Biochemical and Biophysical Research Communications 380 (2009) 127–131

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Congenital long QT syndrome caused by the F275S *KCNQ1* mutation: Mechanism of impaired channel function

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ARTICLE INFO

Article history:
Received 13 December 2008
Available online 22 January 2009

Keywords:
Long QT syndrome
KCNQ1
ER retention
Trafficking

ABSTRACT

Congenital long QT syndrome is characterized by a prolongation of ventricular repolarization and recurrent episodes of life-threatening ventricular tachyarrhythmias, often leading to sudden death. We previously identified a missense mutation F275S located within the S5 transmembrane domain of the KCNQ1 ion channel in a Chinese family with long QT syndrome. We used oocyte expression of the KCNQ1 polypeptide to study the effects of the F275S mutation on channel properties. Expression of the F275 mutant, or co-expression with the wild-type S275 polypeptide, significantly decreased channel current amplitudes. Moreover, the F275S substitution decreased the rates of channel activation and deactivation. In transfected HEK293 cells fluorescence microscopy revealed that the F275S mutation perturbed the subcelluar localization of the ion channel. These results indicate that the F275S KCNQ1 mutation leads to impaired polypeptide trafficking that in turn leads to reduction of channel ion currents and altered gating kinetics.

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Long QT syndrome (LQTS) is a congenital cardiac disease characterized by prolongation of ventricular repolarization and recurrent episodes of life-threatening ventricular tachyarrhythmias, sepecifically torsades de pointes, often leading to sudden death [1]. So far, 11 genes have been identified for LQTS: *KCNQ1* (or *KvLQT1*, LQT1), *KCNH2* (or *HERG*, LQT2), *SCN5A* (LQT3), *Ankyrin-B* (LQT4), *KCNE1* (LQT5), *KCNE2* (LQT6), *KCNJ2* (LQT7), *CACNA1C* (LQT8), *CAV3* (LQT9), *SCN4B* (LQT10), and *AKAP9* (LQT11) [2–8]. Carriers with mutations in *KCNJ2* and *CACNA1C* exhibit not only the LQTS phenotype but other additional phenotypes (respectively designated Andersen syndrome and Timothy syndrome).

The LQT1 variant is the most common subtype of LQTS; *KCNQ1* mutations have routinely been found in this condition [9]. Approximately half of genotyped LQT1 patients were found to carry a mutation in this gene. The *KCNQ1* and *KCNE1* genes, respectively, encode the α (KvLQT1) and β (minK) subunits of the potassium channel that conducts the slowly-activating delayed rectifier current (I_{Ks}) responsible for repolarization of the cardiac action potential [10,11]. Most mutations associated with LQT1 are located within the transmembrane region of the KvLQT1 polypeptide [12]. The *KCNQ1* mutations impair I_{Ks} through reduced channel function, altered channel gating, and/or a dominant-negative effects: the mutations interfere with the function of the normal wild-type form through a 'poison pill' type mechanism [13–15]. To date only a few *KCNQ1* mutations have been associated with impaired trafficking of the encoded polypeptide. This contrasts with *KCNH2* where a substantial proportion of mutations have been linked to defects in polypeptide trafficking [16–20].

We previously reported a *KCNQ1* missense mutation, F275S, in a Chinese family with congenital LQT1 syndrome [21]. The mutation comprises a single amino-acid substitution where serine (Ser, S) is replaced by phenylalanine (Phe, F) at position 275 (F275S) within the S5 transmembrane domain of the polypeptide. We have now studied the functional properties of the mutation in heterologous expression systems. We report that the mutation reduces the channel I_{Ks}, alters channel gating, and impairs protein trafficking.

Materials and methods

Mutagenesis and transcript RNA preparation. The *KCNQ1* and *KCNE1* cDNAs in vector pSP64 were generously provided by Dr. Qing Wang (Center for Cardiovascular Genetics, Department of Molecular Cardiology, Lerner Research Institute, Cleveland Clinic, Cleveland, Ohio, USA). The F275S mutation was generated by overlap extension PCR using *KCNQ1* cDNA as template using the following primers:

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doi:10.1016/j.bbrc.2009.01.051

KCNQ1 forward: 5′-GTAGCAGTCGCTGCGCCGGTGAGCCTAGAC-3′.
KCNQ1 reverse: 5′-TACAGCCTTCCGGATGTAGATCTTCCAGGTG-3′.
Mutation forward: 5′-CCTGGGCCTCATCTCCTCCTCGTACTTTGTG-3′.
Mutation reverse: 5′-CACAAAGTACGAGGAGGAGATGAGGCCCA GG-3′.

Wild-type (WT) KCNQ1 and F275S KCNQ1 cDNAs were linearized by digestion with EcoRI, and transcript RNAs were prepared using the SP6/T7 Transcription Kit (Roche, Switzerland) and SP6 RNA polymerase. RNAs were dissolved in DEPC-H_2O, and RNA size and integrity were evaluated by formaldehyde–agarose gel electrophoresis. RNA concentrations were determined using the Ribogreen RNA Quantitation Kit (Molecular Probes, Eugene, OR, USA) and diluted to the final desired concentration in sterile H_2O before use.

Oocyte preparation and injection. All animal handling procedures were approved by the Animal Research Ethics Committee of the Cardiovascular Institute of the Chinese Academy of Medical Sciences and conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH publication No. 85-23, revised 1996). Female *Xenopus laevis* frogs were anesthetized on ice for 30 min at room temperature. Segments of the ovarian lobes were isolated and follicular layers were removed by digestion with 2 mg/ml collagenase (Sigma, St. Louis, MO, USA) in Ca^{2+}-free ND96 solution for 1 h. ND96 medium is specified below. Stage IV or V oocytes were injected with 50 nl of mixture containing KCNQ1 and KCNE1 transcript RNAs in an equimolar ratio and maintained at 18 °C in ND96 solution supplemented with 100 U/ml penicillin. ND96 medium is 96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 2.5 mM Na pyruvate, 1.8 mM $CaCl_2$, 5 mM HEPES, pH 7.5. RNA concentrations were 0.04 μg/μl (2.0 ng/oocyte) for wild type and mutant KCNQ1 and 0.02 μg/μl (1.0 ng/oocyte) for KCNE1. Wild-type and F275S co-expression experiments employed a constant total quantity of KCNQ1 (mutant and/or wild type).

Electrophysiology and data analysis. Recordings were made 3–5 days after RNA injection at room temperature (about 22–25 °C). Currents were recorded using the 2-electrode voltage-clamp technique using a commercially available amplifier TURBO TEC-03X (NPI Electronic GmbH, Tamm, Germany). Pipettes filled with 3 M KCl had resistances of 0.3–0.8 MΩ in ND-96 buffer. pClamp8 software (Axon Instruments) was used to generate voltage-clamp commands and collect experimental data. The membrane potential was held at −70 mV between test pulses. Currents were recorded during 6 s pulses from a holding potential of −70 mV to test potentials ranging from −70 to +60 mV (10 mV intervals). Deactivating tail currents were elicited by repolarization to −50 mV. A 5 s inter-pulse interval was employed. To determine the voltage-dependence of channel activation, oocytes were depolarized to potentials ranging from −70 to +60 mV (10 mV intervals) for 5 s before repolarizing to −50 mV. The voltage-dependence of the channel deactivation time constant was determined by repolarizing the oocytes to potentials ranging from −20 to −90 mV (10 mV intervals) for 5 s after a 5 s pulse to +60 mV.

Data analysis was carried out using Origin 7.5 software (Microcal Software, Inc. USA) and Clampfit 9.0 software (Axon Instruments, Inc. USA). Voltage-dependence of channel activation was determined by fitting the normalized amplitude of the peak tail currents (G/Gmax) versus test voltage potential with a Boltzmann function (G/Gmax = $1/[(1 + \exp(V - V_{1/2})/\kappa]$; where $V_{1/2}$ is the voltage at which G is half of Gmax, V is the test potential pre-pulse, and κ is the slope factor. Time constants were determined by fitting currents to a single exponential decay function. All Data were presented as means ± SEM; statistical comparisons used the 2-tailed Student t-test.

Cell transfection and confocal microscopy. Plasmid pcDNA3.1-F275S KCNQ1-GFP was constructed by cloning the human F275S KCNQ1 ORF into the pcDNA3.1-CT-GFP-topo vector (Invitrogen), thereby creating a green fluorescent protein (GFP) fusion with the C-terminus of KCNQ1. A similar strategy was used for wild type KCNQ1. pDsRed2-ER, containing the signal sequence of calreticulin, was used as a specific marker for the ER. The full-length cDNA of KCNE1 was subcloned into pcDNA3.1/Zeo(+) (Invitrogen). HEK293 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 IU/ml penicillin and 50 IU/ml streptomycin. Cells were transiently transfected using Lipofectamine 2000 (Invitrogen) and were allowed to grow for 24 h before seeding onto glass coverslips. A confocal microscope (Leica) was used to visualize the subcellular distribution patterns of the GFP and DsRed2 fusion proteins. GFP was excited using an argon laser at 488 nm and emission was recorded using a HQ513/30 filter. DsRed2 was excited using a helium/neon laser at 543 nm and images collected using a HQ590/15 filter. All images were acquired sequentially. Green and red fluorescence images were merged using Leica confocal software. In all the figures the scale bar indicates 5 μm.

Results

Electrophysiological effects of the mutant F275S KCNQ1 on the I_{Ks} currents

To evaluate the functional consequences of the KCNQ1 F275S mutation, mutant or wild-type channel α-subunit polypeptides were co-expressed with the wild-type β-subunit (KCNE1) in Xenopus oocytes. Voltage clamp analysis was performed. Fig. 1A shows the original current traces of the expressed constructs. Expression of mutant F275S KCNQ1, or co-expression with wild type KCNQ1, both significantly decreased channel current amplitudes (Fig. 1B). It is notable that the F275S mutation, compared to the WT, induced a significant decrease in channel current amplitudes at all voltages. Current amplitudes of WT KCNQ1 + KCNE1, F275S + KCNE1 and WT KCNQ1 + F275S + KCNE1 at a clamp voltage of +60 mV were 5.86 + 0.48 μA (n = 10), 2.30 + 0.34 μA (n = 6, P < 0.05 versus WT KCNQ1/KCNE1), and 3.91 + 0.43 μA (n = 6, P < 0.05 versus WT KCNQ1/KCNE1), respectively.

We also observed that F275S shifted the voltage-dependent activation curve of the KCNQ1 channel more positively than did the WT KCNQ1 (Fig. 1C). Half-maximal activation voltages ($V_{1/2}$) of WT KCNQ1 + KCNE1, F275S + KCNE1 and WT KCNQ1 + F275S + KCNE1 channels were 20.00 ± 3.83 mV (k = 18.38 ± 2.35, n = 9), 47.57 ± 6.24 mV, (k = 16.58 ± 1.88, n = 6), and 45.68 ± 6.57 mV (k = 20.18 ± 1.79, n = 7) respectively.

Kinetic analysis revealed that the activation rate of F275S was decreased (Fig. 2A). The activation time constants were strongly increased at all applied voltages between +10 and +40 mV, though the difference between mutant and WT was smaller at higher voltages. In addition, F275S was found to decrease the deactivation rate. As shown in Fig. 2B, deactivation time constants were increased for F275S at all voltages between −70 and −20 mV.

ER retention of KCNQ1

Misfolded proteins are recognized by a quality control mechanism that leads to the retention in the endoplasmic reticulum (ER) and subsequent degradation of the protein [22,23]. We therefore used laser scanning confocal microscopy to address whether the F275S mutation might affect ER retention. HEK293 cells were co-transfected with expression constructs for KCNQ1 and KCNE1. As reported previously, WT KCNQ1 co-expressed with KCNE1 was readily detectable at the plasma membrane (Fig. 3). In contrast, the subcellular localization of the F275S polypeptide was seriously perturbed. Little fluorescence was associated with the

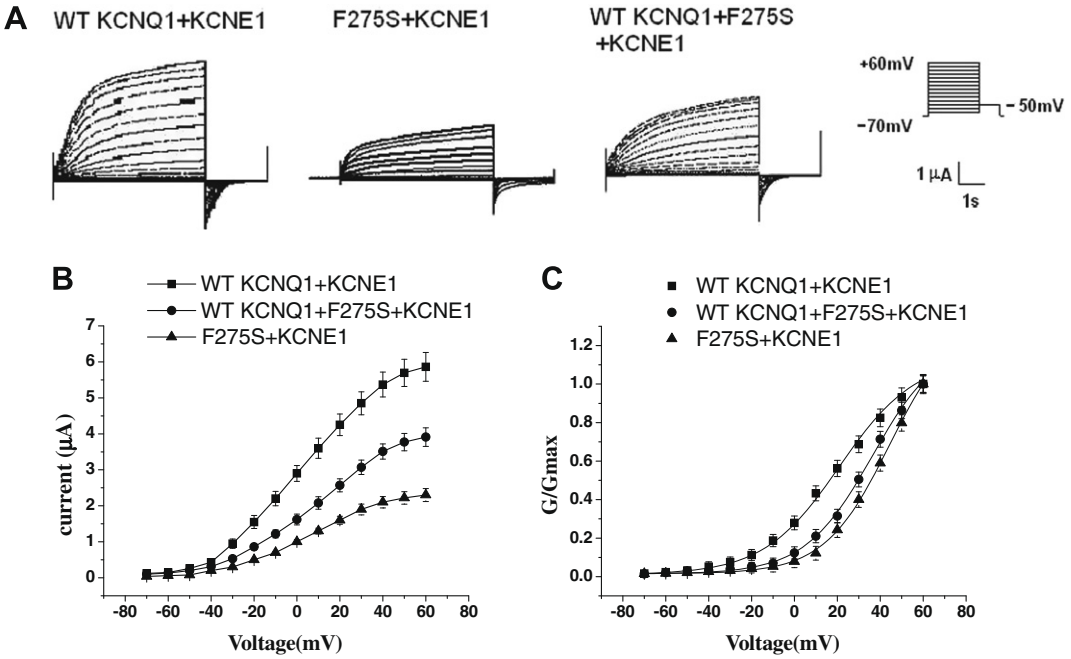

Fig. 1. Voltage-dependence of mutant KCNQ1 + KCNE1 currents. Panel (A) Current traces for WT KCNQ1 and/or F275S co-expressed with KCNE1 in Xenopus oocytes. Currents were recorded after a 6 s depolarizing pulse to potentials ranging from −70 to +60 mV at 10 mV increments from a holding potential of −50 mV. The voltage protocol is shown in the inset. Panel (B) Current–voltage relationships for WT KCNQ1 or F275S channels co-expressed with KCNE1 using the same voltage protocol as in A. The steady-state current amplitudes of the expressed constructs measured after depolarizing test pulses to +60 mV were: KCNQ1 + KCNE1, amp = 5.86 ± 0.48 μA (n = 10); F275S + KCNE1, amp = 2.30 ± 0.34 μA (n = 6); KCNQ1 + F275S + KCNE1, amp = 3.91 ± 0.43 μA (n = 6). Data points are means ± SEM. Panel (C) Relative activation curves of WT and F275S KCNQ1 with KCNE1 determined at the peak of the tail current (same voltage protocol as in A). Experimental data were normalized and fitted to a Boltzmann equation, giving the following $V_{1/2}$ values (in mV) and slope factors k: for KCNQ1 + KCNE1 (■), $V_{1/2}$ = 20.00 ± 3.83 mV, k = 18.38 ± 2.35, n = 9; for F275S + KCNE1 (▲), $V_{1/2}$ = 47.57 ± 6.24 mV, k = 16.58 ± 1.88, n = 6; for KCNQ1 + F275S + KCNE1 (●), $V_{1/2}$ = 45.68 ± 6.57 mV, k = 20.18 ± 1.79, n = 7. Data points are means ± SEM.

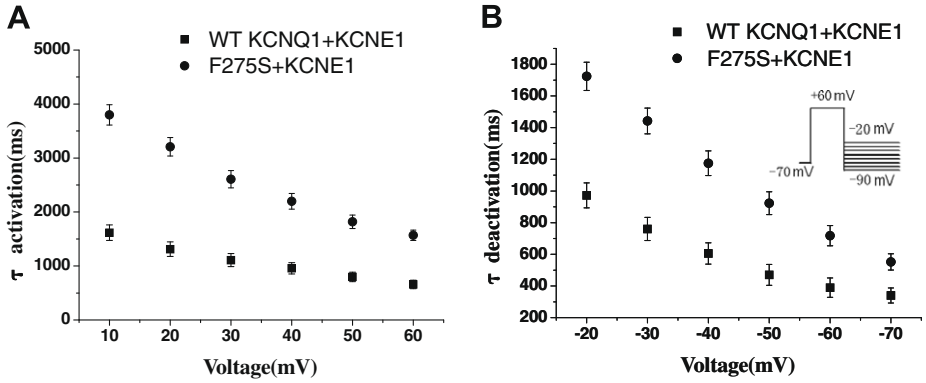

Fig. 2. Kinetic properties of WT KCNQ1 and/or F275S co-expressed with KCNE1 in Xenopus oocytes. Panel (A) Time constant of activation versus membrane potential for WT KCNQ1 and/or F275S co-expressed with KCNE1. Currents recorded after a 6.0 s depolarizing pulse to potentials of −70 to +60 mV at 10 mV increments from a holding potential of −50 mV and were fitted to a single exponential function. Slower activation rate in the presence of F275S was observed at all potentials (10 to 60 mV). Data points are means ± SEM, n = 6–8 cells in each case. Panel (B) Time constant of deactivation versus membrane potential for WT KCNQ1 and/or F275S co-expressed with KCNE1 using the voltage protocol shown in the inset. Decay of tail current was fittted to a single exponential function. F275S increased the deactivation time constants at all voltages between −20 and −70 mV. Data points are means ± SEM, n = 6–7 cells in each case.

plasma membrane and instead intense fluorescence was observed in the cytoplasm. To investigate the precise localization within the cytoplasm, we used a DsRed2-ER marker protein engineered to be retained in the ER. Picture overlay revealed clear co-localization of the mutant channel with the ER marker. These results strongly indicate that the F275S mutation leads to ER retention of the mutant channel subunit. When F275S was co-expressed with WT KCNQ1, however, the extent of ER retention was reduced.

Discussion

We previously identified the *KCNQ1* F275S mutation that co-segregates in a patient diagnosed with LQT1-syndrome and who died from ventricular tachycardia and ventricular fibrillation. Her grandmother also died from arrhythmia apparently triggered by emotional stress. The phenylalanine residue at position 275 lies within the channel S5 transmembrane domain and is highly conserved among different species. Aizawa et al. reported a 14-year-old girl carrying a deletion of the same amino acid (ΔF275) who suffered from syncopal attacks and QT interval prolongation; here there was family history of sudden cardiac death [24]. Other mutations (G269S, G269D) also associated with the S5 transmembrane domain showed severe clinical phenotypes including cardiac arrest and sudden death with prolongation of the QT interval [25,26].

In this study, we used molecular, electrophysiological and imaging techniques to determine the functional characteristics of

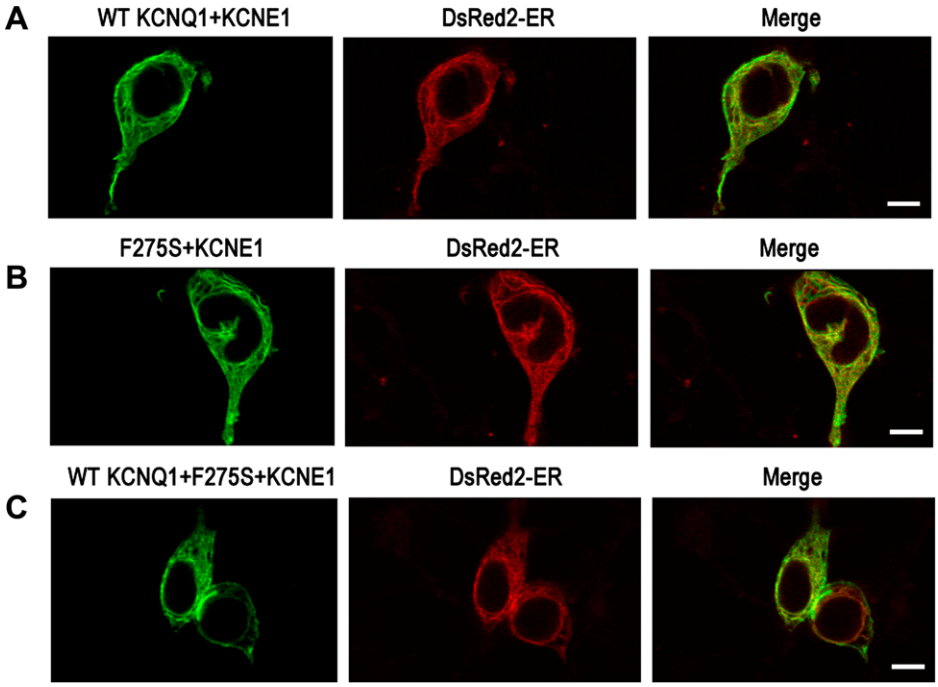

Fig. 3. Subcellular localization of the WT and F275S KCNQ1 polypeptides. WT and mutant F275S KCNQ1 polypeptides marked with a C-terminal GFP tag (green fluorescence) were co-expressed in intact HEK293 cells with KCNE1. pDsRed2-ER used as ER marker (red fluorescence) was co-transfected with WT and mutant F275S KCNQ1. Left panels show fluorescence emitted in the green channel only, while middle panels show fluorescence emitted in the red channel only. In the right panels the red and green channels have been merged. (A) WT KCNQ1 co-expressed with KCNE1 was largely present at the plasma membrane. (B) The mutation F275S leads to ER retention. (C) F275S co-expressed with WT KCNQ1 leads to milder ER retention. The scale bar is 5 μm. (For interpretation of color mentioned in this figure the reader is referred to the web version of the article.)

the F275S missense mutant protein and to examine interactions with the corresponding WT protein. On heterologous expression in microinjected oocytes we found that the F275S mutation significantly decreased *KCNQ1* ion channel current amplitudes. This was also found when the mutant was co-expressed with the WT polypeptide. Moreover, F275S decreased the rates of both channel activation and deactivation. Reduction in the I_{Ks} and altered gating kinetics may lead to a prolongation of cardiac action potentials and QT interval prolongation, consistent with LQTS in members of the Chinese family in which the mutation was described. This observation is reinforced by findings with the ΔF275 *KCNQ1* mutation and other substitutions within the S5 transmembrane domain. Co-transfection of ΔF275 with *KCNE1* produced little to no current; expression of the mutant polypeptide was associated with a potent dominant-negative effect leading to an almost complete loss of function with abolition of I_{Ks}, indicating that this defect underlies LQTS [23]. Other mutations residing within S5 transmembrane domain also reduced I_{Ks} currents either via haploinsufficiency (G269S) or through dominant-negative effects (G269D, L273F, Y281C) [13,25–27].

Several different mutations in *KCNH2* have been shown to cause trafficking defects, these are mostly missense mutations dispersed over the C-terminus of the polypeptide, although some missense mutations were located within the N-terminal, S5, or pore regions [28]. Channel trafficking in *KCNQ1* mutants has not been studied extensively because trafficking defects associated with this channel appear to be rare. Nevertheless, several mutations in *KCNQ1* have been shown to cause trafficking defects that can lead either to haploinsufficiency (ΔS276, T587M and M520R) or to dominant-negative effects (A178fs/105) [29–31]. These variations are often missense mutations at the N-terminal, S5, pore, or C-terminal regions of the polypeptide. Recent studies have indicated that particular regions of *KCNQ1* (specifically the N-terminal juxta-membranous domain and amino acids 610–620 at the C-terminus) are critical for cell-surface expression of the channel [32–35]. However, mutations outside these domains (R243H, E261K) can impair trafficking to varying degrees [16]. In this study, the F275S mutation impaired trafficking to the cell surface. In contrast to F275S, normal cell surface trafficking we found with ΔF275.

In summary, we have examined the functional consequences of the F275S substitution within the *KCNQ1* polypeptide. Analysis revealed that the mutation leads to defective trafficking with retention in the ER, and significantly reduced channel I_{Ks} currents. These impairments are consistent with LQTS in the members of the Chinese family in which the mutation was described.

Acknowledgments

This study was supported by grants from the National Natural Science Foundation of China (No. 30700298) and Doctoral Program Foundation of Institutions of Higher Education of China (No. 20070487148).

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