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ABSTRACT

The study aimed to discover some variables influencing the risk of nicotine dependence in the Anatolian population. We examined 10 candidate genes and analysed 10 single nucleotide polymorphism (SNP) loci in 50 cases and 50 controls in a preliminary study. The main purpose of this work was to provide predicative definition in this regard. Buccal swab samples were collected from 50 cases and 50 controls, who had volunteered to participate in the study. The Fagerström Test for Nicotine Dependence (FTND) score of 4 or more was required for the case group. The control group was strictly chosen from people who had an FTND score of 0. SNP loci were genotyped and variations were observed in five SNP loci (rs16969968, rs806380, rs3733829, rs6474412, rs1329650). Based on these results, polymorphism was identified in the Turkish population in five SNP loci (rs16969968, rs806380, rs6474412, rs37333829, rs1329650). Although our findings indicated a non-significant association between the 10 selected loci and nicotine dependence in the Anatolian population, we believe that with a larger number of cases with five SNP loci, the variance we find will provide significant results for both forensic geneticists and psychiatrists. Since, to our knowledge, this is the first work to explore the potential association of these SNPs with nicotine addiction in the Anatolian population, the results should be expanded with larger sample groups.

Introduction

Addiction is an important field of forensic psychiatry. The progress of deoxyribonucleic acid (DNA) technology in forensic science has led to the emphasis on DNA phenotyping studies. Addiction is the repeated involvement with a substance or activity, despite the substantial harm it now causes, because that involvement was (and may continue to be) pleasurable, and/or valuable [1]. Indeed, addiction is a complex series of diseases affected by genetic and environmental factors. Addiction to both legal and illegal substances is a common public health concern [2]. Addictions are compulsive, uncontrollable behaviours that become chronic, and they can have devastating long-term consequences. Eventhough addictive substances such as narcotics and tobacco are volitive, addiction can cause the person to lose volitive control. Excessive use of alcohol and nicotine are reported as the major causes of preventable morbidity and mortality in Western societies [3]. Predisposition to alchol addiction, nicotine or illegal substances have the same genetic background [4, 5]. Eventhough the factors are substance-specific, all genetic predispositions to different substancess how common characteristics [6, 7]. Important research has been carried out on the genetics of addiction. These studies targeted the mechanisms in the brain that operate via the biogenic amines, such as acetylcholine, dopamine, and serotonin including receptors and carriers [8, 9].

Nicotine addiction is a type of addiction that reduces the quality of life and can even be fatal. It is reported that the reason behind one in every five deaths in the world is nicotine addiction caused by cigarette smoking [10]. Smoking or inhaling the

Table 1. Primers used in the study.

NCBI Rs code	Forward primer	Reverse primer	Amplicon length (bp)
rs1051730	GGCTCTTCCATGAACCTCAA	GCCGGATGTACAGCGAGTAT	139
rs6474412	CAACAGAGCAAGACAGCCTCT	TCAACTCTTCACCATCCCACT	117
rs2184026	TGCAGCAAAAGAAAGCTGAA	AGGAAAGATGCTGGGTCACA	83
rs1801272	CGGGCTTCCTCATCGAC	TGGGTGTTTTCCTTCTCCTG	83
rs16969968	TGAGAGTGGTAGTGGACCAAAA	CCTCACGGACATCATTTTCC	107
rs806380	CCGCCCAGAAAGCAATTAT	CACCTTCCACTTTTAATGACCAA	149
rs1329650	GAATAAGAGGGCCGTGATGA	CTACCCCTTACTGGGCAGGT	127
rs3733829	TGCACTGCTCACTCCCTATG	TGAGGTGGTTGCTCAGAGTG	50
rs3025343	TGAAGTGGTTGCTGTTTCCA	ATGGCTGCAGGCTAAGAAGA	113
rs6265	TGGCTGACACTTTCGAACAC	AGAAGAGGAGGCTCCAAAGG	145

cigarette smoke causes psychological and physical addiction over time [11]. Studies have shown that genetic factors also play an important role in nicotine addiction. These genes are of the nicotine receptor subunits, the dopamine receptors and carriers, and the gamma-aminobutyric acid (GABA) receptors in the brain [12–15].

In the study by Saccone et al. [16] on secondary genes, the research was mostly focused on the α5/α3/β4 nicotinic cholinergic receptor gene complex located on Chromosome 15. Nicotine addiction depends on the changes detected in the α5/α3/β4 gene loci, the number of cigarettes smoked per day, blood cotinine (biomarker for the intake of nicotine) in the blood, and the amount of cigarette-associated carcinogenic substances in the urine. Cholinergic receptors trigger the release of dopamine and other neurotransmitters from the brain. Dopamine, glutamate, and GABA release play an important role in nicotine dependence in brain functions. In order to adapt and tolerate this situation, nicotinic receptors in the brain cause changes in neuronal plasticity [16]. The relationship between genetics and cigarette addiction is important for the fields of forensic science and criminology, which continue their studies based on the hypothesis that people with criminal tendencies share common characteristics. Identification of these characteristics is significant for the preventability of the crime before it is committed. In this regard, identification of characteristics of an individual, primarily in terms of nicotine, and in terms of other types of addiction in the further studies, will be a clue of utmost importance for forensic science. Thus, it is hypothesised that genetic information may give an idea about the psychiatric tendencies of an individual and the characteristics of these tendencies.

The aim of this study was to investigate 10 SNP loci (rs2273504, rs806368, rs2023239, rs806380, rs324420, rs2184026, rs16969968, rs1051730, rs4950, and rs686) in 50 control subjects and 50 nicotine addicts selected at random, to analyse the genetic polymorphisms and to determine whether there is a relationship between these and cigarette addiction. This study will also contribute to the literature in terms of predictive identification.

Subjects and methods

Study design and sample collection

This study was carried out in Istanbul University— Forensic Molecular Genetics Laboratory of Forensic Sciences Institute of Cerrahpasa. In this study, a total of 100 FNS (Fagerstrom Test of Nicotine Dependence) tests were used, including 47 women (23 cigarette addicts, 24 controls) and 53 men (27 cigarette addicts, 26 controls), who gave permission for research and declared themselves as belonging to the population of Anatolia. Swab samples were taken. In order to avoid possible contamination and to obtain the most efficient amount of DNA, the samples were kept in Eppendorf™ tubes at +4° C for a maximum of 24 hours and the DNA isolation step was taken as soon as possible. If DNA isolation was not possible immediately, the samples were incubated at −20 °C. Since the laboratory in which this study was carried out has TSE/IEC 17025 accreditation criteria, necessary precautions were taken in order to prevent contamination.

Ethics statement

The study protocol was approved by the board of the Istanbul University —Cerrahpasa Ethics Committee (2012/1813), and informed consent was obtained from all volunteers.

DNA isolation and quantification

DNA isolation of the samples was performed by the buccal swap procedure of the QIAamp® DNA Mini Kit. Quant-iT™ dsDNA HS (High Sensetive) kit was used to determine the amount of DNA and measurement was made on a Fluorometer (Invitrogen) device.
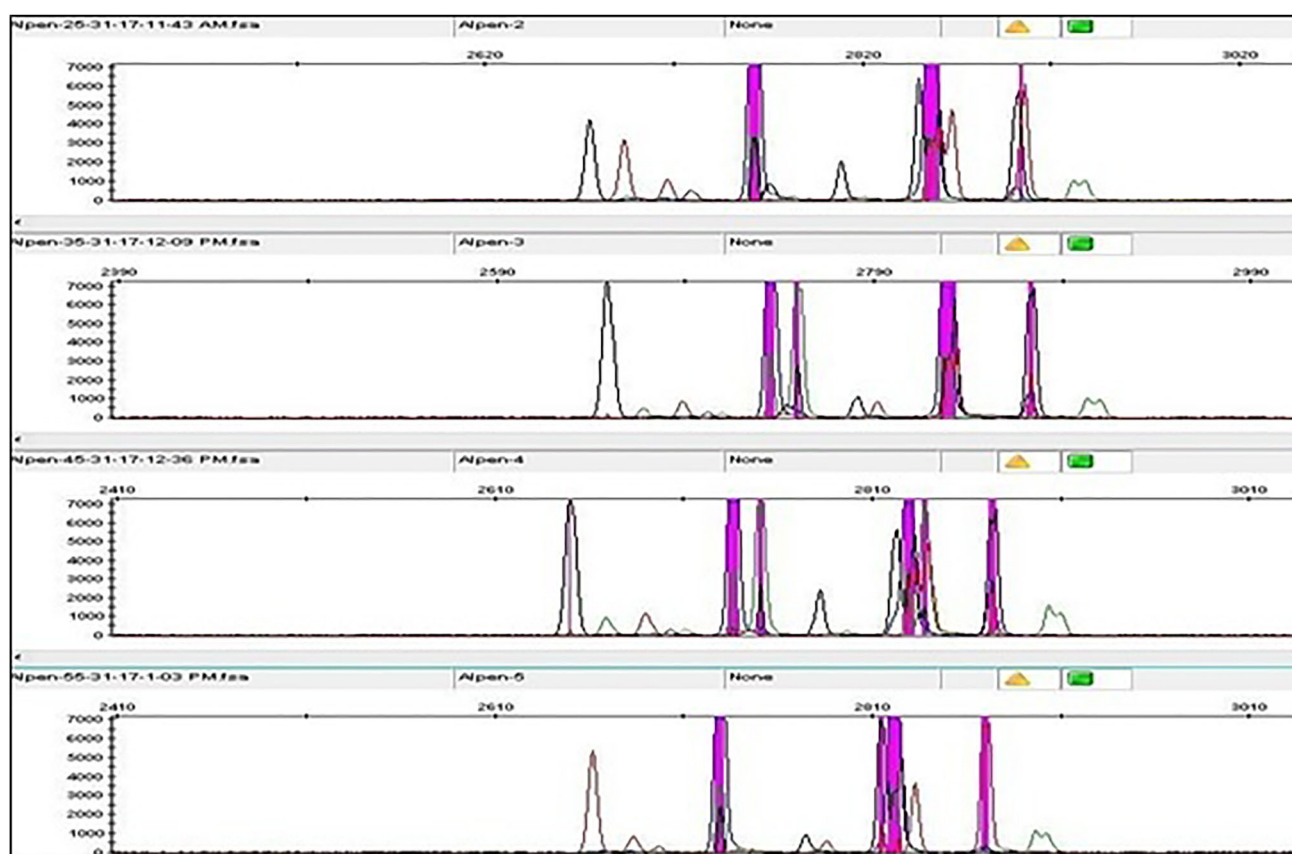
Figure 1. Representative multiplex images for all 10 loci in samples from four active cigarette addicts.
Note: The pink columns represent the pull-up peaks.

Multiplex polymerase chain reaction (PCR)

The respective ranges of the 10 identified SNPs (rs2273504, rs806368, rs2023239, rs806380, rs324420, rs2184026, rs16969968, rs1051730, rs4950, and rs686) were amplified in the first PCR. In the second PCR, a SNaPshot Multiplex Kit was used following purification. Primers designed to be suitable for multiplex work are shown in Table 1. QIAGEN Multiplex PCR kit was used in the study.

Purification of second PCR products

In the purification step, 1 μl Shrimp Alkaline Phosphatase (SAP) (1 U/μL) was used to remove the dideoxynucleotides (ddNTPs). The samples were vortexed after addition of the substance and were centrifuged at 3000 rpm for 30 s (Thermo Scientific™ Heraeus Biofuge). The obtained mixture was incubated at 37 °C for 90 minutes and at 85 °C for 15 minutes.

Electrophoresis and evaluation of secondary PCR products

Sample lists were prepared through Data Collection Software 3.0 (Applied Biosystems) prior to each execution. The GeneScan E5 module parameters were adjusted and Matrix Standards Set DS-02 kit (Applied Biosystems) was used. The samples were loaded on the ABI Prism 310 Genetic Analyzer. For statistical analysis, data were evaluated using the NCSS 2007 (Number Cruncher Statistical System) program. Descriptive statistics methods (Frequency, Ratio) were used for the evaluation of the study data and Fisher's and *chi*-squared tests were used for comparing the qualitative data.

Results and discussion

The multiplex image for all the loci (rs1051730, rs6474412, rs2184026, rs1801272, rs16969968, rs806380, rs1329650, rs3733829, rs3025343, and rs6265) in four active cigarette smokers is shown in Figure 1.

The detailed genotype information about the 10 SNPs in 25 samples randomly selected from the genotype results obtained from 50 passive and 50 active smokers who are non-related, is shown in Table 2. According to these results, polymorphism was identified in the Anatolian population in five SNP loci

Table 2. Genotype information obtained from 25 selected samples.

Sample code	Smoking	rs16969968 A/G	rs806380 A/G	rs3025343 A/G	rs6265 A/G	rs1051730 C/T	rs6474412 C/T	rs3733829 C/T	rs2184026 C/T	rs1329650 A/C	rs1801272 A/T
S14	Active	A/A	A/A	G/G	G/G	C/C	C/C	C/C	C/C	A/A	T/T
S15	Active	G/G	A/A	G/G	G/G	C/C	C/C	C/C	C/C	A/C	T/T
S16	Active	G/G	A/A	G/G	G/G	C/C	C/C	C/C	C/C	A/A	T/T
S17	Active	G/G	A/G	G/G	G/G	C/C	C/C	C/C	C/C	A/A	T/T
S64	Active	G/G	A/A	G/G	G/G	C/C	C/C	C/C	C/C	A/A	T/T
S65	Active	G/G	A/A	G/G	G/G	C/C	T/T	C/T	C/C	.	T/T
S73	Active	G/G	A/A	G/G	G/G	C/C	C/C	C/C	C/C	A/A	T/T
S69	Active	G/G	A/A	G/G	G/G	C/C	C/C	C/T	C/C	A/A	T/T
S70	Active	G/G	.	G/G	G/G	C/C	.	C/T	C/C	A/A	T/T
S71	Active	.	A/A	G/G	G/G	.	C/C	C/T	C/C	.	T/T
S50	Active	G/G	A/A	G/G	G/G	C/C	C/C	C/C	C/C	A/A	T/T
S55	Active	G/G	A/A	G/G	G/G	C/C	C/C	C/C	C/C	A/A	T/T
S59	Active	G/G	A/A	G/G	G/G	C/C	C/C	C/C	C/C	A/A	T/T
C6	Passive	G/G	A/A	G/G	G/G	C/C	C/C	C/C	C/C	A/A	T/T
C7	Passive	G/G	A/A	G/G	G/G	C/C	C/C	C/C	C/C	A/A	T/T
C8	Passive	G/G	A/A	G/G	G/G	C/C	C/C	C/C	C/C	A/A	T/T
C171	Passive	G/G	A/A	G/G	G/G	C/C	C/C	C/C	C/C	A/A	T/T
C172	Passive	G/G	A/A	G/G	G/G	C/C	C/C	C/C	C/C	A/A	T/T
C155	Passive	G/G	A/A	G/G	G/G	C/C	C/C	C/C	C/C	A/A	T/T
C8	Passive	G/G	A/A	G/G	G/G	C/C	C/C	C/C	C/C	A/A	T/T
C10	Passive	G/G	A/A	G/G	G/G	C/C	C/C	C/C	C/C	A/A	T/T
C11	Passive	G/G	A/A	G/G	G/G	C/C	C/C	C/C	C/C	A/A	T/T
C145	Passive	G/G	A/A	G/G	G/G	C/C	C/C	C/C	C/C	A/A	T/T
C160	Passive	G/G	A/A	G/G	G/G	C/C	C/C	C/C	C/C	A/A	T/T
C164	Passive	G/G	A/A	G/G	G/G	C/C	C/C	C/C	C/C	A/A	T/T

(rs16969968, rs806380, rs6474412, rs37333829, and rs1329650).

In terms of smoking, there was no statistically significant difference between the C/C and T/T genotypes of the rs6474412 locus ($p > 0.05$) (Table 3).

Substance dependence is a chronic brain disease caused by neurological changes characterized by the compulsive search for and intake of substance [14]. Dependence is reported as a public health concern with detrimental effects at a familial and social level throughout the world [15]. Various studies have shown that, in addition to the contribution of the environmental factors, multiple genes and genetic factors also play a role in drug addiction. Cigarettes and marijuana are the leading causes of preventable death and are commonly abused. Many researchers have reported the presence of alleles that predict dependence on legal and illegal substances [17, 18].

Multi-SNP associations with cocaine, opioid, nicotine and alcohol dependence have also been studied [19]. In addition to these, it has been reported that various aspects of impulsive personality and/or psychiatric disorders emerge with substance dependence [20]. In these cases, it is important to investigate the behavioural characteristics as an additional field of study towards forensic DNA phenotyping.

Previous studies showed that the A allele of rs16969968 increased the possibility of becoming an addict in both males and females (allele frequency of 38% in nicotine addicts and 32% in non-smokers)

Table 3. Evaluation of the rs6474412 locus in terms of smoking.

rs6474412 locus	Total n	Total %	Active (n = 50) n	Active (n = 50) %	Passive (n = 50) n	Passive (n = 50) %	ap
C/C Genotype	94	98.0	45	95.8	49	100	0.237
T/T	1	1.0	1	2.1	0	0	0.490
C Alleles	188	98.4	90	96.8	98	100	0.114
T	3	1.6	3	3.2	0	0	

[a]Fisher's Exact test.

when compared to non-smokers [16, 21]. The CHRNA5 gene found in Chromosome 15 shows very different allele frequencies among the world populations. The rs16969968 risk variant, which causes amino acid changes, while being widespread in some societies of European origin (minor allele frequency, MAF = 0.42), is rarely found in Asian samples (MAF = 0.01–0.03) and not at all in Sub-Saharan African populations (MAF = 0) [11]. In our study, polymorphism was seen in just two of the 50 samples in this region.

The SNP rs806380 on the CNR1 gene, which is a locus referred to by Corradini et al. [22] in the study on the genetic variants of nicotine dependence, has been associated with symptoms of marijuana dependence in adolescents of the Caucasian population [23]. At the same time, it was proposed that this locus can be associated with multiple substance use in

European-American subjects [24]. In our study, polymorphism was observed in only one subject among 50 nicotine addicts.

Chen et al. [25] investigated rs6474412 found in CHRNA6-CHRNB3 gene set on 8p11, rs3733829 found in EGLN2 in the vicinity of CYP2A6 gene on 19q13, and rs1329650, an intergenic region on 10q23 in 2047 samples of European origin (1062 addicts and 985 control subjects) depending on the distribution and weight of the questions on the FTND test. In their study, rs6474412 and rs3733829 were poorly correlated with dependence, whereas rs1329650 was not correlated. In our study, in terms of smoking, no statistically significant difference was detected between the C and T alleles in the rs6474412 locus, but polymorphism was detected in only one subject. In terms of smoking, no statistically significant difference was detected between C/T, C/C and C/- genotypes in the rs3733829 locus ($p = 0.056$; $p > 0.05$), whereas the C/C genotype was observed in four subjects who smoked. The high ratio of C/T genotype observed in non-smokers was striking. In terms of smoking, no statistically significant difference was detected between C and T alleles in the rs3733829locus ($p > 0.05$). No statistically significant difference was detected between A/C, A/A and A/- genotypes in the rs1329650 locus ($p > 0.05$), but the A/C genotype was detected in only one smoker. No changes were observed in some of the selected regions (rs3025343, rs6265, rs1051730, rs2184026, rs1801272). Failure to obtain any results particularly in regions which are known to have strong association was attributed to the small sample size. In a study conducted in 2017, none of the tested loci significantly influenced the association between rs3025343 and smoking cessation. Overall, marital status, education, depression, alcohol use, self-rated health, and chronic obstructive pulmonary disease (COPD) showed phenotypic associations with smoking cessation, but the association of various phenotypes with smoking cessation did not vary by genotype [26]. Our findings are in agreement with these results.

A comprehensive study investigated the CHRNA3 polymorphism and lung cancer susceptibility of carriers of rs16969968 in CHRNA5 and rs1051730 in the Palestinian population in relation to cigarette smoking [27]. The variant SNP rs16969968 in both heterozygous and homozygous forms appeared to exert a significant effect on nicotine dependence. Nicotine addiction is the world's most common addiction type, which reduces the quality of life and even results in death. The cause of one out of five deaths on Earth is cigarette consumption, which is the result of nicotine

addiction [18]. As expected, smoking was strongly associated with lung cancer. The risk allele rs16969968 in CHRNA5 also showed a significant association with increased lung cancer risk, alone and with smoking as a co-variable [28]. Comparison of an analysis performed with other populations suggested that individuals with the rs16969968 risk allele in the Indian and Caucasian population are more susceptible to lung cancer [28, 29].

Another study carried out in Poland found a strong association between risk allele A of rs1051730 and smoking cigarettes per day [30]. These findings are supported by the results in the present study. Further studies will show how the determination of a strong relationship between a cluster of genes and a phenotype (e.g., CHRNA5-CHRNA3 and nicotine dependence) can help in the elucidation of other genes or phenotypes involved in the pathophysiology of nicotine dependence and related complications [31].

This study has some limitations. These are the small number of samples, the diffculties in obtaining a sufficient amount of DNA from the buccal swab samples, the risk of contamination if the samples were not processed immediately, and the fact that analysis can be conducted according to the weight of each question in the FTND test given to the smokers. Significant results can be obtained if the sample size is increased and more SNP loci associated with dependence are included in the studies performed in this field. It is considered important to perform another study on five SNPs (rs16969968, rs806380, rs3733829, rs6474412, and rs1329650) which showed the least amount of change in a way that they could not give statistically significant results, and on rs1051730, which was studied together with CHRNA5/A3/B4 gene set in other populations [21]. In addition, the data obtained when the study is repeated with the addition of rs578776 in CHRNA3 gene, which was not included in our study, and with a larger sample size, will have a significant contribution to the fields of forensic science, forensic psychiatry and pharmacogenetics of dependence in terms of the Anatolian population. Further studies on the SNPs in association with forensic DNA phenotyping and forensic psychiatry will yield useful results for the professionals in many fields.

Conclusions

To the best of our knowledge, this is the first attempt to seek association between SNPs and nicotine addiction in the Anatolian population. The results from this study neet to be expanded with larger sample groups.

Thus, this study contributes to the knowledge obtained from similar studies conducted in many countries and by adding data about the Turkish population. Further studies will also throw more light on the predisposition of the Anatolian population to cigarette consumption, which is also important in terms of community health as well as forensic sciences.

Disclosure statement

Funding

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