LETTER TO JMG

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


J uvenile myoclonic epilepsy (JME; OMIM 606904) is a subtype of common idiopathic generalised epilepsy (IGE) and affects up to 26% of all individuals with IGE.1,2 JME is characterised by the onset in adolescence of bilateral myoclonic jerks usually affecting the upper limbs.3,4 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 Kᵥ class of potassium channels that regulate neuronal excitability.9–11 They are 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 classified9–11 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.12 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 afterhyperpolarisation13,14 and regulation of intracellular calcium signals by binding with calmodulin.15 In situ hybridisation in rat16 and human brain17 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,18–20 the possibility of its influence on epilepsy phenotypes remains poorly studied.

hSKCa3 encodes a 731 amino acid protein comprising two polyglutamine arrays in its N terminus of which the second polyglutamine repeat is highly polymorphic.19 We investigated 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. 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.22 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 International League Against Epilepsy.25

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.
International League Against Epilepsy. 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 \textit{hSKCa3} was carried out in all subjects. Each reaction comprised of 50 ng of genomic DNA, 20 pmol primers (\textit{hSKCa3-1}: 5'-CAC CGT CAG TGT CAC CAG TCC CCC-3' and \textit{hSKCa3-2}: 5'-Hex-GAA GGG GTT GCT GTC CCG CCG GT-3'), 200 μM 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 μl final volume. Forty cycles of PCR were carried out, each cycle with denaturation at 94°C for 40 s, annealing at 52°C for 10 s, and extension at 72°C for 45 s. 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.

Sixteen distinct \textit{hSKCa3} alleles were observed. Repeat size ranged from 10 to 25. Thirteen alleles (CAG\textsubscript{10–22}) were seen in the controls and 16 alleles (CAG\textsubscript{10–25}) 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 CAG\textsubscript{16}. 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 16 alleles (alleles with frequency less than 0.02 were aggregated) was performed. In this analysis, the three alleles CAG\textsubscript{16} (0.018), CAG\textsubscript{18} (0.019), and CAG\textsubscript{19} (<0.00001) were found to be significant. The distribution of CAG\textsubscript{16} and CAG\textsubscript{18} was higher in JME patients, while allele CAG\textsubscript{19} was quite rare in JME cases but present at a very high frequency in the control population. Among the nine major alleles (CAG\textsubscript{15–21}) observed, relative risk was found to be maximum for allele CAG\textsubscript{16} (1.198) and minimum for allele CAG\textsubscript{19} (0.574) (Table 1). When Bonferroni correction for multiple testing (α at 0.005) was applied, only CAG\textsubscript{19} was found to be significant. Among the nine major alleles (CAG\textsubscript{15–21}) observed, relative risk was found to be maximum for allele CAG\textsubscript{16} (1.198) and minimum for allele CAG\textsubscript{19} (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. 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
<p>| Allele frequencies and pair-wise Z tests of JME cases and controls |
|----------------------|----------------------|----------------------|----------------------|----------------------|----------------------|----------------------|</p>
<table>
<thead>
<tr>
<th>CAG\textsubscript{a}</th>
<th>Cases</th>
<th>Controls</th>
<th>p (cases)</th>
<th>p (controls)</th>
<th>Z value</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>13</td>
<td>28</td>
<td>23</td>
<td>0.063</td>
<td>0.046</td>
<td>1.128</td>
<td>0.259</td>
</tr>
<tr>
<td>14</td>
<td>13</td>
<td>23</td>
<td>0.029</td>
<td>0.046</td>
<td>-1.363</td>
<td>0.1728</td>
</tr>
<tr>
<td>15</td>
<td>13</td>
<td>15</td>
<td>0.020</td>
<td>0.030</td>
<td>-0.967</td>
<td>0.333</td>
</tr>
<tr>
<td>16</td>
<td>80</td>
<td>62</td>
<td>0.180</td>
<td>0.125</td>
<td>2.335</td>
<td>0.018</td>
</tr>
<tr>
<td>17</td>
<td>75</td>
<td>91</td>
<td>0.169</td>
<td>0.183</td>
<td>-0.583</td>
<td>0.559</td>
</tr>
<tr>
<td>18</td>
<td>125</td>
<td>107</td>
<td>0.282</td>
<td>0.216</td>
<td>2.336</td>
<td>0.019</td>
</tr>
<tr>
<td>19</td>
<td>45</td>
<td>102</td>
<td>0.101</td>
<td>0.206</td>
<td>-4.395</td>
<td>&lt;0.00001</td>
</tr>
<tr>
<td>20</td>
<td>40</td>
<td>43</td>
<td>0.090</td>
<td>0.087</td>
<td>0.1832</td>
<td>0.855</td>
</tr>
<tr>
<td>21</td>
<td>11</td>
<td>9</td>
<td>0.025</td>
<td>0.018</td>
<td>0.703</td>
<td>0.4819</td>
</tr>
<tr>
<td>Agg*</td>
<td>18</td>
<td>21</td>
<td>0.041</td>
<td>0.042</td>
<td>-0.138</td>
<td>0.8902</td>
</tr>
</tbody>
</table>

\(\text{CAG}_a\), 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.
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 hSKCa3.7 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.29 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 the coding or regulatory regions of this complex gene that phenotype cannot be ruled out. Additional polymorphisms in hSKCa3 as a numerical CAG counter. The possibility of additional correction. When this correction was applied, CAG 19 was

<table>
<thead>
<tr>
<th>Allele</th>
<th>Relative risk</th>
</tr>
</thead>
<tbody>
<tr>
<td>13</td>
<td>1.047</td>
</tr>
<tr>
<td>14</td>
<td>0.705</td>
</tr>
<tr>
<td>15</td>
<td>0.739</td>
</tr>
<tr>
<td>16</td>
<td>1.198</td>
</tr>
<tr>
<td>17</td>
<td>0.854</td>
</tr>
<tr>
<td>18</td>
<td>1.178</td>
</tr>
<tr>
<td>19</td>
<td>0.514</td>
</tr>
<tr>
<td>20</td>
<td>0.956</td>
</tr>
<tr>
<td>21</td>
<td>1.105</td>
</tr>
</tbody>
</table>

*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.

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 CCR3 protects from HIV infection,30 while HLA-DRB11 and HLA-DRQ0302 alleles are both over-represented in controls versus breast cancer 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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