Novel characteristics of a trafficking-defective G572R-hERG channel linked to hereditary long QT syndrome

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J Lian, N Huang, J Zhou, et al. Novel characteristics of a trafficking-defective G572R-hERG channel linked to hereditary long QT syndrome. *Can J Cardiol* 2010;26(8):417-422.

BACKGROUND: The congenital long QT syndrome is a heterogeneous genetic disease associated with delayed cardiac repolarization, prolonged QT intervals, the development of ventricular arrhythmias and sudden death. Type 2 congenital long QT syndrome (LQT2) results from *KCNH2* or *hERG* gene mutations. *hERG* encodes the $K_{v11.1}$ alpha subunit of the rapidly activating delayed rectifier K^+ current in the heart. Studies of mutant *hERG* channels indicate that most LQT2 missense mutations generate trafficking-deficient $K_{v11.1}$ channels.

OBJECTIVE: To identify the mechanism underlying G572R-hERG by using molecular and electrophysiological analyses.

METHODS AND RESULTS: To elucidate the electrophysiological properties of the G572R-hERG mutant channels, mutant *hERG* subunits were heterologously expressed in HEK293 cells alone or in combination with wild-type (WT)-*hERG* subunits. Patch-clamp techniques were used to record currents, and double immunofluorescence protein tagging and Western blotting were performed to examine the cellular trafficking of mutant subunits. When expressed alone, G572R-hERG subunits were not present in the cell membrane and did not produce detectable currents. When coexpressed with WT-hERG subunits, G572R-hERG decreased current density and altered gating properties of the WT-hERG channel.

CONCLUSION: The *hERG*-associated missense mutation G572R, like most LQT2 missense mutations, generates a trafficking-deficient phenotype. Furthermore, G572R-hERG causes a loss of function in *hERG* by a strong dominant negative effect on the WT-hERG channel.

Key Words: *hERG*; Long QT syndrome; Mutation; Potassium channel; Protein trafficking

The congenital long QT syndrome (LQTS) is a heterogeneous genetic disease associated with delayed cardiac repolarization, prolonged QT intervals, the development of ventricular arrhythmias (torsade de pointes) and sudden death (1,2). To date, 10 gene defects have been identified as being responsible for LQTS (3,4). The *KCNH2* or *hERG* gene encodes the $K_{v11.1}$ protein alpha (α) subunits that underlie the rapidly activating delayed rectifier K^+ current in the heart. Mutations in *hERG* cause a loss-of-function phenotype resulting in the proarrhythmic type 2 LQTS (LQT2) (4-6). More than 200 LQT2-linked mutations have been identified. Missense mutations are the most prevalent mutations and, thus, have been studied most intensively (7,8). Many functional expression studies suggest that the loss of *hERG* channel function in LQT2 mutations is caused by multiple mechanisms including abnormalities in $K_{v11.1}$ synthesis (class 1 mechanism), intracellular transport (protein trafficking) to the cell surface membrane (class 2 mechanism),

De nouvelles caractéristiques du canal G572R-hERG au trafic défectueux liées au syndrome du QT long héréditaire

HISTORIQUE : Le syndrome du QT long congénital est une maladie génétique hétérogène associée à un retard de la repolarisation cardiaque, à des intervalles QT prolongés, à l'apparition d'arythmies ventriculaires et à une mort subite. Le syndrome du QT long congénital de type 2 (QTL2) est causé par des mutations du gène *KCNH2* ou *hERG*. Le gène *hERG* code la sous-unité alpha $K_{v11.1}$ du canal K^+ redresseur retardé du cœur qui s'active rapidement. Des études des canaux *hERG* mutants indiquent que la plupart des mutations faux-sens QTL2 produisent des canaux $K_{v11.1}$ au trafic défectueux.

OBJECTIF : Dépister les mécanismes sous-jacents du canal G572R-hERG au moyen d'analyses moléculaires et électrophysiologiques.

MÉTHODOLOGIE ET RÉSULTATS : Afin d'établir les propriétés électrophysiologiques des canaux mutants G572R-hERG, les sous-unités mutantes *hERG* étaient exprimées de manière hétérologue dans des cellules HEK293, seules ou en combinaison avec des sous-unités *hERG* de type sauvage (WT). Les techniques du patch-clamp ont permis d'enregistrer les courants, tandis que le marquage des protéines par double immunofluorescence et le transfert Western ont permis d'examiner le trafic cellulaire des sous-unités mutantes. Exprimées seules, les sous-unités G572R-hERG étaient absentes de la membrane cellulaire et ne produisaient pas de courants décelables. Coexprimé avec les sous-unités WT-hERG, le canal G572R-hERG réduisait la densité du courant et modifiait les propriétés de synchronisation du canal WT-hERG.

CONCLUSION : La mutation faux-sens du gène G572R associée au gène *hERG*, comme la plupart des mutations faux-sens QTL2, produit un phénotype au trafic défectueux. De plus, le canal G572R-hERG entraîne une perte de fonction du gène *hERG* par son puissant effet négatif dominant sur le canal WT-hERG.

channel gating (class 3 mechanism) and permeation (class 4 mechanism) (8-12). Thus, each mutation should be studied individually to further clarify the mechanisms of *hERG* channel dysfunction. The purpose of the present study was to analyze the mechanism of *hERG* channel dysfunction in G572R-hERG.

The missense mutation G572R-hERG, which had not been characterized previously, was constructed for the present study (13,14). The G572R mutation, causing the substitution of a glycine with an arginine residue at position 572, is located at the end of the S5 transmembrane segment of *hERG*. A combination of molecular, biochemical and electrophysiological methods was used to functionally analyze G572R-hERG as a homotetrameric protein and as a heterotetrameric protein along with wild-type (WT)-*hERG* in HEK293 cells. Our results suggest that the G572R-hERG mutation is a trafficking deficiency and, therefore, may provide new information on additional function of this protein domain.

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Received for publication May 22, 2008. Accepted March 1, 2009

Figure 1 Analysis of hERG protein expression in HEK293 cells. Wild-type (WT)-hERG channels show two single protein bands at approximately 135 kDa and 155 kDa. G572R-hERG channels show a single band at approximately 135 kDa. WT-hERG/G572R-hERG channels show two single protein bands at approximately 135 kDa and 155 kDa, but the 155 kDa protein band is faint

METHODS

Complementary DNA and cell culture

WT-hERG was expressed by using the pcDNA3 vector (Invitrogen, USA) (10). G572R-hERG complementary DNA was generated by site-directed mutagenesis of WT-hERG complementary DNA and then subcloned into WT-hERG pcDNA3 vector at the BstEII and XhoI restriction sites. The functional effects of G572R-hERG were examined through transient expression in HEK293 cells. HEK293 cells were cultured in modified minimum essential media (Gibco/Invitrogen, USA) and maintained at 37°C in 5% CO₂ (15).

Western blotting, immunocytochemistry and confocal imaging

WT-hERG, G572R-hERG and G572R-hERG/WT-hERG protein were isolated from an equally confluent HEK293 cell culture and probed as previously described (16), with the exception that the primary antibody (Santa Cruz Biotechnology Inc, USA) was directed against the N-terminal of the hERG protein. Briefly, cells were lysed in NP-40 buffer, incubated on ice for 30 min and centrifuged at 15,000 rpm for 15 min at 4°C to pellet detergent-insoluble cell debris. The supernatant was stored at -80°C. For Western blotting analysis, protein aliquots were mixed with equal volumes of sample buffer, incubated at 50°C for 20 min, separated through 7.5% sodium dodecyl sulphate-polyacrylamide gel electrophoresis, followed by electrophoretic transfer onto nitrocellulose membranes. Nitrocellulose membranes were incubated with primary antibody (1:3000 dilution), followed by several washes in transfer buffer, and then incubated with a horseradish peroxidase-conjugated secondary antibody. Labelled protein bands were visualized through enhanced chemiluminescence (ECL; Amersham, GE Healthcare, USA) detection.

For immunocytochemical labelling, HEK293 cells transiently expressing WT-hERG, G572R-hERG and G572R-hERG/WT-hERG channels were plated in 35 mm Petri dishes containing collagen-coated coverslips. Cells were fixed with 4% buffered paraformaldehyde, permeabilized with triton X-100 (0.1%) in phosphate-buffered saline (PBS) and rinsed in 0.75% glycine/PBS buffer to quench background fluorescence. The cells were incubated at room temperature in blocking solution (10% goat serum in PBS) for 1 h and then were subsequently supplemented with the hERG antibody (1:500 dilution). Excess antibody was washed off with blocking solution; after that, the cells were incubated with highly cross-absorbed Alexa Fluor 568 goat antirabbit immunoglobulin G (IgG) (heavy and light) antibody (1:500 dilution, Molecular Probes Inc, USA) in blocking solution. After washing with blocking solution, coverslips were mounted in 50% glycerol/PBS. Imaging was performed using a Leica TCS SP2 confocal laser scanning microscope (Leica, Germany). (Excitation and emission wavelengths were 488 nm and 507 nm, respectively.) Cells were then labelled with rabbit polyclonal anti-hERG (1:25 dilution, Alomone, Israel) and mouse monoclonal anticalnexin (1:100 dilution, Chemicon, USA) at 4°C overnight followed by incubation with fluorescein isothiocyanate-conjugated goat antirabbit IgG secondary antibody and tetramethylrhodamine isothiocyanate-conjugated goat antimouse IgG

secondary antibody at 37°C for 2 h. Immunofluorescence staining was also viewed using a Leica TCS SP2 confocal laser scanning microscope. (Excitation and emission wavelengths for fluorescein isothiocyanate were 492 nm and 520 nm, respectively; excitation and emission wavelengths for tetramethylrhodamine isothiocyanate were 492 nm and 520 nm, respectively.)

Patch-clamp recording

All patch-clamp experiments were performed at 22°C to 24°C within 2 h of removing cells from culture conditions. The transfected cells were transferred to a bath mounted on the stage of an inverted microscope (Olympus, Japan). Cells were superfused with HEPES-buffered Tyrode's solution containing 137 mM NaCl, 4 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 10 mM glucose and 10 mM HEPES (pH 7.4; adjusted with NaOH). The internal pipette solution contained 130 mM KCl, 1 mM MgCl₂, 5 mM EGTA, 5 mM Mg-ATP and 10 mM HEPES (pH 7.2 adjusted with NaOH) according to previous reports (8,17-19). Patch electrodes typically had resistances of 2 Mohm to 5 Mohm. Membrane currents were recorded using an Axopatch 200B amplifier (Axon Instruments, USA) and digitized at 2 kHz using an analogue-to-digital converter (DigiData 1200B; Axon Instruments). Computer software (pCLAMP version 8.1; Axon Instruments) was used to generate voltage-clamp protocols, acquire data and analyze current traces.

Statistics

Data are presented as mean ± SEM, where n is the number of cells. Voltage dependence of activation was determined by fitting the peak tail current (I_{tail}) with a Boltzmann equation:

$$y = 1 / (1 + \exp[-(E - E_h)/k]),$$

where E is membrane voltage, E_h is the voltage at which 50% of channels are activated and k is the slope factor. The Student's *t* test was used to test for significance (*P* < 0.05).

RESULTS

Western blotting and immunocytochemistry analyses

To study the mechanisms accounting for dysfunctional mutant channels, hERG channel protein was analyzed by Western blotting. Cellular lysates were probed with an N-terminal hERG antibody. As shown in Figure 1, the lysate isolated from HEK293 cells expressing WT-hERG presented the typical hERG signature of two single protein bands at approximately 135 kDa and 155 kDa. The upper band at 155 kDa represents the complex glycosylated mature form of the hERG channel inserted in the cell membrane, whereas the lower band (135 kDa) represents the immature core glycosylated form of the hERG channel retained in the endoplasmic reticulum (ER) (9,15). In contrast, the band isolated from HEK293 cells expressing G572R-hERG showed an atypical hERG expression pattern with a protein band at approximately 135 kDa and an absent 155 kDa protein band. These results indicate that the G572R-hERG protein displays defective trafficking. Moreover, the lysate isolated from HEK293 cells cotransfected with G572R-hERG/WT-hERG showed two single protein bands at approximately 135 kDa and 155 kDa, but the 155 kDa protein band was fainter than in the case of WT-hERG. These data suggest that the G572R-hERG mutation results in a trafficking-deficient phenotype when coexpressed with WT-hERG.

The subcellular localization of hERG protein in HEK293 cells transfected by WT-hERG, G572R-hERG/WT-hERG or G572R-hERG was analyzed by a permeabilized immunostaining method. Figure 2 shows confocal images of green fluorescent protein-staining patterns for WT-hERG, G572R-hERG/WT-hERG and G572R-hERG protein. WT-hERG mainly labelled at the surface membrane (Figure 2A); in contrast, the trafficking-defective G572R-hERG was mainly expressed in the cytoplasm (Figure 2C) (20,21). Figure 2B shows that G572R-hERG/WT-hERG results in less plasma membrane fluorescence compared with the consequences of WT-hERG. This restricted intracellular distribution pattern is consistent with the results of the Western

Figure 2) Confocal images of wild-type (WT)-hERG and G572R-hERG channels in HEK293 cells. Subcellular localization of green fluorescent protein (GFP)-tagged WT-hERG and G572R-hERG channels. **A** WT-hERG channel is expressed on the cell surface (arrow). **B** Cells cotransfected by a red-shifted variant of enhanced GFP (pEGFP)-WT-hERG and pEGFP-G572R-hERG show less fluorescence in the plasma membrane compared with cells transfected with pEGFP-WT-hERG. **C** The G572R-hERG channel is mainly expressed in the cytoplasm. **D** Double immunofluorescence staining of hERG protein and the endoplasmic reticulum marker protein calnexin. HEK293 cells were transiently transfected with pcDNA3-WT, pcDNA3-G572R, or pcDNA3-WT and pcDNA3-G572R, and were costained with anti-hERG antibody (left column) and with an endoplasmic reticulum marker protein anticalnexin antibody (middle column). The right column shows overlays of the two images (scale bar 20 μ m). The top row shows nontransfected controls. Arrows indicate the cell surface

blotting experiments and shows that the mutant protein is retained intracellularly.

To study whether transport-deficient hERG mutant protein is trapped in the ER, double immunofluorescence staining of hERG protein and ER marker protein calnexin was performed. HEK293 cells transfected by WT-hERG, G572R-hERG/WT-hERG or G572R-hERG were costained with an antibody to hERG (Figure 2D; green images, left column) and antibody to ER marker protein calnexin (Figure 2D; red images, middle column). The right column in Figure 2D shows overlaying of the former two images (scale bar 20 μ m). Confocal images (Figure 2D) clearly show the marked presence of WT-hERG protein in the plasma membrane. In contrast, the image of the G572R-hERG shows that mutant protein is retained in the ER. This was confirmed in the overlaying image, which showed a uniform yellow colour. For G572R-hERG/WT-hERG, the green hERG and red calnexin immunoreactivities were restricted to the perinuclear distribution, indicating that the mutant hERG and calnexin were colocalized. These findings suggest that G572R-hERG results in intracellular protein processing defects and retention in the ER.

Figure 3) Voltage dependence of hERG channel activation. Inset shows the voltage clamp protocol. **A** to **C** Representative current traces in HEK293 cells transfected with wild-type (WT), G572R alone, or coexpression of WT and G572R plasmids. **D** Current-voltage relationships for peak currents during test pulses. **E** Current-voltage relationships for amplitudes of tail currents recorded during test pulses. **F** Amplitudes of tail currents plotted as a function of the test potential and fitted to a Boltzmann function. *Significantly different versus WT-hERG and G572R-hERG/WT-hERG; ** $P < 0.01$

Electrophysiological properties of WT-hERG and G572R-hERG channels

WT-hERG (4 μ g) and G572R-hERG (4 μ g) plasmid (in a 35 mm culture dish) were transfected into HEK293 cells. Channels from cells were assessed after 24 h transient transfection.

Cells were depolarized to test voltages between -60 mV and +60 mV in 10 mV increments for 2 s from a -80 mV holding potential, followed by steps to a level of -40 mV for 5 s to elicit I_{tail} (Figure 3, inset). Figures 3A to 3C show representative current traces from cells transfected by WT-hERG, G572R-hERG/WT-hERG and G572R-hERG. Cells transfected by WT-hERG showed a typical hERG current waveform (Figure 3A), while cells transfected by G572R-hERG did not show I_{tails} above background noise levels (Figure 3C) (eight cells per group). These findings indicate that the G572R-hERG mutation results in a loss of function.

Coexpression of WT-hERG and G572R-hERG

Mutant hERG peptides have been proposed to adversely affect WT-hERG protein function through heteromeric channels assembled from WT and mutant subunits (ie, dominant negative interaction) (6). To determine the dominant negative effects of G572R protein, 4 μ g of WT-hERG plus 4 μ g of mutant hERG plasmid (in a 35 mm culture dish) were transfected transiently into HEK293 cells. The voltage dependence of activation was

Figure 4) Steady-state inactivation and time courses of inactivation of wild-type (WT)-hERG or G572R-hERG/WT-hERG. **A** and **B** Representative current traces showing steady-state inactivation. **C** Normalized steady-state inactivation curves of expressed currents in HEK293 cells transfected by WT-hERG or G572R-hERG/WT-hERG, respectively. Insets show a portion of the voltage-clamp protocol. **D** and **E** Representative current traces of time courses of inactivation and inactivation time constant (τ) values for expressed currents in HEK293 cells transfected by WT-hERG or G572R-hERG/WT-hERG, respectively. **F** τ was measured by fitting inactivating currents during test pulses at each potential with a single exponential function. *Significantly different versus WT-hERG and G572R-hERG/WT-hERG

measured using a standard isochronal I_{tail} protocol (Figure 3, inset). WT-hERG and G572R-hERG/WT-hERG channels showed qualitatively similar current waveforms; however, cells expressing G572R-hERG/WT-hERG channels (Figure 3B) showed reduced current amplitudes compared with those expressing WT-hERG channels alone. Figures 3D and 3F show current-voltage relationships of the activation and I_{tail} obtained from cells expressing WT-hERG and G572R-hERG/WT-hERG, respectively. The membrane potential of maximal current density was observed between 0 mV and 20 mV in cells expressing WT-hERG and G572R-hERG/WT-hERG channels. The maximal current densities were 58.6 ± 5.9 pA/pF and 12.7 ± 0.94 pA/pF for cells expressing WT-hERG and G572R-hERG/WT-hERG channels,

respectively (eight cells per group). The current density of cotransfected cells was decreased by 78.3% ($P < 0.05$) compared with the results of cells expressing WT-hERG alone. The peak I_{tail} density was 89.5 ± 6.6 pA/pF and 32.7 ± 3.3 pA/pF for cells expressing WT-hERG and G572R-hERG/WT-hERG channels, respectively (eight cells per group). The I_{tail} density of cotransfected cells was decreased by 63.5% ($P < 0.05$) compared with the results of cells expressing WT-hERG alone. These findings indicate that the G572R-hERG subunit is capable of interacting with the WT-hERG subunit and that G572R-hERG exerts a dominant negative effect on WT-hERG protein and functional current.

Normalized I_{tail} values of WT-hERG and G572R-hERG/WT-hERG were averaged for each channel variant and plotted as a function of the activating voltage step, then fitted to a Boltzmann function (Figure 3F). The half-maximal activation voltage was -3.3 ± 0.37 mV for WT-hERG and was 4.6 ± 0.43 mV for G572R-hERG/WT-hERG channels (eight cells per group, $P < 0.05$). The slope factor was 9.5 ± 0.32 mV for WT-hERG and was 7.7 ± 0.38 mV for G572R-hERG/WT-hERG (eight cells per group, $P < 0.05$). The relationship was shifted to a statistically significant more positive potential in cells expressing G572R-hERG/WT-hERG compared with values from cells expressing WT-hERG only. Thus, the G572R-hERG mutation decreases the rate of WT-hERG channel activation.

To measure channel inactivation, test potentials between 130 mV and 20 mV in 10 mV increments for 20 ms were applied after a depolarizing pulse to 20 mV for 4 s. Next, a test pulse back to 20 mV for 500 ms was performed, followed by a final return of the voltage to the -80 mV holding potential (Figure 4, top inset). Cells expressing WT-hERG (Figure 4A) and G572R-hERG/WT-hERG (Figure 4B) showed typical steady-state inactivation current traces; however, the latter displayed the remarkable reduced currents. Figure 4C shows the normalized steady-state inactivation curves. The half-maximal inactivation voltages for WT and G572R/WT channels were -19.62 ± 1.94 mV and -44.41 ± 1.43 mV (eight cells per group), respectively ($P < 0.05$). The slope factors were 25.36 ± 1.56 mV and 28.51 ± 1.74 mV, respectively (eight cells per group, $P > 0.05$). The slope factor of steady-state inactivation was augmented slightly for cells expressing G572R-hERG/WT-hERG compared with that for cells expressing WT-hERG. These results indicate that fewer channels are available for opening at the same depolarized potentials for the G572R/WT channel.

To assess the inactivation time course of hERG currents, cells were depolarized from a holding potential of -80 mV to $+60$ mV for 2 s, hyperpolarized to -100 mV for 10 ms to allow the hERG channel to recover from inactivation, and then depolarized to test voltages between -60 mV and $+60$ mV in 10 mV decrements to record inactivating currents (Figure 4, lower inset). Figures 4D and 4E show typical traces of expressed currents for cells expressing WT-hERG and G572R-hERG/WT-hERG channels, respectively, again illustrating the smaller current magnitudes in the latter case. The time constant for channel inactivation was fitted by a single-exponential process. Inactivation time constants were plotted as a function of test voltage (Figure 4F). The inactivation of G572R-hERG/WT-hERG channels was faster than that of WT-hERG channels at all potentials (eight cells per group, $P < 0.05$). Therefore, inactivation in the hERG channel was accelerated.

Recovery from inactivation was measured by depolarizing cells to 50 mV for 1.5 s to activate and inactivate hERG channels, followed by repolarizing test voltages between -120 mV and -30 mV for 3 s to elicit I_{tail} (Figure 5, upper inset). Figures 5A and 5B show the I_{tail} s for cells expressing WT-hERG and G572R-hERG/WT-hERG channels, respectively. The initial phase ('hook') of the I_{tail} (arrow) reflected recovery from inactivation and was fit with a single exponential process. When the time constants of recovery from inactivation were plotted as a function of test voltage (Figure 5C), there were no statistically significant differences between WT-hERG and G572R-hERG/WT-hERG at all potentials. Thus, G572R-hERG mutation does not alter recovery from inactivation in the hERG channel.

Deactivation was analyzed by using the same pulse protocol as recovery from inactivation (Figure 5, lower inset). The fast and

slow deactivation time courses were assessed by fitting deactivating I_{tails} during test pulses to a double exponential function. Figures 5D and 5E display traces for cells expressing WT-hERG and G572R-hERG/WT-hERG channels, respectively (eight cells per group). The mean fast and slow deactivation time constants were plotted as a function of test voltage (Figure 5F). At all test potentials, neither fast nor slow time constants of deactivation for G572R-hERG/WT-hERG were different from those for WT-hERG. These results suggest that the G572R-hERG mutation does not modify the deactivation time course of the hERG channel despite the reduced amplitudes of relevant I_{tails} .

DISCUSSION

Larsen et al (13,14) reported the missense mutation G572R at the end of the S5 transmembrane segment of the hERG channel in a four-generation Danish family. The G572R mutation is associated with a high mortality rate. Three untreated relatives of the proband died suddenly and unexpectedly at 18, 32 and 57 years of age. However, the mechanism underlying the G572R mutation is uncertain.

In the present study, G572R-hERG as a homotetrameric and heterotetrameric protein with WT-hERG in HEK293 cells was functionally characterized by using molecular, biochemical and electrophysiological analyses. Interestingly, G572R-hERG, when expressed alone, shows defective trafficking from the cytoplasm to the surface membrane and an inability to produce any detectable currents. When coexpressed with WT-hERG, the G572R-hERG mutation resulted in an apparent dominant negative effect on WT-hERG current densities, with additional alterations in gating properties of hERG channels.

To date, the phenotypes of LQT2-linked mutations have been characterized by expression of the mutant hERG channels in heterologous systems (6,9,10). These mutations have been postulated to reduce functional rapidly activating delayed rectifier K^+ current through biophysical and/or biogenic mechanisms (16,22-27). In the former case, protein processing is normal and the protein is transported to the cell membrane, but its biophysical properties are altered. In the latter instance, protein processing is abnormal and is not transported to the membrane; thus, such channels were termed 'trafficking deficient'. Anderson et al (9) further proved that the majority of LQT2-linked missense mutations were trafficking defective.

The G572R-hERG mutation represents a protein that is trafficking deficient for LQT2. In the present study, double immunofluorescence staining of hERG protein and the ER marker protein calnexin was used. The G572R-hERG mutant was visible in the cytoplasm labelling, with a notable absence at the surface membrane. G572R-hERG mutant subunits were retained in the ER and failed to be transported to the Golgi apparatus for further processing. Moreover, Western blotting analysis shows that the G572R-hERG mutant generated the immature protein (135 kDa band) within the ER and it was the lack of mature protein (155 kDa band) that formed functional channels in the surface membrane. Thus, the G572R-hERG mutant subunits would not be expected to create a functional channel. The data from the present study further demonstrate the important role of defective trafficking in LQT2. Our results agree with most previous reports on hERG in the S5/P region (G572S, I593R, P596R, G601S, G604S, Y611H, V612L and T613M), which were shown to induce trafficking deficiency (9,28,29). Interestingly, N588D causes a loss of function with the voltage dependence of inactivation shifted to more negative potentials (30). However, the N588K mutation involving the same residue leads to a gain of function produced by positive shifts in the voltage dependence of hERG current inactivation (31). Thus, any mutation should be examined to clarify the mechanisms and effects on hERG channel function.

LQT2 is an autosomal dominant form – one allele is normal hERG and another allele is mutant hERG. We studied G572R-hERG to determine whether there was a dominant negative mechanism effect on WT-hERG channel function. Our results showed that when G572R-hERG was coexpressed with WT-hERG subunits in HEK293 cells, the G572R-hERG/WT-hERG channels were 78.3% reduced in maximum current density and 63.5% reduced in maximum I_{tail} density, compared

Figure 5 Recovery from inactivation and deactivation of wild-type (WT)-hERG or G572R-hERG/WT-hERG. **A** and **B** Representative current traces showing recovery from inactivation as reflected in the initial phase of the tail currents (arrow) in HEK293 cells transfected with WT-hERG or G572R-hERG/WT-hERG. **C** Time constant (τ) values for channel recovery from inactivation plotted as a function of test voltage. **D** and **E** Representative current recordings of deactivation in HEK293 cells transfected by WT-hERG or G572R-hERG/WT-hERG (the phase of deactivation is marked with an arrow). Deactivation time constants were measured by fitting deactivating currents during test pulses at each potential with double exponentials. **F** Fast and slow components of deactivation time constants (τ) as a function of test potential. $P > 0.05$ versus WT-hERG and G572R-hERG/WT-hERG. Insets show the voltage clamp protocol

with WT-hERG channels alone. Thus, G572R-hERG causes a dominant negative effect on WT-hERG channel function.

Previous studies demonstrated that the S5P α -helix is critical for normal inactivation and S5P α -helix charge scan mutants have a very marked effect on the voltage dependence of inactivation (32,33). A number of mutations (W585, L586, H587, L589, G590, D591, I593, G594, G604S, Y611H, V612L and T613M) in the S5-P region have been identified that abolish or substantially reduce inactivation of WT-hERG (9,33-35). We examined properties and kinetics of expressed currents of HEK293 cells transfected

by G572R-hERG/WT-hERG. The G572R mutation results in altered gating properties of WT-hERG channels. Cells coexpressing G572R-hERG/WT-hERG did not show changes in recovery and deactivation compared with cells expressing WT-hERG alone. Cells coexpressing G572R-hERG/WT-hERG showed a voltage dependence of activation that was shifted in a positive direction, a voltage dependence of inactivation that was shifted in a negative direction and a more rapid inactivation process, compared with WT-hERG. G572R-hERG exhibited strong dominant negative current suppression resulting in decreased current density and altered gating properties of the WT-hERG channel. Thus, G572R-hERG subunits can coassemble with WT-hERG subunits to form a heteromultimeric channel with altered gating properties. Therefore, we speculate that the direction of shifts in gating caused by G572R would be expected to induce a decrease in current beyond that resulting from a dominant negative-induced suppression of channel function.

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Our data are the first to provide a functional analysis of the missense mutation G572R-hERG. The G572R-hERG mutant causes a loss of function and a dominant negative effect. Meanwhile, it alters gating properties of heteromultimeric channels. It is also likely that the G572R-hERG mutant forms stable heteromultimeric complexes with WT-hERG subunits. The present study confirms previous reports that LQTS-associated mutations in hERG cause a reduction in current magnitude when mutant and WT subunits are coexpressed, as expected for the common dominant inheritance pattern of the Romano-Ward syndrome.

GRANTS: National Natural Science Foundation of China (30772155); Zhejiang Provincial Program for the Cultivation of High-level Innovative Health Talents, Zhejiang Province Natural Science Foundation (Y206608); and Ningbo Youth and Doctor Foundation (2005A610016), Ningbo Program for the Cultivation of Health Technology Talents.