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Original Article

Genetic polymorphisms of the drug-metabolizing enzyme cytochrome P450 2A6 in a Tibetan Chinese population

Ridong Xia[1,2,3*], Xikai Zhu[1,2,3*], Zhengshuai Chen[4], Yongjun He[1,2,3], Longli Kang[1,2,3], Dongya Yuan[1,2,3], Tianbo Jin[1,2,3,4], Li Wang[1,2,3]

[1]Key Laboratory for Molecular Genetic Mechanisms and Intervention Research on High Altitude Disease of Tibet Autonomous Region, School of Medicine, Xizang Minzu University, Xianyang, Shaanxi, China; [2]Key Laboratory of High Altitude Environment and Genes Related to Diseases of Tibet Autonomous Region, School of Medicine, Xizang Minzu University, Xianyang, Shaanxi, China; [3]Key Laboratory for Basic Life Science Research of Tibet Autonomous Region, School of Medicine, Xizang Minzu University, Xianyang, Shaanxi, China; [4]Key Laboratory of Resource Biology and Biotechnology in Western China (Northwest University), Ministry of Education, Xi'an, Shaanxi 710069, China. [*]Equal contributors and co-first authors.

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Abstract: Detection of CYP2A6 variant alleles, and knowledge about their allelic frequency in Tibetan ethnic groups, is important to establish the clinical relevance of screening for these polymorphisms to optimize pharmacotherapy. We used DNA sequencing to investigate the promoter, exons and surrounding introns, and untranslated region of the CYP2A6 gene in 100 unrelated healthy Tibetan individuals. We also used SIFT and PolyPhen-2 to predict the protein function of the novel non-synonymous mutation in CYP2A6 coding regions. We found 33 different CYP2A6 polymorphisms in the Tibetan population, five of which were novel: -98T > G in promoter region, 1886C > A, 5640C > A, 5827G > T in intron and missense mutation 5011G > A in exon 7. We identified six CYP2A6 alleles (*1, *10, *11, *14, *15 and *18), with a wide frequency range from 1.00% to 86.50% in the population. We also detected six CYP2A6 genotypes, with a wide frequency range from 2.00% to 73.00%. Three of which (*1/*10, *1/*11 and *1/*18) lead to decreased enzyme activity. This study provided new information regarding CYP2A6genetic polymorphisms in Tibetan individuals, which will greatly facilitate studies on the relevance of pharmacogenetics for CYP2A6 with respect to disease risk and to the pharmacokinetics and pharmacodynamics of many drugs.

Keywords: CYP2A6, polymorphisms, haplotype, Tibetan population

Introduction

Cytochrome P450 (CYP) represents a large group of enzymes that localize to the endoplasmic reticulum and play critical roles in the metabolism of endogenous and exogenous molecules, including most carcinogens, procarcinogens and drugs [1]. Among 69 CYP families in animals and approximately 50-100 CYP genes in vertebrates [2], CYP2 family is the largest and most diverse one and metabolize diverse substances, such as xenobiotics, drugs, and arachidonic acid [3]. CYP2A6 was first determined to catalyze coumarin 7-hydroxylation in the human liver [4].

Human CYP2A6 is a phase I enzyme responsible for the metabolism of drugs and chemical compounds such as nicotine, and coumarin [5]. CYP2A6 is also an important enzyme involved in the metabolic activation of procarcinogens in tobacco smoke [6, 7]. The CYP2A6 gene shows inter-individual variation of > 100-fold in both CYP2A6 mRNA and protein levels [5]. It is a highly polymorphic gene and characterized by multiple gene conversions with CYP2A7, and several forms of gene deletions (CYP2A6*4A-F), duplications (CYP2A6*1×2A, CYP2A6*1×2B) and single nucleotide polymorphisms (SNP) in coding and regulatory regions (CYP2A6*9A) [8, 9].

To date, numerous allelic variants of CYP2A6 have been identified, and most of these are dered from single nucleotide polymorphisms (SNPs) in coding and regulatory regions (see

Table 1. Primers used to amplify regions of CPY2A6

Primer name	Primer sequence (5'-3')	DNA size for PCR (bp)
UTR_F	CTCTGGTCTTCCTCCCCTGC	843
UTR_R	CTGCCAAACAGACATCAAGACCAT	
Exon1&2_F	AGGTGAAATGAGGTAATTATGTAATCAG	829
Exon1&2_R	AGACTGGGGACTCTGCCT	
Exon3&4_F	CACCCTACTCCCTCTCACC	829
Exon3&4_R	AGGGTATTGGACATCCATCCT	
Exon5_F	TTCAAATACCTGAAACCTGGATATATGTCT	702
Exon5_R	GCGCAACCATGCCCAAC	
Exon6_F	TGAAGGACAGATGGTCAGCAGG	1004
Exon6_R	TTGGTGTCCTTTTTGACTCTGCG	
Exon7_F	CGTTCCACCGGGTCATCC	743
Exon7_R	TCCTTCTAGGCAGGAGTTTGG	
Exon8_F	GGAGAATCAAACACATGTTCCC	773
Exon8_R	TGTCCTTTACTGCCAGGTAC	
Exon9_F	TGTAACTGGCAGGAAAGGACAT	851
Exon9_R	TTAGGTGAGCGTGCAATGGTT	
UTR_F	GGGGCAGGATGGCGGATA	753
UTR_R	ATGGCTATGTCCTGATCCAGAGTTC	

UTR: untranslated region.

http://www.cypalleles.ki.se/cyp2a6.htm). Genetic polymorphisms in the CYP2A6 gene have been associated with variations in enzyme activity; CYP2A6*2, *4, *5, and *20 exhibit no enzyme activity, whereas CYP2A6*6, *7, *9, *10, *11, *12, *17, *18, and *19 yield enzymes with reduced activity (see http://www.cypalleles.ki.se). Recently, functional and genetic associations of CYP2 family genes to several diseases, such as progressive stages of nonalcoholic fatty liver disease and upper aerodigestive tract cancer, have also been reported [10, 11]. Nonsynonymous single nucleotide polymorphisms (SNPs) can alter the protein sequences of CYP2A6. The substrate preferences of these mutant enzymes are different from those of the wild-type enzymes, and therefore, the mutant enzymes can produce dramatic effects in CYP2A6 metabolism.

Tibetans inhabit a vast area of the Qinghai-Tibetan Plateau, which spans approximately 1.5 million square km and the diet of Tibetan people is characterized by beef and mutton, dairy products, butter tea and milk-tea due to the special geographical environment. The unique diet and hypoxia environment usually lead to dyslipidemia which has been one of the major conditions threatening the health of the Tibetan population [12]. We systematically screened the whole CYP2A6 genes of 100 healthy, unrelated Tibetans for polymorphisms. We performed a retrospective analysis to explore the major factors affecting the tacrolimus blood trough concentration. Because information regarding CYP2A6 polymorphisms is limited in the Tibetan population, the main aim of this study was to investigate the distribution of CYP2A6 genetic variations in Tibetan individuals and the secondary aim was to compare their allele frequencies with previous observations of other ethnic groups. Our results will provide a better understanding of CYP2A6 variants and a potential database for promoting personalized medicine in Tibetan patients.

Materials and methods

Subjects

We recruited a random sample of 100 healthy, unrelated Tibetan (50 males and 50 females) between October and December 2009 from the Xizang Minzu University in Xianyang. All participants were Tibetan Chinese residing in the Tibet Autonomous Region of China, and they had at least three generations of Tibetan paternal ancestry, and their parents were Tibetans. All subjects were deemed healthy based on their 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 Xizang Minzu University.

PCR and DNA sequencing

Genetic polymorphisms of CYP2A6 in the Tibetan study group were screened by DNA sequencing. Briefly, 5 ml venous blood was collected in a tube containing EDTA and genomic DNA was extracted from leukocytes using the GoldMag nanoparticles method (GoldMag Ltd., Xi'an, China) according to the manufacturer's instructions. Primers for PCR were designed to amplify the promoter, exons and the 30-untranslated region of CYP2A6, and their sequences are pro-

Table 2. Positions and frequencies of CY2A6 genetic variants in 100 Tibetan subjects

Position	SNP	Nucleotide change	Allele	Frequencies (%)	Amino-acid effect	Region
-98	/	T > G	Novel	1	No translated	Promoter
-48	rs28399433	T > G	*15	28	No translated	Promoter
22	rs8192720	C > T		36	Leu8=	Exon 1
51	rs1137115	G > A	CYP2A6*14	45	Val17=	Exon 1
86	rs28399435	G > A	CYP2A6*14	2	Ser29Asn	Exon 1
144	rs56283800	G > A		1	Gln48=	Exon 1
1836	rs8192726	G > T		23	No translated	Intron 3
1886		C > A	Novel	1	No translated	Intron 3
1890	rs57607700	G > C		1	No translated	Intron 3
2134	rs199916117	A > G	*15	4	Lys194Glu	Exon 4
3391	rs111033610	T > C	CYP2A6*11	6	Ser224Pro	Exon 5
3492	rs1809811	C > T		5	Arg257=	Exon 5
3570	rs4079369	C > G		5	No translated	Intron 5
4303	rs762258775	T > C		2	No translated	Intron 5
4794	rs8192728	C > A		26	No translated	Intron 6
5011	rs200267449	G > A	Novel	1	Ala347Thr	Exon 7
5573	rs150455365	A > C		2	No translated	Intron 7
5628	rs553913513	C > T		1	No translated	Intron 7
5636	rs550145221	A > C		1	No translated	Intron 7
5640		C > A	Novel	1	No translated	Intron 7
5668	rs1809810	A > T	*18A	2	Phe392Tyr	Exon 8
5684	rs28399461	T > C		1	Ser397=	Exon 8
5738	rs2002977	C > T		1	His415=	Exon 8
5823	rs2002976	C > T		1	No translated	Intron 8
5827	rs113368210	G > T	Novel	2	No translated	Intron 8
5843	rs2002975	G > C		3	No translated	Intron 8
5857	rs72549446	T > A		2	No translated	Intron 8
6389	rs373949046	C > G		2	No translated	Intron 8
6558	rs5031016	T > C	*10	32	Ile471Thr	Exon 9
6600	rs28399468	G > T	*10	13	Arg485Leu	Exon 9
7073	rs2431412	T > C		1	No translated	3'UTR
7082	rs2259219	G > C		1	No translated	3'UTR
7160	rs28742185	A > G		28	No translated	3'UTR

UTR: untranslated region.

vided in **Table 1**. Thermal cycling conditions were as follows: an initial denaturation step at 95°C for 15 min was followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 55-64°C for 30 s, and extension at 72°C for 1 min. A final extension step was performed at 72°C for 3 min. The PCR products were sequenced using the ABI Prism BigDye Terminator Cycle Sequencing Kit version 3.1 (Applied Biosystems, Foster City, CA) on an ABI Prism3100 sequencer (Applied Biosystems, Foster City, CA).

Data analysis

The CYP Allele Nomenclature Database describes CYP2A6 variants according to the NCBI reference sequence NG_008377.1. We compared the differences in allelic frequencies between ethnic populations using the chi-squared test [13]. We used P=0.05 as the threshold of statistical significance. Hardy-Weinberg equilibrium and linkage disequilibrium (LD) between loci pairs were assessed using Haploview software (version 4.2, http:// broad.mit.edu/mpg/

Table 3. CYP2A6 allele and genotype frequencies in 100 Tibetan subjects

CYP2A6	Allele	Number	Phenotype	Frequency (%)
	*1	173	Normal	86.50
	*10	13	Decreased	6.50
	*11	6	Decreased	3.00
	*14	2		1.00
	*15	4		2.00
	*18	2	Decreased	1.00
	Genotype	Number	Phenotype	Frequency (%)
	*1/*1	73	Normal	73.00
	*1/*10	13	Decreased	13.00
	*1/*11	6	Decreased	6.00
	*1/*14	2		2.00
	*1/*15	4		4.00
	*1/*18	2	Decreased	2.00
Total number	100			

haploview). Furthermore, LD was investigated across all SNPs and selected haplotypes. Haplotype blocks were defined based on the Gabriel definition (D' > 0.9; minimum allele frequency, 5%) [14].

Transcriptional prediction

We used the online tools SIFT (http://sift.bii.a-star.edu.sg/) and PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/) to predict protein function of non-synonymous SNPs in CYP2A6 coding regions. Each variant was given a score based on the impact of its mutation on protein function. 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). PolyPhen-2 results were divided into five categories: probably benign (0.000-0.999), borderline (1.000-1.249), potentially damaging (1.250-1.449), possibly damaging (1.500-1.999) and probably damaging (≥ 2.000). PolyPhen-2 utilized two models (HumDiv and HumVar), in which the latter is more rigorous in its false discovery rate. In general, the HumVar dataset was used to predict protein function.

Results

Genetic variants

We identified a total of 33 CYP2A6 polymorphisms in the current Tibetan population, five of which were novel: mutation -98T > G in promoter region, mutation 1886C > A in intron 3, mutation 5640C > A in intron 7 and mutation 5827G > T in intron 8, they have no effect on protein sequence. Missense mutation 5011G > A in exon 7, as a result of Ala347Thr. All of them have not previously been reported in the NCBI database or in the Human CYP Allele Nomenclature Committee tables (**Table 2**).

Allele and genotype frequency

We identified six CYP2A6 alleles in the Uyghur population (**Table 3**). In the present study, the wild-type CYP2A6 allele, the normal CYP2A6*1 was found in 86.50% of the population. The other five mutant type alleles, decreased CYP2A6*10, decreased CYP2A6*11, CYP2A6*14, CYP2A6*15 and decreased CYP2A6*18, were rare with frequencies of 6.50%, 3.00%, 1.00%, 2.00% and 1.00%, respectively.

We also detected six CYP2A6 genotypes, with a wide frequency range from 2.00% to 73.00% in Tibetan population. Individuals with the wild-type *1/*1 genotype have normal enzyme activity and this genotype frequency was 72.00% in our study group. Other identified genotypes included *1/*10 (13%), *1/*11 (6.00%) and *1/*18 (1.00%), which lead to decreased enzyme activity. According to haplotype analysis, all allele and genotype frequencies were in Hardy-Weinberg equilibrium.

Inter-population comparisons

We compared CYP2A6 alleles (*1 and *10) distribution patterns between Tibetans and populations from various countries and ethnic groups in Asians [15-22], Caucasians [15], and Europe [16]. No subjects with variant CYP2A6*4 and CYP2A6*7 allele were identified in our study. We further compared the two alleles distribution patterns between Chinese and other populations. Above all of the results were listed in **Table 4**.

Linkage disequilibrium analysis

Haploview was used to assess LD between pairs of loci. The extent of LD for each pair of SNPs was measured by the D'-value, which was

Table 4. Allele frequencies of CYP2A6 in different populations

Populations	Total number	Allele frequency (%)				Reference
		CYP2A6*1	CYP2A6*4	CYP2A6*7	CYP2A6*10	
Asians						
Tibetan	100	86.6	-	-	6.5	Present study
Chinese	228	90.35	6.6	2.2	0.4*	6
Koreans	288	51*	9.4	-	4.2	1
Koreans	418	83	11	3.6	0.5*	7
Koreans	209	88.5	11	-	-	8
Japanese	182	44.2*	19.3	-	-	5
Japanese	126	68.2*	22.2	6.3	1.6	6
Japanese	163	84	37.4	-	-	2
Japanese	184	70.1*	37	6.5	2	7
Bangladeshi	212	95.28*	4.72	-	-	3
Sri Lanka	270	90.4	9.6	-	-	4
Europe						
Swedes	380	90.2	1.1	-	-	1
Caucasians						
Caucasian	301	97.2*	1	0	0*	6

*P < 0.01, compared with data of the present study. Reference: 1: Comparisons of CYP2A6 genotype and enzyme activity between Swedes and Koreans; 2: Genetic Polymorphisms of CYP2A6 in a Case-Control Study on Bladder Cancer in Japanese Smokers; 3: Lung cancer risk in relation to nicotinic acetylcholine receptor, CYP2A6 and CYP1A1 genotypes in the Bangladeshi population; 4: CYP2A6 gene deletion reduces oral cancer risk in betel quid chewers in Sri Lanka; 5: Effect of Genetic Polymorphism of CYP2A6 on Individual in Japanese Smokers Susceptibility to Colorectal Tumors; 6: An in Vivo Pilot Study Characterizing the New CYP2A6*7, *8, and *10 Alleles; 7: Genetic polymorphisms in human CYP2A6 gene causing impaired nicotine metabolism; 8: Nicotine metabolism and CYP2A6 allele frequencies in Koreans.

most accurate when minor allele frequencies (MAFs) were greater than 5% [23]. The overall LD across the CYP2A6 gene is depicted in **Figure 1**. But haplotype analysis identified no LD block within CYP2A6.

Predicted protein function of the non-synonymous mutation

Analysis using SIFT of the CYP2A6 5011G > A variant indicated that it was tolerated (score =0.63). PolyPhen-2 results for the same loci revealed that 5011G > A was benign with a score of 0.004 (**Figure 2**). The protein function prediction results from SIFT and PolyPhen-2 analysis of the 5011G > A were consistent.

Discussion

As observed in previous studies, enzyme activity of CYP2A6 significantly depends on CYP2A62A6 genetic variability [5, 24]. Genetic polymorphisms in CYP2A6 are highly relevant in the metabolism of clinically prescribed drugs and may influence patient responsiveness and adverse drug reactions. Thus, our results provide a better understanding of CYP2A6 variants and a potential database for promoting personalized medicine in Tibetan patients. Genetic variations in metabolic enzymes can cause dramatic differences in the response to specific drugs. Previous studies reported the genetic polymorphisms of CYP2A6 and proposed their relevance to cancer risk due to variations in nicotine and N-nitrosoamine metabolism [25-27].

In our current study, we analyzed the distribution of CYP2A6 allele frequency in the Tibetan population. Then, we compared these frequencies with those of other populations reported in the literature. Overall, we determined that the allele CYP2A6*1 (86.6%) is a common genetic variant in the Tibetan population and approximately 6.5% of the Tibetan population carried CYP2A6*10 allele potentially associated with decreased CYP2A6 activity. However, CYP2A6*4 and CYP2A6*7 allele are absent in the Tibetan population. Some alleles encode enzymes that are associated with absent (e.g. *4) [28], reduced (e.g. *9) [28], normal (e.g. *8) [29] or increased (e.g. *1B) [28]. Namely, carri-

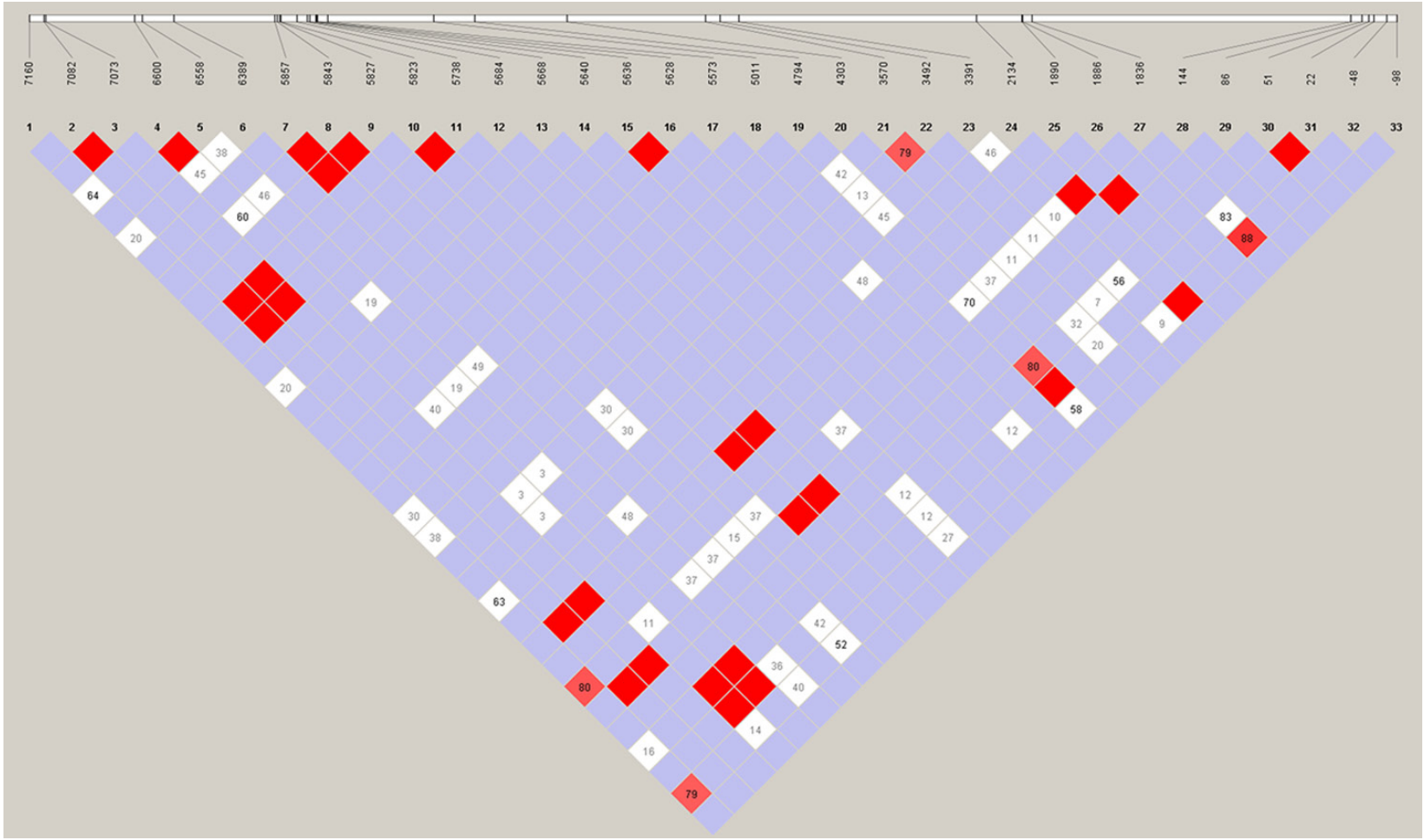

Figure 1. Linkage disequilibrium (LD) analysis of CYP2A6 genetic polymorphisms. LD is indicated by bright red (very strong: LOD > 2, D'=1), light red (LOD > 2, D' < 1), and blue (LOD52, D'=1) for intermediate LD, and white (none: LOD52, D' < 1).

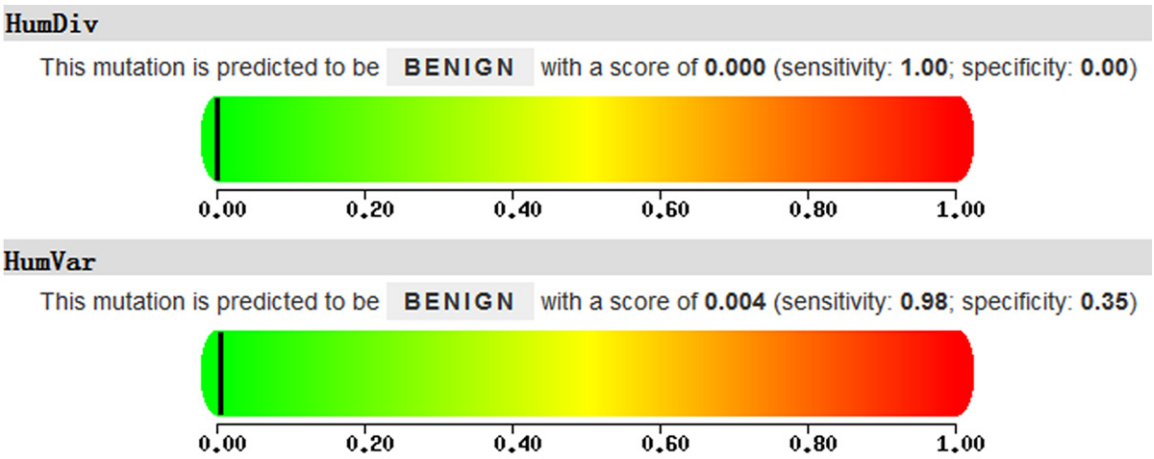

Figure 2. Predicted protein function of the mutation 5011G > A by PolyPhen-2.

ers of CYP2A6*4 (gene deletion) and CYP2A6*5 (Gly479Val) completely lack CYP2A6 [5, 15, 30]. Similarly, CYP2A6*7 (Ile471Thr), CYP2A6*8 (Arg485Leu), CYP2A6*9 (single nucleotide polymorphism in the TATA box), CYP2A6*10 (combination of *7 and *8 allele), CYP2A6*18 (Tyr392Phe) and CYP2A6*19 (combination of *7 and *18 allele) all lead to decreased enzymatic activity [5, 15, 30-32].

The whole-gene deletion variant CYP2A6*4 is one of the most common variants in the Japanese, Korean, and Chinese populations, whereas it is a minor variant in French and Brazilian populations [33, 34]. Overall, two deletion mutants of the CYP2A6, CYP2A6*4A (22.3%) and CYP2A6*4B (0.24%), have been reported in the Japanese population [35]. CYP2A6 activity with markedly different frequencies among ethnic groups [36]. The frequency of the CYP2A6*4 (*4A and *4D) variant is high in Asian populations, ranging from 6.7 to 24.2% in Japanese, Koreans, and Chinese, but is much less prevalent in Caucasians and African-Americans [8, 37].

On the other hand, CYP2A6*1B1 (gene conversion in 3'-UTR) is mostly associated with increased enzyme activity [38], while CYP2A6 duplication corresponds to the presence of three copies of the gene and thus provides about 1.4-fold higher enzyme activity [8, 39]. In addition to genetic variations, activity of CYP2A6 enzyme seems to be affected by a variety of non-genetics factors, including several antiepileptic agents as inducers, and pilocarpine and tranylcypromine as inhibitors [40, 41]. Therefore, it is common that liver content and activity of CYP2A6 vary considerably among individuals [41]. Due to potential clinical importance, this pronounced variation and its possible causes have been widely investigated. Numerous factors affecting CYP2A6 enzyme activity have been described, including ethnicity [42], genotype [31], sex [42], age [43], cigarette smoking [44]. However, the findings were not always consistent [45, 46], warranting further investigations. The data from this study and others demonstrated that the allelic distribution of CYP2A6 gene differs widely in different populations, presumably due to geographically-determined selection pressures.

It is widely accepted that ethnicity represents an important component of inter-individual variability in response to drugs [47]. Due to its crucial role in metabolism of many drugs, toxins and endogenous compounds [5], potential inter-ethnic differences in CYP2A6 levels and activity have been studied in numerous populations. Significantly lower CYP2A6 activity was observed in African Americans and Asians compared to Caucasians [42] as well as in Japanese compared to black, white or Korean population [22].

In our study, the protein prediction results of CYP2A6 5011G > A from the SIFT and PolyPhen-2 analysis were consistent. Missense mutation 5011G > A in exon 7, as a result of Ala347Thr, which suggested that this variant will affect the protein structure and function. 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 [48, 49]. Therefore, the prediction results may be biased and require further experimental data to more reliably predict the effects of variants identified in our study. The genetic polymorphisms of CYP2A6 cannot explain the inter-individual differences reported in CYP2A mediated metabolism. Further studies should conduct a large number of parameters including: mRNA expression, drug-drug interaction as well as external influences in order to more fully understand the factors that determine the metabolic capabilities of the CYP2A family enzymes.

There are several limitations to our study. The number of patients in several genotypic groups was small when the samples were divided into different groups according to the genotype, which could influence the study results because of insufficient statistical power.

In summary, we studied the distribution of CYP2A6 allelic variants and genotypes in the Chinese Tibetan population. Our data provide new information regarding CYP2A6 genetic polymorphisms in Tibetan individuals. This will greatly facilitate studies on the relevance of pharmacogenetics for CYP2A6 genes with respect to disease risk and to the pharmacokinetics and pharmacodynamics of many drugs. Future studies will focus on identifying CYP2A6 variants in a larger sample size of Tibetan, contributing to the enhanced application of personalized medicine in this population.

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Disclosure of conflict of interest

None.

Address correspondence to: Drs. Tianbo Jin and Li Wang, Key Laboratory for Molecular Genetic Mechanisms and Intervention Research on High Altitude Disease of Tibet Autonomous Region, School of Medicine, Xizang Minzu University, 6 East Wenhui Road, Xianyang 712082, Shaanxi, China. Tel: +86-29-33755247; Fax: +86-29-33755247; E-mail: jintianbo@gmail.com (TBJ); wangli_xzmd@163.com (LW)

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