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Phenotypic Traits and Genetic Markers Associated with Smoking

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Abstract

Introduction: Smoking is a complex addiction, with heterogeneous phenotypic traits and marked genetic influence. In this study, we compared the variant rs1137115 of the *CYP2A6* gene, which encodes the enzyme that metabolizes nicotine, and variant rs16969968 of the *CHRNA5* gene, which encodes the receptor to which nicotine binds, between smokers and non-smokers. We assessed any associations between these molecular markers and phenotypes of smokers.

Methods: We surveyed 101 non-smoker individuals and 101 nicotine-dependent individuals according to the NDSS-S scale, paired by age and gender. We identified the rs1137115 and rs16969968 alleles using real-time PCR.

Results: We found associations between the NDSS-S scale scores and age, years of smoking, number of cigarettes per day and number of attempts to quit smoking. The genotype distributions of the rs1137115 and rs16969968 alleles showed no differences between the control and smoker groups. In the smokers, the rs16969968 allele was not associated with any of the phenotypic variables studied, but the rs1137115 marker of the CYP2A6 gene was associated with the NDSS-S scale score (G+GA = 21.4±5.5 vs AA = 15±4.6; p = 0.04). This difference is conserved only among smokers who are also homozygous wild type at the CHRNA gene (haplotypes G/G and G/GA = 22.7±5.2 vs the rest of haplotypes= 19.9±5.6; p = 0.02).

Conclusion: Individuals carrying the wild-type allele of the CYP2A6 gene have higher scores on the nicotine dependence scale. This genotype-phenotype association is conserved only for those who also have the wild-type form of the nicotine receptor.

Keywords: Addiction; CYP2A6 gene; CHRNA5 gene; Genetic Polymorphism; Smoking

Introduction

More than 20% of the world's population suffers from nicotine dependence. According to the World Health Organization, smoking is responsible for the death of approximately 6 million people each year and kills more people than tuberculosis, HIV/AIDS and malaria combined. Moreover, in the next two decades, the annual deaths related to smoking (ischemic heart disease, heart failure, cancer, stroke and COPD) are expected to increase to 8 million people, of which 80% will occur in low- and mid-income countries. These numbers make smoking the most important preventable cause of morbidity and mortality due to cancer, respiratory and cardiovascular diseases [1,2]. In 2013 in Colombia a global rate of cigarette consumption of 12.9% (18.8% men and 7.4% women) was estimated in the last month, suggesting that there were approximately 3 million people smoking at the time of the survey [3].

Many compounds are present in cigarettes, but nicotine is the addictive component. It acts through cholinergic nicotinic receptors that are widely distributed in the peripheral and central nervous systems. The nicotine receptor is formed by 5 protein subunits organized in different combinations around

a central pore that acts as an ion channel. These proteins are encoded by 16 different genes (α1-7, 9, 10, β1-4, δ, ε and γ) [4]. When these receptors are activated by agonist compounds, they allow Ca^{2+} to enter the neurons. The subtypes containing the α3 and α5 subunits are expressed in regions of the brain involved in the rewards, emotions, learning and memory systems of the prefrontal cortex and of the mesolimbic reward pathway, which are associated with addictive behaviors [5]. By other hand, the first step of nicotine metabolism is the 5' oxidation of cytochrome P-450 2A6 (*CYP2A6*), and the product of this reaction is again oxidized to cotinine by the same *CYP2A6* or by the aldehyde oxidase [6].

Like all addictions, smoking is influenced by genetic and environmental factors. While the environmental influence has a very important role in smoking onset, the effect of heritability (the risk fraction attributable to genetic factors) in the persistence and intensity of smoking and in nicotine dependence is estimated at approximately 60% to 70% [7].

There is a controversy regarding the genetic variants influencing nicotine addiction. In fact, a recent study gathering scientific evidence prioritized approximately 220 genes as the most influential in the addiction to nicotine [8]. However, studies have focused on genes encoding for the main enzyme responsible for nicotine's metabolism in the liver (CYP2A6), genes encoding neuronal nicotine receptors (CHRNA5/CHRNA3/CHRNB4) and genes involved in neurotransmitter systems responsible for the reward centers, these genes encode proteins such as D2 (dopamine receptor), SLC6A4 (serotonin transporter), HTR2A (serotonin receptor), CNR1 (cannabinoid receptor) and OPRM1 (mu opioid receptor) [7,9-11].

Despite the great number of candidate genes studied regarding nicotine's pharmacokinetics, the strongest associations with smoking have been found at the metabolism level. For instance, *CYP2A6* is polymorphic, and compared with the wild-type genotype, some alleles are associated with a lower risk of addiction to nicotine, particularly those translated into a "metabolize deficient" phenotype such as those sharing the polymorphism rs1137115 (51G>A) [6,7].

Several studies have identified three genes (*CHRNA5*/*CHRNA3*/*CHRNB4*), which encode the subunits of the nicotine receptor α5-α3-β4 in chromosome 15 (region 15q25.1), that are associated with nicotine dependence, pulmonary adenocarcinoma (although nicotine is not carcinogenic *per se*) and COPD. The rs16969968 variant (1192G>A) of the α5 subunit of the *CHRNA5* receptor has generated great interest because of the consistency of the results yielded by studies related to smoking, even those on the response to smoking cessation programs [2]. This polymorphism changes aspartate for asparagine in position 398 (D398N) of the α5 subunit, resulting in a decreased affinity of the receptor for its agonists [7,13-20]. Interestingly, the combined effect of polymorphisms in the *CYP2A6* gene and the 15q25.1 region on the addictive behaviour of teenagers [21] and on lung cancer [22] has been studied, and although both genotypes have independent effects, each one doubles the risk of these pathologies.

The existence of a genetic marker (rs16969968) as a common denominator in nicotine dependence, lung cancer and COPD affirms that the genetic research on smokers is clinically relevant, even without considering that smoking contributes to other cardiovascular diseases [4]. However, studies show that this association only explains a small proportion of the disease's variance, and therefore, most genetic contributions have not yet been detected. Moreover, the allelic frequencies of polymorphisms in *CYP2A6* and *CHRNA5* vary between ethnic groups [3]. For example, while rs16969968 has a high frequency in the European Caucasian population (the frequency of AA homozygotes is between 12% and 18%), in Mexican and Spanish populations, it is approximately 6% and is less relevant in Afro-American (0.04%), Asian (0.01%) and African populations (0.00%) (http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs=16969968). Given the high prevalence of nicotine dependence in these populations, the possibility of the influence of other polymorphisms outside of the *CHRNA5*/*CHRNA3*/*CHRNB4* cluster on nicotine addiction must be considered in individuals with non-European ancestry [3,24,25]. Therefore, genetic studies on non-Caucasian individuals are desirable to broaden the search for biologically important genetic variants. Greater knowledge of the genetic contribution to smoking may advance treatments and be used to identify new therapies that help to reduce the morbidity and mortality associated with this addiction.

In this study, we aimed to compare the prevalences of rs1137115 variant of the *CYP2A6* gene and the rs16969968 variant of the *CHRNA5* gene in smokers and non-smokers and to find for associations between these genetic markers and phenotypes of smokers.

Materials and methods

Legal aspects. This study was approved from the technical and scientific points of view by the authorities of Fundación Universitaria Autónoma de las Américas and Universidad Tecnológica de Pereira. The bioethics approval was conducted through the Bioethics Committee of the Technological University of Pereira. The informed consent was filled according to resolution 008430 of 1993 of the Health Ministry of Colombia, in the research category of minimum risk.

Participants. We enrolled 101 persons currently smoking (cases) and 101 who have never smoked (controls) of both genders who were 18 years or older, non-related and phenotypically mestizos. We included persons that fulfilled the following criteria: (i) smoking ≥ 1 year and (ii) more than eleven points on the "short nicotine dependence syndrome

scale" (NDSS-S) [26]. We excluded minors, pregnant women and persons with backgrounds of mental diseases or abuse/dependence of psychoactive substances. The participants in the control group had to have been exposed to cigarette smoke but never developed a regular consumption pattern. Using a personal interview, we completed an information recollection form and conducted the Fargerström and NDSS-S tests for each volunteer. A blood sample for genotyping was taken using digital puncture. The persons in charge of the genetic characterization were blind regarding the condition of case or control of the participants.

Phenotypes of the smokers. We examined the following phenotypes, which were previously demonstrated to be heritable in several ethnical groups: a) age at smoking onset and years smoking, determined from the moment the daily consumption started; b) previous attempts to quit smoking; and c) the amount of tobacco consumed, evaluated by self-report of the average number of cigarettes consumed per day (CPD). Although individuals with similar numbers of cigarettes smoked can have different nicotine consumption due to the differences in smoking styles, the types of cigarettes smoked, the proportion of cigarette smoked and the nicotine content in the cigarette, self-report was the chosen method for the evaluation of the number of cigarettes smoked. Nicotine dependence was assessed using the Fargerström (a survey of the severity of nicotine dependence with a range of 0–10, in which higher values indicate higher dependence) [27] and NDSS-S tests (a survey of six items, each one with a value of 1–5 points, for a maximum of 30 points, with a cutoff point of nicotine dependence ≥ 11 points) [26]. It should be noted that both surveys are validated in Spanish [28].

Genotyping. Based on a previous genomic DNA extraction from a blood stain on filter paper using the QIAGEN DNA Minikit following the manufacturer's instructions (QIAGEN), the genotypes at *CYP2A6* (rs1137115, 51G>A) and *CHRNA5* (rs16969968) were determined using real-time PCR. The primers and Taqman probes were designed and supplied by Applied Biosystems with the following assay codes: C_26681694-20 (rs1137115) and C_26000428-20 (rs16969968). Each PCR reaction was performed in a 25 µL reaction with 1–10 ng genomic DNA, 1X TaqMan Genotyping Master Mix and 1X Taqman SNP Genotyping Assay Mix, following the manufacturer's instructions. In each PCR reaction, positive and negative controls were used. The amplification and detection were performed in a SmartCycler machine (Cepheid) under the following conditions: an initial denaturing step at 95ºC for 10 min, followed by 40 cycles of 15 sec at 95ºC and 1 min at 60ºC. The results were analyzed using the SmartCycler software (Cepheid) according to the visualization of the curve specific for each allele. All of the randomly chosen samples were confirmed by sequencing.

Statistical analysis. The Chi squared test was used to establish Hardy-Weinberg equilibrium and for the comparisons between qualitative variables. The quantitative variables were compared with a Student's t-test or ANOVA. We worked with binary correlation analysis and confidence intervals of 95%; p values < 0.05 were considered significant. The SPSS Statistics software v.20 was used for general statistical analysis.

Results

The 202 volunteers (101 cases and 101 controls) were 42.4 ± 15.7 years old (range 18–81 years), and 69.8% (n= 141) were male. There were no differences between cases and controls regarding age (43 ± 16 vs 42 ± 15; p = 0.58) or gender (male 68% vs 70%; p = 0.88).

The smokers (n = 101) exhibited a male:female ratio of 2:1. They started regular consumption of cigarettes at 16.1 ± 4.8 years old, had a trajectory of 27.1 ± 16.5 years smoking, smoked between 11 and 20 cigarettes daily and had performed an average of 3.7 ± 4.8 failed attempts to quit smoking. The mean number of points on the Fargerström scales was 4 ± 2.7, while it was 21.2 ± 5.6 on the NDSS-S scale. When using the Fargerström test criteria, 32.7% were non-dependent smokers (≤ 2 points) and 67.3% were dependent smokers (≥ 3 points), while on the NDSS-S scale, all smokers were nicotine dependent (≥ 11 points) given that this was an inclusion criterion. Men started cigarette consumption at younger ages than women (14.9 ± 3.6 years vs 18.7 ± 6 years; p = 0.001). Table 1 shows that both tests significantly correlate with the number of years smoking, number of cigarettes per day and number of attempts to quit smoking, but not with age of consumption onset.

Table 1. Correlations Fargerström and NDSS-S tests with some phenotypic traits of smoking.

Smoking phenotype	Fargerström test		NDSS-S test	
	Pearson	p	Pearson	p
Age (years)	0.44	0.001	0.18	0.07
Age of first use (years)	-0.62	0.54	-0.56	0.58
Years of smoking	0.45	0.001	0.21	0.04
Cigarretes per day	0.73	0.001	0.43	0.001
Quit attempts	0.33	0.001	0.26	0.01

Table 2 shows the genotypic and allele distributions of both polymorphisms analyzed. The frequencies of both genotypes corresponded to those reported for Colombian mestizos in the 1000 genomes database (www.1000genomes.org), and Hardy-Weinberg equilibrium was maintained in both genotypes, in cases and controls. There were no genotypic nor allelic differences between the control and smoker groups in an additive model (Table 2) or a dominant model (data not shown).

In the smoker group, there was no significant association between allele rs16969968, corresponding to the α5 subunit

Table 2. Association analysis of CHRNA5-rs16969968 and CYP2A6-rs1137115 polymorphisms with nicotine dependence in Colombian.

Polymorhisms			Smoking		Non-Smoking		p
			n	% (CI_{95})	n	% (CI_{95})	
CHRNA5-rs16969968	Genotype	GG	53	54.1 (45-64)	60	60 (50-70)	0.41
		GA	40	40.8 (32-50)	38	38 (28-48)	
		AA	5	5.1 (1-10)	2	2 (0 – 5)	
	Allele	G	146	74.5 (68-81)	158	79.0 (74-84)	0.29
		A	50	25.5 (19-32)	42	21.0 (16-26)	
CYP2A6 - rs1137115	Genotype	GG	58	58.6 (49-69)	62	62.6 (53-72)	0.56
		GA	38	38.4 (28-48)	32	32.3 (23-42)	
		AA	3	3 (0-7)	5	5.1 (1-10)	
	Allele	G	154	77.8 (72-83)	156	78.8 (73-84)	0.81
		A	44	22.2 (17-28)	42	21.2 (16-27)	

of the nicotine receptor, and any of the phenotypic variables studied (age, gender, age at consumption onset, years smoking, quit attempts, cigarettes per day or Fargerström and NDSS-S scores). Regarding the rs1137115 polymorphism in the *CYP2A6* gene encoding the enzyme that metabolizes nicotine, we found that the carriers of the wild-type allele had significantly higher NDSS-S scores than those homozygous for the mutant allele (G+GA = 21.4 ± 5.5 vs AA = 15 ± 4.6; $p = 0.04$). In the haplotype analysis, this difference in the NDSS-S scores was only conserved in smokers that are wild-type homozygous for the *CHRNA* gene (haplotypes G/G and G/GA = 22.7 ± 5.2 vs the rest of haplotypes= 19.9 ± 5.6; $p = 0.02$).

Discussion

Nicotine dependence is the first preventable cause of death in the world and the highest risk factor for COPD and lung cancer. The smoking phenotypic features of the study's smokers, such as gender distribution, age of consumption onset, consumption time, cigarettes per day and number of failed attempts to overcome dependence, were adjusted to those widely described in the literature. In this study other potentially influenial variables on cigarette addiction, such as nicotine exposure *in utero* or during childhood were not evaluated [28].

Several tests can be used to evaluate nicotine dependence, but in this study, we used the most widely used tests (Fargerström and NDSS-S tests), which were already validated in Spanish [29]. These scales evaluate different aspects of the dependence of tobacco: the Fargerström test focuses on the dependence degree, the moment of the day of the first cigarette and consumption despite consequences. 63.4% of our cases (n = 64) were non-dependent smokers or exhibited low dependence according to the Fargerström scale. This test has been questioned due to its debatable psychometric properties because several researchers have demonstrated that the instrument has poor internal consistency, reliability and validity [27]. The NDSS focuses on typical drug dependence symptoms such as craving, withdrawal syndrome, tolerance, consumption priority and use continuity. Therefore, the latter test seems to be the most useful for properly evaluating nicotine dependence [30]. In this study, all volunteers had nicotine dependence according to the NDSS-S scale (≥ 11 points), but according to the Fargerström test criteria, only 67.3% were dependent smokers (≥ 3 points). These data agree with those of other studies [4]. Despite this difference in the tests to diagnose nicotine dependence, scores on both tests correlate with some of the classic smoking phenotypic features (Table 2) but not with others. This result confirms the opinion of some authors that no scale is completely satisfactory in describing tobacco addiction due to the limited knowledge of the psychological, social and biological variables involved in the dependence on nicotine, among other factors [31].

While most evidence suggests that both genetic markers studied here are associated with a higher risk of developing nicotine addiction, higher scores on the addiction scales and a higher number of failed attempts to overcome the addiction [32,33], the lack of differences in both polymorphisms between smokers and non-smokers in our study (Table 3), as well as the lack of an association between allele rs16969968 and the phenotypic variables, agrees with other reports [34]. Such contradictory results are common in these types of studies and confirm the great difficulty in identifying variables associated with complex diseases due to the influence of genetic, clinical and environmental variables [34,35].

By other hand, the finding that smokers bearing the wild-type

allele of the gene encoding the enzyme that metabolizes nicotine have a more severe addiction, based on the NDSS-S scale, agrees with scientific evidence stating that compared to those with wild-type genotypes, the nicotine-deficient metabolizers have higher and longer-lasting concentrations of nicotine [6,7]. Moreover, the fact that such an association is conserved only in individuals that are also homozygous for the wild-type cholinergic nicotinic allele suggests an interesting association between two key genes in nicotine pharmacology. This result could be explained by the limited response of the mutated nicotinic receptor and the fact that, after a certain threshold, it is barely influenced by tissue concentrations of nicotine. This hypothesis, of course, must be confirmed; however, it is interesting that these same interactions between genes have already been identified in smokers with more severe addiction levels and higher risks of lung cancer [21,22].

It is necessary to mention some limitations of this study. First, the sample size limits the power of the study. Second, the data on cigarette consumption were obtained by self-report, with subsequent credibility issues. Third, only two genetic markers were evaluated for a complex disease that produces heterogeneous phenotypes in persons addicted to nicotine. Fourth, the study was performed on Colombian mestizos, and the findings may not be extrapolated to other ethnic groups.

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Author's contributions: Carlos A. Isaza M, conceived and participated in the study design, coordinated the research and participated in the analysis, discussion and final reporting. Julieta Henao B, participated in the study design, was responsible for the laboratory work and participated in the analysis, discussion and final reporting. Leonardo Beltrán A, participated in the study design, was responsible for the laboratory work and participated in the analysis, discussion and final reporting. Sandra Valencia C and Alejandra Quiceno G, were responsible for samples collection and application of phenotyping test.

Conflict of Interest

The authors declare no conflict of interest and that there was not any outside interference in the running of the study nor in the preparation of the manuscript. This project was financied with resources of Fundación Universitaria Autónoma de las Américas and Universidad Tecnológica de Pereira.

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