Abstract. The importance of the local sequence context in determining how efficiently aminoglycosides rescue nonsense mutations has been established previously in disease models. Different stop codons appear to facilitate the termination process with differing efficiencies. Furthermore, the efficiency with which termination is suppressed may also be influenced by the local sequence context surrounding the stop codon. The strongest bias has usually been identified with the nucleotide base that immediately follows the stop codon in the majority of experiments. However, how the sequence context influences the efficiency of aminoglycosides in rescuing the human ether-a-go-go-related (HERG) protein in mammalian cells remains to be fully elucidated. Therefore, the present study was devised to examine the susceptibility of different termination codons on the HERG gene and the +4 nucleotide immediately following them to be suppressed by aminoglycosides in 293 cells. The 293 cells were transiently transfected with the wild-type or mutant genes. The read-through effect was subsequently examined by adding aminoglycoside G418 into the culture medium, followed by incubation of the cells for 24 h. An immunofluorescence method was then used to observe the protein expression of HERG prior to and following drug treatment. Patch clamping was performed to evaluate the function of the HERG protein. These experiments revealed that stop codons TGA and TAA in the R1014X mutant were more susceptible to treatment with the drug G418. Similar results were observed with the W927X-TGA and W927X-TAA mutants. Subsequently, R1014X-TGAC, R1014X-TGAG and R1014X-TGAA mutants were constructed based on the R1014X-TGAT mutant. The level of red fluorescence was observed prior to and following the administration of G418 using antibodies targeting the N- or C-terminus of the HERG protein. However, the tail current density was found only to increase with the R1014X-TGAT mutant following G418 treatment. Taken together, the results of the present study suggest that the type of premature stop codon and the context of the nucleotide immediately following at the +4 position, may determine the pharmacological rescue efficiency of the HERG gene.

Introduction

Long QT syndrome (LQTs) is an inherited cardiac arrhythmia syndrome that is associated with syncope and sudden death (1-3). It has been shown that mutations exist in 15 genes encoding either ion channels or structural proteins associated with LQTs (4-7). A previous study revealed that nonsense mutations, which introduce premature termination codons (PTCs) into the gene of interest, account for ~10% of all mutations (8). When a nonsense mutation occurs in the human ether-a-go-go-related (HERG) gene, which encodes the α-subunit of the rapid delayed rectifier potassium current channel (IKr), it may lead to type 2 LQTs.

Aminoglycosides have been demonstrated to have the ability of read-through on nonsense mutations to produce full-length functional proteins in genetic disease models (9-14). Howard et al (11) showed for the first time that G418 suppressed PTCs to restore the function of mutant proteins in
transiently expressed cDNAs that contained mutations in the cystic fibrosis transmembrane conductance regulator gene in mammalian cells. Our published previously study also demonstrated that G418 and gentamicin partially restored the HERG protein in a dosedependent manner in 293 cells expressing R1014X mutant (15).

The importance of the local sequence context in determining how efficiently aminoglycosides rescue nonsense mutations has been established previously in disease models (16‑18). It appears that different stop codons facilitate the termination process with differing efficiencies. The efficiency with which termination may be suppressed can also be influenced by the local sequence context in the immediate vicinity of the stop codons, and for the majority of the disease models, the strongest bias has been found at the position of the base immediately following the stop codon (i.e., the +4 nucleotide). However, at present, how the sequence context may influence the efficiency of aminoglycosides in terms of rescuing HERG nonsense mutants in mammalian cells remains to be fully elucidated. In an attempt to answer this question, the present study aimed to examine the susceptibility of different termination codons, and the local nucleotide sequence surrounding them, in the process of rescuing HERG nonsense mutants by aminoglycosides in 293 cells.

Materials and methods

**HERG mutant cDNA constructs.** Wildtype (WT) HERG cDNA cloned into the pcDNA3.1 vector was provided by Dr Zhengfeng Zhou (Oregon Health and Science University, Portland, OR, USA). The HERG nonsense mutations R1014X and W927X, containing different stop codons and +4 nucleotides, were generated in the pcDNA3.1 WT HERG plasmid using a polymerase chain reaction (PCR) based mutagenesis method. PCR was performed as follows: 95˚C for 2 min, 94˚C for 20 sec, 55˚C for 10 sec, 68˚C for 2.5 min for 18 cycles, 68˚C for 5 min. Briefly, the plasmid containing WT HERG cDNA was purified using Endo‑Free Plasmid Purification kit (Qiagen, Inc.) according to the manufacture's protocol, and used as the PCR template. The primers with the different mutation sites were added (sequences of primers are shown in Table 1), respectively. Amplification was performed using Fast Alteration DNA Polymerase, and a mutated plasmid with a gap was obtained. The methylated template plasmid was digested with DpnI, leaving the mutant plasmid only. Following transfer to the recipient Escherichia coli bacteria, the gap in the mutant plasmid was repaired. The mutant plasmid was obtained and the sequences of the mutants were subsequently verified using ABI PRISM 377 automated sequencer.

**Cell culture and transfection.** The 293 cells (American Type Culture Collection) were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), 1% glutamine, 100 IU/ml penicillin and 100 µg/ml streptomycin at 37˚C in an incubator in a humidified atmosphere of 5% CO₂/95% air. Transfections were performed with Effectene™ transfection reagent (Qiagen, Inc.) according to the manufacturer's protocol. The 293 cells were transiently transfected either with 0.4 µg WT or an equal quantity of mutant cDNA. The green fluorescent protein (GFP) gene (0.2 µg) was cotransfected as an indicator during patch‑clamp recording. The transfection efficiency was ~65% (Fig. 1A‑C). Pharmacological rescue was examined 24 h after adding G418 (400 µg/ml) into DMEM.

**Immunofluorescence and confocal imaging.** An immunofluorescence method was used to observe the protein expression of HERG prior to and following drug treatment. The 293 cells were washed three times with PBS and were subsequently soaked in 4% paraformaldehyde for 30 min at room temperature, followed by washing with PBS a further three times. Following permeabilization with 0.5% Triton X‑100 for 20 min, the cells were incubated in antisera against the N- or C‑terminal antibody of HERG protein (Alomone, cat. nos. APC‑062 and APC‑109, respectively; 1:400 dilution), initially at 37˚C for 1 h, and then at 4˚C overnight. Subsequently, the cells were washed three times in PBS and incubated with antirabbit Cy3‑conjugated secondary antibody (Beyotime Institute of Biotechnology, P0183; 1:500 dilution) at 37˚C for 30 min, followed by three washes with PBS. Finally, cells were incubated with DAPI (Haoxin Biotech, Inc. or Beyotime Institute of Biotechnology) for 5 min and then were washed three times in PBS. Confocal images were captured using a confocal laser scanning microscope (Zeiss LSM510; Zeiss GmbH, Jena, Germany) and analyzed using Velocity® Demo software (version 6.0; PerkinElmer, Inc.).

**Patch‑clamp recording.** The wholecell patch‑clamp method was used in experiments to record the HERG current. The bathing solution contained the following: 136 mM NaCl, 5.4 mM KCl, 0.33 mM NaH₂PO₄, 2H₂O, 1.8 mM CaCl₂, 1 mM MgCl₂, 6H₂O, 10 mM glucose and 10 mM HEPES. The internal pipette solution contained the following: 140 mM KCl, 0.5 mM MgCl₂, 6H₂O, 5 mM HEPES, 2 mM EGTA and 4 mM K₃ATP. The HERG current was activated by a depolarizing pulse (for 4 sec) to 50 mV from the holding potential of -80 mV. The tail current of the HERG channel was recorded following repolarization to -50 mV for 4 sec. All patch‑clamp experiments were performed at room temperature (22±1˚C).

**Statistical analysis.** All results are expressed as the mean ± SEM, and the data were analyzed using SPSS software, version 17.0 (SPSS, Inc., Chicago, IL, USA). For statistical analyses, Student’s t‑test was performed for comparisons between two data groups. For comparisons of data among more than two groups, one‑way analysis of variance was used, followed by Bonferroni post‑hoc test. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**Construction of HERG nonsense mutants containing different stop codons and different downstream nucleotides.** The present study aimed to investigate whether different types of stop codon and the downstream nucleotide context, can influence the ability of aminoglycosides to efficiently suppress PTCs. To accomplish this, R1014X and W927X mutants, containing TGA, TAG and TAA, were constructed using a siteselect directed mutagenesis technique. Based on the R1014X‑TGAT mutant,
R1014X-TGAA, R1014X-TGAC and R1014X-TGAG mutants were also constructed for the present study (Fig. 2A and B).

Effect of PTCs on the G418-mediated suppression of the R1014X and W927X mutants. Using an immunofluorescence method, the protein expression of HERG in 293 cells transfected with the respective R1014X mutants was detected. Protein expression was detected in the control group (transfection reagent only) and the HERG-WT group prior to and following G418 treatment in 293 cells (Fig. 3). No red fluorescence was detected in the control group with or without G418 treatment (Fig. 3A-D). In the WT group, red fluorescence was detected when antibody against the N-terminal (Fig. 3F), or C-terminal (Fig. 3G) were used prior to G418 treatment, but not when no antibody was added (Fig. 3E). Following treatment with G418, red fluorescence was also detected following addition of the anti-C-terminal antibody (Fig. 3H). An antibody raised against the N-terminal sequence of HERG protein was incubated with the protein prior to the addition of G418. As shown in Fig. 4A-C, red fluorescence was detected in all three groups of cells (i.e., transfected with R1014X-TGA, R1014X-TAA or R1014X-TAG mutant, respectively). By contrast, no red fluorescence was detected when an antibody raised against the C-terminus of the HERG protein was used (Fig. 4D-F). However, following treatment with G418 (400 µg/ml), red fluorescence was observed in each group following incubation with the anti-C-terminal antibody (Fig. 4G-I).

Ionic currents were also recorded for the 293 cells transiently transfected with WT cDNA and R1014X mutant containing the different stop codons (Fig. 5A). The mean peak tail current densities of R1014X-TGA (4.82±0.42 pA/pF; n=8), R1014X-TAA (5.31±0.62 pA/pF; n=6) and R1014X-TAG (4.51±0.68 pA/pF; n=6) were all lower compared with those of the WT channel (46.69±3.74 pA/pF; n=6; P<0.05). However, no significant differences were observed among these three mutants. An increase in the peak tail current was only identified with the R1014X-TGA (21.64±1.94 pA/pF; n=6; P<0.05).

Table I. Primer sequences of the site-directed mutants.

| Site-directed mutant | Primer sequence |
|----------------------|-----------------|
| R1014X-TGAT          | F: 5'-GTACCAGGAGCTCCCTTGTGCGCCCGCCGCCC-3' |
|                      | R: 5'-GGGGGCGGGGCATGAGGTGTCTGCGCTGAC-3' |
| R1014X-TAAT          | F: 5'-GTACCAGGAGCTCCCTTAAAGGGAGGTGTCTGAC-3' |
|                      | R: 5'-GGGGGCGGGGCATGAGGTGTCTGCGCTGAC-3' |
| R1014X-TAGT          | F: 5'-GTACCAGGAGCTCCCTATGGGAGGTGTCTGAC-3' |
|                      | R: 5'-GGGGGCGGGGCATGAGGTGTCTGCGCTGAC-3' |
| R1014X-TGAC          | F: 5'-GTACCAGGAGCTCCCTTAAAGGGAGGTGTCTGAC-3' |
|                      | R: 5'-GGGGGCGGGGCATGAGGTGTCTGCGCTGAC-3' |
| R1014X-TGAG          | F: 5'-GTACCAGGAGCTCCCTTAAAGGGAGGTGTCTGAC-3' |
|                      | R: 5'-GGGGGCGGGGCATGAGGTGTCTGCGCTGAC-3' |
| W927X-TGAG           | F: 5'-CGGGCGGGAGCTCCCTTAAAGGGAGGTGTCTGAC-3' |
|                      | R: 5'-CGGGCCTTCTCCCTACGCGCCCCCCC-3' |
| W927X-TAAG           | F: 5'-CGGGCGGGAGCTCCCTTAAAGGGAGGTGTCTGAC-3' |
|                      | R: 5'-CGGGCCTTCTCCCTACGCGCCCCCCC-3' |
| W927X-TAGG           | F: 5'-CGGGCGGGAGCTCCCTTAAAGGGAGGTGTCTGAC-3' |
|                      | R: 5'-CGGGCCTTCTCCCTACGCGCCCCCCC-3' |

F, forward; R, reverse.

Figure 1. Transfection efficiency of 293 cells. (A) Cells under the light microscope. (B) Expression in the nucleus using DAPI. (C) Cells successfully transfected with GFAP and wild-type plasmids. Scale bar, 100 µm.
STOP CODONS AND THE +4 NUCLEOTIDE INFLUENCE THE EFFICIENCY OF G418

and R1014X-TAA (23.19±3.27 pA/pF; n=6; P<0.05) mutants, and not with the R1014X-TAG mutant, subsequent to the rescuing effect of G418 (Fig. 5B).

To further validate these phenomena, the same treatment was applied to W927X mutants, which featured the PTCs of TGA, TAA and TAG, respectively. The immunofluorescence results (Fig. 6A-I) revealed that red fluorescence was detected in all three mutants bearing the different types of PTC following the administration of G418 using the antibody raised against the C-terminus (Fig. 6). The patchclamp results suggested that the mean peak tail electric current density was increased in 293 cells transfected with W927X-TGA, W927X-TAA and W927X-TAG following treatment with G418. However, the increase in current density with W927X-TAG was smaller compared with that with the other two mutants (Fig. 7A and B; P<0.05).

Role of the tetranucleotide termination signal on the rescuing effect of G418.

To observe the impact of the local sequence context surrounding the PTC, the predominant focus of the present study was on the +4 nucleotide, and based on the R1014X-TGAT mutant, R1014X-TGAC, R1014X-TGAG and R1014X-TGAA mutants were constructed. Prior to the addition of G418, the N-terminal antibody was used to observe the protein expression of HERG among the groups. Using the immunofluorescence method, red fluorescent protein was detected in each group (Fig. 8A-C). However, when the C-terminal antibody was used, no red fluorescence was detected (Fig. 8D-F). The functional expression of
HERG protein in each group without drug treatment was subsequently tested by patch-clamp recording. On comparing the peak tail current densities, no significant differences were identified among the groups (5.58±1.33, 5.16±0.99 and 3.80±1.08 pA/pF for the R1014X-TGAC, R1014X-TGAG and R1014X-TGAA mutants, respectively; Fig. 9A and B).

Following G418 treatment, red fluorescence was detected in all groups using the C-terminal antibody (Fig. 8G-I). For the group transfected with R1014X-TGAT, the peak tail current density was increased in the cells treated with G418 (22.96±1.75 pA/pF; n=5; P<0.05) compared with that in the untreated cells (4.54±0.51 pA/pF; n=6), although
this was still lower compared with that of the WT channel (47.83±4.31 pA/pF; n=5). In the other groups transfected with R1014X-TGAC, R1014X-TGAG or R1014X-TGAA, the tail current density did not alter significantly upon treatment with G418 (5.58±1.33 vs. 5.45±1.84 pA/pF, 5.16±0.99 vs. 5.65±1.50 pA/pF and 3.80±1.08 vs. 5.84±1.46 pA/pF, respectively; all P>0.05; Fig. 9A and B).

Discussion

In the present study, to investigate how stop codons and the +4 nucleotide may influence the efficiency of G418 in rescuing nonsense mutations of the HERG gene, the possible impacting factors were examined, such as the type of PTC and the nucleotide sequences immediately downstream of them. It was revealed that the stop codons TGA and TAA were rescued more easily by the aminoglycoside compared with TAG. In addition, the +4 nucleotide influenced the function of HERG protein following the rescuing effect of the aminoglycoside. This is the first time, to the best of our knowledge, that the effect of the intrinsic sequence on the nonsense mutation rescue of the HERG gene has been observed. The HERG gene was a particularly good candidate for PTC suppression, as it has a higher frequency of nonsense mutations than other genes associated with arrhythmia and sudden death, the majority of which are inactivated by missense mutations.

The read-through effect on nonsense mutations to produce full-length functional proteins has been demonstrated for aminoglycosides in numerous genetic disease models, including Duchenne muscular dystrophy (DMD) and ocular genetic diseases (19,20). In our previous study, the expression of WT and R1014X mutant in 293 cells was confirmed by western blotting and PCR following the transfection of corresponding plasmids, respectively. It was also demonstrated that G418 and gentamicin partially restored the HERG-like currents in 293 cells expressing W927X and R1014X mutants (15). It has also been reported that the type of stop codon can influence the rescuing efficiency of an aminoglycoside. Gentamicin and G418 enabled aminoacyl-tRNA to read through UGA more easily than other PTCs in disease models associated with APC or p53 gene nonsense mutations (18,21). In clinical trials associated with DMD, upon administration of gentamicin, the level of the myotubes was upregulated more markedly in patients with the PTC of UGA, followed by UAA and UAG (22). In the present study, the results confirmed that G418 suppressed UGA and UAA, and the IKr current was restored more readily for these PTCs than for UAG in HERG nonsense mutations.

The context of the stop codon and its surrounding sequences influence the efficiency of translation termination,
Figure 7. Ionic current recorded from W927X mutant HERG channels before and after G418 treatment in transfected 293 cells. (A) Representative families of current traces recorded from cells transfected with WT or the three mutant HERG cDNAs. (B) Peak tail currents from cells transfected with the W927X mutants featuring the different stop codons. *P<0.05. HERG, human ether-a-go-go-related; WT, wild-type.

Figure 8. Protein expression in cells transfected with HERG-R1014X mutant containing different +4 nucleotides downstream of the stop codon TGA before and after G418 treatment. Antibody against the N-terminus of the protein was used before G418 was added to mutants with the +4 nucleotides (A) T, (B) G, (C) A and (D) C, respectively. Antibody against the C-terminus of the protein was used before mutants with the +4 nucleotides (E) T, (F) G, (G) A and (H) C were treated with G418. (I-L) Following treatment with G418, anti-C-terminal antibody was used for the (I) T, (J) G, (K) A and (L) C mutants. Red fluorescence represents expression of the HERG protein. Scale bar, 100 µm. HERG, human ether-a-go-go-related.
Nonsense-mediated decay (NMD) is an RNA quality control mechanism that selectively degrades mRNA harboring PTCs. NMD eliminates abnormal mRNA transcripts harboring PTCs, thereby preventing the production of truncated proteins that often have dominant-negative effects (27). However, the role of NMD in the majority of eukaryotic cells requires at least one intron downstream of the PTCs and the corresponding splicing process. Pre-mRNA splicing results in the deposition of a multi-protein complex, known as the exon-junction-complex (EJC), 20-24 nt upstream of each exon-exon junction. The EJCs are displaced by the ribosome during the pioneer round of translation. If translation terminates at a PTC that is located 50-55 nt upstream of an exon-exon junction, the downstream EJC serves as a binding platform for NMD factors that trigger NMD. If no intron exists, the mRNA can escape NMD. In the present study, cDNA of the HERG gene containing exons was used to rule out the interference of NMD on the experimental results.

The efficiency of translation termination is also affected by upstream and downstream sequences around the PTC, particularly by the +4 nucleotide (28,29). When an adenine nucleotide is located at this position, the highest rescuing efficiency may be detected, followed by a pyrimidine nucleotide at the same position (30-32). Therefore, it was hypothesized that the local sequence context featuring the +4 nucleotide is also involved in the process of translation termination, in addition to the PTC. Previous studies on DMD and other disease models have confirmed the important influence of the +4 nucleotide (33,34). In a QX (N) expression system, it was revealed that the stop codon had a close association with the +4 nucleotide (35). Gentamicin and other aminoglycosides were observed to have different rescuing efficiencies with different types of nucleotide at the +4 position. When the stop codon was TGA, the effectiveness of the +4 nucleotide was in the order of C > T, A > G (35).

The results of the present study suggested that the +4 nucleotide downstream of the stop codon had a significant impact on the functional expression of HERG protein following the rescue of readthrough by the drug. When the original base T was mutated to C, G or A, respectively, red fluorescence remained apparent following the addition of an antibody against the C-terminus of HERG following G418 treatment, indicating expression of the intact HERG protein. However, the peak tail current did not alter significantly following the administration of G418. Amino acid sequence analysis predicted that nucleotide mutation results in the original-site cysteine (corresponding to the nucleotide sequence) being mutated to arginine (corresponding to nucleotide sequence, cgc), serine (corresponding to nucleotide sequence, age) or glycine (corresponding to the codon, ggc). Therefore, a mutation at the +4 nucleotide base position altered the amino acid sequence coded by the nucleotides downstream of the stop codon upon intervention with G418. In this manner, no functional integral protein can be produced. Notably, these results differed from previous results obtained in other disease models (31,34). Due to the lack of suitable technology, it is not possible to analyze and predict the amino acid that is inserted into the site of the PTC. However, the results of our previous study demonstrated that certain HERG nonsense mutants, such as R863X, E698X and W412X, positioned close to the Nterminus, were not rescued upon treatment with an aminoglycoside (15); therefore, the amino acid may differ from the...
original amino acid upon rescue with G418. If the mutation is located in the domain of the HERG gene that is most critical for protein structure, the conformation of the HERG protein may be altered upon rescue with an aminoglycoside, leading to its function not being restored. However, further investigations are required in order to elucidate the specific mechanism.

In conclusion, the present study demonstrated that the type of PTC, and the context of the nucleotides downstream of it, may determine the pharmacological rescue efficiency of the HERG gene. TGA and TAA were more easily rescued by the aminoglycoside compared with TAG. Furthermore, the +4 nucleotide was found to influence the function of the HERG protein following the rescuing effect of the aminoglycoside. By identifying the type of stop codon and the following sequence, clinicians can establish patients who are more sensitive to nonsense mutation rescue therapy among those with LQT2 in the future. Pacemaker implantation, which is expensive and traumatic, may be avoided in these individuals. However, these findings highlight the fact that the mechanism of aminoglycosidemediated suppression in mammalian cells remains to be fully elucidated.

In the present study, GFP and the target gene were not placed in the same plasmid. Therefore, it was only possible to demonstrate successful transfection of the target gene through the expression of GFP indirectly. However, by recording the HERG current and using an antibody against the target gene, the expression of the target gene was confirmed. Additionally, transfection of the 293 cells in the experiments was performed with Effectene transfection reagent according to the manufacturer's instructions. After 24 h, Pharmacological rescue was assessed by adding G418 (400 μg/ml) into DMEM for another 24 h. As there was a period of only 48 h after transfection, it was not possible to examine the stability of HERG protein in different groups. This is an important question and it is sensible to examine the stability of HERG protein with long-term drug administration in nonsense mutation animal models.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author upon reasonable request.

Authors’ contributions

HY performed the patch-clamp experiment, analyzed data and was a major contributor in writing the manuscript. YM performed the patch-clamp experiment, analyzed data and was a major contributor in writing the manuscript. FW, NL and QL analyzed and interpreted the data collected from patch-clamp experiments. YJ contributed to improvements in the manuscript and provided financial support for the study. JP made substantial contributions to conception and design and provided financial support for the study. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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