# Cytochrome P450 2B6 is a Growth-Inhibitory and Prognostic Factor for Prostate Cancer

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**BACKGROUND.** Cytochrome P450s (CYPs) influence the biological effects of carcinogens, drugs and hormones including testosterones. Among them, Cytochrome P450 2B6 (CYP2B6) plays a critical role in the deactivation of testosterone. In the present study, we examined CYP2B6 expression in human prostate tissues and prostate cancer.

**METHODS.** Immunohistochemical analysis was performed in 98 benign and 106 malignant prostate tissues and patients' charts were reviewed for clinical, pathologic and survival data. We also investigated whether stable expression of CYP2B6 in LNCaP (human prostate cancer cell line) influences cellular proliferation.

**RESULTS.** CYP2B6 was abundantly expressed in the normal epithelial cells compared to the prostate cancer cells. Significant immunostaining of CYP2B6 was found in 75 of 106 samples (71%), in the cytoplasm of cancerous tissue samples. CYP2B6 immunoreactivity was inversely correlated with high Gleason score ($P < 0.001$). Decreased immunoreactivity of CYP2B6 significantly correlated with poor prognosis ($P < 0.0001$). Univariate and multivariate hazard analyses revealed a significant correlation of decreased CYP2B6 expression with poor cancer-specific survival ($P = 0.0028$ and 0.0142, respectively). Furthermore, overexpression of CYP2B6 in LNCaP cells significantly decreased testosterone-induced proliferation.

**CONCLUSIONS.** These results demonstrated that decreased expression of CYP2B6 might play a role in the development of prostate cancer, and be useful as the prognostic predictor for human prostate cancer. *Prostate 67: 1029–1037, 2007.* © 2007 Wiley-Liss, Inc.

*KEY WORDS:* **testosterone; proliferation and prognosis**

## INTRODUCTION

Prostate cancer is one of the most common malignancies in the world. There is abundant evidence that androgens influence the development and progression of prostate cancer [1–3]. Since most of prostate cancer is androgen-dependent, standard treatment for metastatic prostate cancer patients is androgen deprivation therapy (ADT). However the beneficial effects of ADT are transient and prostate cancer progresses to recurrent cancer. Recent reports showed that levels of testosterone and its metabolites, dihydrotestosterone (DHT) and androstenediol, were still sufficiently high to activate the androgen receptor in recurrent prostate

Abbreviations: ADT, androgen deprivation therapy; CYPs, Cytochrome P450s; DHT, dihydrotestosterone; GS, Gleason score; IR, immunoreactivity; PSA, prostate-specific antigen.

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cancer during ADT [4,5]. The presence of testosterone and its metabolites in recurrent prostate cancer is likely to be significant in prostate cancer progression.

Cytochrome P450s (CYPs) play an important role in biotransformation of xenobiotics such as pharmaceutical drug, environmental contaminants. In addition, these enzymes metabolize endogenous compounds such as steroid hormones [6]. In the liver CYP2B6 hydroxylates testosterone, which results in the deactivation of the hormonal function. It also metabolizes drugs including anti-cancer prodrugs [7]. CYPs have also been detected in extrahepatic tissues, such as the intestine, lung, kidney and brain [8], and breast cancer tissues [9–11]. It was reported that CYP bioactivates anti-cancer prodrug ifosfamide [9] in breast cancers and that the expression of CYPs including CYP2B6 was lower in the tumor tissue than in the adjacent normal tissue [10,11]. The prostate expresses several enzymes involved in androgen metabolism. Since androgens are substrates for multiple CYPs (e.g., CYP2B6, CYP3A4) we undertook to study the expression of this enzyme family in prostate tissues. There are some data from RT-PCR analysis concerning the expression of CYP1A1, CYP1A2, CYP1B1, CYP2B6, and CYP3A4 in human prostate [12,13]. However the expression of CYPs has not been well studied in benign prostate tissues and prostate cancer at the protein level. Since hepatic CYP2B6 is important in testosterone deactivation, we investigated its expression in human prostate tissues using immunohistochemistry. We examined the potential clinical significance of this expression and the influence of CYP2B6 overexpression on the proliferation of the LNCaP cells.

## MATERIALS AND METHODS

### Antibody and Expression Plasmid Constructs

An anti-CYP2B6 rabbit polyclonal antibody was purchased from Research Diagnostics, Inc. (Flanders, NJ); anti-FLAG M2 antibody and anti-β-actin antibody was from Sigma (St. Louis, MO); and anti-rabbit IgG Alexa Fluor 594 and anti-mouse IgG Alexa Fluor 488 were from Molecular Probes (Invitrogen, Carlsbad, CA). The cDNA encoding amino-terminal FLAG-tagged human CYP2B6 was amplified from the IMAGE-clone, NIH MGC 195 (Open Biosystems), subcloned into a mammalian expression vector pcDNA3 (Invitrogen) and the resulting FLAG-tagged CYP2B6 expression plasmid (pcDNA3-CYP2B6-FLAG) was verified by DNA sequencing.

### Cell Culture and Transfection

COS7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS. LNCaP (Human prostate cancer cell line) was purchased from American Type Culture Collection (Manassas, VA). LNCaP was maintained in RPMI1640 media supplemented with 2 mM glutamine, 1% nonessential amino acids, 100 U/ml streptomycin/penicillin and 10% fetal calf serum (FCS). Transfection was performed by using FuGENE6 (Roche, Indianapolis, IN) according to the manufacturer's instruction.

### Immunofluorescence Staining

Cells were grown on 12-mm circle cover glasses (Fisher) in 24-well plates. After 16 hr, living cells were washed three times with phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde/0.1 M phosphate buffer for 5 min at room temperature, washed once with PBS, and permeabilized with 0.2% Triton-X 100 in PBS for 10 min. After another washing step with PBS and blocking in 3% bovine serum albumin (BSA)/TBST (100 mM Tris–HCl pH 8.0, 150 mM NaCl, 0.05% Tween 20) for 30 min, cells were first incubated with rabbit anti-CYP2B6 antibody (1:200) and mouse anti-FLAG M2 antibody (1:500) in 3% BSA/TBST for 1 hr at room temperature, washed three times with PBS, subsequently incubated with anti-rabbit IgG Alexa Fluor 594 (1:2,000) and anti-mouse IgG Alexa Fluor 488 (1:2,000) in 3% BSA/TBST for 1 hr at room temperature. Nuclei were stained with DAPI (4′,6-diamidino-2-phenylindole). After cells were washed three times with PBS, cover glasses were mounted in 1.25% DABCO, 50% PBS, 50% glycerol and visualized using a digital microscope (VH-8000, Keyence, Japan).

### Western Blot Analysis

Western blot analysis was performed using cellular protein extracts. Cells were rinsed twice with ice-cold PBS and lysed in 200 μl Nonidet P-40 lysis buffer (50 mM Tris–HCl [pH 7.4], 150 mM NaCl, 10 mM NaF, 5 mM EDTA, 5 mM EGTA, 2 mM sodium vanadate, 0.5% sodium deoxycholate, 1 mM dithiothreitol [DTT], 1 mM phenylmethylsulfonylfluoride [PMSF], 2 μg/ml aprotinin and 0.1% Nonidet P-40), and the lysates were cleared by centrifugation at 15,000*g* for 15 min at 4°C. Total protein lysate (20 μg) was fractionated on sodium dodecyl sulfate (SDS)-12.5% polyacrylamide gels, and electrophoretically transferred onto polyvinylidene difluoride (PVDF) membranes (Immobilon, Millipore Co., Bedford, MA).The membranes were blocked in Tris-buffered saline (TBS) with 5% skim milk for 30 min, then incubated with 5 ml each of 1:500 diluted anti-CYP2B6 antibody or 1:1,000 diluted anti-FLAG M2 antibody (Sigma) at room temperature for 3 hr. Each membrane was washed in TBS with 0.1% Tween 20 and incubated with 1:5,000 diluted horseradish peroxidase-conjugated donkey anti-rabbit immunoglobulin G

(Ig G) or 1:5,000 diluted horseradish peroxidase-conjugated sheep anti-mouse Ig G (Amersham Pharmacia Biothech, Arlington Heights, IL) at room temperature for 1 hr. Bands were visualized with the chemiluminescence-based ECL plus detection system (Amersham Pharmacia Biotech). The membranes were exposed to X-ray film. All experiments were performed a minimum of three times.

### Tissue Selections and Patient Characteristics

Formalin-fixed, paraffin-embedded sections were obtained from 106 patients who underwent radical prostatectomy for prostatic adenocarcinoma between 1987 and 2001. We obtained informed consent from all the patients. The age of the patients ranged from 52 to 78 years (mean $66.8 \pm 6.0$), and pretreatment serum PSA (prostate-specific antigen) level ranged from 2.2 to 136 ng/ml (mean $16.9 \pm 19.5$). The pathological stages included B ($n = 33$), C ($n = 59$) and $D_1$ ($n = 14$). Prostatic tissue sections submitted for this study contained 98 benign and 106 cancerous foci. The cancerous lesions consisted of tumors with Gleason score (GS) 6 ($n = 22$), 7 ($n = 41$), 8 ($n = 20$), 9 ($n = 22$), and 10 ($n = 1$), which was evaluated by two trained pathologists. Thirty-five patients (33%) were treated with surgery alone, whereas the remaining patients received adjuvant anti-androgen therapy. Patients were followed post-operatively by their surgeons at 3-month intervals to 5 years and yearly thereafter. Mean patient follow-up period was $82 \pm 39$ months (range 10–192). During the follow-up period, 77 patients (73%) are alive with no evidence of the disease and 12 (11%) are alive with biochemical or clinical recurrence. Eleven patients (10%) died from prostate cancer and 6 (6%) died from other diseases during the follow-up period.

### Immunohistochemistry

Immunohistochemical analysis was performed employing the streptavidin–biotin amplification method using a peroxidase catalyzed signal amplification system: CSA system (DAKO, Carpinteria, CA) as previously described [14]. CSA was used following the manufacturer-supplied protocol. Six μm tissue-sections were deparaffinized, rehydrated through a graded ethanol series, and rinsed in PBS. For antigen retrieval, the sections were autoclaved at 120°C for 15 min in citric acid buffer (2 mM citric acid and 9 mM trisodium citrate dehydrate, pH 6.0). After blocking endogenous peroxidase with 0.3% $H_2O_2$, the sections were incubated in 10% bovine serum for 10 min. Application of the polyclonal antibody for CYP2B6 (1:200 dilution) was followed by sequential 15-min incubations with biotinylated link antibody, streptavidin–biotin–peroxidase complex, amplification reagent, and streptavidin-peroxidase. The antigen-antibody complex was visualized with 3,3′-diaminobenzidine (DAB) solution (1 mM DAB, 50 mM Tris–HCl buffer pH 7.6, and 0.006% $H_2O_2$). For negative controls, normal rabbit IgG was used instead of the primary antibodies. As positive controls, sections of human normal liver were immunoassayed with the primary antibodies in the same manner as described above.

### Immunohistochemical Assessment

Immunostained slides were evaluated for the proportion (0, none; 1, <1/100; 2, 1/100 to 1/10; 3, 1/10 to 1/3; 4, 1/3 to 2/3; 5, >2/3) and the intensity (0, none; 1, weak; 2, moderate; 3, strong) of positively stained cells [15]. The total scores of immunoreactivity (0–8) were obtained as the sum of the proportion and the intensity. For immunohistochemical assessment, two investigators (TF and JK) evaluated the tissue sections independently. If the IR score (immunoreactivity score) differed between the two investigators, a third investigator (ST) evaluated the samples an average IR score was adopted. Since almost all benign foci showed >5 of IR scores for CYP2B6, we defined IR score 5 as a cutoff for positive immunoreactivity of CYP2B6.

### Generation of LNCaP Stably Expressing CYP2B6-FLAG

LNCaP was transfected with an expression vector, pcDNA3-CYP2B6-FLAG or vector alone using FuGENE6. G418 resistant cells were selected and several independent clones were isolated.

### 3-(4,5-Dimethylthiazol-2-yl)-5-(3-Carboxymethoxyphenyl-2-(4-Sulfophenyl)-2H-Tetrazolium (MTS) Assay

Cell growth rate was measured using a MTS proliferation assay (Cell Titer 96 AQueous One Solution Cell Proliferation Assay, Promega, Madison, WI). The assay was performed according to manufacturer's instructions. Five thousand cells were seeded in 96-well plates and cultured in RPMI supplemented with 10% FBS for 48 and 72 hr. Before testing, 10 μl of MTS reagent was added and the cells were incubated for a further 4 hr at 37°C. The optical density (OD) was measured at a wavelength of 490 nm by a microplate reader (Bio-Rad model 550, Japan). Each time point was performed in quadruplicate wells and each experiment was repeated at least three times. To evaluate effects of testosterone on the growth of LNCaP-CYP2B6-FLAG or LNCaP-Vector clones, the cells were cultured in phenol-red free medium with 10% charcoal-stripped FBS for 48 hr

before experiments. Then 5,000 cells were seeded in 96-well plates and cultured with either vehicle control or testosterone at a dose of $10^{-10}$ or $10^{-8}$ M for 48 hr. Percent increase of OD compared with vehicle control was calculated.

### Statistical Analysis

Correlations between IR score and clinicopathological characteristics (age, pretreatment serum PSA level, pathological stage and GS) were evaluated using the Student's *t*-test or chi-square test. Cancer-specific survival curves were obtained by the Kaplan–Meier method and verified by the log rank (Mantel-Cox) test. The comparisons between OD of LNCaP clones were evaluated using the Student's *t*-test. Statistical assessment was analyzed by Stat View-J 5.0 software (SAS Institute, Cary, NC) and *P* values less than 0.05 were regarded as statistically significant.

## RESULTS

### Immunofluorescence Staining of Transfected COS7 Cells

We transiently transfected COS7 cells using pcDNA3-CYP2B6-FLAG and immunostained with anti-CYP2B6 and anti-FLAG M2 antibodies. The anti-CYP2B6 antibody revealed a cytoplasmic staining pattern in CYP2B6-FLAG overexpressed COS7. This expression pattern was shared with the anti-FLAG antibody (Fig. 1A), demonstrating that the protein was located in the cytoplasm.

### Validation of CYP2B6 Antibody by Western Blot Analysis

We next transiently transfected COS7 cells using CYP2B6-FLAG expression plasmid for Western blot analysis. As expected, the CYP2B6 antibody detected a 53-kDa band in pCDNA3-CYP2B6-FLAG transfected COS7. A band of apparently the same size was detected by the FLAG M2 antibody (Fig. 1B arrow head).

### Immunohistochemistry

Diffuse, but intense CYP2B6 immunostaining was detected in the cytoplasm of benign prostate epithelium. In contrast, immunoreactivity of CYP2B6 was low in the cancer cells. In addition, immunoreactivity of high GS prostate cancer was markedly less than low GS prostate cancer (Fig. 2). The results for the expression of CYP2B6 in the human prostate tissues are shown in Figure 3. When an IR score $\geq$5 was defined as positive, positive CYP2B6 immunoreactivity was identified in 96 (98.0%) benign prostate epithelium cases. Among the 63 low GS prostate cancer cases, positive CYP2B6 immunoreactivity (IR score $\geq$5) was observed in 55 cases (87.3%). Of the 43 high GS prostate cancer cases, positive CYP2B6 immunoreactivity was observed in 20 cases (46.5%). Therefore, a strong association exists between high GS prostate cancer and low CYP2B6 immunoreactivity.

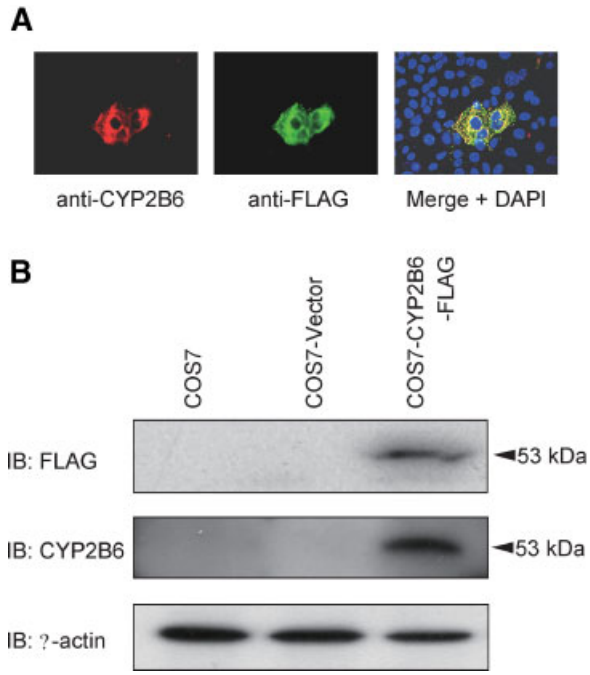



**Fig. 1.** Immunofluorescence staining of transfected COS7 cells and validation of CYP2B6 antibody by Western blot analysis. A: COS7 cells were grown on cover glasses, transiently transfected with CYP2B6-FLAG expression plasmid, fixed with paraformaldehyde and stained with anti-CYP2B6 antibody and anti-FLAG antibody. Nuclei were counterstained with DAPI. Signals from CYP2B6 (left panel) and signals from FLAG (middle panel) antibody shared an identical subcellular distribution. Merged images are shown on the right panel. Scale bars, 20 μm. B: Cell extracts from untransfected, pcDNA3 empty plasmid transfected, and pcDNA3-CYP2B6-FLAG transfected COS7 cells were resolved by SDS-PAGE and transferred to PVDF membrane. Blot was probed with the anti-CYP2B6 polyclonal antibody (1:500), anti-FLAG antibody (1:1,000) and anti-β-actin antibody (1:1,000). CYP2B6 antibody detected a 53-kDa band in pcDNA3-CYP2B6-FLAG transfected COS7 cells, which was coincided with a band detected by anti-FLAG antibody.

### Correlation of CYP2B6 Expression With Clinicopathological Characteristics in Prostate Cancer

This association between CYP2B6 and GS, led us to evaluate the potential correlation between CYP2B6 immunoreactivity and clinicopathological characteristics. Age, pretreatment serum PSA level, GS and pathologic stage) were evaluated (Table I). CYP2B6 immunoreactivity was significantly lower in high GS

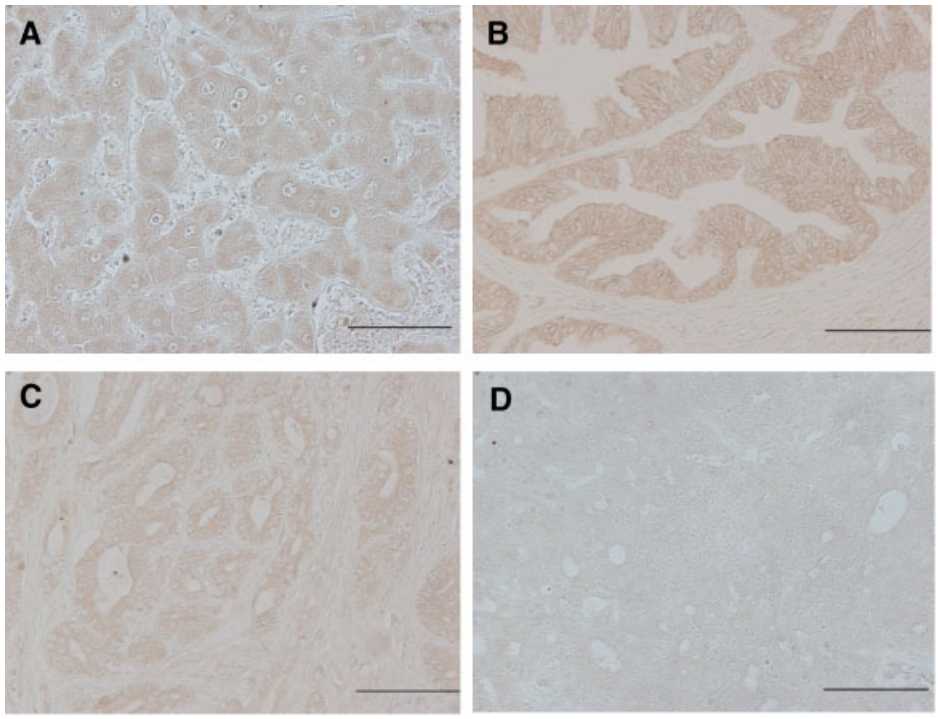



**Fig. 2.** Expression of CYP2B6 in human liver and benign prostate tissue, low GS and high GS prostate cancer. **A**: Strong staining (intensity: 3) of CYP2B6 was identified in liver. **B**: Strong staining (intensity: 3) of CYP2B6 was identified in benign epithelium. **C**: Moderate immunoreactivity (intensity: 2) was identified in low GS prostate cancer (GS 6). **D**: Weak immunoreactivity of CYP2B6 (intensity: 1) was observed in high GS prostate cancer (GS 9). Scale bars, 100 μm.

cancer (GS 8–10) than in low GS cancer (GS 2–7) ($P < 0.001$).

Figure 4 shows a cancer-specific survival curve prepared by the Kaplan–Meier method. Fifteen of 31 (48.4%) CYP2B6-negative cases had died from prostate cancer during the follow-up period. Patients with CYP2B6-negative prostate cancer had significantly worse cancer-specific survival than those with CYP2B6-positive prostate cancer ($P < 0.0001$, log rank test). Table II shows the prognostic value of PSA, pathological stage, GS and CYP2B6 immunoreactivity in univariate and multivariate proportional analyses for cancer-specific survival. In univariate analyses, CYP2B6 immunoreactivity was significantly ($P = 0.003$) related to cancer specific survival as well as the GS ($P = 0.014$) and pathological stage ($P = 0.035$). In multivariate analysis, among four parameters, only CYP2B6 immunoreactivity retained independent prognostic significance ($P = 0.014$). The relative risk for cancer specific mortality was 14.0 (95% CI 1.69–115.4) for patients with CYP2B6-negative prostate cancer.

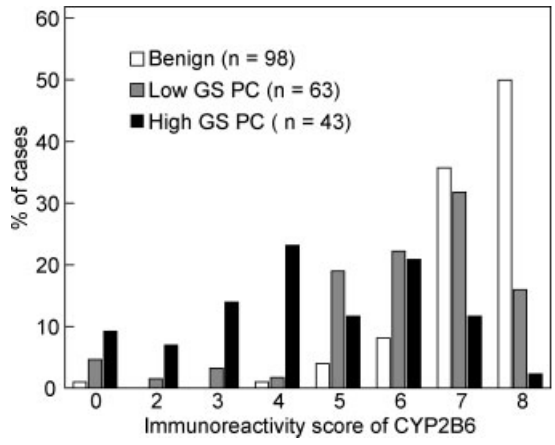



**Fig. 3.** Immunoreactivity score of CYP2B6 in human benign, low GS prostate cancer and high GS prostate cancer. Positive immunostaining (IR score $\geq$5) was observed more frequently in benign prostate than prostate cancer tissue and more frequently in low GS prostate cancer than in high GS prostate cancer.

### Generation of LNCaP Stably Expressing CYP2B6-FLAG

To explore whether constitutive CYP2B6 expression influences cancer cell proliferation in human prostate cancer cell line, we generated LNCaP stably expressing human CYP2B6-FLAG protein and LNCaP clones with the vector (LNCaP-Vector clone #1 and #4). We selected two LNCaP-CYP2B6-FLAG clones #2 and #3 that express CYP2B6-FLAG protein as confirmed by Western blotting using anti-FLAG M2 antibody (Fig. 5A). Further we confirmed protein expression of

**TABLE I. Relationship Between Expression of CYP2B6 and the Clinicopathological Findings in PC (n = 106)**

| | Immunoreactivity of CYP2B6[a] | | |
|---|---|---|---|
| | Negative (n = 31) | Positive (n = 75) | *P* value |
| Age | 66.2 ± 5.6 | 66.9 ± 6.1 | 0.59 |
| Serum PSA (ng/ml) | 19.2 ± 19.3 | 13.7 ± 12.7 | 0.08 |
| Gleason score | | | |
| 2–7 | 8 (12.7) | 55 (87.3) | **<0.001** |
| 8–10 | 23 (53.5) | 20 (46.5) | |
| Pathological stage | | | |
| B, C | 24 (22.6) | 68 (77.4) | 0.07 |
| D1 | 7 (50) | 7 (50) | |

[a]IR score 0–4 and 5–8 were defined as negative and positive immunoreactivity, respectively.

LNCaP-CYP2B6-FLAG stable clones by immunofluorescence staining (Fig. 5B). Almost all stable cells expressed CYP2B6-FLAG protein, and the immunoreactivity detected by the anti-FLAG antibody and anti-CYP2B6 antibody were indistinguishable.

### MTS Proliferation Assay of LNCaP

Proliferation of LNCaP cells was determined by MTS assay. The proliferation of LNCaP-CYP2B6-FLAG stable clones was significantly reduced after 48 and 72 hr incubation compared to vector clones ($P < 0.0001$) (Fig. 5C). The result indicated that stable expression of CYP2B6 decreased the proliferation of cultured prostate cancer cells. This is consistent with the immunohistochemical results that showed decreased expression of CYP2B6 was associated with a poor prognosis.

### Inhibitory Effect of CYP2B6 on Testosterone-Induced Growth

To determine whether effects of testosterone were inhibited by expression of CYP2B6, growth of LNCaP-CYP2B6-FLAG and LNCaP-Vector clones were assayed after testosterone treatment. MTS assay showed that the percent increase of OD was significantly decreased in LNCaP-CYP2B6-FLAG stable clones after 48 hr incubation at doses of $10^{-10}$ M ($P < 0.0001$) and $10^{-8}$ M ($P = 0.012$) of testosterone compared to vector clones (Fig. 5D). These data indicated that testosterone-induced growth was inhibited by CYP2B6 expression clearly.

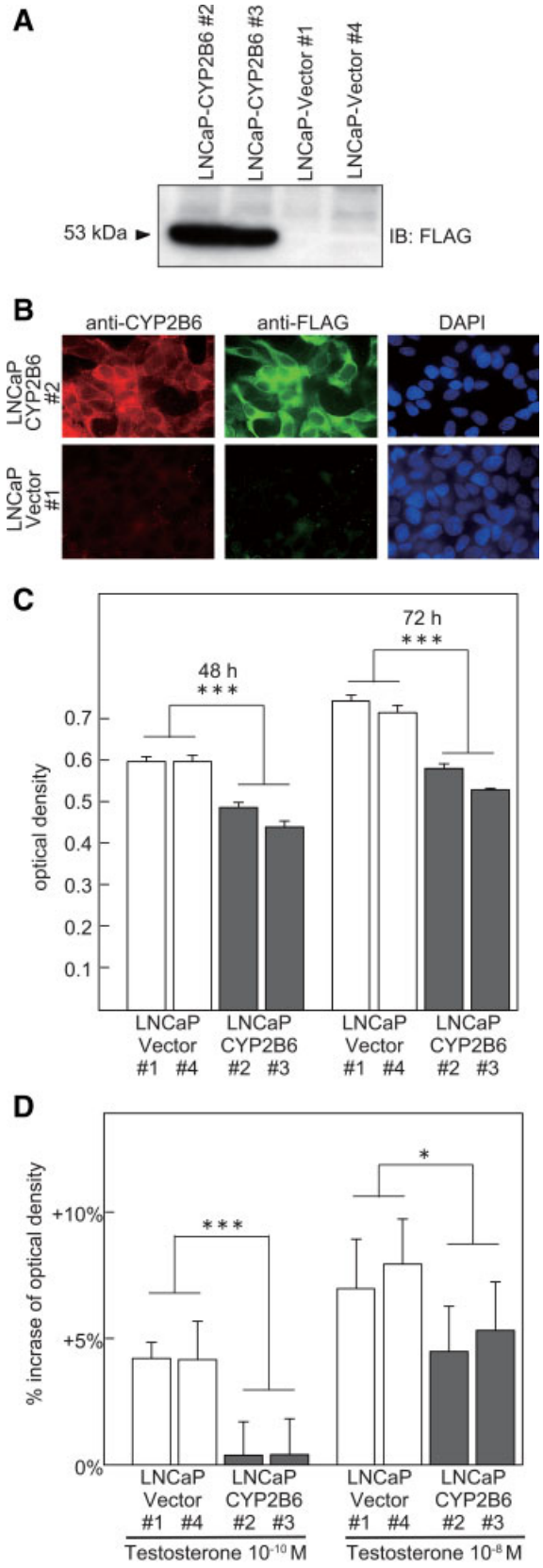
## DISCUSSION

In human liver, CYP2B6 ranges between 2 and 10% of the total P450 content [16]. Moreover, CYP2B6 is involved in the metabolism of nearly 25% of drugs on the market today [17]. CYPs expressed in the liver are well known to play pivotal roles in the metabolism of endobiotics, xenobiotic and pharmaceutical drugs. However, CYPs are also expressed in extra-hepatic tissues. Recent years have seen an increased interest in investigation into the presence, function and regulation of CYPs in such tissues, particularly in tumors. CYP1B1 is reported to be overexpressed in human prostate cancer and it was postulated that it contributes to the

**TABLE II. Univariate and Multivariate Proportional Hazard Analyses of Cancer-Specific Survival (n = 106)**

| | Univariate | | | Multivariate | | |
|---|---|---|---|---|---|---|
| Variable | Hazard ratio | 95% index | *P* value | Hazard ratio | 95% index | *P* value |
| PSA (>10 vs. ≤10) | 0.79 | 0.23–2.8 | 0.73 | 0.75 | 0.17–3.26 | 0.69 |
| Gleason score (high vs. low[a]) | 13.4 | 1.71–105.2 | **0.014** | 5.46 | 0.61–49.6 | 0.13 |
| Pathological stage (D1 vs. B, C) | 3.8 | 1.01–12.9 | **0.035** | 2.02 | 0.47–8.2 | 0.34 |
| CYP2B6 (negative vs. positive[b]) | 23.1 | 2.94–181.5 | **0.0028** | 14 | 1.69–115.4 | **0.014** |

[a]High Gleason score: 8–10, low: 2–7.
[b]IR score 0–4 and 5–8 were defined as negative and positive immunoreactivity, respectively.



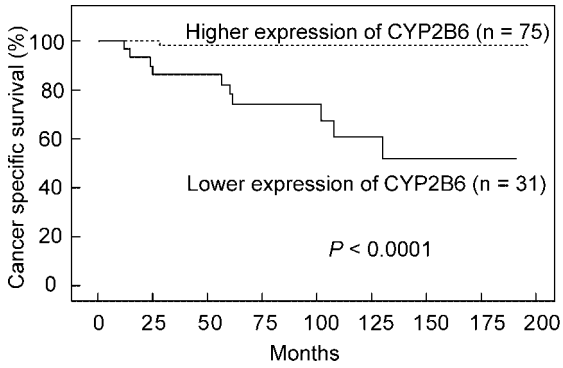




**Fig. 4.** Cancer-specific survival in 106 prostatic cancer patients relative to the immunoreactivity of CYP2B6. Cancer-specific survival of 31 patients with negative CYP2B6 expression (IR score $<5$) was significantly worse than that of 75 positive expression cases (IR score $\geq 5$) ($P < 0.001$).

development or progression of prostate cancer by activating pro-carcinogens [18]. A few reports are available on the expression of CYP2B6 mRNA in prostate [13]. Despite the important role of CYP2B6 in hepatic deactivation of testosterone, extra-hepatic expression of CYP2B6 protein has not been extensively studied, particularly in prostate cancer. Therefore, we studied the expression of CYP2B6 in human prostate tissues using immunohistochemistry and evaluated its clinical significance.

In the present study, we demonstrated CYP2B6 expression in human benign and malignant tissues by immunohistochemical analyses. CYP2B6 immunoreactivity was low in prostate cancer cells, whereas intense and diffuse CYP2B6 immunoreactivity was found in the normal or hyperplastic prostate epithelium. In univariate analysis, CYP2B6 immunoreactivity, GS and pathological stage were significantly related to cancer specific survival, but pretreatment PSA was not associated with cancer-specific survival. Pretreatment serum PSA level is generally an established prognostic

**Fig. 5.** Overexpression of CYP2B6 decreases growth of LNCaP cells. **A**: Western blot analysis of LNCaP stably expressing CYP2B6-FLAG (LNCaP-CYP2B6-FLAG) or empty vector (LNCaP-Vector). CYP2B6-FLAG protein was overexpressed in LNCaP-CYP2B6-FLAG clones. LNCaP-Vector clones were used for control. **B**: Immunofluorescence staining of LNCaP. Almost all cells expressed CYP2B6-FLAG protein in LNCaP-CYP2B6-FLAG clone #2 (upper panel). CYP2B6 immunoreactivity was not detected in LNCaP-Vecter cells as shown in lower panel. **C**: Proliferation of LNCaP stable clones was determined by MTS assay. Cell proliferation was evaluated after 48 and 72 hr incubation. $***P < 0.0001$, compared to vector controls. **D**: Proliferative effects of testosterone were determined by MTS assay. Percent increase of OD was significantly decreased in LNCaP-CYP2B6-FLAG stable clones after 48 hr incubation compared to vector clones at doses of $10^{-10}$ and $10^{-8}$ M testosterone. $***P < 0.0001$, $*P < 0.05$, compared to vector controls.

factor for PSA recurrence, but not for cancer-specific survival after radical prostatectomy [19]. The restricted low range of preoperative PSA in the present study (25th and 75th percentile, 6.2 and 18.1) may have limited the predictive value of preoperative PSA levels on cancer-specific survival. Data reported by other groups were consistent with our results that only pT stage, and GS were significant prognostic factors about cancer specific survival after radical prostatectomy [19,20].

Expression of CYP2B6 has been recently investigated in a few carcinomas, including breast cancer and hepatocellular carcinoma (HCC). In breast cancer, CYP2B6 mRNA was down-regulated in the tumor tissue compared with normal adjacent tissue [10]. In addition, CYP2B6 mRNA was significantly lower in HCC with venous invasion than in HCC without venous invasion [21]. These findings suggest that CYP2B6 expression may be decreased in the development of cancers that are closely related to substrates of CYP2B6.

CYPs sensitize tumor xenograft to anticancer prodrugs such as cyclophosphamide (CPA). The anticancer activity of CPA in cultured tumor cells and in rodent and human xenograft models is substantially increased by introduction of cDNAs encoding CYP2B6, which are major catalysts of CPA activation in rat and human liver, respectively [22,23]. On the other hand Ikezoe et al. demonstrated that ritonavir blocked the docetaxel-induced expression of CYP3A4 at the mRNA level in prostate cancer DU145 cells and enhanced the antitumor effect of docetaxel in vitro and in BNX nude mice bearing DU145 tumors [24]. These findings showed that extrahepatic CYPs also biotransformed drugs like hepatic CYPs and that this biotransformation may be associated with activation or deactivation of anticancer drugs.

Testosterone is inactivated in the liver and the prostate. CYP2B6 are known to function in hepatic testosterone inactivation [7,25]. In the prostate, several enzymes are involved in the metabolism of testosterone. 5α-Reductase converts testosterone to DHT. DHT is converted by the enzymes 3α-and 3β-hydroxysteroid oxidoreductase into 5α-androstane-3α,17β-diol (3α-diol) and 5α-androstane-3β,17β-diol (3βA-diol), respectively. The 3βA-diol is further hydroxylated to 6α-, 6β-, 7α-, and 7β-triols which are inactive as androgens and excreted from the prostate. In addition, rat CYPs catalyze the 6α-, 6β-, 7α-, and 7β-hydroxylation of the 3βA-diol in the ventral prostate [26–28] However little is known about the role of CYPs in testosterone metabolism in human prostate. We hypothesized that, consistent with the role of CYP2B6 in liver and the role of CYPs in rat prostate, CYP2B6 might play an important role in testosterone metabolism in the human prostate. If so, then decreased expression of CYP2B6 might contribute to elevated DHT levels in the prostate, thus promoting cancer progression. Consistent with this hypothesis, our present study showed that decreased expression of CYP2B6 significantly correlated with poor prognosis in human prostate cancer. Moreover, stable overexpression of CYP2B6 in LNCaP reduced their proliferation as well as testosterone-induced proliferation. Taken together, these results support the view that CYP2B6 interferes with proliferation of prostate cancer cells by either catabolize some pro-proliferative substances such as testosterones, or metabolize anti-proliferative substances.

## CONCLUSIONS

In conclusion, our results indicate that decreased CYP2B6 expression was an independent prognostic factor for prostate cancer and that CYP2B6 overexpression interfered with proliferation of LNCaP cells. Immunoreactivity of CYP2B6 may therefore be useful in selecting patients with more aggressive tumor for adjuvant therapy. These results suggest that CYP2B6 has significant anti-tumor effects in prostate cancer. Therefore, compounds which highly induce CYP2B6 such as phenobarbital, rifampicin, clotrimazole, phenytoin and carbamazepine [29–31] might be of clinical benefit in the treatment of recurrent prostate cancer.

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