

Frequencies of two functionally significant SNPs and their haplotypes of organic anion transporting polypeptide 1B1 *SLCO1B1* gene in six ethnic groups of Pakistani population

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ABSTRACT

Objective(s): Organic anion transporter polypeptide 1B1 (OATP1B1) encoded by solute carrier organic transporter 1B1 (*SLCO1B1*) gene; a transporter involved in the uptake of drugs and endogenous compounds is present in hepatocyte sinusoidal membrane. Aim of this study was to investigate the frequencies of functionally significant SNPs (388A>G and 521T>C) and their haplotypes in 6 ethnic groups of Pakistani population through the development of rapid and efficient Tetra amplification refractory mutation system (T. ARMS) genotyping assay.

Materials and Methods: Frequencies of alleles, genotype, and haplotypes of two functionally significant Single nucleotide polymorphism in 180 healthy Pakistani subjects and distributions in six ethnic groups by using a single step T. ARMS genotyping assay.

Results: The allelic frequency for 388A>G SNP was 50% in total Pakistani population with distributions of 9.7%, 15.1%, 19.4%, 16.1%, 18.3%, and 21.5% in Punjabi, Sindhi, Balouchi, Pathan, Kashmiri and Hazara/Baltistan groups respectively; and for 521T>C SNP it was 23.9% in total Pakistani population with distributions of 11.1%, 8.9%, 15.6%, 11.1%, 31.1% and 22.2% in Punjabi, Sindhi, Balouchi, Pathan, Kashmiri, and Hazara/Baltistan groups. Both functionally significant SNPs occurred in four major haplotypes with a frequency of 35.5% for 388A/521T (*1A), 40.5% for 388G/521T (*1B), 14.4% for 388A/521C (*5), and 9.4% for 388G/521C (*15) with varying distributions among six ethnic groups.

Conclusion: The 388A>G and 521T>C genotypes and corresponding haplotypes are present at varying frequencies in various ethnic groups of Pakistani population. Pharmacokinetic and pharmacodynamic profiling is needed to assess and characterize the effects of these haplotypes in our population.

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Introduction

The movement of drugs within a biological system is facilitated by its transport through the transmembrane passages which are not only dependent upon the physicochemical properties of the drug but also on a variety of efflux and uptake membrane transporters (1). Organic anion transporting polypeptide 1B1 (OATP1B1) is an important member of the solute carrier (SLC) super family (2). Their significant importance is as an uptake transporter expressed on the sinusoidal membrane of hepatocytes and their pivotal role in the hepatic uptake of endogenous compounds and several important drugs such as statins, rifampicin, methotrexate, benzyl penicillin, and fexofenadine etc. (3, 4). These act as sodium independent uptake transporters and function through electro-neutral

exchange and coupling of neutralizing anions with organic compounds which result in their cellular uptake (3). Several sequence variations have been discovered in the Solute carrier organic transporter 1B1 (*SLCO1B1*) gene (5) out of which two Single nucleotide polymorphism (SNPs), namely; 388A>G and 521 T>C have been discussed in many studies as they had proved to be the most prevalent and functionally important for the proper transport function of *OATP1B1* transporter (3, 6–13). The 521T>C and 388A>G SNPs cause an amino acid change at positions 174 (from valine to alanine) and position 130 (from asparagine to aspartic acid) respectively. Both SNPs 521T>C and 388A>G have been reported to exist in perfect linkage disequilibrium (LD) (14). Various studies have reported altered plasma concentration and inter-

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Table 1. T. ARMS PCR primer templates

Region		Primer sequence <i>SLCO1B1</i> 388A>G	Primer sequence <i>SLCO1B1</i> 521T>C
Forward	Outer	5'-AATTGACAGAAAGTACTCTGGTAATTTG-3'	5'-CACCATATTGTCAAAGTTTGCAAAGTGA-3'
	Inner	5'-TTACAGGTATTCTAAAGAACTAATAGCG-3'	5'-ATCTGGGTCATACATGTGGATATAGGT-3'
Reverse	Outer	5'-ATTTTGCTAATGAATATCACAACAATTT-3'	5'-TTCAAAAGTAGACAAAGGAAAGTGATCA-3'
	Inner	5'-GTCGATGTTGAATTTCTGATGACTT-3'	5'-ATTCCACGAAGCATATTACCCATGACCG-3'

Table 2. Cocktail for PCR amplification

Constituents	<i>SLCO1B1</i> 388A>G	<i>SLCO1B1</i> 521T>C
Template	50 ng	50 ng
10X PCR buffer	1X	1X
MgCl ₂ (25 mM)	3 mM	5 mM
dNTP's	0.2 mM	0.2 mM
Outer F primer (10 pmol/μl)	1 pmol	1 pmol
Specific F Primer (10 pmol/μl)	1 pmol	1 pmol
Outer R primer (10 pmol/μl)	1 pmol	1 pmol
Specific R primer (10 pmol/μl)	1 pmol	1 pmol
<i>Taq</i> DNA Polymerase (2 U/μl)	10 U	10 U
dH ₂ O (nuclease free)	Upto 50 μl	Upto 50 μl
Total reaction mixture (μl)	50	50

individual variability in therapeutic responses of statins such as simvastatin (10), pitavastatin (9, 11), atorvastatin (13, 15), and cerivastatin (16) and linked them to the two functionally important SNPs (388A>G and 521T>C) and their respective haplotypes.

Statins therapeutic actions and most importantly, adverse effects which include myopathy and rhabdomyolysis have shown great inter-individual and ethnic variations (17, 18). These inter-individual and ethnic variations in the therapeutic response and severity of adverse effects are largely attributed to the genetic variations therefore predisposing to altered drug disposition (16, 19, 20). Identification and characterization of underlying genetic variations will help us towards better dosing regimens and safer drug designs for personalized therapy.

The importance of *SLCO1B1* SNPs on statins pharmacokinetics and pharmacodynamics points towards the necessity for a rapid and efficient genotyping assay. Moreover, there is currently no data available for the presence and subsequent characterization of SNPs and their haplotypes in the ethnic groups of Pakistani population which is crucial for evaluating the significance of *SLCO1B1* SNPs as a determining factor for inter-individual and ethnic variability for statins' pharmacokinetics and pharmacodynamics in our population. This study is therefore an attempt to develop a rapid, efficient, and single step Tetra amplification refractory mutation system (T. ARMS) genotyping assay thus aiding to investigate the frequencies of two functionally important *SLCO1B1* SNPs (388A>G and 521T>C) and their respective haplotypes in six ethnic groups of the Pakistani population.

Materials and Methods

Subjects

This descriptive study was designed with a total population size (N) of 180 normal healthy subjects subdivided into a smaller sample size (n) of 30 into 6

ethnic groups (Punjabi, Sindhi, Balouchi, Pathan, Kashmiri, and Hazara/Balistan). The study sought its approval from Ethics Committee of Centre for Research in Experimental & Applied Medicine (CREAM), Army Medical College, National University of Sciences and Technology (NUST), Pakistan. All subjects were enrolled after asserting their health condition from previous medical history and physical examination. Participation in the study was at their free will and only after explaining the study purpose, procedure, and protocol. Undertaking on consent proforma was followed by a 10 ml blood sample collection from each participant in ethylenediaminetetraacetic acid (EDTA) tube and storage at -20°C.

DNA extraction

Genomic DNA was extracted using standard/conventional kit method (QIAamp DNA kit, Qiagen, Hilden, Germany) according to the protocol provided (21) and analyzed on 1% agarose gel followed by its storage at 4 °C.

T. ARMS genotyping assay

Two SNP variations of *OATP1B1* –encoding gene *SLCO1B1* 388A>G rs2306283 and 521T>C rs4149056 were investigated. The reference sequences were obtained from National Center for Biotechnology Information (NCBI; Bethesda, MD, USA) database (<http://www.ncbi.nlm.nih.gov/>). Genotyping of the DNA samples was performed by using one step T. ARMS polymerase chain reaction (PCR) assay with some modifications on Applied Biosystems, Inc., 96 well thermal cycler (ABI Veriti, Foster City, CA 94404, USA). Primer templates were obtained by using the online software tool specifically designed for T. ARMS primer and accessible on the Cedar Genetics website http://cedar.genetics.soton.ac.uk/public_html/primer1.html (22) (Table 1). The cocktail for PCR amplification is presented in Table 2.

PCR cycling conditions

PCR thermo cycler was preheated to 95°C (hot start) and incubated for 5 min at this temperature (long denaturation). This was followed by 40 cycles of 1 min at 95°C (template denaturation), 1 min at 60°C (primer annealing), and 1 min at 72°C (primer extension). PCR protocol was finalized by the last long extension at 72°C for 10 min. The protocol for both 388A>G and 521T>C SNPs were the same except for the annealing temperature of 55°C for 521T>C during the 40 cycles.

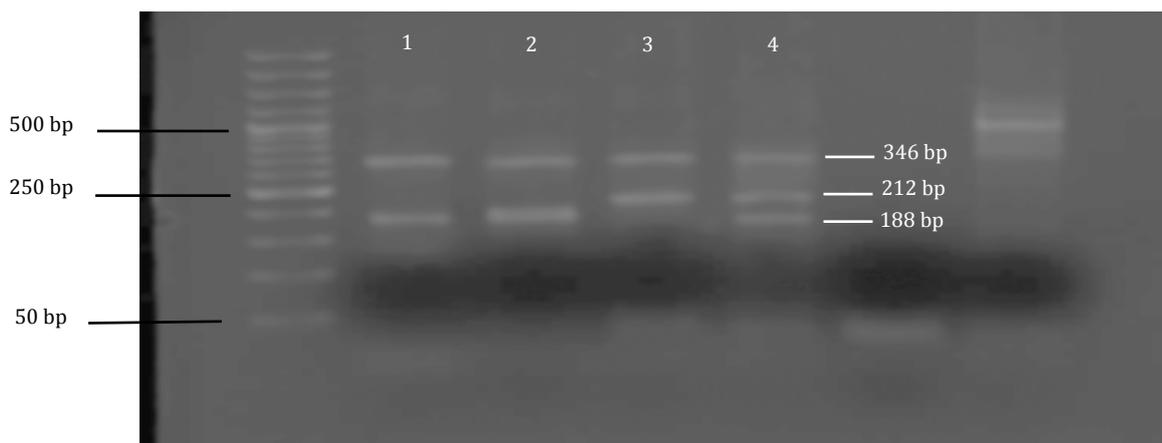


Figure 1. Results of Agarose gel electrophoresis for *OATP1B1* N130D (388A>G). Lanes 1 and 2 indicate AA genotype comprising 346 bp Tetra ARMS control and 188 bp AA fragment. Lane 3 indicates GG genotype comprising 346 bp Tetra ARMS control and 212 bp GG fragment. Lane 4 indicates AG genotype comprising 346 bp Tetra ARMS control, 188bp A fragment and 212 bp G fragment

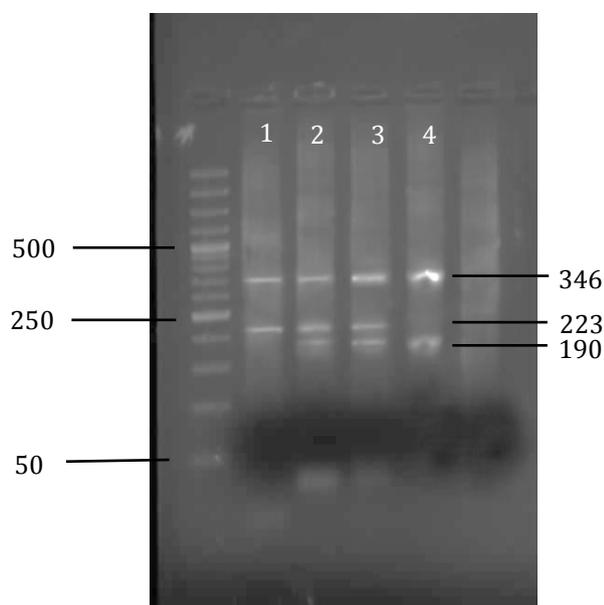


Figure 2. Results of agarose gel electrophoresis for *OATP1B1* V174A (521T>C)

Lane 1 indicates TT genotype comprising 359bp Tetra ARMS control and 223 bp TT fragment. Lanes 2 and 3 indicate TC genotype comprising 359 bp Tetra ARMS control, 223 bp T fragment, and 190 bp C fragment. Lane 4 indicates CC genotype comprising 359 bp Tetra ARMS control and 190 bp CC fragment

The amplified PCR product was analyzed by using agarose gel (2% w/v) electrophoresis. 5 μ l ethidium bromide (10 mg/ml) and 0.25% bromophenol blue solutions were used and samples were loaded into the wells. Electrophoresis was performed at 100 volts for 30 min followed by amplified product's detection on ENDURO™ GDS (Labnet International, Inc., NJ 08837, USA) Gel Documentation System.

Statistical analysis

Computational and Molecular Population Genetics Lab (CMPG) software, Arlequin version 3.5.1.2 <http://cmpg.unibe.ch/software/arlequin3> (Swiss Institute of Bioinformatics, University of Geneva, Switzerland), was used for population

genetic data analysis (23). The allelic and genotypic frequencies of both SNPs were checked for their consistency with the Hardy-Weinberg equilibrium. The distribution of these genotypic frequencies in relation to ethnic groups within the Hardy-Weinberg equilibrium was assessed using Pearson's chi-squared test and graphically analyzed by using triangular ternary plot through "de Finetti" diagram for both the SNPs. Linkage Disequilibrium (LD) for the pair of SNPs was quantified by r^2 and respective D' values (24) using Arlequin.

Genetic diversity between ethnic groups was calculated by Analysis of Molecular Variance (AMOVA) followed by pairwise differentiation with F_{ST} . Mann-Whitney U test was applied for the non-parametric analysis and comparison of haplotypic frequencies (% age) within different ethnic groups using SPSS software version 20.0 (SPSS Inc., Chicago, IL, USA); graphical representation is by Microsoft Excel 2013 with add-on statistical package (Redmond, Washington, USA). All genotype and haplotype frequencies are presented with 95% confidence intervals.

Results

Genotyping

The events produced as a result of T-ARMS PCR for both the 388A>G and 521T>C lead to the complete genotype profiling of the enrolled subjects in a one-step PCR (Figure 1 and Figure 2 respectively).

Alleles and genotypes

The observed allelic frequencies of 388A>G and 521T>C in the total Pakistani population (N=180) were 50% and 23.9% respectively. All six genotypes of both SNPs exhibited a frequency of greater than 10%. The three genotypes of 388A>G SNP, AA, AG, and GG occurred at a frequency of 50%, 28.33%, and 21.66% respectively in the total Pakistani population. Moreover, the genotypes of 521T>C SNP, TT, TC, and CC occurred at a frequency of 76.11%,

Table 3. 388A>G and 521T>C allele and genotype frequencies in six ethnic groups of Pakistani population

Genetic	Ethnicity	Punjabi (n=30)	Sindhi (n=30)	Balouchi (n=30)	Pathan (n=30)	Kashmir (n=30)	Haz/Balt (n=30)
Total population size (N) 180							
388	G Allele	9.7 %	15.1 %	19.4 %	16.1 %	18.3 %	21.5 %
388 A>G Genotype	AA (Control)	24.1 %	18.4 %	13.8 %	17.2 %	14.9 %	11.5 %
	AG (Heterozygous)	11.1 %	16.7 %	20.4 %	16.7 %	16.7 %	18.5 %
	GG (Homozygous)	7.7 %	12.8 %	17.9 %	15.4 %	20.5 %	25.6 %
521	C Allele	11.1 %	8.9 %	15.6 %	11.1 %	31.1 %	22.2 %
521 T>C Genotype	TT (Control)	18.5 %	19.3 %	17 %	18.5 %	11.9 %	14.8 %
	TC (Heterozygous)	15 %	5 %	20 %	15 %	30 %	15 %
	CC (Homozygous)	8 %	12 %	12 %	8 %	32 %	28 %

Table 4. Pairwise group comparison of genotype frequencies among the ethnic groups of Pakistani population

AMOVA	Punjabi	Sindhi	Balouchi	Pathan	Kashmir	Hazara/ Baltistan	Pairwise group average differences (Pxy) P-values
Punjabi		0.854	0.509	0.709	0.181	0.109	
Sindhi	0.846		0.981	1.000	0.427	0.381	
Balouchi	0.504	0.972		1.000	0.545	0.609	
Pathan	0.702	0.990	0.990		0.445	0.436	
Kashmir	0.180	0.468	0.558	0.441		0.900	
Hazara/Baltistan	0.108	0.378	0.630	0.432	0.891		
Pairwise group comparisons (F_{ST}) p values							

Table 5. Haplotype frequencies in six ethnic groups of Pakistani population

Genetic	Ethnicity	Punjabi (n=30)	Sindhi (n=30)	Balouchi (n=30)	Pathan (n=30)	Kashmir (n=30)	Haz/Balt (n=30)
Total population size (N) 180							
Haplotypes	AT [*1A]	29.2 %	20 %	13.8 %	20 %	9.2 %	7.7 %
	GT [*1B]	8.2 %	17.8 %	20.5 %	16.4 %	16.4 %	20.5 %
	AC [*5]	8 %	12 %	12 %	12 %	28 %	28 %
	GC [*15]	17.6 %	5.9 %	17.6 %	11.8 %	29.4 %	17.6 %

Table 6. Pairwise group comparison of haplotypes among ethnic groups of Pakistani population

Mann Whitney U test	Punjabi	Sindhi	Balouchi	Pathan	Kashmir	Hazara/ Baltistan	Haplotype group variations (z values)
Punjabi		1.080	2.064	1.183	2.067	3.058	
Sindhi	0.280		1.156	0.161	2.554	2.477	
Balouchi	0.039*	0.248		0.958	1.507	1.342	
Pathan	0.237	0.872	0.338		2.301	2.210	
Kashmir	0.002*	0.011*	0.132	0.021*		0.345	
Hazara/ Baltistan	0.002*	0.013*	0.180	0.027*	0.730		
Haplotype group comparisons (P- values)							

* P<0.05 (Confidence interval 95%)

10%, and 13.88% respectively in the total Pakistani population. The detected frequencies did not exhibit a significant deviation from the Hardy-Weinberg equilibrium. The allelic and genotypic frequencies were also analyzed among different ethnic groups of the Pakistani population and are summarized in Table 3. Alleles of both SNPs were present in linkage disequilibrium across the six ethnic groups with r^2 values ranging from 0.019 to 0.934. Genetic diversity using analysis of molecular variance (AMOVA) followed by pairwise group comparisons using F_{ST} and average differences Pxy are presented in Table 4. All p values returned by F_{ST} pairwise testing were greater than 0.05 and therefore an insignificant difference among ethnic groups in the population was inferred.

Haplotypes

Four haplotypes from the two SNPs were analyzed. The reference control haplotype having both normal alleles AT (*1A) exhibited a frequency of 35.5% while the highest frequency of 40.5% was presented by the GT (*1B) haplotype. However, the AC (*5) and GC (*15) haplotypes occurred at lower frequencies of 14.4% and 9.4% respectively. The haplotype frequencies were also analyzed among different ethnic groups of the Pakistani population and are summarized in Table 5. Pairwise variations in haplotypes within the ethnic groups were assessed using the Mann-Whitney U test and returned P-values for significant differences in frequencies as summarized in Table 6.

Discussion

Two functionally important and widely studied polymorphic variants of *SLCO1B1*, 388A>G and 521T>C, were investigated in this study. The 521T>C variant has been reported to alter the function of transporter polypeptide (OATP) leading to impaired entry of drug into the hepatocytes followed by increased drug plasma levels and therefore rendering its therapeutic effects and enhancing its toxicity. It was demonstrated in a recent study (19) that the 388A>G variant was responsible for expression of OATP in the hepatocytes. These vitally important variations in the OAT polypeptide led us to selection of these two target SNPs for further investigation in our population. The allelic frequencies of both the SNPs and their genotypes/haplotypes were therefore observed in 6 ethnic groups, namely; Punjabi, Sindhi, Pathan, Balouchi, Kashmiri, and Hazara/Baltistan to find out the extent of genetic variations within these groups and the whole population.

The 388G allele when investigated within the groups showed its highest frequency of 60 % in the Balouchi and Hazara/Baltistan groups with a lowest of 30 % in the Punjabi group. The bi-allelic genotype GG demonstrated a high frequency of 33 % and lowest of 10 % in the Balouchi and Punjabi groups respectively. The frequency of the 521C allele in comparison with the 388G allele was less with highest of 40 % in the Kashmiri group and lowest of 13 % in the Sindhi group with its bi-allelic genotype CC at 27 % and 7 % in Punjabi and in Kashmiri groups respectively. This clearly indicates that the 388G allele responsible for altered expression of OATP is much widely distributed in our population with its elevated focus towards the Kashmir and Hazara/Baltistan regions. The 521C allele responsible for altered function of OATP is less encountered in our population, but, is also concentrated towards the Kashmir and Hazara/Baltistan regions. The Punjabi and Sindhi groups have shown to be least affected by these SNPs therefore exhibited lower frequencies.

Same results extrapolated themselves when computed on full scale population. The highest frequency of 388G allele and its bi-allelic genotype GG were 21.5 % and 25.5 % respectively in the Hazara/Baltistan groups with a lowest frequency trend towards the Punjabi group. A similar behavior was demonstrated by the Kashmiri group for the 521C allele and its bi-allelic genotype CC with highest frequencies of 31.1 % and 32 % respectively. The lowest frequency was exhibited by Sindhi, Punjabi, and Pathan groups. A greater allelic variation in frequency was observed within the groups than within the whole population. This was augmented by the pairwise group comparisons (F_{ST}) using AMOVA resulted none significant P -values (Table 4). The lowest P -values ($P = 0.180, 0.108$) for comparisons

and ($P = 0.181, 0.109$) for average comparisons were found for Kashmir and Hazara/Baltistan groups respectively, when compared with the Punjabi group. No significant genetic variations for *SLCO1B1* within the 6 ethnic groups of Pakistan were inferred from these P -values. It seems that interethnic mobilization and cross linkages/marriages have played a significant role in this distributed frequency pattern. However, the high interethnic group variations may explain the inter-individual differences in statin therapy and the attenuated benefit to risk ratio of statins in these groups.

Among the four haplotypes, *1A is referred to as the reference/control haplotype while all the other diploid haplotypes contain at least one variant allele. The distribution trend in haplotype frequencies among our population was a bit different from what we observed in the allelic and genotypic frequency distribution. When comparing within the groups, the highest frequency was 50 % for *1B in the Balouchi and Hazara/Baltistan groups followed by 43 % for Sindhi, 40 % for Pathan and Kashmir, and lowest of 20 % for the Punjabi group. Except for the reference/control haplotype, all the other haplotypes (*5 and *15) occurred at a relatively lower frequency (< 25 %) within the ethnic groups. A somewhat similar trend was observed when the haplotypes were investigated among the groups in the whole population with the exception of the *5 and *15 haplotype which occurred at a relatively higher frequency in Kashmiri and Hazara/Baltistan groups. The inter-ethnic group variations were also reflected by their significant P -values (Table 6) when compared with lower frequency groups such as Punjabi, Sindhi, and Pathan.

Keeping in view these statistical inferences, Kashmiri, Hazara/Baltistan and to a lesser extent the Balouchi population, are at a higher risk of haplotype induced inter-individual variations to drug disposition and therefore prone to elevated toxicity levels than might be observed in other ethnic groups.

It is evident from these findings that there might exist a significant haplotype variation within the ethnic groups of our population with little to no variation at the allelic and genotype levels. This significant haplotype variation can be explained by the combined effect of both the SNPs and their presence in linkage disequilibrium at more than one loci in our population.

The interpretations of allelic, genotype and haplotype frequency distributions from this study are in agreement with similar population studies that have been carried out in Chinese (10), Tanzanian (25), Brazilian (26, 27), Caucasian, African, and East Asian (28, 29), Canadian (30), Finnish (31), German and Turkish (32), African American and Hispanic populations (20, 33). One large scale study on the global analysis of genetic variations in *SLCO1B1* (5) compared several populations for their allele and

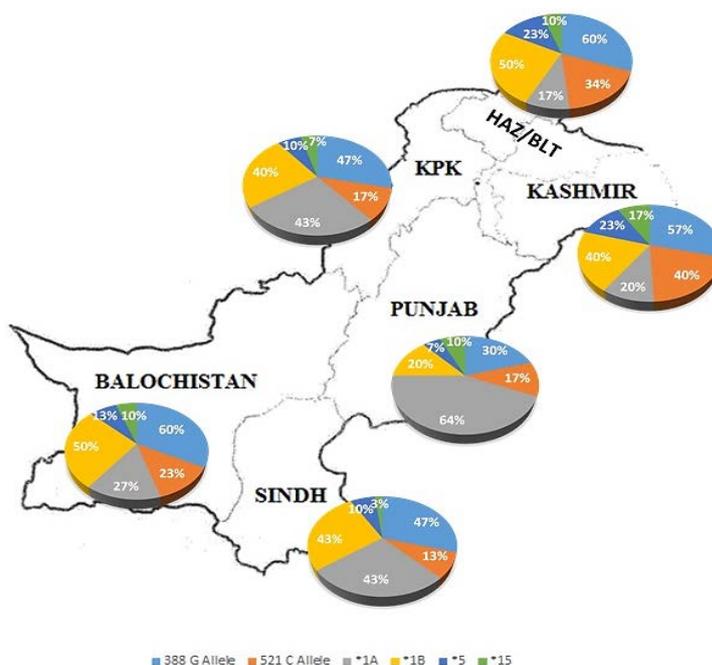


Figure 3. Frequency distribution of *SLCO1B1* alleles and haplotypes within the ethnic groups of Pakistani population

genotype frequencies and found that there was no significant difference within the groups of a population; however, a large variability existed when populations were compared with each other or in such haplotypes where alleles occurred in a linkage disequilibrium with each other. That study is in accordance with our findings and supports the fact of haplotype frequency variations within the groups of our population. Some of these studies have shown the significant effect of these frequencies and related them to the altered drug disposition while others have demonstrated no marked effect on drug PK and PD. Figure 3 delivers an overview of the allelic and their haplotype frequencies distribution within the different ethnic groups of Pakistani population.

Conclusion

Functionally significant sequence variations of OATP1B1 –encoding *SLCO1B1* gene are widely distributed in our population at varying frequencies within the ethnic groups. The most detrimental homozygous variant 521CC genotype and its corresponding GC haplotype are relatively common in Kashmiri and Hazara/Baltistan ethnic groups; however, this diversity is only significant within the groups and not among the total Pakistani population. No significant genetic diversity exists for either SNP among the ethnic groups of our population. Moreover, a rapid and reliable T. ARMS genotyping assay was developed for the extensive analysis of *SLCO1B1* genotype and haplotype variants in a large sample size. More studies can be performed for evaluating the effects of *SLCO1B1*

genetic variants on inter-individual variability in statins pharmacokinetics and pharmacodynamics.

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