RESEARCH ARTICLE

Genetic analysis of drug-metabolizing phase I enzymes CYP3A4 in the Tibetan populations

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Running title: *CYP3A4* polymorphisms in Tibetan

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Abstract

Background: The enzymatic activity of *CYP3A4* results in broad inter-individual variability in response to certain pharmacotherapies. The present study aimed to screen Tibetan volunteers for *CYP3A4* genetic polymorphisms. Previous research has focused on Han Chinese patients, while little is known about the genetic variation of *CYP3A4* in the Tibetan populations.

Material and methods: In the present study, we adopt DNA sequencing to investigate the promoter, exons and surrounding introns, and 3′ -untranslated region of the *CYP3A4* gene in 96 unrelated healthy Tibetan individuals.

Results: We identified 20 different *CYP3A4* polymorphisms in the Tibetan population, including two novel variants (21824 A> G, 15580 G> C). In addition, we determined the allele frequencies of *CYP3A4**1A and *1H were 82.29% and28.13%, respectively. *CYP3A4**1P and *1G were relatively rare with frequencies of only 1.04% and 0.52%, respectively.

Conclusions: Our results provide information on *CYP3A4* polymorphisms in Tibetan individuals, which may help to optimize pharmacotherapy effectiveness by providing personalized medicine to this ethnic group.

Introduction

Environment and hereditary factor can affect the individual multiple drug metabolism. Lots of genes code the drug metabolizing enzymes, so those genes can influence the enzyme activity (DRÖGEMÖLLER *et al.* 2013; FUKUYOSHI *et al.* 2016). Pharmacokinetic polymorphisms divide the population into at least two phenotypes: poor metabolizers (PMs) and extensive metabolizers (EMs). The PM condition can lead to an excessive or prolonged therapeutic effect or drug-related toxicity after a normal dose. While extensive metabolizers populations may not achieve therapeutic levels of the drug administered at a standard dose resulting in the lack of a therapeutic effect.

The CYP3A locus consists of four genes, CYP3A4, CYP3A5, CYP3A7, and CYP3A43, all of which reside in a 231-kb region of chromosome 7q21.1(DRÖGEMÖLLER *et al.* 2013).

CYP3A4 is responsible for the metabolism of approximately 50-60% of clinical drugs used today, including acetaminophen (JIANG *et al.* 2015), codeine (EISSING *et al.* 2012), cyclosporine A (CAI *et al.* 2015), diazepam (REZAEE *et al.* 2014), and erythromycin (BOETSCH *et al.* 2016). The most prevalent polymorphism in *CYP3A4* is (CYP3A4*1B) (rs2740574) occurs in the 5' flanking region of the gene, it involves an A>G transition at -293 position from the transcription start site (LAKHMAN *et al.* 2009). The distribution of frequency of *CYP3A4**1B exists difference in some ethnic. The frequency of *CYP3A4**1B in Caucasians was 0.02, while in Africans was 0.82.(GARSA *et al.* 2005; JIN *et al.* 2005). *CYP3A4**5 and *CYP3A4**18 are the predominant mutations affecting the metabolism of certain drugs in Chinese people.

Tibetans is a minority in China, have big differences in the genetic structure, physiology, diet and lifestyle compared with the Han population. These variable factors may affect the drug metabolism. So, in this study, we want to explore allelic and genotypic pattern of CYP3A4 in healthy Tibetan nationalities of the Chinese population.

Materials and Methods

Subjects

In our study, we recruited 96 healthy Tibetans, including 48 males and 48 females, from the Xizang Minzu University from October and December 2009, based on strict inclusion and exclusion criteria. All participants are from the Tibet Autonomous Region, and had at least three generations of Tibetan descent. The exclusion criteria were: 1) with any type of medical illness, 2) organ transplant, 3) pregnant, 4) smoking, drug/alcohol addiction. The purpose of exclusion criteria was to minimize some factors that may have influenced genetic variation in

the genes of interest.

When collecting samples, we have been informed the purpose and experimental procedures of our study to every participant, and all participants have been signed informed consent. The study protocol was performed in accordance with the Declaration of Helsinki and was approved by The Ethics Committees of Xizang Minzu University.

DNA sequencing of CYP3A4 variants

We adopted GoldMag nanoparticles method (GoldMag Ltd. Xi'an, China) to extract genomic DNA from the 5 ml venous blood, according to the manufacturer's instructions. We designed PCR primers to amplify the promoter, exons and the 3'-untranslated region of CYP3A4, and primers sequences are shown in Table 1. PCR reaction system: 1 μl for genomic DNA (20 ng/μl), 5 μl for Hotstar Taq Master Mix, 0.5 μl for Forward primer, 0.5 μl for Reverse primer, and 3μl for deionized water to make up 10 ml reaction system. The process of PCR reaction: 15 min at 95 °C; 35 cycles of 30 sec at 95 °C, 30 sec at 60 °C and 1 min at 72 °C; 3 min at 72 °C; and 4 °C hold. The result of amplicon was analyzed on agarose gel electrophoresis, and used ABI PrismBigDye Terminator Cycle Sequencing Kit version 3.1 (Applied Biosystems) on an ABI Prism3100 sequencer (Applied Biosystems) to sequenced.

Data analysis

We analyzed the sequencing results of CYP3A4, through Squencher4.10.1 (http://www.genecodes.com/) software. All the position of CYP3A polymorphism loci are reference CYP3A4 gene sequences (Genbank Accession ID): AF208107 and protein sequences: P08684. We calculated the allelic and genotypic frequencies through a statistical method and adopted chi-squared test to compare the differences of allele frequency with other

ethnic populations(ADAMEC 1964). We also compared the frequency of the variants *CYP3A4* with 1000 Genome population frequencies. $P < 0.05$ was considered to represent statistical significance.

Finally, we used HAPLOVIEW 4.1 (http://broad.mit.edu/mpg/haploview) software platform to analysis linkage disequilibrium (LD) and Hardy-Weinberg equilibrium for each genetic variant (BARRETT *et al.* 2005). Meanwhile, we selected tSNPs of CYP3A4 to construct haplotype.

Transcriptional prediction

Sorting intolerant from tolerant (SIFT) (http://sift.bii.a-star.edu.sg/) and Polymorphism phenotyping-2 (PolyPhen-2) (http://genetics.bwh.harvard.edu/pph2/) may be useful in prioritizing changes that are likely to cause a loss of protein function. In our research, these tools were used to predict the function, which non-synonymous SNPs (nsSNPs), in CYP3A4 coding regions. The predicted results of SIFT can be divided into four categories: tolerant (0.201-1.00), borderline (0.101-0.20), potentially intolerant (0.051-0.10) and intolerant (0.00-0.05). Score value of the smaller show that the mutation may have great influence on the function of the protein. The results of PolyPhen-2 can be 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 ($\geqslant$2.000). The score is the lower, the effect on protein is greater.

Results

Genetic variants

Preliminary analysis using Squencher4.10.1 on CYP3A4 sequencing in Tibetan, we

detected twenty polymorphisms on the *CYP3A4* gene, including two new mutations in the coding region (Table 2). The first new mutations occurred in downstream position of 21824 (21824 A> G), which is a non-synonymous mutations. Another mutation is a synonymous mutation, in in exon seven (15580 G> C).

Allele and genotype frequency

We detected four *CYP3A4* alleles in the Tibetan study population, respectively is *CYP3A4**1A, *CYP3A4* *1H, *CYP3A4**1P, and *CYP3A4* *1G (Table 3). The frequencies of *CYP3A4**1A, *CYP3A4* *1H, *CYP3A4**1P, and *CYP3A4* *1G were 0.8229, 0.2813, 0.0104, 0.0052, respectively. The frequency of *CYP3A4**1A allele is the higher than other allele, and *CYP3A4**1P and *1G were relatively rare.

We further compared the frequency of the variants *CYP3A4* with 1000 Genome population frequencies (Table 4). Our results showed that the *CYP3A4**2 and *CYP3A4**18 were absent in our subjects. Compared with 1000 Genome population frequencies, *CYP3A4*2* occurs at a frequency of 1.01% in Finniah individuals from Finland. The frequencies of *CYP3A4**18 in CHS, JPT, and KHV populations were 1.43%, 1.44%, 2.53%, respectively.

We also identified four *CYP3A4* genotypes, *1A/*1A,*1A/*1G,*1A/*1H and *1A/*1P, with frequencies ranging from 1.04% to 64.58% (Table 3). The frequencies of *1A/*1A,*1A/*1G,*1A/*1H and *1A/*1P were 0.6458, 0.0104, 0.3229 and 0.0208. The heterozygous genotype *1A/*1P and *1A/*1H are relatively rare.

LD analysis

We used HAPLOVIEW 4.1 software platform to analysis linkage disequilibrium. The overall LD across the *CYP3A4* gene is depicted in Figure 1, found one LD blocks. In this

block, we found that rs25721 and rs16613, rs20230 and rs16613 were very tightly correlated, and rs25721 and rs20230 linkage degree up to 92.

Protein function prediction

Through the protein function of *CYP3A4* non-synonymous mutation (21824 A> G), SIFT prediction results show that the score is 0.89 and the category is tolerant; PolyPhen-2 analysis predicted that the score is 0.001, and the mutation is benign. (Figure 2)

Discussions

Our research adopts the direct sequencing to screen CYP3A4 gene polymorphism in the Tibetan population, and statistics the frequency of allele and genotype. In this study, we detected twenty polymorphisms on the *CYP3A4* gene, including two new mutations in the coding region (21824 A> G and 15580 G> C)

We further compared *CYP3A4* allele frequencies between our data and previously published data from different countries and ethnic groups. Our results showed that the frequency of the wild-type allele, *CYP3A4**1, in our study group was significantly lower than in Han, Uighur, Hui and Mongolian populations. The frequencies of *CYP3A4**1 in Han, Uighur, Hui and Mongolian populations were 0.8162, 0.8598, 0.8079, 0.7975, respectively (ZUO *et al*. 2012). The differences could be attributed to the ethnic origin and geographical distribution of these populations, their cultural and dietary habits, or other environmental factors. It is of potential clinical importance to identify individuals from different areas of China who have altered pharmacokinetics for *CYP3A4* substrates so that appropriate dosage strategies for these drugs can be adopted and adverse drug reactions can be avoided.

Recently, several *CYP3A4* variant alleles have been identified. *CYP3A4**1B (rs2740574

A> G) is known to be the polymorphism that increases expression by changing the transcription factor binding affinity(AMIRIMANI *et al.* 2003; GEORGITSI *et al.* 2011). we did not detect the *CYP3A4**1B gene in our study, while 0.042 in European-Americans, 0.271 in African-Americans(LEE *et al.* 2013). CYP3A4*1B carriers showed higher drug clearance for anti-cancer agents, such as docetaxel and cyclophosphamide, than wild type subjects (TRAN *et al.* 2006). Midazolam (MDZ), which can be administrated both intravenously and orally, is selectively metabolized by CYP3A4 and CYP3A5 to its primary metabolite, 1′-hydroxymidazolam, and is not a substrate of P-glycoprotein. In healthy volunteers for midazolam content detection results show that CYP3A4*1B homozygous mutant of midazolam clearance rate decreased 30% compared to homozygous wild (MATSUMURA *et al.* 2004).

Additional alleles include *CYP3A4**2(S222P), that occurs at a frequency of 2.75% in Finnish Caucasians, while absent in Black and Chinese subjects, and *CYP3A4**3 (M445T) that occurs with an allele frequency of 2.2% in Dutch Caucasians , and *CYP3A4**4 (I118V), *CYP3A4**5 (P218R) and *CYP3A4**6 (17776A ins) demonstrating frequencies of 1.47%, 0.98% and 0.5%, respectively, in a Chinese population(LAMBA *et al.* 2002). CYP3A4*2 was found in a white population and had altered catalytic activity toward nifedipine and testosterone compared with the wild-type CYP3A4*1 P450. Sata F et al. revealed that the CYP3A4*2 P450 had a lower intrinsic clearance for the CYP3A4 substrate nifedipine compared with the wild-type enzyme, and the clearance rate was reduced by 36% (SATA *et al.* 2000).

CYP3A4*18 variant allele in the exon 10 of CYP3A4 involves T to C transition at

position 169068 (in GenBankNG-000004) that changes leucine -293 to proline, which was identified by direct sequencing in 24 Asians. Dai et al. indicated that CYP3A4*18 showed significantly a higher turnover number for both testosterone and insecticide chlorpyrifos in vitro(DAI *et al.* 2001). YF Hu et al. determined the allelic frequency of the CYP3A5*3 and CYP3A4*18 in a group of 302 Chinese subjects by using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assays, found that the frequency of the CYP3A4*18 variant allele in Chinese population was 0.01(HU *et al.* 2005). Jinliang Zuo et al. compared the frequencies of *CYP3A4* allelic variants in Han (Liaoning), Uighur, Hui and Mongolian people in the Chinese population. The frequencies of *CYP3A4**18 in Han, Uighur, Hui and Mongolian populations were 0.1838, 0.1402, 0.1921, 0.2025, respectively (ZUO *et al.* 2012). The frequencies of CYP3A4*18 in CHS, JPT, and KHV populations were 1.43%, 1.44%, 2.53%, respectively.

Through analysis of the protein function of *CYP3A4* non-synonymous mutation, which results in an amino acid change from Thr to Ala (21824 A> G), we found that this mutation may not influence the protein function. The novel genetic variants identified here should be confirmed by other means in further studies. Another novel polymorphisms is synonymous mutation in exon seven (15580 G> C, Gly178=Gly). Synonymous mutations are mutations that do not change the encoded amino acids. It is generally assumed that synonymous mutations are evolutionary neutral and they have no effects on the phenotype. Therefore, may not affect the function of the encoded protein and not affect the drug metabolism. SIFT uses sequence homology to predict whether an amino acid substitution will affect protein function and hence, potentially alter phenotype. The result predict of SIFT exist 20% false positive

error and 31% false negative error(NG and HENIKOFF 2003). Polymorphism Phenotyping (PolyPhen): a popular structure-/ sequence-based amino acid substitution prediction method. The predict result of PolyPhen exist 9% false positive error and 31% false negative error(NG and HENIKOFF 2006).

Conclusion

In this study, we detected twenty polymorphisms on the *CYP3A4* gene, including two new mutations in the coding region (21824 A> G and 15580 G> C). Our results provide a basic profile of *CYP3A4* in the Tibetan population, and can be used to determine optimal dosage recommendations, leading to individualized medicine.

Competing interests

The authors declare no competing interests in the study.

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Table 1 Primers used to amplify regions of *CYP3A4*

Primer name	Primer sequence (5′–3′)	DNA size for PCR (bp)
Promoter_F	GTGCAGAGACAGCAGCTGAG	882
Promoter_R	TCTCCTCTGAGTCTTCCTTTCA	
Exon1_F	CTTCCAACTGCAGGCAGAG	900
Exon1_R	GTTTGGAATGAGATCCGTCA	
Exon2_F	ATTCCTGCCTGAACCTCTCA	894
Exon2_R	GGTAAATACCTGGGCTCCCTA	
Exon3_F	AAGGATGACAAAGAGATAAAACACTG	898
Exon3_R	AAGACTCCGCAAAACTACAAGC	
Exon4_F	GGAGAATGGCATGGGAAATA	855
Exon4_R	CCACATGGAGACAGAGTGGA	
Exon5_6_F	CGCCCCACACAAATACATC	871
Exon5_6_R	TGTGCACAGGGGAGAAGAT	
Exon7_F	TGAGCCCCTTAGGAAGAGTT	900
Exon7_R	GCAGAAGAAAGAAAATGATACAGAC	
Exon8_F	TCTTGACTACCTACTATTTCTTGAACA	893
Exon8_R	TTGAAATGAGTCTTTACCAATTTATGA	
Exon9_F	CCCTTCAATAAATTGTCAGAGGA	927
Exon9_R	GTGGCTCCTGATTGGATGTT	
Exon10_F	ACATTTTTCTTGGGGGAGAG	928
Exon10_R	TAAGGGGACATCACACACCA	
Exon11_F	CAAAAGTCCTCCTTTTAGTGTGTG	912
Exon11_R	AAAAATATTCATTTGGGGGACA	
Exon12_F	TTCCCCTTCTCCTTCCTCAT	928
Exon12_R	CCAAGTTCTGGTTGGGAAGA	
Exon13_F	TTCAAAAACAGTTTGCCATCA	935
Exon13_R	GAATACTCCAGAGAAAACATGTGA	
3'-UTR_F	TTGGCTCCTCTGCTTCTCAC	880
3'-UTR_R	TTGGGTGTTGAGGATGGAAT	

Table 2 Frequency distribution of *CYP3A4* polymorphisms in 96 Tibetan subjects

Nucleotide change	POSITION	Region	SNP	Allele	Amino-acid effect	Frequencies
A>W(T)	-789	Promoter			Not translated[a]	1.04%
T>Y(C	102	Intron 1	rs373014415		Not translated	2.08%
T>K(G)	186	Intron 1			Not translated	1.04%
C>Y(T)	3858	Intron 1	rs55913187		Not translated	8.33%
A>M(C	4303	Intron 2			Not translated	1.04%
C>Y(T)	5843	Intron 2			Not translated	1.04%
G>S(C	15580	Exon 7		Novel 1	Gly178=	1.04%
G>A	15727	Intron 7	rs55808838	CYP3A4*1P	Not translated	2.08%
T>Y(C	15977	Intron 7	rs2246709		Not translated	43.75%
C>Y(T)	16613	Intron 7	rs4646437		Not translated	17.71%
T>K(G)	17128	Intron 8	rs28371756		Not translated	1.04%
G>R(A)	20230	Intron 10	rs2242480	CYP3A4*1G、1H	Not translated	33.33%
T>K(G)	21734	Intron 10			Not translated	1.04%
A>R(G)	21824	Exon 11		Novel 2	Thr349Ala	1.04%
T>K(G)	22121	Intron 11			Not translated	2.08%
T>Y(C	22140	Intron 11			Not translated	1.04%
A>R(G)	22213	Intron 11	rs34382314		Not translated	1.04%
G>R(A)	23383	Intron 12			Not translated	1.04%
A>R(G)	25721	Intron 12	rs28988600		Not translated	37.50%
C>M(A)	26206	3‘UTR	rs59715127	CYP3A4*1G	Not translated	1.04%

SNP, single nucleotide polymorphism

UTR, untranslated region

[a]Not translated: These synonymous SNP mutations have no effect on protein sequence.

Table 3 *CYP3A4* allele and genotype frequencies in Tibetan individuals

Gene	Allele	Number (%)	Phenotype
CYP3A4	*1A	158(82.29)	Normal
	*1G	1(0.52)	/
	*1H	31(16.15)	/
	*1P	2(1.04)	/
	Genotype	Number (%)	Phenotype
	*1A/*1A	62(64.58)	Normal
	*1A/*1G	1(1.04)	/
	*1A/*1H	31(32.29)	/
	*1A/*1P	2(2.08)	/
Total	96		

Table 4 *CYP3A4* allele frequencies in 1000 Genome population

	ACB	ASW	BEB	CDX	CEU	CHB	CHS	CLM	ESN	FIN	GBR	GIH	GWD
1	0.9844	0.9754	0.9942	0.9946	0.9342	0.9951	0.9809	0.9521	0.9898	0.9241	0.8791	0.9902	0.9779
*2	/	/	/	/	/	/	/	/	/	0.0101	/	/	/
*3	/	0.0082	/	/	0.0152	/	/	0.0213	/	0.0101	0.011	/	/
*4	/	/	/	/	/	/	0.0048	/	/	/	/	/	/
*5	/	/	/	0.0054	/	0.0049	/	/	/	/	/	/	/
*6	/	/	/	/	/	/	/	/	/	/	/	/	/
*7	/	/	/	/	/	/	/	/	/	0.0051	/	/	/
*8	/	/	/	/	/	/	/	/	/	0.0051	/	/	/
*9	/	/	/	/	/	/	/	/	/	/	/	/	/
*10	/	/	0.0058	/	0.0051	/	/	/	/	/	0.0165	/	/
*11	/	/	/	/	/	/	/	/	/	/	0.0055	/	/
*12	/	/	/	/	/	/	/	/	0.0051	/	/	/	/
*15	0.0104	0.0164	/	/	/	/	/	/	0.0051	/	/	/	0.0221
*16	/	/	/	/	/	/	/	/	/	/	/	/	/
*18	/	/	/	/	/	/	0.0143	/	/	/	/	/	/
*19	/	/	/	/	/	/	/	/	/	/	/	0.0049	/
*22	0.0052	/	/	/	0.0455	/	/	0.0266	/	0.0455	0.0879	0.0049	/
	IBS	ITU	JPT	KHV	LWK	MSL	MXL	PEL	PJL	PUR	STU	TSI	YRI
1	0.9532	0.9853	0.9664	0.9545	0.9747	0.9824	0.9844	0.9823	0.9896	0.9327	0.9853	0.9579	0.9537
*2	/	/	/	/	/	/	/	/	/	/	/	/	/
*3	/	/	/	/	/	/	/	/	/	0.0048	/	/	/
*4	/	/	/	0.0202	/	/	/	/	/	/	/	/	/
*5	/	/	/	/	/	/	/	/	/	/	/	/	/
*6	/	/	/	/	/	/	/	/	/	/	/	/	/
*7	/	/	/	/	/	/	/	/	/	/	/	0.0047	/
*8	/	/	/	/	/	/	/	/	/	/	/	/	/
*9	/	/	/	/	/	/	/	0.0059	/	/	/	/	/
*10	0.0047	0.0098	/	/	/	/	/	/	/	0.0048	/	/	/
*11	/	/	/	/	/	/	/	/	/	/	/	/	0.0046
*12	/	/	/	/	0.0202	/	/	/	/	0.0048	/	/	/
*15	0.0047	/	/	/	0.0051	0.0176	0.0078	/	/	0.0048	/	/	0.0417
*16	/	/	0.0192	/	/	/	/	/	/	/	/	/	/
*18	/	/	0.0144	0.0253	/	/	/	/	/	/	/	/	/
*19	/	/	/	/	/	/	/	/	/	/	0.0049	/	/
*22	0.0374	0.0049	/	/	/	/	0.0078	0.0118	0.0104	0.0481	0.0098	0.0374	/

ACB: African Carbbean Barbados; ASW: HapMap African ancestry individuals from SW US;

BEB: Bengali in Bangladesh; CDX: Chinese Dai in Xishuangbanna, China; CEU: CEPH individuals; CHB: Han Chinese in Beijing; CHS: Han Chinese South;

CLM: Colombian in Medellin, Colombia; FIN: HapMap Finniah individuals from Finland; GBR: British individuals from England and Scotland (GBR); GIH: HapMap Gujarati India individuals from Texas; GWD: Gambian in Western Division - Mandinka; IBS: Iberian populations in Spain; JPT: Japanese individuals; KHV : Kinh in Ho Chi minh City, Vietnam; LWK: Luhya individuals; MSL: Mende in Sierra Leone; MXL: HapMap Mexican individuals from LA California; PEL: Peruvian in Lima, Peru; PUR : Puerto Rican in Puerto Rico; STU: Sri Lankan Tamil in the UK; TSI: Toscan individuals; YRI: Yoruba individuals.

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.

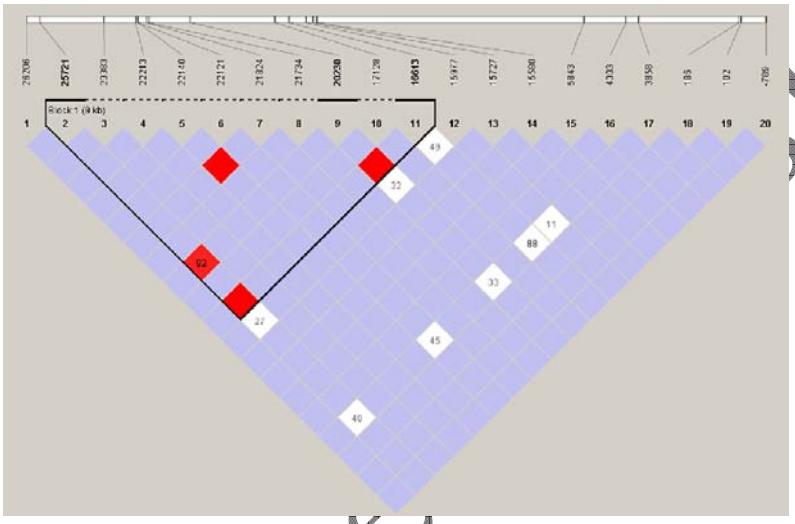

Figure 2 PolyPhen-2 prediction of functional change resulting from an amino acid mutation at position Figure 2 PolyPhen-2 prediction of functional change resulting from an amino acid mutation at position 349.