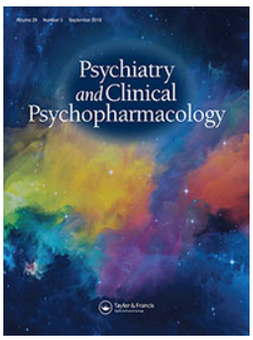

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CYP2A6 gene variants may explain smoking status in a Turkish cohort

Sacide Pehlivan[a], Mehmet Atilla Uysal[b], Tulin Cagatay[c], Ayse Feyda Nursal [d], Cigdem Kekik Cinar[a], Feyza Erkan[c], Ulgen Sever[a], Zuleyha Bingol[c], Mustafa Pehlivan[e] and Sadrettin Pence [f]

[a]Department of Medical Biology, Istanbul University, Istanbul Faculty of Medicine, Istanbul, Turkey; [b]Department of Chest Diseases, Health Sciences University, Yedikule Hospital for Chest Diseases and Thoracic Surgery, Istanbul, Turkey; [c]Department of Chest Diseases, Istanbul University, Istanbul Faculty of Medicine, Istanbul, Turkey; [d]Department of Medical Genetics, Faculty of Medicine, Hitit University, Corum, Turkey; [e]Department of Hematology, Gaziantep University, Faculty of Medicine, Gaziantep, Turkey; [f]Department of Molecular Medicine, Istanbul University, Aziz Sancar Instutute, Istanbul, Turkey

ABSTRACT

OBJECTIVE: Nicotine is the main addictive agent present in tobacco and is principally metabolized by a cytochrome P450-mediated oxidation process. While smoking patterns differ widely among smokers, the metabolization rate of nicotine can also be affected by variations in rates of enzyme activity between individuals. Therefore, we aimed to investigate the significance of *CYP2A* gene variants in the smoking status in a Turkish population using next-generation sequencing (NGS).

METHODS: This case–control study involved 64 subjects with Nicotine dependence (ND) and 36 Non-smoker (NS) subjects. Amplicants designed by "Primer-BLAST" programme were all sequenced using the "Illimuna-MiseqQ-platform".

RESULTS: It was found that there were five SNPs in the *CYP2A6* gene (rs8192725, rs7248240, rs1809810, rs8192733 and rs28399435). *CYP2A6* rs1809810 homozygous TT genotype and T allele were seen in lower percentages in ND group compared to the NS group ($p = 0.045$; $p = 0.021$). Individuals with *CYP2A6* rs1809810 TT genotypes and T allele showed odds ratio of 4.760 and 5.360 for developing protective role ND, respectively. *CYP2A6* rs8192733 CC genotype and C allele were both lower in ND group (respectively $p = 0.001$, $p = 0.023$) while GC genotype was higher in the ND group ($p = 0.004$). *CYP2A6* rs28399435 TT genotype and T allele were more common in the ND group (respectively $p = 0.001$, $p = 0.001$). *CYP2A6* rs28399435 CC genotype was lower in the ND group than in the NS group ($p = 0.010$).

CONCLUSIONS: *CYP2A6* rs1809810, rs8192733, rs28399435 could be genetic risk factors for ND in a Turkish population.

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Smoking; *CYP2A6*; next generation sequencing; variant

Introduction

Tobacco use constitutes an important public health problem in addition to its economic burden. Nicotine is the major compound responsible for the development of cigarette addiction. It leads to addiction by stimulating nicotinic cholinergic receptors (nAChR) that release neurotransmitters into the brain and induce euphoria in the smoker [1]. Nicotine dependence (ND) is regarded as a disease in which genetic factors play a role. These factors include encode proteins in various neurotransmission pathways. Those that have been suggested as taking a part in the nicotine response include nicotinic cholinergic receptors and nicotine metabolic enzymes [2].

CYP2A6 enzyme is a member of an enzyme superfamily known as the cytochrome P450 system (CYP450), whichis classified in the drug metabolizing enzymes group [1]. These enzymes are located in the endoplasmic reticulum of the cells of several tissues in the body, especially in the liver. CYP2A6 metabolizes up to 70% of nicotine into cotinine through C-oxidation [3]. Several other enzymes of CYP450 such as CYP2B6, CYP2A13, CYP2D6 and CYP2E1 also play a minor role in the nicotine metabolism. The CYP2A6 gene (MIM 122720) was mapped to chromosome 19 where it is found within a 350-bp gene cluster along with the CYP2A7 and CYP2A13 genes [4]. The gene contains 9 exons spanning approximately 6 kb and encodes a protein with 494 amino acids [5]. The most common variants of this gene are single nucleotide polymorphisms (SNPs), which make CYP2A6 highly polymorphic, allowing it to produce isoforms that differ in enzymatic activity; hence, the nicotine level in the body differs from person to person. Smokers with distinct CYP2A6 variants manifest different smoking behaviours from those who do not bear these variants. This suggests that smokers regulate their nicotine consumption to maintain a certain drug level in the body [6]. CYP2A6 variability has a heterogeneous distribution in populations worldwide, which

CONTACT Ayse Feyda Nursal feydanursal@hotmail.com, feyda.nursal@gmail.com Department of Medical Genetics, Faculty of Medicine, Hitit University, Corum, Turkey

can explain the various metabolic responses to nicotine that are closely related with ND [1]. Even though some SNPs have been linked with ND in various ethnic populations, they are uncommon in Turkish populations.

Therefore, we conducted a case–control study in a Turkish population to assess the impact of the *CYP2A6* gene variants on smoking status in a Turkish population using next-generation sequencing (NGS). The groups were created as ND and non-smoker (NS) groups.

Methods

Study population

This study is conducted in a single-center, clinic-based, cross-sectional design. We performed a study with a total of 100 individuals enrolled, including 64 smokers (mean age: 47 ± 13) and 36 non-smokers (mean age: 34 ± 12). All subjects were recruited from the Department of Chest Diseases, Yedikule Hospital for Chest Diseases and Thoracic Surgery Training and Research Hospital in Istanbul, Turkey between January 2015 and February 2016. All subjects were matched for age and sex, and recruited from the same institutions during the same time period. The ND group consisted of active smokers. These people were defined as those who had previously smoked more than one cigarette/day but had quit smoking for more than one year. The degree of ND was evaluated by the scores on Heaviness of Smoking Index (HSI) and the Fagerström Test for Nicotine Dependence (FTND) [7]. Subjects in the NS group had never smoked in their life. All groups were selected from subjects who had no major medical and chronic diseases. Neither patients nor control subjects suffered from drug or alcohol abuse/dependence. A written informed consent was obtained from each participant. This study protocol was approved by the Local Ethics Committee (2014/1195) and all the procedures performed in the study were in accordance with the Declaration of Helsinki.

Genotyping

Genomic DNA was isolated from 1.5 mL whole blood collected in EDTA using QIAamp DNA MiniKit (QIAGEN GmbH, Hilden, Germany) and stored at −20°C until analysis. Long range polymerase chain reaction (LD-PCR) was applied for enrichment of *CYP2A6* gene. The primers of amplicants of the *CYP2A6* gene were designed by the "Primer-BLAST" programme. Gel electrophoresis was applied to determine the quality and quantity of the purified DNA. DNA samples amplified using Takara LA Taq Polymerase. Nextera XT DNA Library preparation kit were used for library preparation. DNA library was sequenced on a miSeq instrument with v2 reagent kit (Illumina Inc., San Diego, CA, USA), according to the suppliers' recommendations. Variants in the *CYP2A6* gene were screened by Illimuna-MiseqQ® -platform. Confirmation of the identified variants was done using the Sanger method.

Statistical analysis

We used SPSS 13.0 software package (SPSS, Inc., Chicago, ILUSA) for all analyses and calculation. Odds ratios (OR) and 95% confidence intervals (CI) were calculated. Differences of the CYP2A6 allele and genotype frequencies between groups were assessed using chi-square (χ^2) test, with Fisher's exact test being used when needed. All analyses were two-tailed, with differences being considered as statistically significant when p value < 0.05. Post- hoc power analysis with a preestablished effect size, error probability, and sample size was performed using the G*Power version 3.1.3 programme.

Results

Using *CYP2A6*-specific NGS, we found forty-one different gene variants. Twelve variants were in intronic region while 29 variants were in exonic region of *CYP2A6* gene (Table 1). Thirty-six variants were in the ND group, while 26 variants were found in the NS group. There was a significant difference between the ND and NS groups ($p < 0.05$). There were five SNPs in the *CYP2A6* gene (rs8192725, rs7248240, rs1809810, rs8192733 and rs28399435). It was found that there was a statistically significant difference between the ND and the NS group for 3 SNPs (rs1809810, rs8192733 and rs28399435). Genotype distribution of these SNPs (significant) in the ND and the NS group are summarized in Table 2.

Table 1. *CYP2A6* gene variants in ND and NS groups.

	ND group		NS group	
	Genotypes *n* (%)	Alleles *n* (%)	Genotypes *n* (%)	Alleles *n* (%)
CYP2A6				
1	4 (6.25)	5 (1.059)	2 (5.556)	4 (1.379)
2	6 (9.375)	17 (3.602)	3 (8.333)	10 (3.448)
3	4 (6.25)	15 (3.178)	6 (16.667)	30 (10.345)
4	14 (21.875)	61 (12.924)	7 (19.444)	41 (14.138)
5	6 (9.375)	53 (11.229)	2 (5.556)	17 (5.862)
6	11 (17.187)	95 (20.127)	5 (13.889)	44 (15.172)
7	7 (10.937)	81 (17.161)	4 (11.111)	53 (18.276)
8	3 (4,687)	34 (7.203)	2 (5.556)	19 (6.552)
9	3 (4.687)	37 (7.839)	2 (5.556)	26 (8.965)
10	3 (4.687)	29 (6.144)	2 (5.556)	28 (9.655)
11	3 (4.687)	45 (9.534)	–	–
12	–	–	1 (2.778)	18 (6.207)
Total	**64**	**472**	**36**	**290**

ND: Nicotine dependence; NS: Non-smoker.

Table 2. Genotype and allele distribution of *CYP2A6* variants in ND cases and NS controls.

	ND group	NS group	OR	95% CI	*p**
rs1809810	***n* = 64 (%)**	***n* = 36 (%)**			
Genotypes					
AA	4 (6.25)	0 (0)	1.067	1.001–1.136	0.294
AT	10 (15.63)	2 (5.56)	3.148	0.650–15.248	0.203
TT	50 (78.13)	34 (94.44)	4.760	1.016–22.300	**0.045**
Alleles					
A	18 (14.06)	2 (2.78)			
T	110 (85.94)	70 (97.22)	5.360	1.202–23.913	**0.021**
rs8192733					
Genotypes					
GG	29 (45.31)	16 (44.44)	1.036	0.456–2.354	1.000
GC	28 (43.75)	5 (13.89)	4.822	1.661–14.001	**0.004**
CC	7 (10.94)	15 (41.67)	0.137	0.049–0.381	**0.001**
Alleles					
G	86 (67.19)	37 (51.39)			
C	42 (32.81)	35 (48.61)	0.498	0.275–0.901	**0.023**
rs28399435					
Genotypes					
TT	33 (51.56)	4 (11.11)	8.516	2.699–26.875	**0.001**
TC	13 (20.31)	12 (33.33)	0.560	0.225–1.394	0.240
CC	18 (28.13)	20 (55.56)	0.313	0.133–0.735	**0.010**
Alleles					
T	79 (61.72)	20 (27.78)			
C	49 (38.28)	52 (72.22)	0.231	0.123–0.432	**0.001**

ND: Nicotine dependence; NS: Non-smoker; OR: odds ratio; 95%CI: confidence interval; *Fisher's Exact Test.

rs1809810 (missense variant)

CYP2A6 rs1809810 TT genotype and T allele were lower in the ND group compared to the NS group ($p = 0.045$, OR: 4.760, 95%CI: 1.016–22.300; $p = 0.021$, OR: 5.360, 95%CI: 1.202–23.913, respectively) (Table 2).

rs8192733 (3′ UTR variant)

CYP2A6 rs8192733 CC genotype and C allele were lower in the ND group ($p = 0.010$, OR: 0.313, 95%CI: 0.133–0.735; $p = 0.023$, OR: 0.498, 95%CI: 0.275–0.901) while GC genotype was higher in the ND group ($p = 0.004$, OR: 4.822, 95%CI: 1.661–14.001, respectively) (Table 2).

rs28399435 (missense variant)

CYP2A6 rs28399435 TT genotype and T allele were more common inthe ND group ($p = 0.001$, OR: 8.516, 95%CI: 2.699–26.875; $p = 0.001$, OR: 0231, 95%CI: 0.123–0.432, respectively). CYP2A6 rs28399435 CC genotype was lower in the ND group than the NS group ($p = 0.010$, OR: 0313, 95%CI: 0.133–0.735) (Table 2).

Post-hoc analyses

rs1809819

In the study, while 80% power, 0.05 alpha and 0.2 beta error with at least 16 pieces of samples needed to be taken, we have studied with 36 samples in NS group and 64 samples in ND group, therefore we fulfilled the necessity of this analysis.

rs8192733

While with 80% power, 0.05 alpha and 0.2 beta error, at least 151 samples needed be taken, we have studied 36 samples in NS group and 64 samples in ND group and thus we could not fulfil the necessity of this analysis due to economic deficiencies.

rs28399435

In the study, while 80% power, 0.05 alpha and 0.2 beta error with at least 1 piece of samples needed to be taken, we have studied with 36 samples in NS group and 64 samples in ND group, therefore we fulfilled the necessity of this analysis.

Discussion

In the present study, we aimed to evaluate the relationship between the *CYP2A6* gene variants and smoking status in a Turkish population. To the best of our knowledge, this is the first study that correlates the *CYP2A6* gene variants with smoking status in thispopulation. We showed the significant association between *CYP2A6* gene variants and the smoking status using the NGS method.

Smoking displays a multi-factorial behaviour pattern with both genetic and environmental elements [8]. Even though nicotine is non-carcinogenic, it acts as a psychoactive agent that induces dependence and affects tobacco use characteristics. As in other drugs causing dependence, nicotine elevates dopamine levels in the nucleus accumbens and induces the mesolimbic brain reward pathway [9]. Smokers are known to titrate their nicotine levels through cigarette use, number and volume of puffs, and depth of inhalation, to achieve

and maintain desired levels; therefore, nicotine clearance rate affects smoking behaviour [10]. Nicotine metabolism has many steps and several enzymatic pathways. Upto 75% of nicotine is transformed to cotinine principally by *CYP2A6*, 15% of nicotine is metabolized through other metabolic pathways, and a minor portion (10%–15%) is secreted to urine unchanged. The majority of cotinine is further transformed to 3-hydroxycotinine exclusively by CYP2A6; upto 40% is secreted to urine as 3-hydroxycotinine whereas 10% is further metabolized into 3-hydroxycotinine-glucuronide by UGT enzymes prior to urinary excretion. About 15% of cotinine is transformed to cotinine-glucuronide by UGT enzymes, and the rest is metabolized via other pathways [11].

The *CYP2A6* gene contains nine exons, which are identified by consensus splicing sequences (GT and AG) on the boundaries between intron and exon regions. The transcribed genes belonging to the human *CYP2A* subfamily have many genomic sequence similarities in common. Numerous distinct *CYP2A6* alleles have been defined, such as SNPs, duplications, deletions, and conversions (www.cypalleles.ki.se/cyp2a6.htm). The majority of these *CYP2A6* polymorphisms are non-synonymous SNPs present in exons. *CYP2A6* variations have been phenotypically classified as slow (<50% of activity), intermediate (80% of activity), and normal (100% of activity) metabolizers. *CYP2A6* variability has a heterogeneous distribution in populations worldwide; these ethnic/racial differences have impact on the activity of the enzymes that catalyze nicotine metabolism, thus, the relative distribution of the metabolites excreted may differ among different populations [12]. It was found that African-Americans have a significantly diminished clearance of cotinine, fractional transformation of nicotine to cotinine and metabolic clearance of nicotine to cotinine compared with Caucasians [13]. Furthermore, it was recently reported that Chinese-Americans metabolize nicotine 25% slower than Caucasians and Latinos [14].

CYP2A6 gene polymorphism contains alleles with different structures, varying from deleted alleles to completely functioning alleles, which determine the role their level of enzymatic activity plays in nicotine etiology and the effect they have on ND treatment [15]. The assessment of the enzymatic activities of *CYP2A6* in vitro and in vivo studies has reported that *CYP2A6* polymorphisms can change or increase the pharmacokinetics of nicotine with regard to their structure, thus establishing their tendency to predispose to or protect individuals from ND [16]. In some studies, smokers with *CYP2A6* reduce-of-function genotypes or slower *CYP2A6* activity (lower nicotine metabolite ratio quartiles) manifested lower FTND scores, a measure of the degree of ND, compared to faster nicotine metabolizers [17,18]. On the other hand, many studies showed no difference in FTND among different *CYP2A6* genotype or nicotine metabolite ratio groups [19,20].

Star (*) nomenclature is used to describe different *CYP2A6* alleles, with the "wild type" reference allele defined as *1 [21]. It is known currently that *CYP2A6**6, *CYP2A6**7, *CYP2A6**9, *CYP2A6**10, *CYP2A6**11, *CYP2A6**13 result in reduced enzymatic activities while 5 variants (*CYP2A6**2, *CYP2A6**4, *CYP2A6**5, *CYP2A6**12, *CYP2A6**20) synthesize no functional enzyme [22].

CYP2A6 rs1809810 (*18) exists in exon 8. Tanner and Tyndale identified that *CYP2A6* rs1809810 allele frequency was 1.1–2.1% in white race, 0% in African [23]. Fukami et al. found that the allelic variation in *CYP2A6**18 had an impact on the enzymatic activity and induced reduced metabolism of coumarin, nicotine, and tegafur in vivo and in vitro. They showed that the Km and kcat values of *CYP2A6**18 for nicotine oxidation was elevated compared with those of *CYP2A6**1 [24]. In HaploReg data base, it was reported that this variant causes protein motif changes in RE1 Silencing Transcription factor and Transcription factor 5 [25]. In the HaploReg4 database, it has been reported that this missense variant causes protein motif change in the RE1 Silencing Transcription Factor and Transcription Factor 5 [26]. In the rVarbase data base, it was reported that it functions in the chromatine state (weak transcription, enhancers and strong transcription) [27]. In present study, we found that *CYP2A6* rs1809810 TT genotype and T allele had lower percentage in ND group compared to NS group ($p = 0.045$, $p = 0.021$). Individuals with TT genotype and T allele showed an odds ratio of 4.760 and 5.360 of developing protective role ND, respectively.

CYP2A6 rs8192733, non-coding SNP, is found in the 3' UTR region of *CYP2A6* gene. It is associated with increased protein expression and enzyme activity. It was reported that *CYP2A6* rs8192733 allele frequency was 47–48% in white, 23% in African [28]. A recent unpublished study has demonstrated that Alaska Natives have a higher frequency of the increase-of-function *CYP2A6* rs8192733 than White and African American populations do [23]. In the HaploReg4 database, it was reported that this 3'UTR variant causes protein motif change in LF-A1 and Interferon Regulatory Factor, and acts as a promoter histone marker in 10 tissues and as an enhancer histone marker in 11 tissues [25]. In the rVarbase database, it was reported that it functions in the Chromatin state (Active TSS, Enhancers, Weak Transcription) [27]. We found that *CYP2A6* rs8192733 CC genotype and C allele were lower frequency in ND group compared to the NS group ($p = 0.001$, $p = 0.023$). Also, the subjects carrying GC genotype had 4.822-fold increased risk ND ($p = 0.004$) (Table 2). This was considered a heterozygote disadvantage.

CYP2A6 rs28399435 (*14), in exon 1, is a missense variant. It is significantly associated with reduced in vivo *CYP2A6* RNA expression and slower in vivo metabolism [29]. In HaploReg4 data base, it has been reported that this missense variant causes 6 different protein motif changes, acts as a promoter histone marker in LIV (estrogen regulated) gene and as an enhancer histone marker in GI (G protein subunit alpha) [25]. In rVarbase data base, it was reported that it functions in its chromatin state (Active TSS, Enhancers, weak transciption) [27]. In present study, we also found *CYP2A6* rs28399435 TT genotype and T allele were more common in the ND group than in the NS group ($p = 0.001$; $p = 0.001$, respectively) (Table 2). The subjects carrying the TT genotype had 8.516-fold increased risk of ND. *CYP2A6* rs28399435 CC genotype seems to be a protective factor for ND ($p = 0.010$).

Although there are many studies investigating the frequency of these variants in different ethnic groups, no study has been found investigating its relationship with ND. In present study, there is one value that involve "1" confidence intervals. It was not statistically and clinically significant. The results of post-hoc analysis revealed the achieved power of the study for the sample size.

The present study bears some limitations. First, the present study has a relatively small population size and may not have the necessary strength. Second, this study focuses on only the Turkish population. Third, ND has complex genetic architecture challenges. We may not have captured other minor genetic variations which could have contributed to the nicotine metabolism.

Drug metabolism is the most studied domain in terms of individual treatment and contains the strongest findings [30]. *CYP2A6* belongs to the CYP2A subfamily and plays a role in the biotransformation of many drugs, such as nicotine, halothane, disulfiram and valproic acid as well as the metabolism of carcinogens like nitrosamines and aflatoxin B1. Therefore the variants of *CYP2A6* might be clinically useful. We showed for the first time that *CYP2A6* gene variants are associated with smoking status in a Turkish cohort by NGS. This result suggests that the SNPs (rs1809810, rs8192733 and rs28399435) of the *CYP2A6* gene may affect smoking status in a Turkish population. As allelic differentiations of *CYP2A6* may affect smoking cessation outcome, and therapeutic response, further research with a larger sample of the Turkish population is necessary for the development of personalized approaches.

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ORCID

Ayse Feyda Nursal http://orcid.org/0000-0001-7639-1122
Sadrettin Pence http://orcid.org/0000-0001-9453-4166

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