Characterisation and PCR-based detection of a *CYP2A6* gene deletion found at a high frequency in a Chinese population

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Abstract Cytochrome P450 2A6 is an important human hepatic P450 which activates pre-carcinogens, oxidises some drugs and constitutes the major nicotine *C*-oxidase. In fact, results have been presented in the literature which suggested a relationship between the distribution of defective *CYP2A6* alleles and smoking behaviour as well as cigarette consumption. In the present report, we describe the structure of a novel *CYP2A* locus where the whole *CYP2A6* gene has been deleted, resulting in an abolished cytochrome P450 2A6-dependent metabolism. The origin of this locus is apparently due to an unequal crossover event between the 3′-flanking region of the *CYP2A6* and *CYP2A7* genes. A rapid PCR-based method for the detection of the *CYP2A6del* allele was developed and the allele frequency was 15.1% among 96 Chinese subjects, but only 1.0% in Finns ($n = 100$) and 0.5% in Spaniards ($n = 100$). In the Chinese population, we did not detect any *CYP2A6*2* alleles using an improved genotyping procedure, in contrast to the 11–20% previously reported. It is concluded that genotyping for the *CYP2A6del* allele is of great importance in studies correlating, for example, smoking behaviour, pre-carcinogen activation or drug metabolism to the *CYP2A6* genotype, in particular when oriental populations are investigated.

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Key words: Cytochrome P450; Coumarin; Gene deletion; Unequal crossover; Genotype; Phenotype

1. Introduction

Cytochrome P450 2A6 (CYP2A6) is an important hepatic P450 that can activate many different pre-carcinogens, including *N*-nitrosodiethylamine, aflatoxin B1, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, 1,3-butadiene and 2,6-dichlorobenzonitrile (see [1,2] and references therein). In addition, CYP2A6 is involved in the metabolism of some pharmaceuticals such as methoxyflurane, halothane, (+)-*cis*-3,5-dimethyl-2-(3-pyridyl) thiozolidin-4-one hydrochloride (SM-12502), losigamone, letrozole, valproic acid and disulfiram (see [3] for references). In addition, CYP2A6 catalyses the *C*-oxidation of nicotine to cotinine [4–6], the major metabolic pathway of nicotine, and also carries out the subsequent 3′-hydroxylation of cotinine [7]. Pianezza and coworkers [8] have presented results indicating that inter-individual variability in the CYP2A6 activity affected smoking behaviour. They presented results indicating that a lower number of subjects carrying defective *CYP2A6* alleles were found in a tobacco-dependent group as compared to a never tobacco-dependent group and that smokers carrying a defective *CYP2A6* allele smoked fewer cigarettes. Thus, these results could implicate that carriers of defective *CYP2A6* alleles would be at a lower risk of becoming smokers and thereby be protected from lung cancer and other tobacco-related diseases.

Phenotyping with the probe drug coumarin has revealed a pronounced inter-individual variability in the CYP2A6 activity [9–11]. In Caucasian populations, the prevalence of the poor metaboliser (PM) phenotype for CYP2A6 appears to be very low and there are only a few cases reported where individuals completely lacked CYP2A6 activity [3,12]. By contrast, the frequency of PMs for CYP2A6 among Japanese seems to be higher and in vitro studies using microsomes from Japanese subjects demonstrated that eight out of 30 livers had a very low or no CYP2A6 immunoreactivity and activity [13].

The *CYP2A6* gene has been mapped to chromosome 19 between 19q12 and 19q13.2 [14]. It is located within a 350 kb gene cluster together with the *CYP2A7* and *CYP2A13* genes, two *CYP2A7* pseudogenes as well as genes in the *CYP2B* and *CYP2F* subfamilies [15]. Several variants of the *CYP2A6* gene have been described thus far. The *CYP2A6*2*[1] allele encodes an enzyme with a L160H substitution that does not incorporate heme and is therefore inactive [17]. The original PCR-based method for the detection of this allele [18] has been found to occasionally yield erroneous results. Because of this, we recently developed an improved a genotyping method for the *CYP2A6*2* allele [3], which avoids misclassifying individuals as PMs. Using this method, we determined a *CYP2A6*2* allele frequency of 1–3% in Caucasians populations in contrast to the 4–17% as otherwise reported [18,19]. In addition to the *CYP2A6*2* allele, *CYP2A6*3*, proposed to have been generated through a gene conversion between *CYP2A6* and the *CYP2A7* pseudogene, was also suggested

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Abbreviations: CYP or P450, cytochrome P450; PM, poor metaboliser; PCR, polymerase chain reaction

[1] In this report we have used the nomenclature system for *CYP2A6* alleles proposed by Daly et al. [16]: *CYP2A6*1*, 2A6wt; *CYP2A6*2*, 2A6v1; *CYP2A6*3*, 2A6v2

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to be inactive [18]. Nunoya and coworkers have also briefly described an allele found in Japanese individuals where a part of the *CYP2A6* gene was deleted [20]. As expected, individuals homozygous for this allele completely lacked both CYP2A6 mRNA and protein and were therefore CYP2A6 PMs.

Due to the important role that CYP2A6 has in the nicotine metabolism and the recent report of a deleted *CYP2A6* allele [20], we considered it of interest to investigate the occurrence of defective *CYP2A6* alleles and the *CYP2A* locus in an oriental population. To this end, we have characterised a *CYP2A* locus which lacks the *CYP2A6* gene, developed a rapid PCR-based method for its detection and determined the frequency of this allele in three different ethnic groups.

2. Material and methods

2.1. Subjects

Three different populations of Chinese, Spanish and Finnish origins were used. Genomic DNA was obtained from 96 Chinese individuals and 100 volunteers from the Zaragoza area in northern Spain, which have been used earlier in *CYP2D6* genotype-phenotype correlation studies [21,22]. The Finnish population consisted of a random sample of 100 healthy Finns (27–67 years old) living in the southwestern area of Finland. We also genotyped DNA from 11 Thai individuals that were previously phenotyped with coumarin [23]. This study was approved by the ethical committees at Karolinska Institutet and the University of Oulu.

2.2. Southern blot analysis

Approximately 4 μg of genomic DNA was digested with the restriction endonuclease *Eco*RI and was subjected to electrophoresis for 3 days at 0.85 V/cm using a 0.8% agarose gel. Southern blot was performed using Qiabrane positively charged nylon membranes (Qiagen GmbH, Hilden, Germany). A ^{32}P-labelled *CYP2A6* probe ranging from exon 6 to exon 9 was used to avoid hybridisation with the *CYP2A7PT* and *CYP2A7PC* pseudogenes [18]. Densitometric analysis of the Southern blot fragments was performed using a personal densitometer (Molecular Dynamics, Sunnyvale, CA, USA).

2.3. Long PCR and DNA sequencing

A GeneAmp XL PCR Kit (Perkin-Elmer, Norwalk, CT, USA) was used to amplify the *CYP2A7*/*CYP2A6* hybrid from genomic DNA of individual Ch98 using the *CYP2A7*-specific primer 5′-GCA CGT TGA ACC TCT TCA TTG CA-3′ located in exon 6 and the *CYP2A6*-specific primer 2A6R1 (see Table 1). The PCR fragments and cosmids 19296 and 19019, containing the *CYP2A6* and *CYP2A7* genes, respectively (kindly supplied by Dr. Linda K. Ashworth, LLNL Human Genome Center, Livermore, CA, USA) [15], were partially sequenced on both strands with the ABI PRISM BigDye terminator cycle sequencing kit and analysed on an ABI Prism 377 DNA sequencer.

2.4. CYP2A6 genotyping

Genotyping for the *CYP2A6*2* allele was carried out essentially as previously described [3]. The β-globin primers 5′-GAA GAG CCA AGG ACA GGT AC-3′ and 5′-TAG CCA CAC CAG CCA CCA CT-3′ were, however, also added to the reaction mixture at a final concentration of 0.12 μM in order to amplify a part of the β-globin gene as a positive control for the PCR reaction.

A two step PCR method was used to detect the *CYP2A6del* allele. In the first PCR reaction (PCR I), a region from exon 7 to approximately 420 bp downstream of exon 9 of *CYP2A6* or the *CYP2A7*/*CYP2A6* hybrid was specifically amplified. This PCR product was subsequently used as a template in the second allele-specific PCR reaction (PCR II). PCR I was done using the primers 2Aex7F and 2A6R1 (see Table 1). The reaction mixture contained approximately 50 ng genomic DNA, 0.25 μM of each primer, 0.2 mM of each dNTP, 1.2 mM $MgCl_2$, 0.6 U of Taq polymerase (Advanced Biotechnologies, Epsom, UK) and was carried out in 1×Reaction Buffer IV (Advanced Biotechnologies) in a total volume of 25 μl using a Perkin Elmer GeneAmp PCR System 2400 or 9700. The initial denaturation was performed at 95°C for 1 min, followed by 35 cycles each consisting of denaturation at 95°C for 15 s, annealing at 60°C for 20 s and extension at 72°C for 3 min, followed by a final extension at 72°C for 7 min.

The mixture for the allele-specific reaction (PCR II) contained 0.8 μl PCR I product, 0.25 μM of primer 2A6ex8F or primer 2A7ex8F, 0.25 μM of primer 2A6R2 (see Table 1), 0.1 mM of each dNTP, 1.0 mM $MgCl_2$, 0.3 U of Taq polymerase and was done in buffer consisting of 10 mM Tris-HCl, pH 8.3 and 50 mM KCl in a total volume of 25 μl. The amplification was performed by initial denaturation at 95°C for 1 min, followed by 14 cycles each involving denaturation at 95°C for 15 s, annealing at 52°C for 20 s and extension at 72°C for 2 min. 8 μl of the PCR products was subsequently analysed on a 1.0% agarose gel (Life Technologies, Rockville, MD, USA) stained with ethidium bromide.

3. Results

3.1. The allele frequency of the CYP2A6*2 allele in a Chinese and a Finnish population

We used our previously described method [3] to genotype the *CYP2A6*2* allele on a population of 96 Chinese individuals. In three of the samples, we failed to obtain any amplification product with our *CYP2A6*-specific primers located in exon 1 and exon 4, although the β-globin fragment was amplified in all cases (data not shown). This suggested that these subjects were homozygous for an allele lacking the *CYP2A6* gene, therefore these samples were analysed further in greater detail (see below). The other 93 samples were subjected to the allele-specific second PCR reaction. No *CYP2A6*2* alleles were, however, detected in the Chinese population. Similar analysis of genomic DNA from 100 Finns revealed a *CYP2A6*2* allele frequency of 3.0%, in accordance with our previous results [3].

3.2. The structure of the CYP2A locus in individuals with a deleted CYP2A6 gene

The *CYP2A* locus has previously been mapped with the restriction endonuclease *Eco*RI [15]. We therefore carried out a Southern blot analysis using *Eco*RI on genomic DNA from 15 Chinese individuals, including one of the individuals in which the *CYP2A6* gene could not be amplified (Ch98). Southern blot analysis of cosmids from the *CYP2A* locus revealed that in a normal *CYP2A* locus, the 6.6 kb, 7.5 kb and 7.7 kb fragments correspond to the *CYP2A6*, *CYP2A7* and *CYP2A13* genes, respectively. The faint 15 kb fragment likely represents another part of the *CYP2A13* gene or another related sequence.

Interestingly, individual Ch98 completely lacked the 7.5 kb fragment (Fig. 1A). Our explanation for the Southern blot pattern is that this subject carried two alleles with *CYP2A6* gene deletions. An unequal crossover event which has occurred between the 3′-flanking regions of the *CYP2A6* and *CYP2A7* genes would lead to the loss of both the 7.5 kb and 6.6 kb fragments and generate a novel *CYP2A7*/*CYP2A6* hybrid fragment of 6.6 kb (Fig. 1B). Densitometric

Table 1
Primers used to genotype for the *CYP2A6del* allele

Primer	Sequence
2Aex7F	5′-GRC CAA CAT GCC CTA CAT G-3′
2A6ex8F	5′-CAC TTC CTG AAT GAG-3′
2A7ex8F	5′-CAT TTC CTG GAT GAC-3′
2A6R1	5′-GCA CTT ATG TTT TGT GAG ACA TCA GAG ACA A-3′
2A6R2	5′-AAA ATG GGC ATG AAC GCC C-3′

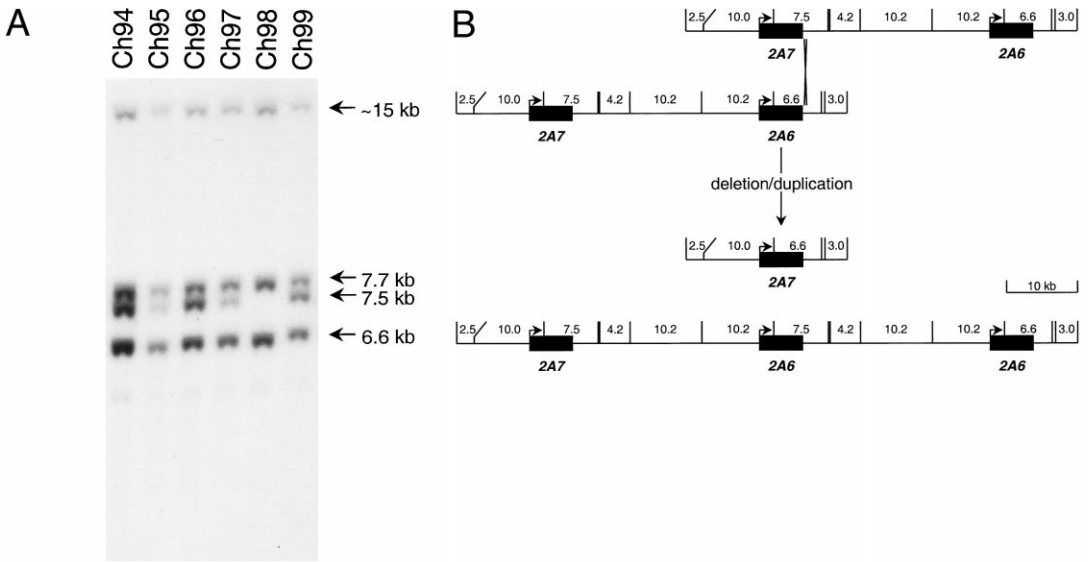

Fig. 1. A: Southern blot analysis of the *CYP2A* genes in genomic DNA from Chinese individuals using the restriction endonuclease *Eco*RI. MW, 1 kb plus DNA ladder (Life Technologies, Rockville, MD, USA). B: The proposed mechanism for the *CYP2A6* gene deletion which was generated through an unequal crossover event. The outcome would be the *CYP2A6del* allele and a hypothetical allele containing two *CYP2A6* genes in tandem. *Eco*RI restriction sites are shown with vertical lines. Fragment lengths generated by *Eco*RI digestion were previously determined by cosmid mapping [15]. Genes are represented by black boxes and the start codons are indicated by bent arrows.

quantification of the relative intensity of the 7.5 kb fragment from 15 different individuals divided them into three distinct groups, which corresponded to having either 0, 1 or 2 copies of the *CYP2A6* gene (see Table 2).

In order to design gene-specific primers in the 3′-flanking regions of the *CYP2A6* and *CYP2A7* genes, we sequenced 520 bp downstream of exon 9 using the cosmids which contain either the *CYP2A6* or *CYP2A7* gene. As expected, the sequences were very similar but a number of regions with differences were found (see Fig. 2). The *CYP2A6*-specific primer 2A6R1, which was designed in one of these regions, was used together with a *CYP2A7*-specific primer in exon 6 to amplify the *CYP2A7*/*CYP2A6* hybrid from individual Ch98. The PCR fragment was partially sequenced and this showed that the proximal 3′ flank consisted of the *CYP2A7* sequence, whereas the region further downstream had the *CYP2A6* sequence (Fig. 2). Thus, the breakpoint for the unequal crossover is likely situated in the 82 bp region of the identical sequence, defined as the 'deletion junction region'.

Table 2
Correlation between the *CYP2A6* genotype determined by a Southern blot and by the allele-specific PCR method

Individual	Relative intensity (7.5 kb band/7.7 kb band)[a]	Genotype determined by PCR
Ch72	1.5	**1/*1*
Ch73	1.5	**1/*1*
Ch78	0.5	**1/del*
Ch79	1.3	**1/*1*
Ch81	0.5	**1/del*
Ch84	0.4	**1/del*
Ch85	1.4	**1/*1*
Ch92	0.5	**1/del*
Ch93	1.3	**1/*1*
Ch94	1.3	**1/*1*
Ch95	0.5	**1/del*
Ch96	1.6	**1/*1*
Ch97	0.5	**1/del*
Ch98	0.0	*del/del*
Ch99	1.4	**1/*1*

[a]The constant 7.7 kb fragment was used as an internal standard for DNA loading.

3.3. PCR-based detection of the deleted CYP2A6 gene

In order to facilitate genotyping for the *CYP2A6del* allele, we developed a PCR-based method as outlined in Fig. 3A. In the first PCR reaction (PCR I), a fragment extending from exon 7 to 420 bp downstream of exon 9 was amplified in both the *CYP2A6*1* and *CYP2A6del* alleles. This reaction yielded a 1.9 kb product from all individuals and therefore also acts as a positive control for the PCR reaction. This fragment was subsequently used as a template for an allele-specific PCR method (PCR II). In this reaction, primers were specifically designed for amplification of either the *CYP2A6*1* or the *CYP2A7*/*CYP2A6* hybrid found in individuals with the *CYP2A6del* allele, allowing for the convenient detection of heterozygous or homozygous carriers of the *CYP2A6del* allele (see Fig. 3). Because the 3′-flanking region from +40 to +120 is polymorphic in certain individuals ([3], M. Oscarson, unpublished observations), we opted to amplify a relatively long fragment to avoid using primers in this region. As shown in Table 2, our PCR method could correctly predict the genotype of all 15 individuals which were analysed by a Southern blot.

3.4. Correlation between genotype and phenotype

The PCR-based genotyping method was validated with respect to the in vivo CYP2A6 activity by genotyping 11 Thai individuals who had previously been phenotyped with coumarin [23]. One individual, that completely lacked CYP2A6 activity in vivo, was found to be homozygous for the *CYP2A6del* allele and two individuals, who excreted only 1.2 and 3.7 mg of 7-OH-coumarin, respectively, were heterozygous for the *CYP2A6del* allele. The other eight individuals were homozygous for the *CYP2A6*1* allele and excreted on average 5.6 mg 7-OH-coumarin (range 1.0–11.9). Thus, the

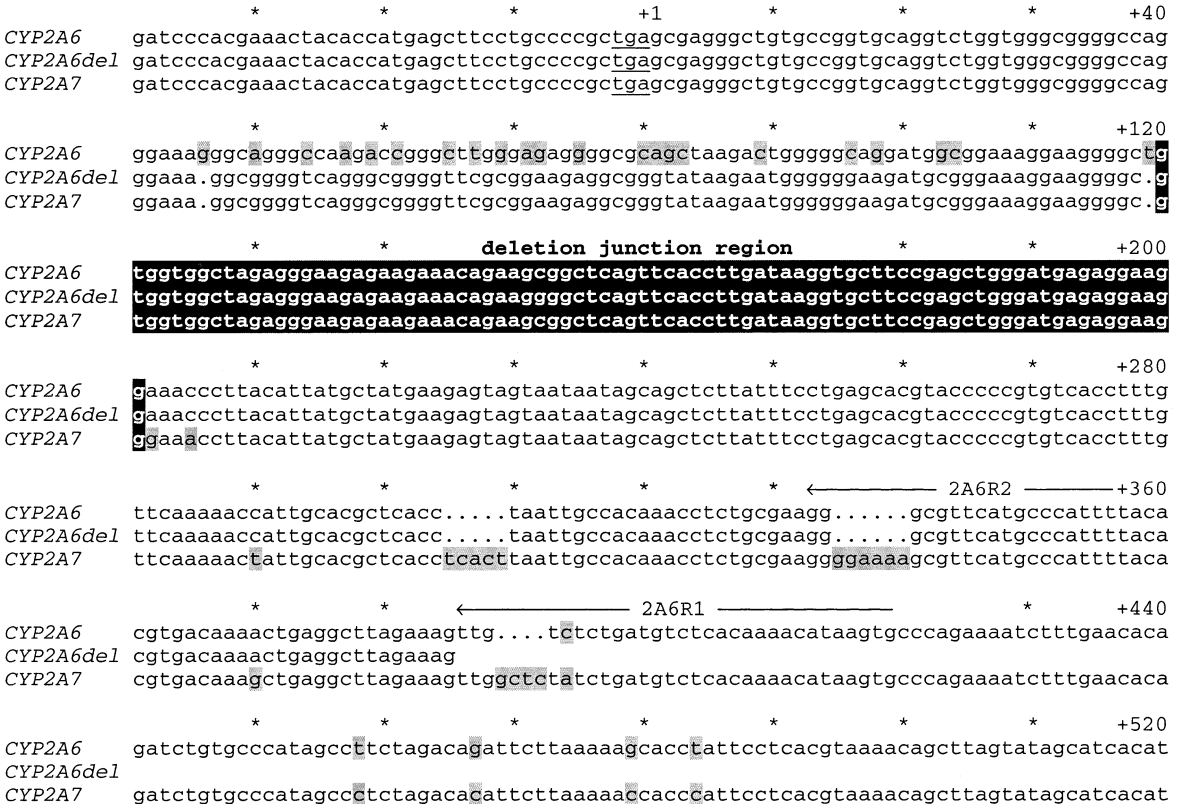

Fig. 2. The DNA sequence of the 3′-flanking region of the *CYP2A6* and *CYP2A7* genes and the *CYP2A6del* allele. The light gray shaded boxes represent differences between the *CYP2A* sequences. The proposed crossover region, in which the *CYP2A6* and *CYP2A7* sequences are identical, is indicated by the black box. Arrows above the sequences indicate the *CYP2A6*-specific reverse primers used for genotyping. Numbering of the sequence begins with the first base pair following the stop codon (underlined).

presence of the *CYP2A6del* allele correlated well with the observed in vivo coumarin-7-hydroxylase activity.

3.5. Allele frequencies of the CYP2A6del allele

To estimate the frequency of the *CYP2A6del* allele, we genotyped almost 300 subjects belonging to three different ethnic groups, namely 96 Chinese individuals, 100 Finns and 100 individuals from northern Spain. In the Chinese population, we observed an allele frequency of 15.1% (Table 3). Three of the 96 individuals were homozygous for the *CYP2A6del* allele, indicative of a PM phenotype. The *CYP2A6del* allele was, however, rare in the two Caucasian populations and only three individuals in total were heterozygous for the *CYP2A6del* allele.

4. Discussion

In the present study, we have characterised a *CYP2A* locus completely lacking the *CYP2A6* gene using a sequence analysis of cosmids, gene fragment analysis by Southern blot and PCR amplification. We have also developed a reliable PCR method for the rapid screening of the *CYP2A6* gene deletion, which accurately predicts the CYP2A6 phenotype. The gene deletion is most likely similar or identical to one of the defective alleles recently described by Kamataki and collaborators [20,24] but the limited amount of data presented by these authors did not allow for a detailed comparison to be made.

A striking inter-ethnic difference in the distribution of the *CYP2A6del* allele was found as a high frequency was observed in the Chinese population, in contrast to the very low frequency seen in Caucasians. This indicates that the *CYP2A6* deletion is a relatively recent genetic event which occurred after the Caucasoid/Asian split 35 000 years ago [25]. In contrast, the *CYP2D6* gene deletion which creates the *CYP2D6*5* allele, appears to be a much older genetic event because the frequency of *CYP2D6*5* is between 2 and 5% in all populations studied [26].

We propose that the *CYP2A6del* allele was generated through an unequal crossover event between non-allelic sequences involving an unequal pairing of homologous chromo-

Table 3
CYP2A6 genotypes and allele frequencies in Chinese, Finnish and Spanish populations determined by allele-specific PCR methods

Population	Genotypes frequency (%)						Alleles frequency (%)		
	*1/*1	*1/*2	*2/*2	*1/del	*2/del	del/del	CYP2A6*1	CYP2A6*2	CYP2A6del
Chinese ($n=96$)	72.9	0.0	0.0	24.0	0.0	3.1	84.9	0.0	15.1
Finns ($n=100$)	92.0	6.0	0.0	2.0	0.0	0.0	96.0	3.0	1.0
Spaniards ($n=100$)	93.0	6.0[a]	0.0[a]	1.0	0.0	0.0	96.5	3.0[a]	0.5

[a]Data are from Oscarson et al. [3].

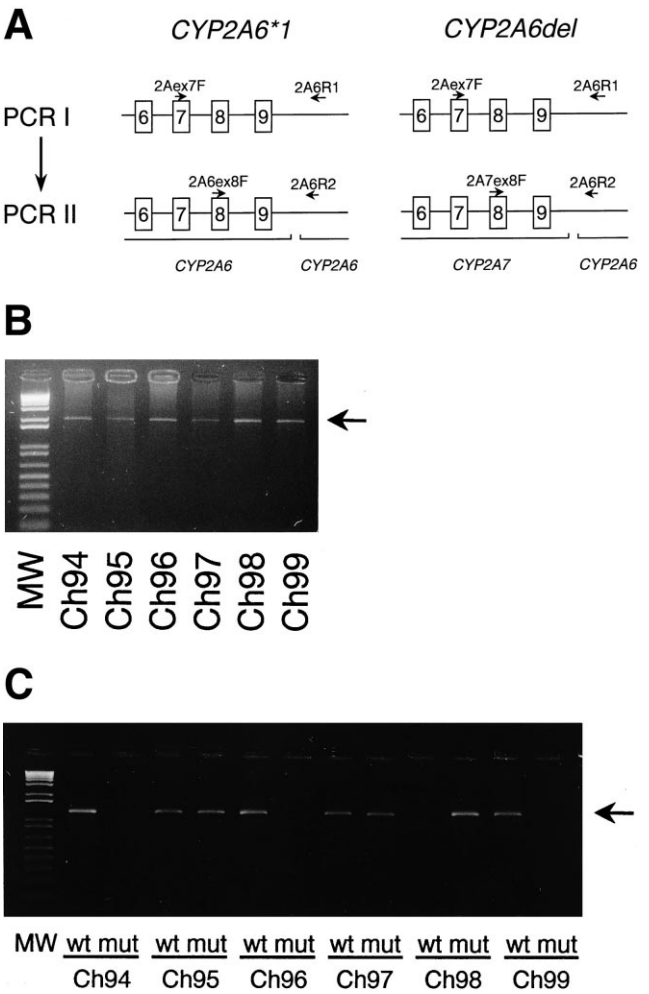

Fig. 3. A: A schematic representation of the strategy used to genotype for the *CYP2A6del* allele. B: Amplification of a part of the *CYP2A6* gene or the *CYP2A7*/*CYP2A6* hybrid from genomic DNA (PCR I). C: Allele-specific PCR for detection of the *CYP2A6del* allele (PCR II). MW, 1 kb plus DNA ladder (Life Technologies, Rockville, MD, USA).

somes or sister chromatids. Our sequence analysis revealed that the deletion junction region of 82 bp is located approximately +150 bp from the *CYP2A6* gene. This region has also recently been proposed by Yokoi and Kamataki [24] to be a breakpoint region. A similar mechanism for gene deletion is involved in the generation of alleles lacking either the *CYP2D6* or *GSTM1* genes [27,28]. The other outcome of an unequal crossover event is an insertion creating an allele with two active genes in tandem, causing more enzyme to be expressed and thereby an increased enzyme activity. Indeed, individuals have been found where either the *CYP2D6* or the *GSTM1* gene is duplicated [29,30] and it is likely that subjects also carrying alleles with duplicated *CYP2A6* genes exist. Such alleles could, however, have been eliminated through an evolutionary pressure.

Our novel genotyping method for the *CYP2A6*2* allele [3] was also used on the Chinese population studied. Interestingly, we did not detect any *CYP2A6*2* alleles in the Chinese population, in contrast to the 11–20% previously reported [18]. One possible explanation for this difference could be that individuals were misclassified in the earlier studies due to the erroneous method used.

The results presented by Pianezza and coworkers [8] indicate a relationship between the distribution of defective *CYP2A6* alleles and the likelihood for an individual to become a smoker as well as the number of cigarettes smoked per day. Thus, CYP2A6 PMs would be at a lower risk of becoming smokers and thereby be protected from lung cancer and other tobacco-related diseases. Many pre-carcinogens found in tobacco smoke are also activated by CYP2A6 [1] and it could be speculated that PMs would activate these pre-carcinogens at a reduced rate, thereby gaining an additional protective effect against tobacco smoke. This could be of particular importance in the oriental populations where the frequencies of PMs and heterozygotes are considerably higher than in Caucasians. The genotyping method presented herein will facilitate molecular epidemiological studies aimed at elucidating the potential impact of the *CYP2A6* genotype on such diseases.

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