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Alcohol Dependence and Polymorphisms of Serotonin-Related Genes: Association Studies

Gordana Mokrović[1], Ana Matošić[2], Dubravka Hranilović[3], Jasminka Štefulj[1], Mislav Novokmet[4], Melita Balija[5], Srđan Marušić[2] and Lipa Čičin-Šain[1]

[1] Laboratory of Neurochemistry and Molecular Biology, Department of Molecular Biology, Institute »Rudjer Bošković«, Zagreb, Croatia
[2] Department of Psychiatry, University Hospital »Sestre milosrdnice«, Zagreb, Croatia
[3] Department of Animal Physiology, Faculty of Science, University of Zagreb, Zagreb, Croatia
[4] Department of Biology, Faculty of Science, University of Zagreb, Zagreb, Croatia
[5] Croatian Institute of Transfusion Medicine, Zagreb, Croatia

A B S T R A C T

Variations in 5HT-related genes contribute to the alterations of serotonergic neurotransmission, which is implicated in the etiopathology of alcoholism. In this preliminary study we have tested polymorphisms of genes involved in 5HT transport and turnover for their association with alcohol dependence. A case group of males with type 2 alcoholism (N=59) and a control group of healthy males (N=282), both of Croatian origin, were analyzed for the frequency distribution of polymorphisms in 5HT transporter (5HTT-VNTR2, 5HTT-LPR), monoamine oxidase A (MAOA-uVNTR) and B (MAOB-A/G) and tryptophan hydroxylase 1 (TPH1 A218C) and 2 (TPH2 G-703T) genes. An increase in the frequencies of 10-repeat allele (p=0.010; OR=1.73; 95% CI=1.14–2.60) and 10/10 genotype (p=0.006; OR=2.57; 95% CI=1.3[illegible]00) of the 5HTT-VNTR2 polymorphism was found in alcoholic patients. No differences between case and control group were observed for the other tested polymorphisms. Present results support earlier studies implicating the role of 5HTT gene in alcoholism. The increase of sample size (in progress) is expected to enable search of more subtle differences, as well as re-evaluation of these preliminary findings.

Key words: *alcoholism, gene polymorphism, monoamine oxidase, serotonin transporter, tryptophan hydroxylase*

Introduction

Alcohol dependence is a complex psychiatric disorder with high heritability and high lifetime prevalence[1]. Among other factors, individual vulnerability to alcoholism has been related to inheritance of genes coding for proteins involved in neurotransmitters pathways of the brain reward system, including serotonergic (5HT) pathway[2]. Serotonergic transmission is regulated by coordinated actions of several 5HT-related proteins: 5HT autoreceptors, enzymes that synthesize (tryptophan hydroxylase, TPH) or degrade (monoamine oxidase, MAO) serotonin and 5HT transporter (5HTT) that cleans synaptic cleft by uptaking released 5HT back into nerve terminals. It was suggested that genetically driven variations in function of these 5HT-related proteins affects 5HT levels in the synaptic cleft, and consequently 5HT neurotransmission[3]. The relationship between hypoactivity of 5HT transmission and development of alcoholism has been well documented[4–7].

Previous studies on the genetic risk factors for alcohol dependence have mainly focused on the 5HTT gene[3]. Only a limited number of studies have tested polymorphisms in other 5HT-related genes providing inconsistent findings. This make further studies warranted, particularly on new, ethnically homogenous samples, as well as on patients subtyped according severity of symptoms.

The aim of this study was to test the association between type 2 alcoholic outcome and polymorphisms in

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several candidate genes from the 5HT-ergic pathway, for the first time in the Croatian population. The study encompassed six polymorphisms: variable number of tandem repeats in the 5HTT (5HTT-VNTR2 and 5HTT--LPR) and MAO-A (MAOA-uVNTR) genes and single nucleotide polymorphisms in the MAO-B (A/G dimorphism), TPH-1 (TPH1 A218C) and TPH-2 (TPH2 G--703T) genes.

Materials and Methods

Patients

The patient sa consisted of 59 male alcoholic inpatients, aging 32 6 years, and the co sample included 282 male blood donors, aging 42 12 years, without personal and family history of alcohol dependence and other psychiatric disorders. All subjects were of Croatian origin. The diagnosis of alcohol dependence and comorbidity was assessed by the structured clinical interview based on DSM-IV criteria. Additionally, patients were subtyped according to criteria for type 1 or type 2 alcoholism[8]. Only patients diagnosed with type 2 alcoholism were included in study. Psychiatric comorbidity within the sample included posttraumatic stress disorder (N=5), personality disorder (N=7) and anxiety/depression (N=3). This study was approved by the Ethics Committee of the »Sisters of Mercy« University Hospital, and written consent has been obtained from all participants.

Genotyping

Genomic DNA was prepared from blood samples using standard phenol chloroform extraction. Information on primers sequences and cycling conditions for PCR reactions (except for TPH-2 polymorphism) were reported previously[9–11].

For genotyping of TPH2 G-703T polymorphic site cycling conditions were: initial denaturation (96 °C for 90 s), 45 cycles of amplification (95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s) and final extension at 72 °C for 5 min. Total volume of 15 μL reaction mixture contained 100 ng of DNA, 1.5 mM $MgCl_2$, 50 μM dNTP, 0.5 U of Amply Taq DNA polymerase and 0.5 μM primers (5'-TTTCCATGATTTCCAGTAGAGAG-3' and 5'-AAGCTTTTTCTGACTTGACAAAT-3'). 5-μL aliquots of PCR products were digested overnight with 5U of XapI (*Fermentas*) in a total volume of 20 μL, and then separated by electrophoresis on 3% agarose gel stained with ethidium bromide. A 309 bp band represented G allele, while T allele was cut into two fragments of 285 bp and 24 bp.

Statistics

Hardy-Weinberg equilibrium and differences between groups genotype distribution were tested by two sided χ^2 test for independence. Comparisons of allele frequencies and genotype per genotype analyses were performed by two-tailed Fisher's exact test. Odd's ratio (OR) with 95% confidence interval (95% CI) was calculated using the approximation of Woolf's method. GraphPad InStat (version 3.01) was used for performing all statistical analyses. The level of significance was set at 0.05.

Results

Genotype and allele frequencies of six examined polymorphisms are presented in Tables 1–3. Genotype frequencies of all tested polymorphisms accorded with Hardy-Weinberg expectations in both, control and patient samples.

The frequency of the 10-repeat allele in 5HTT-VNTR2 polymorphism was significantly higher in patients than in the control group (p=0.010; OR=1.73; 95% CI= 1.14–2.60). The observed difference in genotype distribution (χ^2=8.35, *df*=2; p=0.015) is apparently due to increase of homozygote 10/10 genotype in the alcoholic sample (p= 0.006; OR=2.57; 95% CI=1.32–5.00) in comparison to genotypes 10/12 and 12/12. Because of the small frequency of 9-repeat allele, this allele and genotype containing it were not included in statistical analysis. The

TABLE 1
GENOTYPE AND ALLELE FREQUENCIES OF 5HTT-VNTR2 AND 5HTT-LPR POLYMORPHISMS OF 5HTT GENE IN TYPE 2 ALCOHOLICS AND MATCHED CONTROLS (PROPORTION OF TOTAL NUMBER OF INDIVIDUALS)

		5HTT-VNTR2							
		Genotype frequency					Allele frequency		
	N	12/12	10/12	10/10	9/12	9/10	12	10	9
Controls	282	0.41	0.41	0.14	0.03	0.02	0.64	0.34	0.02
Alcoholics	59	0.31	0.36	0.29	0.05	–	0.51	0.47	0.03

		5HTT-LPR				
		Genotype frequency			Allele frequency	
	N	L/L	L/S	S/S	L	S
Controls	281	0.36	0.49	0.15	0.61	0.39
Alcoholics	58	0.45	0.45	0.10	0.67	0.33

9,10,12 – number of repeats in particular allele, L – long allele, S – short allele

TABLE 2
ALLELE FREQUENCIES OF MAOA-uVNTR IN MAO-A GENE AND OF A/G DIMORPHISM IN MAO-B GENE IN TYPE 2 ALCOHOLICS AND MATCHED CONTROLS (PROPORTION OF TOTAL NUMBER OF INDIVIDUALS)

	MAOA-uVNTR						MAOB A/G		
		Allele frequency						Allele frequency	
	N	2R	3R	3.5R	4R	4.5R	N	A	G
Controls	141	0.01	0.36	0.01	0.62	0.01	141	0.50	0.50
Alcoholics	59	–	0.31	0.05	0.61	0.03	58	0.59	0.41

3R, 3.5R, 4R, 4.5R = number of repeats in particular allele

TABLE 3
GENOTYPE AND ALLELE FREQUENCIES OF SINGLE NUCLEOTIDE POLYMORPHISMS IN TPH-1 AND TPH-2 GENES IN TYPE 2 ALCOHOLICS AND MATCHED CONTROLS (PROPORTION OF TOTAL NUMBER OF INDIVIDUALS)

	TPH1 A218C					
		Genotype frequency			Allele frequency	
	N	A/A	A/C	C/C	A	C
Controls	281	0.16	0.50	0.34	0.41	0.59
Alcoholics	58	0.16	0.52	0.33	0.41	0.59

	TPH2 G-703T					
		Genotype frequency			Allele frequency	
	N	G/G	G/T	T/T	G	T
Controls	91	0.73	0.23	0.04	0.84	0.16
Alcoholics	59	0.69	0.25	0.05	0.82	0.18

allele (p=0.208) and genotype frequencies (χ^2= 1.80, *df*=2; p=0.407) of 5HTT-LPR polymorphism did not differ significantly between case and control samples (Table 1).

In the promoter region of MAO-A gene we have identified five different alleles with 2, 3, 3.5, 4 and 4.5 repeats (Table 2), with genotype/alleles with 3 or 4 repeats being the most common, as expected. The frequencies of MAOA-uVNTR (p=0.739) as well as MAOB A/G (p=0.277) genotype/alleles were not significantly different between alcoholics and controls (Table 2). No differences were obtained also in allele (TPH-1: p=1.00; TPH-2: p=0.751) or genotype frequencies (TPH-1: χ^2=0.058, *df*=2; p=0.971; TPH-2: χ^2=0.165, *df*=2; p=0.921) of tryptophan hydroxylase genes (Table 3).

Discussion

In this study we have examined the possible contribution of structural variations in 5HT-related genes to the etiology of alcohol dependence. Results of the association study for type 2 alcoholics were significant for intron 2 VNTR polymorphism of 5HTT gene, while none of the other results reached the significance level. The main drawback of the present study is a relatively small number of patients for this type of analysis, and, in this sense, our results should be regarded only as preliminary. Advantages of our study are: 1) Highly homogenous patients group regarding diagnosis – only type 2 alcoholics (thought to have more extensively impaired brain 5HT system than type 1 patients[4,5,12]) were included, 2) Several 5HT-related gene polymorphisms were genotyped in the same individual what will enable us to study 5HT--genes interactions as we increase the number of subjects, 3) To our knowledge this is the first study on the relationship between variants in 5HT-related genes and alcoholism in Croatian (Slavic) population. In genetic studies ethnicity represents an important factor, which may, in addition to other factors, contribute to unequivocal results, so replication studies in different ethnic groups are desirable[13].

We have identified increased frequency of the 10-repeat allele in type-2 alcoholic patients, as well as higher presence of homozygote 10/10 genotype of the intron 2 VNTR polymorphism of 5HTT gene. The allele and genotype frequencies of all other examined genes did not differ significantly between alcoholics and controls.

In the literature, we have found only one study on the relationship of 5HTT intron VNTR polymorphism to alcohol abuse, showing no association between them[14]. In contrast to our sample, patients in that study were not subtyped, so the excess of low-expressing 10-repeat allele

in our patient group may be related to the severity of alcoholism. These findings are in agreement with reports on prevalence of alleles with lower number of copies in depression[15], which is highly comorbid to alcoholism, as well as with later onset of mood disorders in patients carrying longer allele[16]. The other examined 5HTT gene polymorphism, 5HTT-LPR, did not show significant association with alcoholism in our sample. Its lower activity, short variant has been repeatedly associated with alcoholism[1,13,17], although there are also studies showing dissimilar results[18]. Enlargement of our patient group, providing higher statistical power, is needed to draw a final conclusion on our sample.

Regarding MAO, the most investigated polymorphism in alcoholism is MAO-uVNTR (which affects transcriptional activity[19]), but with contradictory results. Thus, an association between low-activity 3-repeat allele and alcoholism was described[20,21], but a lack of association[22,23] or association related only to females[24], was also found. No differences between type 1 and type 2 alcoholics were shown[25], but also a higher presence of 3-repeat allele was reported in earlier onset of alcoholism[21].

As to the role of TPH genes in genetic predisposition to alcoholism, there are only few studies suggesting no relation between alcohol dependence and either TPH-1 or TPH-2 gene variations[26–28], and our results are in line with their findings.

In conclusion, our preliminary results support previous studies implicating the role of the 5HTT gene in development of alcoholism, while other examined 5HT gene variants did not seem to be related to alcohol abuse. Future increase of sample size is expected to enable analyses of more subtle differences, as well as a reevaluation of the above-mentioned findings, and these investigations are in course in our laboratory.

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L. Čičin-Šain

Laboratory of Neurochemistry and Molecular Neurobiology, Ruđjer Boskovic Institute, Bijenicka cesta 54, 10000 Zagreb, Croatia
e-mail: cicinsai@irb.hr

OVISNOST O ALKOHOLU I POLIMORFIZMI GENA SEROTONINSKOG SUSTAVA: ASOCIJACIJSKA STUDIJA

S A Ž E T A K

Etiopatologija alkoholizma povezuje se sa varijacijama u genima koji kodiraju 5HT-vezane sinaptičke proteine – transporter, receptore, enzime – koji sudjeluju u regulaciji serotonergične transmisije. U ovom radu istražili smo povezanost alkoholizma i varijacija u genima koji su odgovorni za prijenos i metabolizam serotonina. Uspoređena je distribucija učestalosti polimorfizama gena za 5HT prijenosnik (5HTT-VNTR2, 5HTT-LPR), monoaminoksidazu A (MAOA-uVNTR) i B (MAOB-A/G) te triptofan hidroksilazu 1 (TPH1 A218C) i 2 (TPH2 G-703T) kod pacijenata sa tipom 2 alkoholizma (N=59) i kontrolnih ispitanika (N=282). Kod pacijenata je opažena povećana učestalost alela s 10 ponavljanja (p=0.010; OR=1.73; 95% CI=1.14–2.60) i 10/10 genotipa (p=0.006; OR=2.57; 95% CI=1.32–5.00) 5HTT-VNTR2 polimorfizma. Učestalost ostalih polimorfizama nije se razlikovala između pacijenata i kontrolne skupine. Naši rezultati, dobiveni na hrvatskoj populaciji, u skladu su sa literaturnim podacima koji upućuju na ulogu serotoninskog prijenosnika u nastanaku alkoholizma.