JAGGED1 Expression Is Associated with Prostate Cancer Metastasis and Recurrence

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Abstract
Recent studies suggest that NOTCH signaling can promote epithelial-mesenchymal transitions and augment signaling through AKT, an important growth and survival pathway in epithelial cells and prostate cancer in particular. Here we show that JAGGED1, a NOTCH receptor ligand, is significantly more highly expressed in metastatic prostate cancer as compared with localized prostate cancer or benign prostatic tissues, based on immunohistochemical analysis of JAGGED1 expression in human tumor samples from 154 men. Furthermore, high JAGGED1 expression in a subset of clinically localized tumors was significantly associated with recurrence, independent of other clinical parameters. These findings support a model in which dysregulation of JAGGED1 protein levels plays a role in prostate cancer progression and metastasis and suggest that JAGGED1 may be a useful marker in distinguishing indolent and aggressive prostate cancers.

Introduction
The androgen receptor plays a critical role in prostate development (1). Withdrawal of androgens leads to a rapid decline in prostate cancer growth with significant clinical response, but this response is brief as tumor cells rapidly emerge, which are refractory to hormonal manipulation. Recent work from Chen et al. (2) suggests that increases in androgen receptor mRNA and protein are both necessary and sufficient to convert prostate cancer cells to a hormone-refractory state. Their model proposes that increased androgen receptor expression permits continued signaling even in the face of low levels of agonist ligands, and thereby limits the effectiveness of surgical or chemical castration. Thus, characterization of the androgen receptor ligand-response repertoire may identify downstream targets that contribute to the development of the hormone refractory state. Using a high throughput quantitative proteomic technique, Martin et al. (3) recently identified JAGGED1, a NOTCH receptor ligand, as a highly androgen-responsive gene product in the prostate cancer cell line LNCaP. Dysregulation of the NOTCH signaling pathway, which normally regulates many cell fate decisions, has been described in human T-cell leukemias and mucosideropidermoid carcinoma (4) and has been recently proposed to play a role in epithelial-mesenchymal transitions in cancer (5). In the current study, we describe for the first time the increased expression of JAGGED1 protein in human prostate tumors and its significant association with prostate cancer progression and metastasis.

Materials and Methods
Cell Culture. LNCaP cells (American Type Culture Collection, Manassas, VA) were grown in RPMI 1640 without phenol red (Sigma, St. Louis, MO), supplemented with 10% fetal bovine serum (BioWhittaker, Walkersville, MD), 2 mmol/L l-glutamine, 50 units/ml penicillin, and 50 µg/ml streptomycin. Cells were maintained in a 5% CO2, 95% air-humidified atmosphere at 37°C and cultured in phenol red-free medium with 5% stripped fetal bovine serum (BioWhittaker) 48 hours before the experiments. The cells were plated on 100-mm dishes with 50% density. Forty-eight hours later, the cells were treated with either vehicle control or 1 nmol/L synthetic androgen R1881 (New England Nuclear, Perkin Elmer, Boston, MA). All other cell lines were grown in DMEM containing 10% FCS, 2 mmol/L glutamine, 100 units/ml penicillin G, and 100 µg/ml streptomycin at 37°C under 5% CO2.

Immunoblot Analyses. Washed cells were homogenized in lysis buffer containing 50 mmol/L Tris-HCl (pH 7.4), 1% NP40 (Sigma), and complete protease inhibitor mixture (Roche, Nutley, NJ). Protein concentrations were determined with the Bradford assay (Bio-Rad, Hercules, CA). Protein extracts were mixed with SDS sample buffer and electrophoresed in 10% SDS-polyacrylamide gels under reducing conditions. The separated proteins were transferred onto nitrocellulose membranes (Amersham Pharmacia Biotech, Piscataway, NJ), which were incubated for 1 hour in blocking buffer (TBS containing 0.1% Tween and 5% nonfat dry milk). The blots were incubated in blocking buffer with either affinity-purified JAGGED1 goat polyclonal antibody (AF1277, R&D Systems, Minneapolis, MN) at a 1 µg/ml concentration in blocking buffer overnight at 4°C or rabbit anti-JAGGED1 (H-114, Santa Cruz Biotechnology, Santa Cruz, CA) at 1:200 for 1 hour. After washing three times with TBS with 0.1% Tween buffer, the membrane was incubated with horseradish peroxidase-linked rabbit antigoat IgG antibody (Zymed, South San Francisco, CA) or goat antirabbit antibody (DAKO USA, Carpinteria, CA) at a 1:5,000 dilution for 1 hour at 25°C. The signals were visualized with the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech) and autoradiography. Some blots were restained with rabbit antiprotein-specific antigen antibody (DAKO USA) for 2 hours or with rabbit anti-β-tubulin antibody (1:1,000 dilution; Santa Cruz Biotechnology).

Patient Population, Tissue Collection, and Tissue Microarray Construction. Samples from 236 patients with benign, high-grade prostatic intraepithelial neoplasia and localized and metastatic prostate cancer were obtained from the rapid autopsy program and the radical prostatectomy series, respectively, of the University of Michigan Prostate Cancer SPORE Tissue Bank and assembled on tissue microarrays as described previously (6, 7). A subset of 95 men with clinically localized prostate cancer was evaluated for associations between JAGGED1 expression levels and tumor recurrence (as judged by a rise in prostate-specific antigen level). A total of 1,247 tissue microarray cores were evaluated.

Immunohistochemical Analysis. Immunohistochemistry was done on 5-µm sections prepared from paraffin-embedded tissue microarrays. Slides were successively soaked in xylene; passed through graded alcohols; washed in distilled water; pretreated with 10 mmol/L citrate (pH 6.0; Zymed) in a steam pressure cooker (Decloaking Chamber, BioCare Medical, Walnut Creek, California) at 121°C for 60 minutes; and rehydrated. Incubated with the specific antigen antibody (DAKO USA) for 2 hours or with rabbit antiprotein-specific antigen antibody (DAKO USA) for 2 hours or with rabbit anti-β-tubulin antibody (1:1,000 dilution; Santa Cruz Biotechnology). The slides were then washed three times with TBS with 0.1% Tween buffer, the membrane was incubated with horseradish peroxidase-linked rabbit antigoat IgG antibody (Zymed, South San Francisco, CA) or goat antirabbit antibody (DAKO USA, Carpinteria, CA) at a 1:5,000 dilution for 1 hour at 25°C. The signals were visualized with the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech) and autoradiography. Some blots were restained with rabbit antiprotein-specific antigen antibody (DAKO USA) for 2 hours or with rabbit anti-β-tubulin antibody (1:1,000 dilution; Santa Cruz Biotechnology).
characterization of commercially available JAGGED1 antibodies on Western blots containing whole cell protein extracts from diverse cell types, including LNCaP cells. Immunostaining with a polyclonal rabbit antibody raised against the cytoplasmic domain of JAGGED1 revealed a strong cross-reactive protein of the expected size of JAGGED1 only in LNCaP cells (Fig. 1A, lane 14), human umbilical vein endothelial cells (Fig. 1A, lane 10 and 11), and control 293 cells transfected with a pcDNA3 expression plasmid encoding JAGGED1 (Fig. 1A, lane 2). The levels of JAGGED1 in LNCaP cells increased substantially after incubation with the synthetic androgen analog R1881 for 1 to 3 days (Fig. 1B), as judged by stains done with a second polyclonal JAGGED1 antibody raised in goats, confirming that JAGGED1 increases in LNCaP cells stimulated with androgen (3). Based on pilot studies conducted with control cells embedded in paraffin (data not shown), we elected to use the polyclonal rabbit anti-JAGGED1 antibody for immunohistochemical staining of paraffin sections.

**JAGGED1 Protein Expression Is Associated with Prostate Cancer Progression.** To study the expression of JAGGED1 in human prostate specimens, immunostaining was done on high-density tissue microarrays containing benign prostatic tissues, localized untreated prostatic adenocarcinomas, and hormone-naïve and -refractory metastatic tumors. Two samples from 18 different patients imprinted in duplicate were represented in these tissue microarrays (giving a total of 4 measurements per patient and 72 per category). The staining intensity of JAGGED1 was measured and quantified with the Chromavision Automated Cellular Imaging System II. For each patient a minimum, maximum, and mean staining intensity were measured for the 4 samples.

Representative examples of staining for JAGGED1 in benign prostatic tissue, localized cancer, and metastatic tumor are shown in Fig. 2A. Mean JAGGED1 staining intensity was increased significantly in clinically localized prostate cancer (score = 94.2; SE = 1.8) versus benign prostate tissue (score = 79.6; SE = 2.8; P < 0.001) and again in metastatic tumor (score = 127.5; SE = 4.6) as compared with either clinically localized prostate cancer (P < 0.001) or benign prostate tissue (P < 0.000). There was no significant difference between the mean JAGGED1 staining between hormone naïve (score = 126.2; SE = 3.8) and hormone refractory metastatic prostate cancer (score = 129.1; SE = 9.7; P = 0.79). The data for mean intensity measurements is summarized with error bars with 95% confidence intervals in Fig. 2B. The findings were unchanged when minimum or maximum staining intensities were evaluated. The tissue microarray

![Immuno blot analysis of JAGGED1 expression.](image-url)

*Fig. 1. Immunoblot analysis of JAGGED1 expression. A, rabbit anti-JAGGED1 antibody. Samples used to test the specificity of this antibody included whole cell extracts prepared from cell lines derived from human B cells (BJAB), murine T cells (T6E), and human T-cell lines (TALL-1, ALL-SIL, K3P, DND-41, P12-Ichikawa, and HPB-ALL), human kidney (293T cells), Ewing’s sarcoma (RDES), hormone-responsive human prostate cancer cells (LNCaP), cervical carcinoma (HELA), and neuroblastoma (IMR32). Extracts from primary human umbilical vein endothelial cells (HUVEC), and control human kidney 293T cells transfected with a JAGGED1 expression plasmid were also analyzed. Twenty-five micrograms of protein were loaded per lane. B, goat anti-JAGGED1 antibody. LNCaP cells were treated with synthetic androgen R1881 for 1 to 3 days before extracts were prepared. After JAGGED1 staining, the same blot was subsequently restained for prostate-specific antigen and β-tubulin.*
High JAGGED1 Expression Is Associated with Prostate Cancer Recurrence after Radical Prostatectomy for Clinically Localized Disease. The increased expression of JAGGED1 in localized prostate cancer and the additional up-regulation in metastatic tumor suggested that JAGGED1 might be a useful tissue biomarker to facilitate differentiating indolent from more aggressive prostate cancer. To examine this possibility, an association was sought between JAGGED1 levels and prostate cancer recurrence, defined as an increase in prostate-specific antigen of 0.2 ng/ml after radical prostatectomy or the development of overt metastatic disease. JAGGED1 immunostaining was done on 3 previously validated tissue microarrays. Analysis was restricted to a subset of patients (n = 95) with clinically localized prostate cancer and 3 or more interpretable tissue cores. Patient demographics, which are representative of the total tissue microarray cohort, are presented in Table 1. Among these 95 patients, 26 (27.4%) experienced prostate-specific antigen failure as of January 2004.

The results of univariate analysis are shown in Table 2. Consistent with prior studies, Gleason score, preoperative prostate-specific antigen, extraprostatic extension, seminal vesicle invasion, positive surgical margins, and digital rectal examination were all significantly associated with prostate-specific antigen failure at the univariate level. Interestingly, in univariate analysis, a strong association between prostate-specific antigen recurrence and high levels of JAGGED1 staining was found (relative risks of 2.55 with 1.55 intensity cutoff, and 3.05 with 1.8 intensity cutoff, respectively; Fig. 2C). It should be noted that significant P values were found for JAGGED1 only when maximum intensity values for each patient were used. The variation in staining across cores is consistent with the heterogeneous nature of prostate cancer and suggests that broad tissue sampling may be needed to maximize the predictive power of JAGGED1 staining. A Kaplan-Meier analysis depicting the association between JAGGED1 (cutoff of 1.8) and the probability of prostate-specific antigen free survival is shown in Fig. 2D.

Table 1. Clinical demographics of 95 men with localized prostate cancer

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>n</th>
<th>%</th>
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<tbody>
<tr>
<td>Median age (years)</td>
<td>59</td>
<td>53.3</td>
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<tr>
<td>Preoperative PSA (ng/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; = 4 and &lt; 10</td>
<td>17</td>
<td>17.9</td>
</tr>
<tr>
<td>&gt; 10</td>
<td>56</td>
<td>58.9</td>
</tr>
<tr>
<td>Digital rectal examination</td>
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<td></td>
</tr>
<tr>
<td>Negative</td>
<td>54</td>
<td>56.8</td>
</tr>
<tr>
<td>Positive</td>
<td>41</td>
<td>43.2</td>
</tr>
<tr>
<td>Gleason score</td>
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<td></td>
</tr>
<tr>
<td>6-8</td>
<td>35</td>
<td>36.8</td>
</tr>
<tr>
<td>7</td>
<td>55</td>
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<td>3</td>
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<td>Positive</td>
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<td>32.6</td>
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<td>94.7</td>
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<tr>
<td>Positive</td>
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<td>5.3</td>
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<tr>
<td>Extraprostatic extension</td>
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<tr>
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<td>15.7</td>
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<tr>
<td>No</td>
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<td>72.6</td>
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<tr>
<td>Yes</td>
<td>26</td>
<td>27.4</td>
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</table>

Abbreviation: PSA, prostate-specific antigen.

8 http://rubinlab.tch.harvard.edu/supplemental_data/JAGGED1/index.jsp
The best multivariate model predictive of prostate cancer specific recurrence after radical prostatectomy for clinically localized prostate cancer included extraprostatic extension ($P = 0.0005$; relative risk = 3.94), preoperative prostate-specific antigen ($P = 0.042$; relative risk = 1.97), and JAGGED1 (maximum intensity values were used; $P = 0.016$; relative risk = 3.51). Hence, high JAGGED1 protein level is a strong independent predictor of prostate cancer recurrence.

**JAGGED1 Expression in Other Cancers.** On the basis of these observations, we assessed JAGGED1 expression in other tumors with ONCOMINE II, a bioinformatics platform that provides a compendium of microarray data (9). We interrogated 90 cancer types by ONCOMINE II, a bioinformatics platform that provides a compendium of microarray data (9). We interrogated 90 cancer types (6), and are modulated by androgen in the ventral prostate of orchiectomized rats (15) and in LNCaP cells (16). Of additional interest, JAGGED1 transcript are also increased in metastatic human tumors which we report here. It will be of interest to investigate whether increased levels of JAGGED1 correlate with NOTCH receptor activation in prostate cancer and other tumor types and to determine whether such tumors are sensitive to inhibitors of NOTCH signaling. Conversely, other studies have suggested the existence of a "reverse" JAGGED1 signaling pathway that activates the transcription factor AP-1 (19) and contributes to transformation of RKE cells in culture (20). Our findings lay the groundwork for exploration of the importance of canonical JAGGED1/NOTCH signaling and possible NOTCH-independent JAGGED1 signaling in prostate cancer.

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