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Association of polymorphisms of the serotonergic system with smoking initiation in Caucasians

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ABSTRACT

Background: The serotonergic system may be implicated in susceptibility to nicotine dependence as nicotine increases 5-hydroxytryptamine (5-HT) release in brain and symptoms of nicotine withdrawal may be modulated by diminished serotonergic neurotransmission. We examined the association of polymorphisms of genes involved in release and receptor function of 5-HT with cigarette smoking initiation in subjects of Caucasian origin.
Methods: *5-HTTLPR* polymorphism of the 5-HT transporter gene and *−759C/T* (rs3813929) and *−697G/C* (rs518147) polymorphisms of the *5-HT$_{2C}$* receptor gene were analyzed in 172 smoking initiators and 254 non-initiators, using PCR–RFLP method. Smoking behavior was assessed with a questionnaire about tobacco use.
Results: We found no differences in the frequency of the *5-HTTLPR* genotypes between smoking initiators and non-initiators. However, the frequency of *5-HT$_{2C}$ −759T* allele was significantly higher in non-initiators than smoking initiators (29.5% vs 16.3%, $p = 0.002$) and the same was true for *5-HT$_{2C}$ −697C* allele carriers (48.8% vs 34.9%, $p = 0.004$). Sex-dependent analysis revealed that these increased frequencies of *−759T* and *−697C* allele carriers were present only in males. No association was observed between any quantitative measures of smoking and these three polymorphisms.
Conclusions: *5-HTTLPR* polymorphism was not associated with smoking initiation in either male or female subjects. However, significant association was found between *5-HT$_{2C}$* receptor gene polymorphisms and smoking initiation in male Caucasian subjects.

1. Introduction

Cigarette smoking increases the risk of numerous cardiovascular and pulmonary diseases as well as cancer, and is responsible for approximately 5 million deaths worldwide according to World Health Organization (http://www.who.int/tobacco/health_priority/en/index.html). The majority of smokers (more than 80%) express a desire to quit smoking, however the success rate at doing so is quite low (less than 5%) due to nicotine that is primarily responsible for the highly addictive properties of cigarettes (Ho and Tyndale, 2007). Although environmental factors such as peer influences and advertising may contribute to smoking, a significant determinant of continued tobacco use is dependent on nicotine (Dani and Heinemann, 1996). However, genetic factors may also influence smoking initiation and persistence as well as the ability to quit smoking, according to growing evidence from twin studies (Li et al., 2003).

People smoke cigarettes habitually to maintain nicotine levels in the body, and nicotine plays a role in stimulating brain reward mechanisms via central neuronal dopaminergic pathways (Henningfield and Fant, 1999). The serotonergic system may also be implicated in the susceptibility to nicotine dependence because nicotine increases 5-HT release in brain and symptoms of nicotine withdrawal may be modulated by diminished serotonergic neurotransmission (Ribeiro et al., 1993; Mihailescu et al., 1998). The neurotransmission of 5-HT is modulated by the serotonin transporter (5-HTT) which facilitates removal of 5-HT from the synapse of serotonergic neurons, resulting in serotonin reuptake into the presynaptic neuron (Smeraldi et al., 2006). The transcriptional activity of human *5-HTT* gene is modulated by a repetitive element of varying length in the 5′ flanking region located ∼1.4 kb upstream of the transcription start site, termed *5-HTT* gene-linked polymorphic region (*5-HTTLPR*). Specifically, a 44-bp insertion/deletion polymorphism in the promoter region of *5-HTT* gene has been iden-

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tified with two allelic variants, the "long" (*L*) and the "short" (*S*) and is only present in humans and higher non-human primates (Heils et al., 1996). *S* allele of *5-HTTLPR* is dominant and its presence is associated with lower expression of *5-HTT* gene, resulting in a reduced capability to take up and release 5-HT (Heils et al., 1996). In contrast, the *L* variant of *5-HTTLPR* is associated with an almost threefold increased transcription of the *5-HTT* gene (Heils et al., 1996).

The effects of 5-HT follow its binding to 5-HT receptors, a large and diverse family of G protein-coupled receptors (Cravchik and Goldman, 2000). Electrophysiological data support a preferential control of mesocorticolimbic dopamine pathways by the 5-HT_{2C} receptor subtype (Di Matteo et al., 2002). Furthermore, biochemical studies indicate that pharmacological manipulation of the 5-HT_{2C} receptor significantly affects dopamine transmission in the dorsal stratium as 5-HT_{2C} receptor agonists reduce, while 5-HT_{2C} receptor antagonists enhance, mesocorticolimbic dopamine function (Di Giovanni et al., 1999). The gene of 5-HT_{2C} receptor is located at chromosome Xq24 (Stam et al., 1994). Several polymorphisms of this gene, particularly the ones located within a 600 bp fragment of the promoter region, have attracted substantial interest in genetic studies (Hill and Reynolds, 2007). Three SNPs, *−997G/A* (rs3813928), *−759C/T* (rs3813929) and, *−697G/C* (rs518147) and a dinucleotide GT repeat at −1027 varying from 11 to 21 repeats in length give rise to a series of haplotypes known to regulate gene expression (Reynolds et al., 2005). Two of these SNPs, *−997G/A* and *−759C/T*, are in complete LD (Yuan et al., 2000). The site of the *−759C/T* and *−697G/C* polymorphisms contains regulatory and putative transcription factor binding regions (Shih et al., 1996) that regulate levels of the receptor protein. Altered protein expression could potentially change the neuronal regulation of many physiological processes. Concerning promoter activity, *−997A/−759T* or *−697C* alleles have been reported to result in increased transcription rates (Yuan et al., 2000; Buckland et al., 2005) while GT repeat was found not to influence transcription (Buckland et al., 2005; Meyer et al., 2002).

Until now, genetic studies examining the association of serotonergic system with smoking behavior have focused on *5-HTT* gene and results are not conclusive. We hypothesized that smoking may be associated with diminished 5-HT neurotransmission and binding to 5-HT receptors that are determined by genetic polymorphisms in the serotonergic system. Our aim was to examine the possible influence in cigarette smoking of genes involved in release and receptor function of 5-HT in subjects of Caucasian origin. Particularly, we examined the association between polymorphisms in serotonergic system key molecules and smoking initiation, which has a stronger genetic influence than smoking persistence (Li et al., 2003). Polymorphisms studied included the *5-HTTLPR* polymorphism of the *5-HTT* gene and the *−759C/T* and *−697G/C* polymorphisms of the *5-HT_{2C}* receptor gene.

2. Methods

2.1. Subjects

Study population consisted of 426 subjects. It consisted of 172 smoking initiators (SI) (93 current smokers and 79 ex-smokers) with average age 63.6 ± 1.1 years and 254 non-initiators (NI) with average age 67.7 ± 0.6, all of Greek Caucasian origin. All subjects were outpatients of the Internal Medicine Clinic of the Alexandroupolis Academic General Hospital and received a thorough medical examination, including medical and drug history. NI subjects had never smoked in their lifetime. Individuals who continued to smoke at the time of the study as well as those who reported having smoked at least 100 cigarettes in their lifetime and had successfully stopped smoking (ex-smokers) were defined as SI and grouped together. Smoking behavior was also assessed with a questionnaire about number of cigarettes smoked daily, number of pack years, and age of smoking initiation.

Other clinical variables measured included height, body weight, blood glucose levels, total, LDL- and HDL-cholesterol, triglycerides, systolic and diastolic blood pressure, urea, creatinine, and body mass index (BMI). All subjects participated after being informed about the study by their attending clinician and giving written consent. The study was approved by the Scientific Council and the Ethics Committee of the Academic General Hospital of Alexandroupolis.

Among the subjects included in the present study, no one met the complete diagnostic criteria for alcoholism or drug dependence. Other exclusion criteria were age under 18, a personal history of cancer, and presence of a psychiatric disorder or taking 5-HT agonists or antagonists.

2.2. Genotyping

Genomic DNA was extracted from white blood cells in peripheral venous blood by Puregene DNA Purification System (Gentra, Minnesota, MI, USA) and analyzed for *5-HTTLPR* polymorphism of the *5-HTT* gene and *−759C/T* and *−697G/C* polymorphisms of the *5-HT_{2C}* receptor gene. For the *5-HTTLPR* polymorphism, taking into account previous reports presenting the difficulties in genotyping this polymorphism (Kaiser et al., 2002; Yonan et al., 2006), a novel set of primers was designed with the aid of the Oligo-6 software (NBI, Plymouth, USA) at very high strigency conditions. Using polymerase chain reaction (PCR), DNA was amplified with the following primer set: [5′-GTTTTGTGTTGCCCTTGCCTAT-3′] and [5′-CACCGCCCCTTGTACTTG-′3] to generate 705- or 749-bp fragments. The PCR reaction was performed in a 50 μl aliquot of the reaction mixture with 0.5 μg genomic DNA; 0.2 mM dNTP mix; 60 pmole of each primer; 1.2 mM $MgCl_2$ and 2.5 IU of Taq polymerase. All reagents for PCR amplification were supplied by Invitrogen (Carlsbad, CA, USA). After an initial denaturation step at 94 °C for 10 min, cycling parameters were 42 cycles with denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min, extension at 72 °C for 1 min, and a final extension step at 72 °C for 5 min. All PCR amplifications were carried out in the PCR-engine apparatus PTC-200 of MJ Research (Watertown, MA, USA). The PCR products were visualized on a 1.5% agarose gel stained with ethidium bromide to determine each subject's genotype. The *L* allele of *5-HTTLPR* was denoted by the presence of 749 bp fragment and the *S* allele by the presence of 705 bp fragment. It was reported recently that total magnesium concentration is a fundamental factor in the sensitivity of *5-HTTLPR* genotyping results as the high Mg concentrations used in many previous studies were shown to cause allele-dependent, non-random genotyping errors, with the *L* allele of *5-HTTLPR* amplifying poorly at these concentrations (Yonan et al., 2006). Accordingly, in our study, Mg concentration was reduced to 1.2 mM. The protocol used for the *−759C/T* and the *−697G/C* polymorphisms of the 5-HT_{2C} receptor gene, a modified polymerase chain reaction-restriction fragment length polymorphism (PCR–RFLP) method, has recently been described (Yuan et al., 2000; Iordanidou et al., 2008).

2.3. Statistical analysis

Quantitative data are presented as mean ± standard error of mean (SEM). Relative frequencies of genotypes and alleles were calculated for each group and a chi-square analysis was conducted comparing the distribution of genotypes and alleles between smokers and non-smokers. Comparisons for continuous or categorical