The American Journal of Pathology, Vol. 182, No. 6, June 2013

ELSEVIER

The American Journal of
PATHOLOGY

ajp.amjpathol.org

METABOLIC, ENDOCRINE, AND GENITOURINARY PATHOBIOLOGY

STAT5A/B Gene Locus Undergoes Amplification during Human Prostate Cancer Progression

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Accepted for publication February 28, 2013.

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The molecular mechanisms underlying progression of prostate cancer (PCa) to castrate-resistant (CR) and metastatic disease are poorly understood. Our previous mechanistic work shows that inhibition of transcription factor Stat5 by multiple alternative methods induces extensive rapid apoptotic death of Stat5-positive PCa cells *in vitro* and inhibits PCa xenograft tumor growth in nude mice. Furthermore, STAT5A/B induces invasive behavior of PCa cells *in vitro* and *in vivo,* suggesting involvement of STAT5A/B in PCa progression. Nuclear STAT5A/B protein levels are increased in high-grade PCas, CR PCas, and distant metastases, and high nuclear STAT5A/B expression predicts early disease recurrence and PCa-specific death in clinical PCas. Based on these findings, STAT5A/B represents a therapeutic target protein for advanced PCa. The mechanisms underlying increased Stat5 protein levels in PCa are unclear. Herein, we demonstrate amplification at the *STAT5A/B* gene locus in a significant fraction of clinical PCa specimens. *STAT5A/B* gene amplification was more frequently found in PCas of high histologic grades and in CR distant metastases. Quantitative *in situ* analysis revealed that *STAT5A/B* gene amplification was associated with increased STAT5A/B protein expression in PCa. Functional studies showed that increased *STAT5A/B* copy numbers conferred growth advantage in PCa cells *in vitro* and as xenograft tumors *in vivo*. The work presented herein provides the first evidence of somatic *STAT5A/B* gene amplification in clinical PCas. *(Am J Pathol 2013, 182: 2264–2275; http://dx.doi.org/10.1016/j.ajpath.2013.02.044)*

Organ-confined prostate cancer (PCa) is treated by surgery or radiation, whereas options for disseminated PCa include hormone therapy, radiotherapy, and chemotherapy. The response to hormone therapy is of limited duration, and metastatic PCa inevitably becomes castrate resistant (CR), a stage for which there is no cure.[1,2] Mechanisms underlying PCa progression to metastatic CR disease remain to be identified. Known molecular mechanisms underlying the development of CR PCa include i) gene amplification of androgen receptor (AR),[3] ii) somatic mutations of AR resulting in increased affinity for ligands,[4,5] and iii) development of intracellular capacity to biosynthesize androgens from adrenal steroids and cholesterol.[6,7] In addition, constitutively active alternative splice variants of AR have been recently described in CR xenograft models of PCa and in clinical CR PCa.[8–10] These truncated AR isoforms do not require ligand and may support CR PCa growth. Alternatively, or in addition to alterations in AR, hyperactivation of growth factor signaling may promote proliferation and survival of

Supported by NIH National Cancer Institute grant 1RO1CA113580-01A1 (M.T.N.) and Academy of Finland grant 135655 (T.M.). Shared resources of Kimmel Cancer Center are partially supported by NIH grant CA56036-08.

http://dx.doi.org/10.1016/j.ajpath.2013.02.044

CR PCa through stimulation of AR or independently of AR.[11] STAT5A/B controls PCa growth and tumor progression.[12–18] Nuclear STAT5A/B protein levels are elevated in high-grade organ-confined PCas, hormone therapy–resistant cancers, and distant metastases.[12,17–21] STAT5A/B belongs to the Stat family of transcription factors[22] and comprises two highly homologous isoforms, 94-kDa STAT5A and 92-kDa STAT5B.[22] *STAT5A* (24 kb) and *STAT5B* (77 kb) genes are located juxtaposed in a head-to-head orientation on chromosome 17q21.31.[23] STAT5A/B are latent cytoplasmic proteins that become activated by phosphorylation of a conserved tyrosine residue in the carboxy-terminal domain.[22] Tyrosine phosphorylation leads to STAT5A/B homodimerization or heterodimerization and to translocation to the nucleus, where the dimers bind to specific STAT5A/B response elements of target genes for transcriptional regulation.[22]

Inhibition of STAT5A/B protein expression or transcriptional activity induces extensive apoptotic death of STAT5-positive human PCa cells.[12,14,15] Inhibition of STAT5A/B significantly reduces subcutaneous and orthotopic PCa xenograft tumor growth in nude mice.[12,14,15,18] In addition, nuclear STAT5A/B expression is elevated in PCa compared with normal prostate epithelium.[12,19,24] Increased nuclear STAT5A/B is clustered to PCas of high histologic grades,[19] a finding that we later confirmed in three additional independent sets of human PCas.[20,25] Nuclear STAT5A/B expression is elevated in most CR PCas and distant PCa metastases.[17,18,21] STAT5A/B and AR functionally synergize in AR-positive PCa cells.[21] Consistent with these findings, a recent study suggested that STAT5 may be involved in redirecting AR binding sites in clinical CR PCas.[26] However, STAT5A/B promotes PCa cell viability also through AR-independent pathways/mechanisms because STAT5A/B inhibition triggers extensive apoptosis of PCa cells, which lack AR expression.[15] We recently showed that STAT5A/B promotes metastatic behavior of human PCa cells *in vitro*, including STAT5A/B-induced migration and heterotypic adhesion of PCa cells and down-regulation of E-cadherin.[18,20] STAT5A/B induced a 10-fold increase in metastasis formation in an experimental metastasis assay in nude mice,[18] and STAT5A/B has been shown to control tumor growth and metastatic potential in the autochthonous transgenic mouse PCa model.[27] Consistent with these findings, elevated nuclear STAT5A/B expression in clinical PCas predicted early disease recurrence and PCa-specific death in cohorts of 357[20] and 678[25] organ-confined PCas.

Given the importance of STAT5A/B in PCa growth and progression, it is imperative to determine the mechanisms leading to increased nuclear STAT5A/B protein levels in PCa. One of the key activators of STAT5A/B in PCa is locally produced prolactin,[13,19,28] the expression of which is elevated in high-grade PCas, CR PCas, and distant metastases.[13,19,29,30] Chromosome arm 17q, where the *STAT5A* and *STAT5B* genes reside (17q21), is known to be altered in hereditary and incidental PCa.[31–44] This led us to hypothesize that the *STAT5A/B* gene locus may undergo somatic amplification in PCa during disease progression. Using fluorescence *in situ* hybridization (FISH) analysis of prostate tumor sections and a PCR-based copy number assay, we demonstrate herein that *STAT5A/B* gene copy numbers are increased in a significant fraction of clinical PCas. High copy number gain (HCNG) of *STAT5A/B* genes was clustered to PCas of high histologic grades and to CR metastases. Amplification of the *STAT5A/B* gene locus in PCa was associated with elevated nuclear STAT5A/B protein levels in the tumor samples. Finally, we demonstrate that an increase in *STAT5A/B* copy numbers in PCa cells promoted growth of PCa cells *in vitro* and PCa xenograft tumors *in vivo*. In summary, these data establish the novel concept that *STAT5A/B* genes undergo amplification in high-grade organ-confined PCas, CR local recurrences, and CR distant metastases. Somatic *STAT5A/B* gene amplification may represent a mechanism promoting PCa growth and progression.

Materials and Methods

Prostate Specimens

We evaluated 257 formalin-fixed, paraffin-embedded human prostate specimens obtained from three different institutions divided into four categories, as follows: i) whole tissue sections of benign prostate hyperplasia specimens ($n = 9$) were from the Institute for Pathology, University of Basel (Basel, Switzerland)[45]; ii) whole tissue sections of primary, organ-confined PCa specimens of different histologic grades ($n = 108$) were from Georgetown University (Washington, DC) [$n = 69$; Gleason score (GS) 6, $n = 17$; GS 7, $n = 24$; and GS 8/9, $n = 28$] and from Thomas Jefferson University (Philadelphia, PA) ($n = 39$; GS 6, $n = 14$; GS 7, $n = 18$; and GS 8/9, $n = 7$); iii) recurrent CR PCa specimens ($n = 64$) collected by transurethral resection of the prostate after androgen deprivation therapy in a tissue microarray platform were from the Institute for Pathology, University of Basel; and iv) whole tissue sections of distant PCa metastases ($n = 21$) were from Georgetown University. The metastases were from lymph nodes ($n = 12$) or bone ($n = 9$).

In addition, CR distant PCa metastases in a tissue microarray format were obtained from the Institute for Pathology, University of Basel ($n = 55$). The metastases were from lymph nodes ($n = 27$), bone ($n = 6$), or other organs ($n = 43$). The metastatic lesions were collected at autopsy from patients who had undergone androgen deprivation and had subsequently died of end-stage disease.

Freshly frozen samples of 14 CR PCas (Tampere University Hospital, Tampere, Finland) were from patients who had experienced local progression of the disease during hormone therapy. The specimens were histologically examined for the presence of tumor tissue (>50% of cells) using H&E-stained slides. The therapy modalities were orchiectomy (four cases), luteinizing hormone-releasing hormone analogue (three cases), estrogen (two cases), orchiectomy and estrogen (two cases), combined androgen blockade (two cases), and

unknown (one case). The mean time from the onset of androgen ablation to progression was 40 months (range, 15 to 68 months). DNA was amplified using a GenomiPhi DNA amplification kit (Amersham, GE Healthcare, Little Chalfont, UK) according to the manufacturer's instructions.

The sample-processing procedures for the paraffin-embedded tissue samples in the four institutions were highly consistent with each other. The Thomas Jefferson University Institutional Review Board found this research to be in compliance with federal regulations governing research on deidentified specimens and/or clinical data [45 CFR 46.102(f)].

PCa Cell Lines

DU145, LNCaP, and CWR22Rv1 cell lines were obtained from ATCC (Manassas, VA) and were grown in RPMI 1640 medium (Mediatech Inc., Herndon, VA) and 10% fetal bovine serum (Quality Biological Inc., Gaithersburg, MD), 2 mmol/L L-glutamine (50 IU/mL), and 50 μg/mL of penicillin-streptomycin (Mediatech Inc.). C4-2B and B4 cells were cultured in T-medium in the presence of 5% fetal bovine serum. Chromosomes were prepared from each cell line using a standard protocol.[46]

Fluorescence *in Situ* Hybridization

To evaluate *STAT5A/B* gene copy numbers in PCa cell lines and in the PCa tissue sections, we designed a *STAT5A/B* probe consisting of a contig of four overlapping bacterial artificial chromosome (BAC) clones containing sequences of the *STAT5A/B* gene: RP11-60B4, RP11-1151C17, RP11-1151G10, and RP11-365D24 (BACPAC Resources, Oakland, CA). As a control probe for all the samples, we used a chromosome 17 centromeric BAC clone: RP11-299G20. Correct chromosomal localization of each of the four *STAT5A/B* probe BAC clones to 17q21.1-21.2 and of the control probe BAC clone to 17p11.1 was confirmed by FISH mapping.[47] FISH analysis of the cell lines and tissue sections was performed using a standard protocol.[47–49] In brief, DNA from each BAC clone was prepared and labeled using nick translation, as previously described. The clones of the *STAT5A/B* probe and the control clone were labeled with digoxigenin-11-dUTP and biotin-16-dUTP (Roche Applied Sciences, Indianapolis, IN), respectively, using nick translation.

For the tissue sections, each section was deparaffinized with xylene, followed by pepsin digestion for 90 minutes and fixation in an ethanol series. After denaturing in 70% formamide/2× standard saline citrate at 80°C, the tissue sections or the chromosomal preparations from the cell lines were hybridized with the denatured probe cocktail at 37°C overnight. After stringent washes, the digoxigenin-labeled probe (Stat5) was detected with a mouse anti-digoxin antibody (Sigma-Aldrich, St. Louis, MO), followed by a goat anti-mouse antibody conjugated to tetramethylrhodamine isothiocyanate (Sigma-Aldrich). The biotin-labeled probe (control) was detected with avidin conjugated to fluorescein isothiocyanate (Vector Laboratories, Burlingame, CA). The chromosomes (cell line preparations) and the nuclei (cell line preparations and paraffin sections) were counterstained with DAPI and embedded in antifade reagent [200 mmol/L 1,4-diazobicyclo[2,2,2]-octane 90% v/v glycerol, 20 mmol/L Tris-HCl (pH 8)] to reduce photobleaching.

Scoring of cells and digital image acquisition were performed using a 63× objective mounted on a Leica DMRBE microscope (Leica Microsystems, GmbH, Wetzlar, Germany) equipped with optical filters for DAPI, fluorescein isothiocyanate, and tetramethylrhodamine isothiocyanate (Chroma Technology Corp, Bellows Falls, VT) and a charge-coupled device camera (CV-M4$^+$ CL camera; JAI Ltd., Yokohama, Japan). The Applied Imaging CytoVision 4.5 software package (Genetix, Molecular Devices, San Jose, CA) was used for image acquisition and processing. At least 50 nonoverlapping nuclei were evaluated in each case. *STAT5A/B* amplification was defined as a signal ratio of gene probe to control probe $\geq$2 or five or more copies of the gene signal in $\geq$10% of the tumor nuclei.[48,49]

Immunohistochemical Analysis

Fluorescence Immunohistochemical Analysis Using Automated Quantitative Analysis

Slides were deparaffinized, followed by dehydration and antigen retrieval in Dako PT module using low pH retrieval buffer (Dako, Carpinteria, CA). Immunostaining was performed using Dako Autolink Plus autostainer. Endogenous peroxidase activity was blocked using Dako FLEX peroxidase-blocking reagent for 10 minutes, followed by serum-free protein block for 30 minutes. Slides were incubated for 20 minutes with a mixture of the primary antibodies anti-STAT5A (dilution 1:4000; Advantex BioReagents, Houston, TX), anti-STAT5B (dilution 1:8000; Advantex Bioreagents), and anti-cytokeratin (AE1/AE3) (dilution 1:100; Dako, Carpinteria, CA); washed three times with Dako wash buffer; and subsequently incubated with a mixture of secondary antibodies containing rabbit horseradish peroxidase–conjugated antibody and mouse Alexa Fluor 488 (Molecular Probes Inc., Eugene, OR). The slides were washed three times with Dako wash buffer and were subsequently incubated with tyramide-Cy5 (PerkinElmer, Waltham, MA). The slides were stained with DAPI for nuclear visualization, and automated quantitative analysis (AQUA) was performed using the AQUA/PM2000 imaging platform (HistoRx Inc., Branford, CT). Slides were scanned and images of each area were captured at three different channels detecting fluorescein isothiocyanate/Alexa Fluor 488 (cytokeratin; green), Cy5 (STAT5; red), or DAPI (nuclei; blue). AQUA scores were generated based on the images acquired and the software program used, and the results were validated manually, as described previously.[50]

Chromogenic Histochemical Analysis

Immunostaining was performed as described previously.[12,19,20,28,51,52] The primary antibody recognizing

STAT5A/B (monoclonal antibody) (Santa Cruz Biotechnology, Santa Cruz, CA) was diluted in 1% bovine serum albumin in PBS at a concentration of 0.2 μg/mL. The primary antibody for detection of Ki-67 was from Biogenex Laboratories (San Ramon, CA). Antigen-antibody complexes were detected using appropriate biotinylated goat secondary antibodies (Biogenex Laboratories) followed by streptavidin—horseradish peroxidase complex, using diaminobenzidine as chromogen and Mayer's hematoxylin as counterstain. Apoptotic cells were detected by TUNEL assay, which was performed using the *in Situ* Death Detection Kit (Roche Applied Sciences) as previously described.[53]

Scoring of Levels of Nuclear STAT5A/B in PCa Sections

Individual PCas were scored for STAT5A/B protein levels on a scale from 0 to 3 where 0 represented undetectable; 1, low; 2, moderate; and 3, high levels of STAT5A/B.

Copy Number Assay

Genomic DNA from 14 freshly frozen CR PCas were extracted using a DNeasy blood and tissue kit (Qiagen Inc., Valencia, CA) and were amplified using a GenomePhi DNA amplification kit (Amersham, GE Healthcare) according to the manufacturers' instructions. The copy numbers of *STAT5A* and *STAT5B* were measured using a duplex RT-PCR—based predesigned Taqman copy number assay (Applied Biosystems, Foster city, CA) according to the manufacturer's protocol. The predesigned primers used for *STAT5A/B* were as follows: Hs06414094 (*STAT5A*, intron 17) and Hs05504773 (*STAT5B*, intron 5) labeled with FAM dye. VIC dye—labeled primers for the detection of RNase P H1 RNA gene (*RPPH1*) copy numbers on chromosome 14 was the internal reference copy number, and human genomic DNA from pooled healthy male blood was used as a control. The real-time quantitative PCR reactions were performed in quadruplicate using 20 ng of DNA as a template. The PCR program was 95°C for 10 minutes followed by 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. The PCR products were detected using the ABI PRISM 7900HT sequence detection system, and the data were analyzed using CopyCaller software version 2.0 (Applied Biosystems).

Generation of Adenoviruses for Gene Delivery of *STAT5* or LacZ to PCa Cells

Plasmid cDNA-CMV-Stat5a and pCDNA-CMV-LacZ were cloned into an adenoviral vector using the BD Adeno-X Expression System 2 (BD Biosciences Clontech, Palo Alto, CA) according to the manufacturer's protocol, as described previously.[14,15] This specific cloning system was chosen because it uses Cre-loxP—mediated recombination, which reduces the likelihood of development of replication-competent adenovirus over time. The STAT5A and LacZ BD Creator donor vector (pDNR-CMV) expression cassettes were transferred to BD Adeno-X acceptor vector (pLP-Adeno-X-CMV) (BD Biosciences Clontech) by Cre-loxP—mediated recombination. The recombinant adenoviruses were purified, linearized by PacI digestion, and transfected to QBI-293A cells to produce infectious recombinant adenoviruses. Viral stocks were expanded in large-scale cultures, purified by double cesium chloride gradient centrifugation, and titered side-by-side by a standard plaque assay method in QBI-293A cells as per the manufacturer's instructions.

Solubilization of Proteins, Immunoprecipitation, and Immunoblotting

Cells were lyzed in lysis buffer [10 mmol/L Tris-HCl (pH 7.5), 5 mmol/L EDTA, 50 mmol/L NaCl, 30 mmol/L sodium pyrophosphate, 50 mmol/L sodium fluoride, 1 mmol/L sodium orthovanadate, 1% Triton X-100 (Roche Diagnostics GmbH, Mannheim, Germany), 1 mmol/L phenylmethylsulphonylfluoride, 5 μg/mL of aprotinin, 1 μg/mL of pepstatin A, and 2 μg/mL of leupeptin], and the protein concentrations of the whole cell lysates were determined by the Bradford method (Bio-Rad Laboratories, Hercules, CA). The cell lysates were immunoprecipitated for 2 hours at 4°C with 1.2 μg/mL of anti-STAT5A polyclonal antibody (Advantex Bioreagents, Conroe, TX). Antibodies were captured by incubation with protein A—Sepharose beads (Pharmacia Biotech, Piscataway, NJ) for 60 minutes. The filters were blotted with 1:250 anti-STAT5A/B monoclonal antibody (Transduction Laboratories Inc.) or anti-actin polyclonal antibody (Sigma-Aldrich). The immunoreaction was detected by horseradish peroxidase—conjugated secondary antibodies in conjunction with enhanced chemiluminescence substrate mixture (Amersham, Piscataway, NJ) and was exposed to film.

Clonogenic Survival Assay

DU145 cells were infected with AdStat5a or AdLacZ at a multiplicity of infection (MOI) of 5. Infected cells were trypsinized 24 hours later, and 50, 100, 200, and 400 cells were seeded in triplicate. After 11 days, cells were washed twice with PBS and were stained for 30 minutes in 0.25% crystal violet solution (Sigma-Aldrich), and colonies with >30 cells were counted. For each group, we calculated plating efficiency (PE) as (colonies counted/cells seeded) $\times$ 100 and survival fraction as colonies counted/cells seeded $\times$ (PE/100).

Human PCa Xenograft Tumor Growth Experiments

Castrated male athymic mice (Taconic, Germantown, NY) were cared for according to the institutional guidelines. Sustained-release dihydrotestosterone (DHT) pellets (90-day release, 1 pellet per mouse; Innovative Research of America, Sarasota, FL) were implanted s.c. 3 days before the tumor cell inoculations to normalize the circulating DHT levels. DU145 cells were infected 24 hours before inoculation to the mice with AdStat5a or AdLacZ at an MOI of 5. DU145 cells (20×10^6)

were mixed with half of the total injection volume (0.2 mL) with Matrigel (BD Biosciences, San Jose, CA) and injected s.c. into the flanks of the nude mice (one site per mouse). When the tumors reached 15 to 20 mm in diameter, the mice were sacrificed, and the tumor tissues were harvested. The volumes of the tumors were measured until day 55 and were calculated using the following formula: $V = (\pi/6) \times d_1 \times (d_2)^2$, with d_1 and d_2 indicating two perpendicular tumor diameters.

Statistical Analysis

For comparison of *STAT5A/B* gene amplification frequency in PCas with different histologic Gleason Scores (GSs) or immunohistochemical (IHC) STAT5A staining scores, logistic regression analysis with pairwise comparisons was used. The Mantel-Haenszel χ^2_1 test of correlation was used to determine whether the intensity of STAT5A immunostaining of PCas was associated with the GS of the cancers. Fisher's exact test was used for analysis of the rate of *STAT5A* locus amplification in Gleason grade 3/3 versus 4/4 PCas. For comparison of the tumor volumes between the control group and the group in which the tumors overexpressed STAT5A gene, mixed-effects linear regression analysis was used. Data were log10 transformed to better meet assumptions of normality and constant residual variance. Fixed effects were included for the treatment groups (LacZ versus STAT5A), time, and their interaction. Time was treated as a categorical variable. A first-order autoregressive structure was assumed for the residual variance covariance matrix. The interaction P value was <0.0001, indicating that the difference between groups differed by time. A geometric mean ratio of 1 indicates that the (geometric) mean tumor volumes in the two groups are the same. Significant differences were observed at measurements on days 20 ($P = 0.006$), 27 ($P = 0.004$), 41 ($P = 0.0007$), and 55 ($P = 0.025$). Mixed linear regression analysis was used for comparing Ki-67 or apoptotic cell numbers in LacZ- versus STAT5A-overexpressing prostate tumors. Percentages were logit-transformed before analysis.

Results

HCNG of *STAT5A/B* Genes at 17q21 in Primary Organ-Confined PCas

To determine whether the *STAT5A/B* gene locus undergoes copy number changes in PCa, we used FISH analysis of PCa cell lines and formalin-fixed, paraffin embedded tissue sections of benign prostate hyperplasias and primary organ-confined PCas. First, FISH analysis of metaphase chromosomes prepared from three PCa cell lines using the *STAT5A/B* probe showed two copies of the gene in CWR22Rv1 cells (diploid chromosome content), three copies in DU145 cells (triploid chromosome content), and four copies in LNCaP cells (tetraploid chromosome content) (Supplemental Figure S1). *STAT5A/B* FISH analysis of paraffin-embedded tissue sections of nine benign prostate hyperplasias showed a diploid pattern in all benign prostate hyperplasia samples (Supplemental Figure S2 and Table 1). In organ-confined PCas ($n = 108$), 19% of the cancers showed amplification of the *STAT5A/B* locus. Notably, analysis of whole sections of PCas indicated that the amplification of *STAT5A/B* locus was focal. Figure 1, A and B, shows examples of PCa sections with amplification at the *STAT5A/B* gene locus versus PCas with the *STAT5A/B* diploid pattern (Figure 1C).

STAT5A/B Gene Amplification Is Clustered to PCas of High Histologic Grade

To determine the distribution of HCNG of the *STAT5A/B* gene locus in PCas of different histologic grades, the frequency of *STAT5A/B* gene amplification was analyzed in organ-confined PCas, which were well differentiated (GS 6, $n = 31$), moderately differentiated (GS 7, $n = 42$), or poorly differentiated (GS 8/9, $n = 35$) (Table 1), by FISH. The *STAT5A/B* gene amplification status differed significantly by GS ($P = 0.0017$). As shown in Figure 1D, the percentage of cases with gene amplification was 40% in high-grade (GS 8/9) PCas, 12% in moderately differentiated PCas (GS 7), and 3% in well-differentiated (GS 6) PCas. Pairwise comparisons of logistic regression analysis indicated that the *STAT5A/B* gene locus was amplified in PCas of GS 8/9 significantly more frequently compared with PCas of GS 6 ($P = 0.0053$) or GS 7 ($P = 0.0067$). GS 6 and GS 7 PCas did not differ with respect to percentage of HCNG of *STAT5A/B* genes ($P = 0.21$). Of the 108 PCas analyzed, Gleason grade information was available for 38 cases, of which 13 were Gleason grade 3/3 and 7 were 4/4. The rate of *STAT5A/B* locus amplification for Gleason grade 3/3 was 7.7%, whereas for Gleason grade 4/4 it was 71.4%. This suggests that

Table 1 *STAT5A/B* Amplification Status in Clinical PCas

Amplification status	No. of patients	%
Benign prostate hyperplasia		
Positive	0	0
Negative	9	100
GS 6 primary PCas		
Positive	1	3
Negative	30	97
GS 7 primary PCas		
Positive	5	12
Negative	37	88
GS 8 or 9 primary PCas		
Positive	14	40
Negative	21	60
CR local PCa recurrences		
Positive	10	16
Negative	54	84
Distant PCa metastases		
Positive	4	19
Negative	17	81
CR distant PCa metastases		
Positive	16	29
Negatve	39	71

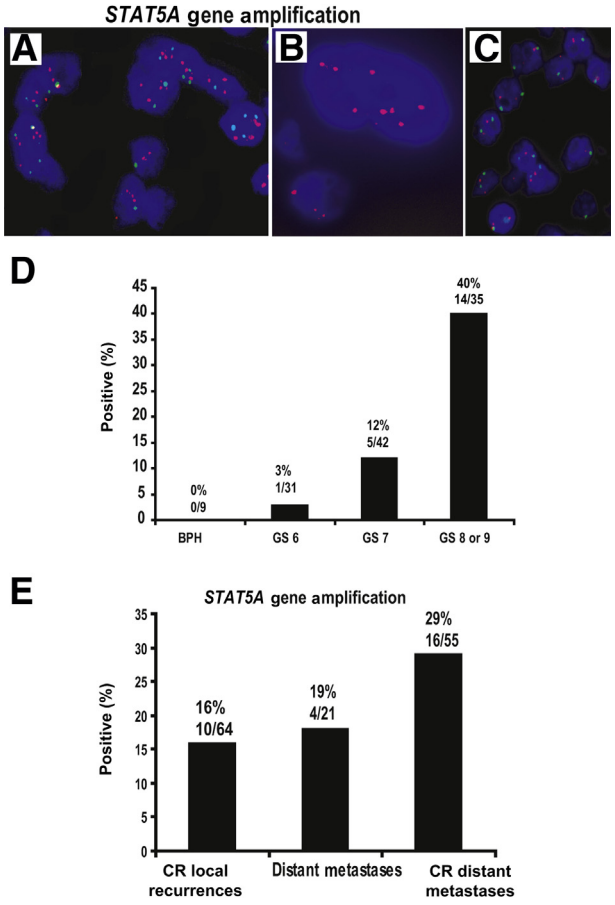

Figure 1 Amplification at the *STAT5A/B* gene locus in clinical human PCas was overrepresented in primary organ-confined PCas of high histologic grades, CR PCas, distant metastases, and CR distant metastases. **A**–**C:** HCNG of the *STAT5A/B* gene locus was more frequent in primary organ-confined PCas of high histologic grades. Paraffin-embedded tissue sections of PCas were analyzed by FISH for *STAT5A/B* gene copy numbers. Representative images of PCa sections show amplification at the *STAT5A/B* gene locus (**A** and **B**) or diploid *STAT5A/B* locus pattern (**C**). *STAT5A* copy numbers were analyzed by FISH in paraffin-embedded tissue sections of 108 organ-confined PCas of GS 6 ($n = 31$), GS 7 ($n = 42$), and GS 8 or 9 ($n = 35$) PCas. The percentages positive for *STAT5A* gene amplification are shown above each column (**D**). *STAT5A/B* gene locus is amplified in PCas of GS 8/9 significantly more frequently compared with PCas of GS 6 ($P = 0.0053$) or GS 7 ($P = 0.0067$). BPH, benign prostate hyperplasia. **E:** Amplification at the *STAT5A/B* gene locus was frequent in CR local recurrences, distant metastases, and CR distant metastases. Paraffin-embedded tissue sections of local CR recurrences obtained by transurethral resection of the prostate ($n = 64$), distant metastases ($n = 21$), and CR PCa metastases ($n = 55$) were analyzed for *STAT5A/B* gene copy numbers by FISH. The percentages positive for HCNG of *STAT5A/B* genes are shown above each column.

STAT5A/B locus amplification may represent a marker of Gleason grade 4 PCa, which warrants further analyses in larger cohorts in the future. In summary, these results indicate that amplification at the *STAT5A/B* gene locus was clustered to primary organ-confined PCas of high histologic grade.

HCNG of *STAT5A/B* Genes Is Frequent in CR Distant PCa Metastases

The observation that increased *STAT5A/B* gene copy numbers were significantly more prevalent in high-grade PCas prompted us to investigate whether *STAT5A/B* genes undergo amplification during progression of PCa to CR and/or metastatic disease. To address this question, we first evaluated *STAT5A/B* copy numbers by FISH in a tissue microarray of specimens obtained by transurethral resection of the prostate from 64 patients with PCa with local recurrence (development of urethral obstruction) after androgen deprivation therapy (Figure 1E and Table 1). Amplification at the *STAT5A/B* gene locus was detected in 16% of the CR local PCa recurrences. Next, we analyzed *STAT5A/B* copy numbers in 14 freshly frozen CR PCas from patients who had experienced local progression of PCa during hormone therapy (Table 2) using a duplex real-time PCR-based detection method TaqMan copy number assay. The samples had been histologically examined by a pathologist for the presence of >50% cancer cells. The average time from the onset of androgen deprivation to recurrence was 40 months. The relative copy number of *STAT5A/B* was normalized to a known copy number of the RNaseP reference gene and was further compared with the calibrator DNA (genomic DNA from pooled healthy male blood) and calculated using Copycaller software version 2.0 (Applied Biosystems). Increase in *STAT5A/B* gene copy numbers was identified in 2 of 14 CR PCa recurrences (14%) (four to six copies) (Table 2).

To assess *STAT5A/B* copy number status in disseminated PCas, we analyzed paraffin-embedded tissue sections of 21 distant metastases (lymph nodes, $n = 12$; bone, $n = 9$) by FISH. The results showed HCNG of *STAT5A/B* locus in 19% of the distant metastases (Figure 1E). Finally, we evaluated *STAT5A/B* copy numbers by FISH in paraffin-embedded tissue sections of CR distant PCa metastases collected at autopsy from patients who had undergone androgen deprivation therapy and subsequently had died of end-stage metastatic PCa (Figure 1E). The *STAT5A* FISH analyses indicated that *STAT5A/B* locus was amplified in 29% of the CR metastatic lesions. Collectively, these data showed HCNG of the *STAT5A* gene locus in 14% to 16% of CR local recurrences and 29% of CR distant metastases. Note that in this study, the CR recurrences and CR distant metastases analyzed were on tissue microarrays, whereas the organ-confined PCas were individual tissue sections on regular slides. Because tissue microarrays provide significantly less tissue to be examined than whole tissue sections on individual slides, the *STAT5A* gene amplification frequency may be underestimated in local and distant CR PCa recurrences owing to limited tumor tissue examined. When emergence of *STAT5A/B* locus amplification was analyzed in the cell culture setting by comparing LNCaP cells versus C4-2B and B4 androgen-independent sublines of LNCaP cells or CWR22Pc cells after prolonged androgen deprivation (32 days) *in vitro*, no apparent amplification at the *STAT5A/B* locus was detected (Supplemental Figures S3 and S4). This may be due to lack of the presence of tumor microenvironment and the consequent critical epithelial-stromal interactions in an *in vitro* setting. Taken together,

Table 2 Amplification at the *STAT5A/B* Locus in CR PCas

Sample ID	*STAT5A/B* locus amplification	Treatment	Diagnosis to sample (months)
1	No	Orchiectomy + estrogen	22
2	No	Estrogen	29
3	No	Orchiectomy + estrogen	68
4	No	Unkown	
5	No	Bicalutamide + estrogen	60
6	No	Orchiectomy	50
7	Yes	Estrogen	27
8	No	LHRH	47
9	No	LHRH + bicalutamide	60
10	No	LHRH	15
11	No	Orchiectomy	50
12	No	LHRH	27
13	No	Orchiectomy	37
14	Yes	Orchiectomy	40

LHRH, luteinizing hormone—releasing hormone.

the data presented herein imply that *STAT5A/B* gene loci undergo amplification during the process of PCa disease progression.

STAT5A/B Gene Amplification Is Associated with Increased STAT5A/B Protein Expression in PCa

To investigate the relationship between HCNG of *STAT5A/B* gene locus and STAT5A/B protein expression in PCas, we performed quantitative fluorescent STAT5A/B IHC analysis and *STAT5A/B* FISH analysis on serial sections of paraffin-embedded tissues of primary, organ-confined PCas (Figure 2A). STAT5A/B immunostaining was quantified by AQUA.[50] Areas of diploid *STAT5A/B* status versus robust *STAT5A/B* gene amplification showed low versus high STAT5A/B protein expression, respectively (Figure 2A), as quantified by AQUA in a single PCa (Figure 2B). Analysis of a large section of each given PCa clearly indicated a focal nature of *STAT5A/B* locus amplification. In summary, the results of these experiments indicate that HCNG of the *STAT5A/B* gene locus in PCa was associated with increased STAT5A protein expression.

To further analyze whether amplification of *STAT5A/B* genes is associated with increased STAT5A/B protein expression, we analyzed serial paraffin-embedded PCa tissue sections by *STAT5A/B* FISH and IHC analyses of STAT5A/B protein in the set of 108 organ-confined PCas investigated for *STAT5A/B* locus amplification distribution versus histologic grade of cancer (Figure 2C). The specificity of the STAT5A/B antibody used for IHC studies has been demonstrated previously.[12,18–21,51–53] The immunostained tissue sections were evaluated using a quantitative scoring method, which was based on the intensity of STAT5A staining where 0 represented negative; 1, weak; 2, moderate; and 3, strong staining (Figure 2C). Immunostaining for STAT5A protein was positively associated with the GS of PCa ($P < 0.0001$), as we have previously shown in other clinical PCa materials.[19,20] Similar to *STAT5A/B* gene amplification, the percentage of PCas with high immunostaining for STAT5A increased as the GS increased, whereas the percentage of PCas with low immunostaining for STAT5A increased as the GS decreased (Figure 2C). When the frequency of HCNG of the *STAT5A/B* gene locus was evaluated in PCas with STAT5A immunostaining scores of 0, 1, 2, or 3, the presence of HCNG of the *STAT5A/B* gene locus differed significantly by STAT5A immunostaining score (logistic regression analysis, $P = 0.034$) (Figure 2D). Pairwise comparisons of the immunostaining score showed that the frequency of *STAT5A/B* gene amplification was significantly higher in the group where STAT5A protein expression was at the highest levels (score 3) compared with STAT5A protein expression score 0 ($P = 0.01$), 1 ($P = 0.02$), or 2 ($P = 0.04$) PCas (Figure 2D). Collectively, these results indicate that *STAT5A/B* gene amplification positively associated with increased STAT5A protein expression in PCa.

Increased Copy Numbers of *STAT5A/B* Genes in PCa Cells Promotes Growth of PCa Cells *in Vitro* and PCa Xenograft Tumor Growth *in Vivo*

Given that *STAT5A/B* genes at 17q21 locus are frequently amplified in high-grade clinical PCas and that STAT5A/B is critical for viability of PCa cells in culture, we hypothesized that the introduction of additional copies of the *STAT5A/B* gene will promote growth of PCa cells in culture and xenograft tumors *in vivo* in nude mice. To test this hypothesis, we cloned *STAT5A/B* into replication-deficient adenovirus (AdStat5) and verified that adenoviral expression of STAT5A resulted in increased STAT5A expression for at least 21 days (data not shown). DU145 cells were infected with AdStat5 or AdLacZ (MOI = 5) as the control group (Figure 3A), and cells were seeded the next day at different densities for clonogenic survival assay (Figure 3B). Introduction of extra copies of *STAT5A* (MOI = 5) increased the fraction of surviving cell clones by 400% to 500% on day 11 compared with control cells expressing LacZ (Figure 3B). To test if the expression of extra copies of *STAT5A/B* affected PCa xenograft tumor growth *in vivo*, DU145 cells were infected with AdStat5 (MOI = 5) 24 hours before inoculation of the cancer cells subcutaneously into the flanks of nude mice (Figure 3C). The mice had been castrated and supplemented with sustained-release DHT pellets to normalize the circulating androgen levels, and the tumor sizes were measured once per week. Introduction of additional copies of *STAT5A/B* genes promoted prostate tumor growth significantly ($P < 0.0001$) compared with the control group (AdLacZ) (Figure 3C). Specifically, size differences between tumors expressing LacZ versus tumors with additional *STAT5A/B* copies were evident at tumor size measurements on days 20 ($P = 0.006$), 27 ($P = 0.004$), 41 ($P = 0.0007$), and 55 ($P = 0.025$). To verify STAT5A protein expression levels in PCa xenograft tumors in the two treatment groups (AdStat5 versus AdLacZ), paraffin-embedded tissue

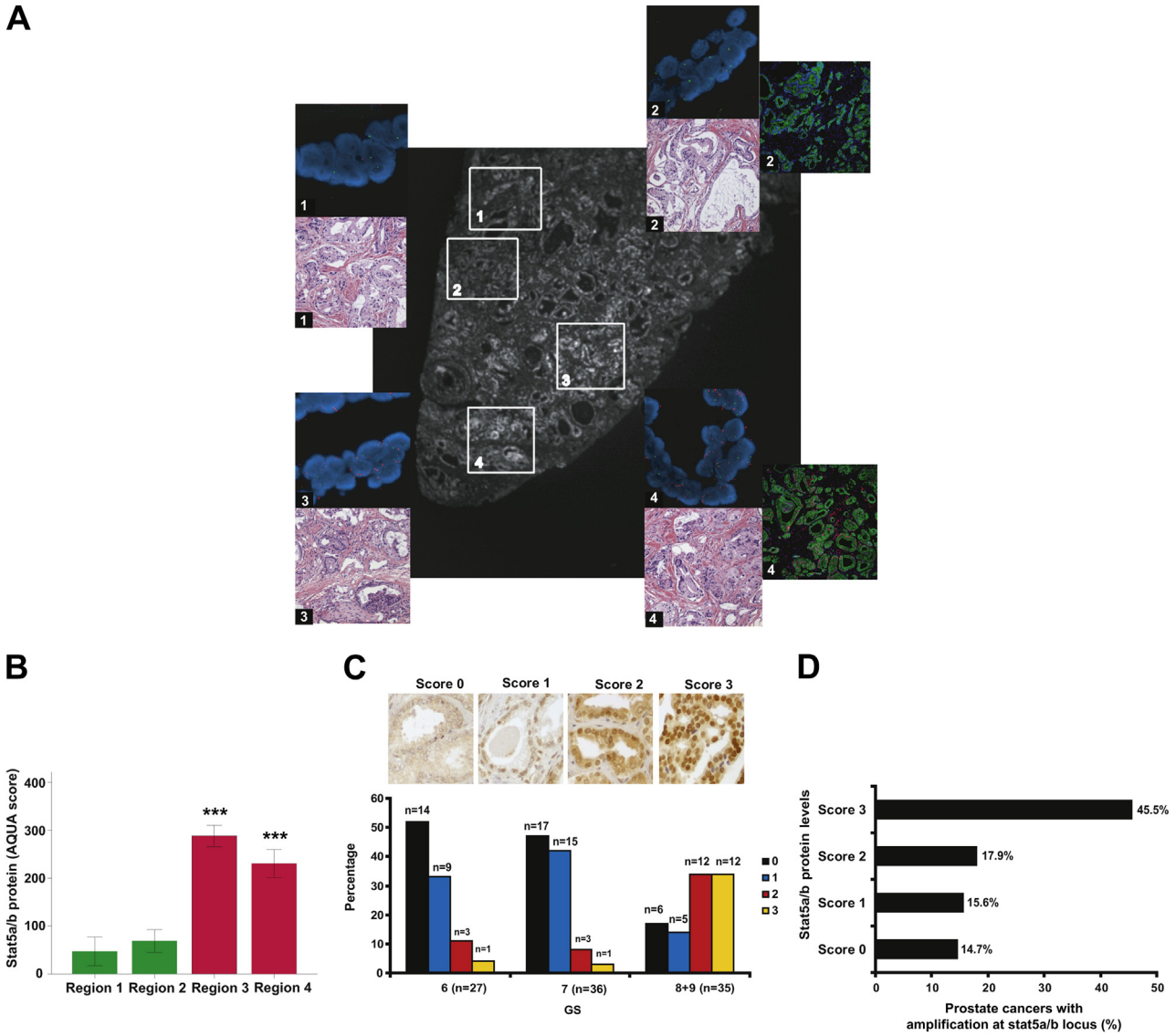

Figure 2 HCNG of the *STAT5A/B* locus positively correlates with high STAT5A protein expression in PCa. **A**: *STAT5A/B* gene amplification is overrepresented in the areas of PCa where STAT5A/B protein expression levels are high. STAT5A/B expression was analyzed in paraffin-embedded tissue sections of primary organ-confined PCas by immunofluorescence immunostaining (red) and was quantified by AQUA. In adjacent serial paraffin-embedded tissue sections, *STAT5A/B* copy numbers were analyzed by *STAT5A/B* FISH, and H&E staining of areas **1**, **2**, **3**, and **4** is also shown. **B**: Tumor regions with FISH-verified gene amplification [regions **3** ($n = 4$) and **4** ($n = 4$)] show significantly elevated STAT5A/B protein compared with regions without detectable gene amplification [regions **1** ($n = 4$) and **2** ($n = 9$); $P < 0.001$ by analysis of variance and Sheffe's post hoc test]. **C**: High STAT5A/B protein expression was associated with high histologic grade of PCa. The set of organ-confined PCas ($n = 108$) analyzed for *STAT5A* gene amplification was assayed for STAT5A/B protein levels by IHC analysis. Immunostaining scores were obtained for 98 of the 108 cases (GS 6, $n = 27$; GS 7, $n = 36$; GS 8 or 9, $n = 35$). Individual PCas were scored for STAT5A/B protein levels on a scale from 0 to 3 where 0 represented undetectable; 1, low; 2, moderate; and 3, high levels of STAT5A/B. Immunostaining for STAT5A/B was significantly more frequent in PCas of GS 8 or 9 compared with GS 7 or 6 (***$P < 0.0001$, Mantel-Haenszel χ^2_1 test). Data are given as means ± SD. **D**: Logistic regression analysis shows a high frequency of *STAT5A/B* gene amplification in PCas with high levels of STAT5A/B protein expression. HCNG of the *STAT5A* gene locus was significantly higher in the group where STAT5A protein expression was at the highest levels (score 3) compared with score 0 ($P = 0.01$), 1 ($P = 0.02$), or 2 ($P = 0.04$) PCas. *STAT5A/B* locus amplification using FISH versus STAT5A/B protein expression.

sections of the tumors were immunostained for STAT5A. As demonstrated in Figure 3D, the increase in STAT5A protein expression was robust in prostate xenograft tumors grown from DU145 cells infected with extra copies of *STAT5A* compared with the control group. The tumors were further analyzed for apoptotic and proliferating cells (Figure 3E). The number of apoptotic cells was eightfold lower in PCa xenograft tumors expressing STAT5A versus the control tumors (LacZ) ($P = 0.006$). At the same time, the number of Ki-67–expressing cells was increased by approximately twofold in STAT5A-overexpressing tumors ($P = 0.009$) versus control tumors (LacZ). In summary, these results support the functional importance of increased *STAT5A* gene expression for PCa growth in an AR-negative PCa tumor model.

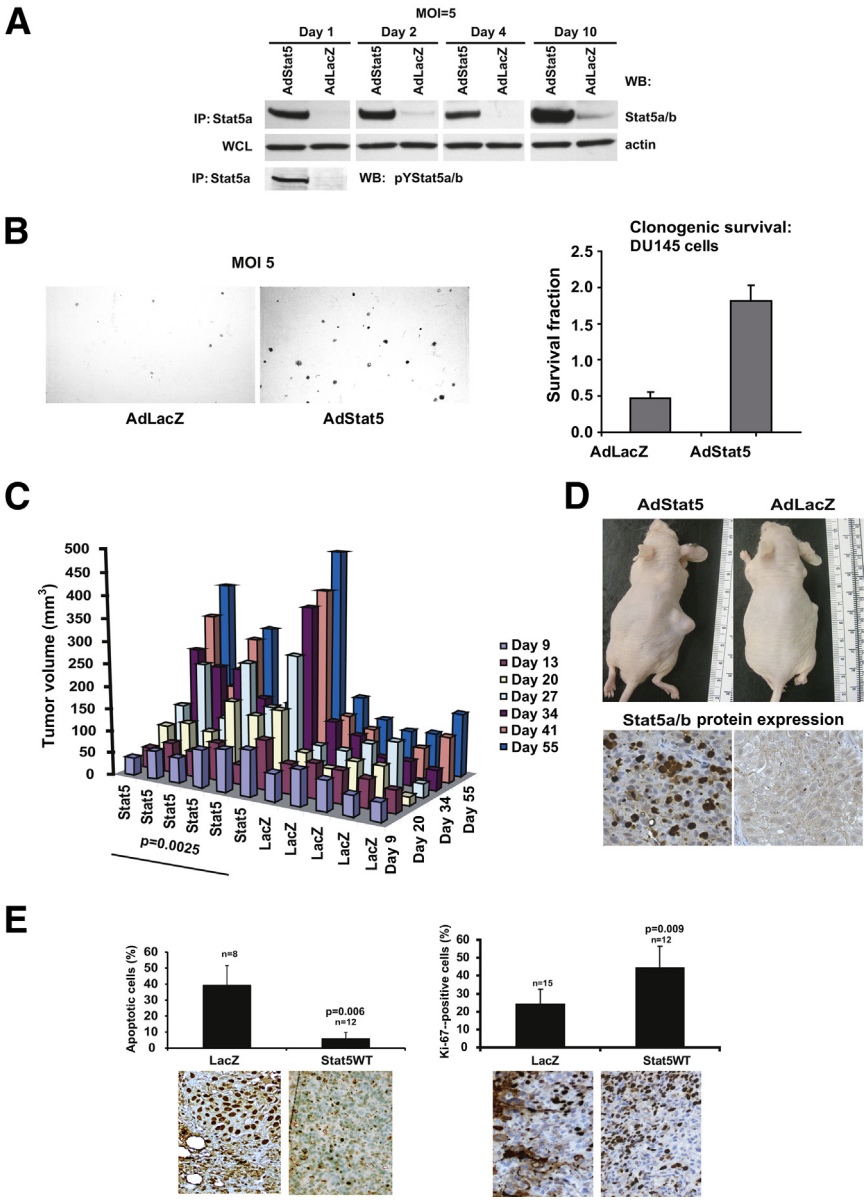

Figure 3 Increased *STAT5A* copy numbers promoted growth of PCa cells and PCa xenograft tumors in nude mice. **A:** DU145 cells were infected with AdStat5 or AdLacZ at an MOI of 5. To verify increased expression of STAT5A in AdStat5-infecetd DU145 cells, STAT5A was immunoprecipitated (IP) using anti-STAT5A polyclonal antibody at different time points and was immunoblotted (WB) with anti-STAT5A/B monoclonal antibody. WCL, whole cell lystate. **B:** For clonogenic survival assay, 50, 100, 200, and 400 cells were seeded in triplicate. After 11 days, cells were stained with 0.5% crystal violet, and colonies with >30 cells were counted. PE [(colonies counted/cells seeded) × 100] and survival fraction [(colonies counted/cells seeded) × (PE/100)] per each group were calculated. Data are given as means ± SD. **C:** Introduction of extra copies of STAT5A to DU145 human PCa cells and subsequent subcutaneous tumor growth in athymic nude mice. DU145 PCa cells infected with adenovirus-expressing STAT5A at an MOI of 5. Twenty-four hours after infection, the cells were inoculated s.c. into the flanks of castrated athymic nude mice supplied with sustained-release DHT pellets (1 tumor per mouse, 20×10^6 DU145 cells per site, 1 DHT pellet per mouse). Tumor growth was measured for 55 days. Tumor volumes were calculated using the following formula: $V = (\pi/6) \times d_1 \times (d_2)^2$, with d_1 and d_2 indicating two perpendicular tumor diameters. Mixed-effects linear regression analysis, measurements on days 20 ($P = 0.006$), 27 ($P = 0.004$), 41 ($P = 0.0007$), and 55 ($P = 0.025$). **D:** IHC analysis of STAT5A/B showed a high level of nuclear STAT5A expression in DU145 xenograft tumors overexpressing STAT5A protein. **E:** The percentage of apoptotic cells determined by TUNEL assay (**left panel**) was decreased ($P = 0.006$) and the percentage of Ki-67–positive cells (**right panel**) was increased ($P = 0.009$) in DU145 xenograft tumors overexpressing STAT5A.

Discussion

PCa typically starts as organ-confined androgen-regulated cancer that later progresses to CR and disseminated disease. The molecular mechanisms underlying uncontrolled growth and metastatic potential are unclear. STAT5A/B signaling cascade represents a therapeutic target pathway in PCa. In the present work, we demonstrate amplification at the *STAT5A/B* gene locus in clinical PCas. We show that HCNG of the *STAT5A/B* gene locus was particularly prevalent in organ-confined PCas of high histologic grades, CR local recurrences, and CR distant metastases. We further demonstrate that *STAT5A/B* gene amplification was spatially associated with high STAT5A/B protein expression and that introduction of additional copies of *STAT5A/B* to PCa cells promoted growth of the cells in culture and as xenograft tumors *in vivo*.

In this study, *STAT5A/B* copy numbers were analyzed by FISH in paraffin-embedded tissue sections and by a PCR-based copy number assay in freshly frozen PCa specimens. By FISH analysis, the *STAT5A/B* gene locus was amplified on average in 16% of the 128 organ-confined PCas analyzed, 16% of CR local recurrences, 19% of distant metastases, and 29% of CR distant metastases. In organ-confined PCas, HCNG of *STAT5A/B* genes was associated with high histologic grade of PCa. In other words, the frequency of HCNG of the *STAT5A/B* gene locus in organ-confined PCas was at the highest (40%) in poorly differentiated GS 8 or 9 PCas, whereas only 3% of well-differentiated GS 6 PCas showed an increase in *STAT5A/B* gene copy numbers. *STAT5A/B* gene copy number analysis of 14 CR local PCa recurrences by a PCR-based method indicated that 14% of the recurrences had HCNG of the *STAT5A/B* locus. To compare these results with the results of

other studies, we focused computational analysis of GEO data sets of PCas (GSE 8026, 3289, 2171, 1439, and 1390) for *STAT5A/B* gene amplification on GSE 8026[54] because it represented an analysis of genome variation profiling of DNA, whereas the others were transcriptomic databases. In the GSE 8026 database, expression levels were found for the *STAT5A* probe set for 20 localized PCas and 17 metastatic CR PCas. Two of the 17 metastatic PCas (12%) showed clear *STAT5A/B* gene amplification, whereas none of the localized PCas exhibited a copy number increase in the *STAT5A/B* locus. Note that the DNA samples analyzed in this data set (GSE 8026) were not from metastases at distant sites but from the actual PCas that had disseminated and become CR; therefore, an analysis of the distant CR metastases would potentially yield higher *STAT5A/B* locus amplification rates.[55] Analysis of the GSE 35988 database indicated a 15% rate for alterations in the *STAT5A/B* gene locus in a cohort of 61 primary and CR PCas. Future studies will need to determine the size of the amplicon at 17q.21 by array comparative genomic hybridization using chromosome 17−specific probe sets.

HCNG of the *STAT5A/B* locus in PCa was associated with high nuclear STAT5A/B expression, suggesting that *STAT5A/B* gene amplification results in increased STAT5A protein expression. This was determined in adjacent tissue sections by FISH and by AQUA analysis of STAT5A protein expression *in situ*. Moreover, in the cohort of 114 PCas where HCNG of *STAT5A/B* genes was more frequent in high GS cancers (Figure 1A), also high nuclear STAT5A protein expression was clustered to the same high GS PCas (Figure 2B). In fact, 45% of the PCas with high nuclear STAT5A expression had HCNG of the *STAT5A/B* gene locus (Figure 2D). Amplification of *STAT5A/B* gene locus in PCa may represent a mechanism that provides certain PCa cell clones an increased supply of STAT5A/B proteins to be activated by the local growth factor tyrosine kinase networks, such as Prl-Jak2, IL-6−Jak2, Src, or EGFR family.[18,56−59]

The finding of amplification at the *STAT5A/B* gene locus in PCa is important because STAT5A/B is crucial for PCa cell growth in culture and for PCa tumor growth *in vivo*.[12−15,17,27] Specifically, data from our laboratory[12−15] and others[17,27] have shown that inhibition of STAT5A/B in PCa cells by RNA interference, antisense oligonucleotides, or adenoviral expression of dominant-negative mutant of STAT5A/B triggers extensive apoptotic death of PCa cells *in vitro* and inhibits PCa tumor growth *in vivo*.[12−15] STAT5A promotes transcriptional activity of AR in PCa cells,[21] and STAT5A/B increases protein stability of the AR in PCa cells.[17] Moreover, a recent study by Sharma et al,[26] suggested that STAT5A and other members of the Stat transcription factor family may redirect AR binding site selection in clinical CR PCas, leading to altered AR transcriptome in CR PCas in patients. Nevertheless, the mechanisms underlying STAT5A induction of PCa growth likely involve AR-dependent and AR-independent pathways. This concept is supported by data showing that STAT5A/B inhibition also induces extensive death of AR-negative PCa cells in culture.[15] Herein, we provide additional data demonstrating that simulation of HCNG of the *STAT5A/B* gene locus in the AR-negative DU145 prostate xenograft tumor model by introduction of additional *STAT5A/B* gene copies using adenovirus as an expression vector resulted in significant promotion of PCa tumor growth in nude mice accompanied by decreased apoptotic cells and increased number of Ki-67−positive cells. The results of this study point out that increased expression of STAT5A/B by the introduction of additional *STAT5A/B* gene copies critically promoted PCa cell and tumor growth through mechanisms independent of the AR.

The frequency of *STAT5A/B* gene locus amplification was high in CR distant metastases of PCas. We have shown previously that STAT5A/B induces metastastic characteristics and behavior of PCa cells in culture and promotes metastases formation in experimental metastases models in nude mice.[15] Moreover, protein levels of STAT5A/B have been shown to be increased in distant PCa metastases.[15] It is possible that HCNG of the *STAT5A/B* gene locus in PCa may provide certain PCa cells with growth advantage, metastatic potential, and increased ability to survive at the distant sites. For understanding of the full biological significance of *STAT5A/B* locus amplification in PCa, it will be crucial to determine whether *STAT5A/B* gene amplification in PCa predicts early recurrence, development of metastatic disease, and PCa-specific death.

In summary, the findings presented in this work show, for the first time, HCNG of the *STAT5A/B* locus in clinical PCas. This finding is important because the JAK2-STAT5A/B signaling pathway represents a therapeutic target for PCa, and pharmacologic inhibitors of this pathway are currently entering clinical trials for PCa. Ongoing work aims to determine whether *STAT5A/B* amplification in PCa predicts the responsiveness of a given cancer to inhibitors of the JAK2-STAT5A/B pathway.

Supplemental Data

Supplemental material for this article can be found at *http://dx.doi.org/10.1016/j.ajpath.2013.02.044*

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