

## Association between variation in the human *KCNJ10* potassium ion channel gene and seizure susceptibility

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### Abstract

**Purpose:** Our research program uses genetic linkage and association analysis to identify human seizure sensitivity and resistance alleles. Quantitative trait loci mapping in mice led to identification of genetic variation in the potassium ion channel gene *Kcnj10*, implicating it as a putative seizure susceptibility gene. The purpose of this work was to translate these animal model data to a human genetic association study. **Methods:** We used single stranded conformation polymorphism (SSCP) electrophoresis, DNA sequencing and database searching (NCBI) to identify variation in the human *KCNJ10* gene. Restriction fragment length polymorphism (RFLP) analysis, SSCP and Pyrosequencing<sup>TM</sup> were used to genotype a single nucleotide polymorphism (SNP, dbSNP rs#1130183) in *KCNJ10* in epilepsy patients ( $n = 407$ ) and unrelated controls ( $n = 284$ ). The epilepsy group was comprised of patients with refractory mesial temporal lobe epilepsy ( $n = 153$ ), childhood absence ( $n = 84$ ), juvenile myoclonic ( $n = 111$ ) and idiopathic generalized epilepsy not otherwise specified (IGE-NOS,  $n = 59$ ) and all were of European ancestry. **Results:** SNP rs#1130183 (C > T) alters amino acid 271 (of 379) from an arginine to a cysteine (R271C). The C allele (Arg) is common with conversion to the T allele (Cys) occurring twice as often in controls compared to epilepsy patients. Contingency analysis documented a statistically significant association between seizure resistance and allele frequency, Mantel–Haenszel chi square = 5.65, d.f. = 1,  $P = 0.017$ , odds ratio 0.52, 95% CI 0.33–0.82. **Conclusion:** The T allele of SNP rs#1130183 is associated with seizure resistance when common forms of focal and generalized epilepsy are analyzed as a group. These

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data suggest that this missense variation in *KCNJ10* (or a nearby variation) is related to general seizure susceptibility in humans.

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## 1. Introduction

Epilepsy is a general term that includes over 40 different types of human seizure disorder (Engel, 1998; ILAE, 1989). Most of these are likely to be the result of interaction between individual genetic variation and environmental influences. Several types of human epilepsy are caused by single gene variations that are inherited in a predictable Mendelian fashion. Such cases are amenable to genetic dissection and causative gene variations have been isolated for some forms of epilepsy including autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE) (Steinlein et al., 1997; De Fusco et al., 2000), benign neonatal familial convulsions (BFNC) (Singh et al., 1998; Charlier et al., 1998), progressive myoclonic epilepsy of the Lafora and the Unverricht-Lundborg types (Minassian et al., 1998; Serratosa et al., 1999; Lalioti et al., 1997), generalized epilepsy with febrile seizures plus (GEFS+) (Wallace et al., 1998, 2001a,b; Escayg et al., 2001; Harkin et al., 2002; Baulac et al., 2001) and others. Although single gene causes of epilepsy have yielded to genetic dissection, they are rare, and when combined, represent perhaps less than 1% of all cases of epilepsy seen in the clinic. Furthermore, an understanding of the basic mechanisms causing these rare seizure disorders is complicated by genetic heterogeneity. For example, there are ADNFLE and GEFS+ families in which affected individuals do not carry the causative variations in the genes previously identified as disease loci; rather they are linked to unknown gene variations (Phillips et al., 1998; Lerche et al., 2001; Gerard et al., 2002). Nevertheless, the success achieved in isolating gene variations involved in several of these rare forms of seizure disorder has broadened the focus of seizure research from studies on GABA and glutamate pathways to studies on ion channels and other genes that directly affect ion flux across excitable membranes. Simultaneously, such studies demonstrated that variation in genes unrelated

directly to ion homeostasis and neurotransmission can cause epilepsy (Minassian et al., 1998; Serratosa et al., 1999; Lalioti et al., 1997; Kalachikov et al., 2002).

In contrast, the search for genetic variation that is associated with common forms of epilepsy, such as juvenile myoclonic epilepsy or temporal lobe epilepsy, has been less successful. The identification of seizure susceptibility genes has been hampered by lack of a genetic animal model that recapitulates common types of human spontaneous seizures, by genetic heterogeneity and by the relatively small numbers of patients studied by either genetic linkage or association analyses. Idiopathic generalized epilepsies (IGE) have been studied intensively with regard to genetic variation associated with illness. To date, about thirty different association studies have been published, examining the relationship between common forms of IGE and variation in a number of plausible candidate genes. The majority of these studies report no association; however, attempts to replicate negative results are rare and it is debatable whether to exclude genes from further consideration based on a single study. Similarly, positive associations either have not been validated in a second study or else replication studies have not yet been attempted. In one case, an association was reported between IGE and variations in the mu opioid receptor (MOR) gene with a nominal *P* value of 0.02 (Sander et al., 2000). A second study replicated this finding in a separate population with a much lower *P* value of 0.0003 (Wilkie et al., 2002). Thus, the MOR gene and neighboring genomic regions warrant further study with respect to association with IGE. Genetic linkage between IGE and markers on human chromosomes 3, 5, 6, 8, and 15 has also been reported. Recently Pal et al. (2003) described strong association between juvenile myoclonic epilepsy (JME) and variation in the BRD2 gene on chromosome 6. The gene is a transcription factor and further work is needed to elucidate the functional consequences of the variations identified. Associations between temporal lobe epilepsy (TLE) and variation

in the interleukin 1 beta (IL-1B) and prodynorphin genes have also been reported (Kanemoto et al., 2000; Stogmann et al., 2002). The IL-1B polymorphism was also found to be associated with febrile convulsions ([FC] Virta et al., 2002), a condition long thought to be associated with some cases of TLE. However, failure to replicate the IL-1B findings in separate populations of TLE patients (Buono et al., 2001; Heils et al., 2000) and FC patients (Tilgen et al., 2002) was subsequently reported. Failure to reproduce the prodynorphin findings in a separate population of TLE patients was also reported (Tilgen et al., 2003). These results exemplify some of the complexities involved with the search for epilepsy susceptibility alleles by association studies in groups of unrelated individuals.

We have taken advantage of a polygenic animal model of seizure sensitivity and resistance in common inbred strains of mice (Ferraro et al., 1997, 1999, 2001, *in press*) and have identified the mouse *Kcnj10* gene as a candidate seizure susceptibility locus (Ferraro et al., *in press*). The homologous human *KCNJ10* gene encodes an inward rectifying potassium ion channel. These channels have widespread expression in the CNS and help regulate extracellular potassium ion concentrations. Of note is a positive association between human IGE and a SNP in the *KCNJ3* inward rectifying potassium ion channel gene (Chioza et al., 2002). The present report describes a case control study testing for association between seizure susceptibility and a SNP (NCBI dbSNP rs#1130183) in the human *KCNJ10* gene. Our findings show that the T allele of the SNP is associated with seizure resistance when patients with common types of focal or generalized epilepsy are pooled together and compared against a collection of ethnically matched controls. These results suggest that variation in (or nearby) *KCNJ10* affects risk for development of common types of epilepsy and defines the *KCNJ10* SNP as a seizure susceptibility allele. This is the first report of seizure resistance being associated with the minor allele of a relatively common variation.

## 2. Methods

All patient samples were collected using proper informed consent and protocols approved by Institutional Review Boards at each clinical site. Clinical in-

clusion criteria for patients collected in Germany by Dr. Sander have been published previously (Sander et al., 2000). Clinical criteria in the US collection were as follows: Inclusion criteria for mesial temporal lobe epilepsy (TLE): (1) medically refractory complex partial seizures and an interictal scalp electroencephalogram (EEG) demonstrating spikes or sharp waves in the temporal leads F7, F8, T4, T3, Sp1 or Sp2; (2) a magnetic resonance imaging (MRI) scan of the brain with evidence of unilateral temporal lobe atrophy. If the MRI did not show unilateral hippocampal atrophy, then intracranial EEG recording should demonstrate mesial temporal lobe origin of seizures using stereotactically implanted depth electrodes; (3) scalp ictal EEG consistent with temporal lobe seizure onset; (4) Neuropsychological testing indicating impairment of verbal and/or visuospatial memory. Patients are excluded from the TLE group if seizures are of extratemporal origin, if interictal spikes are observed from EEG leads over frontal, parietal or occipital lobes, or if seizures are caused by an identifiable brain lesion as a result of trauma, encephalomalacia, stroke, dysplasia, vascular malformation or intra-cranial tumor.

Inclusion criteria for juvenile myoclonic epilepsy (JME) are as follows: (1) normal neurological examination; (2) seizure onset between ages of 11 and 19; (3) history of myoclonus, with or without tonic-clonic seizures, with or without absence seizures; (4) interictal scalp EEG showing generalized spike-wave or polyspike-wave discharges at a frequency of 3–5 Hz and absence of focal slowing in the interictal EEG; (5) normal MRI of the brain. Patients with many of the characteristics of JME but without myoclonus have been categorized as having Idiopathic Generalized Epilepsy not otherwise specified or IGE-NOS.

Inclusion criteria for childhood absence epilepsy (CAE) are as follows: (1) seizure onset between ages of 2 and 9; (2) history of brief episodes of diminished responsiveness and cessation of normal activity with an average duration of 5–20 s. Occipital or frontal, bilaterally symmetrical, intermittent rhythmic delta activity (OIRDA or FIRDA) is often present in CAE patients and not a basis for exclusion; also, a history of an occasional generalized tonic clonic seizure is permissible; (3) a pretreatment EEG showing generalized, high voltage, regular 2.5–5 Hz spike wave discharges superimposed on a normal background; (4) normal cognitive function; (5) decline in seizure frequency

following treatment with ethosuximide, valproic acid or lamotrigine.

DNA was extracted from a whole blood sample using Puregene reagents following manufacturer's protocols (Gentra Systems, Minneapolis, MN). Work on the mouse homolog determined that the *KCNJ10* coding region was intronless (BAC RP23 157J4, NCBI accession # AC074311). Thus, the human *KCNJ10* gene was originally screened for variation with single stranded conformation polymorphism (SSCP) electrophoresis using genomic DNA as a template (primer pairs for SSCP analyses available upon request). Small fragments (200–300 bp) were amplified from human genomic DNA by PCR using primers designed from the cDNA sequences present in the NCBI database for human "KIR4.1" accession # NM002241 and Norway rat # X83585. PCR amplicons were scanned for evidence of variation using SSCP with mutation enhancement detection (MDE) gel electrophoresis as per the manufacturer's protocol (FMC Bioproducts, Rockland, ME). Mobility shifted PCR products were subcloned (TOPO vector as per manufacturer's protocols) and sequenced using the nucleic acid core facility at the Children's Hospital of Philadelphia. SNP 1130183 is a "C > T" transition 1037 bases downstream from the transcription start site (# NM002241 as a reference). The SNP confers an arginine to a cysteine variation at amino acid position 271 and destroys an EarI restriction enzyme site.

Genotyping was performed by three different techniques. Approximately one third of the samples were genotyped using the SSCP assay that initially detected the variation. Primers for SSCP genotyping were designed from the rat sequence (NCBI # X83585) and are as follows: forward primer: 5' AGA CAC AGC CTC TGA TAG 3' (pos 720–737) and reverse primer: 5' CTT AGG ATC AGC ACGAGC 3' (pos 851–834). These primers amplify 131bp of DNA including the C > T transition at position 802. Subsequently, an RFLP assay was used to genotype another one third of the sample. PCR was used to amplify a 622-bp fragment containing the SNP. The "C" allele produces an amplified product that digests into three fragments after incubation with EarI (376, 238 and 8 bp). The "T" allele destroys one restriction site and thus the amplicon is digested into two fragments after EarI incubation (614 and 8 bp). Primers for this RFLP assay were designed from human cDNA sequence (NCBI

# NM002241) and are as follows: forward primer: 5'-GCA AGC CCT GCC TCA TGA-3' (bp position 801 in NM002241) and reverse primer: 5'-AGG GCA TTG GAA GAG AGG-3' (bp position 1422 in NM002241). The remaining one-third of the sample was genotyped using Pyrosequencing (Westborough, MA) following the manufacturer's protocol. Primers for the Pyrosequencing assay were as follows: forward; 5'-biotin-ACA CAG CCT CTG ACA GCC C-3'; reverse: 5'-TGT GAA CTC GTA GCC CCA-3'; reverse sequencing primer: 5'-AGT CAC CCT CAC CAC TG-3'. The amplicon size is 214 bp and the sequencing primer is at position 1039 from the transcription start site, 2 bases away from the C > T transition at position 1037 (using NCBI # NM002241 as a reference).

As one means of standardizing the different genotyping methods, 87 different DNA samples were scored by both SSCP and Pyrosequencing and only one was different between the two techniques. Subsequent analysis proved the SSCP genotype to be correct. Thus, the two techniques have a very high concordance rate for genotype scoring. Additionally, 18 of the same samples were tested by the RFLP analysis and all 18 agreed with the genotype produced by pyrosequencing and SSCP.

Genotypes were tested for deviation from Hardy–Weinberg equilibrium. Genotype frequencies were not compared for association tests since the "TT" genotype was rare. Instead, allele frequencies were calculated and allele distribution in the cases and controls was analyzed by Chi-square contingency tables. A significant portion of the cases (~30%) and controls (~50%) were obtained from Dr. Thomas Sander in Berlin Germany, and the rest from the Philadelphia metropolitan area in the USA. Since baseline allele frequencies were different between the German and US populations (Table 1), the groups were placed in different cells of the contingency table using a Mantel–Haenszel Chi-square test with continuity correction. This test results in one degree of freedom for the analysis.

### 3. Results

All of the populations were found to be in Hardy–Weinberg equilibrium for the R271C geno-

Table 1  
Genotype distribution of dBSNP1130183 in epilepsy patients and controls

Population	# Subjects	Genotype CC	Genotype CT	Genotype TT	Minor allele (%)
German controls	132	106	25	1	10.2
German IGE <sup>a</sup>	138	122	16	0	5.7
US controls	152	134	18	0	5.9
US GE <sup>b</sup>	29	27	2	0	3.4
US JME <sup>c</sup>	34	31	3	0	4.4
US CAE <sup>d</sup>	53	49	4	0	3.7
US TLE <sup>e</sup>	153	143	10	0	3.2
Total controls	284	240	43	1	7.9
Total patients	407	372	35	0	4.2

<sup>a</sup> German IGE patients included those with juvenile myoclonic epilepsy (JME), juvenile absence epilepsy (JAE), and childhood absence epilepsy (CAE).

<sup>b</sup> US GE: patients have generalized epilepsy, but do not meet the typical criteria for JME, JAE or CAE.

<sup>c</sup> JME: juvenile myoclonic epilepsy.

<sup>d</sup> CAE: childhood absence epilepsy.

<sup>e</sup> TLE: temporal lobe epilepsy.

types (Table 1). The US group included both IGE and TLE cases whereas the German group consisted of IGE patients only. The minor allele frequency in the German controls was approximately 10.2% whereas the frequency in German IGE patients was 5.7%. For populations collected in the US, the minor allele frequencies were 5.9 and 3.5% for the controls and epilepsy patients respectively. The allele distribution between all cases (407) versus all controls (284) was significantly different using the Mantel–Haenszel test with continuity correction: chi square = 5.6536, d.f. = 1,  $P = 0.017$ , odds ratio 0.52, 95% CI 0.33–0.82 (Table 2). Chi square analysis of German controls versus German IGE cases resulted in a  $P = 0.058$  using Mantel–Haenszel tests (Table 2). When US IGE or TLE cases were compared to US controls, separately or combined, the

difference in allele distribution was not statistically significant.

#### 4. Discussion

Quantitative trait loci (QTL) mapping was used to identify chromosomal regions harboring genes related to seizure sensitivity and resistance in DBA/2 and C57BL/6 inbred mouse strains respectively. Three separate QTL mapping experiments (using kainic acid, pentylentetrazol and electroconvulsive shock for seizure induction) identified distal mouse chromosome 1 as harboring a gene(s) related to the relative sensitivity of the DBA strain to both focal and generalized seizures (Ferraro et al., 1997, 1999, 2001). The critical region of mouse chromosome 1 has been reduced to 6.6 Mb by production of congenic animals and candidate genes have been systematically studied for differences in primary sequence and expression patterns that differentiate the two inbred strains (Ferraro et al., in press). Thus far the potassium ion channel gene *Kcnj10* is the most promising candidate based on the function and expression patterns of the encoded protein. Furthermore, this gene contains a coding region SNP variation in the mouse that correlates with seizure susceptibility among a variety of inbred strains (Ferraro et al., in press). The murine SNP alters a threonine to a serine in the carboxyl terminus of the protein (T262S), a region documented to

Table 2  
Comparison of allele distribution by Mantel–Haenszel chi square contingency analyses

Comparison	Chi square value	$P$ value
All epilepsy vs. all controls	5.65	0.017 <sup>a</sup>
German IGE vs. German controls	3.61	0.058
US patients vs. US controls	2.64	0.10
US IGE vs. US controls	1.15	0.28
US TLE vs. US controls	2.45	0.18

<sup>a</sup> Mantel–Haenszel with continuity correction for different baseline allele frequencies between the German and US cases and controls.

be involved with ionic conductance, channel subunit dimerization, and anchoring to the plasma membrane (Nishida and MacKinnon, 2002). The human variation R271C occurs just nine amino acids away in this same critical region. Our hypothesis is that the T262S variation in the mouse *Kcnj10* is the causative susceptibility allele underlying the chromosome 1 QTL. Furthermore, we hypothesize that variation in human *KCNJ10* will be associated with multiple seizure phenotypes (combined focal and generalized epilepsy), but not necessarily with any particular type of epilepsy. This hypothesis is strengthened by our QTL studies as well as epidemiological data suggesting that susceptibility factors exist in human epilepsy populations that increase risk for both focal and generalized epilepsy (Miller et al., 1999; Ottman et al., 1998). Our studies and those of others demonstrate that first degree relatives of patients with focal or generalized epilepsy have a 3–4-fold increased incidence of any type of seizure disorder compared to the general population (Buono et al., 2000; Kjeldsen et al., 2001). These data support the notion that there will be some genetic variation that will associate with general seizure sensitivity or resistance. In contrast, there are sound data that demonstrate that some genetic variation will likely associate with a particular clinical type of seizure disorder (Winawer et al., 2002).

The relationship between neuronal excitability and extracellular potassium ion concentration has been well established (Somjen, 2002; Gorji et al., 2001a,b). Neurons become hyperexcitable when extra-cellular potassium concentrations are above 5 mM or below 2 mM. Thus, subtle variation in the inward rectifying channel may have an appreciable effect on extra-cellular potassium ion concentration during and after neuronal excitation. The inward rectifier family contains 16 different genes with an average 42% homology at the amino acid level (based on BLAST alignment and NCBI sequences). The residues surrounding and including R271 are 100% conserved between different vertebrate species with respect to *KCNJ10*, however, the R271 is only 20% conserved between the 16 human gene members of the inward rectifier family.

Results of the present study suggest that the “T” allele of SNP rs#1130183 (R271C) is associated with general seizure resistance. These data exemplify several important aspects of complex trait inheritance and

genetic susceptibility. Natural variation would predict that both seizure resistance and sensitivity alleles exist in the population, and the combination of these alleles make up the genetic contribution for risk of illness. There is currently no accepted convention that the minor allele must be associated with illness in order to be considered a “susceptibility” allele. In the present case, the minor allele leading to the R271C variation in *KCNJ10* is associated with seizure resistance. Thus, the variation in *KCNJ10* can be considered a susceptibility allele, even though it is related to seizure resistance. This is the first documentation of a seizure resistance allele and may help to explain the observation that some patients with seizure disorder have no obvious risk factors (family history, trauma with loss of consciousness, febrile convulsions, etc) while other individuals have many risk factors, but do not develop epilepsy. Furthermore, these data support our hypothesis that some genetic variation is related to general seizure susceptibility, without necessarily being associated with any specific epilepsy subtype.

Although the association between *KCNJ10* variation and common subtypes of epilepsy with a *P* value of 0.017 is statistically significant, it is possible that this result is a false positive and occurred simply by chance. Lander and Schork (1994) proposed that a *P* value of 0.0001 is an appropriate measure of significance for association studies that typically plan to study hundreds of variations over time. This stringent level would allow for correction of multiple testing of the same population using the method of Bonferroni. Alternatively, Plomin et al. (1994) suggested that statistical significance at a value of  $P < 0.05$  is suggestive of association and warrants an attempt to confirm the initial finding in a separate population. This latter alternative seems to have some merit, as the work on association between MOR variation and IGE showed a nominal *P* value ( $P = 0.02$ ) in an initial study (Sander et al., 2000) that was substantially lowered ( $P = 0.0003$ ) in a replication study using a separate population (Wilkie et al., 2002).

It is clear that there are differences in *KCNJ10* allele frequencies in control populations collected in different parts of the world (Table 1). These differences underscore the importance of collecting controls from the same population as that from which patients are collected or to correct statistically for these popula-

tion differences when combining patients and controls collected in different geographical regions. The statistical  $P$  values calculated in the present study would be reduced by three to four orders of magnitude ( $P = 0.0001$ ) if allele frequencies in US patients of European descent were compared to those frequencies reported for controls collected in Europe alone. Similarly, the Celera database identifies this SNP as having a 12% minor allele frequency in healthy controls of European ancestry correlating well to the frequency reported for the German control population we studied. Thus some heterogeneity exists in the US population collected with respect to this variation. For this reason, data from both the US and German populations were analyzed simultaneously using Mantel-Haenszel tests with continuity correction.

In summary, the missense variation R271C at the human *KCNJ10* locus influences risk for acquiring common forms of human epilepsy and identifies *KCNJ10* as a seizure susceptibility locus. These results represent translation of data from mouse QTL studies and demonstrate the usefulness of such a strategy to identify the elusive genetic variations that increase or decrease risk for human complex disease traits. We anticipate that the *KCNJ10* variation R271C can be quickly tested in separate epilepsy populations to confirm the biological significance of the observed association.

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