

# Genetic variants of the *CYP1B1* gene as predictors of biochemical recurrence after radical prostatectomy in localized prostate cancer patients

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## Abstract

Clinically localized prostate cancer is curative. Nevertheless many patients suffered from biochemical recurrence (BCR) after radical prostatectomy (RP). Mounting evidence suggest that estrogen and xenobiotic carcinogens play an essential role in progression of prostate cancer via oxidative estrogen metabolism. *CYP1B1* is an enzyme involved in the hydroxylation of estrogens, a reaction of key relevance in estrogen metabolism. Given the role of *CYP1B1* in the oxidative metabolism of endogenous/exogenous estrogen and compounds, *CYP1B1* polymorphisms have the potential to modify its expression and subsequently lead to progression. We hypothesize that genetic variants of the *CYP1B1* gene may influence clinical outcome in clinically localized prostate cancer patients. In this cohort study, we genotyped 9 tagging single nucleotide polymorphisms (SNPs) from the *CYP1B1* gene in 312 patients treated with RP. For replication, these SNPs were genotyped in an independent cohort of 426 patients. The expression level of *CYP1B1* in the adjacent normal prostate tissues was quantified by reverse transcription and real-time polymerase chain reaction. Kaplan–Meier analysis and Cox proportional hazard models were utilized to identify SNPs that correlated with BCR. *CYP1B1* rs1056836 was significantly associated with BCR (hazard ratio [HR]: 0.69; 95% confidence interval [CI]: 0.40–0.89,  $P=0.002$ ) and relative *CYP1B1* mRNA expression. Our findings suggest inherited genetic variation in the *CYP1B1* gene may contribute to variable clinical outcomes for patients with clinically localized prostate cancer.

**Abbreviations:** BCR = biochemical recurrence, CI = confidence interval, HR = hazard ratio, PSA = prostate-specific antigen, RP = radical prostatectomy, SNP = single nucleotide polymorphism.

**Keywords:** biochemical recurrence, *CYP1B1*, prostate cancer, radical prostatectomy, single nucleotide polymorphism

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## 1. Introduction

Radical prostatectomy (RP) is the gold standard treatment modality for clinically localized prostate cancer. Prostate cancer patients treated with RP have an overall risk of biochemical recurrence (BCR) of approximately 30% at 10 years after surgery.<sup>[1]</sup> Due to the limitations of prostate-specific antigen (PSA) and other clinicopathological parameters, great efforts have been made to identify novel prognostic molecular markers to predict BCR after surgical treatment.<sup>[2]</sup> Such prognostic markers for use in localized prostate cancer would considerably improve disease management.

Previous studies suggest that estrogen and xenobiotic carcinogens play an essential role in progression of prostate cancer via oxidative estrogen metabolism.<sup>[3]</sup> *CYP1B1* is an enzyme involved in the hydroxylation of estrogens, a reaction of key relevance in estrogen metabolism.<sup>[3]</sup> The overproduction of estrogen like E<sub>2</sub> or the bioconversion of E<sub>2</sub> into genotoxic metabolites like estradiol-3,4-quinone (E<sub>2</sub>-3,4-Q) or 4-hydroxyestradiol (4-OH E<sub>2</sub>) by *CYP1B1* lead to generation of reactive oxygen species, which cause DNA adducts and enhance prostate progression.<sup>[4]</sup> Substantial evidence for a potential role of *CYP1B1* in prostate cancer comes from the observations that *CYP1B1* protein is overexpressed in prostate carcinoma whereas no *CYP1B1* protein expression is detected in normal prostate tissue.<sup>[5]</sup>

Based on these observations, single nucleotide polymorphisms (SNPs) of *CYP1B1* have been proposed as candidates for association studies on prostate cancer risk.<sup>[6–15]</sup>

*CYP1B1* is polymorphous and there were 304 SNPs that have been identified.<sup>[3]</sup>

Several *CYP1B1* SNPs altering the estrogen metabolism may modify an individual's prostate cancer risk. The SNP rs1056836, leading to a substitution of an amino acid leucine for valine, is associated with increased *CYP1B1* messenger ribonucleic acid (mRNA) expression,<sup>[3,16]</sup> with a subsequent elevation in 4-OH E2 formation resulting in increased estrogen-mediated carcinogenicity.<sup>[3,17]</sup> By contrast, the SNP rs10012, which is responsible for Arg48Gly transition, has no effect on protein folding or stability.<sup>[3,16]</sup> Another SNPs1800440 that results in Asn453Ser transition leads to a decrease in protein expression caused by degradation of the protein.<sup>[3,18]</sup> Given the role of *CYP1B1* in the oxidative metabolism of endogenous/exogenous estrogen and compounds, *CYP1B1* polymorphisms have the potential to modify its expression and subsequently lead to progression. *CYP1B1* may also be involved in recurrence after RP by altering the tissue response to hormones and other genes that participate in *CYP1B1*-mediated pathways (e.g., estrogen receptor and aryl hydrocarbon receptor signaling pathway).<sup>[3,4]</sup> However, no studies have looked for associations between *CYP1B1* polymorphisms and prognosis in prostate cancer cases. Herein, we evaluated whether genetic variations in *CYP1B1* were correlated with BCR-free survival in localized prostate cancer patients treated with RP.

## 2. Methods

### 2.1. Study population

We retrospectively analyzed 738 men who had undergone RP from 2 independent cohorts in China as described previously.<sup>[19]</sup> The 1st cohort was composed of 312 patients from the Affiliated Hospital of Qingdao University and Fudan University Shanghai Cancer Center, and the 2nd was composed of 426 patients from Fudan University Shanghai Cancer Center. The median follow-up times were 36.3 and 37.7 months. The clinical information were abstracted from the archival medical records. The patients who received adjuvant hormone therapy or radiotherapy were excluded. BCR was defined as 2 consecutive PSA measurements >0.2 ng/mL at an interval of >3 months, and the date of this event was set to the 1st of these 2 test occasions. All participants provided written informed consent. The protocol and consent documents were approved by the Institutional Review Board of Fudan University Shanghai Cancer Center and the Affiliated Hospital of Qingdao University.

### 2.2. SNP selection and genotyping

Tagging SNPs were identified in the *CYP1B1* gene with  $r^2 \geq 0.8$  and a minor allele frequency  $\geq 5\%$  in Chinese using HapMap database. A total of 9 tagging SNPs were chosen. Genomic DNA was extracted from peripheral blood using QIAmp DNA extraction kit (Qiagen) and genotyped by TaqMan real-time PCR method according to manufacturer's instruction. All SNPs were consistent with Hardy–Weinberg equilibrium (HWE), except for rs9341250 ( $P < 0.01$ ), which was excluded from further analysis. Each SNP had greater than 99% completion and the concordance was 100% for duplicated specimens.

### 2.3. *CYP1B1* expression analysis

This analysis included 218 patients whose frozen adjacent normal prostate tissue was available for RNA extraction. Tissue specimens were collected during surgery and stored at  $-80^\circ\text{C}$ . The TRIzol reagent (Invitrogen, CA) was used to isolate total RNA. cDNA was synthesized using PrimeScriptRT Master Mix system (TAKARA, Osaka, Japan). Quantitative PCR was carried out using ABI 7900 Real-Time PCR System (Applied Biosystems, CA). The sequences of the *CYP1B1* specific primers were forward, GCTGCAGTGGCTGCTCCT, and reverse, CCCACGACCTGATCCA AT TCT. The negative controls for each primer consisted of a reaction with no cDNA added. Quantification was achieved by the use of a standard curve for *CYP1B1* and normalization of the corresponding transcripts by the  $\Delta\Delta\text{Ct}$  method and the use of the housekeeping gene GAPDH.

### 2.4. Statistical analysis

Categorical variables were compared by the  $\chi^2$  test or Fisher exact test. HWE for the evaluation of genotype frequencies was performed by the goodness-of fit  $\chi^2$  test. The association between SNPs, clinical characteristics, and BCR was assessed with hazard ratios (HRs) and 95% confidence interval (CI) estimated by Cox proportional hazards regression analysis under different genetic models. All analyses were adjusted for known prognostic factors including age, disease stage, Gleason score, lymph node involvement, and PSA at diagnosis. The BCR-free survival interval was estimated using the Kaplan–Meier method, and the significance was determined using the log-rank test. Differences in distribution of expression levels by genotype were analyzed by Student *t* test. All reported *P*-values are 2-sided. SAS version 9.1 (SAS institute Inc., NC) was used for all analyses.

## 3. Results

### 3.1. Patient characteristics and treatment outcome

Patient characteristics of the study 1 and study 2 are described in Table 1. A total of 79 (25.3%) and 100 (23.5%) patients experienced BCR in the study 1 and study 2, respectively. PSA levels, pathologic stage, lymph node involvement, and Gleason score were significantly associated with BCR in both cohorts ( $P < 0.01$ ).

### 3.2. Genetic analyses of localized prostate cancer

The relative frequencies of the SNPs in patients and their corresponding HRs (95% CI) are shown in Table 2 and Supplementary Table S1, <http://links.lww.com/MD/B109>. After making adjustments for known clinicopathologic variables, *CYP1B1*rs1056836 were found to be significantly associated with time to BCR in both study 1 and study 2 ( $P = 0.028$  and  $0.035$ , respectively), and upon combined analysis (HR: 0.69, 95% CI: 0.40–0.89,  $P = 0.002$ ; Table 2 and Fig. 1).

### 3.3. Relationship between rs1056836 and expression of the *CYP1B1*

The distributions of *CYP1B1* expression in the prostate tissue of patients are shown in Fig. 2. Compared with CG/GG genotype,

**Table 1****Clinicopathologic characteristics of the study populations.**

Variables	Qingdao, N = 312			Shanghai, N = 426		
	BCR	Non-BCR	P	BCR	Non-BCR	P
Age, years	67 (52–79)	68 (46–80)	0.991	68 (58–79)	68 (46–82)	0.951
BMI, kg/m <sup>2</sup>						
<24	60 (77.9)	181 (77.0)	0.870	46 (46.0)	176 (54.0)	0.162
≥24	17 (22.1)	54 (23.0)		54 (54.0)	150 (46.0)	
PSA, ng/mL						
≤20	40 (51.9)	161 (68.5)	0.008	39 (39.0)	210 (64.4)	<0.001
>20	37 (48.1)	74 (31.5)		61 (61.0)	116 (35.6)	
Gleason score						
≤7 (3+4)	20 (26.0)	107 (45.5)	0.002	27 (27.0)	173 (53.1)	<0.001
≥7 (4+3)	57 (74.0)	128 (54.5)		73 (73.0)	153 (46.9)	
Pathologic stage						
T1–2	24 (31.2)	138 (58.7)	<0.001	49 (49.0)	233 (71.5)	<0.001
≥T3	53 (68.8)	97 (41.3)		51 (51.0)	93 (28.5)	
Lymph node involvement						
Negative	59 (76.6)	216 (91.9)	<0.001	75 (75.0)	304 (93.3)	<0.001
Positive	18 (23.4)	19 (8.1)		25 (25.0)	22 (6.7)	
Follow-up, months	36.3			37.7		

BCR = biochemical recurrence, BMI = body mass index, PSA = prostate-specific antigen.

rs1056836CC genotype was significantly associated with higher expression levels of CYP1B1 mRNA ( $P=0.025$ ).

#### 4. Discussion

In this hypothesis-driven study, we observed significant associations between CYP1B1 rs1056836 and BCR in patients with localized prostate cancer. Our results are biologically plausible considering that elevated CYP1B1 gene expression was associated with more aggressive cancers.<sup>[20]</sup> These results support the hypothesis of a link between the genetic variants of the CYP1B1 gene and prostate cancer progression.

The SNP rs1056836 is a nonsynonymous polymorphism in exon 3, which encodes the heme-binding domain, and results in a G-to-C and subsequent amino acid substitutions of valine-to-leucine. A number of studies have suggested that the rs1056836 may increase prostate cancer risk, but the evidence has not been entirely consistent.<sup>[6–15]</sup> When stratifying by ethnicity, a meta-analysis 12 independent studies revealed rs1056836 was significantly associated with an increased risk of prostate cancer in Asians.<sup>[21]</sup> In contrast, no association was found between the polymorphism and prostate cancer risk in either the Caucasian or mixed subject subgroup. The apparent inconsistency of these

results may underlie differences in distribution of allele frequencies among ethnicities, lifestyle, and disease prevalence. In the current study of Chinese origin, the minor allele frequency of rs1056836 was lower than Caucasian or mixed population, which is in accordance with Asian studies.<sup>[13,15]</sup>

Five previous studies have investigated the relationship between the CYP1B1 rs1056836 and prostate cancer aggressiveness, but the definitions of low and high aggressiveness vary among these research reports.<sup>[8,10,12,13,15]</sup> In spite of these limitations, there is some evidence that the CYP1B1 rs1056836 showed a significant decrease in the risk for disease aggressiveness (OR: 0.45, 95% CI: 0.24–0.84,  $P=0.01$ ).<sup>[8]</sup> Overall, our data combined with previous observations reinforce the importance of this critical gene in cancer progression at numerous disease stages.

We speculated that rs1056836 in the coding region of CYP1B1 may lead to increases in consequent expression levels, thus increasing the risk of cancer by elevating the generation of bioactivated carcinogenic metabolites. Subsequent RT-PCR assays consistently showed CYP1B1 rs1056836 was associated with mRNA level. Transcription level of CYP1B1 in prostate cancer was readily increased more than in normal prostate cells.<sup>[22]</sup> In prostate cancer, CYP1B1 activity is regulated via

**Table 2****Associations between CYP1B1 rs1056836 and biochemical recurrence.**

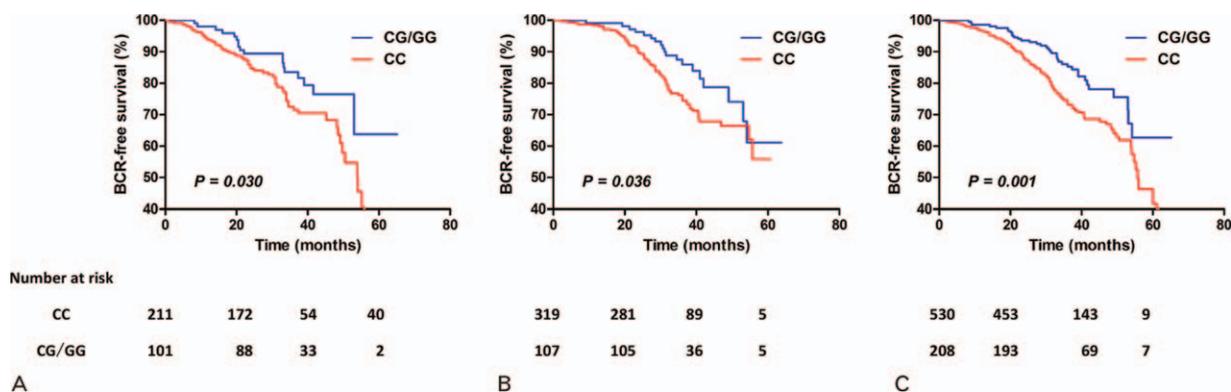
SNP	Qingdao, N = 312				Shanghai, N = 426				Combined, N = 738			
	BCR, n, %	Non-BCR, n, %	HR (95% CI)*	P†	BCR, n, %	Non-BCR, n, %	HR (95% CI)*	P†	BCR, n, %	Non-BCR, n, %	HR (95% CI)*	P†
rs1056836												
CC	60 (75.9)	151 (64.8)	1.00		81 (81.0)	238 (73.0)	1.00		141 (78.8)	389 (69.6)	1.00	
CG	18 (22.8)	73 (31.3)	0.61 (0.36–1.05)	0.073	17 (17.0)	83 (25.5)	<b>0.65 (0.33–0.93)</b>	<b>0.025</b>	35 (19.6)	156 (27.9)	<b>0.66 (0.39–0.83)</b>	<b>0.003</b>
GG	1 (1.3)	9 (3.9)	0.22 (0.03–1.57)	0.130	2 (2.0)	5 (1.5)	1.19 (0.29–4.85)	0.807	3 (1.6)	14 (2.5)	0.55 (0.18–1.73)	0.307
GG/CG vs CC			<b>0.55 (0.33–0.94)</b>	<b>0.028</b>			<b>0.68 (0.35–0.96)</b>	<b>0.035</b>			<b>0.69 (0.40–0.89)</b>	<b>0.002</b>
CC/CG vs GG			0.25 (0.03–1.78)	0.164			0.74 (0.18–3.00)	0.671			0.63 (0.20–1.98)	0.431

BCR = biochemical recurrence, CI = confidence interval, HR = hazard ratio, PSA = prostate-specific antigen, SNP = single nucleotide polymorphism.

\* Adjusted by age, PSA at diagnosis, Gleason score, pathologic stage, and lymph node involvement.

† P value obtained from Cox proportional hazards regression.

The result were in bold, if the 95% CI excluded 1 or  $P < 0.05$ .



**Figure 1.** Kaplan–Meier survival curves for biochemical recurrence (BCR)-free survival by rs1056836 dominant model in (A) study 1, (B) study 2, and (C) combined analysis. *P* value obtained from log-rank *t* test.

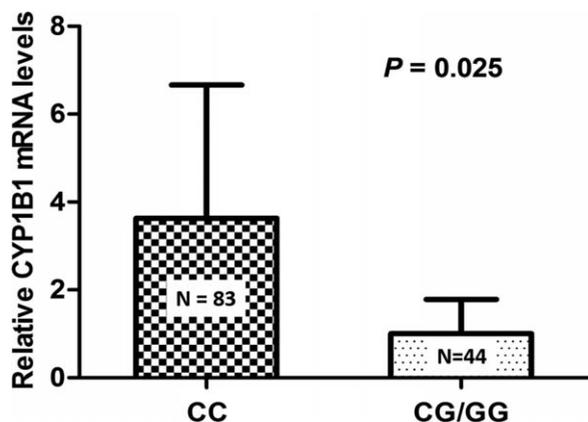
several factors including aryl hydrocarbon receptor (AhR), endogenous estrogen levels, and ERs.<sup>[4,23]</sup> AhR is constitutively active in advanced prostate cancer cell lines.<sup>[20]</sup> The overproduction of estrogen such as E2 via ERs into carcinogenic metabolites such as estradiol-3,4-quinone or 4-hydroxyestradiol by CYP1B1 can induce proliferation and malignant transformation of prostate cells.<sup>[4]</sup> Taken together, our data suggest that rs1056836 alters expression of CYP1B1, which in turn influence AhR and estrogen-mediated carcinogenic activity, consequently contributing to the more aggressive phenotype and poorer clinical outcome in prostate cancer. Further biological and functional studies should be accompanied to determine the role of this SNP/gene during prostate cancer progression.

A limitation of our study is that RP case number and the relatively short follow-up period may have limited the power to detect existing associations between the other SNPs studied and prostate cancer outcomes. Due to the multiple comparisons in our data analysis, we could not completely rule out the possibility of false positive findings. However, the replication of data in an independent population reduced the possibility of chance findings. Furthermore, because our analysis was limited to homogeneous Chinese Han population, the generalize ability to other ethnic groups is uncertain.

In conclusion, we found that an SNP located in the coding region of the *CYP1B1* gene was associated with prostate cancer clinical outcome. Men with *CYP1B1* rs1056836 CC genotype may be at higher risk of recurrence and thus genotyping *CYP1B1* has the potential of improving risk stratification and adjuvant therapeutic decision making in localized prostate cancer. Future epidemiological and functional studies are warranted to confirm these findings for translational application and to uncover the mechanistic basis of the *CYP1B1* genes in affecting cancer progression.

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**Figure 2.** The relative CYP1B1 messenger ribonucleic acid (mRNA) expression levels in prostate tissue by rs1056836 dominant model.

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