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Original Article

Genetic polymorphisms and phenotypic analysis of *CYP2A6* in the Sherpa population

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Abstract: Objectives: *CYP2A6* is a highly polymorphic gene and CYP2A6 enzyme results in broad inter-individual variability in response to certain drugs and carcinogens, while little is known about the genetic variation of *CYP2A6* in Sherpa population. The aim of this study was to identify different *CYP2A6* mutant alleles and determine their frequencies, along with genotype frequencies, in the Sherpa population. Methods: We used DNA sequencing to investigate promoter, exons, introns, and 3'UTR of the *CYP2A6* gene in 100 unrelated healthy Sherpa individuals from southwestern Tibet, China. We also used SIFT and PolyPhen-2 to predict the protein function of the non-synonymous mutation in *CYP2A6* coding regions. Results: We identified a total of 25 different *CYP2A6* polymorphisms in the Sherpa population, including one novel variants (-98T > G). The allele frequencies of *CYP2A6**1, *4, *11, *14 and *18 were 81%, 10%, 0.5%, 7.5%, and 1%, respectively. The common genotype combinations were *1/*1 (62%), *1/*4 (20%) and *1/*14 (15%). We also determined rare genotypes *1/*11 (1%) and *1/*18 (2%) in Sherpas, which have decreased CYP2A6 activity. Additionally, the mutation I471T was predicted to be intolerant and probably damaging by SIFT and PolyPhen-2, respectively. Conclusion: Our results shed new light on *CYP2A6* polymorphisms in Sherpa individuals, which may help to optimize pharmacotherapy effectiveness by providing personalized medicine to this ethnic group.

Keywords: Genetic polymorphism, CYP2A6, Sherpa population, ethnic groups

Introduction

The cytochrome P450 (CYP450) superfamily is a large and diverse group of enzymes, mainly localized in the endoplasmic reticulum, that metabolize many common therapeutic drugs [1]. Among 69 CYP families in animals and approximately 50-100 CYP genes in vertebrates [2], CYP2 subfamily is the largest and most diverse one and metabolize diverse substances, such as xenobiotics, drugs, and arachidonic acid [3]. CYP2A6 is a member of the CYP2A subfamily and involved in biotransformation of a lot of drugs, including nicotine, halothane, disulfiram and valproic acid as well as the metabolism of carcinogens such nitrosamines and aflatoxin B1 [4, 5].

As observed in previous studies, the enzyme activity of CYP2A6 significantly depends on *CYP2A6* genetic variability [6, 7]. The gene encoding CYP2A6 is highly polymorphic (http://www.cypalleles.ki.se/cyp2a6.htm), and substantial interethnic and inter-individual variation in *CYP2A6* allele frequencies and resulting activity exists [8-10]. The wild-type allele, *CYP2A6*1A,* have normal CYP2A6 activity. Some alleles such as *CYP2A6*2* [11], **4A* [12], **4B* [13], **4D* and **5* [14] were found to be associated with inactive enzyme activity; by contrast, a lot of *CYP2A6* alleles were reported to have decreased activity, including *CYP2A6*6* [15], **7* [16], **9A* [17], **10* [18], **11* [19], **12A* [20], **17* [21], **20*, **21* [22], **23* [23], **24A*, **26* [22] and **35* [24]. In contrast, only three

Table 1. Primers used to amplify regions of *CYP2A6*

Primer name	Primer sequence (5'–3')	PCR product size (bp)
5'UTR_F	CTCTGGTCTTCCTCCCCTGC	843
5'UTR_R	CTGCCAAACAGACATCAAGACCAT	
Exon 1&2_F	AGGTGAAATGAGGTAATTATGTAATCAG	829
Exon 1&2_R	AGACTGGGGACTCTGCCT	
Exon 3&4_F	CACCCTACTCCCTCTCACC	829
Exon 3&4_R	AGGGTATTGGACATCCATCCT	
Exon 5_F	TTCAAATACCTGAAACCTGGATATATGTCT	702
Exon 5_R	GCGCAACCATGCCCAAC	
Exon 6_F	TGAAGGACAGATGGTCAGCAGG	1004
Exon 6_R	TTGGTGTCCTTTTTGACTCTGCG	
Exon 7_F	CGTTCCACCGGGTCATCC	743
Exon 7_R	TCCTTCTAGGCAGGAGTTTGG	
Exon 8_F	GGAGAATCAAACACATGTTCCC	773
Exon 8_R	TGTCCTTTACTGCCAGGTAC	
Exon 9_F	TGTAACTGGCAGGAAAGGACAT	851
Exon 9_R	TTAGGTGAGCGTGCAATGGTT	
3'UTR_F	GGGGCAGGATGGCGGATA	753
3'UTR_R	ATGGCTATGTCCTGATCCAGAGTTC	

Table 2. Primers used to genotype for the *CYP2A6*4* (deleted) allele

Primer name	Primer Sequence (5'–3')
2Aex7F	GRCCAACATGCCCTACATG
2A6R1	GCACTTATGTTTTGTGAGACATCAGAGACAA
2A6ex8F	CACTTCCTGAATGAG
2A7ex8F	CATTTCCTGGATGAC
2A6R2	AAAATGGGCATGAACGCCC

alleles, *CYP2A6*1B* [25], **42* and **44* [26] were reported to may be have increased activity. Moreover, previous studies have revealed that there are significant differences in the frequency of *CYP2A6* variants in different ethnicities. For example, the allele frequencies of *CYP2A6*4* and *CYP2A6*7* are 18-20% and 6.3% in Japanese [7, 16], but have only 0-4% in Caucasians [27, 28]; and some rare alleles such as **12*, **17* and **20* were determined only in a few populations [20].

Sherpa is a minority ethnic population and has resided for almost 500 years in the Himalayan region. In China, most Sherpas live in the certain areas between the northern Tibetan Plateau and the southern mountains and plains in Nepal, where the elevation ranges from 1600 to 4000 m [29-31]. To adapt to the low oxygen environment of the highlands, Sherpas are known for their extraordinary mountaineering ability and power of endurance. In these years, many researches have been done to study the genetic mechanism of highland adaptation in Sherpas, but little information is about the pharmacogenetics. Because information regarding *CYP2A6* polymorphisms is limited in the Sherpa population, the aim of this study was to investigate the distribution of *CYP2A6* genetic variations in Sherpa individuals and compare their allelic frequencies with previous data of other ethnic groups. We hope that our result could be useful for personalized medicine in Sherpas.

Materials and methods

Subjects

One hundred unrelated Sherpa volunteers (50 males and 50 females, age range 20-48) were recruited from southwestern Tibet. All participants were judged to be of good health and had at least three generations of paternal ancestry within this ethnic group. All patients gave a detailed medical history and underwent a physical examination prior to the study, including gynecological examinations and clinical laboratory tests.

Blood samples were obtained with signed informed consent from every participant enrolled. This research was approved by the Clinical Research Ethics of Xizang Minzu University and Northwest University and was in compliance with the Department of Health and Human Services (DHHS) regulations for the protection of human research subjects.

Genotyping of CYP2A6

Genetic polymorphisms of *CYP2A6* in the Sherpa study group were screened by DNA sequencing. A blood sample (5 mL) was taken from each subject into an EDTA tube and genomic DNA was extracted using the GoldMag-Mini Whole Blood Genomic DNA Purification Kit (GoldMag Ltd., Xi'an, China) according to the manufacturer's instructions. Primers listed in **Table 1** were designed to amplify the flanking regions, all exons, and all introns of the *CYP2A6* gene. Primers listed in **Table 2** were designed

Table 3. Frequency distribution of *CYP2A6* polymorphisms in 100 Sherpa subjects

#	SNP	Position	Nucleotide change	Region	Allele	Frequencies (%)	Amino-acid effect
1	Novel	-98	T > G	Promoter		2	No translated
2	rs28399433	-48	T > G	Promoter		32	No translated
3	rs8192720	22	C > T	Exon 1		30	Leu8Leu[a]
4	rs1137115	51	G > A	Exon 1	*CYP2A6*14*	51	Val17Val[a]
5	rs28399435	86	G > A	Exon 1	*CYP2A6*14*	15	Ser29Asn[b]
6	rs56283800	144	G > A	Exon 1		3	Gln48Gln[a]
7	rs57607700	1890	G > C	Intron 3		3	No translated
8	rs111033610	3391	T > C	Exon 5	*CYP2A6*11*	1	Ser224Pro[b]
9	rs1809811	3492	C > T	Exon 5		2	Arg257Arg[a]
10	rs4079369	3570	C > G	Intron 5		2	No translated
11	rs55971367	4718	C > T	Intron 6		1	No translated
12	rs549902541	4721	G > A	Intron 6		3	No translated
13	rs150455365	5573	A > C	Intron 7		2	No translated
14	rs1809810	5668	A > T	Exon 8	*CYP2A6*18A*	2	Phe392Tyr[b]
15	rs28399461	5684	T > C	Exon 8		1	Ser397Ser[a]
16	rs2002977	5738	C > T	Exon 8		3	His415His[a]
17	rs2002976	5823	C > T	Intron 8		3	No translated
18	rs113368210	5827	G > T	Intron 8		1	No translated
19	rs2002975	5843	G > C	Intron 8		5	No translated
20	rs72549446	5857	T > A	Intron 8		2	No translated
21	rs373949046	6389	C > G	Intron 8		6	No translated
22	rs5031016	6558	T > C	Exon 9		25	Ile471Thr[b]
23	rs2431412	7073	T > C	3'UTR		3	No translated
24	rs2259219	7082	G > C	3'UTR		3/100	No translated
25	rs28742185	7160	A > G	3'UTR		45/100	No translated

[a]synonymous mutations; [b]non-synonymous mutations.

Table 4. Allele and genotype frequencies of *CYP2A6* in Sherpa population

Allele	Total (n = 200)	Frequency	Phenotype
*1	162	81.00%	Normal
*4	20	10.00%	Deleted
*11	1	0.50%	Decreased
*14	15	7.50%	/
*18	2	1.00%	Decreased
Genotype	**Total (n = 100)**	**Frequency**	**Phenotype**
*1/*1	62	62.00%	Normal
*1/*4	20	20.00%	Deleted
*1/*11	1	1.00%	Decreased
*1/*14	15	15.00%	/
*1/*18	2	2.00%	Decreased

to genotype for the deleted allele *CYP2A6*4*. Polymerase chain reaction (PCR) for all single nucleotide polymorphisms (SNPs) was performed in 10 μL reactions with 5 μL HotStar Taq Master Mix (QIAGEN, Germantown, MD), 1 μL of template DNA, 0.5 μL each primer (5 μM) and 3 μL deionized water. Thermal cycling conditions were as follows: an initial denaturation step of 15 min at 95°C, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 57-62°C for 30 s, extension at 72°C for 1 min, and a final extension at 72°C for 3 min. The PCR products were sequenced using the ABI PrismBigDye 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 Sequencher 4.10.1 (http://www.genecodes.com/) software (Gene Codes Corporation, Ann Arbor, MI) was used for our initial analysis of the sequences including base calling, fragment assembly, and detection of SNPs, insertions, and deletions. We named the *CYP2A6* variants based on the NCBI Reference Sequence: NG_008377.1 and CYP

Table 5. Comparison of *CYP2A6* alleles in different populations

Allele(s)	Allele Frequencies (%)					
	Sherpa (n = 200)	Chinese (n = 226)	Japanese (n = 184)	Koreans (n = 418)	Caucasians (n = 374)	Africans (n = 352)
*CYP2A6*1*	81.0	51.7**	45.1**	53.5**	84.5*	74.4*
*CYP2A6*2*					1.1	0.3
*CYP2A6*4*	10.0	6.7	19.0*	10.8		0.9
*CYP2A6*5*		0.5				
CYP2A6*7		3.1*	9.8**	9.8**		
CYP2A6*8		3.6*	1.1	1.2		
CYP2A6*9		15.6*	19.0**	19.6**	8.0**	8.5**
*CYP2A6*10*		0.4	2.2	1.0		
*CYP2A6*11*	0.5		0.5	0.7		
CYP2A6*12						
CYP2A6*13			1.1	0.2		
*CYP2A6*14*	7.5				3.5*	1.4**
*CYP2A6*15*			2.2	1.2		
*CYP2A6*16*					0.3	1.7
*CYP2A6*17*						10.5**
*CYP2A6*18*	1.0			0.5	2.1	
*CYP2A6*19*				1.0		
*CYP2A6*20*						1.7
*CYP2A6*21*					0.5	0.6

**P < 0.01, compared with the data of the present study; *P < 0.05, compared with the data of the present study.

allele nomenclature (http://www.cypalleles.ki.se/). Allelic frequency comparisons between Sherpa population and other populations were performed using the Chi-squared test with a significance level set at P = 0.05 [32]. Haploview software (version 4.2) was used to assess linkage disequilibrium (LD) and Hardy-Weinberg equilibrium for each genetic variant [33].

Transcriptional prediction

We analyzed non-synonymous SNPs in the *CYP2A6* coding regions to predict their potential effects on transcription. Two algorithms, SIFT (Sorting Intolerant From Tolerant, http://sift.bii.a-star.edu.sg/) and PolyPhen-2 (Polymorphism Phenotyping v2, http://genetics.bwh.harvard.edu/pph2/), were used to perform the functional prediction of non-synonymous SNPs [34]. Each variant was given a score based on the impact of its mutation on protein function. The SIFT divided results 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 three categories: benign, possibly damaging and probably damaging.

Results

Genetic variants

We sequenced *CYP2A6* from our study subjects and successfully identified a total of 25 *CYP2A6* polymorphisms in this population. One of the polymorphisms (-98T > G in promoter region) had not previously been reported in either the NCBI database or the Human Cytochrome P450 Allele Nomenclature Committee tables (**Table 3**). We also determined four non-synonymous mutations in exons: 86G > A, 3391T > C, 5668A > T and 6558T > C.

Allele and genotype frequency

We identified five *CYP2A6* alleles in the Sherpa study group: the wild-type *CYP2A6* allele, *CYP2A6**1, which found in 81% of the population. The deleted *CYP2A6* allele, *CYP2A6**4, has a frequencies of 10% in Sherpas. Three rare alleles, *CYP2A6**11, *14 and *18, with frequencies of 0.5%, 7.5% and 1.0%, respectively (**Table 4**).

We also detected five *CYP2A6* genotypes, with a wide frequency range from 2% to 62% in this Sherpa population. Individuals with the wild-type *1/*1 genotype have normal enzyme activity, and this genotype was the most prevalent (62%) in our study group. Other identified genotypes included the heterozygous genotype *1/*11 (1%) and *1/*18 (2%), which lead to decreased enzyme activity, the deleted genotype *1/*4 (20%) and the less studied *1/*14 genotype (15%). According to Haplo-

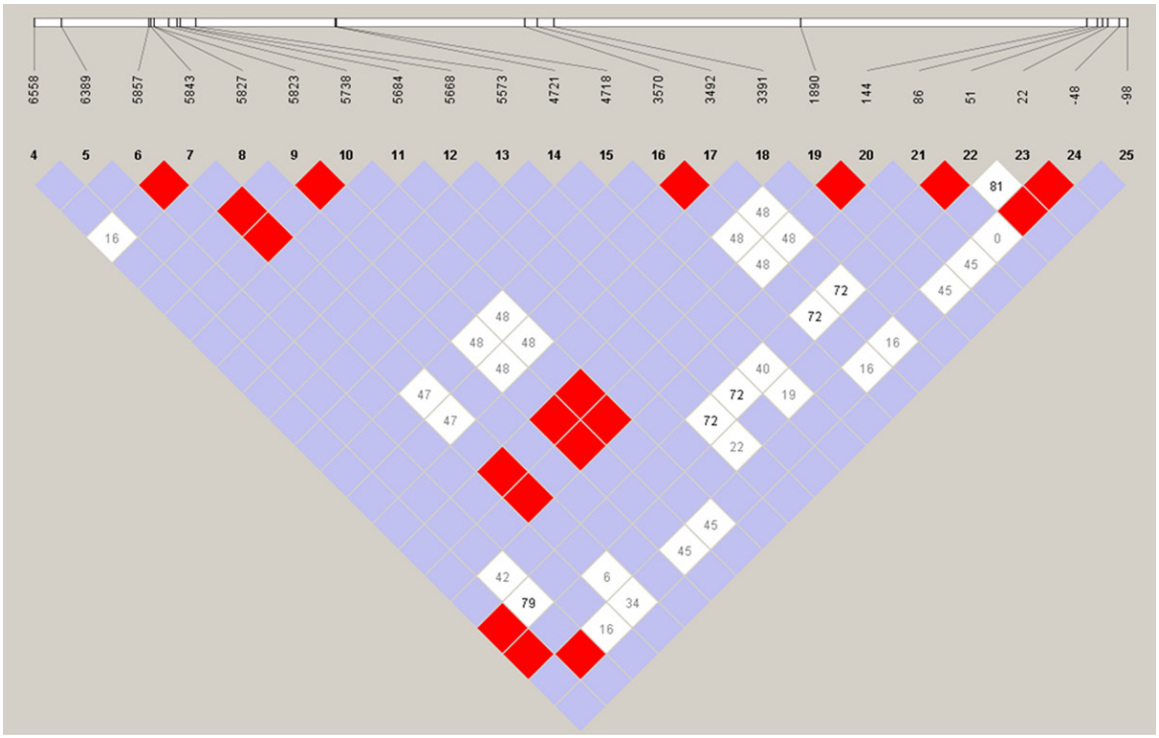

Figure 1. Linkage disequilibrium analysis of *CYP2A6*. LD is displayed by standard color schemes, with bright red for very strong LD (LOD > 2, D' = 1), pink red (LOD > 2, D' < 1) and blue (LOD < 2, D' = 1) for intermediate LD, and white (LOD < 2, D' < 1) for no LD.

Table 6. Prediction results of four non-synonymous mutations in *CYP2A6* using SIFT and Polyphen-2

SNPs	Substitutions	SIFT		Polyphen-2	
		Score	Results	Score	Results
rs28399435	S29N	0.49	Tolerant	0.078	Benign
rs111033610	S224P	1.00	Tolerant	0.057	Benign
rs1809810	F392Y	0.40	Tolerant	0.084	Benign
rs5031016	I471T	0.00	Intolerant	0.996	Probably damaging

SIFT, Sorting Intolerant from Tolerant; PolyPhen-2, Polymorphism Phenotyping v2.

view analysis, all allele and genotype frequencies were in Hardy-Weinberg equilibrium (**Table 4**).

Inter-population comparisons

We further compared *CYP2A6* allele frequencies between our data and previously published data of different ethnic groups from Asia, Europe and Africa [10, 35]. A total of 19 different *CYP2A6* alleles were analyzed in those populations. We found that the allele frequency of *CYP2A6*1* was significantly higher ($P < 0.05$) in Sherpas compared with Asian populations including Chinese Han, Japanese and Koreans. The allele frequency of *CYP2A6*4* in Sherpa was similar to Asian populations and have significant difference with Africans. Similarly, The frequency of *CYP2A6*11* in Sherpas was similar to other Asian group, but was not found in Caucasians and Africans. In contrast, *CYP2A6*14* was not found in other Asian group, but the frequency of *CYP2A6*14* in Sherpas was even significantly higher ($P < 0.05$) than Caucasians and Africans. Additionally, *CYP2A6*18* was also found in Koreans and Caucasians, and the frequency is similar (**Table 5**).

Linkage disequilibrium analysis

We performed LD analysis using Haploview with confidence intervals to define LD blocks (**Figure 1**). The extent of LD for each pair of SNPs was measured by the D' value, which was most accurate when minor allele frequencies (MAFs) were greater than 5%. Unfortunately, we failed to Haplotype analysis identified any LD blocks within *CYP2A6*.

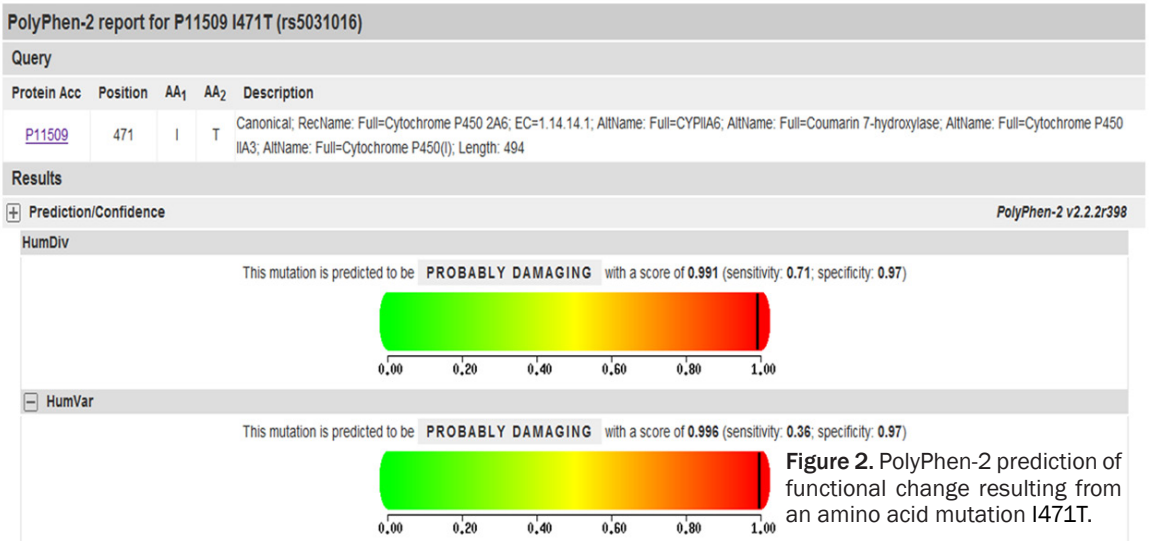

Figure 2. PolyPhen-2 prediction of functional change resulting from an amino acid mutation I471T.

Predicted protein function of the non-synonymous mutation

We identified four non-synonymous mutation of *CYP2A6* in our study group: S29N, S224P, F392Y and I471T. The protein function prediction results from SIFT and PolyPhen-2 analysis were highly consistent (**Table 6**). S29N, S224P and F392Y were identified as benign; but I471T was determined as probably damaging (**Figure 2**).

Discussion

It is widely accepted that ethnicity represents an important component of inter-individual variability in response to drugs [36]. Due to its crucial role in the metabolism of many drugs, exogenous and endogenous compounds, potential inter-ethnic differences in *CYP2A6* alleles and CYP2A6 activity have been studied in several populations. To better understand the distribution of *CYP2A6* allele frequency in the Sherpa populations, we systematically screened the whole *CYP2A6* genes of 100 healthy, unrelated Sherpa subjects for polymorphisms. We identified 25 genetic variants including one novel polymorphism, four alleles, and four genotypes of *CYP2A6* in our study Sherpa population, and compared these data with previous observations of other ethnic groups. We hope this could provide some useful information for future personalized medicine in the Sherpa population.

To date, more than 50 variants of the *CYP2A6* gene have been identified (http://www.cypalleles.ki.se/cyp2a6.htm). In our study, we compared 19 major allelic polymorphisms in Sherpa and five other populations including Chinese, Japanese, Koreans, Caucasians and Africans. We found that allele frequencies identified in Sherpas were different from neither Asian populations nor Caucasians or Africans. The lack of *CYP2A6*2* allele in the Sherpa group is in accordance with its reported absence in Asian populations. However, the variants *CYP2A6*5*, **7*, **8* and **10*, which were common variants in Asian populations, were not found in our Sherpa group. Moreover, variants *CYP2A6*14* and **18*, which were usually identified in Caucasians and Africans, have relatively high frequency in Sherpas. In terms of polymorphism distribution, these differences could be attributed to the origin and geographical isolation experienced by different ethnic populations, as well as their dietary habits and lifestyles, all of which may affect *CYP2A6* polymorphisms.

Analysis of genetic variants in the coding region revealed variant rs5031016/I471T will affect the protein structure and function, and the results of SITF and PolyPhen-2 were highly consistent. Previous study have shown that SNP rs5031016 was associated with reduced nicotine metabolism and cigarette consumption in Japanese population [37], which is consistent with our study. However, the prediction accuracy of SIFT and PolyPhen-2 is 63% and 75%, and the false positive rate is 19% and 9%, respectively [34, 38]. Therefore, the results identified here should be confirmed by further functional studies.

In our current study, we determined that the allele *CYP2A6*14* is a common genetic variant in the Sherpa population, previous literatures have no information about the phenotype of this allele, which we prepared to study on its function in our further study. Individuals who are homozygous carriers for the *CYP2A6*11* and *CYP2A6*18* alleles show decreased enzyme activity compared to the wild type. Overall, approximately 3% of the Sherpa population carried genotypes potentially associated with decreased *CYP2A6* activity. Previous study have shown that decreased CYP2A6 activity is closely associated with metabolism of nicotine [35], but the smoking status data of our samples is absent, we could not explore how *CYP2A6* polymorphisms and tobacco consumption interact with each other. This limitation of our study also reminds us that we should collect more specific clinical information of participants next time.

Taken together, our results provide a basic profile of CYP2A6 in the Sherpa population, and the novel allele and genotype would be useful for determining metabolic phenotypes of CYP2A6 substrate drugs in Sherpas, and provide a basis for safer drug administration and better therapeutic treatment among Sherpa population.

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Disclosure of conflict of interest

None.

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