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Original Article

Cytochrome P450 2C9 (*CYP2C9*) polymorphisms in Chinese Li population

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Abstract: Background: The frequencies of Cytochrome P450 2C9 (*CYP2C9*) genotypes were various between populations. The aim of this study was to investigate the frequencies of the major variants of the *CYP2C9* in Chinese Li minority populations. Methods: The promoter, exons and surrounding introns, and 3'-untranslated region of the *CYP2C9* gene was detected by DNA sequencing to investigate in 100 unrelated healthy Chinese Li subjects. The protein function prediction was used the online tools: Sorting Intolerant From Tolerant (SIFT) and Phenotyping Version 2 (PolyPhen-2). The comparison of *CYP2C9* allele frequencies in different populations were analyzed by Chi-square (χ^2) test. Linkage disequilibrium (LD) analysis was performed using Haploview software. Results: We identified 17 different *CYP2C9* single nucleotide polymorphisms (SNPs) in the Li population, including two missense mutations (3549 G > A and 42614 A > C) and two silent mutations (3514 T > Cand 50298A > T). The protein function prediction revealed the two missense mutations result in protein damaging. In addition, we detected the allele frequencies of CYP2C9*1, CYP2C9*3 and CYP2C9*42 were 98%, 1%, and 1%, respectively. Finally, we compared three major allelic frequency (CYP2C9*1, CYP2C9*2, and CYP2C9*3) between Li and other populations. We found that our results were similar to East Asians and Africans.

Keywords: CYP2C9, allele frequencies, Chinese Li minority populations, CYP2C9*1, CYP2C9*3, CYP2C9*42

Introduction

Cytochrome P450 genes is a super family of cysteine-heme enzymes, which catalyze the oxidation of various drugs and endogenous substrates, such as vitamin D, steroids, and fatty acids, including arachidonic acid (AA) [1]. CYP enzymes of the P450 2C9 (CYP2C9) subfamily are found in the liver, vascular smooth muscle, endothelial cells of human aorta and coronary artery [2-4]. Cytochrome P450 2C (CYP2C) subfamily of enzymes form 18-30% of human CYPs and metabolism nearly 20% of all therapeutic drugs commonly prescribed in clinical practice [5]. CYP2C gene is made up of four isoforms, *CYP2C8*, *CYP2C9*, *CYP2C18* and *CYP2C19* which are located together on chromosome 10q24 [6]. For example, *CYP2C9* polymorphisms have been associated with an increased risk of bleeding in patients treated with standard doses of warfarin while phenytoin toxicity has also been reported in some patients [7].

The population of China consists of Han Chinese and 55 ethnic minorities currently recognized by the People's Republic of China. The Li population, which is one among the 55 minority ethnic groups in China, exceeds 1.3 million and resides primarily in the Li and Miao Autonomous Prefecture in the center and southwest regions of the Hainan Province. So far, the data of *CYP2C9* polymorphisms is lacked in the Li population, the main aim of this study was to investigate the CYP2C9 genetic polymorphisms in Li populations, and the secondary aim was to compare their allelic frequencies with previous observations of other studies. Our results will

Table 1. Primers used to amplify regions in CYP2C9

Primer name	Primer sequence (5'-3')	DNA size for PCR (bp)
PromoterF	GCAGTGATGGAGAAGGGAGA	924
PromoterR	AATTCGGTGTGTGCCTCTTT	
Exon1_F	GAACCATCTGGGTTAACATTTG	925
Exon1_R	AGTGCATTCTTGGCACCAT	
Exon2_3_F	TGCCTTGAACATCACAGGCCATC	826
Exon2_3_R	TGGCTCTCAGCTTCAAACCCCC	
Exon4_F	TCTTGCCCTTTCCATCTCAG	911
Exon4_R	TGCCACATAATACGACAAAGTG	
Exon5_F	TGCTGTCATCTACAAAACGTGA	908
Exon5_R	CAGGGATTTGACTTCTTCCTTG	
Exon6_F	AATCCCAGGATGGGGTCTAC	880
Exon6_R	CAATTGATTCCAGTGCCTCA	
Exon7_F	TTTGATTGGAGATTTTATTCCATTT	925
Exon7_R	GATTCAGTTCTTTCCAAACTAGCC	
Exon8_F	TACTGCCCTTCTTTGGAACGGGAT	809
Exon8_R	ACCTCCCAACCCCCAACAGC	
Exon9_F	AGAATGTGAGGGTCCAGATCA	913
Exon9_R	TTCAGGGAAGGGAAAATGTG	
3'-UTR_F	TGTGGGAGAAGCCCTGGCCG	737
3'-UTR_R	AGAGCTGCCCCTGGACACAG	

PCR: polymerase chain reaction.

add some data to support the understanding of *CYP2C9* variants and expect to promote personalized medicine in Li patients.

Materials and methods

Subjects

The study subjects consisted of 100 unrelated healthy Li subjects (including 50 males and 50 females) from Hainan province of China. All participants were recruited between October and December, 2014 from the People's Hospital of Hainan Province. All participants were Li Chinese residing in the Hainan Provincial which is located southwest of China, and they had at least three generations of Li paternal ancestry. All subjects were deemed healthy based on medical history and a physical examination. The purpose and experimental procedures of the study were explained to all individuals, and written informed consent was obtained from all participants prior to sample donation. The study protocol was performed in accordance with the Declaration of Helsinki and was approved by the Ethics Committees of People's Hospital of Hainan Province.

DNA sequencing of CYP2C9 variants

Genetic polymorphisms of *CYP2C9* were screened by DNA sequencing. Briefly, 5 ml blood samples in tubes containing ethylene diaminetetraacetic acid (EDTA) were collected from the subjects and kept under -20°C until DNA was extracted. We used the GoldMag® nanoparticles method (GoldMag® Ltd. Xi'an, China) to extracted DNA from blood according to the manufacturer's instructions genomic. The DNA samples were stored at -80°C until use. When used, the DNA was diluted to 50 ng/ul concentration. Primers for polymerase chain reaction (PCR) were designed to amplify the promoter, exons and the 3'-untranslated region of *CYP2C9*, and their sequences are provided in **Table 1**. Thermal cycling conditions were as follows: 95°C for 5 min; followed by 35 cycles of denaturation at 95°C for 30 s; annealing at 60°C for 1 min; and extension at 72°C for 1 min. A final extension step was performed at 72°C for 10 min. Preparation of DNA for sequencing included incubation of PCR products with 0.1 U of shrimp alkaline phosphatase (Roche, Basel, Switzerland) and 0.5 U of exonuclease I (New England Biolabs, Inc., Beverly, MA, USA) at 37°C for 45 min, followed by heat inactivation at 85°C for 20 min. The PCR products were sequenced using the ABI PrismBigDye Terminator Cycle Sequencing Kit version 3.1 (Applied Biosystems) on an ABI Prism3100 sequencer (Applied Biosystems).

Data analysis

The Human Cytochrome P450 (CYP) Allele Nomenclature Database describes *CYP2C9* variants according to the National Center of Biotechnology Information (NCBI) reference sequence NG_008385.1. Allelic frequency comparisons between Li and other populations were performed using the Chi-squared test with a significance level set at $P = 0.05$ [8]. Hardy-Weinberg equilibrium calculations were performed using the Arlequin program (http://anthropologie.unige.ch/arlequin). Linkage disequilibrium (LD) between loci pairs were assessed using Haploview software (version 4.2) (Mark Daly's Laboratory, Massachusetts Institute of Technology/Harvard Broad Institute, Cambridge, MA, USA) [9]. Furthermore, LD was investigated across all single nucleotide polymorphisms (SNPs) and selected haplotypes. Haplotype blocks were defined based on the

Table 2. Frequency distribution of *CYP2C9* polymorphisms in 100 Li subjects

SNP	Region	Position	Nucleotide change	Amino-acid effect	Frequency % (n)
rs148342296	Promoter	-477	A > G	No translated[a]	11/99
rs9332105	Intron 1	486	G > C	No translated	52/98
rs201856860	Exon 3	3514	T > C	Ile112 = silent[b]	1/100
rs12414460	Exon 3	3549	G > A	Arg124Glnmissense[c]	2/100
rs9332127	Intron 3	9032	G > C	No translated	3/99
rs9332172	Intron 5	33349	A > G	No translated	5/100
rs148303924	Intron 5	33622	T > C	No translated	1/100
rs1057910	Exon 7	42614	A > C	Ile359Leumissense[c]	2/99
rs17847029	Intron 7	42726	C > T	No translated	7/99
rs93321230	Intron 8	47545	A > T	No translated	2/100
rs2298037	Intron 8	47639	C > T	No translated	54/100
rs147375734	Intron 8	47947	T > C	No translated	3/98
rs28371688	Intron 8	50053	G > A	No translated	2/100
rs1934669	Intron 8	50056	A > T	No translated	56/100
rs1057911	Exon 9	50298	A > T	Gly475 = silent[b]	2/100
rs9332244	3'-UTR	50566	A > G	No translated	9/100
rs9332245	3'-UTR	50742	T > A	No translated	2/100

SNP, single nucleotide polymorphism; [a]Non translated: These synonymous SNP mutations have no effect on protein sequence; [b]silent mutation; [c]missense mutation.

Table 3. *CYP2C9* allele and genotype frequencies in 100 Li individuals

Allele	Number	Phenotype	Frequency
*1	196	Normal	98.00%
*3	2	Decreased	1.00%
*42	2	/	1.00%
Total	200		100.00%
Genotype	Number	Phenotype	Frequency
*1/*1	96	Normal	96.00%
*1/*3	2	Decreased	2.00%
*1/*42	2	/	2.00%
Total	100		100.00%

*indicated genotype typewhich according database of "Human Cytochrome P450 Allele Nomenclature".

Gabriel definition (D' > 0.9; minimum allele frequency > 5%) [10].

Transcriptional protein function prediction

We used the online tools UniProt Knowledgebase (UniProtKB) [11] (http://www.uniprot.org/uniprot/), Polymorphism Phenotyping v2 (PolyPhen-2) (http://genetics.bwh.harvard.edu/pph2/) and Sorting Intolerant From Tolerant (SIFT) (http://sift.bii.a-star.edu.sg/) to predict protein function of non-synonymous SNPs in CYP2C9 exon regions. Each variant was given a score based on the impact of its mutation on protein function. PolyPhen-2 results were divided into three categories: benign, potentially damaging, and probably damaging [12]. We referenced the previous reported that the SIFT output was then divided into four categories based on these scores: tolerant (0.201-1.00), borderline (0.101-0.20), potentially intolerant (0.051-0.10) and intolerant (0.00-0.05) [13].

Result

Genetic variants

In our study, 17 different SNPs were identified among Li subjects. We detected two missense mutations (3549 G > A and 42614 A > C) and two silent mutations (3514 T > C, and 50298 A > T). The two missense variants were identified in exon 3 and 7, respectively. And the two silent mutations located on exon 3 and 9, respectively (**Table 2**). However, these mutations have been reported in the NCBI database or in the Human *CYP*Allele Nomenclature Committee tables previously [14].

Allele and genotype frequency and non-synonymous mutation protein function predicted

As **Table 3** showed, we identified three *CYP2C9* alleles: the wild-type CYP2C9 allele, CYP2C9*1, which found in 98% of the population. One common allele, CYP2C9*3, comprised only 1% of the variants, while no subjects with CYP2C9*2 were identified. Furthermore, the rare allele CYP2C9*42, which was first reported in Li Chinese population, was present in 1% of our study subjects. We also calculated three CYP2C9 genotypes in the Li population. We found 96 individuals with the wild-type (*1*1) genotype have normal enzyme activity. Other

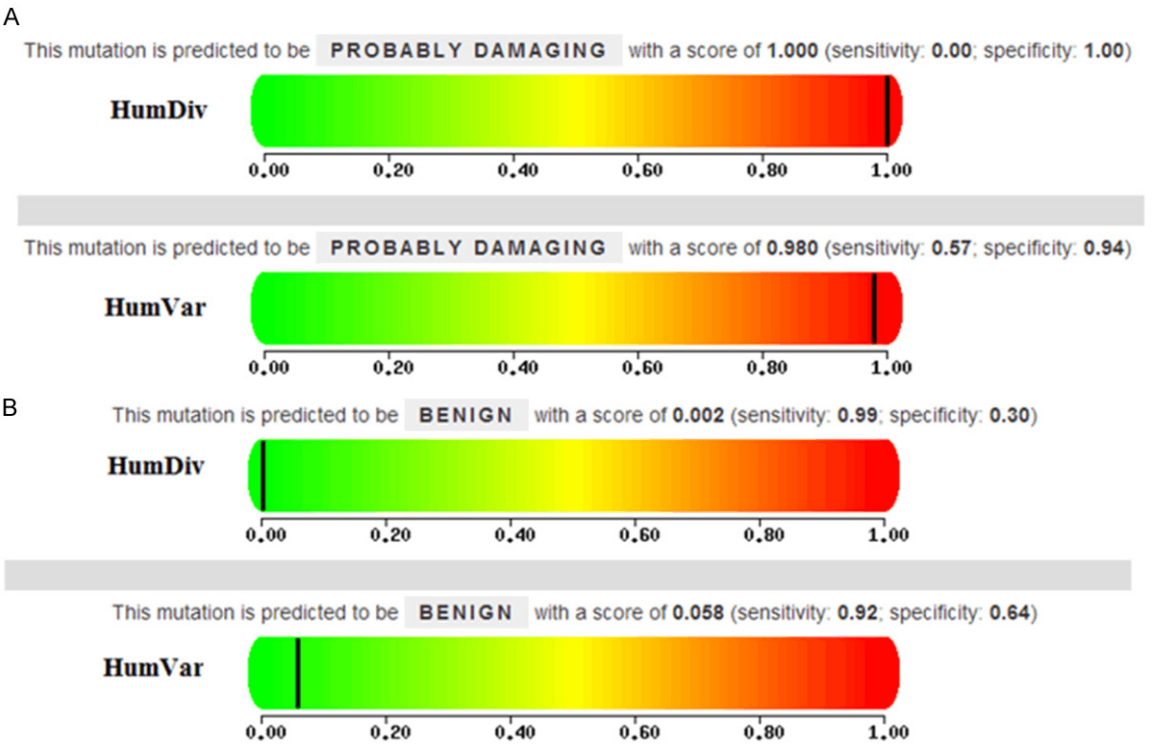

Figure 1. Protein function predicted by PolyPhen-2. A. Prediction of the mutation 3549 G > A. B. Prediction of the mutation 42614 A > C. HumDiv: is Mendelian disease variants vs divergence from close mammalian homologs of human proteins (5564 deleterious + 7539 neutral mutations from the same set of 978 human proteins). HumVar: is all human variants associated with some disease (except cancer mutations) or loss of activity/function vs common human polymorphism with no reported association with a disease of other effect (22196 deleterious + 21119 neutral mutations in 9679 human proteins).

identified genotypes included 2 individuals with the heterozygous genotype (*1*3), which leads to decreased enzyme activity. Additionally, we also found 2 individuals with the rare genotype (*1*42). Furthermore, we used the online tools PolyPhen-2 and SIFT to predict protein function of non-synonymous SNPs in *CYP2C9* exon regions. PolyPhen-2 utilized two models (HumDiv and HumVar), in which the latter is more rigorous in its false discovery rate (**Figure 1**). PolyPhen-2 results revealed that rs1241-4460 (Arg124Gln) was probably damaging in both models. The SIFT protein function prediction results (score = 0 intolerant) were consistent with PolyPhen-2 which also revealed probably damaging (**Figure 1A**). However, the protein function prediction results of the rs1057910 (Ile359Leu) was inconsistentwith-PolyPhen-2 and SIFT. The result of SIFT prediction was scored 0.04, which indicted the protein function was intolerant, while PolyPhen-2 results showed benign (**Figure 1B**).

Comparisons of inter-population

We further analyzed the distribution patterns of *CYP2C9* allele between Li and other populations (involved East Asian, Middle East Arab, Africans, and Caucasian) around world (**Table 4**). Three major alleles, the wild-type allele CYP2C9*1, the prevalent allele CYP2C9*2 and CYP2C9*3 were compared. We found that the allele frequency of CYP2C9*1, CYP2C9*2 and CYP2C9*3 in our study was close to majority East Asian populations. In summary, the frequency of CYP2C9*1 was significant ($P < 0.05$) higher than Uyghur Chinese populations and Tamil Nadu. In contrast, CYP2C9*2 was significant ($P < 0.05$) lower than Uyghur Chinese, CYP2C9*3 was no significant with other East Asians. In Middle East Arab, the frequency of CYP2C9*1, CYP2C9*2 and CYP2C9*3 were significant different with all most Middle East Arabs. In Africans, we found the allele frequency of CYP2C9*1, CYP2C9*2 and CYP2C9*3 in

Table 4. Three types of *CYP2C9* allele frequencies in different populations

Races	N	*CYP2C9**1	*p*	*CYP2C9**2	*p*	*CYP2C9**3	*p*	Ref
Li	100	0.98	/	0	/	0.01	/	Current study
East Asians								
Tibetan Chinese	96	0.938	0.259	0	/	0.057	0.150	[13]
Tibetan Chinese	107	0.972	0.938	0	/	0.028	0.664	[19]
Han Chineese	2127	0.945	0.128	0.001	/	0.029	0.416	[16]
Korean	358	0.934	0.077	0	/	0.06	0.074	[20]
Korean	574	0.98	0.69	0	/	0.011	0.664	[21]
Japanese	218	0.979	0.714	0	/	0.021	0.815	[22]
Uyghur Chinese	214	0.902	0.013*	0.096	0.001*	0	0.318	[23]
Bai Chinese	132	0.955	0.501	0	/	0.045	0.246	[19]
Hui Chinese	164	0.954	0.448	0.046	0.073	0	0.379	[23]
Mongolian Chinese	560	0.97	0.820	0	/	0.03	0.422	[24]
Vietnamese	157	0.978	0.737	0	/	0.022	0.819	[25]
Malay	209	0.957	0.488	0.019	0.397	0.024	0.694	[26]
Tamil Nadu	135	0.907	0.022*	0.026	0.280	0.067	0.070	[27]
Middle East Arab								
Saudi (Al-Ahsa)	131	0.844	< 0.001*	0.13	< 0.001*	0.023	0.809	[28]
Saudi (Riyadh)	192	0.792	< 0.001*	0.117	< 0.001*	0.091	0.007*	[29]
Egyptian	247	0.820	< 0.001*	0.12	< 0.001*	0.06	0.082	[30]
Jordanian	263	0.797	< 0.001*	0.135	< 0.001*	0.068	0.026*	[31]
Lebanese	161	0.792	< 0.001*	0.112	< 0.001*	0.096	0.005*	[32]
Omani	189	0.897	0.01*	0.074	0.012*	0.029	0.535	[33]
Africans								
Ethiopian	150	0.934	0.171	0.043	0.090	0.023	0.785	[34]
African-American	600	0.867	0.001*	0.028	0.180	0.02	0.775	[35]
African-American	490	/	/	0.011	1	0.018	0.888	[36]
Beninese	111	0.955	0.530	0	/	0	0.474	[37]
Ghanaian	204	/	/	0	/	0	0.329	[6]
Iranian	200	/	/	0.13	< 0.001*	0	0.333	[38]
Caucasians								
Turkish	499	0.794	< 0.001*	0.106	< 0.001*	0.1	0.003*	[39]
Brazilian	103	0.83	< 0.001*	0.097	0.004*	0.073	0.059	[40]
Mexican	98	0.86	0.002*	0.08	0.012*	0.06	0.125	[41]
Ecuadorian	194	0.93	0.069	0.054	0.042*	0.015	0.855	[42]
Swedish	430	0.819	< 0.001*	0.107	< 0.001*	0.074	0.017*	[43]
Russian	352	0.831	< 0.001*	0.119	< 0.001*	0.05	0.136	[44]
Italian	157	0.796	< 0.001*	0.112	< 0.001*	0.092	0.007*	[34]
British	100	0.79	< 0.001*	0.125	< 0.001*	0.085	0.031*	[45]
Portuguese	135	0.788	< 0.001*	0.132	0.0002*	0.08	0.015*	[46]
Spanish	1076	0.766	< 0.001*	0.156	< 0.001*	0.078	0.012*	[47]
French	151	0.77	< 0.001*	0.15	< 0.001*	0.08	0.015*	[48]
Bolivian	778	0.922	0.034*	0.048	0.048*	0.03	0.410	[49]
Cuban	132	0.72	< 0.001*	0.17	< 0.001*	0.11	0.002*	[50]
American	100	/	/	0.08	0.011*	0.06	0.124	[51]
Croatian	200	0.74	< 0.001*	0.165	< 0.001*	0.095	0.005*	[52]
German	118	0.81	< 0.001*	0.14	< 0.001*	0.05	0.196	[53]
Greek	283	/	/	0.129	< 0.001*	0.081	0.012*	[54]
Belgian	121	0.822	< 0.001*	0.1	0.001*	0.074	0.050*	[37]

*Indicated $P < 0.05$. Ref: Reference.

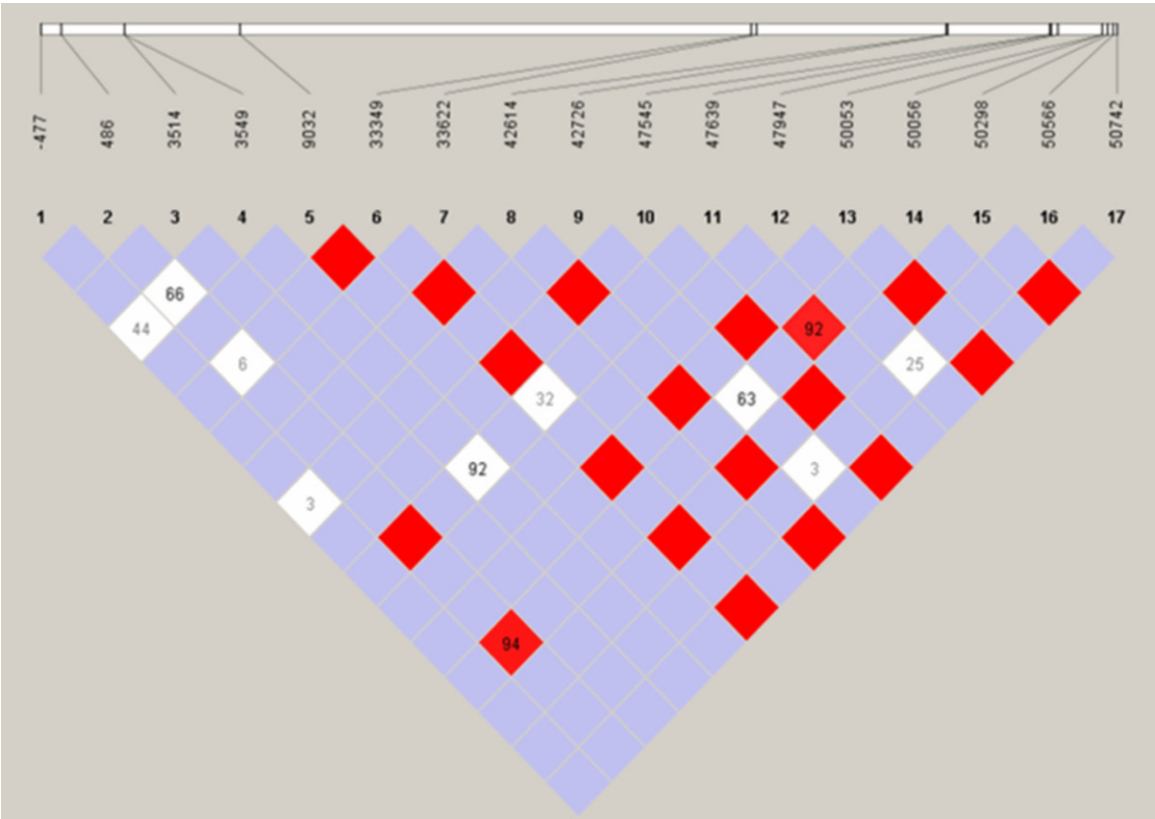

Figure 2. 17 SNPs linkage disequilibrium (LD) analysis of *CYP2C9* in Li population. Abbreviations: LD: Linkage disequilibrium; LOD: likelihood of odds; LD is indicated by bright red (very strong: LOD ≥ 2, D' = 1), light red (LOD ≥ 2, D' < 1) and blue (LOD < 2, D' = 1) for intermediate LD, and white (none: LOD < 2, D' < 1).

our study was very similar to our study, the allele frequency of CYP2C9*1 was higher than African-American (P < 0.05). In Caucasians, we found that the allele frequency of *CYP2C9**1 was significantly higher (*P* < 0.05) in Li compared with almost all Caucasian populations except Ecuadorian. In contrast, the allele frequency *CYP2C9**2 in Li was significantly lower (*P* < 0.05) than all Caucasians which we involved in this study. The allele frequency of *CYP2C9**3 was also obvious lower (*P* < 0.05) than Caucasian populations except for Brazilian, Mexican, Ecuadorian, Russian, Bolivian and American.

Linkage disequilibrium analysis

We further used Haploview software to assess LD between pairs of loci. The overall LD across the *CYP2C9* gene is depicted in **Figure 2**. We found 19 red LD points (17 bright red and 2 light red) between two SNPs. But there was no LD block within *CYP2C9* among this Li population study.

Discussion

This is the first study to screen the DNA sequence of *CYP2C9* in Li populations, we found 17 different SNPs among 100 Li subjects. *CYP2C9* genetic polymorphisms are highly relevant in the metabolism of clinically prescribed drugs and may influence patient responsiveness and adverse drug reactions. While previous studies have analyzed *CYP2C9* genetic polymorphisms focus on Chinese Han, Tibetan, Bai, and Hui populations, butto date no study focused on Chinese Li population. Thus, our results provided a better understanding of *CYP2C9* variants and added some database for promoting personalized medicine in Li patients.

Three major allelic polymorphisms (CYP2C9*1, CYP2C9*2, and CYP2C9*3) were compared between Li and other ethnic populations. In general, we found that allele frequencies identified in Li were most similar to East Asian and African populations but were different from

those of Caucasian and Middle East Arab populations. For example, the frequencies of CYP2C9*2, and CYP2C9*3 among our Li were similar to East Asian and African populations which significantly lower than Middle East Arab and Caucasian, this was consistent with previous research [15]. The phenomenon of lacking*CYP2C9*2 allele in the Li group is in accordance with its reported absence in the majority of East Asian populations. However, the variant of CYP2C9*2 was also identified in certain Asian populations, only found in Uyghur Chinese ($P < 0.05$). The Uyghur are an ethnic group of Central Asia. They are one of China's 56 officially recognized ethnicities. Throughout the history of Central Asia, they left a lasting imprint on both the culture and tradition. Today in China, Uyghur live primarily in the Xinjiang Uygur Autonomous Region in the northwest of China. There are also existing Uygur communities in Kazakhstan, Kyrgyzstan, Mongolia, Uzbekistan, and Turkey. It is known that the genetic polymorphisms were influenced by environmental and genetic factors. We inferred that these differences may be attributed to the origin and geographical isolation experienced by different ethnic populations, as well as their dietary habits and lifestyles, or other factors, all of which may affect *CYP2C9* polymorphisms. In addition, we also detected a rare mutations *CYP2C9*42*, which had be designated by the Human CYP Allele Nomenclature Committee [16].

We detected two missense mutations and two silent mutations in protein coding region. The non-synonymous mutation protein function predicted showed *CYP2C9*42* (3549G > A result Arg124Gln amino acid alteration) result in protein damaging. The protein mutation prediction result of *CYP2C9*3* (42614 A > Cinvolved Ile359Leu changed) was inconsistent between PolyPhen-2 and SIFT. SIFT scored 0.04 showed intolerant probably damaging but the PolyPhen-2 prediction result was benign. Although our result of *CYP2C9*3* (42614 A > C result Ile359Leu amino acid alteration) protein function predicted was not uniform, but previous studies indicated that homozygous mutationshow 95% decreased the enzyme activity compared to the wild type [17]. However, the protein function prediction results of the variant *CYP2C9*3* were highly inconsistent, since the accuracy of SIFT and PolyPhen-2 prediction typically reaches 63% and 75%, with false positive rates as high as 19% and 9%, respectively [18]. Therefore, the prediction results may be require and biased further experimental data to more reliably predict the effects of variants identified in our study.

Conclusions

This study provides the first pharmacogenomics information of *CYP2C9* in Chinese Li population and the comparision of *CYP2C9* allele frequencies with other populations worldwide. Our study provides a limited theoretical basis for safer drug administration and better therapeutic treatments in this unique population.

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Disclosure of conflict of interest

None.

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