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**Lack of association between dopamine receptor D4 variable numbers of tandem repeats gene polymorphism and smoking**

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## **Abstract**

Nicotine addiction, related to cigarette smoking, develops as a product of the complex interactions between social, environmental and genetic factors. Genes encoding the components of the dopaminergic system are thought to be associated with smoking. Literature data showed an association, but also a lack of association between variable number of tandem repeats (VNTR) polymorphism located in the third exon of dopamine D4 receptor (DRD4) gene and smoking. Repetitive sequence of DRD4 VNTR is 48 bp long and maximum 11 tandem copies were reported in humans. Presence of alleles with 6 and more repeats (i.e. long alleles) was associated with greater tendency to novelty seeking and addictive behaviors than the presence of 5 and less alleles (short alleles). The aim of this study was to determine the association between VNTR in DRD4 gene and present smoking status in ethnically homogenous Caucasian population from the Eastern European (Croatian) origin. Genotyping was done in 565 healthy subjects, 511 men and 54 women, respectively, who were subdivided into 176 smokers and 389 nonsmokers. Logistic regression analyses, adjusted for age and sex, revealed the lack of significant ( $p>0.05$ ) effect of the 4/4, 4/7 and 7/7 genotypes, or carriers of the long and short allele, or all genotypes of the DRD4 VNTR on smoking status. The results of this study failed to confirm the hypothesis that long allele of the DRD4 VNTR is associated with smoking status in Caucasian subjects.

**Keywords:** smoking, healthy subjects, D4 dopamine receptor, variable number of tandem repeats polymorphism, genetics

## **Abbreviations<sup>1</sup>**

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<sup>1</sup> DRD4-dopamine D4 receptor; VNTR-variable numbers of tandem repeats

## Introduction

Nicotine addiction develops under the influence of social, environmental and genetic factors. Smoking increases the risk of cancers, cardiovascular and pulmonary diseases, reduces the quality of life and causes untimely death [8]. According to World Health Organization (WHO), there are more than 1.3 billion regular smokers worldwide and smoking causes around 5 million deaths every year. The estimated smoking prevalence in Croatia is 34.1% among men and 26.6% among women. In addition, during a one year period around 10 000 people in Croatia die as a consequence of smoking-related diseases (The Ministry of Health of the Republic of Croatia). Initiation of smoking is highly influenced by social factors, while smoking persistence is affected by the genetic factors [15, 31]. Nicotine dependence candidate genes are genes coding the components of the serotonergic and dopaminergic systems, nicotine receptors and enzymes involved in nicotine metabolism [2, 28].

Dopamine is a catecholamine neurotransmitter involved in the reward pathways and development of addictive behaviors. Addictive substances activate reward pathways and increase dopamine release in the brain [5], while nicotine binds to nicotinic acetylcholine receptors (nAChR) in the brain and stimulates dopamine release [19]. There are five subtypes of dopamine receptors divided into two classes: D1-like receptors (D1 and D5) and D2-like receptors (D2, D3 and D4). Dopamine receptor D4 (DRD4 or D4DR) gene is located on chromosome 11 (region 11p15.5). Location of the DRD4 gene in the telomeric region of the chromosome might be the reason of multiple polymorphisms existence inside the gene and 5' regulatory region [16]. Variable number of tandem repeats (VNTR) polymorphism in the third exon of DRD4 gene consists of 48 bp long repetitive sequence with 1-11 copies in humans [7, 37]. The most common variants are those with 4 (64 %) and 7 repeats (20 %) [22]. The VNTR region codes for amino acids in the third cytoplasmic loop of the receptor, a region of G-protein coupled receptors thought to have functional importance with respect to G-protein coupling [14]. The influence of VNTR on receptor function is still not clear. It is assumed that VNTR can affect dopamine binding, connection with second messengers and signal transduction [22]. The effect of DRD4 receptor on cAMP is two-fold lower in long allele carriers than in short allele carriers [1, 37].

Individuals with 7 repeats allele (long allele carriers) have a higher tendency of novelty seeking [3, 9], which is often associated with addictive behaviors. There is an association of DRD4 long allele (DRD4L) with greater urge for drinking among alcoholics [12], greater heroine uptake among heroin addicts [33] and greater urge for food cues among individuals with eating disorders [35]. The results showing the association between nicotine addiction and DRD4 VNTR are conflicting. No association between DRD4 VNTR polymorphism and smoking [17, 20, 23, 34, 38], or significant association between DRD4 VNTR and nicotine dependence [6, 11, 18, 24, 29] was previously reported.

Given the fact that genetic variations in components of the dopaminergic system might alter the risk of smoking [32, 34], and in an effort to elucidate the role of DRD4 VNTR in smoking, we performed the current study with the aim of evaluating the association between

dopamine receptor D4 gene VNTR polymorphism and nicotine addiction in medication-free Caucasian healthy subjects of European origin.

## **Material and methods**

### ***Research subjects***

The study included 511 (159 smokers and 352 nonsmokers) men and 54 (17 smokers and 37 nonsmokers) women who were unrelated, medication-free Caucasian healthy subjects of Croatian origin and were recruited in the period between 2006 and 2010 at the University Hospital Centre Zagreb, Zagreb, Croatia. They filled in the questionnaire answering the questions about their detail medical history, smoking and drinking habits. Inclusion criteria were: no current medication therapy; no previous or current psychiatric disorders other than nicotine addiction; no drug or alcohol abuse, no suicidal attempts and no family history of psychiatric disorders (determined according to the answers of participants about the mental health status of their parents, grandparents, siblings and children). Smokers were classified as subjects smoking  $\geq 10$  cigarettes per day and non-smokers were classified as never-smokers and ex-smokers. There were only 9 ex-smokers, categorized into non-smokers, since they quit smoking more than 10 years ago. Men were  $39.9 \pm 12.1$  and women were  $42.4 \pm 13.1$  years old. Written informed consent was obtained from all participants, after explaining the aims and procedures of the study, under procedures approved by the Ethics committee of the Clinical Hospital Centre Zagreb, Zagreb, Croatia. All human studies have been carried out with the full cooperation of participants, adequate understanding, and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

### ***Genotyping***

Subjects' blood samples (8 ml) were drawn using plastic syringes with 2 ml of acid citrate dextrose anticoagulant. Genomic DNA was extracted from peripheral blood using the salting out method [30]. The 48 bp repeats polymorphism in the third exon of the DRD4 gene was detected by polymerase chain reaction (PCR) using Gene Amp PCR system 2700 (Applied Biosystems, USA). Primers used were 5'-AGGACCCTCATGGCCTTG-3' and 5'-GCGACTACGTGGTCTACTCG-3'. PCR was performed in total volume of 15  $\mu$ l containing 200 ng of template DNA, 0.8  $\mu$ M of each primer (Sigma-Aldrich, USA), 0.2 mM of each dNTP (dATP, dCTP, dTTP, 50% 7-deaza-GTP, 50% dGTP, Roche, Switzerland), 1 unit of Tfi polymerase (Invitrogen, USA), 1X Tfi buffer (Invitrogen, USA), 1X Q-Solution (Qiagen, Germany) and 1  $\mu$ M MgCl<sub>2</sub> (Invitrogen, USA). Cycle conditions consisted of an initial denaturation at 94°C for 10 min, followed by 35 cycles of denaturation (94°C for 30 s), annealing (54°C for 30 s) and extension (72°C for 1 min). Final extension was carried out at 72°C for 10 min. PCR products were separated by 2% agarose gel electrophoresis, and visualized with ultraviolet light after SYBR® Safe (Invitrogen, USA) staining. All laboratory procedures were performed blind to subject status.

### **Data analysis**

Subjects were grouped according to their DRD4 genotype in three groups: group A (4/4, 4/7 and 7/7 genotypes), group B (all genotypes) and group C (carriers of 2-5 repeats (short; s) and 6 or more repeats (long; l) DRD4 alleles). Logistic regression analysis was used to elucidate the association of the smoking status (smokers vs. non-smokers) as the dependent variable with particular DRD4 genotype as independent variable. All analyses were adjusted for age and sex. Following the idea that the greatest functional differences are found between 4 and 7 repeats alleles, it was assumed that any influence of DRD4 genotype to nicotine addiction would be found comparing 4 and 7 repeats carriers. Since heritability of functional effect of DRD4 third exon VNTR is still unknown, both dominant and co-dominant effect of 7 repeats allele were examined as suggested by Lettre et al. [21]. For dominant effect, 7 repeats allele carriers (7r+) were compared with subjects with no 7 repeats allele (7r-). For co-dominant effect, 7 repeats allele homozygotes were compared separately with 7 repeats allele heterozygotes and no 7 repeats allele homozygotes. For group B, only dominant effect was investigated. The results were evaluated with SigmaStat 3.1 statistical software (Jandell Scientific Corp. San Raphael, California, USA). The Hardy–Weinberg (HWE) analysis was used to test the equilibrium of the study group. The level of significance was set to  $\alpha=0.05$ .

### **Results**

No deviation from the HWE was found in the group of smokers ( $\chi^2=3.74$ ;  $df=1$ ;  $p=0.053$ ) However, deviation from HWE was detected in the group of nonsmokers ( $\chi^2=29.89$ ;  $df=1$ ;  $p=0.000$ ). All subjects ( $n=565$ ) were analyzed for the DRD4 genotype and alleles with 2 to 8 copies of the repeated sequence were found. The most abundant alleles were those with 2 (11.7%), 4 (71.0%) and 7 repeats (13.0%). Alleles with 3, 5, 6 and 8 repeats had frequencies of 2.8, 0.8, 0.4 and 0.3%, respectively. Table 1 shows the counts and frequencies of the DRD4 genotypes in all subjects subdivided according to smoking status into smokers and non-smokers. Non-smokers included also a small group ( $N=9$ ) of ex-smokers, with 6 of them carrying 4/4, 1 with 4/7, 1 with 4/3 and 1 with 6/8 genotype, respectively. Results of logistic regression analyses, adjusted for age and sex, are shown in Table 2. This analysis revealed no significant ( $p>0.05$ ) effect of DRD4 exon 3 VNTR polymorphism on smoking status (Table 2).

### **Discussion**

The major finding is a lack of association between the DRD4 VNTR gene variants and nicotine addiction. Our results concur with some [17, 20, 23, 34, 38], but disagree with other [6, 11, 18, 24, 29] results. Although there are findings showing an association of long DRD4 gene allele and smoking only in men [17], the majority of previous studies did not find sex differences in the distribution of long and short alleles [6, 24, 34] and our data are in line with these findings. DRD4 VNTR gene variants were in HWE in the group of smokers, while in non-smoker group

DRD4 VNTR deviated from HWE. The reason for this surprising finding is at present unclear, and might be explained by the region-specific sampling, error in selection of subjects sampled at the University Hospital Centre where non-smokers predominate, or the presence of population substructure. This is a limitation of the study. The association between the DRD4 gene variants and smoking is controversial. The inconsistencies might be explained by the ethnic differences, since an association of long DRD4 allele with smoking was found in 403 African-Americans but not in 72 Caucasian subjects [34]. DRD4 genotype frequencies vary among different ethnical groups [36]. It was shown that smokers with depression who are short allele carriers, smoke more cigarettes in order to reduce negative feelings caused by depression [20]. Hypothesis about the association of long DRD4 gene allele (7 and more repeats) with greater urge for smoking is confirmed on 68 smokers from USA [11]. The study carried out on 384 German adolescents showed an association of long DRD4 gene allele (7 and more repeats) with smoking for males but not for females [17]. The same research group confirmed an association of long DRD4 gene allele, long term smoking and lower rates of smoking cessation. Opposite of these findings, study including 769 subjects from Australia did not confirm the association of long DRD4 gene allele and smoking [23]. Besides that, it has been shown that Caucasian long allele carriers (more than 5 repeats) can endure the abstinence crisis more easily and exhibit less urge for smoking than Caucasian short allele carriers [38]. The influence of VNTR in the third exon of DRD4 gene on smoking cessation was also studied in order to develop an individualized nicotine replacement therapy [6]. A study with 720 subjects of European ancestry showed that DRD4 gene long allele carriers (7 and more repeats) withstood the abstinence crisis for 3 months with more difficulties than short allele carriers. However, these results were not confirmed 6 months after quitting smoking.

The influence of VNTR in the third exon of DRD4 gene on the brain of smokers is confirmed with neuroimaging findings. Differential activation of brain parts was observed for smokers, long and short allele carriers, after exposure to different smoking-related cues [29]. This study was performed on 15 smokers from USA. Long allele carriers had a greater activity in brain areas involved in response to urge (right frontal gyrus and right insula). It was assumed that in these brain areas DRD4 receptors are expressed in high amount [29]. In Caucasian subjects, long allele carriers who smoked, needed more time for solving the Stroop test after the exposure to smoking-related cues, than smokers which were DRD4 gene short allele carriers [24]. Binding of nicotine to nicotinic receptors (nAChR) located on dopaminergic neurons in ventral tegmental area (VTA), enhanced dopamine release in nucleus accumbens [19]. Nicotinic receptors are also located on GABA-ergic, glutamatergic and cholinergic neurons. GABA-ergic interneurons provide inhibitory control over dopaminergic neurons, whereas glutamatergic neurons from prefrontal cortex (PFC) and cholinergic neurons from tegmental pedunculo pontine nucleus (TPP) have stimulatory effects on VTA. After nicotine exposure, inhibitory effects of GABA are reduced and positive effects of glutamatergic and cholinergic projections are increased, resulting in increased dopamine release from VTA [19, 27]. Smokers, DRD4 short allele carriers, who have low resting dopamine tone, had greater smoking-induced dopamine release than individuals



with other DRD4 genotypes [4, 32]. Brody et al. [4] reported an association between decreased craving and increased dopamine concentration with smoking. In DRD4 long allele carriers, the ability of dopamine to inhibit cAMP formation is decreased, and these individuals have a greater resting dopaminergic tone, and lower smoking-induced dopamine release than DRD4 short allele carriers [4]. Therefore, DRD4 long allele carriers are expected to have greater craving for nicotine and higher vulnerability to smoking.

Negative results about the association of DRD4 gene variant and smoking are probably due the influence of ethnicity, since there was an association between DRD4 gene long allele and smoking in African-Americans, but not in Caucasian subjects [34]. It is assumed that the presence of DRD4 long allele does not predispose the development of addictive behavior, however long allele can influence the severity of addictive behavior [25]. In addition, DRD4 VNTR can influence only particular endophenotype (a narrowly defined phenotype closely associated with biological mechanism) in smoking behavior. Endophenotype studies should prevent inaccurate results in genetic studies [10]. In the case of smoking, candidate for endophenotype might be a “cue-elicited craving”. Although clinical importance of craving is controversial, previous studies confirmed that craving has an important role in the long-term addictions [33]. Namely, studies about the influence of smoking and alcohol cues on individuals show a greater urge at individuals with DRD4 gene long allele [11, 12, 26]. In line with this, pharmacogenetic study [13] revealed that urge for drinking can be reduced by a DRD4 antagonist. It was also proposed that DRD4 is a regulator of “cue-elicited craving” and controls this endophenotype at nicotine addiction [13].

## **Conclusion**

In conclusion, the results of the study did not confirm a significant association between DRD4 VNTR alleles and smoking in healthy medication-free Caucasian subjects. Further studies should focus on the association between DRD4 alleles and “cue-elicited craving for nicotine”, or the influence of DRD4 long allele on the severity of addiction among smokers in a larger sample.

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Table 1: Dopamine receptor D4 genotype counts and frequencies in healthy subjects subdivided according to smoking status

DRD4 genotype	Counts	Frequencies (%)	Smoking status	
			Smoking (N=176)	Non-smoking (N=389)
2/2	31	5.5	8 (4.5)	23 (5.9)
2/4	64	11.3	17 (9.7)	47 (12.1)
2/7	1	0.2	0 (0)	1 (0.3)
2/n	5	0.9	1 (0.6)	4 (1.0)
4/4	308	54.5	99 (56.3)	209 (53.7)
4/7	95	16.8	36 (20.5)	59 (15.2)
4/n	24	4.2	3 (1.7)	21 (5.4)
7/7	22	3.9	6 (3.4)	16 (4.1)
7/n	9	1.6	5 (2.8)	4 (1.0)
n/n	6	1.1	1 (0.6)	5 (1.3)

n = 3-, 5-, 6-, 8- repeats allele

Table 2. Logistic regression analyses predicting smoking status.

		Group A <sup>a</sup> 7r- vs. 7r+	Group B <sup>b</sup> 7r- vs. 7r+	Group C <sup>c</sup> s vs. l
Dominant effect	OR	0.83	0.71	0.76
	95% CI	0.53-1.31	0.47-1.08	0.50-1.15
	<i>p</i>	0.429	0.113	0.196
		Group A <sup>a,*</sup>		
		4/4 vs. 7/7	4/7 vs. 7/7	
Co-dominant effect	OR	1.27	1.67	
	95% CI	0.48-3.37	0.59-4.70	
	<i>p</i>	0.632	0.333	

<sup>a, b</sup> 7 repeats allele as a reference category; <sup>c</sup> long allele as a reference category; \* 7/7 genotype as a reference category. All analyses were adjusted for age and sex.