostate cancer as a marker would be for identification of a subgroup of prostate cancers of intermediate Gleason grades that are likely to progress earlier and, therefore, would benefit from active treatment. Specifically, primary prostate cancers of Gleason grades 3 or 4 that are positive for active Stat5 in the initial biopsy should not remain treated with watchful waiting only but should be subjected to active and extensive treatment regimens.

The biological mechanisms that may explain Stat5 activation as a marker of early progression of prostate cancer remain to be identified. In the present work, which included 357 primary human prostate cancer specimens of different Gleason grades, we show that activation of Stat5 was associated with high histologic grade of prostate cancer (R = 0.11, P = 0.033). These data confirm our previous finding of increased activation of Stat5 with high-grade human prostate cancer based on analysis of an independent material of 114 primary prostate cancer specimens of different histologic grades (3). Collectively, these results indicate that Stat5 is involved in dedifferentiation of prostate cancer cells. Loss of normal differentiation in tumor cells is known to be particularly prominent at the transition from localized and surgically curable cancer to metastatic disease (21). Furthermore, epithelial-to-mesenchymal dedifferentiation is required for migration of cancer cells (22). Therefore, in the present study, we further investigated the association of active Stat5 with cell surface E-cadherin in prostate cancer (Table 3). Immunostaining for cell surface E-cadherin in these prostate cancer specimens has been done, analyzed, and reported previously by Zellweger et al. (13). E-cadherin is a transmembrane glycoprotein of 120 kDa that is linked to the actin cytoskeleton via catenins (23). Loss of functional E-cadherin disrupts normal homotypic adhesiveness of prostate epithelial cells and is therefore a critical early step in invasion and metastatic progression. Based on our analysis, activation of Stat5 in prostate cancer was associated with loss of cell surface E-cadherin expression (P = 0.0234; Table 3). We propose that activation of Stat5 in primary prostate cancer could represent a tumor progression event that contributes to increased risk of peritumoral invasion and cancer cell dissemination. Furthermore, mechanistic testing of Stat5 as a stimulator of prostate cancer invasion and metastasis is now warranted.

A second mechanism that could underlie the increased likelihood of prostate cancers with active Stat5 to progress earlier compared with those without active Stat5, may be related to Stat5 serving as a critical survival factor in prostate cancer cells. Specifically, we have shown previously that inhibition of Stat5 induces apoptotic death of human prostate cancer cells (1). This was later confirmed by others in the mouse TRAMP prostate cancer model (2). Moreover, in the present study, activation of Stat5 was significantly more

Table 3. Association between active Stat5 and cell surface E-cadherin/positive immunostaining for p53 in prostate cancer

	Active Stat5		*P*
	Negative	Positive	
E-cad			
Negative	46	96	0.0234
Positive	95	119	
P53			
Negative	124	162	0.0084
Positive	15	45	

NOTE: Statistical analysis was done by a two-sided χ^2 test.

frequent in human prostate cancers that had positive immunostaining for p53 (13) indicating p53 mutation in these cases (P = 0.0084; Table 3). This is an interesting association, especially because wild-type but not mutant p53 suppressed the transcriptional activity of Stat5 in reporter gene assays (24). The importance of p53 status for biological function of Stat5 in prostate cancer will therefore be subject of future studies.

Several limitations of the work presented here will be addressed in follow-up investigations. First, the patient material was heterogeneous in terms of adjuvant treatment. Specifically, 18% of the patients whose prostate cancers showed activation of Stat5 received androgen deprivation therapy and 10% received radiation therapy for prostate cancer. It will therefore be important to validate the conclusions of this report in prospective studies with well-defined adjuvant therapies or in prostate cancers that have been treated with watchful waiting. Second, whereas analysis of full tissue sections of prostate tumors indicated that Stat5 activation was homogenous within a tissue area of a certain Gleason grade (3), the present tumor microarray studies need to be repeated on full-size tumor sections in correlation with Gleason score of each prostate cancer. Moreover, the predictive value of active Stat5 in prostate cancers of intermediate and low histologic grades may be improved by analysis of other prognostic markers in conjunction with active Stat5 such as Ki67, P53, Bcl-2, Syndecan-1, CD10 (13), p27, hepsin, and EzH2. Finally, it will be important to validate our initial observations in a large prospective cohort.

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