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Original Research

Frequency Evaluation of T6235C (m1) and A4889G (m2) Polymorphisms of *CYP1A1* Gene in a Healthy Population from the west of Mazandaran Province, Iran

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Abstract: CYP1A1 is an important phase I xenobiotic metabolizing enzyme involved in the metabolism of numbers of toxins, endogenous hormones and drugs. Polymorphisms in this phase I gene can alter enzyme activity and induction, also are known to be associated with cancer susceptibility related to environmental toxins and hormone exposure. The present study was aimed to determine the frequencies of commonly known functional polymorphismsof *CYP1A1* gene including *CYP1A1* m1 (MspI), and *CYP1A1* m2 (Ile-Val) in a healthy population from the west of Mazandaran province, Iran. A total of 200 unrelated healthy subjects from Mazandaran province, residing in Tonekabon city, coming for blood donating at Tonekabon Blood Transfusion Center were enrolled. Genomic DNA was extracted from peripheral blood lymphocytes of each subject. All subjects were genotyped for *CYP1A1* m1 (T>C) and m2 (A>G) by polymerase chain reaction-restriction fragment length polymorphism method. The frequencies of the TT(wt/wt), TC(wt/mt) and CC(mt/mt) genotypes were as 65.5%, 32.0% and 2.5% respectively for m1 and frequencies of the AA(wt/wt), AG(wt/mt) and GG(mt/mt) genotypes were as 84.5%, 15% and 0.5% respectively for the m2. The frequencies of T and C alleles in the population were 81.5% and 18.5% respectively and the frequencies of A and G alleles were 92% and 8% respectively. Results of the present study might be important in understanding the distribution of *CYP1A1* (m1) and *CYP1A1* (m2) polymorphisms in Mazandaran province of Iran. Moreover, these results may determine the susceptibilities of individuals towards environmental procarcinogens that result in several cancers.

Key words: Cytochrome P450, *CYP1A1*, polymorphism, Iranian population, toxicogenetic.

Introduction

Cytochrome P450 (CYP) includes a superfamily of enzymes that act in phase I of xenobiotic metabolic transformation. Phase II enzymes then process the metabolites produced by these reactions (1,2). The CYP enzymes are responsible for the metabolism of diverse range of xenobiotics, carcinogens, toxins, therapeutic drugs and steroid hormones (3-6). The CYP metabolically activates carcinogens, such as polycyclic aromatic hydrocarbons and N-nitrosamines, to reactive intermediates. These intermediates are capable of binding covalently to DNA to form DNA adducts, potentially initiating the carcinogenic process (7). Individual susceptibility to cancer from environmental agents may be influenced by polymorphism in such metabolic gene families (6).

The *CYP1A1* gene is located in chromosome 15q22–q24, is 5987 base pairs long and encodes a protein of 512 amino acids (8)and in addition to the lung, it is also expressed in the liver, gastrointestinal tract, brain, lymphocytes and macrophages (9,10).The *CYP1A1* gene have been shown to be expressed predominantly in the extra-hepatic organs and encodes a phase I cytochrome P450 enzyme that plays an important role in the bioactivation of procarcinogenic xenobiotics, such as benzo(a)- pyrene (B(a)P) and other polycyclic aromatic hydrocarbons (PAHs) which are constituents of cigarette smoke(11-13). *CYP1A1* polymorphisms have been shown to be associated with moderate to high risk of lung cancer in Asians (14) and in Caucasian and Hawaiian population (15,16) making it a strong candidate gene in susceptibility to smoking-related cancers of larynx, mouth, esophagus, urinary bladder and kidney (17). CYP1A1 enzyme also hydroxylates estradiol for its elimination and thus can increase the risk of estrogen-related cancers (18). The human *CYP1A1* gene is polymorphic, and this has provided a basis for numerous assessments of its role in chemical carcinogenesis, by enhancing susceptibility of individuals, possibly through increased bioactivation of carcinogens (11). Four different polymorphisms of *CYP1A1* gene, m1, m2, m3 and m4 have been described (19). The m1 and m2 polymorphisms are more widely studied due to not only their higher genotype frequency but also their possible involvement in lung carcinogenesis (20).

CYP1A1 m1 (MspI) is a T→C transition located downstream of exon 7, in 3′ noncoding region (21). It does not exert any effect on *CYP1A1* induction (19) but increases the microsomal enzyme activity (22,23). *CYP1A1* m2 (Ile-Val), an A→G transition in exon7, leads to an amino acid substitution of Val for Ile in the heme binding region (21) and is significantly associated with *CYP1A1* inducibility and increased enzyme activity (22 - 24). Polymorphism in human xenobiotic metabolizing

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genes displays parallelism in racial, ethnical and geographical distribution and the ethnic-specific effect of *CYP* genes is well known (25). Therefore, studies are required to report the frequencies in different populations of gene polymorphisms that might possibly be associated with risk of various cancers. No study has reported frequencies of this gene polymorphisms in normal, healthy Iranian population. Hence, the present study was aimed to analyze the frequencies of commonly known polymorphisms of *CYP1A1* gene in a sample of Iranian population from the west Mazandaran province.

Materials and Methods

Study population

The present study included 200 (100 females and 100 males, ages between 20-60 years) randomly selected, healthy, unrelated individuals from Mazandaran province, residing in Tonekabon, coming for blood donating at Tonekabon Blood Transfusion Center during the period of Apr 2014– Sep 2014 fulfilling our inclusion and exclusion criteria. Individuals were eligible for study if they had no history of any diseases such as thyroid disease, AIDS, hepatitis B, hepatitis C, diabetes mellitus, anemia and any type of cancer. They were enrolled in the study after a written informed consent was obtained. Ethnicity was recorded by self-report.

The protocol of the study was approved by the research ethics committee of Mazandaran University of Medical Sciences.

Sample collection

Blood samples (about 2mL) were collected from all subjects in disposable presterilized K_2EDTA-coated vacutainer tubes (Soran-sanat-azma, Iran) with the help of a trained technician. These samples were stored at -20˚C.

DNA Extraction

Genomic DNA was extracted from the peripheral blood using the Blood/Tissue DNA Extraction Mini Kit (DynaBio™, Iran) according to the manufacturer's instruction. These isolated DNA were stored at -20˚C for genotype analysis.

Genotyping of CYP1A1

CYP1A1 genotypes at m1 and m2 were analyzed by polymerase chain reaction-restriction fragment length polymorphisms (PCR-RFLP) method. The primers were selected according to previous study by (Hussein et al., 2014) and were *m1 F 5′ CAG TGA AGA GGT GTA GCC GCT 3′ and m1 R 5′ TAG GAG TCT TGT CTC ATG CCT 3′, m2 F 5′ TTC CAC CCG TTG CAG CAG GAT AGC 3′ and m2 R 5′ CTG TCT CCC TCT GGT TAC AGG AAG 3′*(26). PCR were carried out in a volume of 25 µL containing 2µL of genomic DNA, 12.5 µL 2X PCR reaction mix (included 2X PCR buffer, 0.4mM dNTP, 32mM MgCl2, 0.02% bromophenol blue (Yekta-tajhiz-azma, Iran), 0.5µl of Taq DNA polymerase (2.5U/µL)(Yekta-tajhiz-azma, Iran), 1µL of each primer (BIONEER, Korea), and 8µL nuclease-free water. The PCR protocol was carried out in the thermocycler (BIO-RAD, USA) with an initial denaturation at 94˚C for 5 min, followed by 35 cycles of 45s at 94˚C, 1min at 61˚C and 45s at 72˚C and a final elongation step of 5min at 72˚C (to find the best annealing temperature for each set of primers, we used PCR gradient programs).The PCR product size were 340 and 204bp for m1 and m2 respectively.

Polymorphism detection

The PCR product for m1 and m2 were digested with Msp1 (Fermentas, Lithuania) and BsrD1 (Fermentas, Lithuania) respectively.

Digestion conditions were as follow: 10µL of PCR mixture, 2µL of buffer (10X), 1µL of enzyme and 18µL of de-ionized water to reach the final volume of 31µL. The reaction was incubated for 16h in 37˚C for Msp1 enzyme and 16h in 55˚C for BsrD1 enzyme. The restriction digested products were analyzed by electrophoresis on 2.5% agarose gel containing ethidium bromide and visualized under UV illumination.

Gain of a Msp1 restriction site occurs in the polymorphic allele, the wild type allele showed a single band representing the entire 340bp fragment and variant allele resulted in two fragments of 200bp and 140bp. The restriction enzyme BsrD1 was distinguishing the m2 polymorphism; the cleavage sites were lost in the case of the mutation and resulted in a single band (204bp), whereas the wild type alleles generated 149 and 55bp bands (table 1)(27).

Agarose gel electrophoresis

PCR products and digested products with enzyme were analyzed using agarose gel electrophoresis. The gel was prepared with 2.5% agarose (Merck, Germany), in Tris Borate EDTA (TBE) 1X. The conditions were in constant voltage at 120V for 40min in TBE 1X buffer.

Statistical analysis

Data obtained from our study was analyzed using the SPSS version 16.0. Group comparisons for categorical variables were carried out using the chi-square (*x2*) and Fisher's exact test. A p-value of less than 0.05 was considered statistically significant. The observed genotype frequencies of *CYP1A1* were compared with expected frequencies according to Hardy-Weinberg law.

Results

The present study was carried out on a group of 200 unrelated healthy individuals including 100 females and 100 males ages 20-60 years and genotypic frequencies of two main *CYP1A1* gene polymorphisms were studied

Table 1. Restriction endonucleases for the recognition of m1 and m2 polymorphisms

Mutation	Restriction enzyme	Transition	Recognition sequence	Digestion pattern (bp)		References
				Wild	Variant	
CYP1A1 m1	MspI	T→C	C^CGG	340	200,140	(27)
CYP1A1 m2	BsrDI	A→G	GCAATGNN^	149,55	204	(27)

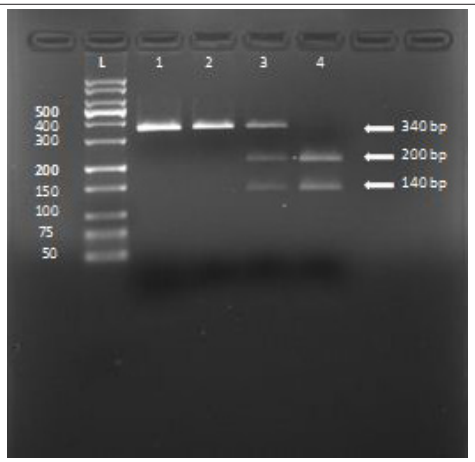

Figure 1. The PCR-RFLP analysis of *CYP1A1* m1 polymorphism. The DNA from healthy individuals was subjected to PCR followed by RFLP using Msp1 digest. The reactions were resolved on ethidium bromide-stained 2.5% agarose gel electrophoresis. Lane L shows 50bp Molecular Weight(MW) DNA Ladder, lane 1 represent PCR product, lane 2 represent homozygous wild-type allele(wt/wt), lane 3 represent heterozygous allele(wt/mt), lane 4 represent homozygous mutant-type allele(mt/mt).

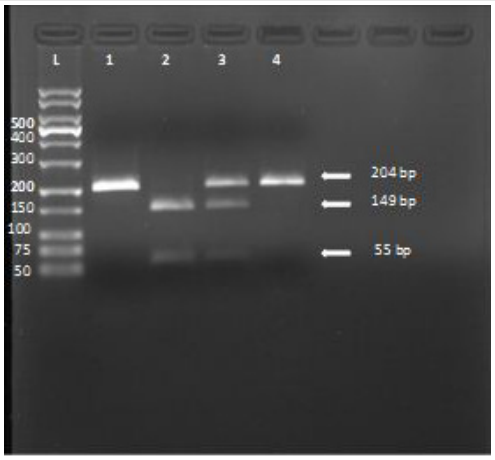

Figure 2. The PCR-RFLP analysis of *CYP1A1* m2 polymorphism. The DNA from healthy individuals was subjected to PCR followed by RFLP using BsrD1 digest. The reactions were resolved on ethidium bromide-stained 2.5% agarose gel electrophoresis. Lane L shows 50bp Molecular Weight(MW) DNA Ladder, lane 1 represent PCR product, lane 2 represent homozygous wild-type allele(wt/wt), lane 3 represent heterozygous allele(wt/mt), lane 4 represent homozygous mutant-type allele(mt/mt).

in the Iranian population (Mazandaran province, Tonekabon).The PCR-RFPL analysis and pattern of each genotype detection of these two polymorphisms are indicated in figure 1and 2. The genotypic frequencies were 65.5%(TT), 32%(TC) and 2.5%(CC) for *CYP1A1* m1 and 84.5%(AA), 15%(AG) and 0.5%(GG) for *CYP1A1* m2 (Table 2). Frequency of T and C alleles was 81.5% and 18.5% and frequency of A and G alleles was 92% and 8% respectively. Gender distribution of the alleles are shown in Table 3. Accordingly, the T and A wild-type alleles were more frequent in both genders. Distribution of the C mutant allele was statistically more frequent in males (22%) compared to females (15%) (p= 0.05). Similarly, distribution of the G mutant allele was more frequent in males (8.5%) compared to females (7.5%), but this difference was not statistically significant (p>0.05). The observed frequencies of all studied alleles were not significantly different from expected frequencies, indicating that they were in Hardy-Weinberg equilibrium. In our studied population, *CYP1A1* m1 and m2 mutant alleles frequencies (C:18.5% , G:8%) were lower than the Chinese population (C:35.5%,G:25.6%), the north Indian population (Chowk) (C:27.9% ,G: 16.6%), the Haryana population (C:29.65% , G:24.15%), the Egypt population (C:27.3% , G:24.1%), and the Jordanian population (C:28.57% , G:15.71%); also it was significantly lower than in another north Indian population (Kanpur) study (C:54% , G:48.75%), but our results showed higher frequencies than Spanish (C:9.8%, G:1.5%), German (C:7.7%, G:2.8%) and Russian population (C:11.21%, G:4.55%), whereas the prevalence of *CYP1A1* m1 and m2 were closest to the one reported for the Turkish population (C:18.1%, G:8.9%) (Table 4) (21,26-38).

Table 3. Allele frequencies of *CYP1A1* m1 and m2 polymorphisms in Iranian population, Mazandaran province.

Gender	m1Allele frequency (%)		m2Allele frequency (%)	
	T	C	A	G
Male	78	22	91.5	8.5
Female	85	15	92.5	7.5

Discussion

The Present study investigated the frequency of commonly known genetic *CYP1A1* polymorphisms in the Iranian population (Mazandaran province, Tonekabon). Cytochrome P450 are phase I xenobiotic metabolizing enzyme and their genotypes may display ethnicity dependent population frequencies. *CYP1A1* is a member of the Cytochrome P450 enzymes multigene family having ability to metabolize endogenous and exogenous substances. Polymorphisms in these genes have been shown to be associated with cancer susceptibility rela-

Table 2. Gender distribution of *CYP1A1* polymorphisms in the population.

Polymorphism	Genotype[a]	Gender	
		Male (n=100)	Female (n=100)
CYP1A1 m1*	*wt/wt (TT)*	61	70
	wt/mt (TC)	34	30
	mt/mt (CC)	5	0
CYP1A1 m2†	*wt/wt (AA)*	83	86
	wt/mt (AG)	17	13
	mt/mt (GG)	0	1

[a] *wt* wild type; *mt* variant, * *p=0.05*, †*p>0.05.*

Table 4. Comparison of allele frequencies of *CYP1A1* reported from different ethnic populations.

Population		Allele frequencies				References
	n	T	C	A	G	
Iranian	200	81.5%	18.5%	92%	8%	Current study
Asians:						
Chinese	404	64.5%	35.5%	74.4%	25.6%	27
North Indian(Chowk)	208	72.1%	27.9%	83.4%	16.6%	28
India (Haryana)	290	70.35%	29.65%	75.85%	24.15%	29
North Indian (Kanpur)	200	46%	54%	51.25%	48.75%	30
India (Kashmir)	163	69.7%	30.3%	73.4%	26.6%	31
Jordanian	70	71.43%	28.57%	84.29%	15.71%	32
Egypt	110	72.7%	27.3%	75.9%	24.1%	26
Caucasians:						
Spanish	265	90.2%	9.8%	98.5%	1.5%	33
Turkish	271	81.9%	18.1%	91.1%	8.9%	34
German	880	92.3%	7.7%	97.2%	2.8%	21
Russian	451	88.79%	11.21%	95.45%	4.55%	35
Russian (Tatars)	333	84.98%	15.02%	94.14%	5.86%	35
Russian (Bashkir)	171	78.36%	21.64%	89.47%	10.53%	35
Africans	445	78.2%	21.8%	99.4%	0.6%	36
African-American	278	76.3%	23.7%	97%	3.0%	37
Mexicans	40	60%	40%	50%	50%	38

ted to environmental toxins and hormone exposure (39). *CYP1A1* plays an important role in the metabolism of procarcinogens present in the tobacco smoke to DNA-binding carcinogenic metabolites (40). Thus *CYP1A1* polymorphisms can be associated with smoking-related human cancers of larynx, mouth, esophagus, urinary bladder and kidney (17). *CYP1A1* alleles (m1 and m2) are known as "higher risk alleles" and frequencies of these alleles are reported to be eight to eighteen times higher in Asians than in Caucasian population (41).

The association of gene polymorphisms of *CYP1A1* with susceptibility to several types of cancers, e.g. lung, bladder, pancreatic, breast, ovarian, prostate, oral and gynecological malignancies including cervical cancer, has been evaluated in different populations (42-50). Suggawara et al. however, did not find any relation between *CYP1A1*gene polymorphisms and gynecological malignancies (51). A strong correlation was found between homozygous m2 polymorphism and lung cancer among Japanese, unlike Caucasians (16). Anantharaman et al. did not find any significant association of *CYP1A1* m1 and m2 polymorphisms with risk of oral cancer in Indian population (52). Abbas et al. suggests that C allele of m1 (T>C) and G allele for m2 (A>G) may be the risk alleles for cervical cancer susceptibility (28).Sabitha et al. found elevated frequencies of *CYP1A1* m1 and m2 homozygous variants and their association with elevated risk of head and neck cancer in tobacco smokers in Hyderabad (53). Morita et al. reported an increased prevalence of the *CYP1A1* m2 homozygous variant among Japanese patients with head and neck cancers (54). Nimura et al. found a threefold frequency of the *CYP1A1* m2 homozygous variant in esophageal cancer patients in China who were heavy smokers (55). Tanimoto et al. reported that Japanese individuals homozygous for the *CYP1A1* m1 variant were at significantly increased risk for oral squamous cell carcinoma after exposure to low concentrations of PAH (56). But, two Japanese studies did not find any association of *CYP1A1* polymorphism with urinary bladder (57) and esophageal (58) cancer. Based on our investigations in Iranian population for *CYP1A1* m2 polymorphism, there was no significant difference for G mutant allele between males (8.5%) and females (7.5%). Therefore, genetic m2 polymorphism probably has no interference with gender-specific cancer susceptibility. But for *CYP1A1* m1 polymorphism, C mutant allele frequencies in males (22%) was significantly more than females (15%). This is an intriguing find. A meta-analysis by shaik et al. suggests that although the *CYP1A1* m1 polymorphism is likely to increase the risk of sporadic prostate cancer considerably on a wide population basis, the m2 polymorphism may not influence this risk (59). Acevedo et al. suggest that the *CYP1A1*2A* genotypes may be good prognosis markers for Chilean patients with prostate cancer, particularly in patients with high-risk tumors (60). Ding et al. in a meta-analysis suggest an important role of the *CYP1A1* MspI polymorphism in the risk of developing prostate cancer, especially in Asians (61). Suzuki et al. suggest that metastatic prostate cancer has a significant association with mutated alleles of m1 and m2 and *CYP1A1* polymorphisms has an association with prostate cancer risk, especially with progression of prostate cancer (62). Kristiansen et al. suggest that polymorphisms in the *CYP1A1* gene may contribute to variability of Norwegian Caucasian susceptibility to testicular cancer (63). Based on the studies which were referred above and our findings, this can direct us to investigate the association between this polymorphism and prevalence of male specific malignancies such as prostate and testicular cancer in our population in the future. Also due to significant frequency of C (22%) and G allele (8.5%) in Iranian men, this population may have increased susceptibility of encountering with other malignancies in their life._Moreover, our female population with C and G allele may have increasing cervical cancer suscep-

tibility. The association of polymorphisms, however, may be significant with respect to smoking history, diet habits, ethnicity, and race.

Another interesting finding of this research is that people residing in our studied region, West of Mazandaran, have the intermediate frequency of m1 and m2 mutant alleles between Asian and European groups. As referred in the result section, our prevalence of m1 and m2 polymorphisms is close to the Turkish population. Mazandaran Province is located in the north of Iran and has close proximity to the 2 European-Asian countries, Turkey and Russia. So, it will be expected that our frequencies are between European and Asian groups.

The results of this research, along with the results of other researchers, requires that those carrying the mutant alleles and are at a higher risk of developing certain malignancies should be monitored regularly. Moreover, they could be educated to notice and report any signs that may be related to early stages of cancers. Identification of the common genetic variants is relevant, as their assessment may provide opportunities for screening and reducing possible environmental risk factors. Genetic polymorphisms are known to be the basis of frequently observed variations in activity of the metabolizing enzymes among different ethnic human populations. This might lead to a higher risk of development of toxins and hormone related cancers during a lifetime as a result of fatal gene-environment interaction. Therefore, undoubtedly, there is a need to have knowledge of the distribution of high-risk alleles of metabolizing genes in different ethnic population. Results of the present study might be important in understanding the distribution of *CYP1A1* m1 and m2 polymorphisms in Mazandaran province of Iran. Moreover, these results may determine the susceptibilities of this population towards environmental procarcinogens that result in several cancers.

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