Original Article
Corrective action of aminoglycoside antibiotic G418 on defective F463L-hERG channels

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Abstract: Long QT syndrome (LQTS) is an inherited arrhythmia syndrome characterized by a propensity to recurrent ventricular tachyarrhythmias or torsades de pointes. Ion channel gene mutations associated with LQTS can be corrected by chemical or physical agents. The aim of this study was to evaluate the potential of the aminoglycoside antibiotic G418 to improve function of defective hERG channels due to F463L. WT-hERG and F463L-hERG plasmids instantaneously were transfected into the HEK293 cells to simulate heterozygous mutant (WT+F463L-hERG). Whole-cell patch clamp and laser confocal scanning microscopy were used to evaluate electrophysiological consequences and the membrane distribution of hERG protein. The density of tail currents was 56.75±2.113 pA/pF and 54.56±2.05 pA/pF for WT-hERG (P>0.05), 23.63±1.52 pA/pF and 39.08±2.32 pA/pF for heterozygous F463L-hERG (P<0.05), under control condition and exposed to G418, respectively. Comparison of these data from the 4 groups determined the potential of G418 to increase the mutant hERG currents due to F463L, resulting in an increase of around 65.4%. In addition, F463L-hERG also altered the potential of G418 on the biophysical of hERG channels although the underlying mechanisms require further study. Moreover, G418 showed potential to improve the distribution of mutant hERG proteins and increase hERG expression in HEK293 cells. Therefore, the aminoglycoside antibiotic G418 can partly restore functional expression of defective hERG channels due to F463L.

Keywords: HERG channels, mutation, whole-cell patch clamp, laser confocal scanning microscopy, aminoglycoside antibiotic

Introduction

Long QT syndrome (LQTS) is an inherited arrhythmia syndrome characterized by prolongation of the QT interval, abnormal T-wave or obvious U-wave on electrocardiograms, and a propensity to recurrent ventricular tachyarrhythmias or torsades de pointes, which may lead to cardiovascular events such as syncope or sudden death. So far, 15 genes have been identified to be linked to LQTS [1-3]. Human ether-a-go-go-related gene (hERG or KCNH2) is a member of the voltage-gated potassium channel ether-a-go-go (EAG) family, located on chromosome 7q35-36. The hERG gene encodes the α-subunit of rapidly activated delayed rectifier potassium channel (IKr) and interacts with the β-subunit which is encoded by KCNE2 gene (MiRPI) to form the IKr channel participating in the third stage of repolarization in cardiac cells [4, 5]. Mutations in the hERG gene directly or indirectly affect the function of IKr channel and reduce the IKr current by loss-of-function

underlying the Long QT2 syndrome (LQT2) [6-9]. Furthermore, this subtype accounts for approximately 40%-45% of LQTS. The molecular defects caused by hERG gene mutations may occur in the following aspects: protein synthesis (transcription and translation), transport process, altered channel gating, and abnormal permeability [5, 7, 8, 10].

Previous studies have found that ion channel gene mutations associated with LQTS can be corrected by chemical or physical agents [8, 10, 11]. The most important molecular mechanism associated with LQT2 caused by hERG channel missense mutation is a transport defect and can be corrected by physical hypothermia such as culturing cells at 27°C or hERG channel blockers (E-4031 and Cisapride) and other drugs (β-thapsigargin) [4, 10-14]. Effective pharmacological rescue is manifested by an increase in the functional expression of IKr current and the appearance of mature hERG protein on the cell membrane.

Aminoglycoside antibiotics are potential drugs that not only have bactericidal activity, but also reduce the accuracy and fidelity of translation, avoiding mutation genes translating to toxic proteins, and further allowing the translation proceed forward resulting in full-length, functional proteins [10, 15, 16]. In the most common mutation at position 508, which causes 70% of cystic fibrosis cases, aminoglycoside antibiotics not only show positive results in both lines of tissues and transgenic mice, but also can alleviate clinical symptoms in patients with cystic fibrosis [18]. As one of the most commonly used drugs in the clinic, aminoglycoside antibiotics not only can be used in diseases caused by gram negative bacteria, also have a powerful effect on improvement of read through efficiency [9, 17].

F463L mutation leads to loss of function in hERG through a dominant-negative effect caused by impaired trafficking of the channel [9]. The aim of the present study was to identify the potential of the aminoglycoside antibiotics G418 to improve function of defective hERG channels due to F463L.

Materials and methods

Cell culture and transfection

All plasmids in this present study were kindly provided by the Shaanxi Key Laboratory of Molecular Cardiology, China. The potentials of G418 on hERG channels were determined in HEK293 cells transiently expressing hERG channels. Equal wide type (2 μg) and mutant (2 μg) hERG plasmids were transiently transfected into HEK293 cells to simulate heterozygous mutation as previously described. Cell lines were regularly maintained. Cells in the present study were cultured for a minimum of two days prior to any experiment. All transfections were performed by X-tremeGENE HP DNA Transfection Reagent (Germany, Roche). Additional GFP plasmids were co-transfected to detect transfection efficacy.

Electrophysiology

Whole-cell patch clamp was performed to determine the hERG currents. Cells were superfused with the following solutions (mM): NaCl 140, KCl 4, $CaCl_2$ 2, $MgCl_2$ 1, glucose 5, hydroxyethyl piperazine ethanesulfonic acid (HEPES) 10; adjusted to pH 7.4 with NaOH. Pipettes

were filled with the following solutions: (mM): KCl 20, K-Aspartate 115, $MgCl_2$ 1, Na2ATP 2, ethyleneglycol bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) 5, HEPES 10; pH adjusted to 7.2 with KOH. A Multi Clamp 700B amplifier instrument (Axon Instruments) was adopted to record membrane currents. Cell capacitance (in pF) was made from whole-cell capacitance compensation. The data were analyzed with p-CLAMP 9.2 software (Molecular Devices, Sunnyvale, CA, USA). All experiments were performed at the environmental temperature of 22-25°C.

Confocal microscope analysis

The distribution of hERG channel in cells was analyzed by laser scanning confocal microscope. The WT-hERG and F463L-hERG channels were GFP-labeled at the N-terminus by insertion of cDNA fragments into pEGFP-C2. Cells were cultured in 35 mm glass bottom culture dish (NEST Biotechnology, Hong Kong) and were then transiently transfected with WT-hERG plasmids. WT-hERG and an equal amount of F463L-hERG-hERG plasmids were transiently co-transfected to mimic the heterozygous genotype. PDsRed2-ER plasmids were co-transfected to indicate the endoplasmic reticulum. The images were obtained using laser scanning microscope (Olympus, Japan) and analyzed by FV10-ASW Viewer 4.0.

G418 preparation and treatment

Similarly to previous studies described [10], G418 (Sigma, USA) was initially dissolved in dimethylsulfoxide (DMSO) to 50 mg/ml as stock in -20°C, and further diluted in physiological saline to the required concentration. The final content of DMSO in saline during experiments was lower than 0.05%. At 24 hours after transfection, HEK293 cells were continuously incubated for 24 hours with G418 before whole-cell patch clamp and laser scanning confocal microscope experiments.

Statistical analysis

All analyses were performed using SPSS version 18.0 (SPSS Inc., Chicago, IL, USA). All values are presented as mean ± SEM. Statistical comparisons between two groups were performed with a two sample t-test. The voltage-dependence of current activation was determined by fitting the values of the normalized tail currents to a Boltzmann function.
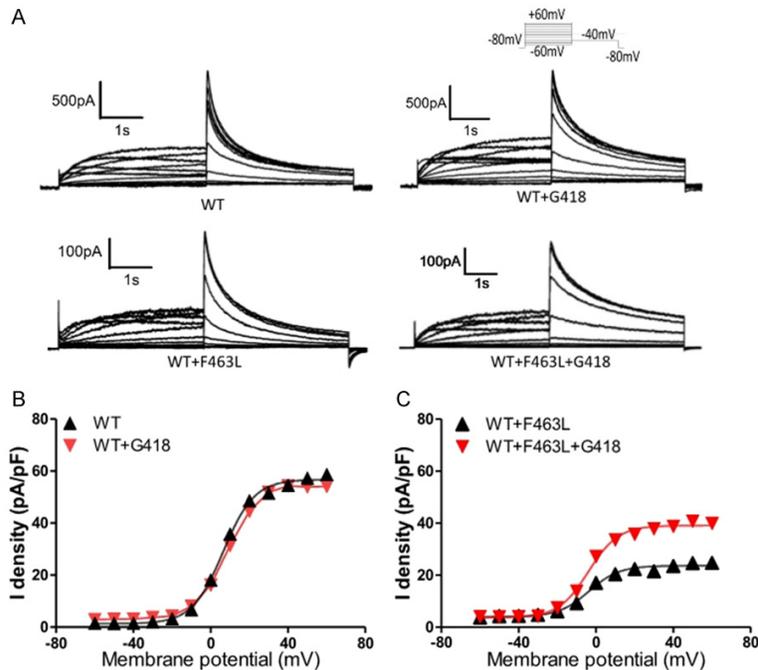
Figure 1. Currents obtained from cells expressing WT-hERG and heterozygous F463L-hERG. A. Representative whole cell current traces were obtained from cells expressing homozygote WT-hERG and heterozygous F463L-hERG both under control condition and exposed to G418, respectively. B and C. I-V relationships of the maximal density of tail currents obtained from WT-hERG and heterozygous F463L-hERG under control condition and exposed to G418.

Results

G418 suppressed hERG currents

Whole-cell currents were recorded in cells expressing homozygote WT-hERG and heterozygous F463L-hERG with and without G418 (400 µg/ml). IKr was elicited by the protocol shown in the inset of **Figure 1**. The holding potential was maintained at -90 mV, and tail currents were recorded at a level of -40 mV for 4 seconds after depolarizing pulses from -60 mV to +60 mV in 10 mV increments for 2 seconds. Representative traces were from cells expressing homozygote WT-hERG and heterozygous F463L-hERG with and without G418, respectively (**Figure 1A**). Current-voltage (I-V) relationships of the maximal density of tail currents from WT-hERG and WT+F463L-hERG under control and in the presence of G418 were displayed in **Figure 1B**, **1C**, respectively. The density of tail currents was 56.75±2.113 pA/pF and 54.56±2.05 pA/pF for WT-hERG (P>0.05), 23.63±1.52 pA/pF and 39.08±2.32 pA/pF for heterozygous F463L-hERG (P<0.05), under con-

trol condition and exposed to G418, respectively.

The density of tail currents from WT+F463L-hERG without G418 corresponded to a decrease of 58.36% when compared with the ones from WT, however the decrease was 31.1% after G418 treatment. Comparison of these data from the 4 groups determined the potential of G418 to increase the mutant hERG currents due to F463L, resulting in an increase of about 65.4%.

Kinetics of activation

The normalized tail currents of WT-hERG were plotted as a function of the test potential and were then fitted to a Boltzmann function [1]. As shown in **Figure 2A**, **2B**, the voltage to achieve half activation ($V_{1/2}$) were 6.37±0.41 mV and 8.44±0.47 mV for WT-hERG, -3.69±0.71 mV and -4.04±0.01 mV for WT+F463L-hERG, respectively, under control and with G418. G418 resulted in a shift of around 2.07 mV for the voltage to achieve half activation ($V_{1/2}$) of WT-hERG and 0.35 mV for the counterparts of WT+F463L-hERG. To summarize, F463L-hERG resulted in alterations of the activation properties for hERG channels exposed to G418.

Kinetics of inactivation

To analyze steady-state inactivation, test potentials between -130 and 20 mV in 10 mV increments for 20 ms were applied after a depolarizing pulse to 20 mV for 4 seconds. This was followed by a test pulse back to 20 mV for 500 ms before a final return of the voltage to the -80 mV holding potential. The protocol is shown in the inset of **Figure 3**, which is a standard protocol used to study hERG currents [7]. The resulting normalized steady-state inactivation curves are shown in **Figure 3A**, **3B**, respectively. The voltages to achieve half inactivation ($V_{1/2}$) were -44.97±3.13 mV and -44.06±0.04 mV for WT-hERG channels, -48.70±1.67 mV and -46.72±
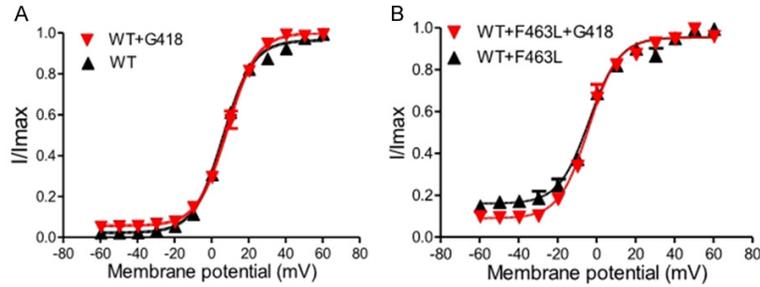
Figure 2. A and B. The voltage to achieve $V_{1/2}$ for WT-hERG and heterozygous F463L-hERG, respectively, under control condition and exposed to G418.
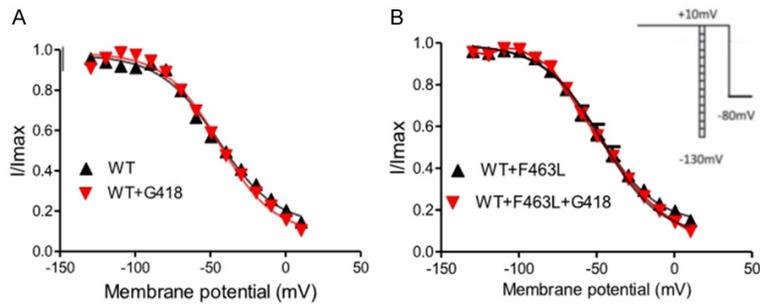

Figure 3. Steady-state inactivation. A and B. The normalized steady-state inactivation curves for WT-hERG and heterozygous F463L-hERG under control condition and exposed to G418, respectively.

2.48 mV for heterozygous F463L-hERG channels, under control condition and in the presence of G418, corresponding to a shift of around 0.91 mV and 1.98 mV, respectively. To summarize, F463L-hERG altered the gating properties of steady-state inactivation for hERG channels with G418.

Expression and location of hERG protein in HEK293 cells

To evaluate the potential of G418 on the distribution of hERG proteins in HEK293 cells, pEG-FP-C2-WT-hERG plus pEGFP-C2-F463L-hERG were co-transfected into cells to simulate the WT+F463L-hERG channels. PDsRed2-ER (red) was co-transfected into cells with pE-GFP-C2-WT-hERG, pE-GFP-C2-WT-hERG plus pEGFP-C2-F463L-hERG, respectively. The effect of G418 on the subcellular localization of WT-hERG was compared with the WT+F463L-hERG channels in cells that were transfected with pEGFP-C2-WT-hERG. Furthermore, pEGFP-C2-WT-hERG plus pEGFP-C2-F463L-hERG plasmids were tested with and without G418, respectively. GFP in cells transfected with WT-hERG plasmids without G418 (**Figure 4**, first

panel) were found predominantly on the cell surface, indicating efficient trafficking of hERG proteins from the endoplasmic reticulum to the plasma membrane as described previously [6, 18]. Subcellular localization of GFP in cells expressing WT-hERG plasmids with G418 (**Figure 4**, second panel) was observed both on the cell membrane and in the cytoplasm, indicating the potential of G418 to influence the trafficking of hERG proteins from endoplasmic reticulum to the plasma membrane. The subcellular localization of GFP in cells expressing WT+F463L-hERG plasmids without G418 (**Figure 4**, third panel) was observed to be more in the cytoplasm when compared with cell membrane, indicating the potential of F463L-hERG to bring about dysfunctional trafficking as shown previously [1]. The subcellular localization of GFP in cells co-transfected with WT-hERG and F463L-hERG plasmids in the presence of G418 (**Figure 4**, fourth panel) was more observed on the cell membrane.

Discussion

Mutations in hERG can induce LQT2 in a variety of ways, in which subunit folding abnormalities and abnormal transport are the most important pathogenesis. Mutation F4631-HERG can lead to hERG channel dysfunction by mainly affecting channel protein transport which can be alleviated by changing culturing environment or drug intervention. Effective drug rescue can be manifested as partially or fully recovery of functional mutant hERG channel, appearance or increase of hERG channel protein in cell membrane, and decrease of proteins retained in the endoplasmic reticulum. This study found that G418 could enhance the functional expression of F463L+WT-HERG and significantly increase the tail current amplitude. The maximal tail current densities of F463L+WT-hERG were 39.08±2.32 pA/pF after cultured with 400 μg/ml G418. The average maximal tail current den-
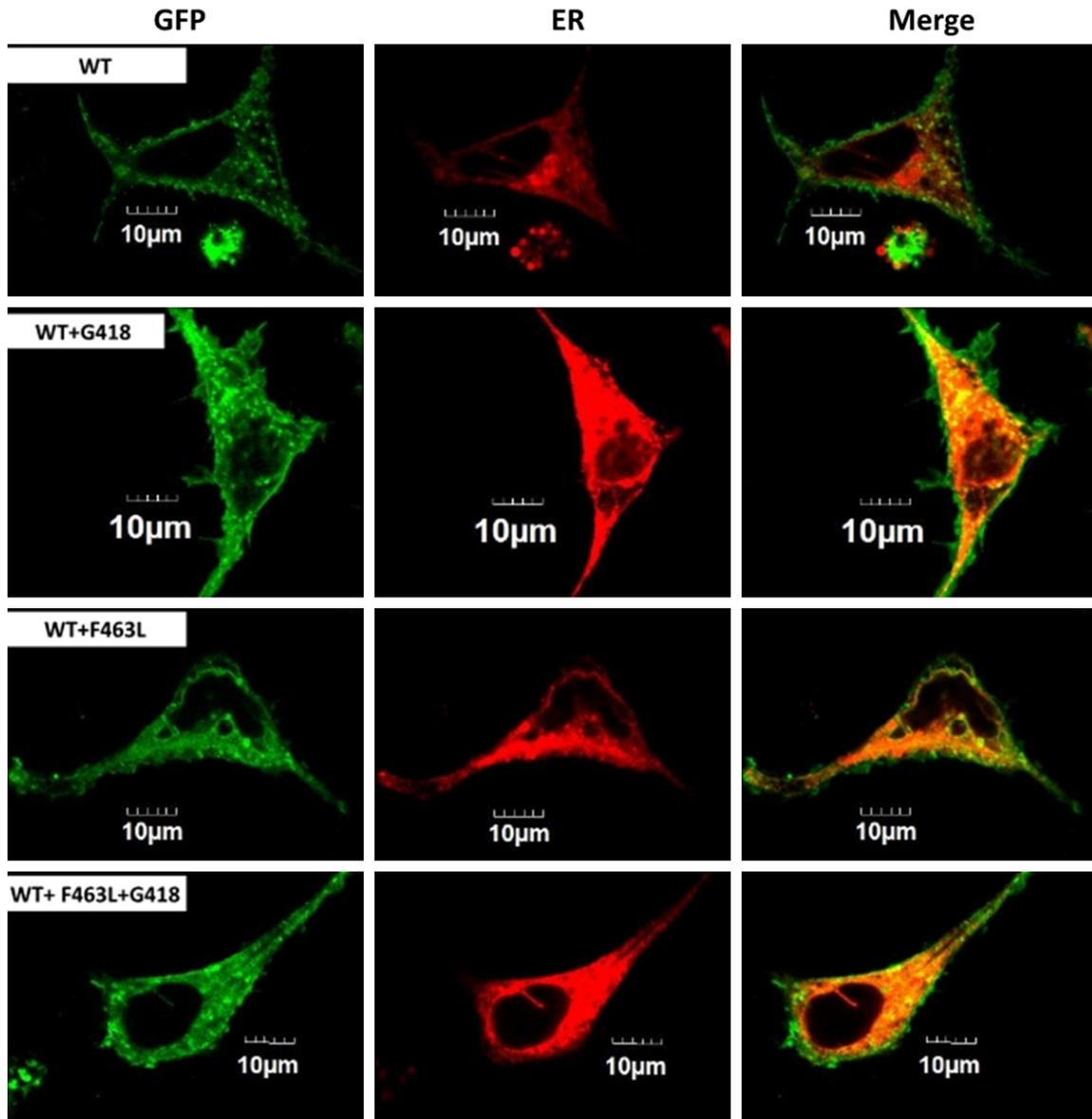
Figure 4. Representative images of subcellular localization of hERG protein expressed in HEK 293 cells. The panels from top to bottom are the representative images of HEK 293 cells expressing pEGFP-C2-WT and pEGFP-C2-WT-hERG plus pEGFP-C2-F463L-hERG under control condition and with G418, respectively. The first column is the hERG protein tagged with green fluorescence (green). The second column shows the images of pDsRed2-ER (red). The third column is merged versions (scale bar indicates 10 μm).

sity of F463L+WT-hERG was 46.73% of WT-HERG, and increased to 68.86% after G418 intervention. There was no statistically significant difference between WT-HERG and F436L+WT-HERG with G418 intervention.

The loss of hERG ionic current has been attributed to the following several mechanisms, including abnormalities in Kv11.1 synthesis (class 1 mechanism), intracellular transport (protein trafficking) to the cell surface membrane (class 2 mechanism), channel gating (class 3 mechanism), or permeation (class 4 mechanism) [4, 8].

The most important molecular mechanism of HERG gene mutations leading to LQT2 is a trafficking defect [8]. About 68% of trafficking defect could be rescued. Normal HERG gene encoding protein is transcribed and translated in the nucleus and then glycosylated, folded and assembled in the endoplasmic reticulum to form immature protein precursors with a relative molecular weight of 135 KDa. Trafficking-

deficient mutant channels are retained in the endoplasmic reticulum and can't be transported into Golgi for complex agglutination. It can be speculated that trafficking-deficient mutant channels are rescued after translation by drug intervention. Drug intervention changes the conformation of the mutant channels protein and enhances its stability to facilitating its transport out of the endoplasmic reticulum to further forming a mature channel expressing in the cell membrane. It is also worth noting that different interventions may act on different processes, so different interventions may be used in different mutations and the rescue consequences may be different [8]. Gong [12] has found that culturing N470D with WT-HERG could form heterozygous channel protein which was retained intracellularly and failed to traffic into the plasma membrane. However, after culturing cells in E-4031, channel proteins could be expressed in the plasma membrane, the functional current of heterozygous channel could be significantly increased and the ion-gating characteristics of channel restored. The above description shows that transport defect of homozygous and heterozygous mutant channel can be rescued.

Aminoglycosides antibiotics are a well-studied, highly potent class of drugs which not only have bactericidal activity, but also can inhibit the early appearance of stop codon and promote proper reading and translation of ribosomes, by reducing the accuracy and fidelity of translation and making the incompletely matched aminoacyl-S-RNA entering the polypeptide chain. In this situation, aminoglycosides can avoid the nonsense mutation transforming into the stop codon and allow translation to continue resulting in the production of a full length functional protein. This phenomenon has been confirmed in some nonsense mutations causing inherited diseases, such as cystic fibrosis, Duchenne muscular dystrophy and Hurler's syndrome [19, 20]. Burke [21] has studied CAT gene carrying amber mutations with G418, with a concentration of 0-2000 µg/ml. When G418 concentration increased to 50 µg/ml, the activity of mutant increased significantly, but the activity of wild type was not affected. But higher concentration of G418 could reduce the activity of both CAT mutant and wild type. Thus, G418 might inhibit protein synthesis at higher concentration. Subsequently, Bidou [21] found that a linear relationship between read through effi-

ciency and drug concentration was obtained when the concentration reached 600 mg/ml. When the concentration was above 600 µg/ml, the increase of read through efficiency was similar. Sermet-Gaudelus [22] has found that the maximum of read through efficiency could be achieved with 600 µg/ml gentamicin without any toxic reaction. In a recent work conducted by Yan [10], aminoglycoside antibiotics at the concentration of 400 µg/ml restored functional expression of mutant hERG channels. In this study, the final concentration of aminoglycoside antibiotics was 400 µg/ml, at which not only cytotoxicity was low and HEK293 cells grown well, but also read through efficiency was significant.

In conclusion, aminoglycoside G418 could partly restore functional expression of heterozygous mutant WT+F463L-HERG channel. This study may provide new ideas for the treatment of inherited arrhythmias. However, due to the toxicity of G418, it is currently only used in scientific research. More studies should be conducted on other less toxicity and safety aminoglycoside drugs and more *in vivo* research should be carried on, providing experimental basis for clinical treatment of LQTS.

Acknowledgements

Disclosure of conflict of interest

None.

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References

[1] Schwartz PJ, Ackerman MJ, George AL Jr and Wilde AAM. Impact of genetics on the clinical management of channelopathies. J Am Coll Cardiol 2013; 62: 169-180.

[2] Zhang L, Timothy KW, Vincent GM, Lehmann MH, Fox J, Giuli LC, Shen J, Splawski I, Priori SG, Compton SJ, Yanowitz F, Benhorin J, Moss AJ, Schwartz PJ, Robinson JL, Wang Q, Zareba W, Keating MT, Towbin JA, Napolitano C and Medina A. Spectrum of ST-T-wave patterns and repolarization parameters in congenital long-

QT syndrome: ECG findings identify genotypes. Circulation 2000; 102: 2849-2855.

[3] Schwartz PJ, Crotti L and Insolia R. Long-QT syndrome: from genetics to management. Circ Arrhythm Electrophysiol 2012; 5: 868-877.

[4] Zhou Z, Gong Q, Epstein ML and January CT. HERG channel dysfunction in human long QT syndrome. Intracellular transport and functional defects. J Biol Chem 1998; 273: 21061-21066.

[5] Sanguinetti MC and Tristani-Firouzi M. HERG potassium channels and cardiac arrhythmia. Nature 2006; 440: 463-469.

[6] Chen G, Guo H, Song Y, Chang H, Wang S, Zhang M and Liu C. Long non-coding RNA AK055347 is upregulated in patients with atrial fibrillation and regulates mitochondrial energy production in myocardiocytes. Mol Med Rep 2016; 14: 5311-5317.

[7] Zhang A, Sun C, Zhang L, Lv Y, Xue X, Li G, Cui C and Yan GX. L539 fs/47, a truncated mutation of human ether-a-go-go-related gene (hERG), decreases hERG channel ion currents in HEK 293 cells. Clin Exp Pharmacol Physiolo 2013; 40: 28-36.

[8] Anderson CL, Delisle BP, Anson BD, Kilby JA, Will ML, Tester DJ, Gong Q, Zhou Z, Ackerman MJ and January CT. Most LQT2 mutations reduce Kv11.1 (hERG) current by a class 2 (trafficking-deficient) mechanism. Circulation 2006; 113: 365-373.

[9] Splawski I, Shen J, Timothy KW, Lehmann MH, Priori S, Robinson JL, Moss AJ, Schwartz PJ, Towbin JA, Vincent GM and Keating MT. Spectrum of mutations in long-QT syndrome genes. KVLQT1, HERG, SCN5A, KCNE1, and KCNE2. Circulation 2000; 102: 1178-1185.

[10] Yao Y, Teng S, Li N, Zhang Y, Boyden PA and Pu J. Aminoglycoside antibiotics restore functional expression of truncated HERG channels produced by nonsense mutations. Heart Rhythm 2009; 6: 553-560.

[11] Zhou Z, Gong Q and January CT. Correction of defective protein trafficking of a mutant HERG potassium channel in human long QT syndrome. Pharmacological and temperature effects. J Biol Chem 1999; 274: 31123-31126.

[12] Rajamani S, Anderson CL, Anson BD and January CT. Pharmacological rescue of human K (+) channel long-QT2 mutations: human ether-a-go-go-related gene rescue without block. Circulation 2002; 105: 2830-2835.

[13] Chen MX, Sandow SL, Doceul V, Chen YH, Harper H, Hamilton B, Meadows HJ, Trezise DJ and Clare JJ. Improved functional expression of recombinant human ether-a-go-go (hERG) K+ channels by cultivation at reduced temperature. BMC Biotechnol 2007; 7: 93.

[14] Rossenbacker T, Mubagwa K, Jongbloed RJ, Vereecke J, Devriendt K, Gewillig M, Carmeliet E, Collen D, Heidbuchel H and Carmeliet P. Novel mutation in the Per-Arnt-Sim domain of KCNH2 causes a malignant form of long-QT syndrome. Circulation 2005; 111: 961-968.

[15] Wilschanski M, Yahav Y, Yaacov Y, Blau H, Bentur L, Rivlin J, Aviram M, Bdolah-Abram T, Bebok Z, Shushi L, Kerem B and Kerem E. Gentamicin-induced correction of CFTR function in patients with cystic fibrosis and CFTR stop mutations. N Engl J Med 2003; 349: 1433-1441.

[16] Tran NM and Chen S. Mechanisms of blindness: animal models provide insight into distinct CRX-associated retinopathies. Dev Dyn 2014; 243: 1153-1166.

[17] Burke JF and Mogg AE. Suppression of a nonsense mutation in mammalian cells in vivo by the aminoglycoside antibiotics G-418 and paromomycin. Nucleic Acids Res 1985; 13: 6265-6272.

[18] Babcock JJ and Li M. HERG channel function: beyond long QT. Acta Pharmacol Sin 2013; 34: 329-335.

[19] Lai CH, Chun HH, Nahas SA, Mitui M, Gamo KM, Du L and Gatti RA. Correction of ATM gene function by aminoglycoside-induced readthrough of premature termination codons. Proc Natl Acad Sci U S A 2004; 101: 15676-15681.

[20] Schulz A, Sangkuhl K, Lennert T, Wigger M, Price DA, Nuuja A, Gruters A, Schultz G and Schoneberg T. Aminoglycoside pretreatment partially restores the function of truncated V(2) vasopressin receptors found in patients with nephrogenic diabetes insipidus. J Clin Endocrinol Metab 2002; 87: 5247-5257.

[21] Bidou L, Hatin I, Perez N, Allamand V, Panthier JJ and Rousset JP. Premature stop codons involved in muscular dystrophies show a broad spectrum of readthrough efficiencies in response to gentamicin treatment. Gene Ther 2004; 11: 619-627.

[22] Sermet-Gaudelus I, Renouil M, Fajac A, Bidou L, Parbaille B, Pierrot S, Davy N, Bismuth E, Reinert P, Lenoir G, Lesure JF, Rousset JP and Edelman A. In vitro prediction of stop-codon suppression by intravenous gentamicin in patients with cystic fibrosis: a pilot study. BMC Med 2007; 5: 5.