

RESEARCH ARTICLE

A Single Nucleotide Polymorphism in the *RASGRF2* Gene Is Associated with Alcoholic Liver Cirrhosis in Men

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Abstract

Background

Genetic polymorphisms in the *RAS* gene family are associated with different diseases, which may include alcohol-related disorders. Previous studies showed an association of the allelic variant rs26907 in *RASGRF2* gene with higher alcohol intake. Additionally, the rs61764370 polymorphism in the *KRAS* gene is located in a binding site for the *let-7* micro-RNA family, which is potentially involved in alcohol-induced inflammation. Therefore, this study was designed to explore the association between these two polymorphisms and susceptibility to alcoholism or alcoholic liver disease (ALD).

Methods

We enrolled 301 male alcoholic patients and 156 healthy male volunteers in this study. Polymorphisms were genotyped by using TaqMan[®] PCR assays for allelic discrimination. Allelic and genotypic frequencies were compared between the two groups. Logistic regression analysis was performed to analyze the inheritance model.

Results

The A allele of the *RASGRF2* polymorphism (rs26907) was significantly more prevalent among alcoholic patients with cirrhosis (23.2%) compared to alcoholic patients without ALD (14.2%). This difference remained significant in the group of patients with alcohol dependence (28.8% vs. 14.3%) but not in those with alcohol abuse (15.1% vs. 14.4%). Multivariable logistic regression analysis showed that the A allele of this polymorphism (AA or GA genotype) was associated with alcoholic cirrhosis both in the total group of alcoholics (odds ratio [OR]: 2.33, 95% confidence interval [CI]: 1.32–4.11; *P* = 0.002) and in the group of

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patients with alcohol dependence (OR: 3.1, 95% CI: 1.50–6.20; $P = 0.001$). Allelic distributions of the *KRAS* polymorphism (rs61764370) did not differ between the groups.

Conclusions

To our knowledge, this genetic association study represents the first to show an association of the *RASGRF2* G>A (rs26907) polymorphism with ALD in men, particularly in the subgroup of patients with AD. The findings suggest the potential relevance of the *RAS* gene family in alcoholism and ALD.

Introduction

Genetic predisposition plays a major role in susceptibility to alcoholism [1] or ethanol-induced organ damage [2]. Many association studies have been conducted to identify specific genetic variants in these conditions. Most studies of alcohol use disorders and alcohol dependence (AD) have focused on candidate genes involved in brain reward pathways [3]. Meanwhile, genetic studies related to alcoholic liver disease (ALD) have centered on genes coding for detoxifying enzymes [4,5] or inflammatory response mediators [6,7]. A recent meta-analysis of genome-wide association studies (GWAS) provided new insights into genes associated with AD [8]. Nevertheless, the complete profile of genetic variants underlying susceptibility to alcohol use disorders or ALD remains unknown.

In this setting, genetic polymorphisms of members of the *RAS* gene family have been associated with alcohol-related problems, through their roles in several biological processes, such as neurotransmission and inflammation [9,10]. Specifically, the single nucleotide polymorphism (SNP) rs26907 in the Ras-specific guanine nucleotide-releasing factor 2 gene (*RASGRF2*) was linked to higher alcohol consumption [11] and variability in alcohol-induced reward response. These findings suggest a potential effect of this SNP on alcoholism susceptibility [12]. This is reinforced by the role of Ras protein, which is regulated by RasGRF2, in synaptic transmission [13]. In addition, RasGRF2 has an implied role in inflammatory pathways, through its activation of mitogen-activated protein kinases (MAPKs) and extracellular signal-regulated kinases (ERKs) [14,15]. This observation suggests a link of this protein with ethanol-induced inflammation and, thus, with other alcohol-related problems.

Other polymorphisms within the *RAS* gene family have been associated with human diseases [16]. The rs61764370 polymorphism of the GTPase *KRAS* gene is located in a micro-RNA (miRNA) binding site for members of the *let-7* miRNA family. Possession of the G allele hampers binding of *let-7* and has been associated with different diseases [17]. This polymorphism may also be relevant in alcoholism, as one GWAS showed a relationship between alcohol craving and K-ras activity in an animal model [18]. In addition, *let-7*, which is overexpressed in alcoholic brains, has been associated with the alcohol-triggered inflammatory response [19,20]. The regulatory functions of *let-7* miRNA family members also affect hepatic stellate cell activation [19] and liver disease [21], which may imply a function in ALD development.

In light of these data, the present study was designed to analyze the potential relationships between alcohol-related problems and the SNPs *KRAS* 3377 T>G (rs61764370) and *RASGRF2* 113808 G>A (rs26907).

Materials and Methods

Patients and controls

For this study, we enrolled 301 male alcoholic patients from the Alcoholism Unit of the University Hospital of Salamanca (Spain), with an average age of 52.2 years (standard deviation [SD] = 12.5 years; range = 24–80). All patients reported a daily alcohol consumption of more than 100 g for at least 10 years. According to the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV) [22] criteria, which were valid during the study period and implemented through the use of semistructured interviews conducted by trained staff members, 189 patients were diagnosed of AD and 112 patients of alcohol abuse (AA). Patients with addictions to other drugs (apart from nicotine) or with major Axis I disorders (i.e. schizophrenia, mood disorders, or major anxiety disorders), established by a comprehensive psychiatric examination, were excluded.

As we previously described for our cohort [23,24], the diagnosis of alcoholic cirrhosis (AC) in 103 of the 301 alcoholic patients was established by liver biopsy in 93 patients or by the presence of clinical, analytical, endoscopic, and/or radiological criteria in the other 10 patients, in whom biopsy was contraindicated. The other 198 alcoholic patients were not considered to have ALD according to clinical, analytical, and radiological criteria. All patients had negative results on hepatitis C and hepatitis B tests. Other causes of liver disease (e.g., haemochromatosis or autoimmune liver disease) were excluded by appropriate tests. Within cirrhotic patients, 43 (41.7%) had AA and 60 (58.3%) AD; these percentages were 34.8% and 65.2%, respectively, in the group of alcoholic patients without ALD.

A control group of 156 healthy male volunteers with mean age of 47 years (SD = 19.6 years, range: 21–88) was also enrolled. Participants in the control group consumed less than 10 g of ethanol per day. Neither the control group participants nor their first- or second-degree relatives had a history of AA or AD.

All patients and controls were at least third-generation Spaniards, born in northwestern Spain. All participants provided informed written consent for study participation. The study was carried out with approval from the Ethics Committee of the University Hospital of Salamanca.

Genetic analysis

Genomic DNA was extracted from nucleated peripheral blood cells by using standard proteinase K digestion, phenol-chloroform extraction, and ethanol precipitation. Samples were stored at -20°C until use. Samples from healthy controls, alcoholic patients with AC (AC patients), and alcoholic patients without ALD (AWLD patients) were mixed on PCR plates to ensure simultaneous detection and analysis in a blinded fashion. The rs61764370 and rs26907 polymorphisms were genotyped by using TaqMan MGB[®] assays for allelic discrimination with the StepOnePlus[®] System (Applied Biosystems, Foster City, CA, USA), according to the manufacturers' protocols. Genotyping rates were 99.3% among cases and 99.4% among controls for KRAS 3377 T>G (rs61764370) polymorphism and 95.7% among cases and 97.4% among controls for RASGRF2 113808 G>A (rs26907) polymorphism. The consistency of genotyping process was checked by genotyping 25% of the samples in duplicate, showing consistent results.

Statistical analysis

As previously described [23,24], differences in allelic and genotypic frequencies between groups were compared by the χ^2 or Fisher's exact test, as appropriate (when the expected frequency value was < 5). Deviations from Hardy-Weinberg equilibrium in genotype

frequencies among controls were assessed by the χ^2 test. A *P* value < 0.05 was considered significant. Alcoholic patients were compared to controls, and AC patients were compared to AWLD patients.

The independent contribution of genetic markers to the phenotype was tested by using regression logistic analysis, in order to evaluate recessive versus dominant inheritance of specific alleles. Considering the analysis of the *RASGRF2* G>A polymorphism, arbitrary effects for heterozygous individuals were included in the general model. The three possible genotypes (GG, AG, and AA) were denoted by three dummy variables (0, 1, and 2). The dominant model was based on the hypothesis that the AG and AA genotypes contributed equally to the risk of AC, and the genotypes were coded as follows: GG = 0 and AG and AA = 1. The recessive model was based on the hypothesis that only the AA genotype contributed to the risk of AC, and the genotypes were coded as follows: AG and GG = 0 and AA = 1. Akaike's information criterion (AIC), which combines goodness of fit with the number of parameters, was used to select the best model. The model with the lowest AIC value was considered to have the best fit [25]. Strength of association was calculated with the odds ratio (OR) and 95% confidence interval (CI). A *P* value < 0.05 was regarded as significant. All statistical analyses were performed by using SPSS version 20.0 (IBM SPSS Statistics).

The statistical power of this study to detect a polymorphism conferring an OR of 2 for the presence of alcoholism or AC was calculated by the Power and Sample Size Calculations software, version 3.0.43 (available at <http://biostat.mc.vanderbilt.edu/twiki/bin/view/Main/PowerSampleSize>) [26]. For alcoholism, assuming an α of 0.05 and a prevalence of the less-frequent allele among controls of 0.2, the statistical power was 81% for the total study population (alcoholic patients, n = 301; healthy controls, n = 156). For AC, with the same assumptions, the statistical power was 71.4% for patients with alcoholism (alcoholics with AC, n = 103; alcoholics without liver disease, n = 198). Post-hoc power calculation was performed with ClinCalc post-hoc power calculator (available at <http://clincalc.com/stats/power.aspx>) [27].

Results

Genotypic and allelic distributions of both polymorphisms among alcoholic patients and healthy controls are shown in Tables 1 and 2 and supplementary Table 1. The genotypic

Table 1. Genotypic and allelic frequencies of the *KRAS* and *RASGRF2* polymorphisms in alcoholic patients vs. controls and in patients with alcoholic cirrhosis vs. patients without alcoholic liver disease.

		Alcoholic patients vs. controls		<i>P</i>	AC vs. AWLD patients		<i>P</i>
		Patients (n = 301)	Controls (n = 156)		AC (n = 103)	AWLD (n = 198)	
<i>KRAS</i> rs61764370	TT	232 (77.6)	110 (71)	0.280	84 (81.6)	148 (75.5)	0.063
	GT	63 (27.7)	43 (27.7)		16 (15.5)	47 (24)	
	GG	4 (1.3)	2 (1.3)		3 (2.9)	1 (0.5)	
	MAF (G)	71 (11.9)	47 (15.2)	0.121	22 (10.7)	49 (12.5)	0.234
<i>RASGRF2</i> rs26907	GG	196 (68.1)	109 (71.7)	0.523	57 (57.6)	137 (73.7)	0.022*
	AG	84 (29.2)	41 (27)		38 (38.4)	45 (24.2)	
	AA	8 (2.8)	2 (1.3)		4 (4)	4 (2.2)	
	MAF (A)	100 (17.4)	45 (14.8)	0.429	46 (23.2)	53 (14.2)	0.005*

Data are presented as absolute frequencies (%). AC: alcoholic cirrhosis; AWLD: alcoholics without liver disease. MAF: minor allele frequency. Some subjects could not be genotyped for technical reasons.

* Genotypic and allelic frequencies of the *RASGRF2* polymorphism showed a significantly different distribution in patients with ALC compared to those without ALD (*P* = 0.022 and 0.005, respectively).

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Table 2. Genotypic and allelic frequencies of the *KRAS* and *RASGRF2* polymorphisms in patients with AA, AD and controls.

	AA (n = 112)	AD (n = 189)	P	Controls (n = 156)	P*	P**
<i>KRAS</i>						
TT	88 (79.3)	144 (76.6)		110 (71)		
GT	21 (18.9)	42 (22.3)	0.693	43 (27.7)	0.240	0.497
GG	2 (1.8)	2 (1.1)		2 (1.3)		
MAF (G)	25 (11.3)	46 (12.2)	0.591	47 (15.2)	0.125	0.237
<i>RASGRF2</i>						
GG	75 (73)	121 (65.4)		109 (71.7)		
AG	26 (25)	58 (31.4)	0.410	41 (27)	0.891	0.299
AA	2 (1.9)	6 (3.2)		2 (1.3)		
MAF (A)	30 (14.6)	70 (18.9)	0.241	45 (14.8)	0.847	0.216

Data are presented as absolute frequencies (%). AD: alcoholic dependence; AA: alcohol abuse. MAF: minor allele frequency. Some subjects could not be genotyped for technical reasons.

*P-value for the comparison of AA patients vs. controls.

**P-value for the comparison of AD patients vs. controls.

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distribution among healthy controls was similar to that previously reported among healthy Caucasians [28–30]. No significant deviation from Hardy–Weinberg equilibrium was found.

The genotypic distribution of the *KRAS* rs61764370 polymorphism showed no significant differences between groups (alcoholic patients vs. healthy controls, or AC patients vs. AWLD patients; Tables 1 and 2), apart from a nominally significant difference in genotypic but not allelic distribution between patients with AC and those without liver disease in the subgroup of patients with AD (supplementary Table 1).

In contrast, the genotypic distribution of the *RASGRF2* rs26907 polymorphism showed significant differences between AC patients and AWLD patients ($P = 0.022$, Table 1). Allelic analysis of this polymorphism also showed that the prevalence of the A allele was significantly higher among AC patients (23.2%) compared to AWLD patients (14.2%) ($P = 0.005$). These differences between AC and AWLD patients for this SNP were also found when the analysis was restricted to patients with AD, but not in the AA group (supplementary Table 1). No differences were found when comparing alcoholic patients with healthy controls or patients with AA vs. those with AD, and neither when comparing patients with AA or AD with controls (Table 2).

The analysis of the different models of inheritance for the *RASGRF2* polymorphism and ALD is shown in Table 3 and the results of multivariable logistic regression analyses of the relationship between the *RASGRF2* polymorphism and ALD are presented in Table 4. After adjustment for age, allele A carriers (AA and GA genotypes combined) showed an OR of 2.33 (95% CI: 1.32–4.11; $P = 0.002$) for the presence of AC. We also performed this analysis in the subgroup of patients with AD, in which allele A carriers showed an OR of 3.34 (95% CI: 1.60–6.96; $P = 0.001$) for the presence of liver disease. According to the AIC scores, the dominant model for the A allele of this polymorphism was the most parsimonious model of inheritance on both analyses.

Post-hoc power calculations for our study showed a power of 16.4% for detecting differences between minor allele frequency of *RASGRF2* SNP between alcoholics and controls and a power of 77.9% for differences between alcoholics with AC and those without liver disease.

Table 3. Genotypic frequencies of the *RASGRF2* 113808 G>A (rs26907) polymorphism by disease status and model of inheritance for the A allele.

Model for the A allele	Alcoholic patients			Alcoholic patients with AD		
	AC patients	AWLD patients	P	AC patients	AWLD patients	P
General						
GG	59 (57.8)	137 (73.7)		28 (47.5)	93 (73.8)	
AG	39 (38.2)	45 (24.2)	0.022	28 (47.5)	30 (23.8)	0.002
AA	4 (4)	4 (2.2)		3 (5)	3 (2.4)	
Recessive						
AG + GG	98 (96)	182 (97.8)		56 (95)	123 (97.6)	
AA	4 (4)	4 (2.2)	0.382	3 (5)	3 (2.4)	0.385
Dominant						
GG	59 (57.8)	137 (73.7)		28 (47.5)	93 (73.8)	
AG + AA	43 (42.2)	49 (26.3)	0.006	31 (52.5)	33 (26.2)	<0.001

Data are presented as absolute frequencies (%). AC: alcoholic cirrhosis. AWLD: alcoholics without liver disease. Some subjects could not be genotyped for technical reasons.

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Discussion

We found an association of the *RASGRF2* G>A polymorphism (rs26907) with the presence of AC in male alcoholic patients, but not with the presence of alcoholism itself (defined as AA or AD, according to DSM-IV criteria). Possession of the A allele of this SNP (genotype AA or AG) was associated with a higher risk of AC. Of note, this association remained significant among patients with AD, but not among those with AA. Conversely, the *KRAS* T>G polymorphism (rs61764370) showed no consistent association with the presence of ALD or alcohol use disorders. To our knowledge, this is the first study to describe a relationship between ALD and polymorphisms within the *RAS* gene family.

The association of *RASGRF2* polymorphisms and ALD is biologically plausible when we consider that RasGRF2 is involved in the inflammatory response through the activation of the MAPK/ERK pathway [15,31]. This pathway participates in the ethanol-induced inflammatory

Table 4. Results of the multivariable logistic regression analysis of the dominant model for the A allele of *RASGRF2* 113808 G>A (rs26907) polymorphism in cirrhotic patients.

Alcoholic patients	Multivariable analysis				
	AC patients	AWLD patients	OR	95% CI	P
Genotype					
GG	59 (57.8)	137 (73.7)	1	Reference	
AG + AA	43 (42.2)	49 (26.3)	2.33	1.32–4.11	0.002
Age	59 (11.8)	48.7 (11.3)	1.1	1.05–1.10	<0.001
Alcoholic patients with AD					
	AC patients	AWLD patients	OR	95% CI	P
Genotype					
GG	28 (47.5)	93 (73.8)	1	Reference	
AG + AA	31 (52.5)	33 (26.2)	3.1	1.50–6.20	0.001
Age	56.2 (10.8)	48.5 (9.6)	1.1	1.05–1.10	<0.001

Data are presented as absolute frequencies (%) for genotype and mean for age (SD). OR = odds ratio; CI = confidence interval; AC: alcoholic cirrhosis; AWLD: alcoholics without liver disease; AD: alcohol dependence. Some subjects could not be genotyped for technical reasons.

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response mediated by Toll-like receptor 4 (TLR4) and nuclear factor kappa B (NF- κ B) [32]. Additional experimental data regarding the MAPK/ERK pathway reinforce the potential association of RasGRF2 with ALD development. On the one hand, this pathway is involved in ALD development through negative regulation of hepatocyte differentiation and proliferation [33]. On the other hand, ERK activation by alcohol has been associated with macrophage stimulation [34] and ERK phosphorylation has been described in the peritoneal macrophages of ALD patients [35], which could be associated with an elevated inflammatory response. In light of these previous data and our own results, we could hypothesize that the presence of the A allele in the *RASGRF2* polymorphism may potentiate the RasGRF2-mediated ethanol-induced inflammatory response, leading to macrophage activation and an increased risk of liver fibrosis. Despite the lack of functional data for this polymorphism or information to perform an eQTL analysis, this hypothesis could explain the higher frequency of the A allele among AC patients compared to AWLD patients. Although subgroup analysis may be prone to type I error, the finding of a different risk in patients with AA or AD may reflect a higher level of alcohol consumption in patients with AD, which leads to an increased risk of AC development in the presence of the A allele.

In contrast, our negative result for the association with alcoholism (AA or AD) was unexpected, because previous findings have pointed towards an association of this polymorphism with alcohol use disorders [30]. This relationship was initially suggested by a GWAS showing an association between higher alcohol consumption and the presence of the G allele of rs26907 [11]. Reinforcing this finding, a haplotype containing the G allele of this polymorphism was associated with a different reward sensitivity related to alcohol exposure and, in adolescent males, with a higher number of drinking episodes [12]. A relationship between the *RASGRF2* G>A polymorphism and alcoholism itself is also biologically plausible. RasGRF2 can activate the MAPK/ERK pathway, which is involved in neurotransmission (especially through dopamine receptors and transporters) and thus potentially associated with reward mechanisms in alcoholism [36,37]. In mouse models, ethanol administration increased ERK activity in the *nucleus accumbens*, and inhibition of ERK activity influenced ethanol self-administration [38,39]. In addition, *RASGRF2* knockout mice showed decreased alcohol consumption owing to a diminished neuronal excitability, which was mediated not only by dopaminergic pathways [12], but also by a decrease in noradrenergic activation [40].

Given this background, it is difficult to reconcile the previous findings showing an association of the G allele of the *RASGRF2* SNP with higher alcohol intake and our own data showing an association of the A allele with ALD, particularly in patients with dependence. In this context, we have to consider the differences between the studies. Our large cohort of alcoholic patients was comprised of individuals with AA or AD. In contrast, previous works [12,13,40] analyzed patients with different alcohol-related phenotypes. Thus, our study is the first to explore the association of this polymorphism among patients with a diagnosis of alcohol-related disorders. Although we did not find an association with this SNP, further association studies will be necessary to clarify this relationship. Furthermore, our Spanish population may have a different genetic background compared to sample populations in other studies. This makes plausible the hypothesis that the discordant results may be due to a different pattern of linkage disequilibrium with other SNPs within this gene.

Regarding *KRAS* 3377 T>G (rs61764370) polymorphism, we did not find a consistent association of this SNP with ALD or alcoholism and we only observed, in the subgroup of patients with AD, a nominally significant higher frequency of the GT genotype in patients with AC when compared with those without liver disease. Although this result may be a false positive, we cannot dismiss the possibility that this gene plays a role in alcoholism, considering previous

results from animal models [18]. Furthermore, this polymorphism is a target of the *let7-e* miRNA, which is potentially involved in both alcoholism and ALD development [20,41,42].

Despite the fact that we have controlled several potential confounders that could affect the validity of our findings, like addiction to other drugs, major psychiatric disorders, and age; we have to acknowledge several limitations of our work, such as the lack of detailed information regarding alcohol consumption levels. In addition, although we have limited the risk of population stratification, this bias is still possible. Another of the main limitations of our work is that our results can only be extrapolated to male subjects. The small number of patients with the AA genotype of the *RASGRF2* polymorphism makes it difficult to completely discard an additive model of inheritance for this SNP. Finally, the possibility of a type I error is an important concern in genetic studies, particularly when subgroup analysis are performed, and therefore our results should be interpreted with caution until they are independently replicated.

Conclusions

To the best of our knowledge, our genetic association study represents the first to show an association of the *RASGRF2* G>A (rs26907) allelic variant with alcoholic liver cirrhosis in men, which was restricted to patients with AD. However, we did not find that this polymorphism was associated with alcohol use disorders. Our findings suggest the potential relevance of the *RAS* gene family in alcoholism and, more specifically, in ALD development. These results are of particular interest given previous findings linking this SNP to alcoholism. Further investigations are needed to clarify the role of these genetic variants in alcohol-related diseases.

Supporting Information

S1 Table. Supplementary Table 1. Genotypic and allelic frequencies of the *KRAS* and *RASGRF2* polymorphisms in patients with and without liver disease according to the presence of alcohol abuse or dependence.

(DOCX)

S2 Table. Supplementary database. Clinical and genotypic data from all included patients.

(XLS)

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