Identification and functional characterization of KCNQ1 mutations around the exon 7–intron 7 junction affecting the splicing process

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ARTICLE INFO

ABSTRACT

Background. KCNQ1 gene encodes the delayed rectifier K^+ channel in cardiac muscle, and its mutations cause long QT syndrome type 1 (LQT1). Especially exercise-related cardiac events predominate in LQT1. We previously reported that a KCNQ1 splicing mutation displays LQT1 phenotypes. *Methods and results.* We identified novel mutation at the third base of intron 7 (IVS7 +3A>G) in exercise-induced LQT1 patients. Minigene assay in COS7 cells and RT-PCR analysis of patients' lymphocytes demonstrated the presence of exon 7-deficient mRNA in IVS7 +3A>G, as well as c.1032G>A, but not in c.1022C>T. Real-time RT-PCR demonstrated that both IVS7 +3A>G and c.1032G>A carrier expressed significant amounts of exon-skipping mRNAs (18.8% and 44.8% of total KCNQ1 mRNA). Current recordings from *Xenopus* oocytes injected cRNA by simulating its ratios of exon skipping displayed a significant reduction in currents to $64.8 \pm 4.5\%$ for IVS7 + 3A>G and to $41.4 \pm 9.5\%$ for c.1032G>A carrier, respectively, compared to the condition without splicing error. Computer simulation incorporating these quantitative results revealed the pronounced QT prolongation under beta-adrenergic stimulation in IVS7 +3A>G carrier model. *Conclusion.* Here we report a novel splicing mutation IVS7 +3A>G, identified in a family with mild form LQT1 phenotypes, and examined functional outcome in comparison with three other variants around the exon 7–intron 7 junction. In addition to c.1032G>A mutation, IVS7 +3A>G generates exon-skipping mRNAs, and thereby causing LQT1 phenotype. The severity of clinical phenotypes appeared to differ between the two splicing-related mutations and to result from the amount of resultant mRNAs and their functional consequences.

1. Introduction

Long QT syndrome (LQTS) is characterized by prolongation of the cardiac action potential, syncopal attacks, torsades de pointes arrhythmias and sudden cardiac death [1–3]. The slow component of delayed rectifier K^+ current (I_{Ks}) in the heart modulates repolarization of cardiac action potential. The I_{Ks} channel is formed by the co-assembly of KCNQ1 α-subunits and KCNE1 β-subunits [4,5]. Mutations in the KCNQ1 cause the most frequent form of inherited LQT1 [6]. Exercise-related cardiac events dominate the clinical picture of LQT1 patients.

Pre-mRNA processing is an important aspect of gene expression and consists of the precise recognition of exons and removal of introns in such a way that the exons are joined to form mature mRNAs with intact translational reading frames [7,8]. Disruption of normal splicing as a result of genetic mutation can lead to the generation of abnormal proteins or the degradation of aberrant transcripts through nonsense-mediated decay, and thus to the pathogenesis of a variety of human diseases [9].

We previously reported three LQTS families, in whom a G to A change in the last base of KCNQ1 exon 7 (c.1032G>A) was identified [10]. The mutation alters the 5′ splice-site of intron 7, resulting in the production of exon-skipping transcripts, but not to alter the coded alanine (A344A) [11,12], since it involves the characteristic consensus sequence of the splicing donor site, A**G**/GUAAGU. The vicinity of junction around the KCNQ1 exon 7–intron 7 appeared to be a hot area for genetic variants that may potentially cause aberrant splicing, and we identified a novel mutation that changes an A to G at the third base of intron 7 (IVS7 +3A>G) in LQTS family with mild clinical phenotypes. In contrast, another neighboring KCNQ1 mutation, c.1022C>T (p. A341V) is known to produce severe clinical phenotypes [13].

To test the potential influence of these mutations that may affect the KCNQ1 splicing, we established a minigene assay system in which a respective mutant construct is transcribed in COS7 cells and examined the genetic and biophysical characterization of the novel IVS7 +3A>G

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mutation. For comparison, we also investigated two other mutations around the exon 7–intron 7 junction; c.1022C>T and c.1032G>A. We quantitatively analyzed the aberrant splicing and its functional consequences and then carried out a computer simulation to explore how this mutation could be associated with exercise-induced QT prolongation and tachyarrhythmias.

2. Materials and methods

2.1. Genomic DNA isolation and mutation analysis

Mutation analysis was carried out as previously described [10]. Genomic DNA was prepared from peripheral blood leukocytes. Sixteen exons of the KCNQ1 gene were amplified by PCR. Genetic screening was performed for KCNQ1 by denaturing high-performance liquid chromatography (DHPLC) using a WAVE System Model 3500 (Transgenomic: Omaha, NE). We optimized the running optimum temperature at 64.6 °C. Abnormal conformers were amplified by PCR and sequencing was performed on an ABI PRISM3130 DNA sequencer (Applied Biosystems: Foster City, CA). We also carried out a complete screening for other LQTS-causing genes; KCNH2, SCN5A, KCNE1, KCNE2, and KCNJ2.

2.2. Construction of splicing minigene and transfection

Exon 7 of the KCNQ1 gene (111 bps) and its flanking introns (507 bps at 5′ arm and 453 bps at 3′ arm) were amplified by PCR using genomic DNA from control and patients. PCR fragments were cloned into the pSPL3 exon trapping vector (Gibco BRL) digested with *EcoRV* within the multiple cloning site. The pSPL3 vector contains the HIV-1 tat exons and the intervening intron with *EcoRV* site. COS7, CHO and HL-1cells were transfected with 0.25 μg plasmid DNA using Lipofectamin transfection reagent (Invitrogen). Cells were harvested 48 h post-transfection.

2.3. RNA extraction and RT-PCR

Total cellular RNA was isolated with QIAamp RNA Blood Mini Kits (Qiagen). Subsequently, total RNA was reverse-transcribed by use of the SuperScriptIII FirstStrand Synthesis System (Invitrogen: Carlsbad, CA), and was used as a template for subsequent PCR reactions. We used the forward primer (5′-TCTGAGTCACCTGGACAACC-3′) and the reverse primer (5′-ATCTCAGTGGTATTTGTGAGC-3′), both of which anneal to the pSPL3 vector sequence.

Total RNA was extracted from leukocytes of fresh blood and was reverse-transcribed using the same methods described above. Using the cDNAs as templates, PCR amplification was performed with the exon 5-F forward primer (5′-GGGCATCCGCTTCCTGCAGA-3′) and the exon10-R reverse primer (5′-CCATTGTCTTTGTCCAGCTTGAAC-3′) to amplify KCNQ1 cDNA from exons 5 through 10.

Measurements of normal and mutant mRNA levels were performed by real-time RT-PCR by use of an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). The reaction mixture contained SYBR Green PCR Master Mix (Applied Biosystems), cDNA template, and PCR primers. In order to selectively amplify these splicing variants, PCR primers were designed so that they spanned the adjacent exons: exon 6.8-F: 5′-CTGTGGTGGGGGGTG-GGGATT-3′, exon 6.9-F: 5′-TGTGGTGGGGGGGTG-ACCGCAT-3′, and exon 7.9-F: 5′-CTTTGCGCTCCCAGCG-ACCG-3′ (all the hyphens inside the primer sequence indicate the boundaries of exons). In all cases, the dissociation curves showed that there was no significant contribution of relatively short by-products to the measured fluorescence intensities.

All the samples were tested in duplicate. A standard curve for each primer pair was obtained using serial dilutions of a recombinant plasmid containing cDNA. The threshold cycle (Ct) was subsequently determined. Relative mRNA levels of splice mutants were calculated based on the Ct values and normalized by the GAPDH level of each sample. The amounts of mutant cDNA were expressed as a percentage of the total KCNQ1 mRNA, for which exons 9 through 10 were amplified with the exon 9-F forward primer (5′-CGCATGGAGGTGC-TATGCT-3′) and the exon 10-R reverse primer.

2.4. Oocyte isolation and electrophysiology

Xenopus laevis oocytes were prepared and current recordings were carried out as described previously [14]. Wild-type (WT) cRNA plus mutant-cRNA (total 10 ng) was injected into *Xenopus* oocytes. All the current recordings in the present study were performed in the presence of KCNE1 β-subunits (1 ng). An axoclamp-2B amplifier (Axon Instruments: Union City, CA) was used to record currents at 25 °C in oocytes 3–4 days after cRNA injection, using standard two-electrode voltage-clamp techniques. To decrease the interference from endogenous Cl^- current, we used a low-Cl^- bath solution (mM): NaOH 96, KOH 2, $CaCl_2$ 2, $MgCl_2$ 1, MeS 101, HEPES 5 (pH titrated to 7.6 with methanesulfonic acid). Currents were sampled at 10 kHz and filtered at 2 kHz. Voltage steps were applied with 3-second pulses in 10 mV increments from a holding potential of −80 mV to voltages from −70 to +30 mV, and then to −30 mV. Current amplitudes were measured at 1.8-second after the initiation of 3-second pulse applied to a +30 mV test potential, followed by the subtraction of background I_{Ks} current (22.9 nA).

2.5. Computer simulation

We conducted simulations of paced propagation in a one-dimensional (1D) bidomain myocardial model of 9.0-mm length with transverse conductivity, mimicking transmural section of left ventricular free wall. Membrane kinetics was represented by the Priebe–Beuckelmann model [15], which can simulate human ventricular action potentials.

To obtain the ventricular transmural gradient, we defined endocardial, mid-myocardial, and epicardial tissues of lengths (thicknesses) 0.6 mm, 6.0 mm, and 2.4 mm, respectively, and then we incorporated modifications of ion channel conductance (Table 1), based on the previous studies [16,17]. Pacing stimuli of 3-ms duration and strength twice-diastolic threshold were applied transmembranously to the endocardial end at a cycle length of 1000 ms. To get ECG similar to the left precordial ECG, a unipolar recording electrode was located 3 cm above the epicardial end of the tissue. Other model parameters, such as the tissue conductivities and the boundary conditions, can be found elsewhere [18,19].

To achieve the beta-adrenergic stimulation, we set the parameters as previously described [20–22]: (1) shifting the fast and slow inactivation curves of the sodium current (I_{Na}) −3.4 mV, (2) increasing the L-type calcium current (I_{CaL}) 3 times and slowing the time constant of inactivation 1.13 times, (3) increasing the half-point concentration for the calcium-dependent inactivation (f_{Ca}) from 0.7 to 0.9 μM, and setting its non-zero minimum value to 0.03, (4) increasing the slowly

Table 1
Model modification values for ventricular transmural gradient.

	Endo	M	Epi
G_{Ks}	208%	52%	280%
G_{K1}	82%	83%	100%
G_{NaCa}	72%	108%	100%
G_{to}	25%	87%	100%
G_j	100%	100%	76%

G_{Ks}, conductance of slowly activating component of delayed rectifier potassium channel; G_{K1}, conductance of inward rectifier potassium channel; G_{NaCa}, conductance of sodium–calcium exchanger; G_{to}, conductance of transient outward potassium channel; G_j; gap junctional conductance. All values are expressed in percentage compared to original values [15]. Endo; endocardial cell, M; midcardial cell, Epi; epicardial cell.