

GABA Receptors Genes Polymorphisms and Alcohol Dependence: No Evidence of an Association in an Italian Male Population

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Objective: The genes encoding for gamma-aminobutyric acid (GABA) A and B receptors may be considered as candidates for alcoholism; genetic alterations at this level may produce structural and functional diversity and thus play a role in the response to alcohol addiction treatment. To investigate these aspects further, we conducted a preliminary genetic association study on a population of Italian male alcohol addicts, focusing on GABA A and B receptors.

Methods: A total of 186 alcohol-dependent subjects (in the first phase 139, then 47 more samples) and 182 controls were genotyped for 25 single nucleotide polymorphisms (SNPs) of genes encoding the alpha-1 subunit of GABA A receptor (GABRA1) and subunits 1 and 2 of GABA B receptor (GABBR1 and GABBR2). The chi-squared test for allele and genotype distributions and Hardy-Weinberg equilibrium analysis of both subjects and controls were performed. Bonferroni's correction for multiple comparisons was applied.

Results: Preliminary results comparing 139 alcohol-dependent subjects and 182 controls showed differences in genotype distribution in the former for SNP rs29253, located in the intron region of the GABBR1 gene. In order to clarify the meaning of this association, 47 more samples from alcohol-dependent subjects were tested for this SNP only: the previously found association was not confirmed.

Conclusion: The lack of significant differences between the two groups does not provide evidence that GABRA 1 and GABBR1 and 2 genes are candidates for alcoholism in this population. Further studies with larger samples are needed, together with investigation of other components of the GABA pathway.

KEY WORDS: Alcohol dependence; γ -Aminobutyric acid receptors; GABRA1; GABBR1; GABBR2.

INTRODUCTION

Alcohol consumption has been associated with personal, family, social and medico-legal problems, including dropping out of school, productivity losses at work, and driving impairment with road accidents.^{1,2)}

The development of alcohol dependence and, in general, of alcohol use disorder has been linked to environmental and biological factors.³⁾ Among the latter, the neurobiological pathway of gamma-aminobutyric acid (GABA) has been related to alcohol dependence.⁴⁻⁶⁾ This relation is supported by evidence that the GABA system is involved in the acute and chronic behavioral effects of

ethanol,⁷⁾ including defective motor coordination, anxiety, sedation, withdrawal signs, and ethanol preference.^{8,9)} The relation between alcoholism and the GABA system has also been analysed in studies of GABA receptors. The most frequent subtypes of GABA receptors are type A and type B.

The relation between GABA A receptor and alcoholism has been examined from the pharmacological and genetic points of view in both animal and human studies.^{9,10)} The GABA A receptor is a trans-membrane ligand-gated ion channel which has been shown to be directly modulated by ethanol, which specifically potentiates ligand-gated currents. Acute ethanol exposure causes ion channel opening, with increased GABA-gated chloride ion uptake.¹¹⁾ The agonists of GABA A receptor tend to enhance the behavioral effects of alcohol, whereas its antagonists attenuate them.⁹⁾ Human genetic studies have reported conflicting results about the role of the genetic region coding for this receptor and the development of alcoholism.¹²⁻¹⁵⁾

Received: January 22, 2014 / **Revised:** March 10, 2014

Accepted: April 15, 2014

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GABA B receptor, involved in the pathophysiology of several neuropsychiatric disorders,^{16,17} has also been studied in relation to alcoholism.¹⁸ The role of the direct agonists of this receptor, including baclofen and positive allosteric modulators, on various alcohol-related behaviors,¹⁹ again supports the relation between GABA pathways and alcohol. The drugs acting on this receptor constitute a novel class of potentially effective substances against alcohol dependence.

Genetic studies on DNA regions coding for GABA B receptor performed on GABBR1 gene highlight an association with alcoholism and electroencephalographic abnormalities²⁰ but do not reveal any relation with diagnosis of alcoholism or withdrawal seizures.¹⁸

As GABA is related to alcohol dependence, the drugs acting on GABA A and B receptors (e.g., baclofen or gamma hydroxybutyric acid) may be used to treat alcohol dependence, so that the genes encoding for these receptors may be viewed as candidates for alcoholism and potentially important in modulating the response to alcohol addiction treatment.

To examine these aspects further, we carried out a case-control study to explore the association between the single nucleotide polymorphisms (SNPs) of three GABA receptor genes (GABRA1, GABBR1, GABBR2) and alcohol dependence in a quite homogeneous population of subjects from the Veneto Region, North-East Italy.

METHODS

Participants and Phenotype Assessment

The total cohort analysed contained 368 Italian subjects from the same region, subdivided into two groups: group 1, n=186, subjects with alcohol dependence according to the Diagnostic and Statistical Manual of Mental Disorders 4th edition text revision (DSM-IV-TR) criteria,²¹ recruited from the substance addiction centers of Dolo, Rovigo, Cittadella, and the Section of Forensic Toxicology, University Hospital of Padova; group 2, n=182, recruited from donors from the blood donor centers of Padova Hospital and the nearby "Ai Colli" Hospital.

Participants were all male Italian subjects with grandparents born in the Veneto Region, over the age of 25 and, for group 1, with the onset of alcohol dependence before the age of 25. Exclusion criteria for both groups were psychiatric, neurological and/or medical disorders.

After the aims of the study had been explained to the subjects, together with information on the strict con-

fidentiality and anonymity of recorded data, subjects' written informed consent was obtained. After examination of medical documentation (including chemo-toxicological analyses), anamnesis, and Audit and Cage clinical-behavioral tests, all subjects were subjected to a general objective and toxicological clinical examination.

At the end of the interviews and examinations, EDTA-anticoagulated peripheral blood samples were obtained from each participant for DNA extraction.

The study protocol was approved by the local Ethics Committee for clinical trials of the province of Padova, Hospital of Padova.

SNP Selection and Genotyping

SNPs were selected from the dbSNP database of National Center for Biotechnology Information, United States (NCBI; <http://www.ncbi.nlm.nih.gov>) and from literature findings for DNA regions encoding for GABA A receptor, alpha-1 subunit (GABRA1 gene) and GABA B receptor, subunit 1 (GABBR1 gene) and subunit 2 (GABBR2 gene).

For primary screening of polymorphisms in GABA receptor genes, non-synonymous SNPs located in the coding regions were first selected, as amino-acid changes by SNPs within exon regions may affect protein structure and/or function. Exon SNPs responsible for silent mutation without amino-acid changes were also included in the study. In addition, SNPs in intron regions and promoters were selected, because they may affect mRNA splicing and gene expression, respectively.

A total of 25 SNPs were genotyped: 5 SNPs of GABRA1 gene were located in intron regions (rs 12653365, rs 1026447, rs 10042696, rs 12188495, rs 7701394). Seven SNPs were selected for GABBR1 gene: two SNPs in exon regions (rs 29225, rs 29230) and five in intron regions (rs 3025632, rs 29253, rs 29267, rs 2267635, rs 29259). Thirteen SNPs were genotyped in GABBR2 gene: two in 3'UTR (rs 10124070, rs 9696283) regions, five in coding regions (rs 2304389, rs 3750344, rs 74856364, rs 10985765, rs 80294096) and six in intron regions (rs 3780428, rs 13295101, rs 7865648, rs 12337255, rs 2900512, rs 3780421).

Genome DNA was isolated from venous blood and extracted in an automated 96-well plate system (MultiPROBE II HTFEX automated system; Perkin Elmer, Shelton, CT, USA). SNPs were genotyped on the GenomeLab SNPStream high-throughput 12-plex genotyping platform (Beckman Coulter, Fullerton, CA, USA) according to methods previously described by Terranova

et al.⁶⁾

SNP analysis were carried out in two phases. First, genotyping was performed, comparing 139 alcohol-dependent subjects and 182 controls. Then 47 other samples from alcohol-dependent subjects were compared with the same

controls.

Results were confirmed by direct sequencing on an ABI Prism 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Table 1. Allele and genotype frequencies and exact *p* value of alcoholics and controls for the 23 SNPs resulted polymorphic (MAF>0.01).

ID SNPs	Gene	Location	Group	Allele frequencies		Genotype frequencies			Allele exact <i>p</i> value	Genotype exact <i>p</i> value
				C	T	CC	CT	TT		
rs 12653365	GABRA1	Intron	AD	0.910	0.089	0.849 (118)	0.122 (17)	0.029 (4)	0.3873	0.0694
			Controls	0.931	0.069	0.863 (157)	0.137 (25)	-		
rs 1026447	GABRA1	Intron	AD	0.316	0.683	0.093 (13)	0.446 (62)	0.460 (64)	0.7228	0.6829
			Controls	0.332	0.667	0.121 (22)	0.423 (77)	0.456 (83)		
rs 10042696	GABRA1	Intron	AD	0.547	0.453	0.288 (40)	0.518 (72)	0.194 (27)	0.6690	0.9247
			Controls	0.533	0.467	0.280 (51)	0.505 (92)	0.214 (39)		
rs 12188495	GABRA1	Intron	AD	0.352	0.647	0.101 (14)	0.503 (70)	0.396 (55)	0.7243	0.5747
			Controls	0.338	0.662	0.115 (21)	0.445 (81)	0.439 (80)		
rs 7701394	GABRA1	Intron	AD	0.547	0.453	0.288 (40)	0.518 (72)	0.194 (27)	0.9323	0.2759
			Controls	0.549	0.450	0.335 (61)	0.428 (78)	0.236 (43)		
rs 29225	GABBR1	Exon	AD	0.155	0.845	0.043 (6)	0.223 (31)	0.734 (102)	0.3167	0.0917
			Controls	0.124	0.876	0.005 (1)	0.236 (43)	0.758 (138)		
rs 29230	GABBR1	Exon	AD	0.208	0.791	0.086 (12)	0.244 (34)	0.670 (93)	0.7021	0.2756
			Controls	0.222	0.777	0.060 (11)	0.324 (59)	0.615 (112)		
rs 10985765	GABBR2	Exon	AD	0.136	0.863	0.029 (4)	0.216 (30)	0.755 (105)	0.1183	0.3354
			Controls	0.178	0.821	0.060 (11)	0.236 (43)	0.703 (128)		
rs 13295101	GABBR2	Intron	AD	0.395	0.604	0.144 (20)	0.503 (70)	0.352 (49)	0.3007	0.5827
			Controls	0.357	0.643	0.126 (23)	0.461 (84)	0.412 (75)		
rs 7865648	GABBR2	Intron	AD	0.414	0.586	0.208 (29)	0.410 (57)	0.381 (53)	0.9307	0.9789
			Controls	0.415	0.585	0.214 (39)	0.401 (73)	0.385 (70)		
rs 12337255	GABBR2	Intron	AD	0.216	0.784	0.029 (4)	0.374 (52)	0.597 (83)	0.6457	0.7991
			Controls	0.231	0.769	0.044 (8)	0.374 (68)	0.582 (106)		
rs 2900512	GABBR2	Intron	AD	0.834	0.165	0.705 (98)	0.259 (36)	0.036 (5)	0.7514	0.9736
			Controls	0.827	0.173	0.692 (126)	0.269 (49)	0.038 (7)		
rs 29253	GABBR1	Intron	AD	0.126	0.874	0.043 (6)	0.165 (23)	0.791 (110)	0.821	0.0129
			Controls	0.134	0.865	0.005 (1)	0.258 (47)	0.736 (134)		
rs 29267	GABBR1	Intron	AD	0.859	0.140	0.748 (104)	0.223 (31)	0.029 (4)	0.8289	0.9398
			Controls	0.852	0.148	0.731 (133)	0.242 (44)	0.027 (5)		
rs 2267635	GABBR1	Intron	AD	0.054	0.946	0.029 (4)	0.050 (7)	0.921 (128)	0.0219	0.1621
			Controls	0.104	0.895	0.077 (14)	0.055 (10)	0.868 (158)		
rs 3780421	GABBR2	Intron	AD	0.115	0.885	0.007 (1)	0.216 (30)	0.777 (108)	0.8961	0.9559
			Controls	0.115	0.885	0.011 (2)	0.209 (38)	0.780 (142)		
rs 29259	GABBR1	Intron	AD	0.216	0.784	0.072 (10)	0.288 (40)	0.640 (89)	0.8516	0.1813
			Controls	0.222	0.777	0.038 (7)	0.368 (67)	0.593 (108)		
				A	G	AA	AG	GG		
rs 10124070	GABBR2	3' UTR	AD	0.514	0.485	0.237 (33)	0.554 (77)	0.208 (29)	0.2234	0.1183
			Controls	0.560	0.439	0.335 (61)	0.450 (82)	0.214 (39)		
rs 9696283	GABBR2	3' UTR	AD	0.852	0.147	0.748 (104)	0.208 (29)	0.043 (6)	0.3368	0.3944
			Controls	0.824	0.176	0.687 (125)	0.275 (50)	0.038 (7)		
rs 2304389	GABBR2	Exon	AD	0.187	0.813	0.014 (2)	0.345 (48)	0.640 (89)	0.6635	0.2000
			Controls	0.173	0.827	0.038 (7)	0.269 (49)	0.692 (126)		
rs 3750344	GABBR2	Exon	AD	0.849	0.151	0.720 (100)	0.259 (36)	0.021 (3)	0.7364	0.7496
			Controls	0.841	0.159	0.698 (127)	0.286 (52)	0.016 (3)		
rs 3025632	GABBR1	Intron	AD	0.083	0.917	0.021 (3)	0.122 (17)	0.856 (119)	0.5395	0.1571
			Controls	0.068	0.931	-	0.137 (25)	0.863 (157)		
rs 3780428	GABBR2	Intron	AD	0.324	0.676	0.187 (26)	0.273 (38)	0.539 (75)	0.2711	0.5030
			Controls	0.365	0.635	0.203 (37)	0.324 (59)	0.472 (86)		

MAF, minor allele frequency; ID SNPs, identified single nucleotide polymorphisms; C, cytosine; T, thymine; AD, alcohol dependent; GABRA1, gamma-aminobutyric acid A receptor, alpha-1 subunit; GABBR1, gamma-aminobutyric acid B receptor, subunit 1; GABBR2, GABBR subunit 2; UTR, untranslated region; A, adenine; G, guanine; UTR, untranslated region.

Statistical Analysis

To evaluate study feasibility, a power analysis was performed with software available at <http://pngu.mgh.harvard.edu/~purcell/gpc/>. Power was set at >80%, significance $\alpha=0.05$.

Statistical analyses were carried out by Powermaker (North Carolina State University, Raleigh, NC, USA).²²⁾ The allele frequencies and genotype distributions of each polymorphism were compared in subjects and controls with the χ^2 test, $p \leq 0.05$, to detect statistically significant SNPs.

Hardy-Weinberg equilibrium (HWE) was tested in all 368 subjects, i.e., both groups (186 alcohol-dependent subjects and 182 controls). Bonferroni's correction for multiple comparisons was applied.

Linkage disequilibrium (LD) and haplotype block structure were revealed by Haploview software version 4.1.²³⁾

RESULTS

Twenty-five SNPs located in the GABRA1, GABBR1 and GABBR2 gene regions were genotyped in two phases, according to the number and type of samples. First, 139 alcohol-dependent and 182 controls were genotyped. Table 1 lists the 23 SNPs which turned out to be polymorphic. HWE for the 23 SNPs which were polymorphic (minor allele frequencies >1%) was tested separately

in subjects and controls with the chi-squared test. Deviations from HWE were found for the following SNPs in alcohol-dependent subjects: rs 12653365, rs 29230, rs 2267635, rs 29253, rs 9696283, rs 3780428, rs 3025632. In controls, the SNPs involved were rs 2267635, rs 10985765, rs 7865648 and rs 3780428. Allele and geno-

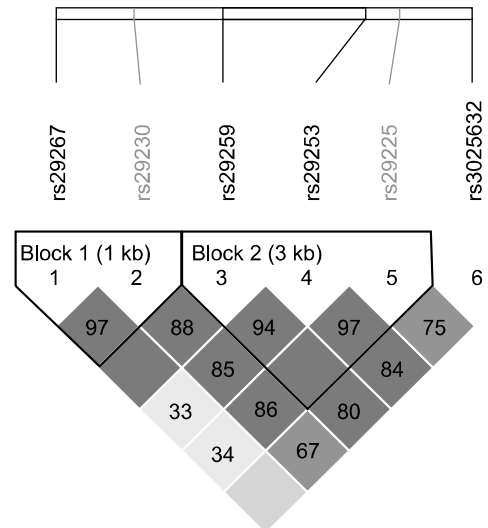


Fig. 2. Linkage disequilibrium (LD) plot in GABBR1 gene identified by SNPs 1 to 6 in control group. Numbers within diamonds are D' values for the respective SNP pairs. Empty diamonds indicate an absolute LD ($D'=1$). GABBR1, gamma-aminobutyric acid B receptor, subunit 1.

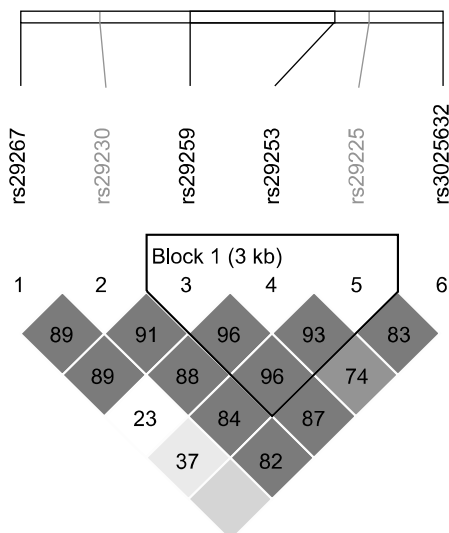


Fig. 1. Linkage disequilibrium plot in GABBR1 gene identified by SNPs 1 to 6 in AD group. Numbers within diamonds are D' values for the respective SNP pairs. GABBR1, gamma-aminobutyric acid B receptor, subunit 1; AD, alcohol dependent.

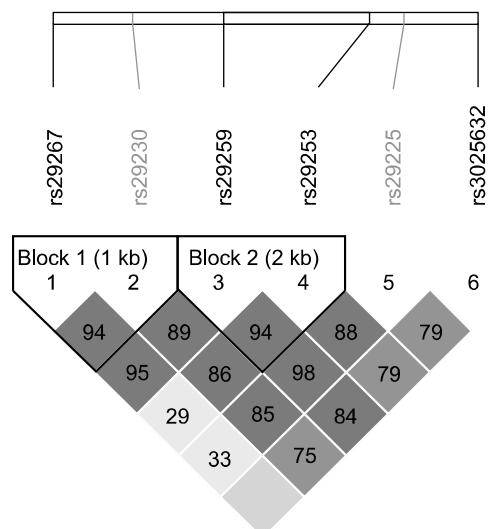


Fig. 3. Linkage disequilibrium plot in GABBR1 gene identified by SNPs 1 to 6 in AD and control groups. Numbers within diamonds are D' values for the respective SNP pairs. GABBR1, gamma-aminobutyric acid B receptor, subunit 1; AD, alcohol dependent.

type frequencies were also calculated, to test the association between the 23 SNPs and alcohol dependence. No significant differences were found, except for SNP rs 29253 ($p=0.0129$; Table 1) located in the intron region of GABBR1 gene. However, this association did not resist Bonferroni correction for multiple testing (p -threshold < 0.002). LD plot strengths between 6 SNPs and haplotype blocks in GABBR1 were tested for alcohol dependent and controls, separately and for the whole sample. Haplotype blocks for alcohol dependent samples are shown in Fig. 1. SNP rs 29253 was found with high LD in intron SNP rs 29259 ($D'=0.96$) and exon SNP rs 29225 ($D'=0.93$).

Similar LD patterns were observed in the control group. In this case, a second LD block was observed, with SNPs rs 29267 and rs 29230 ($D'=0.97$). Results are shown in Fig. 2.

LD haplotype blocks for SNPs in the whole sample (alcohol-dependent and controls) are shown in Fig. 3. Two LD blocks were defined: one consisting of SNPs rs 29267 and rs 29230 ($D'=0.94$) and the other SNPs rs 29259 and rs 29253 ($D'=0.94$).

The haplotypes identified for the two SNPs in each block are listed in Table 2, which also shows haplotype frequencies, calculated for the whole sample and for subjects and controls independently.

Table 2. Haplotype frequencies, χ^2 and p value observed for SNPs rs 29267 and rs 29230 (block 1) and for SNPs rs 29259 and rs 29253 (block 2)

Haplotype	Frequencies			χ^2	p value
	Cases and controls	Cases	Controls		
Block 1					
CT	0.777	0.780	0.774	0.032	0.858
TC	0.138	0.129	0.145	0.340	0.560
CC	0.078	0.080	0.077	0.010	0.921
Block 2					
TT	0.775	0.780	0.771	0.070	0.791
CC	0.126	0.122	0.129	0.063	0.802
CT	0.094	0.094	0.094	0.000	0.997

C, cytosine; T, thymine.

As the Bonferroni correction could be extremely conservative, leading to a high rate of false negative, we decided to clarify the possible significance of the SNP rs 29253 in a second sample of alcohol dependent subjects. After a power analysis based on the first sample (power was set at $> 80\%$), 47 additional alcohol dependent subjects were genotyped. Allele and genotype frequency distributions for these 47 and the 182 controls are listed in Table 3. No significant differences were found ($p=0.766$).

DISCUSSION

This research is the first Italian genetic study evaluating the association between SNPs of GABRA1, GABBR1 and GABBR2 genes and alcohol dependence in a population of subjects all coming from the same region of Italy.

The candidate gene approach was considered suitable for our study, as the trait analysed—alcohol dependence—is relatively homogeneous. In proposing the study, the authors are aware of the low sample size, considering the low risk associated with common variants in relation to psychiatric disorders. However, taking into account sample size, the statistical test at level $\alpha=0.05$ has sufficient power (at least 80%) to demonstrate differences of 15-20% compared with a proportion in the control groups of around 60%.

Association studies of SNPs of GABA A and B receptor DNA regions have already been performed, more frequently analysing subunits of GABA A.²⁴ In order to focus on DNA regions less frequently studied in relation to alcohol dependence, we chose to analyse only five SNPs of GABRA1 gene and 20 SNPs of GABA B receptor genes (7 SNPs of GABBR1, 13 SNPs of GABBR2 gene), although pharmacological studies indicate that GABA A rather than GABA B receptor is more strongly implicated in alcohol dependence. Most of the chosen SNPs have never been investigated in relation to diagnosis of alcohol dependence or related phenotypes.

Specifically, Dick *et al.*²⁵ tested various SNPs on the GABA A receptor gene cluster and 16 SNPs of the

Table 3. Allele and genotype frequencies and exact p value of 47 alcoholics and 182 controls for SNP rs 29253

ID SNPs	Gene	Location	Group	Allele frequencies		Genotype frequencies			Allele exact p value	Genotype exact p value
				C	T	CC	CT	TT		
rs 29253	GABBR1	Intron	AD	0.117	0.883	-	0.234 (11)	0.766 (36)	0.727	0.766
			Controls	0.134	0.865	0.005 (1)	0.258 (47)	0.736 (134)		

ID SNPs, identified single nucleotide polymorphisms; C, cytosine; T, thymine; GABBR1, gamma-aminobutyric acid B receptor, subunit 1; AD, alcohol dependent.

GABRA1 gene for their association with several alcohol-related behaviors. They observed an association across multiple SNPs in GABRA1 with alcohol-related phenotypes, but not for SNP rs 1026447 (which we also studied). The lack of association for GABRA1 in our sample is not in contrast with the above study, and is mainly due to the fact that the studied SNPs were different, with a different population, and only the diagnosis of alcohol dependence was tested, not that of the alcohol-related phenotype.

SNP rs 29230 in the exon regions of GABBR1 gene has been associated with alcoholism and EEG abnormalities,²⁶⁾ although Köhnke *et al.*¹⁸⁾ in 2006 did not find any evidence for considering this SNP as a candidate for alcoholism or alcohol withdrawal seizures. Our findings confirm the lack of association between rs 29230 and alcohol dependence.

For the same GABBR1 gene, we analysed six other SNPs, and found a statistical association between intron SNP rs 29253, never previously studied, and the condition of alcohol dependence. Polymorphism rs 29253 is located in an intron region and does not affect the amino acid sequence of the receptor GABA B subunit 1, responsible for ligand binding in heterodimer configurations.²⁷⁾ To clarify this association further, we extended analysis of this SNP to 47 other alcohol-dependent samples, using the same number of controls. The results showed no evidence of association between SNP rs 29253 and alcohol dependence, and neither did we find any evidence of association between GABBR1 gene and alcohol dependence. However, because recent studies¹⁹⁾ report that GABA B receptor is involved in alcoholism and alcohol withdrawal, future research should explore the existence of other still unknown SNPs, particularly functional polymorphisms, of the GABBR1 gene which may be significantly associated with alcohol dependence.

Five SNPs in GABBR2 (rs 3750344, rs 13295101, rs 7865648, rs 12337255, rs 2900512) have been found to be significantly associated with nicotine dependence,^{28,29)} a condition which frequently coexists and shares some genetic factors with alcohol dependence. We did not find any association between the SNPs examined in GABBR2 and alcohol dependence.

Despite the lack of significant differences between the compared groups, further analysis of more SNPs in a larger sample of subjects would provide new information on the relationship between GABA receptor genes and alcohol dependence.

In conclusion, future developments of our study might

include analysis of variants in GABA receptor genes, not only of their association with alcohol dependence but also as moderators of the response to alcohol addiction treatment. Potential future genetic characterization may suggest the influence of DNA variations on the response to such treatment.

Lastly, we are interested in further assessments to ascertain whether genes of GABA receptors can moderate the effect of environmental factors (such as alcohol availability, parental attitudes, peer pressure, underage drinking and childhood maltreatment) on vulnerability to developing alcohol dependence.

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