Class III β-Tubulin Expression Predicts Prostate Tumor Aggressiveness and Patient Response to Docetaxel-Based Chemotherapy

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Abstract

Expression of class III β-tubulin (βIII-tubulin) correlates with tumor progression and resistance to taxane-based therapies for several human malignancies, but its use as a biomarker of tumor behavior in prostate cancer (PCa) remains largely unexplored. Here, we describe βIII-tubulin immunohistochemical staining patterns of prostate tumors obtained from a broad spectrum of PCa patients, some of whom subsequently received docetaxel therapy for castration-resistant PCa (CRPC). Elevated βIII-tubulin expression was significantly associated with tumor aggressiveness in PCa patients with presumed localized disease, as it was found to be an independent marker of biochemical recurrence after treatment. Additionally, βIII-tubulin expression in tumor cells was an independent predictor of lower overall survival for patients receiving docetaxel-based chemotherapy for CRPC. Manipulation of βIII-tubulin expression in human PCa cell lines using a human βIII-tubulin expression vector or βIII-tubulin small interfering RNA altered cell survival in response to docetaxel treatment in a manner that supports a role for βIII-tubulin expression as a mediator of PCa cell resistance to docetaxel therapy. Our findings suggest a role for βIII-tubulin as candidate theranostic biomarker to predict the response to docetaxel-based chemotherapy as well as to target for treatment of docetaxel-resistant CRPC.

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Introduction

Prostate cancer (PCa) is the most common solid malignancy and the second leading cause of death attributable to cancer in men (1). Despite the widespread use of prostate-specific antigen (PSA) testing to screen for early-stage PCa, men continue to be diagnosed with locally advanced or metastatic disease, and ~30% of newly diagnosed patients treated with curative intent will eventually relapse during follow-up (2). Treatments at this advanced stage usually include androgen deprivation therapy to deplete systemic androgens in patients, thus reducing the levels of a known PCa growth factor. This treatment is most often only transiently effective because prostate tumor cells can progress to a seeming androgen-independent (AI) growth phase now diagnosed as castration-resistant PCa (CRPC; ref. 3). Once at this stage, docetaxel-based chemotherapy has been shown to improve, although the survival advantage is relatively limited (4, 5). At this time, several molecular markers have been proposed to have dependent or independent utility for PCa patient prognostic assessments (6, 7), but these latter biomarkers remain unproven. Here, we discuss our efforts to evaluate the potential utility of class III β-tubulin for purposes of prognostication of PCa patient response to early, localized therapy or to late-stage, taxane-based chemotherapy.

Taxanes constitute an important group of chemotherapeutic agents that specifically target the β-tubulin subunit of microtubules. By targeting microtubule activity, taxanes can block cell mitosis and induce apoptosis, especially in tumor cells (8). A growing body of preclinical and clinical data now suggests that increased expression of one particular β-tubulin isoform, class III β-tubulin (βIII-tubulin), confers cancer cell resistance to taxanes (9–11) and that βIII-tubulin resistance to taxanes is clinically relevant for human lung, breast, and ovarian cancers (12). For these human malignancies, high tumor cell expression of βIII-tubulin was associated with significantly poorer survival rates in patients treated with taxane-based chemotherapy (13–17). For lung cancer, one study suggested that tumor cell βIII-tubulin expression
was a prognostic factor for men who did not receive adjuvant chemotherapy (18).

For PCa, despite evidence from in vitro studies showing that β-tubulin isotype expression was altered in paclitaxel-resistant cells, there has been only limited informative clinical data evaluating the prognostic or predictive value of βIII-tubulin expression in patient tumor cells (19, 20). Previously, we showed that expression of βIII-tubulin was increased in CRPC and that expression of this tubulin isoform might have a role in progression to CRPC (21). The aim of the present study was to determine whether βIII-tubulin expression might have prognostic value for hormone-naive PCa (HNPC) patients treated by surgery or for CRPC patients treated with taxane-based therapy (docetaxel). Here, we evaluated clinical PCa specimens for expression levels of βIII-tubulin by immunohistochemistry and manipulated βIII-tubulin expression in PCa cell lines to determine the effects of this manipulation on in vitro responsiveness to docetaxel.

Materials and Methods

Cell culture

Human PCa cell lines LNCaP (clone FGC), 22Rv1, and DU145 were obtained from the American Type Culture Collection. Cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin. For this study, low-passage cells were used (<20 passages). The LNCaP-AI variant (passages 16–20) was derived from LNCaP cultures maintained >18 months of growth in androgen-depleted medium (phenol red–free RPMI 1640 supplemented with 10% charcoal-stripped FBS). LNCaP and 22Rv1 were authenticated through cell morphology monitoring, response to androgen treatment, and expression of androgen receptor (AR), which harbor mutations in these lines (22–24). DU145 was authenticated by lack of AR expression, expression of mutant TP53 (24), and assessment of the invasive behavior using Boyden chamber assays.

Western blot analysis

Protein lysates were prepared in the radioimmunoprecipitation assay lysis buffer supplemented with protease inhibitor cocktail (Roche Diagnostics) and phosphatase inhibitors (25 mmol/L orthovanadate and 50 mmol/L NaF; Sigma-Aldrich). The total protein concentration of the soluble extract was determined using the bicinchoninic acid kit (Sigma-Aldrich). Each protein sample (30 μg) was resolved to SDS-PAGE, transferred onto a polyvinylidene difluoride membrane (Millipore), and incubated with a monoclonal antibody against βIII-tubulin (1:10,000; clone TUJ1; Covance) or β-actin (1:16,000; AC-15; Sigma-Aldrich). Primary antibodies against the other β-tubulin isotypes were from Abcam. Immune complexes were visualized by enhanced chemiluminescence detection (ECL Plus kit, GE Healthcare).

cDNA synthesis and real-time PCR

Quantitative PCR was carried out using SYBR Green dye on an Applied Biosystems 7000 Real Time PCR system. The conditions for reverse transcription-PCR (RT-PCR) have been described previously (25). The amount of βIII-tubulin mRNA levels relative to the housekeeping gene ribosomal protein, large, P0 (RPLP0) was determined on the basis of the comparative threshold cycle Cₜ method (2⁻ΔΔCₜ). The primer sequences for βIII-tubulin and RPLP0 have been described previously (25, 26).

Construction of βIII-tubulin expression vector and generation of stable βIII-tubulin–overexpressing cells

The open reading frame (ORF) encoding βIII-tubulin was purchased from Invitrogen (Ultimate ORF clone, clone ID IOH3755, NM 006082). The βIII-tubulin ORF was provided in the Gateway entry vector pENTR 221. The βIII-tubulin expression was constructed by recombining the βIII-tubulin pENTR 221 into the destination vector pcDNA 3.2/V5-DEST (Invitrogen) via the LR reaction according to the manufacturer’s instructions (Gateway LR clonase II enzyme mix, Invitrogen). The resulting vector was designated as pcDNA-TUBB3. LNCaP and DU145 cells were seeded at the density of 1.5 × 10⁵ in 100-mm culture dishes in RPMI 1640 supplemented with 10% FBS. The next day, cells were transfected with 4 μg of pcDNA-TUBB3 vector using Lipofectamine 2000 reagent (Invitrogen) following the manufacturer’s instructions. Cells were selected in genetin (G418, 400 μg/mL) for 3 weeks. Resistant colonies were isolated and allowed to grow as monoclonal population. Clones expressing the different levels of βIII-tubulin were selected, as determined by Western blot analysis, for further studies.

Small interfering RNA transfection

Small interfering RNA (siRNA) against TUBB3 and control nontargeting siRNA were obtained from Invitrogen, Inc. Three specific Stealth RNAi sequences were tested: TUBB3HS115886 (5′-GACAUCUCUUCAAGCCUGAACAUUU-3′), TUBB3HS115887 (5′-GCAUAGAACCUCACUCGCUCCG-3′), and TUBB3HS175392 (5′-CAGCUUGAGCCAGAUACGCGUCACU-3′). The nonsilencing control siRNA, which has no sequence homology to any known human gene sequence, was used as a control for nonsequence-specific effects in all experiments.

Subconfluent human prostate cells were transfected with siRNA by using Lipofectamine 2000 (Invitrogen) following the manufacturer’s instructions. Seventy-two hours after the transfection, the efficacy of the siRNA knockdown was assessed by quantitative RT-PCR and immunoblotting. The optimal amount of siRNA used for transfection was determined as being 50 nmol/L, and the best siRNA sequence allowing to reduce >70% of βIII-tubulin expression was identified as the sequence TUBB3HS115887.

Docetaxel dose-response curve

To assess the effect of βIII-tubulin overexpression on chemoresistance, 1 × 10⁴ cells were seeded in 96-well microtiter plates. The next day, cells were treated with docetaxel at growing concentrations for 72 hours. Cell viability was determined by MTT assay.

To assess the effect of the combination treatment of βIII-tubulin silencing plus docetaxel, 22Rv1 cells were transfected with 50 nmol/L of Stealth siRNA against βIII-tubulin or
IC50 values were defined as the concentration of drug surviving cells versus the concentration of docetaxel. The then determined by MTT assay.

The cell survival curve was presented as the percentage of control vector as described above and then treated with docetaxel at various concentrations for 3 days. Cell viability was then determined by MTT assay.

The cell survival curve was presented as the percentage of surviving cells versus the concentration of docetaxel. The IC50 values were defined as the concentration of drug required for 50% cell survival, and were calculated using a logarithmic regression. Results were expressed as means ± SE. Each assay was done in triplicate and repeated on three separate experiments.

**Patients and tissue samples**

Written informed consent was obtained. The study included 258 patients who had undergone radical prostatectomy for localized PCas between November 1988 and May 2007. Demographics, clinical and biological data, pathologic parameters, and outcomes in terms of PSA were collected prospectively in a database and reviewed in a retrospective manner. Clinical, biological, and pathologic parameters and follow-up data are listed in Table 1. No patient had received neoadjuvant therapy.

The study also included 37 patients with CRPC stage and analyzable initial hormone-naive tissue who received docetaxel-based chemotherapy as first-line treatment between January 2002 and July 2008. Demographics, clinical and biological data, pathologic parameters, and outcomes in terms of PSA were collected prospectively in a database and reviewed in a retrospective manner. The mean age was 68.1 years, and the mean PSA level was 135.0 ng/mL at PCa diagnosis. The median Gleason score was 8 at diagnosis. Bone metastases were present in 21 patients at PCa diagnosis. Docetaxel-based chemotherapy was administered for clinical and/or biochemical progression in patients with CRPC after a mean duration of androgen ablation therapy of 33.2 months. The patient’s hormone-refractory status was defined as a progressive increase in PSA level after androgen blockade. Hormonal castration had to be biologically confirmed. Chemotherapy was combined with low-dose orally administered prednisone.

Formalin-fixed, paraffin-embedded specimens were obtained from the Department of Pathology at the Henri Mondor Hospital (Créteil, France). Immunostaining was performed (a) on the initial prostatic biopsies of 37 patients who had received first-line docetaxel-based chemotherapy and (b) on tissue microarrays (TMA) for the HNPC patient’s cohort. When considering TMAs, for each PCa case, four replicate cores (diameter, 0.6 mm) were obtained from cancer foci and four additional cores were also taken from nonneoplastic areas as previously described (25).

**Immunohistochemistry (Supplementary Materials and Methods)**

Immunostaining was done on 5-μm tissue sections mounted on silane-coated slides. βIII-tubulin protein expression was evaluated using a monoclonal antibody specific for the βIII-tubulin isotype (1:500; clone TUJ1).

A numerical score was assigned for the epithelial cells of each specimen. Samples with no stained tumor cells were scored as 0. A score of 1, 2, or 3 was assigned to samples with weak, moderate, or strong staining, respectively, independently of the proportion of stained tumor cells. The proportion of immunostained tumor cells was also assessed. For TMA analysis, the mean of staining in the four neoplastic areas was considered. Only cytoplasmic staining was taken into account. All of the slides were independently evaluated by three observers (G. Ploussard, S. Terry, and Y. Allory). Observers were blinded to the patients’ adjuvant treatment, final pathologic assessment, and outcome. Interrater reproducibility was 95%. Different scores were reassessed and consensus between observers was defined. Staining intensity seen in nerves and axons served as an internal positive control. The absence of immunostaining in red cells was used as negative control (27). Photomicrographs were taken using a Zeiss Axioplan2 microscope (Carl Zeiss) from imaging platform (INSERM, U955, UPEC).

Table 1. Patient’s cohort characteristics of the HNPC cohort

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
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<tbody>
<tr>
<td>Age (y)</td>
<td>64.5 (65.0)</td>
</tr>
<tr>
<td>Median</td>
<td>65.0 (14.2)</td>
</tr>
<tr>
<td>Range</td>
<td>47.1–75.0 (9.4)</td>
</tr>
<tr>
<td>PSA (ng/mL)</td>
<td>47.1–75.0 (9.4)</td>
</tr>
<tr>
<td>Median</td>
<td>55.6 (10.0)</td>
</tr>
<tr>
<td>Range</td>
<td>61.5 (113.0)</td>
</tr>
<tr>
<td>Clinical stage, no. (%)</td>
<td>62 (24.0)</td>
</tr>
<tr>
<td>T1</td>
<td>175 (67.8)</td>
</tr>
<tr>
<td>T2 or more</td>
<td>83 (32.2)</td>
</tr>
<tr>
<td>Gleason score, no. (%)</td>
<td>123 (47.7)</td>
</tr>
<tr>
<td>6 or 7</td>
<td>73 (28.3)</td>
</tr>
<tr>
<td>≥8</td>
<td>62 (24.0)</td>
</tr>
<tr>
<td>pT stage, no. (%)</td>
<td>150 (58.1)</td>
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<tr>
<td>≥2</td>
<td>86 (33.4)</td>
</tr>
<tr>
<td>pT4</td>
<td>22 (8.5)</td>
</tr>
<tr>
<td>Extraprostatic extension, no. (%)</td>
<td>96 (37.2)</td>
</tr>
<tr>
<td>Positive margins, no. (%)</td>
<td>56 (21.7)</td>
</tr>
<tr>
<td>Seminal vesicle invasion, no. (%)</td>
<td>56 (21.7)</td>
</tr>
<tr>
<td>Positive lymph nodes, no. (%)</td>
<td>23 (8.9)</td>
</tr>
<tr>
<td>Duration of follow-up (mo)</td>
<td>61.5 (48.3)</td>
</tr>
<tr>
<td>Mean</td>
<td>61.5 (0.6–170.7)</td>
</tr>
<tr>
<td>Median</td>
<td>48.3 (0.6–170.7)</td>
</tr>
<tr>
<td>Range</td>
<td>24.0 (62)</td>
</tr>
</tbody>
</table>
Statistical analysis

Null expression (0% of stained cells) and weak to strong expression (scores 1–3 and ≥5% of stained cells) were grouped separately as dichotomic variables for statistical analysis. The Student’s t test was used for continuous data. The Mann-Whitney and Kruskal-Wallis tests were used when data were not normally distributed. Qualitative data were tested using a \( \chi^2 \) or Fisher’s test as appropriate. The Gleason score was dichotomized according to the definition of high-risk PCas as follows: Gleason score <8 versus ≥8 (2). Survival curves were generated by the Kaplan-Meier method and compared using the log-rank test. The Cox proportional hazards model was used to evaluate the independent value of βIII-tubulin expression among commonly used prognostic factors. Hazard ratios (HR) were presented with 95% confidence interval (CI).

In the CRPC cohort, the starting point of the analysis was the first cycle of chemotherapy. The PSA Working Group criteria were used to evaluate PSA responses: we chose a decrease of 75% from the baseline PSA level as the criterion for PSA response (28). The primary endpoint was overall survival (OS) defined as the time from the start of chemotherapy until death from any cause or last follow-up for censored patients. Time to progression was defined as the time between the first cycle of chemotherapy and an elevated PSA finding.

In the HNPC cohort, Biochemical recurrence-free survival (RFS) was analyzed. The day of surgery was reported as the starting point of analysis. Recurrence was defined as the first detectable elevation of PSA above 0.20 ng/mL (at least two consecutive measurements).

A value of \( P < 0.05 \) was considered statistically significant, and all \( P \) values were two-sided. SPSS 13.0 software was used for statistical analyses.

Results

Prognostic value of βIII-tubulin expression for PCa recurrence in HNPC patients treated by radical prostatectomy

We had previously reported that βIII-tubulin was expressed significantly lower in HNPC compared with CRPC. In our previous study, however, the small patient sample size in the HNPC group (\( n = 74 \)) prevented us from deriving any statistically reliable association with other patient prognostic factors (21). Here, we extended our assessment of βIII-tubulin expression to PCa-containing specimens obtained from 258 PCa patients that were treated by radical prostatectomy (Table 1). We identified βIII-tubulin expression in 43 of the 258 specimens from patients with HNPC (16.7%; Table 2). Strong βIII-tubulin immunostaining (score of 3.0) was detected in 6.2% of tumors. When βIII-tubulin expression was observed, the percentage of positively stained tumor cells showed a wide range of variability (range, 5–100%; mean, 25.8%; median, 10%). Representative examples of immunostaining are shown in Fig. 1A to C. βIII-tubulin expression was not detected in nonmalignant prostate basal or luminal epithelial cells adjacent to the tumor. Correlations of βIII-tubulin immunostaining with histoprognostic parameters are described in Table 2. We found that positive immunostaining was significantly associated with a Gleason score of ≥8 \( [P = 0.001; \text{odds ratio (OR), 3.17}] \), a primary Gleason grade of 4 or 5 \( [P = 0.013; \text{OR, 2.28}] \), a pT stage of ≥3 \( [P = 0.042; \text{OR, 1.97}] \), an extraprostatic extension \( [P = 0.028; \text{OR, 2.10}] \), and positive lymph nodes \( [P = 0.034; \text{OR, 3.05}] \). It is noteworthy that βIII-tubulin expression was also significantly associated with a high risk for biochemical recurrence \( [P = 0.029; \text{OR, 2.15}] \). The intensity of immunostaining was also correlated with the histoprognostic parameters. A strong immunostaining, defined by a staining score of 3, was also markedly associated with a Gleason score of ≥8 \( [P < 0.001], \text{extraprostatic extension} \( [P < 0.001], \text{positive surgical} \text{margins} \( [P = 0.025], \text{pT} \text{stage} \( [P < 0.001], \text{and positive lymph} \text{nodes} \( [P = 0.043] \) when compared with a null-to-moderate staining \( [\text{score, 0, 1, or 2}] \). The 3- and 5-year RFS was 84.5% and 75.4%, respectively, in patients with no βIII-tubulin expression in the prostate tumor. By contrast, the 3- and 5-year RFS was 72.4% and 59.4%, respectively, in patients expressing βIII-tubulin in prostate tumor cells. The log-rank test was significant with a \( P \) value of 0.002 (Fig. 1D). In patients with favorable pathologic features (Gleason <8, pT2 cancer, and negative surgical margin), the 5-year RFS was 91.7% in βIII-tubulin-negative patients versus 79.6% in βIII-tubulin–positive patients \( [P = 0.006] \). Furthermore, in multivariate analysis using a Cox model taking into account Gleason score, pT stage, and surgical margin status, βIII-tubulin expression was an independent predictor of biochemical recurrence \( [P = 0.029; \text{HR, 1.95}; \text{95% CI, 1.07–3.35}] \).

Increased expression of βIII-tubulin in PCa cell lines in response to in vitro docetaxel treatment

We previously reported that βIII-tubulin expression was increased in LNCaP cells (21), which were grown in androgen-depleted medium or in tumor xenografts from these cells after castration of the host mouse. In the present work, we tested whether docetaxel treatment could affect βIII-tubulin expression in two AI PCa cell lines: LNCaP-AI that expresses low levels of βIII-tubulin or 22Rv1 that endogenously expresses higher levels of βIII-tubulin. LNCaP-AI variant cells were treated with docetaxel at 3 nmol/L for up to 30 days. As shown in Fig. 2A, using Western blots to assess the levels of expressed βIII-tubulin in PCa cell lines in vitro
docetaxel, we observed a significant increase in βIII-tubulin expression in both cell lines compared with untreated controls. The increase in βIII-tubulin expression was associated with a significant decrease in androgen receptor (AR) levels, as determined by Western blot analysis. These findings suggest that docetaxel treatment may induce AR downregulation in PCa cells, potentially contributing to the clinical effectiveness of docetaxel in treating androgen-independent PCa.

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manner (23). Consistent with earlier observations, upregulation of βIII-tubulin protein (Fig. 2B; Supplementary Fig. S2A) as well as mRNA levels (Supplementary Fig. S2B) was evident in docetaxel-treated 22Rv1 cells. The βII and total protein levels were similarly increased in docetaxel-treated cells in contrast to that found for βI and βIV isotypes. Together, these data show that both acute and chronic exposure of AI PCa cells to docetaxel upregulates βIII-tubulin along with a seeming general increase of total β-tubulin.

Stable overexpression of βIII-tubulin in PCa cell lines confers resistance to docetaxel

To assess whether overexpression of βIII-tubulin was sufficient to confer resistance to docetaxel in PCa cells, we established PCa cell clones, from LNCaP or DU145 cells, stably expressing the human TUBB3 gene (βIII-tubulin–transfected clones) under the control of a cytomegalovirus promoter. The AR-negative DU145 line is derived from a brain metastasis. These cells might represent a very aggressive stage of PCa as reflected by their growth rate and their invasive behavior in vitro and in vivo (30). LNCaP cells are androgen-sensitive PCa cells originated from a lymph node metastasis. Lymph nodes are the most common and earliest sites for PCa metastasis, thus rendering this model particularly attractive for the research community (22). Differential expression of βIII-tubulin protein between control vector–transfected LNCaP cells and βIII-tubulin–transfected clones was established by Western blot analysis (Fig. 2C). Despite some decrease of βII isotype was seen in βIII-tubulin–transfected LNCaP cells (βIII-tubulin clone 1), forced expression of βIII-tubulin did not seem to affect consistently the expression of the β-tubulin isotypes. Interestingly, the βIII-tubulin–overexpressing LNCaP cells had a neuroendocrine-like appearance (Supplementary Fig. S3), suggesting that forced expression of βIII-tubulin is associated with a transdifferentiation process that has been frequently described for these cells (31, 32). To examine the effects of βIII-tubulin overexpression, we measured the half-time inhibitory concentration (IC50) of docetaxel assessed by measuring cell viability at 72 hours after exposure. The cell viability assays showed that βIII-tubulin–transfected

<table>
<thead>
<tr>
<th>Table 2. Association between βIII-tubulin expression and clinicopathologic parameters</th>
<th>Patients with no TUBB3 expression (n = 215)</th>
<th>Patients with TUBB3 expression (n = 43)</th>
<th>Univariate analysis</th>
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<tbody>
<tr>
<td>Age (y), no. (%)</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>&lt;65</td>
<td>105 (48.8)</td>
<td>19 (44.2)</td>
<td>0.577</td>
</tr>
<tr>
<td>&gt;65</td>
<td>110 (51.2)</td>
<td>24 (55.8)</td>
<td>0.399</td>
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<tr>
<td>Mean</td>
<td>64.6</td>
<td>63.8</td>
<td>0.068</td>
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<td>Clinical stage, no. (%)</td>
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<tr>
<td>T1</td>
<td>151 (70.2)</td>
<td>25 (58.2)</td>
<td>0.180</td>
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<tr>
<td>T2 or more</td>
<td>64 (29.8)</td>
<td>18 (41.9)</td>
<td>0.066</td>
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<td>PSA (ng/mL), no. (%)</td>
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<tr>
<td>&lt;10</td>
<td>119 (55.3)</td>
<td>19 (44.2)</td>
<td>0.001</td>
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<tr>
<td>&gt;10</td>
<td>96 (44.7)</td>
<td>24 (55.8)</td>
<td>3.17 (1.59–6.30)</td>
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<tr>
<td>Mean</td>
<td>13.1</td>
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<td>&lt;7</td>
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<td>≥8</td>
<td>43 (20.0)</td>
<td>19 (44.2)</td>
<td>3.17 (1.59–6.30)</td>
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<td>Primary Gleason grade 4 or 5, no. (%)</td>
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<tr>
<td>&lt;50</td>
<td>88 (40.9)</td>
<td>22 (51.2)</td>
<td>0.004</td>
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<tr>
<td>&gt;50</td>
<td>127 (59.1)</td>
<td>21 (48.9)</td>
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<tr>
<td>Mean</td>
<td>55.9</td>
<td>54.2</td>
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<td>pT stage, no. (%)</td>
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<tr>
<td>pt2</td>
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<td>74 (34.4)</td>
<td>22 (51.2)</td>
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<td>Positive surgical margins, no. (%)</td>
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<td>43 (20.0)</td>
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<td>Seminal vesicle invasion, no. (%)</td>
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<td>42 (19.5)</td>
<td>14 (32.6)</td>
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<td>Positive lymph nodes, no. (%)</td>
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<td>15 (7.0)</td>
<td>8 (18.6)</td>
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<tr>
<td>Recurrence, no. (%)</td>
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<tr>
<td>46 (21.4)</td>
<td>16 (37.2)</td>
<td>0.029</td>
<td></td>
</tr>
<tr>
<td>Duration of follow-up, mo (mean)</td>
<td>62.1</td>
<td>58.4</td>
<td>0.603</td>
</tr>
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NOTE: Clinical data, pathologic features, and PSA failure: correlations with βIII-tubulin expression in HNPC samples (univariate analysis).
LNCaP cells (βIII-tubulin clone 1 and βIII-tubulin clone 7) were significantly more resistant to docetaxel treatment than control vector–transfected LNCaP cells with a 6.6-fold increase in the IC₅₀ (33.1 versus 5.0 nmol/L; \( P < 0.001 \); Fig. 2D). A significant difference between clones and control cell survival was already achieved at 2 nmol/L docetaxel and persisted up to 50 nmol/L docetaxel treatment (\( P < 0.001 \)).

βIII-tubulin–transfected LNCaP cells were again poorly sensitive to docetaxel when higher doses were tested (Supplementary Fig. S4). Having established that βIII-tubulin has severe implications for docetaxel resistance in LNCaP cells, we sought to determine if this could be due in part to an effect of βIII-tubulin on cell proliferation. To this end, cell doubling times for vector-transfected LNCaP and βIII-tubulin transfectants were estimated at 31 and 44 hours, respectively (Supplementary Table S1). Two βIII-tubulin–transfected DU145 clones were also selected for subsequent treatment, including one clone expressing high levels of βIII-tubulin (clone 7) and one clone expressing moderate βIII-tubulin (clone 5) at a level similar to that found in parental or control vector–transfected DU145 cells (Fig. 3A). The cell viability assays showed that βIII-tubulin–transfected clone 7 was significantly more resistant to docetaxel treatment compared with clone 5 or control vector–transfected DU145 cells with a 2-fold increase in IC₅₀ (11.1 versus 5.1 nmol/L; \( P < 0.001 \); Fig. 3B). A significant difference in cell viability between clone 7 and control cells was already achieved at 5 nmol/L (\( P < 0.001 \)). There were no noticeable morphologic differences between the parental vector–transfected DU145 and either of the two sublines. Nor were there any evident differences in growth rates noted during standard passage and cell proliferation assays of the sublines compared with the vector-transfected or parental cells (Supplementary Table S1).

**βIII-tubulin silencing increases sensitivity to docetaxel**

To further ascertain a role for βIII-tubulin in chemoresistance to docetaxel, siRNAs directed against βIII-tubulin were used to knock down endogenous βIII-tubulin expression in 22Rv1 cells. The targeting siRNA with the highest reduction of βIII-tubulin was selected for further study based on RT-PCR and Western blot analysis for effects on βIII-tubulin mRNA and protein. βIII-tubulin mRNA levels were decreased by 70% using this siRNA, and βIII-tubulin knockdown was confirmed by Western blot analysis after 3 and 6 days (Fig. 3C). siRNA-mediated loss of βIII-tubulin did not seem to affect the other βIII-tubulin isotypes (Supplementary Fig. S5). An assessment of the doubling times revealed that concomitantly with the loss of βIII-tubulin on βIII-tubulin siRNA treatment, there was a trend toward an increase in cell growth (Supplementary Table S1). Docetaxel was then added to the growth medium of control cells or targeting siRNA-treated cells 72 hours after the transfection. Seventy hours later, targeting siRNA-transfected 22Rv1 cells were found to be more sensitive to docetaxel treatment at all doses tested compared with the control 22Rv1 cells. Overall, βIII-tubulin siRNA-transfected 22Rv1 cells were found to be 2-fold more sensitive to docetaxel (IC₅₀ of 4.4 versus 8.5 nmol/L; \( P < 0.001 \); Fig. 3D) than controls.
These results indicate that βIII-tubulin silencing sensitizes AI PCa cells to docetaxel.

Predictive value of early βIII-tubulin expression in a docetaxel-treated patient cohort

By examining the βIII-tubulin status in the initial prostatic biopsies of patients subsequently treated for CRPC disease with docetaxel chemotherapy, we investigated the usefulness of βIII-tubulin expression as a potential predictive tumor biomarker for response to docetaxel. Of the 37 cases, 17 were positive for βIII-tubulin (9 with moderate and 8 with strong staining), whereas 20 were negative. Representative examples of staining are shown in Fig. 4A and B. In this cohort, βIII-tubulin expression was significantly correlated with a Gleason score of >7 at diagnosis; 29.4% of βIII-tubulin–negative cancers were graded 8 or more compared with 63.2% of βIII-tubulin–positive cancers (P = 0.043). PSA responses were observed in 32% of βIII-tubulin–negative patients compared with 35% of βIII-tubulin–positive patients (P = 0.337). Patients with βIII-tubulin–positive tumors experienced shorter time to progression. Median time to progression was 4.7 months in βIII-tubulin–positive patients compared with 9.8 months in βIII-tubulin–negative patients (Breslow P = 0.149; log-rank P = 0.522).

Figure 2. Links between βIII-tubulin expression and the acquisition of docetaxel resistance in androgen-sensitive and androgen-insensitive PCa cells. A, time course expression of βIII-tubulin, total β-tubulin, and β-tubulin isotypes in LNCaP-Al cells cultivated at 3 nmol/L docetaxel. B, increased protein expression was confirmed by Western blotting: untreated 22Rv1 compared with 22Rv1 cultivated in the presence of 15 nmol/L docetaxel. C, differential expression of βIII-tubulin between control vector–transfected cells and βIII-tubulin–transfected clones was assessed by immunoblotting. A significant increase in levels of βIII-tubulin protein was observed in the βIII-tubulin–transfected LNCaP cells (βIII-tubulin clone 1 and βIII-tubulin clone 7) compared with the control vector–transfected LNCaP cells. D, dose-response curve assessing the effect of βIII-tubulin overexpression in LNCaP cells. Cell viability assays showed that βIII-tubulin–transfected LNCaP cells (clones 1 and 7) were significantly more resistant to docetaxel treatment than control vector–transfected LNCaP cells. Points, mean; bars, SE.
Overall, the median survival time for the entire cohort was 25.3 months (95% CI, 16.5–34.2), and the observed cumulative probabilities at years 1, 2, and 3 were 79.5%, 57.8%, and 43.7%, respectively. Median OS was significantly shorter for patients with βIII-tubulin–positive tumors than those with βIII-tubulin–negative hormone-naive tumors (13.5 versus 41.6 months; \( P = 0.019 \); Fig. 4C). Finally, baseline PSA levels measured before the first cycle of chemotherapy in these patients were also significantly related to OS. Multivariate analysis taking βIII-tubulin expression, baseline PSA, age, and duration of androgen deprivation therapy into account showed that βIII-tubulin expression (HR, 2.93; \( P = 0.037 \)) and baseline PSA level (HR, 4.09; \( P = 0.012 \)) were independent predictors of OS.

**Discussion**

In PCa, clinical data and histoprognostic parameters, separated or integrated into nomograms, still fail at the individual level to accurately determine the risk of biochemical or clinical relapse after local treatment (33, 34). At CRPC stage, docetaxel-based chemotherapy has proven to have some effectiveness in terms of overall response rates and survival in CRPC patients (4, 5). However, any selection of patients likely to benefit most from this form of chemotherapy is difficult and is often based simply on patient age or the presence of comorbidities in individuals.

Numerous preclinical studies have reported that the selective overexpression of βIII-tubulin constitutes an important
βIII-tubulin is associated with non-organ-confined disease, metastatic lymph nodes, and PSA failure. Thus, tumor cell βIII-tubulin expression might characterize a general subclass of PCa patients with aggressive behavior and poor prognosis. This could give reason to consider adjuvant treatments for patients with βIII-tubulin-positive tumors.

Characteristics of our HNPC patient’s cohort differed slightly from characteristics of patients who actually undergo a radical prostatectomy. The advent of PSA testing has led to a considerable stage migration with an increase of low-risk PCa. In our HNPC cohort study, we included patients who underwent radical prostatectomy since 1988. This constitutes a selection bias due to a more important proportion of high-risk PCa before the PSA era. These discrepancies may limit the study of βIII-tubulin expression, as the number of high-risk PCa decreases over time. However, we showed that the prognostic effect of βIII-tubulin remained significant in organ-confined PCa, and therefore, we posit that the βIII-tubulin is of additional value to well-established histoprognostic parameters even in the assessment of presumed low-risk PCa.

We also observed here that the βIII-tubulin expression in AI PCa cell lines was increased in response to acute or chronic exposure to docetaxel. In line with our findings, Ranganathan and colleagues (19) previously showed an increase in βIII-tubulin expression in response to paclitaxel treatment of DU145 cells. The exact mechanism by which increased expression of βIII-tubulin mediates drug resistance remains open to debate. In some instances, we found that alterations of βIII-tubulin expression were associated with changes in cell morphology and/or cell proliferation rate. Thus, there is reason to believe that these events might be key determinants of drug resistance. Additionally, evidence is accumulating that microtubules containing βIII-tubulin exhibit an aberrant dynamicity. These microtubules are less stable than microtubules composed of other β-tubulin isotypes (9, 10, 40, 41). Because the primary effect of taxane is to bind microtubules, thereby enhancing the microtubule polymerization and decreasing microtubule dynamicity, it has been suggested that βIII-tubulin-containing microtubules are more prone to overcome the suppressive effects of taxanes on microtubule dynamics. Although it should be noted that all the above studies (12, 13, 42) have focused on the assembly of purified microtubules in cell-free systems and therefore remain controversial given contradictory reports obtained in intact cells, and hence more biologically relevant, that βIII-tubulin did not intrinsically affect microtubule dynamic, at least in some instances (38, 43). Interestingly, in these last studies, the effects of paclitaxel on microtubule dynamics were altered, suggesting a role for βIII-tubulin in drug-microtubule interactions. Consistent with this, Mozzetti and colleagues (42) have reported that βIII-tubulin overexpression was the prominent mechanism of paclitaxel resistance in ovarian cancer patients compared with other mechanisms of drug resistance such as overexpression of MDR-1 and point mutations in tubulin at the binding site of paclitaxel. Although many studies have focused on paclitaxel, such characteristics might also be pertinent for docetaxel. Interestingly, recent reports further showed that

![Figure 4](image_url)

**Figure 4.** Predictive value of βIII-tubulin expression for response to docetaxel in CRPC patients. A and B, representative staining for βIII-tubulin in prostate biopsy cores: absence of staining (A) and staining positivity (B). Photomicrographs are taken at ×20 objective magnification (A and B, insets). Scale bar, 100 μm. C, OS in docetaxel-treated patients: stratification by βIII-tubulin expression in prostatic tissue (negativity versus positivity; P = 0.019, log-rank test). Expression of βIII-tubulin in PCa tissue is significantly predictive for poorer survival in docetaxel-treated patients.

![mechanism](image_url)

Mechanism for resistance to tubulin-binding agents in various cancer cell lines (19, 35–38). Several clinical studies have shown that high levels of βIII-tubulin expression in tumor cells are associated with low response rates and poorer survival in patients treated with taxane-based chemotherapies (13–17, 39). Our findings in PCa were consistent with the previous studies and highlight that the expression of
\(\beta\)III-tubulin confers resistance to microtubule-destabilizing agents such as vinorelbine in breast cancer cells (44). In the setting of non–small cell lung cancer, \(\beta\)III-tubulin was shown to affect significantly the response to both tubulin-targeting agents and DNA-damaging agents (45). The investigators proposed that \(\beta\)III-tubulin may serve as a survival factor to rescue tumor cells from death signals triggered by chemotherapeutic agents. Therefore, it is conceivable that elevated expression of \(\beta\)III-tubulin can exert similar effects in other malignancies, including PCa, and future work should explore this question.

Another interesting open question about anti-microtubule drug resistance associated with changes in \(\beta\)III-tubulin expression in cancer cells is: what is the contribution of other isotypes in response to taxane-based regimens? Renganathan and colleagues (20) have previously noted that class IVb \(\beta\)-tubulin was increased collaterally in \(\beta\)III-tubulin–transfected DU145 cells in response to paclitaxel treatment. In a separate study, wherein paclitaxel-resistant DU145 variants were selected after chronic exposure to the drug, examination of the \(\beta\)-tubulin isotype composition revealed increased expression of \(\beta\)III-tubulin, but no changes were observed for IVb \(\beta\)-tubulin. Instead, some increase in IVa \(\beta\)-tubulin expression was noted (19). Although this previous work in DU145 has suggested that \(\beta\)III-tubulin expression is upregulated in response to paclitaxel treatment, our survey using docetaxel treatment of two AI AR-positive PCa cells, 22Rv1 and LNCaP-AI, provides additional evidence that this situation might be relevant in clinical settings. These studies further indicate that expression of the class \(\beta\)I
tubulin can be altered in PCa cells, and highlight a potential role for this isotype in the emergence of docetaxel resistance. The ectopic overexpression or silencing of \(\beta\)III-tubulin resulted in a seeming specific upregulation or downregulation of the \(\beta\)II-tubulin expression, respectively. Surprisingly, under these conditions, we failed to find any significant changes in the expression of other \(\beta\)-tubulin isotypes. These instances strengthened the role of \(\beta\)III-tubulin in contributing to chemo sensitivity and illustrate the importance of monitoring the \(\beta\)-tubulin isotype profile to confirm the contribution of the target isotype in drug response studies. Although previous work has suggested that manipulation of \(\beta\)II- and IVb-tubulin expression did not cause changes in paclitaxel sensitivity of Chinese hamster ovary and lung cancer cells (46, 47), these cell lines differ from PCa cells, and future work is warranted to determine the full functional implications of each \(\beta\)-tubulin isotype in the emergence of drug resistance in PCa cells.

Our findings in experimental PCa cell lines were consistent with our clinical observations that \(\beta\)III-tubulin expression in prostate tumors correlated significantly with outcomes of docetaxel chemotherapy for CRPC patients. Patients expressing \(\beta\)III-tubulin at diagnosis had reduced survival. Thus, it is tempting to speculate that the assessment of \(\beta\)III-tubulin expression could determine which CRPC patients might benefit from taxane-based chemotherapy. Studies exploring potential molecular markers in response to chemotherapy are currently limited by the difficulty in obtaining tissues from patients with CRPC. Many changes occur during the evolution to CRPC, and cancer tissue at diagnosis does not always reflect accurately the cancer tissue in its more advanced stage. In fact, obtaining tumor tissue at CRPC stage, or before and during therapy, although rarely feasible in clinical practice, would certainly be the best way to investigate the effect of biomarker on response to therapy. Nevertheless, by examining \(\beta\)III-tubulin expression in the tumors of HNPC patients, which are easily accessible to tumor sampling, it seems possible to evaluate their response to docetaxel-based therapy once the disease has progressed to CRPC. If these findings are confirmed, the use of novel tubulin-targeted agents, such as epothilones, could be useful for \(\beta\)III-tubulin–positive PCa patients (11, 48, 49). This might also offer the opportunity for therapeutic intervention by an anti–\(\beta\)III-tubulin treatment in such patients.

Collectively, our results suggest that the functional and clinical biomarker aspects of \(\beta\)III-tubulin expression are linked. Consistent with our previous findings and other studies reporting an increase in the \(\beta\)III-tubulin isotype as a result of anti-microtubule drug treatments (19, 20, 50), it seems likely that adjuvant treatments other than the current taxane-based chemotherapy regimen may be required for this group of patients. More importantly, our results now support the idea that \(\beta\)III-tubulin expression may be linked to multiple forms of PCa: progression to recurrence in HNPC patients, progression to CRPC in hormone-treated patients, and, finally, progression to docetaxel resistance in docetaxel-treated CRPC patients. In the area of cancer treatment, clinicians have to deal with two limitations: the difficulty to predict accurately the relapse after local treatment, and the ability to anticipate ineffective adjuvant or systemic therapy. To date, in PCa, new molecular markers are needed to better define the subset of patients who are most likely to benefit from an adjuvant strategy after radical treatment, and from chemotherapy at CRPC stage. Our findings were consistent with the role of \(\beta\)III-tubulin expression as prognostic marker of biochemical recurrence at HNPC stage. Assessment of the \(\beta\)III-tubulin expression in cancer tissue therefore could be useful to identify and monitor PCa at high risk for recurrence after radical prostatectomy. If longer follow-up and rates of specific mortality confirm these results, the \(\beta\)III-tubulin expression might be interesting for predicting recurrence rates and for proposing adjuvant therapy.

Functional overexpression or knockdown of \(\beta\)III-tubulin modulates the PCa cell line sensitivity to docetaxel. The tissue \(\beta\)III-tubulin expression status also has predictive value in terms of OS in patients receiving docetaxel-based chemotherapy for hormone-independent disease. These findings underline the importance of \(\beta\)III-tubulin expression, which could be used in addition to clinicopathologic characteristics to select patients for docetaxel-based chemotherapy. Prospective studies incorporating \(\beta\)III-tubulin immunohistochemistry are warranted to determine the clinical relevance of routine use of this assay.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.
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