

Original Article

CYP2E1 gene rs6413420 polymorphism was first found in the Bouyei ethnic group of China

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Abstract: Background: China is a multinational country. The relationship between gene polymorphisms of xenobiotic metabolizing enzymes and national ethnicity has not previously investigated among Chinese people. The aim of this study was to investigate distributions of CYP1A1 and CYP2E1 gene polymorphisms in five ethnic groups of China. Methods: 829 blood samples were collected from five ethnic groups (Han, Shui, Miao, Zhuang, Bouyei). Taqman-MGB probe was used in Real-time PCR to test the gene polymorphisms of CYP1A1 (rs1048943 and rs4646903) and CYP2E1 (rs2031920 and rs6413420). We further validate the SNP genotyping results through DNA sequencing. Results: The genotype distribution of all four SNPs was in accordance with Hardy-Weinberg equilibrium except the genotype distribution of rs4646903 in Han and Bouyei ethnic groups ($p=0.013$ and 0.0005 , respectively). CYP2E1 gene rs6413420 polymorphism was first found in the Bouyei ethnic group in China. The results of DNA sequencing were entirely in line with the SNP genotyping assay. Conclusions: The CYP1A1 and CYP2E1 genetic polymorphisms were different in different ethnic groups in China. CYP2E1 gene rs6413420 polymorphism was first found in the Bouyei ethnic group of China.

Keywords: CYP1A1, CYP2E1, Taqman-MGB probe, Real-time PCR, SNPs

Introduction

Cytochrome P450 enzymes (CYP450s) are important phase I xenobiotic metabolizing enzymes (XMEs). Most CYP450s are polymorphic, because of gene deletions, single nucleotide polymorphisms (SNPs), gene duplications and mutated alleles. CYP1A1 and CYP2E1 are two of the main cytochrome P450 isoforms involved in the metabolism of endogenous compounds and xenobiotics considered to be responsible for the development of several human diseases. The human enzyme CYP1A1 is the most active among the CYPs in metabolizing procarcinogens, particularly, the polycyclic aromatic hydrocarbons (PAHs), into highly reactive intermediates [1]. A functional role has been previously assigned to two nonsynonymous polymorphisms in the CYP1A1 gene. The first one is an adenine (A) to guanine (G) substitution at codon 462 in exon 7 (Ile462Val, rs1048943).

The second one is a thymine (T) to cytosine (C) transition (rs4646903) [2]. Rs4646903 is a commonly studied SNP in the CYP1A1 which has been implied to associate with cancer risk [3]. The SNP located at nucleotide 3801 in the 3' non-coding region containing a single T to C substitution that results in a polymorphic restriction site for the MspI enzyme (MspI or CYP1A1 *2A polymorphism, rs4646903). The MspI restriction site polymorphism results in three genotypes: a predominant homozygous m¹ allele without the MspI site (type A, TT), the heterozygote (type B, TC) and a homozygous rare m² allele with the MspI site (type C, CC) [4]. The CYP2E1 enzyme is responsible for the metabolism of alcohol and some tobacco carcinogens such as low-molecular weight nitrosamines [5-7]. The most extensively studied SNPs of CYP2E1 are RsaI/PstI site in the 5' flanking region and the DraI site in intron 6 [8]. Two linked polymorphisms (CYP2E1 *5B) have been

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Table 1. The TaqMan-MGB primer sequences and probe

SNP site	Primer/probe	base sequence (5'-3')	Product length
rs1048943	primer	F: 5'-GGCAAGCGGAAGTGTATCG-3' R: 5'-CAGGATAGCCAGGAAGAGAAAGAC-3'	66 bp
	probe	W: 5'-VIC- TGAGACCATTGCCC-MGB-3' M: 5'-FAM-TGAGACCGTTGCCC-MGB-3'	
	primer	F: 5'-GCACTGGTACCATTTTGTTCCTACT-3' R: 5'-GCTGAGGTGGGAGAATCGT-3'	
probe	W: 5'-VIC- CACCTCCTGGGCTCA-MGB-3' M: 5'-FAM- ACCTCCCGGGCTCA-MGB-3'		
rs2031920	primer	F: 5'-TGACTTTTATTTTCTTCATTCTCATCATATTTCTATTATACAT-3' R: 5'-GTTTTTCATTCTGTCTTCTAACTGGCAATAT-3'	135 bp
	probe	W: 5'-VIC- AGGTTGCAATTTTGTACTTT-MGB-3' M: 5'-FAM- AGGTTGCAATTTTATACTTT-MGB-3'	
	rs6413420	primer	
probe		W: 5'-VIC-TCACCCTCCTTCTCAGAACACATTATAAAA-MGB-3' M: 5'-FAM-TCACCCTCCTTCTCATAACACATTATAAAA-MGB-3'	

Table 2. The reactive condition of Real-time PCR

SNP site	reactive condition
rs1048943	95 °C pretreatment for 20 s, 95 °C degeneration for 15 s, 56 °C anneal for 20 s, 60 °C extend and collect florescence for 34 s
rs4646903	95 °C pretreatment for 20 s, 95 °C degeneration for 15 s, 60 °C anneal, extend and collect florescence for 34 s
rs2031920	95 °C pretreatment for 20 s, 95 °C degeneration for 20 s, 60 °C anneal, extend and collect florescence for 34 s
rs6413420	95 °C pretreatment for 20 s, 95 °C degeneration for 15 s, 60 °C anneal, extend and collect fluorescence for 34 s

described in the *CYP2E1* gene at nucleotides -1259 and -1019. They are located in the 5' regulatory region and are detectable by *RsaI* or *PstI* restriction enzyme digestion [*RsaI* is 21053C > T (rs2031920), and *PstI* is 21293G > C (rs3813867), resp] [9-10].

TaqMan-MGB probe has the advantages of low fluorescence background, high resolution, strong hybridization specificity, good reproducibility, shorten probe length and low cost. It can be monitored in real-time and has no nonspecific diffusion phenomenon. It has become the internationally recognized method for detecting nucleic acids qualitatively and quantitatively.

Genetic susceptibility tends to be race-related, and this is due to the different races have different gene frequency. There have been some reports of *CYP1A1* rs1048943, rs4646903 and *CYP2E1* rs2031920, rs6413420 about gene frequency, but not for Chinese ethnic group. Therefore, we conducted SNPs classification experiment for *CYP1A1* gene rs1048943, rs4646903 and *CYP2E1* gene rs2031920,

rs6413420 using TaqMan-MGB probe for the first time in different ethnic group in China.

Subjects and methods

Blood sample

829 blood samples were collected from health individuals of different ethnic groups from different provinces, including 193 from the Zhuang in Guangxi Province, 108 from the Han in Guangdong Province, 169 from the Shui, 179 from the Miao and 180 from the Bouyei ethnic group in Guizhou Province. There were no genetic relationships, no smoking and drinking histories and no hereditary diseases among these participants. This study protocol was reviewed and approved by the Department of Science and Technology of Shenzhen City and the local Ethics Committees.

DNA extraction and agarose gel electrophoresis (AGE)

We extracted DNA from the blood through three types of methods (standard phenol and chloro-

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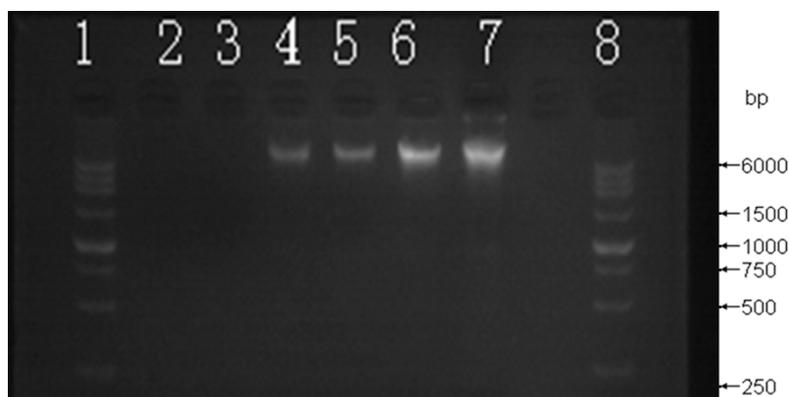


Figure 1. Agarose gel (1.6%) Electrophoresis of DNA extracted by standard phenol and chloroform, blood stain and QIGEN Mini Kit. Lane 1, 8: DNA markers; Lane 2, 3: Blood stain method; Lane 4, 5: Standard phenol and chloroform method; Lane 6, 7: QIGEN Mini Kit method.

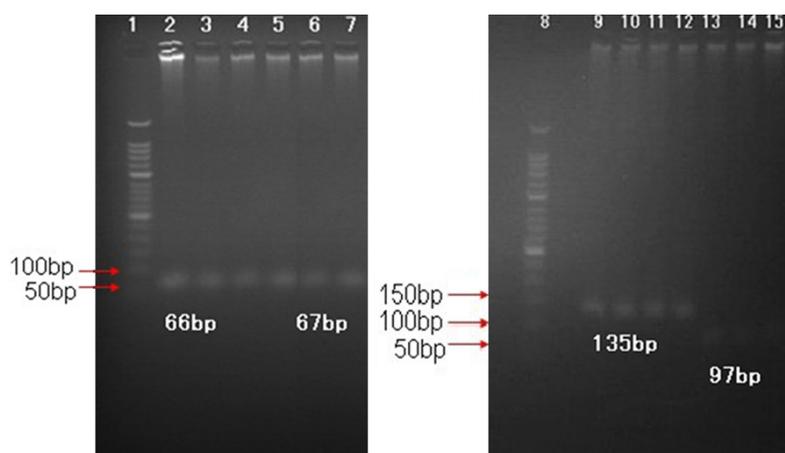


Figure 2. PCR products of rs1048943, rs4646903, rs2031920 and rs6413420 by agarose gel (2%) Electrophoresis. Lane 1, 8: DNA markers; Lane 2, 5, 9, 10, 13: Results of blood stain; Lane 3, 6, 11, 14: Results of standard phenol and chloroform; Lane 4, 7, 12, 15: Results of QIGEN Mini Kit. Lane 2, 3, 4: Stands for rs1048943; Lane 5, 6, 7: Stands for rs4646903; Lane 9, 10, 11, 12: Stands for rs2031920; Lane 13, 14, 15: Stands for rs6413420.

form, blood stain and QIGEN Mini Kit) according to the blood volume. Then we perform 1.6% AGE to observe the results. On the basis of DNA template extracted by these three methods, we conducted a PCR reaction to observe the difference, which was showed in 2% AGE.

TaqMan-MGB primers and probes

Each SNP was genotyped by using conventional TaqMan minor groove binding (MGB) probes, which were commercially supplied by Shanghai GeneCore Biotechnologies. TaqMan SNP genotyping was performed by Taq amplification.

Each SNP genotype required two unlabeled PCR primers and two allele-specific probes. Each probe was doubly labeled with reporter dyes at the 5' end. In the present study, the 5' end of the two probes was labeled with FAM and VIC respectively. We designed the primers and probes of rs1048943 and rs4646903 in *CYP1A1* and rs2031920 and rs6413420 in *CYP2E1* through Primer Premier 5, and confirmed the specificity by BLAST. The base sequence showed on **Table 1**.

Real-time PCR for SNPs

The reactions were performed in 5 μ L, using 2.5 μ L of TaqMan Master Mix, and 6.25 nmol/L of primer, 18 nmol/L of probe and 20-300 ng of DNA template. The reactions were performed under 40 Cycling Standard Conditions, showed in **Table 2**. FAM and VIC were selected as fluorescence labeling perssads, which had similar wavelength. FAM was for wild type probe, while VIC was for mutant type probe.

Sequence validation

To verify the reliability of the results from Real-time PCR SNPs experiment, we conduct a PCR amplified reaction to the sequence primer. For rs1048943 polymorphism, the primers were: forward: 5'-CTGCTTGCCTGTCTCTA-3', reverse: 5'-CAGCCCAGATAGCAAAC-3'. The length of target fragment was 522 bp. For rs2031920 polymorphism, the primers were: forward: 5'-CAAGTGATTTGGCTGGATTG-3', reverse: 5'-ACAGACCCCTCTCCACCTTCT-3'. The length of target fragment was 243 bp. For rs6413420 polymorphism, the primers were: forward: 5'-CCGTTGTCTAACCAAGTGCC-3', reverse: 5'-TGATGGGAA-GCGGAAA-3'. The length of target fragment was 486 bp. Rs4646903 sequencing was

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Table 3. The genotype and allele frequency of rs1048943 in five ethnic groups

ethnic group	sample size	genotype	Actual number (theoretical number)	Frequency (%)	Allele	Frequency (%)	χ^2 (p)
Han	108	A/A	61 (62.3)	56.5	A	75.9	0.44 (0.51)
		A/G	42 (39.5)	38.9	G	24.1	
		G/G	5 (6.3)	4.6			
Zhuang	193	A/A	85 (81)	44.0	A	64.8	1.63 (0.20)
		A/G	80 (88.1)	41.5	G	35.2	
		G/G	28 (24.0)	14.5			
Shui	169	A/A	102 (100.0)	60.4	A	76.9	0.75 (0.39)
		A/G	56 (60.0)	33.1	G	23.1	
		G/G	11 (9.0)	6.5			
Miao	179	A/A	110 (107.9)	61.5	A	77.7	0.79 (0.37)
		A/G	58 (62.1)	32.4	G	22.3	
		G/G	11 (8.9)	6.1			
Bouyei	180	A/A	113 (109.7)	68.9	A	78.1	2.10 (0.15)
		A/G	55 (61.7)	25.4	G	21.9	
		G/G	12 (8.7)	5.7			

Table 4. The genotype and allele frequency of rs4646903 in five ethnic groups

ethnic group	sample size	genotype	Actual number (theoretical number)	Frequency (%)	Allele	Frequency (%)	χ^2 (p)
Han	108	T/T	47 (40.9)	43.5	T	61.6	6.60 (0.013*)
		T/C	39 (51.1)	36.1	C	38.4	
		C/C	22 (15.9)	20.4			
Zhuang	193	T/T	51 (45.8)	26.4	T	48.7	2.25 (0.13)
		T/C	86 (96.4)	44.6	C	51.2	
		C/C	56 (50.8)	29.0			
Shui	169	T/T	58 (56.3)	34.3	T	57.7	0.30 (0.58)
		T/C	79 (82.5)	46.7	C	42.3	
		C/C	32 (30.3)	19.0			
Miao	179	T/T	77 (73.2)	43.0	T	64.0	1.49 (0.22)
		T/C	75 (82.5)	41.9	C	36.0	
		C/C	27 (23.2)	15.1			
Bouyei	180	T/T	72 (60.7)	40.0	T	58.1	12.03 (0.0005*)
		T/C	65 (87.7)	36.1	C	41.9	
		C/C	43 (31.7)	23.9			

using general primers of TaKaRa PMD-20T Vector.

The reaction product was detected by 2% AGE. The target stripe was cut off and purified by QIAquick PCR Purification Kit. The purified product was bi-directional sequenced using up and down primer. The results of sequence were compared with the corresponding DNA sequence of SNP site. If a doublet was appeared, it was heterozygote; if a simple spike appeared, it was wild type or mutation in the homozygous type.

Statistical analysis

Differences in the allele and genotype frequencies of rs1048943, rs4646903, rs2031920 and rs6413420 among different ethnic groups were calculated using a χ^2 test. The χ^2 test was used to test for deviation of genotype distribution from the Hardy-Weinberg law of genetic equilibrium. Descriptive statistical analysis was performed using SPSS Statistics version 13.0. Differences were considered to be statically significant when $p < 0.05$.

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Table 5. The genotype and allele frequency of rs2031920 in five ethnic groups

ethnic group	sample size	genotype	Actual number (theoretical number)	Frequency (%)	Allele	Frequency (%)	χ^2 (p)
Han	108	C/C	69 (66.1)	63.9	C	78.2	2.66 (0.10)
		C/T	31 (36.8)	28.7	T	22.8	
		T/T	8 (5.1)	8.4			
Zhuang	193	C/C	123 (124.5)	63.7	C	80.3	0.46 (0.50)
		C/T	64 (61.0)	33.2	T	19.7	
		T/T	6 (7.5)	3.1			
Shui	169	C/C	106 (107.4)	62.7	C	80.2	0.41 (0.52)
		C/T	59 (56.3)	34.9	T	19.8	
		T/T	4 (7.4)	2.4			
Miao	179	C/C	107 (102.6)	59.8	C	75.7	3.24 (0.07)
		C/T	57 (65.9)	31.8	T	24.3	
		T/T	15 (10.6)	8.4			
Bouyei	180	C/C	127 (125.8)	70.6	C	83.6	0.40 (0.53)
		C/T	47 (49.3)	26.1	T	16.4	
		T/T	6 (4.8)	3.3			

Results

The results of DNA extracted by standard phenol and chloroform, blood stain and QIGEN Mini Kit

In this study, we adopted three methods (standard phenol and chloroform, blood stain and QIGEN Mini Kit) to extract DNA from blood. The stripe which was extracted by QIGEN Mini Kit was better than the standard phenol and chloroform, blood stain, showed in **Figure 1**.

We took DNA extracted by standard phenol and chloroform, blood stain and QIGEN Mini Kit as template, and conducted a Real-time PCR SNPs experiment to compare the difference among these three methods, showed in **Figure 2**. We can see the AGE results of PCR product of 4 SNPs site extracted by three methods was the same. The PCR product of rs1048943 was at 66 bp, and rs4646903 at 67 bp, rs2031920 at 135 bp, rs1048943 at 97 bp.

CYP1A1 (rs1048943 and rs4646903) genotyping

We detected genotype and allele gene frequency of CYP1A1 gene 1048943 and 4646903 in 829 samples of five ethnic groups. In CYP1A1 gene 1048943, the genotype distribution of five ethnic groups was conformed to Hardy-Weinberg genetic equilibrium law, as shown in **Table 3**. There was statistical significance be-

tween the Zhuang ethnic group and other four ethnic groups. In CYP1A1 gene 4646903, only the genotype distribution of the Shui, Miao, and Zhuang ethnic groups was accord with the Hardy-Weinberg genetic equilibrium law, as shown in **Table 4**. There was statistical significance between the Zhuang and Han ethnic group ($\chi^2=9.40$, $p=0.009$), the Zhuang and Bouyei ethnic group ($\chi^2=7.77$, $p=0.021$), the Zhuang and Miao ethnic group ($\chi^2=15.66$, $p < 0.001$).

CYP2E1 (rs2031920 and rs6413420) genotyping

In CYP2E1 gene 2031920, the genotype distribution of five ethnic groups was conformed to Hardy-Weinberg genetic equilibrium law, as shown in **Table 5**. There was statistical significance between the Miao and Bouyei ethnic group ($\chi^2=6.53$, $p=0.038$), the Miao and Shui ethnic group ($\chi^2=6.13$, $p=0.047$). Interestingly, we found only the Bouyei ethnic group had the SNP site of CYP2E1 gene 6413420. The frequency of the gene was 1.4%, as shown in **Table 6**.

Sequence validation

To verify the reliability of the results of Real-time PCR SNPs experiment, we conduct a PCR amplified reaction to the sequence primer. Taken rs4646903 for example, we randomly selected a DNA sample of CYP1A1 gene

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Table 6. The genotype and allele frequency of rs6413420 in five ethnic groups

ethnic group	sample size	genotype	Actual number (theoretical number)	Frequency (%)	Allele	Frequency (%)
Han	108	G/G	108	100	G	100
		G/T	0	0	T	0
		T/T	0	0		
Zhuang	193	G/G	193	100	G	100
		G/T	0	0	T	0
		T/T	0	0		
Shui	169	G/G	169	100	G	100
		G/T	0	0	T	0
		T/T	0	0		
Miao	179	G/G	178	99.4	G	99.7
		G/T	1	0.6	T	0.3
		T/T	0	0		
Bouyei	180	G/G	175	97.2	G	98.6
		G/T	5	2.8	T	1.4
		T/T	0	0		

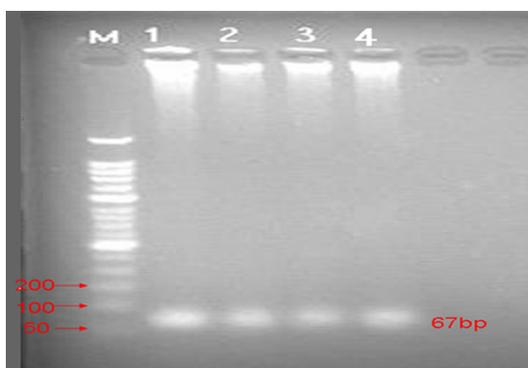


Figure 3. PCR products of *CYP1A1* gene 4646903 by agarose gel (2%) Electrophoresis. Lane M Represents 100 bp DNA Molecular Weight Markers.

4646903 which had wild type (W/M), heterozygosis genotype (W/M) and homozygosis mutant genotype (M/M), and conduct a PCR amplified reaction to the sequence primer. The reaction product was detected by 2% AGE. The stripe located at between 50 bp and 100 bp, which was conformed to 67 bp target stripe, showed in **Figure 3**. The results of sequence validation of rs1048943, rs2031920 and rs6413420 were displayed in [Supplementary materials](#).

The sequence results of W/W genotype of rs4646903 showed that No.69 bp on the forward direction was T, and the peak was singlet. We also validated on reverse direction. No.115 bp was A, and the map also was unimodal. So it

was with W/M and M/M, showed in **Figure 4**. The sequence analysis results were consistent with Real-time PCR, which indicated that studying genetic polymorphism using Tapman-MGB technology was reliable.

Discussion

China is a multinational state, including the Han ethnic group and other 55 minorities. Different ethnic groups have different origin and genetic background. And this feature is quite different with other countries. So it is necessary to investigate the distribution of gene frequency and genotype among 56 ethnic groups in our country.

Now repercussion study of gene and environment is most focused on phase I, II exogenous metabolic enzymes [11]. The enzyme activity and balance relationship among them determines the susceptibility of different crowd to disease. *CYP1A1* is an important phase I metabolic enzymes. Polycyclic aromatic hydrocarbons (PAHs) in tobacco and occupational environment can induce the expression of *CYP1A1* gene. Nowadays, there are mainly four SNPs in research of *CYP1A1* gene, which are rs1048943, rs4646903, rs4986883 and rs1799814. Rs1048943 locates at Exon 7. A-G exchange takes place on No.4889 bp, so the Ile is instead by Val on No.462 amino acid at ferroheme combination zone of the protein. There were some reports indicated that the polymorphism of rs1048943 could cause inducibility of enzymes and enhancement of biological activated ability and is associated with a variety of disease [12]. Another rs4646903 located at 246th bases at the downstream of the 3' end. T changed to C, formed MspI site, which enhanced catalytic activity [13] and was associated with hydrophobic DNA adduct [14-15].

In this study, we detected SNPs of *CYP1A1* gene 1048943 and 4646903 in 829 samples of five ethnic groups, which were the Han ethnic groups, the Bouyei, the Shui, the Miao and the

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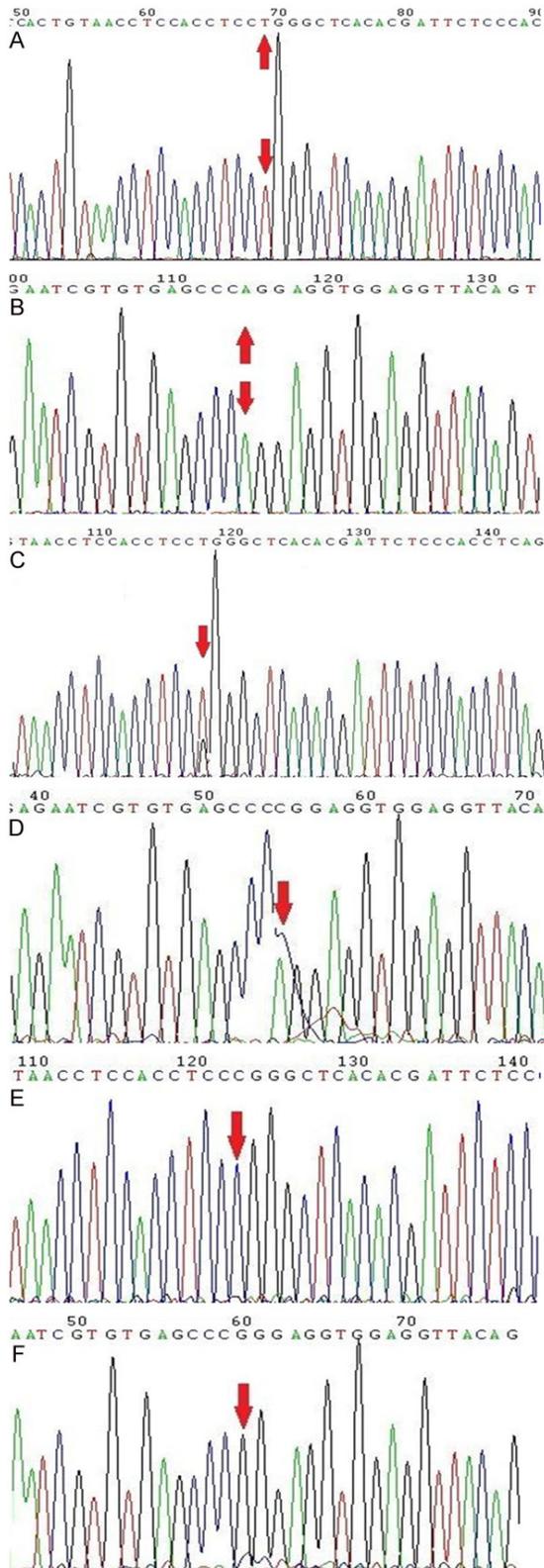


Figure 4. Sequencing analysis of *CYP1A1* gene 4646903. Red arrow was rs4646903 site. A, B: Sequencing results of W/W; C, D: Sequencing results of W/M; E, F: Sequencing results of M/M. A, C, E: Positive-sense strand; B, D, F: Antisense strand.

Zhuang ethnic groups. In *CYP1A1* gene 104-8943, the genotype distribution of five ethnic groups was conformed to Hardy-Weinberg genetic equilibrium law. The gene frequency of five ethnic groups (W: 0.648-0.781; M: 0.219-0.352) was close to Japanese [16] and Indian [17], lower than American [18] and European [19-20], but had larger difference with Saudi Arabian [21]. The gene frequency of rs4646903 in five ethnic groups (W: 0.640-0.687; M: 0.360-0.512) was close to Indian [22], but lower than American [18], European [19-20], and Saudi Arabian [21].

Like most other metabolic enzymes, the gene frequency of *CYP2E1* was different among different ethnic and racial. There were mainly five SNPs in research of *CYP2E1* gene, that were rs2031920, rs6413432, rs6413419, rs6413420 and rs3813867. Rs2031920 and rs6413420 were both located in the 5' end. The mutations of the former site created disappear of RsaI site. The polymorphism of RsaI site can significantly affect the transcription level of *CYP2E1* gene [23-24]. The latter could increase gene transcription activity [25].

In our research, we found that the genotype distribution of rs2031920 in five ethnic groups was conformed to Hardy-Weinberg genetic equilibrium law. The frequency of M gene in the Miao ethnic group, the Han, the Shui, the Zhuang and the Bouyei ethnic group was 24.3%, 22.8%, 19.8%, 19.7% and 16.4%, respectively. The gene frequency of rs2031920 in five ethnic groups (W: 0.757-0.836; M: 0.164-0.243) was close to Japanese [16], but lower than American [26] and European [20, 27]. Interestingly, we found only the Bouyei ethnic group existed the SNPs site of rs6413420. This was the first time found the site in Chinese. The gene frequency was lower than German [20], Norwegians [28] and Turks [29].

In our study, we validate the SNPs classification results using Taqman-MGB probe by designing and compounding sequencing primers of *CYP1A1* gene rs1048943, *CYP2E1* gene rs2031920 and rs6413420. *CYP1A1* gene rs4646903 was validated by molecular cloning methods because of multiple repeat fragments near this site. The sequencing results of the present study were in complete accord with the SNPs classification results, which verify the reliability of the experimental system.

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In summary, the *CYP1A1* and *CYP2E1* genetic polymorphism were different in different places as well as different ethnic groups in China. *CYP2E1* gene rs6413420 polymorphism was first found in the Bouyei ethnic group of China. These results can not only understand the genetic relationship among various ethnic groups and the ethnic origin, but also predict the risk of SNPs site related disease of different ethnic groups.

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Disclosure of conflict of interest

None.

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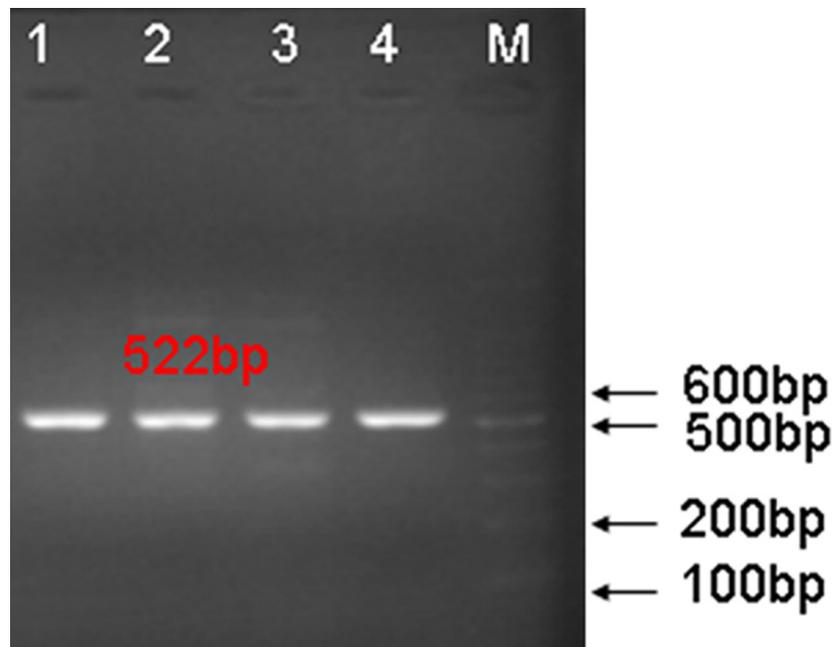
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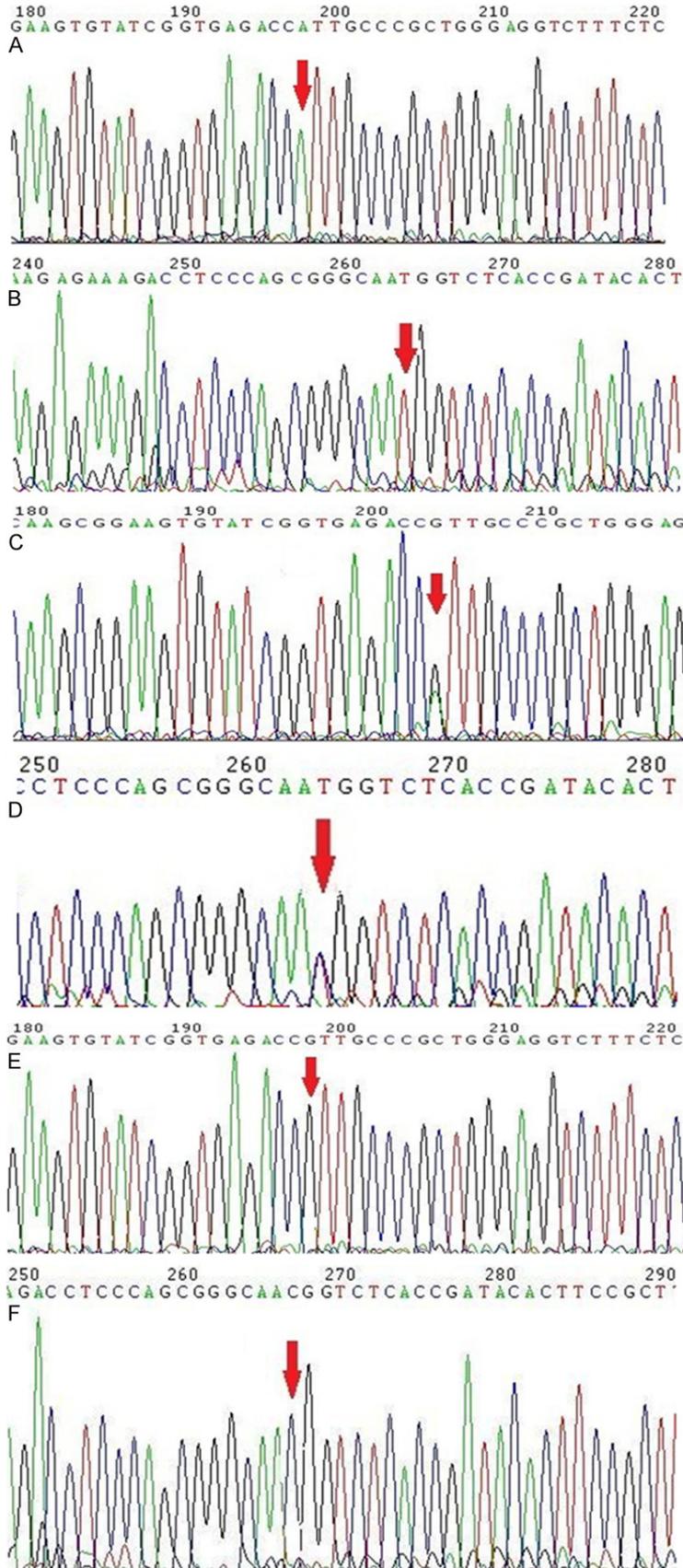
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Supplementary Figure 1. Agarose gel (1.6%) Electrophoresis for PCR products of *CYP1A1* gene 1048943. Lane M Represents 100 bp DNA Molecular Weight Markers.

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Supplementary Figure 2. Sequencing analysis of *CYP1A1* gene 1048-943. Red arrow was rs1048943 site. A, B: Sequencing results of W/W; C, D: Sequencing results of W/M; E, F: Sequencing results of M/M; A, C, E: Positive-sense strand; B, D, F: Antisense strand.