RESEARCH NOTE

Association of the *GRM4* gene variants with juvenile myoclonic epilepsy in an Indian population

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Introduction

Juvenile myoclonic epilepsy (JME; MIM: 254770), an idiopathic form of epilepsy (IGE), accounts for nearly 10% of all forms of epilepsies (Panaviotopoulos et al. 1994; Ganesh and Singh 2005; Berg and Scheffer 2011). JME is genetic in nature, and both monogenic and polygenic forms have been reported (Ganesh and Singh 2005). The latter form is more common among the two and several susceptibility genes for JME have been reported (Ganesh and Singh 2005). Variations in the GRM4 gene, coding for the metabotropic glutamate receptor 4 (mGluR4), was earlier reported to confer risk for IGE in Caucasians (Muhle et al. 2010). Alterations in the functions of the *GRM4* gene are known to cause epilepsy in murine models (Wang et al. 2005). Therefore, we carried out the genetic association study in an Indian population for five single-nucleotide polymorphisms (SNPs) spanning the GRM4 gene to test their possible role in JME. A case-control design was used to screen 249 JME patients and 186 unrelated healthy control individuals. Here, we report that one of the SNPs (rs2029461) showed statistically significant association (after Bonferroni correction) with the JME phenotype in the study population.

JME is characterized by myoclonic jerks, absence and generalized tonic–clonic or clonic–tonic–clonic seizures (Panayiotopoulos *et al.* 1994; Delgado-Escueta 2007). The typical age of onset JME is in late childhood but the seizures may persist into adulthood (Delgado-Escueta 2007). Though believed to be genetic in nature, the mode of inheritance

of JME is not clearly understood (Panayiotopoulos and Obeid 1989; Gardiner 2005; Pal *et al.* 2006). Available information suggests that mutations in one or more genes can cause and/or increase the susceptibility JME (Greenberg *et al.* 1988; Delgado-Escueta *et al.* 1990; Mulley *et al.* 2005). Linkage studies have identified a number of chromosomal loci for JME (Ganesh and Singh 2005; Kapoor *et al.* 2007), and mutations in at least five genes have shown to independently cause JME (Delgado-Escueta 2007). Amongst these, the *EFHC1* gene appears to be more frequently mutated gene (Suzuki *et al.* 2004). Besides such major genes, several susceptibility genes for JME have also been suggested. These include the *BRD2* (Pal and Greenberg 2012), *TAP1* (Layouni *et al.* 2010) and *GRM4* genes (Barbon *et al.* 2000; Izzi *et al.* 2003; Muhle *et al.* 2010).

The GRM4 gene, which codes for the metabotropic glutamate receptor 4 (mGluR4), regulates the glutamatergic neurotransmission and chemicals that activate mGluR4 are shown to have anticonvulsant properties in animal models of epilepsy (Wang et al. 2005). The Grm4 gene knockout mice exhibit epileptic phenotype due to the aberrant excitation and inhibition of neurotransmission (Wang et al. 2005), and modulation of mGluR4-mediated pathways, is known to result in epileptic seizures (Bertaso et al. 2008), thereby suggesting a functional importance for the GRM4 gene in the genesis of epilepsy. An association for the GRM4 gene variants in idiopathic generalized epilepsies was tested by Muhle and colleagues (Muhle et al. 2010), and a positive association for five SNPs spanning the GRM4 gene with the IGE phenotype, including the JME, was indeed established in a Caucasian population. In the present study, we performed a replication

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study to evaluate whether the *GRM4* locus shows association with the JME in an Indian population or not.

Materials and methods

Subject selection

The present study includes 249 unrelated JME patients (120 males and 129 females) as case, and 186 healthy individuals (100 males and 86 females), who did not show any epileptic or neurological deficit until the age of 40 years, as control. The average age of the control group was much higher than the case group, and therefore the individuals in this group were unlikely to develop JME at the later stage. These two groups were used for the case-control association study. Additionally, for a family-based association study, we analysed 56 parent-patient trios representing 56 independent families. This study was approved by the ethical committees of the participating institutes, and the blood samples were collected from the subject and/or parents upon informed consent. The diagnostic criteria for JME were based on the classification proposed by the International League Against Epilepsy (ILAE 1989).

Genotyping

Genomic DNA was extracted from whole blood lymphocytes using the QIAamp blood DNA purification kit (Qiagen, Valencia, USA) as per the manufacturer's protocol. We genotyped the five SNPs (rs937039, rs2499697, rs745501, rs2451334 and rs2029461) present across the *GRM4* gene by polymerase chain reactions (PCR) followed by the restriction fragment length polymorphism (RFLP) analysis to genotype the allele. The details of the primer sequences, PCR conditions, restriction enzymes used and the diagnostic fragments obtained after digestion are summarized in table 1 in electronic supplementary material at http://www.ias.ac.in/ jgenet/.

Statistical analysis

Allele and genotypic frequencies were calculated by direct counting and the statistical analysis was carried out using the χ^2 test (SPSS, Chicago, USA) to test deviation from the Hardy–Weinberg equilibrium (HWE). For haplotype analysis, a web-based statistical program (SNPAnalyzer 2.0; http:// snp.istech21.com/snpanalyzer/2.0/) (Yoo *et al.* 2008) was used. For the transmission disequilibrium test (TDT) and linkage disequilibrium (LD) analysis, the Haploview software (www.broad.mit.edu/personal/jcbarret/haploview) was used. Standard parameters were used for the TDT analysis. We used expectation-maximization (EM) algorithm for the haplotype analysis and the pair-wise LD was calculated using the four gamete rule (Bush *et al.* 2009). Bonferroni corrections were applied for multiple testings for case–control association analysis, and for the haplotype analysis. Therefore,

P values were considered significant when less than 0.05 and 99.5% confidence interval were applied. Monte Carlo simulation was used to assign P values wherever required.

Results

A total number of 249 JME cases and 186 healthy controls were taken to test whether the *GRM4* gene could serve as a genetic risk factor for JME. For this we chose five SNPs falling in the 5'-region of the *GRM4* gene and some of these alleles were previously shown to be associated with JME (Muhle *et al.* 2010). Three of the five SNPs chosen fall in the intronic region (first and second intron) and the remaining two fall in the 5' noncoding exons (figure 1). These SNPs were tested for both case–control association and for the family-based association.

Case-control association analysis

We genotyped the five SNPs by PCR-RFLP and calculated the allele and genotype frequencies for both case and control groups. The distribution of genotypes for each of the five SNPs tested did not deviate from HWE in the control group. We calculated odds ratio for each studied SNP using logistic regression analysis with 95% confidence interval by considering the major allele as the reference. The genotype and allele frequencies obtained for five SNPs in the study population are shown in table 1. We found significant risk association with JME for all five SNPs of the *GRM4* gene tested. However, only one SNP showed statistically significant association after Bonferroni correction (rs2029461) (see table 1). For SNP1 (rs2029461), the AG genotype showed significant association with JME.

We inferred haplotypes from the genotype data obtained for the five SNPs to assess the possible combinatorial effect of SNPs on the JME phenotype. Here we considered only those haplotypes whose frequency were >1% in case as well as control groups (see table 2). The ATAAG and ACACA haplotype combinations were significantly higher in cases suggesting them to be the risk haplotypes. Similarly, the haplotype combinations, ACAAA and GCACA, were significantly lower in cases suggesting them to be the protective haplotypes (table 2). We also measured LD for the SNPs (see figure 1B; table 2 in electronic supplementary material). The LD-measured D' was higher (>0.33) in controls for the SNP pairs SNP3/SNP5 and SNP2/SNP4. Similarly, in cases, the SNP pair SNP1/SNP3 showed higher D' measure (see figure 1B; table 2 in electronic supplementary material).

Family-based association analysis

We also analysed the genotype for all the five SNPs in the 56 families recruited for the trio study. For the analysis, families with at least one informative parent (heterozygous for

SNP	Genotype	C (n =	ases = 249)	Co (<i>n</i> =	ntrols = 186)	Odds ratio	P value (after logistic regression	P value (after Bonferroni
name /ID	per allele	N	%	N	%	(95% CI)	analysis)	correction)
SNP1	AA	128	51.41	78	41.94	1 (reference)		
rs2029461	AG	94	37.75	75	40.32	1.641 (1.238–2.175)	0.001*	0.005*
	GG	27	10.84	33	17.74	1.253 (0.925–1.698)	0.145	
	А	350	70.28	231	62.09	1 (reference)		
	G	148	29.72	141	37.91	1.515 (1.283–1.789)	0.011*	0.056
SNP2	CC	113	40.16	105	56.45	1 (reference)		
rs2451334	СТ	116	50.60	70	37.63	1.076 (0.825–1.404)	0.588	
	TT	20	4.42	11	5.92	1.657 (1.232–2.229)	0.122	
	С	326	65.50	280	75.27	1 (reference)		
	Т	172	34.50	92	24.73	1.221 (1.043–1.430)	0.013*	0.065
SNP3	AA	213	85.54	169	90.86	1 (reference)		
rs745501	AT	36	14.46	17	9.14	1.260 (1.030-1.542)	0.025*	0.125
	TT	0	0.00	0	0.00	_		
	А	462	92.77	355	95.43	1 (reference)		
	Т	36	7.23	17	4.57	0.615 (0.340-1.112)	0.108	
SNP4	AA	131	52.61	109	58.60	1 (reference)		
rs2499697	AC	101	40.56	70	37.63	1.202 (0.932-1.550)	0.156	
	CC	17	6.83	7	3.77	1.443 (1.064–1.957)	0.018*	0.091
	А	363	72.89	288	77.42	1 (reference)		
	С	135	27.11	84	22.58	1.607 (1.224-2.110)	0.128	
SNP5	AA	105	34.94	95	51.08	1 (reference)		
rs937039	AG	116	52.61	81	43.55	1.105 (0.837–1.459)	0.480	
	GG	28	12.45	10	5.38	1.432 (1.078–1.902)	0.013*	0.066
	А	326	61.24	271	72.85	1 (reference)		
	G	172	38.76	101	27.15	1.125 (0.956–1.326)	0.157	

Table 1. Genotype and allele frequencies observed for the five SNPs ID in the study population.

*Statistically significant.

the SNP) were considered and the ratio between the transmitted versus nontransmitted alleles was calculated (see table 3 in electronic supplementary material). Since the sample size was smaller, we could not confidently claim a statistical significance for the data generated in this analysis. We nonetheless found a trend suggestive of biased transmission of the risk allele in SNP1.

Functional impact tested SNPs on the GRM4 gene

We utilized bioinformatic tools to test if the risk-associated SNPs could modulate some functional properties of the *GRM4* gene. For SNPs that are located in the 5' noncoding exons (SNP1 and SNP2; see figure 1A) we used MAT inspector tool (Cartharius *et al.* 2005) to test whether any of these sequence variants alter the potential transcription factor-



Figure 1. (A) Schematic diagram (drawn not to scale) showing the exon/intron organization for the *GRM4* gene positions of the SNPs screened in the present study. The open boxes represent the untranslated exonic region and the filled box denotes the coding region of the exons. The exons are numbered (1 to 10) and the orientation of the gene is identified by the 5' and 3' positions. (B) Haplotype analysis of five SNPs spanning the *GRM4* gene in the case and control groups as indicated. Numbers within the squares refer to the pair-wise D' values. For details, see table 2 in electronic supplementary material.

	Free	quency			
Haplotype	Cases (alleles = 498)	Controls (alleles = 372)	Odds ratio (0.95 CI) [#]	<i>P</i> value (<i>P</i> value*)	
ACAAA	14.05	25.02	0.4907 (0.3475-0.6927)	<0.0001	
ACACA	12.6	5.29	2.5490 (1.5119-4.2973)	0.00047 (0.00236)	
ACAAG	12.33	10.34	1.2269 (0.7986–1.8849)	0.409587	
ATAAA	10.66	11.36	0.9358 (0.6092-1.4374)	0.84181	
ATAAG	9.73	2.06	4.8533 (2.2672-10.3895)	<0.0001	
AAACG	8.72	14.3	0.5688 (0.3711-0.8718)	0.01228	
GCAAG	5.13	7.35	0.7039 (0.4036-1.2275)	0.27133	
GTAAA	4.29	2.52	1.7757 (0.8037-3.9232)	0.211665	
ATACG	4	1.86	2.1817 (0.9127-5.2152)	0.109599	
GCACA	3.58	8.36	0.4125 (0.227-0.7495)	0.004525 (0.022625)	
ACTAA	2.41	0	NC	NC	
GTAAG	2.35	1.02	2.2716 (0.7267-7.1005)	0.231764	
GCACG	2.16	0	NC	NC	
ATTAA	1.77	0	NC	NC	
ACACG	1.61	1.93	0.8513 (0.3059-2.3689)	1	
GTACA	1.56	0	NC	NC	

Table 2. GRM4 gene SNP haplotypes and their association with risk of JME.

Values in bold font indicates statistical significance. NC, not calculated. *After Bonferroni correction.

binding sites. Intriguingly, this tool predicted the possible presence of the core promoter motif ten elements (MTE) but only in the minor allele of the SNP1 (see table 4 in electronic supplementary material). MTEs are known to promote transcription by RNA polymerase II and are recognized by the transcription factors (Lim *et al.* 2004). We have also checked if any of the intronic SNPs could possibly affect the splicing pattern of the *GRM4* gene transcripts. For this we used a splice site predictor tool, NNSPLICE (Reese *et al.* 1997), but this search did not suggest any possible defect.

Discussion

The present set of investigation was carried out to test whether the GRM4 gene variants could confer risk for developing JME in an Indian population or not. In their original study, Muhle et al. (2010) have tested 17 SNPs spanning the GRM4 gene and found that five of them showed significant association with IGE, a subset of which also included the JME cases, in a German population. Therefore, the present case-control study was carried out to check the association of GRM4 gene locus with the JME phenotype in an Indian population. We found that only one of the five SNPs tested showed a moderate but statistically significant association (for the genotype frequency) with the JME. This association was further strengthened by our observations on the haplotype association and the biased transmission of risk alleles in the JME probands. Though the size of the population used in the present study is relatively small, the findings are however compelling enough to suggest that the GRM4 gene could possibly be a risk factor for JME in the Indian population as well.

How variations in the *GRM4* gene modify the risk of JME? While it is hard to predict the effect of SNPs on gene

function, it could be suggested that the risk alleles could bring about subtle changes in the GRM4 gene function, which in association with other genetic and nongenetic factors, could result in the epileptic seizures. In this regard it is interesting to note that the minor allele of the SNP1 (rs2029461), located in the 5' noncoding exon of the GRM4 gene which showed significant association with the JME phenotype in the present study, is predicted to gain MTE. Since such elements are known to promote transcription (Lim et al. 2004) and that this potential element was absent in the major allele, it would be interesting to see if this allele could alter the expression level of the GRM4 gene. Alternatively, the SNPs that showed significant association with JME could be in LD with some other sequence variants that were not discovered and investigated in the present study. Thus, further studies are required to understand the functional significance of these gene variants, and these findings should also be replicated in a larger cohort.

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