Splicing Mutations in \( \text{KCNQ1} \)

A Mutation Hot Spot at Codon 344 That Produces In Frame Transcripts

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**Background**—Long-QT syndrome is a monogenic disorder that produces cardiac arrhythmias and can lead to sudden death. At least 5 loci and 4 known genes exist in which mutations have been shown to be responsible for the disease. The potassium channel gene \( \text{KCNQ1} \), previously named \( \text{KVLQT1} \), on chromosome 11p15.5 is one of these.

**Methods and Results**—We initially analyzed one family using microsatellite markers and found linkage to \( \text{KCNQ1} \). Mutation detection showed a G to C change in the last base of exon 6 (1032 G\( \rightarrow \)C) that does not alter the coded alanine. Restriction digest analysis in the family showed that only affected individuals carried the mutation. A previous report suggested that a G to A substitution at the same position may act as a splice mutation in \( \text{KCNQ1} \), but no data was given to support this hypothesis nor was the transcription product identified. We have shown by reverse-transcription polymerase chain reaction that 2 smaller bands were produced for the \( \text{KCNQ1} \) gene transcripts in addition to the normal-sized transcripts when lymphocytes of affected individuals were analyzed. Sequencing these transcripts showed a loss of exon 7 in one and exons 6 and 7 in the other, but an in-frame transcript was left in each instance. We examined other families in whom long-QT syndrome was diagnosed and found another unreported splice-site mutation, 922-1 G\( \rightarrow \)C, in the acceptor site of intron 5, and 2 of the previously reported 1032 G\( \rightarrow \)A mutations. All these showed a loss of exons 6 and 7 in the mutant transcripts, validating the proposal that a consensus sequence is affected in the exonic mutations and that the integrity of the base at position 1032 is essential for correct processing of the transcript.

**Conclusions**—The 6 cases already reported in the literature with the 1032 G\( \rightarrow \)A transition, the novel 1032 G\( \rightarrow \)C transversion, and a recent G\( \rightarrow \)T transversion at the same base show that codon 344 is the second most frequently mutated after codon 341, suggesting at least two hotspots for mutations in \( \text{KCNQ1} \). (Circulation. 1999;100:1077-1084.)

**Key Words** long-QT syndrome • mutation • ion channels

Long-QT syndrome (LQTS) is an inherited form of cardiac arrhythmia that can lead to sudden death.\(^1\)\(^2\) A recent report on 33 000 newborns suggested an increased risk for those shown to have prolonged QTc on day 3 or 4 of life.\(^3\) Inheritance is commonly autosomal-dominant, where the condition is known as Romano-Ward Syndrome (RWS),\(^1\)\(^2\) but there is also a rare recessive form, Jervell-Lange-Nielsen syndrome,\(^4\) with associated deafness. The genetic pattern is not simple, however, as incomplete penetrance occurs in the dominant mode,\(^5\) and a recent report has shown a family with RWS but recessive inheritance.\(^6\) Four genes are known to cause LQTS, all of which encode ion channels or associated proteins. At loci \( \text{LQT1} \), \( \text{LQT2} \), \( \text{LQT3} \), and \( \text{LQT5} \) are the genes \( \text{KCNQ1} \),\(^7\) \( \text{HERG} \),\(^8\) \( \text{SCN5A} \), and \( \text{KCNE1} \),\(^10\)\(^11\) respectively. The first 2 code for potassium-channel proteins, the third for a sodium channel, and the last for a protein that interacts with potassium channels.\(^12\)\(^-\)\(^15\) \( \text{LQT4} \) is a genetic locus\(^16\) for which linkage has been shown for only 1 family. About 40 mutations have been described for \( \text{KCNQ1} \), but the phenotypic effect of these changes must be dependent on environmental or other genetic factors to explain the high degree of non-penetrance. Mutations in both \( \text{KCNE1} \) and \( \text{KCNQ1} \) produce Jervell-Lange-Nielsen syndrome and RWS,\(^10\)\(^11\)\(^17\)\(^-\)\(^21\) and in one case, amino acid changes in the latter gene showed RWS clinical features with a recessive inheritance.\(^6\) The designation of an inheritance pattern may, in some cases, depend on whether the stimulus that provokes the symptoms is strong enough to show an effect in the heterozygous or homozygous state. Some mutations are likely to prove of greater pathological importance than others, and the degree of non-penetrance and the number of asymptomatic individuals in families with different mutations will provide evidence in

Received February 12, 1999; revision received June 7, 1999; accepted June 15, 1999.

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this respect. In this study, we report on 3 splice mutations involving exons 6 and 7.

Methods

Microsatellite Analysis

Microsatellites were examined using either silver staining on native or denaturing polyacrylamide gels or incorporation of \(^{3}P\)-\(\alpha\) deoxy-
citidene triphosphate (\(^{3}P\)-CTP) on denaturing sequencing gels. Polymerase chain reaction (PCR) for the nonradioactive gels was

performed in a total volume of 15 \(\mu\)L of a solution containing 60 \(\mu\)g of genomic DNA, 1.5 mmol/L MgCl\(_2\), 0.2 mmol/L dNTP, 10 pmol/L

primers, and 1 U of Taq polymerase (Red Hot) and buffer supplied

by the manufacturer (Advanced Biotechnology). Amplification condi-
tions were an initial denaturation at 94°C for 3 minutes followed by

27 cycles at 94°C for 30 s, 55°C for 75 s, 72°C for 15 s, and a

6-minute extension at 72°C. A total of 3 \(\mu\)L of PCR product was run on either 8% to 10% polyacrylamide gels for 3 hours or 6% to 8% denaturing polyacrylamide gels for 50 to 90 minutes, depending on size.

Linkage Analysis

Linkage analysis was done using the M-LINK program of the LINKAGE software, version 5.03. Penetration was assumed to be 0.80. Multipoint analysis was performed by comparing the lodscores obtained when the long-QT locus was positioned between markers D11S1318 and D11S1323 with that when it was placed outside these flanking microsatellites.

PCR and SSCP

PCR for single-strand conformational polymorphism (SSCP) analy-
sis was carried out in a total volume of 25 \(\mu\)L of solution using the same concentrations as above. Primers and primer conditions were as detailed previously. SSCP was performed using 3 \(\mu\)L of PCR product in 8 \(\mu\)L of formamide buffer. After denaturing for 4 minutes, the result was loaded onto a 0.8\(\times\) MDE (mutation detection enhancement) gel (National Diagnostics), run overnight, and then visualized using silver stain.

Sequencing

DNAs giving normal and abnormal SSCP patterns were reamplified by PCR in 50 \(\mu\)L of solution. The result was then cleaned using a Qiagen PCR purification column, and eluted with 30 \(\mu\)L of elution buffer (10 mmol/L Tris, pH 8.5); 5 \(\mu\)L of it was then mixed with 4 \(\mu\)L dRhodamine Terminator Cycle Sequencing Ready Reaction (ABI) and 1 \(\mu\)L of primer (10 pmol/L). The mixture was then cycled as per instructions, purified again through a Qiagen column, and analyzed on the ABI 377 automated sequencer.

After reverse-transcription (RT) PCR, the PCR products were run out in low-melting-point agarose, and the bands were cut out and extracted using a freeze-squeeze method. An equal volume of 1\(\times\) Tris/borate/EDTA (TBE) was added to the agarose, which was then melted, frozen on dry ice for 5 minutes, and then spun through a Spin-X column (Costar). After the DNA was precipitated, it was reamplified and sequenced as above.

Restriction Site Determination and Restriction Fragment Length Polymorphism Analysis

Sequences were investigated for restriction endonuclease sites using “map” (GCC program, Human Genome Mapping Project). The G to C and the G to A mutations abolish an AcI site and, therefore, all individuals were genotyped by PCR followed by enzyme digestion with AcI. A total of 5 \(\mu\)L of the PCR products that were used for SSCP were digested overnight at 37°C with 5 U of enzyme in a total volume of 10 \(\mu\)L. This was then run on a 2% agarose gel.

RT-PCR

For family 10738, RNA was isolated from 300 \(\mu\)L of fresh blood using the Purescript RNA Isolation Kit from Gentra Systems Inc.

Blood for RNA should be as fresh as possible, but it can be kept at 4°C for up to 24 hours (A. Murray, BSc, unpublished data, 1999). The RNA obtained was then resuspended in 20 \(\mu\)L of TBE; 5 \(\mu\)L of this was used in the BRL Superscript Preamplication System, and 5 \(\mu\)L of the resulting cDNA was used with the nested primer pairs 1L-18R and 5L-16R (shown below). Touch-down amplification conditions were from 70°C to 60°C as above. For the other families, total RNA was obtained from lymphoblastoid cell lines (RNA Plus, Bioprobe Systems), and cDNA was prepared using the First-Strand cDNA synthesis kit from Pharmacia. Band sizes were estimated on agarose gels.

The primers (5’ to 3’) for RT-PCR were as follows: 1L, GAG ATC GTG CGT GTG GTG TTC T; 18R, GTC TCC CCT TCC AGG TCC; 5L, GGG CAT CCG CCT CTT GCC GA; and 16R, TCC AGC CGC CGC TCT TCT.

Results

Clinical Details

In family 10738 (Figure 1), the proband came to our attention because of episodes of fainting between 4 and 6 years of age, often associated with swimming. The initial diagnosis was possible epilepsy, but when his sister, who is 3 years younger, began to have similar problems, the proband was seen as a pediatric outpatient. He was found to have a QTc of 440 ms, which increased to 526 ms after exercise. Re-examination of previous ECGs that had been passed as normal showed similar, but less pronounced, abnormalities. His sister had a resting QTc of 480 ms, which rose to 550 ms after exercise. Both children are now on \(\beta\)-blockers and have had no further symptoms.

Examination of other family members revealed 3 with ECG abnormalities. The mother of the proband had a QTc of 470 ms, the maternal uncle of the proband had a QTc of 446 ms and was presumed by the cardiologists to be affected, and the grandmother had a QTc of 500 ms. No family members other than the proband and his sister had any clinical symptoms.

For family 10683 (Figure 1), the proband was an 18-year-old boy who died suddenly while swimming. The father had a QTc of 450 ms but was asymptomatic. The sister of the proband experienced several syncopes with seizures triggered by stress during childhood, which ceased spontaneously during adolescence. At the death of her brother, she was found to have a QTc of 522 ms. Her 3 children had prolonged QTcs of 470, 472, and 451 ms. Only the first child had symptoms (stress-induced syncope at 3 years of age), but all are on atenolol because of the family history.

In family 2915 (Figure 1), no history of sudden death existed, and both parents had normal QTcs. The proband experienced a syncopal event at 6 years of age, and resuscitation was necessary; an ECG showed a QTc of 452 ms. The child was given nadolol and has remained asymptomatic.

Family 9350 (Figure 1) also has no history of sudden death, and both parents had normal QTcs. The proband had a syncope while about to dive during swimming lessons and had other events until the age of 12 years induced by stress. At that time, he was diagnosed with a QTc of 475 ms. His father had a QTc of 462 ms but is asymptomatic.

Molecular Genetics

Microsatellite markers were analyzed in family 10738, who originated from England. The haplotype of the LQTI markers
cosegregating with the disease is shown in Figure 1.24 The two-point lod scores for D11S1318 and D11S1323 were 0.83 and 1.3, with both maximum at $\theta = 0$. Analysis comparing the likelihood of the locus lying between these markers as opposed to outside them gave a lod score of 2.09. The other known loci for LQTS, HERG,25 KCNE1,26 SCN5A,23 and LQT4,16 all gave negative lod scores in this family with the closest known microsatellites (data not shown). The linkage data clearly suggested that the mutated gene in this family was KCNQ1, and mutation analysis on all exons was carried out using SSCP. The only band shift was in exon 6 (Figure 2), and all individuals classed as affected showed this mobility change. Sequencing of this exon showed a G $\rightarrow$ C change at position 1032 (Figure 2). The amino acid coded at this position is alanine, and this silent nucleotide change was in the third base of the codon. The mutation abolishes an Aci site, and digestion with this enzyme confirmed that all individuals with the SSCP band-shift carried the mutation (data not shown).

Abnormal SSCP patterns of exon 6 PCR products were also observed in the probands of 3 French families (9350, 10683, and 2915). By direct sequencing, a G to C transition at position 922 minus 1 in the splice-acceptor site of intron 5 was identified in the proband of family 9350 (Figure 3). This novel mutation was inherited from his father. In families 10683 and 2915, a G to A transition was found at position 1032 (Figure 2). The amino acid coded at this position is alanine, and this silent nucleotide change was in the third base of the codon. The mutation abolishes an Aci site, and digestion with this enzyme confirmed that all individuals with the SSCP band-shift carried the mutation (data not shown).

In families 10683 and 2915, a G to A transition was found at position 1032. This transition of the last base of codon 344 has already been described in 5 families.27,28 As in family 10738 above, it should not affect the encoded alanine; however, because it is the last base of the exon, it has been suggested to cause abnormal splicing, although this has not been demonstrated.27,28

To test the hypothesis that these base changes at position 1032 did indeed affect splicing, we took fresh blood samples from the proband in family 10738 (III-2) and his mother (II-6) and performed RT-PCR. The PCR was nested (Figure 4) to improve the signal. As shown in Figure 4, a major band was produced from 3 unrelated normal individuals for both first and second rounds of PCR, but in both affected individuals, 2 smaller bands of equal intensity also existed. Band sizes are given in the legend to Figure 4. DNA from all bands produced by RT-PCR was sequenced. In control lymphocytes and normal cardiac tissue, the major band is the normal transcript and the minor band is a transcript lacking exon 7. In affected patients, the smallest band showed an absence of exons 6 and 7, whereas the larger one was missing exon 7 (Figure 4). Examination of the transcripts induced by the 2 other mutations in lymphoblastoid cell lines (1032 G $\rightarrow$ A and 922-1C) from affected patients showed an absence of both exons 6 and 7 only (data not shown).

In family 2915, the mutation 1032 G $\rightarrow$ A was not present in either parent. Paternity was confirmed by the analysis of 5 microsatellites in the 3 family members, suggesting that it was a de novo mutation that occurred in the proband. In contrast, in family 10683, the same mutation was transmitted by the grandfather to 3 of his grandchildren.

**Discussion**

In the present study, we report 3 mutations that have been found in 4 families and that cosegregated with LQTS. Two of them are new, 1032 G $\rightarrow$ C, which changes the last base of
codon 344 (the last codon of exon 6), and 922-1 G→C, which affects the acceptor site of intron 5. In addition, another variation of nucleotide 1032, 1032 G→A, which was previously described in 5 families,27,28 was identified in 2 French families. Both symptomatic and asymptomatic individuals carrying a mutation showed a prolongation of the QT interval, with QTc values >440 ms. Among the 14 mutation carriers, half of them were symptomatic, and one sudden death occurred at the age of 18. Five adults remained asymptomatic, as did 2 children who were treated by β-blockers.

Figure 2. SSCP gel of exon 6 PCR products and sequence for mutation for family 10738. The 5 triple-band patterns are from affected individuals shown in Figure 1. Last base of exon is shown as G in the unaffected individuals (top sequence), and G→C heterozygous change in affected cases is shown as N.

About 15% of all point mutations are believed to result in abnormal splicing of pre-mRNA.29 The 3 mutations described here were thought to be splicing mutations because they involve the characteristic consensus sequences of donor and acceptor sites, which are G/GTRAGY and YNYAG/, respectively.30 The 2 guanines involved are shown in bold in the above sequences. The donor site base changes that we found in 3 families concern the last base of exon 6 (G1032A and G1032C), and a G at this position was reportedly present in 78% of exons.31 Its change is responsible for several disease
In the Cardiff Mutation Database, although not the most frequent splice defects (only 7%), there are 106 base changes in the last base of an exon that produce splicing changes, and all but 2 of these involve a G.35 Of the 104 such substitutions, 64 are G→A, 21 are G→C, and 17 are G→T (P. Stenson, PhD, personal communication, 1998). In the fourth family, 1 of the 2 invariant bases of the acceptor site (AG) at the intron/exon junction is affected (922-1 G→C), and such mutations account for ≈88% of point mutations in these regions.29

Evaluation of the mutations described in this report using the method of Shapiro and Senepathy30 gave scores of 68.6%, 67.5%, and 61% for 1032 G→A, 1032 G→C, and 922-1 G→C, respectively. Complete abolition of normal splicing is the most common consequence of a mutation of a G of the consensus splice donor or acceptor sites.30 To determine whether this occurs with these mutations and what the aberrant pattern of splicing might be, we examined lymphocyte RNA using RT-PCR. Transcripts were produced from all 3 mutations lacking exons 6 and 7. In addition, a transcript lacking exon 7 was found in normal cardiac tissue at a low level, and in lymphocytes from a patient with the 1032 G→C base change. Such a transcript was not determined for the 1032 G→A and 922-1 G→C mutations, but this may be due to the fact that the cDNA from the latter was from lymphoblastoid cell lines. For these 2 abnormal transcripts, the reading frame remained intact, resulting in the deletion of part of the pore or S6 domain, which are the major functional regions of all potassium channels.36 In addition, this region in KCNQ1 is involved in the interaction with minK.37 Therefore, these various mutants could not form functional potassium channels. Of course, the changes seen in lymphocytes or lymphoblastoid cell lines may not reflect exactly what occurs in cardiac tissues but, as discussed above, similar mutations are implicated in numerous studies.31–34 A small proportion of transcripts lacking exon 7 was observed in lymphocytes and cardiac tissue from controls. A minor in-frame deleted transcript has also been reported in normal brain cDNA for KCNQ4.38 Interestingly, the 3 mutations in KCNQ1 induce the loss of exons 6 and 7 on the mutated allele (with no equivalent transcript in normal cDNA) and, in addition, at least for 1032 G→C, induce a higher proportion of normal alleles lacking exon 7.

The most frequent mutations described to date in KCNQ1 are those occurring at codon 341. These are a transition (1022 C-T), A341V, and a transversion (1022 C-A), A341E, numbered according to the isoform-1 sequence.39 In 65 families with a KCNQ1 mutation, alanine at position 341 was mutated 16 times,7,21,25,40–42 The mutation A341V is consistent with the accidental deamination of 5-methylcytosine in a CpG dinucleotide. In higher vertebrates, the majority of the cytosines are 5-methylated, and spontaneous deamination leads to a transition to thymine that is not excised by the DNA repair system, thus leading to an irreversible point mutation and the replacement of a CG by either TG or CA. Analysis of the factor IX gene has shown such transitions to be elevated 26-fold.43 Mutation 1032 G→A in codon 344 is also consistent with the accidental deamination of 5-methylcytosine in a CpG dinucleotide. In higher vertebrates, the majority of the cytosines are 5-methylated, and spontaneous deamination leads to a transition to thymine that is not excised by the DNA repair system, thus leading to an irreversible point mutation and the replacement of a CG by either TG or CA. Analysis of the factor IX gene has shown such transitions to be elevated 26-fold.43 Mutation 1032 G→A in codon 344 is also consistent with deamination of 5-methyl cytosine on the noncoding strand of the gene. Two other mutations of the same nucleotide, 1032 G→C (reported here) and 1032 G→T (Le Marec, MD, PhD, personal communication, 1999) are also silent transversions inducing abnormal splicing. In human factor IX mutations, transversions at CpG dinucleotides are elevated by ≈7-fold relative to other transversions, reinforcing the fact that methylated CpG dinucleotides are mutation hotspots.43
Figure 4. RT-PCR of RNA from lymphocytes or cardiac tissue and sequence data for 2 aberrantly spliced transcripts. Lanes 1 and 12 in agarose gel are molecular weight markers. Lanes 2 to 6 are first-round PCR products, and lanes 7 to 11 are nested PCR products. Lanes 2 and 7 are from cardiac cDNA; 3, 4, 8, and 9 are from control blood samples; and 5, 6, 10, and 11 are from blood of 2 affected individuals of family 10738 (II6 and III2; Figure 1). Band sizes (in bp) determined by sequencing are as follows: for first round PCR, 1007, 911, and 800; for nested PCR, 678, 582, and 471. The sequences show that exon 7 is skipped in the larger mutant transcript, and exons 6 and 7 in the smaller.
The CpG dinucleotide of codon 344 has the characteristics of a mutation hotspot: independent occurrence of the typical transition, 2 transversions at the same locus, and a high frequency of these mutations (10 of 66 families with known KCNQ1 mutations, including 1 case that was erroneously reported as a polymorphism\(^\text{44}\)). The discovery of a de novo 1032 G→A mutation in 1 of the French families reported here serves to underline the mutability of this codon. Many previous studies on mutations in KCNQ1 did not cover the entire gene, because full primer sets were not available. It is, therefore, probable that the proportion of mutations accounted for by changes at position 1032 will be less than at present when the C-terminal region is better explored. Nonetheless, these results indicate that codon 344 is the second most frequently mutated after codon 341, suggesting at least 2 hotspots for mutations in KCNQ1.

Acknowledgments

A. Murray was funded by the Cone Power Foundation, and P. Syrris by the British Heart Foundation. The work was supported by the Institut National de la Santé et de la Recherche Médicale (PROGRES No 4P009D), the Association Française contre les Myopathies, and a BIOMED grant (BMH4-CT96-0028). We would like to thank Dr. Igor Splawski for helpful advice and discussion.

References

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Circulation. 1999;100:1077-1084
doi: 10.1161/01.CIR.100.10.1077

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