

Protective and susceptibility effects of *hSKCa3* allelic variants on juvenile myoclonic epilepsy

J Vijai, A Kapoor, H M Ravishankar, P J Cherian, G Kuruttukulam, B Rajendran, R Sridharan, G Rangan, A S Girija, S Jayalakshmi, S Mohandas, K S Mani*, K Radhakrishnan, A Anand

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Juvenile myoclonic epilepsy (JME; OMIM 606904) is a subtype of common idiopathic generalised epilepsy (IGE) and affects up to 26% of all individuals with IGE.^{1–3} JME is characterised by the onset in adolescence of bilateral myoclonic jerks usually affecting the upper limbs.^{1,4} Patients also have generalised tonic-clonic seizures and about one third experience absence seizures. Genetic factors are known to play an important role in the etiology of JME.^{3,4} While identification of genes underlying predisposition to JME has been relatively slow due to clinical and genetic heterogeneity,^{5,6} progress made so far in the isolation and characterisation of genes associated with other monogenic subtypes of IGE, provides evidence that most idiopathic epilepsy syndromes are caused by mutations in genes encoding ion channels.⁷ The implications of these findings in monogenic subtypes of the disorder for the complex polygenic subset are now being increasingly appreciated.⁸

Two types of voltage gated potassium channels have been associated with seizure disorders, the KCNQ channels and the K_v channels. Loss of function mutations for the potassium channels *KCNQ2* and *KCNQ3* have been identified in families with a rare autosomal dominant subtype of IGE called benign familial neonatal convulsions (BFNC).^{9,10} Allelic association of JME with *KCNQ3* has been suggested in a South Indian cohort of JME patients.¹¹ Mutation in human *KCN1A* predisposes to episodic ataxia and partial epilepsy.¹² These findings emphasise the importance of potassium channels in controlling neuronal excitability and thus make potassium channel genes potentially interesting candidates for idiopathic epilepsy syndromes.

The calcium activated potassium channels are an interesting class of potassium channels that regulate neuronal excitability.^{13,14} These are gated by intracellular calcium ions and their activity is responsible in part for the afterpolarisation that follows a single action potential or a train of action potentials in the neurons. According to their single channel conductance in symmetrical potassium solutions, these channels are classified^{13,14} as big (BK), intermediate (IK), or small (SK). Biophysical and pharmacological analysis, single cell mRNA, and protein expression profiling strongly suggest that SK3 channels mediate the calcium dependent afterhyperpolarisation in neurons.¹⁵ The neuronal small conductance calcium activated potassium channel (*hSKCa3*) plays a critical role in determining the firing pattern of neurons through the generation of slow afterhyperpolarisation^{16,17} and regulation of intracellular calcium signals by binding with calmodulin.¹⁸ In situ hybridisation in rat¹⁹ and human brain²⁰ revealed that mRNAs encoding the SK family subunits are widely expressed in the brain and show distinct but overlapping patterns. These physiological attributes make *hSKCa3* an interesting candidate gene for investigation in an IGE syndrome such as JME. While its role in schizophrenia and bipolar disorders has been investigated,^{16,20–23} the

Key points

- Genetic factors are known to play an important role in the etiology of juvenile myoclonic epilepsy (JME), a subtype of common idiopathic generalised epilepsy.
- Most idiopathic epilepsy syndromes are caused by mutations in genes encoding ion channels.
- The length variation in the second polyglutamine stretch of the neuronal small conductance calcium activated potassium channel gene *hSKCa3* was investigated and the allele frequency distribution was compared between 222 well characterised JME patients of South Indian origin and 248 ethnically matched normal subjects.
- Alleles CAG₁₆ and CAG₁₈ were common while CAG₁₉ was rare in the studied JME patients with relative risks of 1.198, 1.178, and 0.514, respectively.

possibility of its influence on epilepsy phenotypes remains poorly studied.

*hSKCa3*¹⁶ encodes a 731 amino acid protein comprising two polyglutamine arrays in its N terminus of which the second polyglutamine repeat is highly polymorphic.¹⁶ We investigated the length variation in the second polyglutamine stretch of *hSKCa3* and compared the allele frequency distribution between 222 well characterised JME patients of South Indian origin²⁴ and 248 ethnically matched normal subjects. Several genetic studies have been conducted on the South Indian populations and it has been found that the genetic distance between even the tribal populations is small, signifying a close genetic relationship.²⁵ Through this case-control design, we intended to investigate whether variations in the length of the expressed polyglutamine tract of *hSKCa3* show allelic association with JME and thereby possibly influence expression of the JME phenotype.

METHODS

Patients and controls

A total of 222 unrelated JME probands were recruited through epilepsy centres situated in South India.²⁴ Patient samples were collected from specialty neurology clinics, referral centres, and medical camps for seizure disorders which were part of the rural outreach programs of the referral centres. All the patients were unambiguously diagnosed cases of JME with classification based on the published criteria of the Commission on Classification and Terminology of the

Abbreviations: BFNC, benign familial neonatal convulsions; HWE, Hardy-Weinberg equilibrium; IGE, idiopathic generalised epilepsy; JME, juvenile myoclonic epilepsy; PCR, polymerase chain reaction

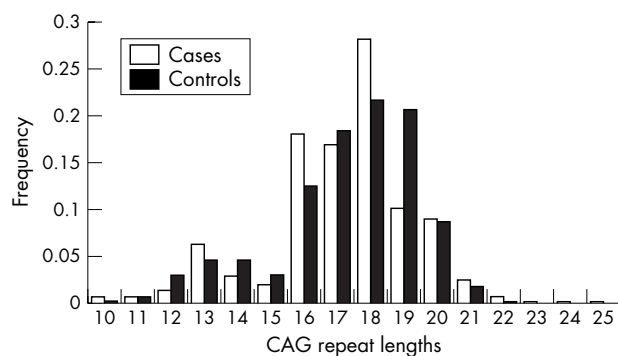


Figure 1 *hSKCa3* allele frequencies in cases and controls.

International League Against Epilepsy.² A total of 248 subjects of South Indian origin without a family history of epilepsy, ataxia, unexplained blackouts, or other chronic neurological disorders were used as controls in this study. All patients and controls provided written informed consent and the study was approved by Institutional Bioethics Review Board.

Genetic analysis

Genomic DNA was isolated from peripheral venous blood by phenol-chloroform method. Polymerase chain reaction (PCR) mediated amplification of the second polyglutamine CAG tract of *hSKCa3* was carried out in all subjects. Each reaction comprised of 50 ng of genomic DNA, 20 pmol primers (*hSKCa3*-F: 5'-CAC CGT CAG TGT CAC CAG TAG TCC CC-3' and *hSKCa3*-R: 5'-Hex-GAA GGG GTT GCT GTC CCG CCG GT-3'),¹⁶ 200 μ M of each dNTP, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl₂, and 0.5 U Taq DNA polymerase in 10 μ l final volume. Forty cycles of PCR were carried out, each cycle with denaturation at 94°C for 40 s, annealing at 52°C for 40 s, and extension at 72°C for 45 s.²³ Genotyping was performed by a person unaware of the affection status using an ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). Allele sizing was done using Genotyper version 2.0 (Applied Biosystems).

Statistical analysis

Descriptive statistics were calculated for patient and control data. Statistical tests were done as follows. Z tests of proportions were computed in controls and cases in order to obtain information on each individual allele. Relative risks of the major alleles were calculated by computing the ratio of presence and absence of each allele in the cases and controls. Hardy-Weinberg equilibrium (HWE) was calculated in this study to test for genotype frequencies, especially in the

control population as a measure to check for population stratification or genotyping errors.

RESULTS

The mean (SD) age at onset of the JME probands was 14.9 (1.4) years. The triad of myoclonus, absences, and generalised tonic-clonic seizures was observed in 27.9%, the combination of myoclonus and generalised tonic-clonic seizures in 64.7%, and myoclonus alone in 7.4% of patients. Of the patients 84% were receiving treatment with sodium valproate. Scalp electroencephalogram (EEG) during wakefulness and sleep exhibited generalised epileptiform abnormalities in about 70% of the patients, while 30% were seizure free for 2 or more years and therefore exhibited normal EEG. Clinical details of a large subset of these patients were recently reported.²⁴

Sixteen distinct *hSKCa3* alleles were observed. Repeat size ranged from 10 to 25. Thirteen alleles (CAG₁₀₋₂₂) were seen in the controls and 16 alleles (CAG₁₀₋₂₅) were seen in the patients. The frequency distribution of the observed alleles in cases and controls is shown in fig 1. The modal repeat size was CAG₁₈. Statistical tests for alleles with a frequency >0.02 were carried out to find out if one or more of these allelic variants showed significant frequency differences between the control and the patient group. Z test of proportions for the 10 alleles (alleles with frequency less than 0.02 were aggregated) was performed. In this analysis, the three alleles CAG₁₆ (0.018), CAG₁₈ (0.019), and CAG₁₉ (<0.00001) were found to be significant. The distribution of CAG₁₆ and CAG₁₈ was higher in JME patients, while allele CAG₁₉ was quite rare in JME cases but present at a very high frequency in the control group (table 1). When Bonferroni correction for multiple testing (α at 0.005) was applied, only CAG₁₉ was found to be significant. Among the nine major alleles (CAG₁₃₋₂₁) observed, relative risk was found to be maximum for allele CAG₁₆ (1.198) and minimum for allele CAG₁₉ (0.574) (table 2). We found the control population to be in HWE at the 5% significance level ($\chi^2 = 0.0154$, $p = 0.9012$, $df = 1$), while the cases deviated from HWE at the 5% significance level ($\chi^2 = 5.5049$, $p = 0.0189$, $df = 1$).

DISCUSSION

Genetic association studies are one of the useful approaches to understanding the etiology of complex disorders.²⁶ Increased or decreased allele or genotype frequencies in cases or controls implicate sequence variants that either increase or decrease the risk of a disease or are in strong linkage disequilibrium with a disease causing mutation. The biological effects of a specific risk or protective allele under study are usually small.

We tested the association of JME with allelic variants at an expressed polymorphic CAG repeat tract in a functionally important calcium activated potassium channel gene

Table 1 Allele frequencies and pair-wise Z tests of JME cases and controls

CAG _n	Cases	Controls	p (cases)	p (controls)	Z value	p Value
13	28	23	0.063	0.046	1.128	0.259
14	13	23	0.029	0.046	-1.36316	0.1728
15	9	15	0.020	0.030	-0.967	0.333
16	80	62	0.180	0.125	2.358	0.018
17	75	91	0.169	0.183	-0.583	0.559
18	125	107	0.282	0.216	2.336	0.019
19	45	102	0.101	0.206	-4.395	<0.00001
20	40	43	0.090	0.087	0.1832	0.855
21	11	9	0.025	0.018	0.703	0.4819
Agg*	18	21	0.041	0.042	-0.138	0.8902

CAG_n, number of CAG repeats in the polymorphic marker alleles observed; p (case), frequency in cases; p (control), frequency in controls. Agg*, aggregate of alleles with frequency less than 0.02.

Table 2 *hSKCa3* allele-wise relative risk

Allele	Relative risk*
13	1.047
14	0.705
15	0.739
16	1.198
17	0.854
18	1.178
19	0.514
20	0.956
21	1.105

*If R1: A/(A+B), R2: C/(C+D), then R1/R2 is the relative risk of the allele where A is alleles present in cases, B is alleles present in controls, C is alleles not present in cases, and D is alleles not present in controls.

(*hSKCa3*). The allele CAG₁₉ was found to be present at significantly different frequencies in cases and controls, implying its association with the JME phenotype. A previous report from a German population investigated 126 IGE patients (78 JME and 48 childhood absence epilepsy or juvenile absence epilepsy cases) and found no evidence for association between IGEs and *hSKCa3*.²⁷ No evidence for association between IGEs and *hSKCa3* was found in this study. We have obtained results which are different from this published work. These differences are perhaps due to the use of different ethnic populations, different sample sizes, or differences in the subtypes of the clinical samples that were studied. The sample size used in our study was comparatively large and homogeneous and consisted only of JME cases of South Indian origin and a control sample from the same population. We found significant differences in the distribution of allele CAG₁₆, CAG₁₈, and CAG₁₉ among South Indian JME probands and ethnically matched control subjects. These three alleles were significant without Bonferroni correction. When this correction was applied, CAG₁₉ was the only allele found to be significant. However, we are concerned about the astuteness of the Bonferroni correction in this scenario as there is no logic in an a priori universal null hypothesis for all the alleles tested or in the study populations being identical on all the alleles analysed. This point has been addressed by Perneger.²⁸ Bonferroni adjustments imply that a given comparison will be interpreted differently according to how many other tests were performed.

CAG₁₆ and CAG₁₈ were common while CAG₁₉ was rare in the JME patients studied. The relative risks due to CAG₁₆, CAG₁₈, and CAG₁₉ were found to be 1.198, 1.178, and 0.514, respectively. The most probable role of CAG₁₆, CAG₁₈, and CAG₁₉ *hSKCa3* variants may be in modulating the channel function. Alternatively, the role of *hSKCa3* may not be simply as a numerical CAG counter. The possibility of additional sequences within *hSKCa3* that may influence the disease phenotype cannot be ruled out. Additional polymorphisms in the coding or regulatory regions of this complex gene that spans about 163 kb and has a complex promoter with binding sites for over 10 transcription factors may be involved.²⁹

Dynamic CAG repeat expansions have been implicated in many neurological diseases. We did not observe a major expansion in the CAG repeat polymorphism in the probands studied. However, at least in two reported scenarios, CAG repeat length polymorphisms within the normal reported range have resulted in a disease state. These are Kennedy's disease³⁰ and spinocerebellar ataxia type 6.³¹

We found observation of a possible protective effect on the JME phenotype interesting. Protective alleles are important modifiers of the phenotype. Unlike the alleles of susceptibility genes that are over-represented in affected individuals

(cases) versus unaffected individuals (controls), protective alleles occur preferentially in healthy individuals, implying that their presence prevents disease despite the presence of other disease promoting (susceptibility) alleles at the same gene or genes elsewhere in the genome. Several reports have highlighted the importance of protective alleles in various disease conditions: deletion of *CCR5* protects from HIV infection,³² while HLA-DRB11 and HLA-DRQ03032 alleles are both over-represented in controls versus breast cancer³³ cases. In the light of these findings, our results on the *hSKCa3* alleles suggest it should be carefully analysed further in JME families.

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Authors' affiliations

J Vijai, P J Cherian, K Radhakrishnan, Department of Neurology, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Trivandrum, India

A Kapoor, H M Ravishankar, A Anand, Molecular Biology and Genetics Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore, India

G Kuruttukulam, Lourdes Hospital, Cochin, India

B Rajendran, West Side Hospital, Cochin, India

R Sridharan, Apollo Hospital, Chennai, India

G Rangan, Sri Satya Sai Hospital, Bangalore, India

A S Girija, Department of Neurology, Medical College, Calicut, India

S Jayalakshmi, S Mohandas, Department of Neurology, Nizam's Institute of Medical Sciences, Hyderabad, India

K S Mani, Neurological Clinic, Bangalore, India

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*Since deceased.

Correspondence to: Dr Anuranjan Anand, Molecular Biology and Genetics Unit, Jawaharlal Nehru Centre for Advanced Scientific Research, Bangalore 560 064, India; anand@jncasr.ac.in

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