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Association of $GABA_A$ receptor α_2 subunit gene (GABRA2) with alcohol dependence-related aggressive behavior

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Abstract

Alcohol dependence is a common chronic disorder precipitated by the complex interaction between biological, genetic and environmental risk factors. Recent studies have demonstrated that polymorphisms of the gene encoding the $GABA_A$ receptor α_2 subunit (GABRA2) are associated with alcohol dependence in different populations of European ancestry. As aggression often occurs in the context of alcohol dependence, the aim of this study was to examine the allelic and haplotypic association of GABRA2 gene with alcohol dependence and related aggressive behavior in subjects of Eastern European (Croatian) origin.

Genotyping of the 3 single nucleotide polymorphisms (SNPs) across the GABRA2 gene (rs567926, rs279858 and rs9291283) was performed in patients with alcohol dependence (N = 654) and healthy control subjects (N = 574). Alcohol-dependent participants were additionally subdivided according to the presence/absence of aggressive behavior and type of alcohol dependence according to the Cloninger's classification.

The association of rs279858 with alcohol dependence yielded nominal significance level. Haplotype analysis revealed a high degree of linkage disequilibrium (LD) for rs567926 and rs279858, but not for rs9291283 polymorphism in the GABRA2 gene. In patients with alcohol dependence, the A-C (rs567926 and rs279858) haplotype carriers were more likely to demonstrate aggressive behavior. The same haplotype (present only in 1.6% of all subjects) was significantly more often present in patients with a combination of early onset alcohol abuse and aggression, corresponding to the Cloninger's type II alcoholism subgroup. These findings support the involvement of GABRA2 gene in alcohol dependence-related aggressive behavior.

Keywords: aggression, alcohol dependence, Cloninger's classification, GABRA2, haplotype, polymorphism

1. Introduction

Alcohol dependence is a highly prevalent chronic disorder associated with a wide range of physical, mental, social, functional, legal and economic consequences. This complex disease, precipitated by the interaction between biological, genetic and environmental risk factors, is heterogeneous in its etiology and phenotype characteristics, such as the age of onset, drinking behavior, as well as comorbid disorders (Köhnke, 2008). The findings that alcoholism shows no classic pattern of inheritance, and that alcohol affects various targets in the brain, suggest that genetic vulnerability to alcoholism is likely to be due to multiple contributing genes encoding proteins in many neurotransmitter systems and signal transduction pathways (Enoch, 2008). $GABA_A$ receptors, the major inhibitory neurotransmitter receptors in the brain (Korpi et al., 2002), are implicated in the acute and chronic effects of alcohol, including sedation, anxiolysis, lack of motor coordination, ethanol preference, tolerance, dependence and withdrawal (Kumar et al., 2009).

Genomewide linkage studies suggest that several $GABA_A$ receptor gene clusters might influence the susceptibility for alcohol dependence, including the cluster of $GABA_A$ receptor subunit genes (α_2, α_4, β_1, γ_1) located on chromosome 4p13-12 (Reich et al., 1998). Different studies demonstrated that various polymorphisms in the genes encoding $GABA_A$ receptor subunits are associated with alcoholism in different populations of European ancestry (Covault et al., 2004; Enoch et al., 2006; Lappalainen et al., 2005; Li et al., 2014; Sander et al., 1999; Soyka et al., 2008). However, recent findings suggest that the association of alcohol dependence with markers and haplotypes in the middle and 3’ region of the GABRA2 gene might also be due to the linkage disequilibrium (LD) with risk-related variants in the adjacent GABRG1 gene (Covault et al., 2008; Drgon et al., 2006).

Alcohol consumption has been often linked to violence and aggression (Beck and Heinz, 2013), while aggression-related personality traits have been suggested to mediate individual responses to alcohol (Bjork et al., 2004). Alcohol-related aggression also occurs frequently in the context of chronic alcohol consumption and dependence (Beck and Heinz, 2013) and various studies estimate that up to 50% of alcohol-dependent men display violent behavior (Giancola et al., 2009). Research conducted on the involvement of GABRA2 gene in alcohol use and impulse control behavior, suggested its role in hyperexcitability, impulsivity, aggression or externalizing spectrum disorder (Dick et al., 2006, 2009; Heitzeg et al., 2014; Simons et al., 2013; Villafuerte et al., 2013, 2014). These findings are in line with the results of many studies demonstrating the involvement of GABAergic system in aggressive behavior (Takahashi and Miczek, 2014).

The aim of the present study was to determine the allelic and haplotypic association of the GABRA2 gene with alcohol dependence and alcohol dependence-related aggressive behavior in Caucasian subjects of East European (Croatian) origin. Therefore, three single nucleotide polymorphisms (SNPs) located in the 5’and central region of the GABRA2 gene and in the intergenic region between GABRA2 and GABRG1 genes were analyzed: rs567926 (3’ flanking region), rs279858 (exon 5) and rs9291283 (intron 3).

All these SNPs have been previously reported to be associated (individually and in haplotype block with other SNPs) with alcohol dependence (Covault et al., 2004; 2008; Edenberg et al., 2004; Fehr et al., 2006; Ittiwut et al., 2008; Lappalainen et al., 2005; Soyka et al., 2008). The distribution of the GABRA2 gene variants in alcohol-dependent patients and healthy subjects, corrected for gender, age and nicotine dependence was determined. In order to elucidate possible behavioral effects of GABRA2 variations, the group of participants with alcohol dependence was additionally subdivided according to the presence/absence of aggressive behavior and type of alcohol dependence according to the Cloninger’s classification.

2. Materials and methods

2.1. Subjects

A total of 1228 unrelated Caucasian subjects of East European (Croatian) origin, including 574 healthy control subjects and 654 medication-free patients with alcohol dependence, were enrolled in the study. All subjects were recruited during the period between 2005 and 2009 from the Centre of Alcoholism and Other Addictions, Psychiatric Hospital Vrapce and University Hospital Centre Zagreb, Croatia.

The diagnosis of alcohol dependence was done using the Structured Clinical Interview (SCID), based on the DSM-IV criteria (American Psychiatric Association, 1994). The interview and the blood sampling were conducted after hospital admission prior to starting treatment. In addition to the SCID and a psychiatric interview, aggressive behavior was assessed using the Brown–Goodwin Assessment of Lifetime Aggression (Brown–Goodwin Scale; Brown et al., 1979). The questionnaire adapted from Brown–Goodwin Scale consisted of seven behavioral categories (Buydens-Branchey et al., 1989), translated into Croatian: problems with discipline in the armed forces; problems with discipline at work; assaults on other persons; property damage; incarceration for assaultive behavior; incarceration for other crimes; crimes that did not result in incarceration. These categories were evaluated with a 0–4 scale. The total maximum score was 28, and according to Buydens-Branchey et al. (1989), a cut-off score of 8 was designated for the presence of aggressive behavior (Buydens-Branchey et al., 1989). The alcohol-dependent patients with a combination of early onset of alcohol abuse (occurring before 25 years of age) and presence of aggressive behavior corresponded to type II alcoholism subgroup, while the patients with the late onset of alcohol abuse (occurring after 25 years of age) and without aggression corresponded to type I alcoholism subgroup, according to the Cloninger’s classification (Cloninger et al., 1988).

Healthy control subjects completed questionnaires regarding their medical history, drinking and smoking habits. Inclusion criteria were no current medication therapy, no previous or current psychiatric disorders, no drug or alcohol abuse, no suicide attempts, no family history of psychiatric disorders (determined according to participants' self-report about the mental health status of their parents, grandparents, siblings and children), unrelated to other study participants, and belonging to the native ethnic group with at least three generations living in the region.

All participants were Caucasians of the East European origin from the same geographic area (i.e., from the Zagreb County, Croatia). The relative genetic homogeneity of the enrolled subjects was confirmed by the principal component analysis (PCA) performed with program PAST, Version 3.06 (Hammer et al., 2001). All participants agreed to give a blood sample, to participate in the study, and gave their written informed consent. The study was approved by the local Ethics committees and was carried out in accordance with the Helsinki declaration (1975).

Demographic and clinical sample characteristics of the control and alcohol-dependent individuals are listed in Table 1. The group of subjects with alcohol dependence was significantly older than the control group ($t = 14.78$, $df = 1226$, $p < 0.0001$; Student'*t*-test). Following ANOVA ($F(3,1224) = 75.58$; $p < 0.001$), Tukey test revealed that both alcohol-dependent males ($p < 0.001$) and females ($p < 0.001$) were significantly older than healthy men and women. Although both healthy and alcohol-dependent participants were predominantly male (89.02% and 81.19% respectively), the group of alcoholics included more females than control group ($\chi^2 = 14.59$, $df = 1$, $p = 0.0001$). Patients with alcohol dependence smoked more frequently ($\chi2 = 99.96$, $df = 1$, $p < 0.0001$) than healthy participants.

2.2. Genotyping

Blood samples (8 ml) from alcohol-dependent patients and control subjects were drawn in a plastic syringe with 2 ml acid citrate dextrose anticoagulant. Genomic DNA was isolated from peripheral blood leukocytes according to standard procedures by a salting out method. Three SNPs located in the 5’and central region of the GABRA2 gene and in the intergenic region between GABRA2 and GABRG1 genes on chromosome 4 were analyzed. Namely, rs567926 (3’ flanking region), rs279858 (exon 5) and rs9291283 (intron 3) were genotyped using Taqman^{TM} probe-primer combinations (Cat. No. C_7537087_10, C_2073557_1_ and C_8262290_10), available from the Applied Biosystems Assay-on-Demand^{TM} human SNP genotyping collection (Applied Biosystems, Foster City, CA, USA). Taqman-based allele-specific polymerase chain reactions and post-PCR fluorescence plate reads were performed according to the procedure described by Applied Biosystems, using an ABI Prism 7000 Sequencing Detection System apparatus. Briefly, 20 ng of genomic DNA was PCR amplified in 96-well plates using a 10 μl reaction volume for 40 cycles at 92°C for 15 s followed by 60°C for 60 s. Allele nucleotide designation of the analyzed SNPs refers to the chromosome plus strand sequence.

2.3. Data analysis

Statistical analyses were performed with GraphPad Prism version 4.00 (GraphPad Software, San Diego, CA, USA) and MedCalc version 12.0 (MedCalc Software, Mariakerke, Belgium). Age (expressed in years as mean ± SD) was analyzed by Student’*t*-test, or with one-way analysis of variance (ANOVA) followed by Tukey’s test. Possible deviations from Hardy-Weinberg equilibrium (HWE) were tested using goodness of fit χ^2 test. Genotype and allele frequencies (presented as numbers and percents) were evaluated by a χ^2 test of independence. Logistic regression analysis was used to derive corrected measures for association of individual SNPs with alcohol dependence in which age, sex and smoking were used as covariates, while the most frequent genotype was taken as the reference group. Odds ratios (ORs) and 95% confidence intervals (CIs) were reported as a measure of the effect size.

Haploview version 4.2 software (Barrett et al., 2005) was used to produce linkage disequilibrium (LD) matrices with D’ set to 0.80 and to compute haplotype block structure. Best-estimate haplotype pairs for each subject were generated using PHASE version 2.0.2 software, which incorporates a Bayesian statistical method for reconstructing estimated haplotypes from population data (Stephens and Donnelly, 2003). Estimated haplotype frequencies were compared between different groups using a series of 2 x 2 contingency tables for each haplotype compared to the sum of all other haplotypes.

G*Power 3 Software (Faul et al., 2007) was used for conducting power analyses, i.e. to determine a priori sample size and to post hoc compute the achieved power. For the analysis of the genotype frequency of the 3 studied SNPs with a $\chi2$-test (with $\alpha = 0.0166$; power $(1 - \beta) = 0.800$; and with a small effect size ($\omega = 0.15$; df = 5), total sample size was 727 and actual total sample size was 1228. Post-hoc computed achieved power $(1 - \beta)$ was 0.998. In a haplotype analysis of the 2 SNPs with a $\chi2$-test (with $\alpha = 0.025$; power $(1 - \beta) = 0.800$; a small effect size ($\omega = 0.15$; df = 1), total sample size was 423, and actual total sample size was 654. Post-hoc computed achieved power $(1 - \beta)$ was 0.949.

3. Results

Genotype distributions in healthy as well as in alcohol-dependent subjects for rs567926 (controls: $\chi2 = 0.273$, $p = 0.601$; AD subjects: $\chi2 = 0.294$, $p = 0.587$), rs279858 (controls: $\chi2 = 0.846$, $p = 0.358$; AD subjects: $\chi2 = 0.040$, $p = 0.842$) and rs9291283 (controls: $\chi2 = 0.139$, $p = 0.709$; AD subjects: $\chi2 = 1.196$, $p = 0.274$) polymorphisms were in Hardy–Weinberg equilibrium (HWE). No significant differences in the frequency of the genotypes or alleles for all three investigated SNPs between alcohol-dependent and control individuals were detected using $\chi2$-test (Table 2). However, logistic regression analysis with age, gender and nicotine dependence as covariates revealed nominal significance for rs279858 polymorphism (that disappeared after correction for multiple testing (i.e. $p = 0.0166$)), suggesting that individuals carrying the T allele might have lower risk of alcohol-dependence (OD = 0.68, 95% CI = 0.47 to 0.98, p = 0.0386), when compared to carriers of CC homozygous genotype.

The genotype and allele distribution, for any specific SNP tested, was not significantly different between aggressive and non-aggressive alcohol-dependent patients, as well as between patients with a combination of early onset of alcohol abuse and aggressive behavior (Cloninger's type II alcoholism subgroup) and patients with the late onset of alcohol abuse without aggression (Cloninger's type I alcoholism subgroup) (Table 2).

To further examine the association of GABRA2 gene with alcohol dependence and related aggressive behavior, a haplotype analysis was performed. A high degree of linkage disequilibrium (LD) was revealed for rs567926 and rs279858, located in the central and 3' region, respectively, but not for rs9291283 located in the 5' region of the GABRA2 gene (Fig. 1). Similar patterns of LD were observed for the control group and the group with alcohol dependence when examined separately (data available on request).

The computation of the best-estimated haplotypes of rs567926 and rs279858 using PHASE, identified four most common 2-marker haplotypes (Table 3). There were no significant differences in the frequency of haplotypes between healthy and alcohol-dependent individuals. However, following a correction for multiple testing, alcohol-dependent A-C haplotype carriers were significantly more likely to demonstrate aggressive behavior (χ^2 = 5.774, df = 1, p = 0.0163). The same haplotype was significantly ($\chi2$ = 6.296, df = 1, p = 0.0121) more frequently present in patients with type II of alcohol dependence according to the Cloninger's classification, characterized with the early onset of alcohol abuse and aggressive behavior.

4. Discussion

The main findings of our study are nominal association (un-corrected for multiple testing) of rs279858 polymorphism with alcohol dependence, as well as significant higher frequency (following correction for multiple testing) of the A-C (rs567926 and rs279858) haplotype in alcohol-dependent individuals with aggressive behavior and with Cloninger's type II of alcohol dependence.

The rs279858 polymorphism is synonymous coding variant (Lys132Lys) in exon 5, which has previously been reported to be associated (individually and in haplotype block with other SNPs) with alcohol dependence (Covault et al., 2004; Edenberg et al., 2004; Fehr et al., 2006; Lappalainen et al., 2005) and subjective effects of alcohol (Haughey et al., 2008). Furthermore, rs279858 was one of the markers at the GABRA2 locus with nominal significant association with alcohol dependence in GWAS (Bierut et al., 2010) and in recent meta-analysis (Zintzaras, 2012). In the study using convergence of GWA and candidate gene studies for alcoholism, among candidate loci available for analysis, only rs279858 in GABRA2 (p = 0.0052, OR = 1.16) demonstrated an association with alcoholism (Olfson & Bierut, 2012). However, some studies have not confirmed these findings (Drgon et al., 2006; Enoch et al., 2006; Soyka et al., 2008).

Our results suggesting that individuals carrying the T allele might have lower risk of alcohol dependence, when compared to carriers of CC homozygous genotype, are in line with studies reporting that C allele was more frequent in the group of subjects addicted to alcohol (Covault et al., 2004; Fehr et al., 2006; Lappalainen et al., 2005), and was associated with a higher daily probability of drinking and heavy drinking in patients with alcohol dependence (Bauer et al., 2007). Moreover, some studies indicated that carriers of the C allele experience greater stimulatory and euphoric effects of alcohol (Arias et al., 2014).

Furthermore, we observed a high degree of linkage disequilibrium (LD) for rs279858 and rs567926 located in the central GABRA2 region and intragenic region between GABRA2 and GABRG1 genes, respectively, indicating that these two SNPs are good surrogates for each other, likely to be transmitted together, and therefore likely to capture similar genetic variance. Our findings of a haplotype block within GABRA2, which extends from the central area of the gene to the 3' end, and probable existence of another haplotype block in the 5' area of the gene, are consistent with the data of previous studies (Enoch, 2008). These results also suggest that the 3' GABRA2 haplotype block might extend to the 5' promoter region of GABRG1 gene, supporting previous reports about alcoholism risk variants located in the gene encoding the γ_1 subtype of $GABA_A$ receptors (Covault et al., 2008).

In contrast to the studies that reported that less frequent of the two common yin-yang GABRA2 haplotypes accounts for increased risk of alcohol dependence (Covault et al., 2004; Edenberg et al., 2004; Fehr et al., 2006; Lappalainen et al., 2005), our study demonstrated no significant differences in the frequency of the two-marker (rs567926 and rs279858) haplotypes between alcohol-dependent subjects and controls.

The lack of association of this risk haplotype with alcohol dependence in our study might be explained by the differences in the origin of participants, as the role of genetic and environmental risk factors may be different in Croatian compared to other reported populations. Specifically, common environmental risk factors, such as level of exposure to alcohol and average alcohol consumption, may differ between various countries, potentially reflecting either a reduced or increased significance of genetic risk factors for alcohol dependence. In line with this hypothesis, population differences in association studies between the GABRA2 gene and alcoholism have also been observed (Covault et al., 2008; Enoch et al., 2006). In addition to ethnic differences, our results could be explained by the sample size, selection of subjects, or the population stratification, but not with sexual dimorphism, since we conducted logistic regression correction for gender.

The GABRA2 gene variants have been associated with alcohol drinking behavior (Bauer et al., 2007), alcoholism-related β-EEG endophenotype (Edenberg et al., 2004), level of response to alcohol (Schuckit et al., 2004), subjective effects of alcohol (Haughey et al., 2008), co-occurring psychiatric disorders (Dick et al., 2006; Enoch et al., 2006), severity of alcohol withdrawal (Soyka et al., 2008), and potential treatment outcome (Bauer et al., 2007; Soyka et al., 2008). In addition, reported association of GABRA2 variants with impulsiveness (Villafuerte et al., 2013, 2014), childhood conduct disorder (Dick et al., 2006), and externalizing behavior (Dick et al., 2009) in alcohol-dependent subjects strongly suggests the role of GABRA2 gene in a general predisposition toward behavioral disinhibition processes and lack of impulse control.

Impulsivity has been identified as an important determinant of alcohol use and alcohol-related problems (Lejuez et al., 2010). High levels of impulsiveness (Lejuez et al., 2010), low stress tolerance (Daughters et al., 2008) and lack of control under distress (Villafuerte et al., 2013) can reinforce the moody and temper tantrum behavior and the inclination to behave aggressively when under the influence of alcohol (Beck and Heinz, 2013). Externalizing behaviors such as aggression and rule breaking (e.g., defiance, theft, and vandalism) seems to be influenced by both genetic and environmental factors (Simons et al., 2012).

It has been demonstrated that individuals with specific GABRA2 genetic variants displayed more aggression and hostility toward their romantic partners when they had been subjected to harsh parenting (Simons et al., 2013). On the other hand, to the best of our knowledge, this is the first report to show the haplotypic association of the GABRA2 gene with aggressive behavior in alcohol-dependent patients, as well as with Cloninger's type II alcoholism, characterized by early onset of alcohol abuse and aggression. However, as A-C haplotype is rare (present only in 1.6% of all subjects enrolled in the study), its contribution to the overall phenotype of aggressive behavior in alcohol-dependent population or Cloninger's type II alcoholism is probably limited. It is now generally accepted that genetic risk for complex disorders, such as alcoholism and aggression, is likely to be due to numerous genetic variants, each of small effect, as well as to interaction of various genetic and environmental factors (Enoch, 2013).

Cloninger's classification (Cloninger et al., 1988) is one of the various methods of subtyping which have been proposed in order to overcome the biological, sociological and psychopathological heterogeneity of alcohol dependence and to more precisely define specific subtypes (endophenotypes) of alcoholism (Leggio et al., 2009). This classification distinguishes type I alcoholism with a relatively late onset, neurotic symptoms and minimal criminality, from the type II alcoholism with a relatively early onset (in the early 20s), elevated levels of antisocial behavior and delinquency which often begins during adolescence. Type I alcoholism, found in both female and male offspring of alcohol-dependent biological parents, is often cited as only moderate heritable (less than 40%), and influenced by postnatal environmental effects. In contrast, type II or "male-limited" alcoholism is suggested to be strongly heritable (estimated heritability of 90%), transmitted primarily from father to son, and showing moderate environmental influence (Cloninger et al., 1988). Early onset of alcoholism often reflects greater severity, including a higher risk for recurrence, as well as comorbid antisocial personality disorder (ASPD) and conduct disorder (Dick et al., 2006), which are seen significantly more often in Cloninger's type II than in type I alcohol-dependent patients. The GABRA2 genotype has been associated with drug dependence and antisocial behavior, which have been related to poor outcome (Dick et al., 2006).

In contrast to Cloninger's classification, Lesch's typology (Lesch et al., 1990) which distinguishes four types of alcohol-dependent subjects, depending on the family history of alcoholism, patients’ drinking patterns, previous personal psychopathology, origin of substance craving and hypothetical neurobiological background, is based mostly on environmental criteria. Hence, perhaps it is not surprising that no genetic background of this classification has been found thus far, including no association of GABRG1 and GABRA2 genes with Lesch’s typology reported by Grzywacz et al. (2012).

Our results support a growing body of evidence linking GABA_A receptors with the development of alcohol tolerance, dependence and withdrawal symptoms (Korpi et al., 2002; 2009; Kumar et al., 2004; 2009; Staley et al., 2005; Enoch, 2008). Furthermore, the involvement of GABA_A receptors in aggressive behavior seems to be well established, although the effects may vary depending on the receptor subtype, its localization, and the type of aggressive behavior studied, possibly resulting in the individual differences in the propensity for escalated aggression induced by alcohol (Takahashi and Miczek, 2014).

GABA system exerts inhibitory effects on dopaminergic function in the nucleus accumbens (NAcc) (Steffensen et al., 1998), which increased activation has been associated with behavioral traits such as impulsiveness (Forbes et al., 2009), sensation seeking (Bjork et al., 2008), and externalizing behaviors (Yau et al., 2012). GABRA2 gene variation has been associated with individual differences in NAcc activation of adolescents during incentive anticipation, related to dopamine-specific motivated behaviors (Heitzeg et al., 2014). Moreover, aggression has been attributed to imbalance between glutamatergic excitation and GABAergic inhibition in limbic areas (Miczek et al., 2007), while modulation of GABAergic system by 5-HT in corticolimbic neurons is suggested as particularly relevant mechanism underlying specific forms of escalated aggressive behavior such as alcohol-heightened aggression (de Almeida et al., 2005).

Although the $\alpha_1\beta_2\gamma_2$ GABA_A receptor, the most abundant and widespread subtype in the adult brain, originates from the chromosome 5 gene cluster, the GABA_A receptor genes on chromosome 4 are highly expressed in the mesolimbic dopamine reward pathway including hippocampus and dopaminergic neurons in the substantia nigra and ventral tegmental area (Okada et al., 2004). The α_2 subunit, which has been identified as the primary α $GABA_A$ receptor subunit in these limbic regions (McKernan & Whiting, 1996), has been implicated in the development of addictions and is stimulated by stress (Enoch, 2008). The α_2 subunit plays a major role in the anxiolytic action of benzodiazepines and barbiturates (Löw et al., 2000) and in the hypnotic effects of combined exposure to ethanol and benzodiazepines (Täuber et al., 2003).

The observed haplotypic association of GABRA2 and alcohol dependence-related aggressive behavior might be due to the linkage disequilibrium of markers in GABRA2 and GABRG1 genes which has been reported in various populations of European ancestry (Covault et al., 2008; Drgon et al., 2006). Results from diverse cultural or ethnic groups might be useful in order to further characterize the genetic variations of $GABA_A$ receptors associated with the alcohol dependence and related aggressive behavior, and to define the alcohol-related functional changes in GABRA2 and GABRG1 genes. Namely, no functional polymorphisms in these genes have yet been identified (Tian et al., 2005); however the results elucidating the functional significance of alternative splicing isoforms have been accumulating. Namely, Haughey et al. (2008) demonstrated that rs279858 variant resulted in changes of the GABRA2 mRNA levels in post-mortem prefrontal cortical tissue. Although numerous alternative splicing isoforms (Tian et al., 2005), as well as distant gene enhancers and suppressors on chromosome 4 (Enoch et al., 2008) may be potentially implicated in function, additional research is required to identify functional loci in this gene cluster.

The limitation of the study is that it analyzes only 3 SNPs in GABRA2 gene. However, given the evidence that genetic effects are likely to be small, the candidate gene approach has an advantage over GWAS due to the relative lack of multiple comparisons and much lower threshold for significance (Holliday et al., 2013). In order to maximize the sample size and power to detect robust association, many GWAS include subjects of different ethnic origin or race, although a substantial number of variants across the genome differ in frequency between various populations (Rosenberg et al., 2010; Gelernter al., 2014). In addition, subjects enrolled in GWAS are often drawn from large consortia with distinct ascertainment design (Bierut et al., 2010; Gelernter al., 2014), resulting in potential genetic heterogeneity and decreased likelihood that GWAS would identify association to genes contributing specifically to particular phenotypes. The discovered loci reaching genome-wide significance have weak additive predictive power for specific phenotypes, which limits their clinical relevance for some traits (Ward & Kellis, 2012).

Moreover, another limitation of our study is that enrolled control subjects were younger than patients with alcohol-dependence. Therefore, we cannot exclude the possibility that these healthy subjects may develop alcohol dependence when getting older. This might explain the lack of detecting a significant genotype and/or haplotype association of the investigated SNPs with alcoholism. However, the only significant findings in this study were obtained in the alcohol-depended group of patients subdivided according to aggressive behavior and Cloninger's type of alcoholism.

On the other hand, the advantages of our study lie in the large number of enrolled ethnically homogenous subjects (1228 Caucasian Croatian subjects from Zagreb County), County), confirmed by the principal component analysis, carefully determined specific alcohol-related phenotypes, correction for multiple testing, and a priori determined sample size and post hoc achieved power. For smaller numbers of variants, it is also possible to consider the joint effects of markers via haplotype association tests (Holliday et al., 2013), which was also performed in our study for rs567926 and rs279858.

In conclusion, besides supporting a moderate GABRA2 involvement in alcohol dependence, to the best of our knowledge, this is the first study to show a significant haplotypic association of this gene with aggression in alcohol-dependent subjects. In line with these findings, our results also suggest a possible role of GABRA2 gene in a more severe form of alcoholism, characterized by the early onset of alcohol abuse and presence of aggressive behavior. Our findings are consistent with studies showing that the GABAergic system is a potential target for promising novel therapeutics in the treatment of alcohol dependence, such as baclofen, gabapentin, topiramate and neuroactive steroids (Enoch, 2008; Korpi et al., 2002; Leggio et al., 2008). However, further studies should investigate whether GABRA2 genetic variants are associated with an increased risk of aggression in the general population.

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Tables

Table 1. The demographic and clinical characteristics of the samples

Subjects	Male	Female	Total
Healthy controls: n (%)	511(89.02%)	63(10.98%)	**574(100%)**
Age: mean ± SD	40.01 ± 12.22	42.63 ± 12.62	40.30 ± 12.28
Smokers: n(%)	161	21	182 (31.71%)
Non-smokers: n(%)	350	42	392 (68.29%)
Patients with alcoholism: n(%)	531(81.19%)	123(18.81%)*	**654(100%)**
Age: mean ± SD	49.30 ± 9.77	51.67 ± 11.38	49.75 ± 10.12*
Smokers: n(%)	327	67	394(60.25%)*
Non-smokers: n(%)	204	56	260(39.75%)
Patients with aggressive behavior: n(%)	156	14	170(25.99%)
Patients without aggressive behavior: n(%)	375	109	484(74.01%)
Cloninger’s type I patients: n(%)	450	119	569(87.00%)
Cloninger’s type II patients: n(%)	81	4	85(13.00%)

******p*< 0.0001 vs. control**

Table 2. Genotype and allele counts and frequencies of GABRA2 SNPs in control and alcohol-dependent subjects as well as in alcohol-dependent patients subdivided according to presence/absence of aggressive behavior and type of alcohol dependence according to Cloninger's classification

GABRA2 SNP	**Genotype count (frequency)**				**Allele count (frequency)**		
rs567926	**AA**	**AG**	**GG**	**df = 2**	**A**	**G**	**df = 1**
Healthy controls	196(34.15%)	284(49.48%)	94(16.38%)	χ^2=0.128	676(58.85%)	472(41.15%)	χ^2=0.125
Alcohol-dependent patients	218(33.33%)	325(49.69%)	111(16.97%)	*p*=0.938	761(57.00%)	574(43.00%)	*p*=0.724
Aggression	57(33.53%)	89(53.35%)	24(14.12%)	χ^2=1.432	203(59.71%)	137(40.29%)	χ^2=0.439
No aggression	161(33.26%)	236(48.76%)	87(17.97%)	*p*=0.489	558(57.65%)	410(42.35%)	*p*=0.507
Cloninger's type I	188(33.04%)	281(49.38%)	100(16.78%)	χ^2=1.132	657(57.73%)	481(42.27%)	χ^2=0.721
Cloninger's type II	30(35.29%)	44(51.76%)	11(12.94%)	*p*=0.568	104(61.18%)	66(38.82%)	*p*=0.396
rs279858	**CC**	**TC**	**TT**	**df = 2**	**C**	**T**	**df = 1**
Healthy controls	75(13.07%)	279(48.61%)	220(38.33%)	χ^2=1.789	429(37.37%)	719(62.63%)	χ^2=1.118
Alcohol-dependent patients	103(15.75%)	310(47.40%)	241(36.85%)	*p*=0.409	516(39.45%)	792(60.55%)	*p*=0.290
Aggression	24(14.12%)	88(51.76%)	58(34.12%)	χ^2=1.777	136(40.00%)	204(60.00%)	χ^2=0.058
No aggression	79(16.32%)	222(45.87%)	183(37.81%)	*p*=0.411	380(39.26%)	588(60.74%)	*p*=0.809
Cloninger's type I	93(16.34%)	268(47.60%)	208(36.55%)	χ^2=1.171	454(39.89%)	684(60.11%)	χ^2=0.726
Cloninger's type II	10(11.76%)	42(49.41%)	33(38.82%)	*p*=0.557	62(36.47%)	108(63.53%)	*p*=0.394
rs9291283	**AA**	**AG**	**GG**	**df = 2**	**A**	**G**	**df = 1**
Healthy controls	29(5.05%)	207(36.06%)	338(58.88%)	χ^2=2.319	265(23.08%)	883(76.92%)	χ^2=1.292
Alcohol-dependent patients	34(5.20%)	209(31.96%)	411(62.84%)	*p*=0.314	277(21.18%)	1031(78.82%)	*p*=0.256
Aggression	7(4.12%)	52(30.59%)	111(65.29%)	χ^2=0.871	66(19.41%)	274(80.59%)	χ^2=0.858
No aggression	27(5.58%)	157(32.44%)	300(61.98%)	*p*= 0.647	211(21.80%)	757(78.20%)	*p*=0.354
Cloninger's type I	30(5.27%)	185(32.51%)	354(62.21%)	χ^2=0.745	245(21.53%)	893(78.47%)	χ^2=0.649
Cloninger's type II	4(4.70%)	24(28.23%)	57(67.06%)	*p*=0.689	32(18.82%)	138(81.18%)	*p*=0.421

Table 3. Counts and frequencies of four most common GABRA2 gene 2-SNP (rs567926 and rs279858) haplotypes in control and alcohol-dependent subjects, aggressive and non-aggressive alcohol-dependent patients and patients with type I and II alcohol dependence according to Cloninger's classification

Haplotype	**Control**	**Alcoholics**	**χ^2; *p* value, df = 1**
	Count (Frequency)	**Count (Frequency)**	
A-C	13(1.20%)	23(1.83%)	1.659; 0.198
A-T	662(57.60%)	742(56.66%)	0.291; 0.639
G-C	414(36.00%)	492(37.54%)	0.632; 0.426
G-T	59(5.20%)	51(3.97%)	2.198; 0.138
Haplotype	**Aggressive alcoholics**	**Non-aggressive alcoholics**	**χ^2; *p* value, df = 1**
	Count (Frequency)	**Count (Frequency)**	
A-C	11(3.37%)	12(1.305%)	**5.774; 0.016***
A-T	193(56.63%)	547(56.56%)	0.002; 0.964
G-C	125(36.63%)	366(37.83%)	0.135; 0.713
G-T	11(3.37%)	41(4.305%)	0.700; 0.413
Haplotype	**Cloninger's type I alcoholics**	**Cloninger's type II alcoholics**	**χ^2; *p* value, df = 1**
	Count (Frequency)	**Count (Frequency)**	
A-C	16(1.46%)	7(4.49%)	**6.296; 0.012***
A-T	644(56.53%)	98(57.28%)	0.067; 0.795
G-C	437(38.34%)	55(31.98%)	2.305; 0.129
G-T	41(3.66%)	10(6.25%)	2.051; 0.152

Figure Legends

Figure 1. LD plot for 3 GABRA2 SNPs in the entire Croatian sample. Pairwise SNP (D') values (x 100) of linkage and haplotype block are identified using the four-gamete rule. Darkened block indicate SNP pair without evidence of extensive recombination**.**