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Original Article

Polymorphisms and phenotypic analysis of cytochrome *P450 3A4* in the Uygur population in northwest China

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Abstract: Purpose: Genetic polymorphisms in CYP3A4 can change its activity to a certain degree, thus leading to differences among different populations in drug efficacy or adverse drug reactions. Methods: The study was intended to validate the genetic polymorphisms in *CYP3A4* in Uygur Chinese population, we sequenced and screened for genetic variants including 5'UTR, promoters, exons, introns, and 3'UTR region of the whole *CYP3A4* gene in 100 unrelated, healthy. Results: Twenty-one genetic polymorphisms in *CYP3A4*, and nine of them were novel. We detected CYP3A4*8, a putative poor-metabolizer allele, with the frequency of 0.5% in Uygur population. Tfsitescan revealed that the density of transcription factor varied in the different promoter regions, among which some were key regions for transcription factor binding. Conclusion: our results provide basic information about *CPY3A4* alleles in Uygur and suggest that the enzymatic activities of CPY3A4 may differ among the diverse ethnic populations of China.

Keywords: *CPY3A4*, genetic polymorphism, haplotype, Uygur, ethnic difference

Introduction

The cytochrome P450 (CYP450) super-gene family codes more than 500 kinds of enzymes can mainly be found in the liver and small bowel in vivo. *CYP450* enzymes exist many isozymes which can be divided into a number of genes/subgenes families, the enzymes of the same family own similar functions [1-3]. Notably, *CYP450* enzymes show extensive structural differences due to genetic polymorphisms in the corresponding genes, thus giving rise to different enzymatic activities and leading to great intra- and inter-population differences in drug efficacy and adverse reactions.

CYP3A4, a subfamily of CYP450, expanding on chromosome 7q22.1, included 13 exons and 12 introns [4]. It is the most important drug metabolizing enzymes in the liver which accounts for 30-40% of total P450 enzymes and involved in metabolism of more than 50% of clinical commonly used drugs [5, 6]. CYP3A4 is a polymorphic enzyme, the expression of CYP3A4 among individuals varied as much as 40 times, to understand the expression of CYP3A4 may determine drug efficacy and safety, thus helping people make the right due to dose [7, 8].

Uygur is an ethnic with a population of 10,069,347 (according to the sixth population survey of China in 2011). They live mostly in the Xinjiang Autonomous Region. Places of residence are relatively stable in the Uygur population and there is little migration [9]. We systematically screened the whole *CYP3A4* genes in 100 healthy, unrelated Uygurs for polymorphisms, hoping to find corresponding phenotypes and offer recommendations pertaining to the drug substrates of *CYP3A4* in the Uygur population.

Materials and methods

Subjects

We recruited a random sample of 100 healthy, unrelated Uygurs (including 50 males and 50

Table 1. Polymorphisms and frequency distribution of *CYP3A4* in Uyghur population

#	SNP ID	Position	Region	Allele	Effect	Flanking Sequence	MAF
1	/	-747C > G	Promoter	CYP3A4*1F	/	TGTACAGCAC S CTGGTAGGGA	0.025
2	/	-667C > T	Promoter	CYP3A4*1W	/	GGGAAACAGG Y GTGGAAACAC	0.005
3	new1	-549C > G	Promoter		/	AGGAAAGACT S TAAGAGAAGG	0.005
4	rs2740574	-392A > G	Promoter	CYP3A4*1B	/	GACAAGGGCA R GAGAGAGGCG	0.015
5	/	-62C > A	5'UTR	CYP3A4*1D	/	CATAGCCCAG M AAAGAGCAAC	0.005
6	rs55913187	3858C > T	Intron1		Not translated	TAATCATTGC Y GTCAGAGTTA	0.015
7	new2	5939A > T	Intron5		Not translated	TTTTCATCCC W ATTAGAGGCA	0.005
8	/	13908G > A	Exon5	CYP3A4*8	130R > Q	AAGAGATTAC R ATCATTGCTG	0.005
9	new3	15380C > T	Intron6		Not translated	TCACACCCAG Y GTAGGGCCAG	0.015
10	/	15727G > C	Intron7	CYP3A4*1P	Not translated	TAAGTATGTG S ACTACTATTT	0.015
11	/	15753T > C	Intron7	T15781G*	Not translated	TATTTATCTT Y CTCTCTTAAA	0.025
12	/	15977T > C	Intron7	C78013T*	Not translated	AACTTTCTGC Y TCTATGGATT	0.740
13		16613C > T	Intron7	C78649T*	Not translated	ACTGCTGTAG Y GGTGCTCCTT	0.115
14	new4	16934A > T	Exon8		264T > S	CCTCGAAGAT W CACAAAAGGT	0.005
15	new5	17864A > C	Intron9		Not translated	AGAGGCATGC M GGCATAGATA	0.005
16	rs2242480	20230G > A	Intron10	CYP3A4*1G	Not translated	TGAGTGGATG R TACATGGAGA	0.115
17	rs4986910	23172T > C	Exon12	CYP3A4*3	445M > T	TGCATTGGCA Y GAGGTTTGCT	0.005
18	new7	25721A > G	Intron12		Not translated	ATTACTCCAT R GAGATCAGAA	0.130
19	new8	25925C > T	Exon13		499T > T	GGGATGGCAC Y GTAAGTGGAG	0.010
20	new9	25931T > C	Exon13		501S > S	GCACCGTAAG Y GGAGCCTGAA	0.005
21	new10	26759G > C	3'UTR		/	TGGTGGACTC S CCTGTAATCT	0.005

MAF: minor allele frequency.

Table 2. Alleles and frequencies of *CYP3A4* in Uyghur population

Allele	*Alleles (N)*	*Frequency (%)*	*Enzyme Activity*
CYP3A4*1A	162	81%	Normal
CYP3A4*1F	5	2.5%	/
CYP3A4*1W	1	0.5%	/
CYP3A4*1B	2	1.5%	/
CYP3A4*1D	1	0.5%	/
CYP3A4*1P	3	1.5%	/
CYP3A4*1G	23	11.5%	/
CYP3A4*3	1	0.5%	Decreased
CYP3A4*8	1	0.5%	Decreased

females) between October 2010 and December 2011 from Xinjiang University in Xinjiang Autonomous Region for population genetics research. All of the chosen subjects were Uygur Chinese living in the Xinjiang Autonomous Region of China and had at least three generations of paternal ancestry in their ethnic. We used detailed recruitment and exclusion criteria excluding subjects with chronic diseases involving vital organs (heart, lung, liver, kidney, and brain) and other related diseases. The purpose of the exclusion procedures was to minimize the known environmental and therapeutic factors that influence genetic variation in the gene *CYP3A4*.

We informed all of the participants about the experimental procedures and the purpose of the study. The Human Research Committee of the Xinjiang University and Northwest University for Approval of Research Involving Human Subjects approved the use of human blood in this study. We also obtained signed informed consent from each study participant.

PCR and DNA sequencing

We used GoldMag-Mini Whole Blood Genomic DNA Purification Kit (GoldMag Ltd.) according to the manufacture's protocol to extract genomic DNA from peripheral blood. Our PCR primers were designed using primer-Blast (http://frodo.wi.mit.edu/primer3/) to amplify about 900bp of the 5' flanking regions, all exons, all introns, and 3'UTR of the CYP3A4 gene. We performed the PCR in a total volume of 10 μl containing 1 μl genomic DNA (20 ng/μl), 5 μl Hot Star Taq

Table 3. Comparison of CYP3A4 alleles in different populations

Ethnicity	*N*	**1B*	**3*	**4*	**5*	**6*	**8*	**11*	**13*	**14*	**15*	**16*	**17*	**18*	**21*	*Ref*
Uyghur	100	1.5%	0.5%				0.5%									/
Han Chinese	100	0.5%			0.5%	1%								1%	0.5%	[19]
Chinese Han, She, Dong	60	1%							1%	7%	16%		2%	1%		[18]
Taiwan Chinese	102			2.9%	1.9%	0.5%										[32]
Japanese	416					0.1%		0.2%				1.4%		2.8%		[33]
Malaysian	121			0%	0%									2.1%		[28]
European	95	4%	2%													[34]
African	95	82%	0%													[34]

Table 4. Genotype and frequency distribution in Uyghur population

Genotype	*Subjects (N)*	*Frequency (%)*	*Phenotype*
*1A/*1A	66	67%	Normal
*1A/*1G	19	19%	Normal
*1A/*1F	5	5%	Normal
*1A/*1P	3	3%	Normal
*1A/*1W	1	1%	Normal
*1G/*1D	1	1%	Normal
*1G/*1B	1	1%	Normal
*1A/*1B	1	1%	Normal
*1G/*3	1	1%	PM
*1G/*8	1	1%	PM

Master Mix, 0.5 μl each primer pair (5 μM), and 3 μl deionized water. The cycling protocol consisted of denaturation at 95°C for 15 min, followed by 35 cycles of 95°C for 30 s, 55-64°C for 30 s, 72°C for 1 min, and a final extension at 72°C for 3 min to hold. The PCR products were purified by incubating them with 0.5 μl shrimp alkaline phosphate (Roche, Basel, Switzerland), 1.5 μl deionized water, and 8 μl HotStar PCR product, for a total volume of 10 μl, at 37°C for 30 min, followed by heat inactivation at 80°C for 15 min. We directly sequenced the purified PCR products using an ABI Prism BigDye Terminator Cycle Sequencing Kit version 3.1 (Applied Biosystems), on an ABI Prism 3100 sequencer (Applied Biosystems).

Data analysis

We used the Squencher 4.10.1 (http://www.genecodes.com/) software for analysis of the sequences including base calling, fragment assembly, and detection of SNPs, insertions, and deletions. We named the *CYP3A4* variants based on the nucleotide reference sequence AY545216 and CYP allele nomenclature (http://www.cypalleles.ki.se/). We assessed linkage disequilibrium (LD) and Hardy-Weinberg equilibrium for each genetic variant using HAPLOVIEW 4.1 (http://broad.mit.edu/mpg/haploview) [10]. We constructed haplotypes from the selected tag SNPs and derived the haplotype frequencies for the Uygur population.

Transcriptional prediction

We analyzed variants in the *CYP3A4* promoter region to predict their potential effects on transcription. We used the online tool Tfsitescan (www.ifti.org/cgi-bin/ifti/Tfsitescan.pl) and the transcription factor binding sites database to investigate the effects that promoter-region variants have on transcription [11]. We analyzed the wild-type and allelic variants separately. Depending on the metabolic activity of *CYP3A4*, we divided the subjects into three phenotypic groups: poor metabolizer (PM), extensive metabolizer (EM), and ultra-rapid metabolizer (UM), according to CYP allele nomenclature (http://www.cypalleles.ki.se/) [12].

Functional prediction of nonsynonymous mutation

We analyzed nonsynonymous mutations in the *CYP3A4* exon region to predict their potential effects on protein function. We used online tool SIFT (http://sift.bii.a-star.edu.sg/) and PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/) to evaluate the effect on exon-region variants have on protein function [13-15]. The results of SIFT prediction can be classified in four categories: tolerant (0.201-1.00), borderline (0.101-0.20), potentially intolerant (0.051-0.10), and intolerant (0-0.05). The results of PolyPhen-2 can be classified in five categories: probably benign (0-0.999), bordline (1.000-1.249), potentially damaging (1.250-1.499),

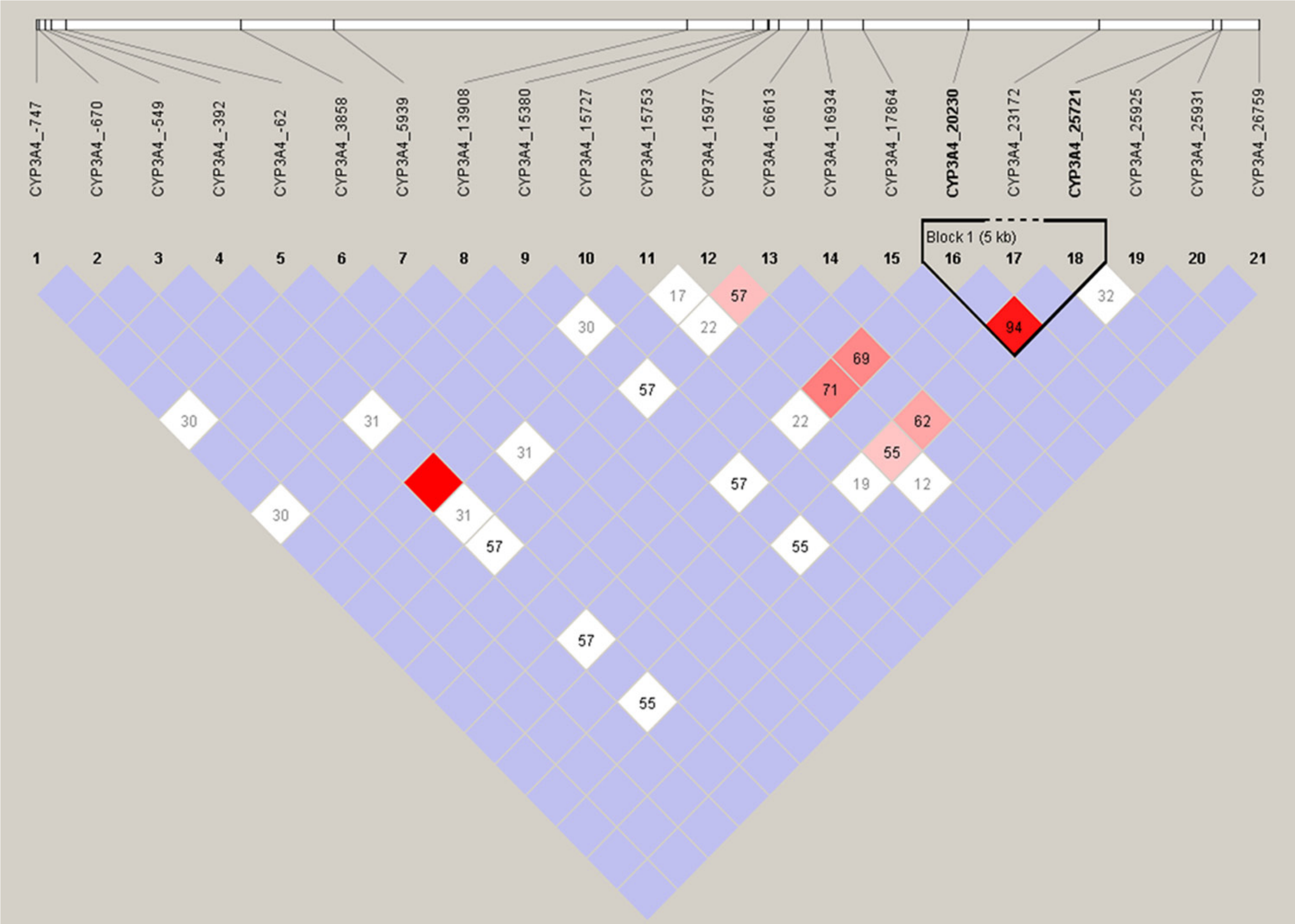

Figure 1. Linkage disequilibrium analysis of *CYP3A4*. LD is displayed by standard color schemes, with bright red for very strong LD (LOD > 2, D' = 1), pink red (LOD > 2, D' < 1) and blue (LOD < 2, D' = 1) for intermediate LD, and white (LOD < 2, D' < 1) for no LD.

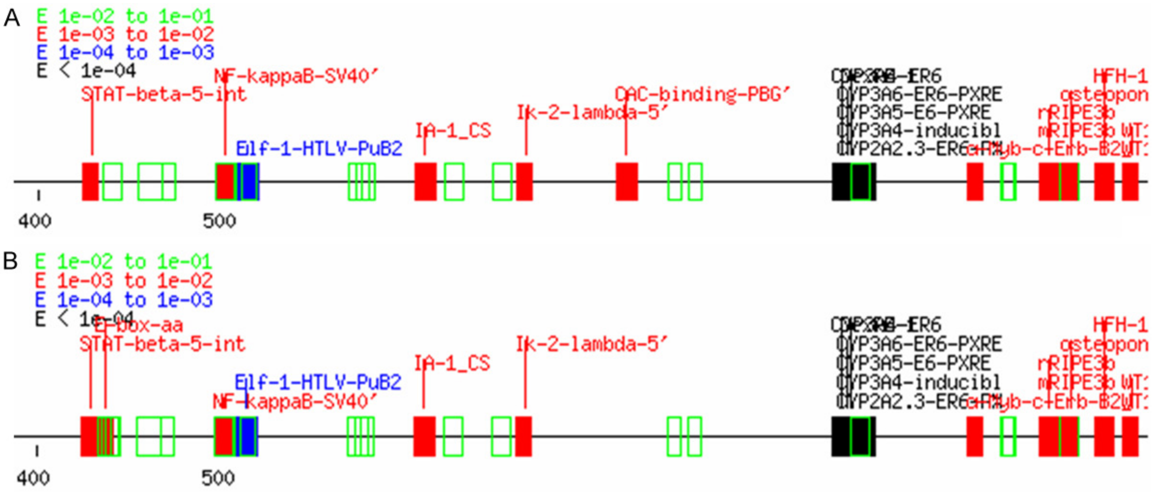

Figure 2. Transcription factor binding sites prediction of genetic variants in promoter region. A. Prediction map of TfsiteScan with the original sequence. B. Prediction map of TfsiteScan with the mutantre sequence.

possibly damaging (1.500-1.999) and probably damaging (≥2).

Results

Genetic variants

We successfully sequenced *CYP3A4* from 100 volunteer subjects, including 50 males and 50 females. We identify a total of twenty-one CYP3A4 polymorphisms in the current Uygur population, among which nine of the polymorphisms are not previously reported in the NCBI database nor the Human Cytochrome P450 Allele Nomenclature Committee tables, most variants have the frequency of less than 5%, besides four variants in introns showed relatively high frequencies of 74% (15977T > C, intron7), 11.5% (16613C > T, intron7), 11.5% (20230G > A, intron10) and 13% (25721A > G, intron12) (**Table 1**). One of the novel polymorphisms is within the promoter region, three are in exons among which one is nonsynonymous mutations, four are in the introns, and one is in 3'UTR region. We did not find any *CYP* duplications or deletions. The primers of Promoter, 13 exons and 3'UTR region were seen in Table S1.

Allele frequency and genotype frequency

We detected nine *CYP3A4* alleles in the Uygur population (**Table 2**). The *CYP3A4*1A* represented the wild type *CYP3A4* allele and had the highest frequency (81%), followed by the *CYP3A4*1G* (11.5%), *CYP3A4*1F* (2.5%), *CYP3A4*1B* (1.5%) and *CYP3A4*1P* (1.5%) allele. The rest alleles: *3A4*1W*, **1D,* *3 and **8*; were relatively rare with frequencies of 0.5%. We also compared the allle frequency in different populations and we found that the allele frequency of *CYP3A4*1B* in the current study was 1.5%, which was as much as 82% in African population (**Table 3**).

We identified ten *CYP3A4* genotypes in the Uygur population, with frequencies ranging from 1% to 67%, listed in **Table 4**. Eight of the genotypes have normal enzyme activity and two have decreased enzyme activity.

LD analysis

We performed LD analysis using Haploview with confidence intervals to define blocks. By default, our method ignores markers with MAF < 0.05; SNPs with lower frequencies have little power to detect LD (**Figure 1**). We found the twenty-one genetic polymorphisms in *CYP3A4* rendered a relatively weak linkage relationship since the D' value (a measure of the extent of LD for each pair of SNPs) on the square is relatively small. We identified one LD block comprised of three haplotypes of GA, AG and GG with the frequencies of 87%, 11% and 2% respectively.

Genetic variants in the transcription factor binding site

We found four polymorphisims including -747C > G, -667C > T, -549C > G, and -392A > G in

Table 5A. Results of SIFT predictions of nsSNPS

SNPs	*Coordinates*	*Codons*	*Ensemble Transcript ID*	*Substitution*	*dbSNP*	*Score**	*Prediction*
13908 G > A	7,99367788 C/T	CCA CaA	ENST000 00336411	R130Q	novel	0	DAMAGING
16934 A > T	7,99364762 T/A	ACA tCA	ENST000 00424826	T180S	novel	0.51	TOLERATED
23172 T > C	7,99358524 A/G	ATG AcG	ENST000 00424826	M361T	novel	0	DAMAGING

Table 5B. Results of PolyPhen predictions of nsSNPS

RS#	*Position*	*SNPs*	*HumVar*	*HumDiv*	*Prediction*
/	Exon5	13908G > A R130Q	1.000 (sensitivity:0.00; specificity:1.00)	1.000 (sensitivity:0.00; specificity:1.00)	Probably damaging
new	Exon8	16934A > T T264S	0.001 (sensitivity:0.99; specificity:0.09)	0.000 (sensitivity:1.00; specificity:0.00)	Benign
rs4986910	Exon12	23172T > C M445T	0.909 (sensitivity:0.69; specificity:0.90)	0.987 (sensitivity:0.73; specificity:0.96)	Probably damaging

promoter region. The search for transcription factor binding domains by Tfsitescan revealed that the -747C > G, -667C > T and -392A > G mutation altered the original binding sites of transcription factors thus potentially affecting the binding proteins and resulting in alteration of the corresponding gene expression, the new polymorphism -549C > G mutation makes no effect on the binding of the transcription factors (**Figure 2**).

Nonsynonymous mutation in the functional change of protein function

We found three polymorphisims includin-g16934A > T, 13908G > A and 23172T > C caused nonnsynonymous mutation in exon region. SIFT and PolyPhen showed high consistency that novel variant 16934A > T had little effect on the change of protein function, but for 13908G > A and 23172T > C, the function were changed to a damaging direction (**Table 5**).

Discussion

Our study, for the first time, systematically screened for variants of *CYP3A4* by direct sequencing among the Uygur population and compared the results with other ethnic populations around the world. We found twenty-one genetic variants including nine novel polymorphisms, nine alleles and ten genotypes. One of the novel genetic variants within the promoter region and makes no effect on the binding of transcription factors. Among the three variants within exons resulted in nonsynonymous mutation, the novel one makes little effect on protein function, but the other two changed the protein function to a damaging direction. In conclusion, our results provide a basic profile of *CYP3A4* in the Uygur population, and thus can be used to inform optimal dosage recommendations and individualized medicine.

CYP3A4 is an important member of phase I drug metabolism enzymes cytochrome P450 (CYP450) and plays an important role in the metabolism of exogenous compounds and the transformation of internal compounds. It involved in more than 50% commonly clinically used drug [5, 7]. The metabolic activity of CYP3A4 exhibit up to 40-folds individual differences, and the metabolism ability of substrate drugs expands from 10 to 20-folds, such a large difference was the co-function of genetic polymorphism, regulation of gene expression and the surrounding environment of the drug or chemical substance interaction, among which 90% of the difference was caused by inherited factors [7, 8, 16, 17].

The 15977T > C, 16613C > T and 25721A > G variants were reported in Chinese population for the first time, enriching the contents of *CYP3A4* polymorphisms in China. Especially for the variant 15977T > C, with the frequency of as high as 74%, we have not found such a high frequency in other *CYP3A4* polymorphisms. The 20230G > A (*CYP3A4*1G*) frequency in the current study was 11.5%, lower than two studies centered on Chinese Han polymorphisms of CYP3A4 with the frequency of 20230G > A (*CYP3A4*1G*) was 25% and 37% [18, 19].

As for allele, we for the first time identified *CYP3A4*1B* in Chinese population. *CYP3A4*1B* (A-392G) was considered to be the most commonly see mutations around the world with the frequency of 4%, 6%, 6.9%, 12.5%, 40% and 72% in Caucasian, Chilean, Jordanian, Central Americans, African Americans and Africans respectively [20-25]. The frequency of

*CYP3A4*1B* in the current study is only 1.5%. The activity of CYP3A4*1B was found to be controversial in previous studies, some found the allele had the decreased activity while some were normal [26, 27]. Whether the function of the allele was normal or not deserved further validation.

We for the first time identified *CYP3A4*8*, a decreased-activity metabolizers in Asia. The allele was the 13908G > A mutation in Exon5, leading to amino change Arg130Gln. It was first identified in Caucasus with the frequency of 0.33% [28]. Tamoxifen, metabolized by CYP3A4 and other CYPs, is a common anti-cancer drug used in breast cancer [29, 30]. We prompt doctors should cautiously consider the dosage of Tamoxifen and other drugs metabolized by CYP3A4 since CYP3A4*8, the decreased-activity metabolizer, is firstly identified in Asian populations in Uygurs.

The LD distribution provides a basic profile of the genomic structure of CYP3A4 in the Uygur population. A haplotype on a block is set of closely linked genetic markers present on one chromosome that tends to be inherited together. The twenty-one genetic polymorphisms in *CYP3A4* rendered a relatively weak linkage relationship and we found only one block comprised of three SNPs. The linkage relationship may be an explanation of why most of the commonly see alleles in CYP3A4 pose no threat on the change of enzyme activity.

For the analysis of novel genetic variants in the promoter region, -747C > G, -667C > T, and -392A > G altered the transcription binding site in promoter region. The -667C > T mutation, leading to the loss of BRE' binding site and appearance of a series new transcription binding sites such as E-box-aa, E47_CS', PTF-7SK-RNA_PSE', E2A_CS, E2A_site_consen', E47-MyoD_CS', MyoD-MCK-right_', C/EBP-SV40-1', AP-3_CS and so on. This is an indicator that the mutation makes great effect on the regulation of promoter region and thus explaining why CYP3A4 content differed interindividually. Some studies reported that the -667C > T mutation makes no effect on the CYP3A4 content [7, 24, 31]. This inconsistency may be explained by the complexity of the regulation network in promoter regions.

SIFT and PolyPhen prediction in coding region revealed that the novel variant 16934A > T makes no effect on the construction and function of the protein, but for 13908G > A (*CYP3A4*8*) and 23172T > C (*CYP3A4*3*), the prediction were damaging, which was in accordance with the reported results that the two alleles represented decreased-activity enzymes.

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Disclosure of conflict of interest

None.

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Table S1. *CYP3A4* primers and amplifications

Region	*Primer pair*	*Primer sequence (5' to 3')*	*Len (bp)*	*GC (%)*	*Anneal temp*	*Fragment size (bp)*
Promoter	CYP3A4_P_F	GTGCAGAGACAGCAGCTGAG	20	60.00	60.08	882
	CYP3A4_P_R	TCTCCTCTGAGTCTTCCTTTCA	22	45.45	45.45	
Exon1	CYP3A4_Exon1_F	CTTCCAACTGCAGGCAGAG	19	57.89	59.71	900
	CYP3A4_Exon1_R	GTTTGGAATGAGATCCGTCA	20	45.00	58.49	
Exon2	CYP3A4_Exon2_F	ATTCCTGCCTGAACCTCTCA	20	50.00	59.80	894
	CYP3A4_Exon2_R	GGTAAATACCTGGGCTCCCTA	21	52.38	59.34	
Exon3	CYP3A4_Exon3_F	AAGGATGACAAAGAGATAAAACACTG	26	34.62	59.20	898
	CYP3A4_Exon3_R	AAGACTCCGCAAAACTACAAGC	22	45.45	59.95	
Exon4	CYP3A4_Exon4_F	GGAGAATGGCATGGGAAATA	20	45.00	59.72	855
	CYP3A4_Exon4_R	CCACATGGAGACAGAGTGGA	20	55.00	59.66	
Exon5	CYP3A4_Exon5_6_F	CGCCCCACACAAATACATC	19	52.63	59.79	871
Exon6	CYP3A4_Exon5_6_R	TGTGCACAGGGGAGAAGAT	19	52.63	59.20	
Exon7	CYP3A4_Exon7_F	TGAGCCCCTTAGGAAGAGTT	20	50.00	58.00	900
	CYP3A4_Exon7_R	GCAGAAGAAAGAAAATGATACAGAC	25	36.00	57.77	
Exon8	CYP3A4_Exon8_F	TCTTGACTACCTACTATTTCTTGAACA	27	33.33	57.09	893
	CYP3A4_Exon8_R	TTGAAATGAGTCTTTACCAATTTATGA	27	25.93	59.41	
Exon9	CYP3A4_Exon9_F	CCCTTCAATAAATTGTCAGAGGA	23	39.13	59.49	927
	CYP3A4_Exon9_R	GTGGCTCCTGATTGGATGTT	20	50.00	59.93	
Exon10	CYP3A4_Exon10_F	ACATTTTTCTTGGGGGAGAG	20	45.00	58.09	928
	CYP3A4_Exon10_R	TAAGGGGACATCACACACCA	20	50.00	59.81	
Exon11	CYP3A4_Exon11_F	CAAAAGTCCTCCTTTTAGTGTGTG	24	41.67	59.28	912
	CYP3A4_Exon11_R	AAAAATATTCATTTGGGGGACA	22	31.82	59.44	
Exon12	CYP3A4_Exon12_F	TTCCCCTTCTCCTTCCTCAT	20	50.00	60.01	928
	CYP3A4_Exon12_R	CCAAGTTCTGGTTGGGAAGA	20	50.00	60.08	
Exon13	CYP3A4_Exon13_F	TTCAAAAACAGTTTGCCATCA	21	33.33	59.19	935
	CYP3A4_Exon13_R	GAATACTCCAGAGAAAACATGTGA	24	37.50	57.73	
3'UTR	CYP3A4_3'-UTR_F	TTGGCTCCTCTGCTTCTCAC	20	55.00	60.68	880
	CYP3A4_3'-UTR_R	TTGGGTGTTGAGGATGGAAT	20	45.00	60.17	