Novel KCNQ4 gene mutations contribute to susceptibility of genetic febrile seizures

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Abstract: Objective: To identify genes potentially accounting for pathogenesis of genetic febrile seizures. Methods: 31 febrile seizures families and 200 healthy patients were included in this study. A high throughput gene capture approach was used to identify candidate genes which were then validated using Sanger sequencing. Pedigree analysis was applied to evaluate phenotype and genotype. Sequencing PCR products was used to screen gene mutations in the control group. Results: We found three KCNQ4 gene missense mutations c.1609C>T (P537S), c.1207G>A (A403T), and c.1177G>T (G393C) in exons 9, 10, and 13, respectively. Each patient only had one of these mutations among three of all 31 febrile seizures families. KCNQ4 gene mutations were not found in the other 28 febrile seizures families and the healthy control group. We also found a missense SCN5A gene mutation c.2893C>T (R965C) in proband 1 family but not the other two febrile seizures families. Pedigree analysis indicated that some mutation-carriers in these members were not febrile seizures. However, the proband 1 and his elder brother carried both the KCNQ4 and SCN5A gene mutations, and probands 2 and 3 carried KCNQ4 gene mutation. Conclusion: Novel KCNQ4 gene mutations and SCN5A gene mutation were not single causal factors for febrile seizures. They most likely interacted with other gene mutations to promote a susceptibility to febrile seizures. Our findings add to the literature that KCNQ4 gene mutations are previously found to be only associated with hearing loss. Our finding provides a new clue to investigate the genetic etiology of febrile seizures.

Keywords: Genetic febrile seizures, KCNQ4, SCN5A, gene mutation, pedigree analysis

Introduction

Febrile seizures have been defined as any seizure associated with >38°C (rectal or tympanic) fever without CNS infection in a child aged 6 months to 5 years. Febrile seizures occur in 2-5% of infants in Europe and North America and in 6-9% in Japan. It is the most common convulsive event in children younger than 5 years of age [1-3]. Most febrile seizures are self-limited with age, but people who have had febrile seizures have a higher risk of developing spontaneous afebrile seizures which defines epilepsy when they recur [4]. The cause of febrile seizures is multifactorial in nature. Several susceptibility genes have been identified, including (1) genes encoding ion channels, such as SCN1A, SCN1B, and SCN2A genes that encode neuronal voltage-gated sodium ion channels and GABRG2 genes that encode ligand-gated chloride ion channel of the GABAA receptor γ2 subunits [5-7]. (2) genes encoding proinflammatory and anti-inflammatory cytokines, such as interleukin 1α (IL-1α), IL-1 receptor antagonist (IL-1Ra) IL-1β, IL-6, and tumor necrosis factorα (TNF-α) [8-11]. Mutations or polymorphisms in these genes are associated with development of febrile seizures. We hypothesized that other genes may also account for the pathogenesis of febrile seizures.

The KCNQ gene family encodes five Kv7 K+ channel subunits (Kv7.1-Kv7.5), which are voltage-gated potassium ion channels. Kv7 subunits are involved in reducing membrane excitability [12-14]. Kv7.2-Kv7.5 are expressed in the nervous system [15]. Studies have shown that Kv7.2 and Kv7.3 mutations are implicated in
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Table 1. Primers for KCNQ4 gene and SCN5A gene

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Tm</th>
</tr>
</thead>
<tbody>
<tr>
<td>KCNQ4_E13-F</td>
<td>5'-TACTTGTCAGGTCTGTGTTGG-3'</td>
<td>56.62</td>
</tr>
<tr>
<td>KCNQ4_E13-R</td>
<td>5'-GAGGGGCTCTTAGGTCTCTT-3'</td>
<td>56.1</td>
</tr>
<tr>
<td>KCNQ4_E10-F</td>
<td>5'-GCTAACCTTGCTCTCCTCC-3'</td>
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<tr>
<td>KCNQ4_E9-F</td>
<td>5'-GAACCTGGGGTAAAGGGA-3'</td>
<td>58.1</td>
</tr>
<tr>
<td>KCNQ4_E9-R</td>
<td>5'-AGGCAGGCTCTAGAGAGGATG-3'</td>
<td>58.2</td>
</tr>
<tr>
<td>SCN5A_E-17-F</td>
<td>5'-TAAAGGTCTCTGCGCTCA-3'</td>
<td>60</td>
</tr>
<tr>
<td>SCN5A_E-17-R</td>
<td>5'-TCATCTGTGTCTGACTCGC-3'</td>
<td>60</td>
</tr>
</tbody>
</table>

Table 2. KCNQ4 and SCN5A gene mutations in 31 febrile seizures families

<table>
<thead>
<tr>
<th>Proband</th>
<th>Genes</th>
<th>Site</th>
<th>Site</th>
<th>Exon</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>KCNQ4</td>
<td>chr1:41303362</td>
<td>c.1609C&gt;T</td>
<td>13</td>
</tr>
<tr>
<td>2</td>
<td>KCNQ4</td>
<td>chr1:41296832</td>
<td>c.1207G&gt;A</td>
<td>10</td>
</tr>
<tr>
<td>3</td>
<td>KCNQ4</td>
<td>chr1:41289815</td>
<td>c.1177G&gt;T</td>
<td>9</td>
</tr>
<tr>
<td>1</td>
<td>SCN5A</td>
<td>Chr3:38622757</td>
<td>c.2893C&gt;T</td>
<td>17</td>
</tr>
</tbody>
</table>

Benign familial neonatal convulsions [16-20]. KCNQ4 gene is located on human chromosome 1p34 and consists of 14 exons, encoding Kv7.4 with 695 amino acids [21]. KCNQ4 is expressed mainly in the central auditory pathway, such as Cochlear nucleus, lateral lemniscus nucleus, and the hypothalamus [22]. KCNQ4 gene mutations can result in human hereditary nonsyndromic progressive hearing loss [23, 24]. The association of KCNQ4 gene with febrile seizures remains unknown.

In this study, we identified three KCNQ4 and SCN5A gene mutations in febrile seizures patients using a high throughput screening approach. These mutations were not present in the healthy population and not single causal factors for febrile seizures clinical manifestation in three pedigrees. They may interact with other mutations to cause febrile seizures. Our findings suggest that KCNQ4 mutations may contribute to susceptibility of febrile seizures, providing a novel clue to investigate the genetic background of epileptogenesis.

Materials and methods

Study subjects

Thirty-one febrile seizures families were included in the study in Guangdong General Hospital from January 2012 to November 2015. The inclusion criteria were based on the clinical guideline published by American Academy of Pediatrics in 2008 and clinical diagnosis. The included patients had a history of febrile convulsions before the age of 5. Exclusion criteria were: (1) patients had a history of afebrile seizures; (2) patients with progressive or degenerative nervous diseases, or inherited metabolic diseases; (3) patients with heart, liver, kidney, blood, and other essential organ disease; (4) patients with intracranial infection [25].

A total of 104 subjects, including 31 probands and their family members, were included in the study. 31 probands consisted of 19 males and 12 females, average age 4.73 ± 2.94, onset from 5 months to 5 years old. Among 31 probands, 29 cases were diagnosed as generalized seizures (28 cases in generalized tonic seizures and 1 case in atonic seizure), 1 case was focal seizures, and 1 case was mixed seizures. 200 healthy subjects were collected as the control group with male: female ratio 1.4:1, average age 11.27 ± 2.56. They did not have a family history of neurological disease. Detailed clinical data were collected and long-range video EEG and cranial MRI examination were performed on all subjects. Venous blood samples were collected from each subject into an anticoagulant EDTA-containing tube. All study subjects or their legal guardians signed informed consent. Our study was approved by the ethics committee of Guangdong General Hospital.

Extraction of DNA samples

DNA samples were extracted from blood samples using genomic DNA Extraction Kit (TIANamp genomic DNA kit, Shanghai, China) following the manufacturer’s protocol and stored in -80°C until use. The concentration of DNA was determined using a NanoDrop 2000/2000c spectrophotometer (Thermo Fisher Scientific, life science reagents, USA). The quality of DNA was confirmed by the 260/280 OD value between 1.8 and 2.0, and by 1% agarose gel electrophoresis.

Capture of target genes

A total of 485 genes related to genetic epilepsy based on OMIM and HGMD databases were
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Probes for exons and the flanking sequences (±10 bp) were designed using the online tool SureDesign (Agilent, USA). The target gene capture kit was custom made by Agilent, USA, containing 60605 probes in a total of 1.961 Mbp. The target gene library was constructed from the DNA sample of febrile seizures patient using the Agilent SureSelect Target Enrichment System Kit (Agilent, USA) by following the manufacturer’s instructions, including broken-down of DNA fragments using ultrasonic probe, library construction, hybridization of probes, amplification and purification of the library. Quality was controlled using DNA chips (Agilent Cat# 5067-1522), DNA 1000 reagent (Agilent Cat# 5067-1504), and an Agilent 2100 Bioanalyzer (Agilent). The library was sequenced using a NEXTSEQ 500 sequencer (Illumina, USA) by following the manufacturer’s protocol. The raw sequencing data Fastq files were processed using RTA software (real-time analysis Illumina), Casava software v1.8.2 (Illumina), BWA, Genome analysis Toolkit (GATK). The sequencing quality was evaluated

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Based on total reads number, the proportion of the reads that match the human genome sequence, the proportion of the reads that was located within the target gene sequence, average depth of sequencing, and the ratio of the fragments where sequencing depth greater than 20x, and uniformity analysis of coverage. The report on single nucleotide variants (SNV) was generated in VCF format and then annotated using PolyPhen2.2.2 and ANNOVAR software, HGMD, dbSNP, and 1000 Genome databases. The candidate variants were verified using the Sanger method.

**Screening of KCNQ4 and SCN5A gene mutations in population**

KCNQ4 and SCN5A gene mutations in the population were screened by sequencing PCR products. Primer pairs for KCNQ4 and SCN5A genes were designed (Table 1) based on genome sequence (NC_000002 and NM_001099405, NCBI GenBank) and the KCNQ4 and SCN5A gene mutations found in “2.3 Capture of target genes” section using Primer Premier V5.0. The primers were synthesized in the Guangzhou Gene Denovo Co., Ltd. PCR products of the DNA

Figure 2. SCN5A gene mutation (c.2893C>T) in Proband 1 family. Proband 1 SCN5A c.2893C>T. Control 1 (The healthy group).
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Figure 3. Pedigree of febrile seizures Proband 1 family with KCNQ4 and SCN5A gene mutations. III-3 experienced two febrile convulsions at temperature 39°C before the age of three years old, who is now asymptomatic and studies in middle school. Gene mutation in III-3 is uncertain. III-2 father, IV-1 brother and III-2 proband have gene mutations in SCN5A. III-2 did not have history of FS. ○ normal male; ◼ normal female; ▲ febrile seizures male proband; ▼ febrile seizures female proband; ■ febrile seizures male patient with gene mutation; □ normal male with gene mutation; ○ normal female with gene mutation.

samples from 31 febrile seizures families and 200 healthy subjects were sequenced and analyzed by alignment to sequence of chr1:41249684-41306124 (GRCh37, GenBank) and chr3:38591812-38674798 (GRCh37/hg19, GenBank).

Results

KCNQ4 and SCN5A gene mutations in febrile seizures patients

We found three KCNQ4 gene mutations c.1177G>T, c.1207G>A, and c.1609C>T in exons 9, 10, and 13, respectively, in febrile seizures patients through high-throughput target gene capture (Table 2; Figure 1). Each patient only had one of these mutations among three febrile seizures families (Table 2; Figure 1). These mutations were missense and may cause the changes p.Gly393Cys, p.Ala403Thr, and p.Pro537Ser, respectively, in the KCNQ4 peptide (Table 2). We did not find any KCNQ4 gene mutation in other 28 febrile seizures families.

We also found a missense SCN5A gene mutation c.2893C>T in exon 17, resulting in a p.Arg965Cys change, in proband 1 family but not in the other two families (Figure 2).

Pedigree analysis of KCNQ4 and SCN5A gene mutation in three febrile seizures families

We performed pedigree analysis of KCNQ4 and SCN5A gene mutations in three febrile seizures families. The results demonstrated that the proband 1’s elder brother and mother carried KCNQ4 c.1609C>T (Figure 3). No other member carried
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Table 3. Clinical data of probands in three febrile seizures families

<table>
<thead>
<tr>
<th>Probands</th>
<th>Sex</th>
<th>Age</th>
<th>Onset age</th>
<th>Attack</th>
<th>T</th>
<th>Duration a</th>
<th>Total attack</th>
<th>Family history</th>
<th>Head MRI</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>2 y</td>
<td>1y9m</td>
<td>Generalized tonic/tonic seizure</td>
<td>1</td>
<td>1</td>
<td>&gt;10</td>
<td>+ #</td>
<td>Normal</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>3 y</td>
<td>6m</td>
<td>Generalized tonic</td>
<td>1</td>
<td>1</td>
<td>7</td>
<td>-</td>
<td>Thin corpus callosum</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>4 y</td>
<td>1y8m</td>
<td>Generalized tonic</td>
<td>2</td>
<td>1</td>
<td>3</td>
<td>-</td>
<td>subependymal cysts</td>
</tr>
</tbody>
</table>

T, Highest body temperature during fever; 1<38.5°C, 2≥38.5°C; Duration, 1<15 min; 2≥15 min; Family history, “+” yes; “-” no; #, The brother of this patient had an onset of convulsion with fever 38°C at 2 years old.

Figure 5. The EEG of proband 1 at 2 years old. The Video-EEG recorded multiple generalized seizures which arose from the central and temporal lobe and spread to all the brain area during monitoring time. There are continuously moderate to extreme high amplitude sharp waves, sharp and slow wave complex, spike and slow wave complex, and multiple spike and slow wave complex in all leads during ictal phase.

cDNA sequence analysis identified a KCNQ4 gene mutation in this family. The proband 1 and his elder brother and father carried SCN5A c.2893C>T (Figure 3). No other member carried the SCN5A gene mutation in this family. Except for proband 1 and his brother, who were febrile seizures patients, no other member in this family had febrile seizures (Figure 3). This suggests that either KCNQ4 c.1609C>T or SCN5A c.2893C>T mutations were not a single causal factor for febrile seizures. Combination of KCNQ4 c.1609C>T and SCN5A c.2893C>T mutations may contribute to febrile seizures.

The proband 2’s mother carried c.1207G>A, and the proband 3’s father and paternal grandmother carried c.1177G>T (Figure 4). No other member carried any KCNQ4 gene mutation in these two febrile seizures families (Figure 4). Clinical information on the febrile seizures patients with KCNQ4 gene mutation was listed in Table 3 and Figures 5-8. No member carried the SCN5A c.2893C>T gene mutation in these families. Except for the probands 2 and 3, no other member in these two families was febrile seizures patient. These results suggested that the KCNQ4 c.1207G>A or c.1177G>T gene...
Mutation was not a single causal factor for febrile seizures, and may contribute to febrile seizures.

No KCNQ4 and SCN5A gene mutation in the healthy control group

We examined whether each KCNQ4 and SCN5A gene mutations was found in the healthy control group. The results showed that no KCNQ4 and SCN5A gene mutation was found in the healthy control group of 200 patients.

Discussion

In the current study, we identified three KCNQ4 gene mutations c.1177G>T, c.1207G>A, and c.1609C>T in exons 9, 10, and 13, respectively, in three febrile seizures families. Each patient only had one of these mutations among 3 febrile seizures families. Each patient only had one of these mutations among 3 febrile seizures families. Each patient only had one of these mutations among 3 febrile seizures families. Each patient only had one of these mutations among 3 febrile seizures families. Each patient only had one of these mutations among 3 febrile seizures families.

Pedigree analysis indicated that these KCNQ4 gene mutations and SCN5A gene mutation were not single causal factors for febrile seizures as some mutation-carriers in these members were not febrile seizures.

Previous studies showed that SCN1A and SCN2A gene mutations are associated with genetic epilepsy with febrile seizures (GEFS+) and epilepsy, and that SCN5A is associated with epilepsy [13, 26, 27]. A recent study showed that SCN5A mutation is related to the pathogenesis of epilepsy, long QT syndrome, and Jervell and Lange-Nielsen syndrome alone or alongside KCNQ1 and KCNH2 gene mutations [28]. In the current study, we found that the missense SCN5A gene mutation c.2893C>T in exon 17 results in a p.Arg965Cys change, in proband 1 family but not the other two families. The proband 1 and his elder brother carried both the KCNQ4 gene mutation and SCN5A gene mutation, the probands 2 and 3 carried KCNQ4 gene mutation, suggesting that they probably interacted with other gene mutations to promote susceptibility to febrile seizures.
KCNQ<sub>4</sub> gene encodes Kv7.4 that has six transmembrane domains (S1-S6) with intracellular NH2 and COOH ends. Like other Kv7 subtypes, KCNQ<sub>4</sub> is characterized by a long COOH end, which determines its physical and functional interactions with other molecules and calcium regulatory proteins [29, 30] such as PIP2 (2-phosphate - inositol phosphate) and PKC (protein kinase C) [31, 32]. KCNQ<sub>4</sub> mutations (Gly-393Cys, p.Ala403Thr, and p.Pro537Ser) identified in the current study are located in the COOH end. In the wild type which contains Gly, Ala, and Pro, Gly does not have a side chain, Ala has a hydrophobic side chain, and Pro has a conformational rigidity due to a specialized side chain. In the mutants which contains Cys, Thr, and Ser, Cys has a sulfhydryl group, and Thr and Ser have polar and uncharged side chain with hydroxyl. Therefore, these mutations may change the structure of the COOH end of the KCNQ<sub>4</sub> protein and affect the KCNQ<sub>4</sub> functions according to structure-activity correlation. In addition, the sulfhydryl and hydroxyl groups in the side chains of Cys, Thr, and Ser are subjected to modification, such as phosphorylation, thereby altering the signaling systems for KCNQ<sub>4</sub> functions. Moreover, Kv7.4 can form hetero-dimers with Kv7.3 in addition to form homotetramer in the regulation of the membrane potential [21, 33, 34]. Kv7.3 is widely expressed in the cerebral cortex, cerebellum, basal ganglia and, hippocampus [15]. We speculate that KCNQ<sub>4</sub> mutations identified in the current study may cause disorders in the structural and functional interactions between Kv7.4 and Kv7.3, and interact with other unknown factors to induce abnormal excitability of neurons and neural networks in the cerebral cortex, cerebellum, basal ganglia, hippocampus, leading to epilepsy. The underlying mechanism remains to be investigated further.

It has been well known that KCNQ<sub>4</sub> gene mutations cause human hereditary nonsyndromic progressive hearing loss [23, 24], such as one-base deletion in exon 1, c.211delC which gen-

Figure 7. The EEG of proband 2 at 3 years old. There are many single asynchronous sharp waves, sharp and slow wave complex in anterior frontal lobe, anterior temporal, right parietal lobe, and bilateral posterior temporal area during sleeping period.
erated a profoundly truncated protein without transmembrane domains (p.Q71fsX138) [35], and the changes of the copies of intron between exon 2 and exon 3 of KCNQ4 [36]. Adding to the literature, we found KCNQ4 gene mutations may contribute to febrile seizures in the current study, suggesting that KCNQ4 is an essential gene in regulating nervous system and a patient predisposition for both hearing loss and febrile seizures. Even though some people with KCNQ4 mutation in the three family have no phenotype of febrile seizures, it might be due to variable penetrance. Syndromes expressed by individual members would be determined by other genes or environmental factors in febrile seizures [37]. How different KCNQ4 gene mutations contribute to either hearing loss or febrile seizures is an important research goal and deserves further investigation.

In summary, we have identified three novel KCNQ4 and SCN5A gene mutations which were not single causal factors for febrile seizures. They most likely interact with other gene mutations to promote susceptibility to febrile seizures. This adds to the literature that KCNQ4 gene mutations are previously found to be only associated with hearing loss. Our finding provides a new clue to investigate the genetic etiology of febrile seizures.

Acknowledgements

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Disclosure of conflict of interest

None.

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Figure 8. The EEG of proband 3 at the age of 4. There are some paroxysmal short to moderate range theta activities mixed with sharp waves, sharp and slow wave complex, spike and slow wave complex during sleeping period, which are obvious in central and frontal lobe.
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References

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