Original Article

Genetic Variability of *CYP2B6* Polymorphisms in Southeast Iranian Population: Implications for Malaria and HIV/AIDS Treatment

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Abstract

Background: Genetic polymorphisms in the cytochrome P450 2B6 (CYP2B6) gene could influence therapeutic outcomes of CYP2B6-metabolized drugs such as artemisinin, nevirapine (NVP), and efavirenz (EFV). The main objective of the present study was to analyze the frequency of the most common allele of *CYP2B6*1* to **7* and **9* in Iranian Baluchi population and also to compare the frequencies of these polymorphisms with those reported in different ethnic groups.

Methods: A total of 206 healthy, unrelated, subjects were participated in this study. *CYP2B6*1, *2, *3, *4, *5, *6,*7,* and **9* polymorphisms were investigated, using PCR-RFLP followed by sequencing analysis.

Results: High frequency of 516T (35.7%) was found among the studied subjects. Also, the three most frequent genotypes were *CYP2B6**1/*6 (28.1%), *CYP2B6**1/*1 (16%) and *CYP2B6**1/*9 (14.6%). The frequency of *CYP2B6**6/*6 (4.8%) was not different from Caucasian, Japanese and Chinese populations, but it was lower than West African (17%) and Papua New Guinean (43%) populations.

Conclusion: Allele frequencies for *CYP2B6* in the examined population were markedly different from those African, Caucasian, and Southeast Asian populations. *CYP2B6*2, *4, *5, *6,* and **7* were found in the Iranian Baluchi that may affect the response to artemisinin and its derivatives. High frequency of G516T (35.7%) was detected among the examined subjects that might cause greater efavirenz plasma exposure and more central nervous system side effects. Therefore, characterization of pharmacologically relevant polymorphisms in CYP2B6 has a great potential to improve drug efficacy and reduce toxicity.

Keywords: Allele frequency, cytochrome P450 2B6, genetic polymorphisms, Southeast Iran

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Introduction

The human hepatic cytochrome P450 2B6 (CYP2B6) is a key enzyme in the metabolism of clinically important drugs (such as anti-malarial and anti-HIV/AIDS compounds), environmental chemicals and endogenous substances. The expression and activity of this enzyme vary widely among individuals, probably due to environmental factors, polymorphisms in the *CYP2B6* gene and drug interactions that contribute to the variable response to these drugs primarily metabolized by this enzyme.[1,2] Human CYP2B6 is highly inducible by several drugs, including artemisinin[3–5] and its derivatives: artesunate (b-arteether)[4,6] efavirenze (EFV), nevirapine,[7,8] metamizole, cyclophosphamide, rifampicin and some other drugs.[7,9–11] In Iran, due to CQ resistance, the national drug policy for malaria control recommended SP-artesunate as the first-line treatment for uncomplicated *P. falciparum* cases in 2007. As a result, with an increase in the use of SP-artesunate in the country, *P. falciparum* isolates are increasingly being exposed to drug pressure of SP-artesunate. Therefore, study on the role of CYP2B6 polymorphisms in case of artemisinin drugs may be pharmacologically important. Different studies have shown inter-individuals variations in the concentration of artimisinin, artesunate, DHA and their anti-malarial effect among malaria patients.[11–14] In this regards, Angus, et al.,[15] reported that doses of artesunate up to 2 mg/kg are correlated with both plasma DHA and parasite and fever clearance. However, based on pharmacokinetics and pharmacodynamics study in various populations, the larger dose (>2 mg/kg) was suggested and needed to clear the parasites.[15] Although the interpretation of the results based on the available information is not easy, one of the possibilities for such variability could be the presence of the polymorphisms in CYP2B6 gene.

In vitro study showed that EFV is primarily metabolized to 8-hydroxyefavirenz by CYP2B6.[7] Notably, the most frequent variant allele CYP2B6*6 (516G>T; Q172H and 785A>G; K262R) has shown a 65% reduction in the mean protein expression and 50% reduction in the mean enzyme activity in the homozygous state.[16] Also, in AIDS clinical studies in various individuals of different ethnicities, the CYP2B6*6 allele was associated with 2–4 fold higher plasma EFV and NVP concentrations that were associated with increased neuropsychiatric adverse effects.[17–20]

So far, no published information is available for CYP2B6 variation in Iranian population; however, such genotype data is mostly available from African, Papua New Guinean, Southeast Asian, Egyptian, and American populations.[16,21–25] On the other hand, the CYP2B6 genetic variation from one population may not be generalized to other populations due to the ethnic differences in

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drug response. Therefore, the main objective of the present study was to analyze the prevalence of the most known and common SNPs 64C>T, 516G>T, 777C>A, 785A>G, and 1459C>T of the *CYP2B6* gene in the Iranian Baluchi ethnic group from areas where malaria, HIV/AIDS and also TB are a significant public health problem. Moreover, the frequencies of these polymorphisms with those reported in different ethnic groups were compared. Such a data in this population on gene coding for the main drug metabolizing enzymes of anti-malaria and anti-HIV/AIDS could provide valuable information on therapeutic responses of CYP2B6-metabolized drugs and further would be helpful for global development, evaluation, and use of therapeutic drugs.

Materials and Methods

Subjects

The studied population was comprised of 206 healthy, unrelated individuals from Sistan and Baluchistan Province, Iran, where both malaria and HIV/AIDS are significant public health threats. Among the samples, 151 and 55 were males and females, respectively with a median age of 27, ranging 5–70 years. The samples were collected in 2005–2008. Two ml venous blood sample was obtained for genotyping from each individual, transferred into vacuum EDTA tubes and stored at -20ºC until DNA extraction could be performed. A written informed consent from adults, as well as the parents or a legal guardian of children was obtained. The study was approved by the Ethical Review Committee of Research in Pasteur Institute of Iran.

Molecular analysis

Genomic DNA was isolated from buffy coats or whole blood (250 UL) by using the commercially available DNA purification kit (Promega, Madison, WI, USA) according to the manufacturer's guidelines and kept at -20ºC until use. For amplification of exons 1, 4, 5, and 9 of *CYP2B6* gene, the primers that were designed based on *CYP2B6* genomic DNA sequence [GenBank: NM-000767] in our laboratory and Lang, et al.,[16] were used (Table 1). In addition, sequence homology and specificity of all primers sequences were checked for CYP2B7 psedogene,[26] using BLAST.

Different SNPs were identified by PCR-RFLP analysis (Table 1). All amplifications were carried out in a final volume of 25 µL including 1 µL of the genomic DNA template. The primers were used at a final concentration of 250 nM and the reaction mixture contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, each of the four deoxynucleotide triphosphates at a concentration of 125 µM, and 0.2 U of Taq polymerase (Invitrogen, Carlsbad, CA). The PCR program was 94ºC for 5 min, followed by 30 cycles of 94ºC for 1 min, 60ºC for 1 min and 72ºC for 1 min, with a final extension step of 72ºC for 5 min. Digestions were done in 20 UL reactions containing 10 UL of PCR fragments according to the manufacturer's instructions (Table 1). The DNA fragments, obtained following PCR amplification or RFLP analysis, were electrophoresed on 2 and 2.5% agarose gels (Invitrogen, Carlsbad, CA), respectively.

To verify the results obtained by RFLP, all PCR products were sequenced using the primers described in Table 1. The amplified fragments were gel-purified using the QIAquick gel extraction kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Direct sequencing of the DNA fragments was performed in both directions for each PCR product using the dideoxy chain termination procedure (Chemistry V3.1, Applied Biosystems) as well as the 3730XL DNA analyzer (Applied Biosystems) by MilleGen sequencing service (Labege, France).

Statistical analysis

The sample size for proportion was calculated using OpenEpi software with 95% confidence level.[27] The statistical analysis was performed with SPSS software for windows, version 15.0 (SPSS Inc., Chicago, USA). Allele (haplotype) and genotype (diplotype) frequencies were calculated by direct counting. Deviations from Hardy-Weinberg equilibrium expectations were determined using the chi-squared test. For all statistical analyses, a P value of < 0.05 was considered to be significant.

Results

Allele frequency

Allele designations were made in accordance with the full-length

Table 1. Primers and profiles used for PCR-RFLP of the *CYP2B6* gene.

SNPs (exon)	Allele	Primer /Sequence	Restriction Enzyme (company)	Cut Product Size (bp)
C64T (1)	CYP2B6*2	**CYP64F:** AGTGGGTAAAGGGATAGGC **CYP64R:** TGTGACCAAGTAAGGCAAGC	*HaeII* BioLabs	**C**: 458 + 276 =734
G516T(4)	CYP2B6*9	**CYP516F**: ATAGCTGTGTTGCCTGGG **CYP516R:** TTCTCGTGTGTTCTGGGTG	*BseNI:* Fermentas	**G**: 235+ 196+102 = 533 **T**:431+ 102= 533
C777A(5)	CYP2B6*3	**CYP777-785F**: TCACCACCCCTTCTTTCTTG **CYP777-785R**: AATTCCTTCCTCAGCCAGTC	*HaeII* BioLabs	**C**: 329 + 157 = 486
A785G(5)*[16]	CYP2B6*4	**CYP2B6-5F**:GACAGAAGGATGAGGGAGGAA* **CYP2B6-5R**:CTCCCTCTGTCTTTCATTCTGT*	*StyI** Fermentas	**A**: 116+56+171+297 = 640 **G**: 116+56+468 = 640
C1459T(9)	CYP2B6*5	**CYP1459F:** TCCTAAAAGTCCACCCTG **CYP1459R:** TCGATAATCTCACTCCTGC	*Bgl II* Fermentas	**T**: 309 + 208= 517

Table 2. Allele frequencies of the *CYP2B6* gene among 206 Iranian individualsin comparison with published data in various populations.

Allele	Mutations	Exon	No of alleles (n = 412)	Frequency of alleles (%) in 206 subjects (95% CI)	Asian-American[11] n=122	Caucasian-American[11] n=120	Hispanic-American[11] n=154	African-American[11] n=186	West-African[11] n=332	PNG[11] n=348	Japanese[12] n=530	Vietnam[26] n=144	Caucasian-German[7] n=430	Caucasian-British[14] n=270	Egyptian[23] n =240
*1	None	All	196	47.6 (0.428–0.524)	64	56	53	56	45	33	68.5	64.6	50.7	53.7	66.3
*2	64 C>T	1	16	3.9 (0.024–0.063)	7	3	3	3	4	—	4.7	—	5.3	3.7	—
*3	777 C>A	5	—	—	—	—	—	—	—	—	—	—	0.5	—	—
*4	785 A>G	5	43	10.4 (0.078–0.138)	4	6	3	2	—	—	9.3	8.3	4	2.2	2.1
*5	1459 C>T	9	10	2.4 (0.013–0.045)	3	3	5	5	2	2	1.1	—	10.9	12.2	3.7
*6	516 G>T 785 A>G	4,5	95	23.1 (0.192–0.274)	23	28	30	34	42	62	—	27.1	25.6	28.1	27.9
*7	516 G>T 785 A>G 1459 C>T	4,5,9	10	2.4 (0.013–0.045)	—	3	2	1	—	1	—	—	3	—	—
*9	516 G>T	4	42	10.2 (0.076–0.135)	—	1	5	1	8	1	—	—	—	—	—

Allele *1 is defined as wild type with no detected mutations in all exons 1, 4, 5, and 9.[26]

Table 3. *CYB2B6* genotype frequencies of 206 Iranian subjects in comparison with published data in different ethnic groups.

Genotypes	No of subjects *n*=206	Observed Frequency (%) (95% CI)	Predicted Frequency (%) by HW	X^2Test	*P*-value	Chinese[13] *n*=193	Japanese[12] *n* =265	West-African[11] *n*=166	PNG[11] *n*=174	Caucasian-British[14] *n*=135	Caucasian-America[11] *n*=60	Asian-American[11] *n*=61	African-American[11] *n*=93	Hispanic-American[11] *n*=77	Egyptian[23] *n*=120
***1/*1**	33	16 (0.116–0.217)	12.6	0.969	0.399	50.8	52.8	18	13	29.6	28	46	28	25	44.2
***1/*2**	14	6.8 (0.040–0.112)	1.1	9.364	0.004*	2.6	4.5	2	—	5.97	2	5	3	3	—
***1/*4**	14	6.8 (0.040–0.112)	15.6	7.929	0.007*	7.2	6.8	—	—	0.75	7	5	2	3	0.83
***1/*5**	8	3.9 (0.019–0.076)	1.6	2.335	0.220	0.5	1.5	—	2	9.6	7	5	5	6	5.83
***1/*6**	58	28.1 (0.224–0.347)	17.5	6.671	0.013*	24.3	18.5	42	36	31.9	33	21	44	39	37.5
***1/*7**	6	2.9 (0.012–0.063)	2.2	0.410	0.751	—	—	—	2	—	7	—	1	4	—
***1/*9**	30	14.6 (0.103–0.201)	14.1	0.020	1.000	1	—	9	—	—	—	—	—	3	—
***2/*2**	1	0.5 (<0.0001–0.03)	0.03	1.002	0.500	—	—	—	—	—	—	—	—	—	—
***4/*4**	5	2.4 (0.009–0.057)	4.6	1.183	0.416	0.5	2.6	—	—	—	—	—	—	—	0.83
***4/*6**	15	7.3 (0.044–0.117)	5.2	0.657	0.544	4.7	4.5	—	—	0.75	2	2	—	3	1.67
***4/*7**	4	1.9 (0.006–0.051)	0.6	1.822	0.372	—	—	—	—	—	—	—	—	—	—
***5/*5**	1	0.5 (<0.0001–0.03)	0.1	1.002	0.500	—	—	—	1	2.99	—	—	1	—	—
***6/*6**	10	4.8 (0.025–0.088)	1.5	3.892	0.087	2.1	3.8	17	43	7.4	8	8	11	6	7.5
***9/*6**	2	1 (0.0004–0.037)	4.9	5.493	0.036*	2.1	—	6	2	—	2	—	1	3	—
***9/*9**	5	2.4 (0.009–0.057)	4	0.715	0.575	—	—	—	—	—	—	—	—	—	—

Allele *1 is defined as wild type with no detected mutations in all exons 1, 4, 5 and 9.[26] **P*-values< 0.05 were Considered statistically significant.

cDNA sequence published by Yamano, et al.[26] The *CYP2B6* SNP C64T, G516T, A785G, and C1459T were observed in this Iranian population (Table 2) with a lower frequency than the wild-type alleles. The frequencies of 64T, 516T, 785G, and 1459T SNPs were 3.9%, 35.7%, 35.9%, and 4.8%, respectively. Interestingly, a novel SNP, C779T (A260V), in exon 4 was identified, for the first time, in three examined samples using sequencing analysis. This SNP was found in combination with either A785G alone (n = 1) or G516T and A785G SNPs (n = 2) (data not shown).

Considering exons (1, 4, 5, and 9) analysis,[26]*CYP2B6*1* allele were detected in 47.6% of the examined samples (Table 2) as it was also found in Papua New Guinean (33%), Caucasian British (53.7%), Caucasian German (50.7%), African American (56%), West African (45%), Asian Americans (64%),[25] Egyptian (66.3%),[23] and Japanese (68.5%),[26] populations (Table 2). However, *CYP2B6*3* was not found in any of examined samples (Table 2). Among the variant alleles, *CYP2B6*1* (47.6%) and *CYP2B6*4* (10.4%) had the highest prevalence in the examined samples. Moreover, *CYP2B6*9* allele (10.2%) was also reported in West African (8%), African American (1%), Caucasian American (1%), Papua New Guinean (1%),[21] and Hispanic American (5%),[21] but not in Asian American,[21] Japanese,[25] Vietnamese,[28] Caucasian German,[16]Caucasian British,[25] and Egyptian[23] (Table 2).

Genotyping frequency

The genotype frequencies of the *CYP2B6* gene are shown in Table 3. The three most frequent genotypes were *CYP2B6**1/*6 (28.1%), *CYP2B6**1/*1 (16%), and *CYP2B6**1/*9 (14.6%), accounting for 58.7 % of the studied population. A comparison of observed and expected numbers of genotypes showed that *CYP2B6**1/*6, *CYP2B6**1/*2, *CYP2B6**1/*4, and *CYP2B6**9/*6 genotypes were significantly different from the expected frequencies which were deviated from HWE (Table 3). This could be explained by genetic drift, non-random mating or an indication of selection acting on specific genotype and at the present, we do not have any evidence to prove any of them in this population.

Discussion

Data from the present study provide, for the first time, the distribution of CYP2B6 allelic variants and genotypes in the Iranian Baluchi population. The results document the presence of polymorphism in this gene and provide evidence that the allele frequencies for *CYP2B6* in Iranian Baluchi are different from those of other populations. Such differences might cause variability in response to drugs that are metabolized via CYP2B6.

In this work, analysis of the *CYP2B6* SNPs showed that in Iranian Baluchi ethnic group 516G>T (35.7%) and 785A>G (35.9%) were highly prevalent similar to European-American, African-American, Kenya and Hispanic-American populations.[29] *CYP2B6*3* was not found in any of the examined samples that it is in agreement with the data reported for Japanese,[22] Han Chinese, Korean,[25] Caucasian American, Caucasian British,[24] Egyptian,[23] and African American[25] populations. However, *CYP2B6*5* variant was identified in 10 (2.4%) individuals that was associated with CYP2B6 catalytic activities, eight-fold lower than those in wild-type homozygotes (C1459C).[16] The frequency of this variant (2.4%) was different from those reported in Caucasian, German (10.9%),[25] and British (12.2%)[24] populations.

Concerning the allele frequencies of *4 (785A>G) and *9 (516G>T), we found more than 10% prevalence for both alleles that were not reported from different populations of diverse ancestry. This might be due to inter-ethnic variability of different populations that might be specific in this ethnic group. Therefore, to verify this postulation, it is worthy of carry out further similar study in different Baluchi ethnic groups who are living in Afghanistan and Pakistan in the Southwest Asia.

As mentioned before, genetic variability in *CYP2B6* gene might change the pharmacokinetics of several drugs, such as efavirenz (anti-HIV/AIDS) and cyclophosphamide (anticancer and immunosuppressant prodrug), both of which require metabolic activation, primarily by CYP2B6, to form its active metabolite.[7,30] The *CYP2B6* G516T SNP has been associated with increased cyclophosphamide activity and clearance compared to the wild-type gene.[31,32] Regarding efavirenz drug, the presence of homozygosity for *CYP2B6*6* are associated with five-fold higher drug concentration as a result of the increase risk of neurotoxicity.[17,18,33] This allele is one of the most prevalent variant allele in ethnically diverse populations, ranging from about 15% in Koreans to 42% in the West-African, and over 62% in Papua New Guinean[21] and this allele (23.1%) was the second prevalent among the studied population in the current investigation. Recently, *CYP2B6* genotyping in HIV/AIDS outpatients in Japan[34] confirmed the association of high efavirenz plasma concentrations and the poor metabolizer genotype (T516T). In addition, the presence of G516T genotype is a good indication of EFV metabolism in the liver that predicts the increased risk of developing drug resistance after discontinuation of EFV-containing regimens.[35] In the present study, high frequency of 516T (35.7%) among the Iranian Baluchi population might causes greater efavirenz plasma exposure and perhaps more central nervous system side effects, as shown by others in India.[20,36] Therefore, similarly, the present data might be used to predict optimal dosing before commencement of EFV-containing therapy in the examined population to prevent reduction in the efficacy of the drug, toxicity and also drug-resistant viruses.

Furthermore, Ariyoshi, et al.,[37] demonstrated that the *CYP2B6* A785G polymorphism results in increased metabolic activity toward efavirenz and decreased activity toward cyclophosphamide. However, when both SNPs (*CYP2B6* A785G and G516T, 23.1% in the present study) are simultaneously present the metabolic profile is the reverse suggesting that the *CYP2B6* G516T can inverse the effects produced by the *CYP2B6* A785G polymorphisms for certain drugs.[37]

Moreover, in this study, *CYP2B6*2, *4, *5, *6,* and **7* (frequencies 3.9%, 10.4%, 2.4%, 23.1%, and 2.4%, respectively) were found in the Iranian Baluchi, who are living in malaria endemic region. However, *CYP2B6*2, *5, *6,* and **9* (frequencies 4%, 2%, 42%, and 8%, respectively) as well as *CYP2B6*5, *6, *7,* and **9* (frequencies 2%, 62%, 1%, and 1%, respectively) were reported from malaria endemic regions of West Africa and PNG, respectively.[21] As these polymorphisms can alter the enzyme expression level and/or activity, the significant allele and genotype differences among these three populations might cause differences in response to drugs that are metabolized via CYP2B6, such as the novel anti-malarial drug artemisinin and its derivatives (Artesunate, Arthemeter, Dehydroartemisinin, etc).[3] It should be noted that the major disadvantage of this drug in mono-therapy is high recrudescence rates, which have been attributed to the remarkable decrease of artemisinin plasma concentrations[38] during multiple dosing, probably by auto-induction of CYP2B6 enzyme

by this medication.[39]

Interestingly, a novel SNP, C779T (A260V), in exon 4 was identified, for the first time, in three examined samples through sequencing analysis. This SNP was also found in combination with either A785G alone (n = 1) or G516T and A785G SNPs (n = 2) (data not shown). Moreover, the presence of this variant mutation in Iranian and the absence of this variant in African, Caucasian, Japanese and Southeast Asian populations may suggest the presence of a particular haplotype in the Middle East region or Southwest Asia that might show a local evolutionary pressure on *CYP2B6* polymorphisms. Further studies are warranted to elucidate the functional impact of this new SNP (C779T) on the CYP2B6 expression level and activity.

In general, the present results on the *CYP2B6* SNP/allele/genotype frequency provide novel information on the prevalence of polymorphisms in key enzymes involved in several drugs within the Iranian Baluchi population. Our findings of high frequency of G516T polymorphism would be also useful for clinicians to optimize antiretroviral dose adjustments in HIV/AIDS patients in this region, accordingly, reduce associated CNS symptoms along with economic advantage. Therefore, it is worth noting that study on antiretroviral (such as EFV) pharmacokinetics, clinical response and toxicity is highly needed in this population. Finally, allele frequencies for *CYP2B6* in our examined population were markedly different from other populations and such differences might cause variability in response to drugs that are metabolized via CYP2B6. This data might also concern for optimizing drug effectiveness, while minimizing toxicity.

Disclosure Statement

No competing financial interests exist.

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