Detection of Cytochrome P450-2A6, -3A5 and -4B1 with Real-Time Polymerase Chain Reaction in Prostate Tissue

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Z. Naturforsch. **63c**, 780–784 (2008); received February 25/April 10, 2008

Cytochrome P450 (CYP) is a heme-containing enzyme superfamily metabolizing a wide variety of xenobiotics, including drugs and carcinogens. The majority of *CYP* genes are expressed in the liver, however, some *CYP* isoforms are also reported for a number of extra hepatic tissues. We analyzed *Cytochrome P450–2A6, -3A5* and *-4B1* mRNAs using real-time reverse-transcriptase polymerase chain reaction (RT-PCR) in a total of 21 homogenized prostate tissues with or without malignancy. We detected a consistent expression of *CYP2A6* and *CYP3A5* in all, and of *CYP4B1* in some (11/21) of the samples at mRNA level. Neither the histopathological status nor the smoking habit of the individuals affected *CYP4B1* expression. Our results reflect possible roles for these particular CYPs in therapy and protection of prostate tissue.

Key words: Prostate, Cytochrome P450 Expression

Introduction

Human Cytochrome P450 (CYP) comprises a multigene family of microsomal enzymes that carry out the oxidative metabolism of numerous endogenous and exogenous compounds (Gonzales, 1992). About 20 individual CYP forms, capable of metabolizing xenobiotics, have been identified in man. Although most of the *CYP* gene family is expressed most abundantly in the liver, some CYP isoforms were also reported in a number of extra hepatic tissues (Vondracek *et al.*, 2001; Ding and Kaminsky, 2003; Sarikaya *et al.*, 2007). Over the past years there has been an increased number of researches on the relationship between CYP expression and cancer because altered CYP isoforms could either decrease or increase the metabolism of certain anticancer drugs leading to cytotoxicity in some patients (Purnapatre *et al.*, 2008). The human prostate is one of the most common sites of pathology among the male population. It is the fifth most common cause of male cancer mortality in the world and the second most common cancer in men (Al-Buheissi *et al.*, 2006). The therapeutical strategy of prostate cancer (PC) frequently employs pharmaceuticals that suppress androgens (Finnstrom *et al.*, 2001). Because androgens may serve as the substrates for CYPs, the expression of CYP isoforms in prostate is important. The presence or absence of certain CYP isoforms can determine the rate of activation of potential carcinogens and the outcome of therapy involving P450 substrates (Ragavan *et al.*, 2006). Moreover, some CYP isoforms are polymorphic, which might contribute to differences in interindividual susceptibility to environmental carcinogens (Topcu *et al.*, 2002; Agundez, 2004). The aim of this study was to analyze the expression of the individual forms of *CYP* genes at the mRNA level in human prostate tissue. Individual forms of CYP mRNAs were detected by real-time reverse-transcriptase polymerase chain reaction (RT-PCR) using specific primers for the *CYP2A6*, *-3A5* and *-4B1* genes.

Materials and Methods

Tissue samples RNA extraction and cDNA synthesis

Prostate needle biopsies (10 to 50 mg) were obtained from a total of 21 patients who visited Cu-

kurova University Hospital, Adana, Turkey. A written informed consent was obtained from each patient and the study was approved by the Ethics Committee of the University. Characteristics of the subjects, covered in this study are summarized in Table II.

The samples were frozen immediately in liquid nitrogen and homogenized in a microtube using a cell lyser for RNA isolation (Retsch, Haan, Germany). Total RNA was isolated using the "High-pure RNA Tissue Kit", and complementary DNA was synthesized using the RNA-PCR kit according to the manufacturer's specifications (Roche, Penzberg, Germany). Reverse transcription was carried out at 65 °C for 10 min, followed by incubation at 55 °C for 30 min and 85 °C for 5 min. The products were exclusively evaluated for GAPDH using conventional PCR to check the fidelity of cDNA synthesis. The primers for detecting *CYP3A5*, *-4B1* and *GAPDH* were purchased as "Human Cytochrome P 450 Competitive RT-PCR Kit" (Takara, Tokyo, Japan). The *CYP2A6* primer set was designed using GenBank database (accession numbers: NM 00762 and NG 000008); 5′-CCCTCATGAAGATCAGTGAGC-3′ forward and 5′-GCGCTCCCCGTTGCTGAATA-3′ reverse primer, giving a band of 200 bp upon PCR amplification. Polymerase chain reactions were carried out in a solution of 20 μL containing 0.1 μg of cDNA, 1 unit *Taq* DNA polymerase and 20 pmol of each primer. The PCR conditions consisted of an initial denaturation at 94 °C for 2 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 30 s and polymerization at 72 °C for 1 min (Sarikaya *et al.*, 2007). Reaction products were analyzed on 2% agarose gels in Tris-Borate-EDTA (TBE) buffer (5 V/cm), and gels were photographed under UV light after staining with ethidium bromide (0.5 μg/mL). To control the reproducibility, all amplifications were repeated at least once. A visible band in agarose gel was regarded as a sign of the presence of corresponding mRNA.

Real-time PCR analysis

RT-PCRs were carried out using a LightCycler instrument (Roche Diagnostics, Mannheim, Germany) and a "LightCycler FastStart DNA Master SYBR Green I Kit" (Roche Applied Science, Indianapolis, IN, USA). For each reaction, the optimized reaction mixture contained 2 μL of LightCycler FastStart Master SYBR Green I, 12.4 μL of PCR-grade H_2O, 1.6 μL of 25 mmol/L $MgCl_2$, 2 μL of 10 mmol/L primer set, and 2 μL of template cDNA. Amplification melting curve analysis was performed thereafter using the program run for one cycle at 95 °C for 1 s hold followed by a slow decrease in the temperature (–0.1 °C/s) and set at continuous acquisition mode. Negative controls without cDNA template were included with every PCR run and were always negative (not shown). All RT-PCR protocols used in this study are presented in Table I.

Table I. The PCR programs for real-time PCR with LightCycler FastStart DNA Master SYBR Green I for the CYP isoforms tested in this study.

Program	Gen		
	CYP2A6	*CYP3A5*	*CYP4B1*
Pre-incubation	95 °C/30 s	95 °C/30 s	95 °C/30 s
Amplification	95 °C/0 s	95 °C/0 s	95 °C/0 s
	58 °C/5 s	60 °C/7 s	55 °C/5 s
	72 °C/17 s	72 °C/18 s	72 °C/15 s
Melting curve	95 °C/20 s	95 °C/20 s	95 °C/20 s
	55 °C/20 s	56 °C/20 s	50 °C/20 s
	95 °C/0 s (slope = 0.1 °C/s)	95 °C/0 s (slope = 0.1 °C/s)	95 °C/0 s (slope = 0.1 °C/s)
Cooling	40 °C/30 s	40 °C/30 s	40 °C/30 s

Results and Discussion

The aim of this study was to detect the mRNAs for *CYP2A6*, *CYP3A5* and *CYP4B1* in the target tissue. The expression of *CYP*s can be determined by various methods including immunoblotting, immunohistochemistry and RT-PCR (Senft and LeVine, 2005). Given that the interpretation of immunoblotting and immunohistochemistry methods is complicated because of the possible non-specific binding of antibodies, RT-PCR is generally considered as a sensitive and powerful method for detection and amplification of low levels of mRNA expression (O'Connell, 2002).

Serum measurement of prostate-specific antigen (PSA), an androgen-regulated serine protease, has been widely used as tumour marker for the early detection of PC and monitoring cancer progression (Gsur *et al.*, 2004). The patients who participated in this study were compared on the basis of PSA as well as age parameters using Mann-Whitney U test (SPSS version 10.0). The ages shown as means ± SD of subjects with malign tumour (5/21) and of subjects without malignancy (16/21)

Table II. Characteristics of the subjects in age and PSA parameters.

Parameter		Mean (± SD)	Median (min–max)	Evaluation
Age	Cancer	65.1 ± 8.2	66.5 (52 – 77)	$p = 0.303$
	Normal	59.6 ± 8.7	58.0 (50 – 70)	
PSA	Cancer	64.6 ± 55.6	100 (3.38–115)	$p = 0.231$
	Normal	16.2 ± 11.8	14.6 (0.3 – 39.4)	

were (65.1 ± 8.2) years (range, 52–77) and (59.6 ± 8.7) years (range, 50–70), respectively (Table II). There was no statistically significant difference either in the mean age ($p = 0.303$) or PSA values ($p = 0.231$) between the subjects with malign tumour and the subjects without malignancy (Table II). RT-PCR gene expression analyses were carried out without prior knowledge of the histopathological findings.

To check the reliability of our method, we first used a positive control primer set for *GAPDH* in the conventional PCR after the synthesis of cDNAs from isolated total RNA from the tissue samples. The results for a representative number of samples (14 out of 21) are given in Fig. 1. Our results showed the bands for amplified GAPDH at the expected molecular weight (546 bp) (Fig. 1, lanes 1 to 14). No amplification was observed in the negative control reaction, which was set by omitting template cDNA (data not shown).

The gene expression of 3 different *CYP* genes, *CYP2A6*, *CYP3A5* and *CYP4B1,* were detected by using RT-PCR combined with melting curve analysis in a total of 21 human prostatic tumour and non-tumour samples. A representative melting curve analysis of *CYP3A5* gene expression is given in Fig. 2. The melting temperatures of *CYP2A6, CYP3A5* and *CYP4B1* were determined to be 78.0 °C, 84.2 °C and 84.0 °C, respectively (Fig. 2 and data not shown). *CYP2A6* and *CYP3A5* mRNAs were detected in all the 21 prostate samples, while *CYP4B1* expression was seen in 11 out of the 21 samples (Table III). Therefore, our statistical evaluation using Fischer's χ^2 test covered only *CYP4B1* expression. As seen in Ta-

Table III. *CYP4B1* gene expression in relationship to the histopathological status and smoking habit of the subjects.

Subject	*CYP4B1* (+)	*CYP4B1* (–)	Number of individuals
Cancer	3 (60%)	2 (40%)	5
Normal	8 (50%)	8 (50%)	16
Total	11 (52%)	10 (48%)	21
$p = 1.00$ ($p > 0.05$)			
Smoker	8 (67%)	4 (33%)	12
Non-smoker	3 (33%)	6 (67%)	9
Total	11 (52%)	10 (48%)	21
$p = 0.198$ ($p > 0.05$)			

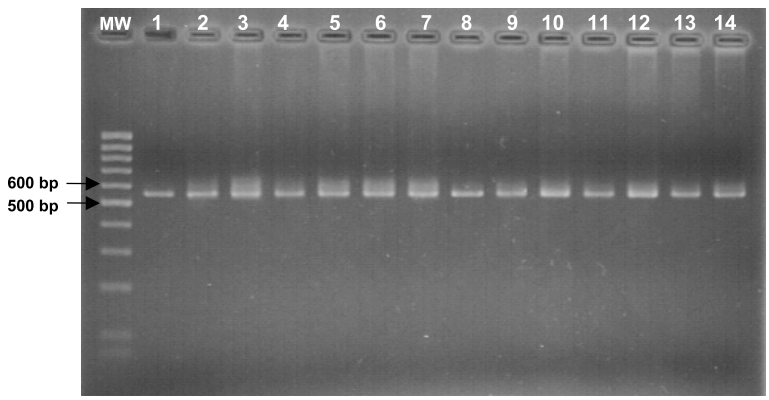

Fig. 1. A representative gel photograph of the amplified *GAPDH* primer (see text for details). MW, 100 bp molecular weight markers covering from 1031 bp to 80 bp; lanes 1–14, amplified *GAPDH* for the samples from 1 to 14.

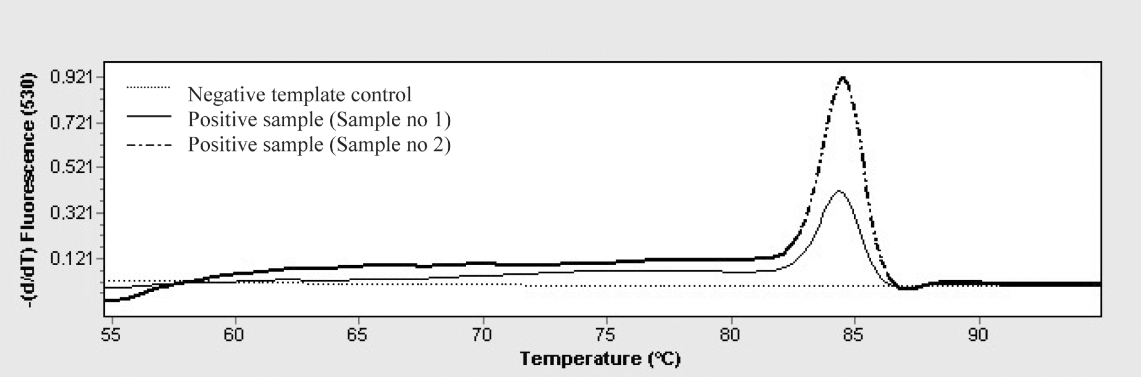

Fig. 2. Melting curve analysis of amplified samples with cDNA for the detection of *CYP3A5* gene expression. Template cDNA was replaced by PCR-grade water as negative control. The samples showed a characteristic sharp decrease in fluorescence at about 84 °C by melting curve analysis obtained by plotting the negative derivative of fluorescence over temperature (-d/d*T*) versus temperature (*T*).

ble III, 60% of the PC samples were found to have *CYP4B1* mRNAs while 50% of the control subjects were positive for *CYP4B1* (Table III). When the results were analyzed on the basis of the smoking habit of the subjects, 67% of the smokers were found to be positive for the detection of *CYP4B1* mRNA while only 33% of non-smokers were *CYP4B1* positive (Table III). Statistical evaluation of the results showed that neither histopathological status nor the smoking habit of the subjects differed from each other significantly in *CYP4B1* expression ($p = 1.00$ and $p = 0.198$, respectively). Because only one of the total 21 subjects had alcohol consumption, we did not carry out a statistical analysis on the basis of this variable.

The expression of *CYP3A5* was reported in prostate and other extrahepatic tissues by a number of researchers using the RT-PCR methodology (Murray *et al.*, 1995; Yamakoshi *et al*., 1999; Finnstrom *et al.*, 2001; Nishimura *et al.*, 2003). A more recent study reported the *CYP3A5* expression in normal prostate cells but not in prostate cancer cells (Leskelä et *al*., 2007). The presence of CYP3A5 was attributed to the excretion of testosterone and progesterone from prostate by altering them to polar metabolites in these studies. The latter group also reported the presence of *CYP4B1*, but not *CYP2A6*, in prostate tissue (Nishimura *et al.,* 2003). Another study reported *CYP4B1* mRNAs in the majority of 28 prostate tissue samples (Finnstrom *et al.,* 2001). This CYP isoform participates in the metabolism of certain xenobiotics that are protoxic, including valproic acid, 3-methylindole, 4-ipomeanol, 3-methoxy-4-aminoazobenzene, and numerous aromatic amines (Finnstrom *et al.*, 2001). Therefore, the presence or absence of CYP4B1 is important as we found that not all prostate samples resulted in *CYP4B1* expression (Table III). CYP2A6, on the other hand, represents a relatively minor component (~4%) of the human CYPs, and it is known to catalyze the metabolic activation of several procarcinogens like nitrosamines, clinically used drugs and nicotine (Topcu *et al.,* 2002). Besides liver, *CYP2A6* expression was also reported in human nasal and bronchial mucosa, trachea and lung (Su *et al.*, 1996; Ding and Kaminsky, 2003).

Taken together, our results agree with the reports of previous literature in terms of *CYP3A5* and *CYP4B1* detection but differ from these reports on detecting *CYP2A6* mRNAs. To our knowledge, this is the first study reporting the presence of CYP2A6 in prostate tissue. However, the expressions of individual CYPs, we and others reported, need further analyses in an extended research to understand the contribution of human extrahepatic tissues to drug clearance and relative importance in chemical toxicity of hepatic clearance versus extrahepatic target tissue metabolic activities. The clinical significance of CYPs in the risk of developing PC is in the focus of our future work.

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