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Significant association of *DRD2* and *ANKK1* genes with rural heroin dependence and relapse in men

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Abstract

Introduction. Substance abuse significantly influences human health and may induce problems with social functioning worldwide. Numerous genetic and environmental risk factors, as well as their interactions, accelerate the development of drug addiction. Etiologically, the dopaminergic mesocorticolimbic reward pathways are related to psychoactive substance addiction, and the reward properties of heroin are connected with changes in the mesolimbic dopaminergic system.

Objective. The aim of this study is a haplotypic analysis of subjects addicted to polysubstance. However, with the knowledge that this is not a homogenous subgroup, it was decided to separate and analyze homogenous subgroups of subjects in order to find specific haplotypic variants among them. The subjects in the subgroups were addicted to heroin, and subjects with more than two relapses in the past two years.

Materials and method. The study group comprised of 301 polysubstance addicted rural male subjects. From this group, 2 homogenous subgroups of subjects were isolated and additionally analyzed: (1) a group of heroin addicted subjects (n=61), and (2) a group of heroin-addicted subjects with at least two relapses in the last two years (n=21). The group consisting of all polysubstance addicted rural subjects and both homogenous subgroups were analyzed against a control group of non-addicted subjects (n=300), matching gender and age. Five polymorphisms in the *DRD2/ANKK1* region were analyzed: rs1076560, rs1800498, rs1079597, rs6276 in the DRD2 gene, and rs1800497 in the *ANKK1* gene.

Results. A statistically significant haplotype association was found in analysis of the heroin addicted subjects, compared to controls, and two possible trends – when comparing the whole group of addicted subjects to controls, and in relapse subgroups, compared to the controls.

Conclusion. The results obtained showed that haplotypes indicate a part of the biological component of addiction.

Key words

Addiction, heroin, dopamine, haplotypes

INTRODUCTION

The worldwide substance use and abuse significantly influences human health and may induce problems with social functioning. It is believed that numerous genetic and environmental risk factors, as well as their relations, may accelerate the development of drug addiction [1]. However, it is still not clear which group of genes may be definitely connected with susceptibility. Analysis of heroin properties allow to relate its reward influence with changes in the mesolimbic dopaminergic system [2]. Additionally, dopamine receptor expression may be of importance in the development of heroin dependence (HD), specifically in relation to the D2 dopamine receptor (DRD2) [3, 4]. Dopamine-related and dopamine-nonrelated mechanisms facilitate the reinforcing effects of drugs of the opioid group. Etiologically, the dopaminergic mesocorticolimbic reward pathways are related to psychoactive substance addiction [5, 6, 7]. Addictive substances temporarily increase the level of extracellular dopamine in the ventral striatum, which increases the speed of the learning process and facilitates compulsiveness of substance abuse [8]. The mesolimbic dopaminergic pathway is activated by substance of abuse, and dopamine is involved in all stages of the development of addiction [9].

The current study investigates the association between genes which influence dopamine production and heroin addiction. Although different ancestral populations are also

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at risk of developing drug addiction, differences in linkage disequilibrium (LD) and allele frequencies may indicate the presence of some specific genetic risk factors. The genes selected for this analysis include encoding DA (dopamine) receptor and ANKK1 (Ankyrin repeat and kinase domain containing 1) protein kinase. The dopamine receptor 2 gene was chosen for this study due to the importance of dopamine in the addiction process, which is supported by many studies [10, 11, 12]. Since both genes are located next to one another and are possibly clustered together with *NCAM1* (Neural cell adhesion molecule 1) and *TTC12* (Tetratricopeptide Repeat Domain 12 gene) genes due to the regulatory functions that are to be maintained across evolution to preserve phenotypic integrity [13], the *ANKK1* gene was also analyzed in this study.

Earlier observations indicate that *DRD2* may be treated as one of the most interesting candidate gene in studies on heroin dependence (HD). Previously, the human *DRD2* gene was thought to be located on chromosome 11q22–23 [14], now it is known that it is shorter and on chromosome 11q23.2. Interestingly, one of the most often analyzed polymorphic sites, Taq1A (rs1800497), is located in the *ANKK1* gene and not in *DRD2*. The *Taq1A* is in linkage disequilibrium with variants located in the dopamine receptor 2 gene, which confirms its functional relation, and both genes are often analyzed together in the context of addictions [15, 16]. It is also of great importance that the *ANKK1* gene in the central nervous system is expressed only in astrocytes which, due to bilateral communication with synapse, are thought to be part of the synapse [17]. ANKK1 belongs to the RIP (Receptor-Interacting Protein) serine/threonine kinase family. These kinases are important in cell proliferation, differentiation and activate transcription factors [18]. *DRD2* gene is probably regulated by ANKK1 through NF-kappaB (Nuclear Factor-kappaB). ANKK1 is activated by apomorphine-dopaminergic agonist, which indicates another link with the dopaminergic system [19].

Other polymorphisms studied in the *DRD2* gene region in the context of addiction include: the *TaqIB* (rs1079597), *TaqID* (rs1800498) polymorphisms, and the deletion of cytosine 141 (-141C Ins/Del, rs1799732). There is high linkage disequilibrium (LD) between *TaqIA* and *TaqIB* in Asian, American, and European populations [20, 21]. However, low LD has been noted in relation between these two polymorphisms and the -141C Ins/ Del polymorphism [22]. The *TaqIA* polymorphism is located in *ANKK1*, whereas the *TaqIB*, *TaqID* and -141C Ins/Del variants are located in the intron and promoter regions of *DRD2*, respectively. Some studies have noted the relationship between addictive behaviours and TaqIA minor allele and heroin, cocaine, and alcohol and tobacco dependence [1, 23, 24, 25, 26, 27]. The *TaqIB* minor allele appears more often in the HD context, alcohol dependence and poly-substance abuse [1, 28, 29, 30]. As mentioned before, *ANKK1* is located very close to *DRD2*, and the LD between SNPs in both genes is high. This is the coding region of *ANKK1* where the *TaqIA* polymorphism (rs1800497) is located. Potentially, *ANKK1* and the dopaminergic system are interrelated [31]. There have been several supporting reports on the association between dopamine-connected genes and heroin or cocaine addiction. The relationship between *ANKK1/DRD2* SNPs and heroin or cocaine addiction/abuse has been observed in different populations [1, 28, 32, 33, 34, 35, 36, 37, 38, 39].

The biological basis of addiction is still not well understood, and although 50% of the risk factors has been attributed to the genetic component, the roles of the myriad of individual candidate genes in conferring genetic risk are not well characterized. Haplotype analyses may shed light on the collective risk profile in this context. Therefore, the current study focuses on the analysis of haplotypes in people addicted to heroin. A thorough analysis was also carried out of this genetic relationship in connection with 5 genetic markers for the *DRD2* and *ANKK1* genes to assess the influence of *DRD2/ANKK1* variants in the pathogenesis of heroin dependence.

MATERIALS AND METHODS

Subjects. After the approval of the Bioethics Committee of the Pomeranian Medical University (KB-0012/106/16), as well as an informed, written consent of the subjects had been granted, the study was carried out in the Independent Laboratory of Health Promotion at the Pomeranian Medical University in Szczecin, Poland. The study involved 601 subjects – 301 men from rural areas (mean age = 28, SD = 6.45) addicted to more than two psychoactive substances (polysubstance addiction), staying in a rehabilitation centre, and 300 healthy, non-addicted men (mean age = 22, SD = 4.57). The research and control groups were examined by a psychiatrist. For the control group, addiction and other mental diseases were excluded by applying the M.I.N.I. Mini International Neuropsychiatric Interview. The main aim of this study was haplotypic analysis of polysubstance addicted subjects; however, knowing that this is not a homogenous subgroup, it was decided to separate homogenous subgroups of subjects and analyze them separately in order to find specific haplotypic variants among them. The subgroups comprised subjects addicted to heroin and subjects with more than two relapses in the past two years.

Genotyping. Blood from the ulnar vein was collected from all subjects. DNA was isolated using standard procedures. The PCR method was used to genotype samples. Genotyping was performed by fluorescence resonance energy transfer using the real-time PCR method on the LightCycler ® 480 II System (Roche Diagnostic, Basel, Switzerland). PCR was performed according to standard procedures. The fluorescence signal was plotted against temperature to provide melting curves for each sample. Peaks for rs1800497 were obtained at 58.95 °C for the T allele and 67.17 °C for the C allele. For rs6276, they were at 59.14 °C for the G allele and at 67.66 °C for the A allele. For rs1076560 – 57.13 °C for the A allele and 64.40 °C for the C allele. For rs1800498 – 57.87 °C for the T allele and 66.34 °C for the C allele. For rs1079597 – 57.41 °C for the G allele and 62.25 °C for the A allele. Figure 1 shows selected polymorphic loci.

Statistical analysis. Haplotype analysis was carried out using R software with Bioconductor packages haplo.stats and genetics. Linkage Disequilibrium and graphical image of the LD were performed by Haploview ver. 4.2 (Fig. 2).

Location of 5 polymorphisms in the *DRD2/ANKK1* genes region is shown in the upper panel; output of Haploview version 4.2 is shown in the lower panel.

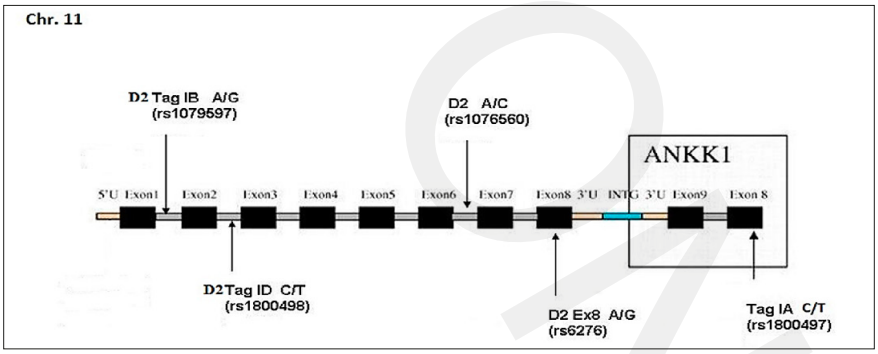

Figure 1. Localization of polymorphisms applied within *DRD2* and *ANKK1* gene. 5 selected markers provided a coverage of DRD2/ANKK1 as a result of the random SNP selection method.

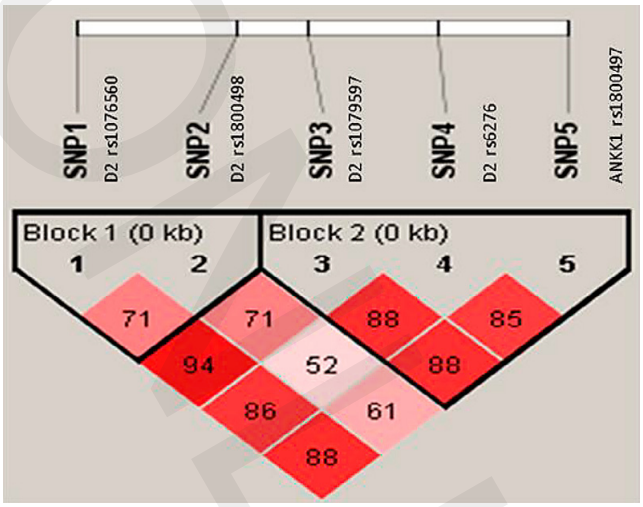

Figure 2. Linkage Disequilibrium structure of 5 polymorphisms in the *DRD2/ANKK1* genes region.
D' – Lewontin's D'; SNP1 – polymorphism *DRD2* rs1076560; SNP2 – polymorphism *DRD2 TagID* rs1800498; SNP3 – polymorphism *DRD2 TagIB* rs1079597; SNP4 – polymorphism *DRD2* Ex8 rs6276; SNP5 – polymorphism *ANKK1 TagIA* rs1800497

RESULTS

Haplotype analysis. Figure 2 shows the haplotype blocks and Linkage Disequilibrium tructure of the *DRD2* and *ANKK1*, as well as the D' and r2 values (for all variants). Two main blocks were detected from 5 polymorphisms. Block 1 (rs1076560, rs1800498) included two SNPs from the intron 6 and intron 2 of *DRD2*. Block 2 (rs1079597, rs6276 and rs1800497) included two SNPs within intron 1 and exon 6 of *DRD2* and exon 8 of *ANKK1* (Fig. 2). Four associations were noted during haplotypic analysis, including possible trend (p=0.05626) C-T-A-A-C (Tab. 1). The Hap-Score of this haplotype equals -1,90899, which suggests that this is a protective haplotypic variant.

During haplotype analysis of selected rural heroin-dependent patients, a statistically more significant frequency of haplotype C-T-G-A-T was observed in the addicted group than in the control group (p = 0.03534) (Table 2). Similarly, when analyzing heroin addicts with more than two relapses, haplotype C-T-G-A-C was more frequent in cases showing a possible **…..?** (Tab. 3).

Table 1. Frequency of haplotypes built from polymorphisms: *DRD2* rs1076560 (A/C), *DRD2 Tag 1D* rs1800498 (C/T), *DRD2 Tag 1B* rs1079597 (A/G), *DRD2* exon 8 rs6276 (A/G) and *Taq1A* rs1800497 (C/T) of the *ANKK1* gene in rural individuals addicted to psychoactive substances and in the control group

Haplotype	Hap-Score	p.val	Overall frequency	Control (n=300)	Case (n=301)
C-T-A-A-C	-1.90899	0.05626	0.00517	0.00950	NA
C-T-G-G-C	-1.25506	0.20946	0.08429	0.09393	0.07208
A-C-A-A-C	-0.92437	0.35529	0.00514	0.00703	0.00204
A-T-A-A-T	-0.81511	0.41501	0.01455	0.01801	0.01079
A-T-G-A-C	-0.45171	0.65147	0.00540	0.00625	0.00418
C-C-G-G-T	-0.14250	0.65147	0.00485	0.00532	0.00462
C-C-G-G-C	0.13657	0.89137	0.25167	0.24972	0.25189
C-T-G-A-C	0.34437	0.73056	0.44086	0.43744	0.44892
C-C-G-A-C	0.45921	0.64608	0.00491	0.00392	0.00581
C-T-G-A-T	0.55053	0.58195	0.02131	0.01811	0.02416
A-C-A-A-T	1.01456	0.31031	0.14176	0.13103	0.15230

NA – data not available

Table 2. Frequency of haplotypes built from polymorphisms: *DRD2* rs1076560 (A/C), *DRD2 Tag1D* rs1800498 (C/T), *DRD2 Tag1B* rs1079597 (A/G), *DRD2* exon 8 rs6276 (A/G) and *Taq1A* rs1800497 (C/T) of the *ANKK1* gene in rural individuals addicted to heroin and in the control group.

Haplotype	Hap-Score	p.val	Overall frequency	Control (n=300)	Case (n=61)
C-C-G-G-C	-1.07326	0.28315	0.24330	0.25016	0.20492
C-T-A-A-C	-0.93538	0.34959	0.00791	0.00954	NA
A-T-A-A-T	-0.93538	0.50307	0.01618	0.01808	0.00820
A-C-A-A-T	-0.53418	0.59322	0.12883	0.13148	0.11475
C-T-G-G-C	-0.19909	0.84219	0.09316	0.09407	0.09011
C-T-G-A-C	1.89991	0.05745	0.45186	0.43787	0.52465
C-T-G-A-T	2.10446	**0.03534**	0.02188	0.01585	0.04912

NA – data not available

Table 3. Frequency of haplotypes built from polymorphisms: *DRD2* rs1076560 (A/C), *DRD2 Tag1D* rs1800498 (C/T), *DRD2 Tag1B* rs1079597 (A/G), *DRD2* exon 8 rs6276 (A/G) and *Taq1A* rs1800497 (C/T) of the *ANKK1* gene in rural individuals addicted to heroin with relapses (more than 2 treatments) and in the control group

Haplotype	**Hap-Score**	**p.val**	**Overall frequency**	**Control (n=300)**	**Case (n=21)**
C-T-G-G-C	-1.36089	0.17355	0.08956	0.09407	0.02381
A-T-A-A-T	-0.75358	0.45110	0.01684	0.01808	NA
A-C-A-A-T	-0.69545	0.48677	0.12911	0.13148	0.09524
C-T-A-A-C	-0.54900	0.58301	0.00891	0.00954	NA
C-C-G-G-C	-0.43307	0.66496	0.24886	0.25016	0.21429
C-T-G-A-T	1.50559	0.13217	0.01877	0.01585	0.04762
C-T-G-A-C	1.95937	**0.05007**	0.44700	0.43787	0.59524

NA – data not available

DISCUSSION

The study focused on the analysis of haplotypes composed of polymorphic variants located in the *DRD2/ANKK1* region in subjects addicted to psychoactive substances, and in a group of patients from a control group. During the first analysis of 301 rural patients from the drug rehabilitation centre, the result at a level of possible trend was observed, which indicated the probable role of a protective haplotype. Interestingly, the significance or possible trend appeard during the analysis of all HD subjects and those with two or more relapses in the last two years. Haplotypes C-T-G-A-T and C-T-G-A-C are typical of the HD group. Despite the small number of subgroups, a specific haplotype was obtained at a significant level. This may be related to the selection of polymorphic sites building those haplotypes, and their association with the addiction by means of the dopaminergic system in the brain.

Because dopamine is a key factor in the transmission of signal by neurons in the reward system, a research study on the variants in addictive disorders is of great importance. It is worth emphasizing that both the limbic and cortical areas are the locations of D1 receptors expression, whereas the ventral tegmental area, nucleus accumbens (D2), and in the Islands of Calleja (D3) are the sites of D2 and D3 receptors expression; however, they both facilitate the addictive processes [40].

The *ANKK1 TaqIA* is included in the group of key variants involved in the process of addiction, although recent research has indicated that variants found within *ANKK1* and *TTC12* may be treated as phenotypes influencing addiction [15, 19, 41, 42]. The protective effect of the G-C-A haplotype, the A-T-G haplotype (comprising opposite alleles) in the same three variants (rs11604671-rs4938015-rs2303380) has been observed to increase risk of nicotine dependence in two different populations [43]. Moreover, haplotypes in the same region influence the risk of developing alcohol dependence [44].

The presence of *T* allele (*TaqIB B1* allele) in the rs1079597 among people of Caucasian origin was associated with an undoubtedly lower number of binding sites, and a decreased density of dopamine D2 receptors, compared to individuals without this variant [45, 46]. Wang et al. observed that *DRD2* was expressed in opiate-dependent subjects on a lower level when compared with controls. In the current study, a higher number of the T allele was found in the heroin-dependent individuals, which allows the conclusion that the T allele of rs1079597 increases the probability of developing heroin dependence [47]. The same conclusions are found in other studies [1, 28].

It was also observed that heroin dependence remained in significant association with *DRD2* rs6275. A lower frequency of heroin dependence was found in subjects with the AA genotype, which shows that the predisposition of rs6275 AA carriers to develop a heroin addiction is decreased. Variant rs6275 is a His313 synonymous polymorphism in the sixth exon of the *DRD2* gene. It was mentioned as a factor having loose association with disease vulnerability in the Indian population, and as a polymorphic locus which modulates methadone treatment [48, 49]. Methadone is effective in treating opioid dependence. Nevertheless, its influence is conditioned with a personalized approach to methadone maintenance with basis in genetics. There is evidence of a strong correlation between bias in synonymous codon usage and the level of gene expression with the rs6275 SNP [50]. The minor *T* allele of rs6275 is found close to the synonymous rs6277 SNP (Pro319Pro), which results in a modified mRNA folding, decrease in mRNA stability and protein synthesis, as well as decreased expression of D2 receptor in the context of dopamine regulatory system [51]. Taking into account the relationship between rs6275 and rs6277, the molecular functioning according to which they act may be of a high similarity [49].

CONCLUSIONS

This study shows the association of haplotype built from polymorphic variants located in *DRD2/ANKK1* region with heroin dependence, and possible trends with substance dependence and heroine relapse. At this stage, it is not possible to state whether the statistically significant haplotypes discovered are typical of HD or a higher relapse rate. Studies by other researchers, as mentioned above, are differ from those obtained in the current study. However, they do indicate a similar research direction; therefore, it is necessary to carry out larger-scale studies. Nevertheless, the significance obtained may determine the direction of further research, both in the group of subjects dependent on psychoactive substances (e.g. polysubstance drug abuse) and other homogeneous subgroups.

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CONFLICTS OF INTEREST

The authors declare they have no conflict of interest.

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