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ORIGINAL ARTICLE

Functional analysis of *KCNH2* gene mutations of type 2 long QT syndrome in larval zebrafish using microscopy and electrocardiography

Yoshihiro Tanaka[1] · Kenshi Hayashi[1] · Noboru Fujino[1] · Tetsuo Konno[1] · Hayato Tada[1] · Chiaki Nakanishi[1] · Akihiko Hodatsu[1] · Toyonobu Tsuda[1] · Yoji Nagata[1] · Ryota Teramoto[1] · Shohei Yoshida[1] · Akihiro Nomura[1] · Masa-aki Kawashiri[1] · Masakazu Yamagishi[1]

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Abstract
Heterologous expression systems play a vital role in the characterization of potassium voltage-gated channel subfamily H member 2 (*KCNH2*) gene mutations, such as E637K which is associated with long QT syndrome type 2 (LQT2). In vivo assays using zebrafish provide a means for testing genetic variants of cardiac disease; however, limited information on the role of the E637K mutation is available from in vivo systems and their utility has yet to be fully exploited in the context of LQT2. We sought to evaluate the ability of the E637K mutant channel to restore normal repolarization in larval zebrafish with a human *KCNH2* orthologue, *kcnh2a*-knockdown. A morpholino (MO) targeting *kcnh2a* was injected alone or with wild type (WT) or E637K KCNH2 cRNA into zebrafish embryos at the 1–2 cell stage. Cardiac repolarization phenotypes were screened using light microscopy and the QT interval was measured by single lead electrocardiograph (ECG) analysis at 72-h post-fertilization. In the MO alone group, 17% of zebrafish had a normal phenotype; this rate increased to 60% in the WT KCNH2 cRNA injected zebrafish and to 35% in the E637K injected zebrafish. The ECG of larval zebrafish revealed that QTc was significantly prolonged in the MO alone group compared to the control group. Co-injection of WT KCNH2 cRNA shortened the QTc interval, however, that of the E637K did not. We suggest that this in vivo cardiac assay using microscopy and ECG in larval zebrafish offers a reliable approach for risk discrimination of *KCNH2* mutations.

Keywords Long QT syndrome · KCNH2 · In vivo cardiac assay · Zebrafish · Electrocardiography

Introduction

Congenital long QT syndrome (LQTS) is an inherited heart disease that causes syncope, torsade de pointes, and sudden cardiac death. LQTS is classified into 15 different subtypes (LQT1–LQT15) [1–4] and LQT2 is reported to be the second most common variant of LQTS and accounts for approximately 30% of mutation-positive LQTS [5]. The causal gene of LQT2 is *KCNH2* which is the official symbol for the *human ether-a-go-go related gene* (*hERG*). *KCNH2* encodes the pore-forming subunit of a voltage-gated K^+ channel with characteristics similar to those of the rapidly activating delayed rectifier K^+ current (I_{kr}) [6]. Identification of *KCNH2* mutations are important because a reduction in I_{kr} current causes QT interval prolongation, which can result in torsade de pointes and ventricular fibrillation depending on the severity of the *KCNH2* mutation.

Currently, the gold-standard for functional analysis of LQTS mutations is electrophysiological measurement by a patch-clamp method in cell expression systems such as HEK293 or CHO cells [7, 8]. Although these heterologous expression systems (HES) are useful for studying the physiological function of the ion channel and pathophysiological changes in gene variants, the advantage of HES is limited because the heterologous cellular signaling components might be different from those of cardiomyocytes [9]. Therefore, the need for an in vivo cardiac assay has risen in recent years [10, 11].

✉ Kenshi Hayashi
kenshi@med.kanazawa-u.ac.jp

[1] Department of Cardiovascular and Internal Medicine, Graduate School of Medical Science, Kanazawa University, 13-1 Takara-machi, Kanazawa, Ishikawa 920-8640, Japan

Zebrafish is an emerging model for studying LQTS in addition to the standard cell expression systems as their cardiomyocytes include the orthologs of genes involved in cardiac ion channels in humans [12]. For example, *kcnh2a* is orthologous to *KCNH2* in humans and essential for cardiac repolarization in the embryonic zebrafish ventricle. Previous work showed that complete loss of functional I_{Kr} in embryonic hearts lead to ventricular depolarization, inability to generate action potentials, and a submaximal I_{Kr} blockade of prolonged ventricular action potential duration, which caused a 2:1 atrioventricular block (AVB) [13]. An LQT2 model in larval zebrafish has been established using a *kcnh2a*-specific morpholino oligonucleotide (MO) and the morphological and electrophysiological changes have been evaluated at early stages post-fertilization [48–72 h post-fertilization (hpf)] [11]. Repolarization phenotypes can be classified into three categories depending on their severity: normal conduction, 2:1 AVB, or ventricular asystole (VA) [11, 13]. Microscopic assessment can be used to evaluate and categorize repolarization phenotypes. Dhillon et al. used ECG measurements in drug-induced QT prolongation in a larval zebrafish model and reported that QT interval evaluation was possible even at early stages of larval development [12].

We previously reported the electrophysiological characteristics of a *KCNH2* gene mutation (E637K) in patients with Type 2 LQTS. The E637K mutation was found in a family that exhibited significant QT prolongation and repetitive syncope due to torsades de pointes (TdP) [7]. Cellular electrophysiological studies using HES demonstrated that E637K has a dominant negative suppression on the wild type (WT) hERG channel. The purpose of this study was to elucidate the utility of the LQT2 larval zebrafish model for functional analysis of the E637K mutation using microscopy and electrocardiography (ECG).

Materials and methods

Ethics statement

This research protocol was approved by the Bioethical Committee on Medical Researches of Kanazawa University School of Medicine (Kanazawa, Japan), and written informed consent was obtained from all subjects or their guardians.

Animal model

Zebrafish were raised and maintained at 28 °C on a 14-h light/10-h dark cycle. The hSPGFF3A zebrafish strain expresses the green fluorescent protein (GFP) in its heart tissue and was used in this experiment [14, 15]. Embryos were collected after mating at 9 o'clock in the morning and then transferred to E3 embryo medium for zebrafish. They were maintained in an incubator at 28 °C and medium was changed daily until 72 hpf.

Morpholino design and construction of KCNH2 cRNA

We used an MO targeted against the translational site of human *KCNH2* orthologue, *kcnh2a* with the following sequence: 5′-CGCGTGGACAGATTCAAGAGCCCT C-3′ (Gene Tools LLC, Philomath, OR). The MO does not interfere with the translation of co-injected KCNH2 RNA because the construct contains a 5′-untranslated region (UTR) β-globin sequence.

KCNH2 cDNA in the PGH19 vector was provided by Dr. Gail Robertson (University of Wisconsin). *KCNH2* E637K cDNA was constructed by an overlap extension strategy [7, 15]. Wild type (WT) *KCNH2* cDNA and E637K cDNA were linearized by digestion with NotI. Capped and tailed RNAs were synthesized using a mMessage mMachine T7 Ultra kit (Life Technologies, Carlsbad, CA) and were purified using the MEGAclear™ Kit (Life Technologies, Carlsbad, CA).

Injection of *kcnh2a* MO and KCNH2 cRNA into zebrafish embryos

In a preliminary study, we optimized the amount of MO for use in microinjection. An adequate repolarization phenotype was identified microscopically after microinjection of 2 ng MO. Zebrafish embryos (hspGFF3A strain) at the 1–2 cell stage were microinjected with *kcnh2a* ATG MO alone or co-injected with WT or mutant KCNH2 cRNAs. A five-mismatch control MO (mm MO, sequence: 5′-CGC CTGCACACATTCAACACCCCTC-3′) was injected in the same manner as a control (Fig. 1). The experiments were repeated more than three times on separate days. After injection, embryos were raised in an incubator at 28 °C. Cardiac repolarization phenotypes (normal conductions, AVB, or VA) were evaluated using light microscopy (Nikon, SMZ 745T, Tokyo, Japan) at 72 hpf at room temperature. At least 50 embryos were screened and evaluated in each group.

Anesthesia

Zebrafish was anesthetized with 100–250 mg/L tricaine for ECG recording as previously described [16]. In contrast, we did not use tricaine in the microscopic evaluation of the repolarization phenotype because tricaine can act as a cardiodepressant and reduce the heart rate.

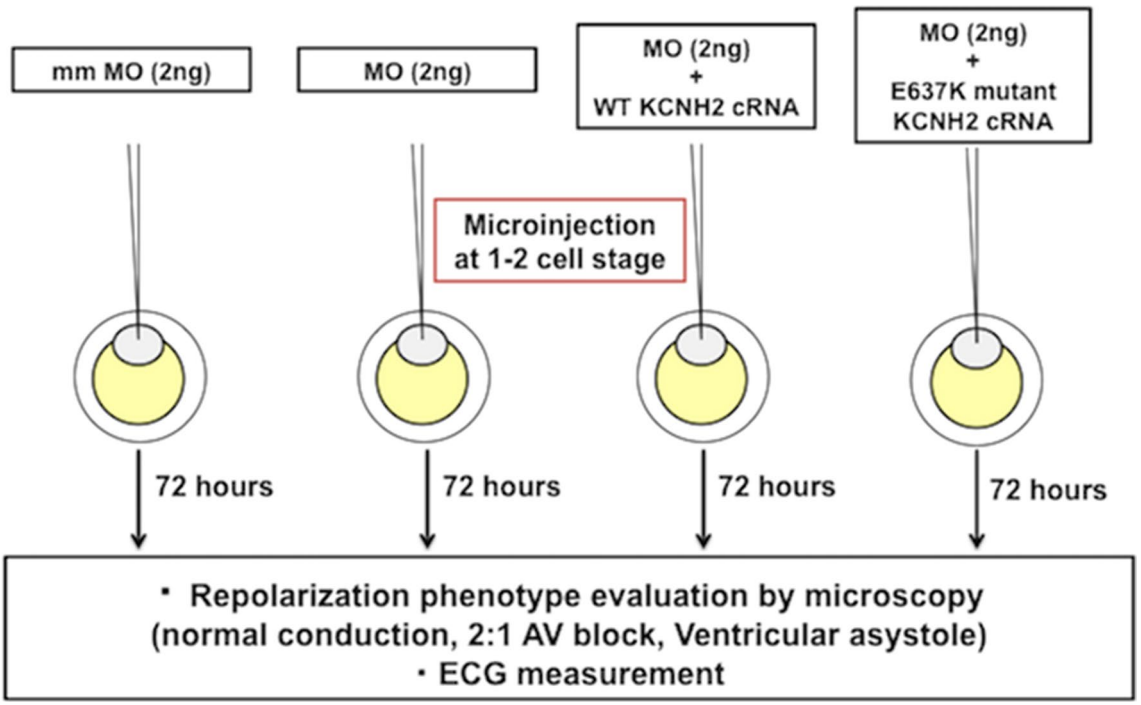

Fig. 1 Injection and measurement schematic for analyzing *KCNH2* gene mutations of type 2 long QT syndrome in a larval zebrafish model. Morpholino oligonucleotide (MO) was injected alone or with either wild type (WT) KCNH2 cRNA, or E637K mutant KCNH2 cRNA. MO against *kcnh2a,* the *KCNH2* orthologue in humans, was used in this study and a 5-mismatch control MO (mm MO) was also injected as control. After microinjection, embryos were raised in the incubator at 28 °C for 72 h. The repolarization phenotype and electrophysiological characteristics were evaluated using microscopy and electrocardiography (ECG) at 28 °C

ECG recording

ECG recording was performed as described previously [12]. The embryo (3 dpf) was positioned ventrally within a groove in a 3% agarose gel and the tips of glass capillaries (GD-1, Narishige, Tokyo, Japan) were positioned on the skin surface in the middle of the ventricle and atrium using manipulators (MMN-8, Narishige, Tokyo, Japan), which were then observed under a microscope (SMZ 745T, Nikon, Tokyo, Japan) equipped with a video recording device (EOS-ID X, Canon, Tokyo, Japan). The capillaries were filled with 3 M potassium acetate solution (Sigma-Aldrich, St. Luis, USA) and methylene blue (Sigma) to easily visualize the tips during recording. A chloridized silver wire that carried the electrical signals to the amplifier was inserted into the capillaries and a second reference electrode was placed in the surrounding gel during recordings. The differential amplifier (Axopatch 200A, Molecular Devices, CA) used for recording was operated in DC mode with the high pass filter set at 0.1 Hz. The raw ECG signals were digitized (PowerLab; ADI Instruments, Dunedin, New Zealand) and viewed using LabChart 7 (ADI Instruments). All the recording equipment was housed on an air table within a grounded Faraday cage to minimized background noise. All experiments were performed at room temperature (22 °C). For the optimization experiments, ECG recordings were taken for up to 5 min. To verify the diagnostic values of ECGs in the larval zebrafish, we simultaneously contrasted the heart movements and electrophysiological activities using a live-mode camera (EOS-ID X, Canon, Tokyo, Japan) and ECGs.

ECG data analysis

ECG analyses were performed for the normal conduction phenotype of each group. Analysis of the digitized ECG was carried out using LabChart. More than 20 waves were averaged to produce a signal average ECG and the mean intervals for the ECG parameters such as heart rate and QT interval were measured. The QT interval was manually measured with the tangent method, which is defined as the time between the beginning of the QRS complex and the point at which the line of the maximal downslope of the T wave crosses the isoelectric line. QTc was corrected by Fridericia's formula. Two independent cardiologists (Y.T. and K.H.) evaluated all signal averaged ECGs. They independently defined the P wave, QRS complex, and T wave in each ECG and measured QT interval manually, producing consensus reports afterward that were evaluated in the present study. Finally, independent QT intervals were averaged for the calculation of QTc.

Statistical analysis

Statistical analysis was performed using JMP® 11 (SAS Institute Inc., Cary, NC, USA). Continuous data were expressed as mean ± standard deviation (SD). Continuous variables were compared with an independent Student's *t* test. For the microscopic assessment, the distribution patterns of repolarization phenotypes were evaluated with Fisher's exact test with the MO alone group defined as a reference. The QTc interval of each group was compared with Tukey's HSD method. A p value < 0.05 was considered statistically significant.

Results

Microscopic evaluation of the repolarization phenotype in larval zebrafish at 72 hpf

The distribution patterns of repolarization phenotypes in each morphant group are presented in Fig. 2. The five-mismatch control (the mm MO) group had normal conduction in 100% of the larval zebrafish, but there were significant differences among the other four groups for the rate of normal conductance ($p < 0.05$). VA was more frequently observed in the MO alone group compared to the mm MO group (74.6 vs 0%, $p < 0.01$). The rate of VA was reduced by co-injection of WT KCNH2 cRNA from 74.6 to 23.8% ($p < 0.01$) with a concomitant increase in normal conduction (60%). Interestingly, in the MO + E637K KCNH2 injection group the rate of normal conduction significantly decreased from 60 to 35.2% compared to the MO + WT KCNH2 cRNA group ($p < 0.01$).

Evaluation of heart rate and QT interval in larval zebrafish ECGs

ECG measurements were performed on larval zebrafish at 72 hpf. A representative digitized ECG is shown in Fig. 3a. The ECG consisted of three major components including the P wave, QRS complex, and T wave. Signal averaging was performed to eliminate the baseline fluctuation (Fig. 3b). QT interval was measured using the tangent method as shown in Fig. 3b and was found to be considerably prolonged in the MO alone group and the E637K KCNH2 + MO group compared to the mm MO group. In contrast, QT interval in the WT KCNH2 + MO group was almost the same level as that of the mm MO group.

Heart rates in the mm MO group (114 ± 18 bpm) and WT KCNH2 groups (121 ± 10) were higher than in the MO group (110 ± 15 bpm) and E637K KCNH2 (109 ± 29 bpm), but there was no statistical difference between each group.

The QT and QTc measurements were performed in the normal conduction phenotype of each group. The inter-observer correlation coefficient for QT intervals was 0.98, and the absolute value of the QT interval difference between the two observers was 5 ms (interquartile range 1–14). The QT intervals were 350 ± 66 ms in the control group ($n = 22$), 432 ± 67 ms in the MO alone group ($n = 10$), 320 ± 20 ms in the MO + WT KCNH2 group

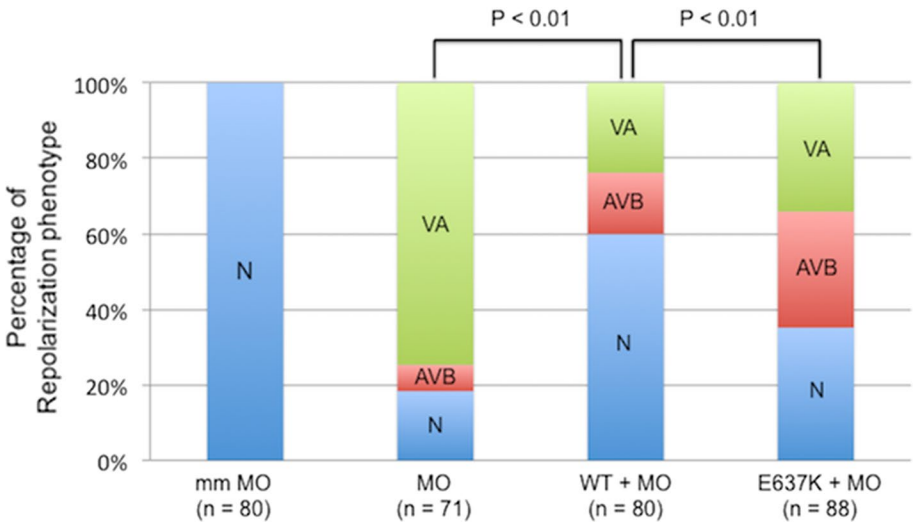

Fig. 2 Assessment of the repolarization phenotype in larval zebrafish using microscopy. Repolarization phenotype [normal conduction, 2:1 atrioventricular block (AVB), or ventricular asystole (VA)] was evaluated using microscopy at 72-h post-fertilization (hpf). Normal conduction was always observed in the control group, whereas AVB and VA were observed in the MO alone group, the MO + WT KCNH2 cRNA injected group, and the MO + E637K injected group depending on the severity of the repolarization abnormality. The distribution patterns of repolarization phenotypes were statistically evaluated with Fisher's exact test with the MO alone group used as the reference

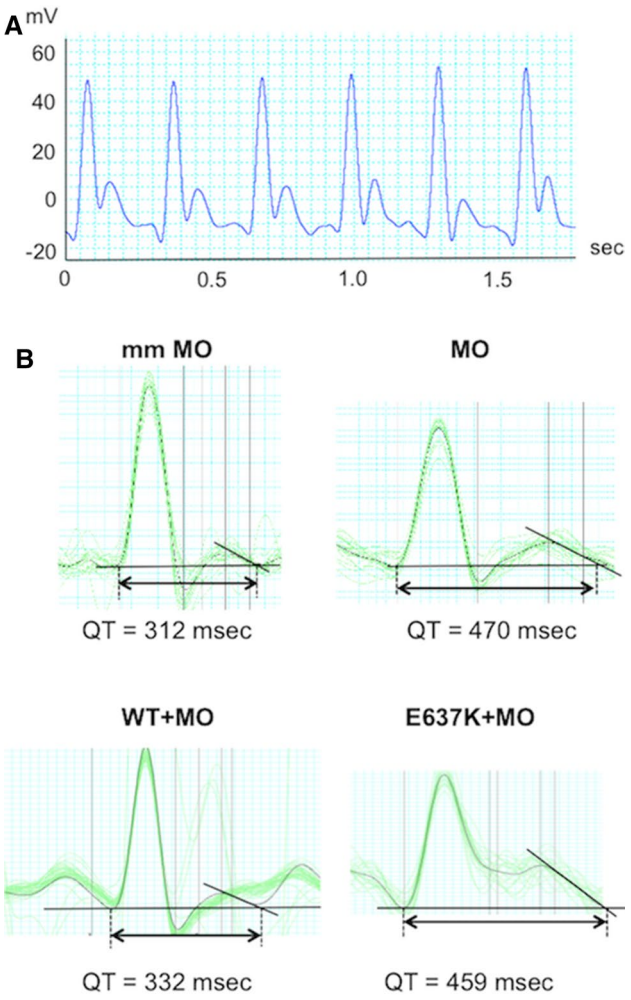

Fig. 3 A representative digitized electrocardiogram (ECG) and signal averaged electrocardiogram (SAECG). Raw ECG signals were digitized using PowerLab and viewed using LabChart 7. The digitized ECG consists of three major components (P wave, QRS complex, and T wave). **a** A baseline fluctuation was observed in the digitized ECG and the end of the T wave was difficult to define because amplitudes and shapes of the T waves varied from beat to beat. **b** An SAECG stabilized the baseline, making it possible to define the end of T wave clearly. The QT duration was measured in each group for the evaluation of repolarization abnormalities

($n = 5$), and 430 ± 113 m in the MO + E637K KCNH2 group ($n = 6$). The QTc distributions for each group are summarized in Fig. 4. QTc in the MO alone group (525 ± 69 ms) was significantly longer than that in the mm MO group (431 ± 73, $p < 0.01$) and MO + WT KCNH2 (403 ± 25 ms, $p < 0.05$) groups. Co-injection of WT KCNH2 shortened the QTc interval to the control level. In contrast, co-injection of E637K KCNH2 (524 ± 144 ms) did not shorten the QTc duration, which was longer than that of the MO + WT KCNH2 group.

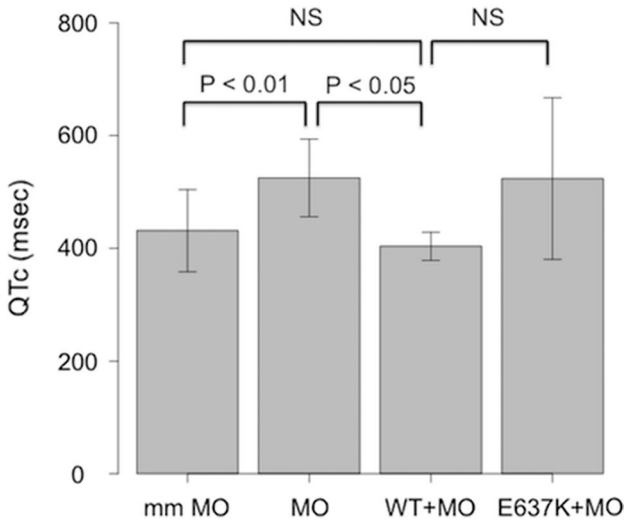

Fig. 4 Comparison of QTc interval in mm MO, MO, WT KCNH2 + MO, and E637K KCNH2 + MO group. The QTc was measured for the normal conduction phenotype. The QTc in MO alone group was significantly longer than that in the mm MO group ($p = 0.02$). In contrast, QTc in the MO + WT KCNH2 cRNA injected group recovered to a level comparable to the mm MO. The QTc in the MO + E637K injected group was longer than that measured in the MO + WT injected group, but was not statistically significant

Discussion

In this study, we evaluated the E637K *KCNH2* mutation that was identified in LQT2 patients with a severe phenotype using an in vivo zebrafish cardiac assay. The first point to note is that according to our microscopic repolarization analysis, the injection of a *kcnh2a* antisense MO into zebrafish embryos caused a severe repolarization phenotype that was rescued by the co-injection of WT KCNH2 cRNA, but not by the co-injection of the E637K KCNH2. The second important finding is that QTc measurements in zebrafish could discriminate between the mutant and wild type forms of *hERG*. In conclusion, these findings were consistent with both the clinical phenotype of the LQT2 family with E637K *KCNH2* and the cellular electrophysiological findings in HES. To our knowledge, this is the first study to perform a functional analysis of the *KCNH2* mutation E637K using LQT2 and ECG measurement in a larval zebrafish model. The difference between the in vivo zebrafish cardiac assay and HES is that the former evaluated the repolarization-deficient phenotype (AVB or VA) via light microscopy and the QTc of the larval zebrafish ECG, while the latter evaluated Kv11.1 current by whole-cell patch-clamp. We believe that these different methods are both useful and essential for evaluating the pathogenesis of the *KCNH2* mutation, because they can evaluate the cardiac repolarization through different mechanisms that complement each other.

Repolarization analysis using microscopy

Jou et al. reported the utility of microscopic repolarization analysis in *KCNH2* mutations using a larval zebrafish model of LQT2 [11]. According to their study, the rate of the normal conduction phenotype depended on the severity of the *KCNH2* mutation co-injected with MO. Indeed, the rate of normal conduction was 55% in zebrafish expressing WT *hERG*, which was slightly lower than the result of the present study (60%). The same trend was observed for E637K *hERG* mutants. The E637K mutation was identified in the pore-S6 loop of the transmembrane domain and was expected to have a considerably lower normal conduction rate (10–30%) than found in the study by Jou et al. [11]. However, our present study demonstrates that the normal conduction rate in E637K zebrafish was 35.2%. This difference can be explained by the difference the stage of development of the larvae: the study by Jou et al. was performed at 48 hpf, which was 24 h earlier than in the present study. Although the effect of MO persists for up to 5 days, its expression is time-dependent [17]; therefore, the effect of MO in the present study may be less than that in the study by Jou et al. However, the heart is known to develop to the adult level in zebrafish at 96 hpf, and the heart at 72 hpf is considered better for evaluation than that at 48 hpf given the stage of heart maturation. Furthermore, most embryos are still within an embryo sac at 48 hpf and come out of their sac by themselves at around 72 hpf. Therefore, the method described in the present study could distinguish the repolarization phenotype in each treatment group without artificial sac removal which may result in the physical stress on the embryos.

ECG evaluation in larval zebrafish

ECG analysis of adult zebrafish is an established method for the assessment of electrophysiology and drug-induced QT prolongation [18–20]. A distinct P wave, QRS complex, and T wave have been identified in adult zebrafish ECG recordings [19]. The heart rate and QT interval of adult zebrafish are 110–130 beats min^{-1} and 200–290 ms, respectively. It is important to optimize embryonic ECG measurement in zebrafish for high throughput and less laborious evaluation of drug-induced QT prolongation or MO induced gene knockdown. A recent study showed that ECG in the larval zebrafish is a viable alternative for evaluation of drug-induced QT prolongation [12]. The present study highlights the utility of ECGs for QT evaluation, not only in drug-induced QT prolongation, but also in a morphant (E637K *KCNH2*) at an early developmental stage in larval zebrafish. Additionally, we used a novel strategy to validate the linkage between ECG findings and actual heart movements by simultaneous video recording and ECGs. The QTc interval of the control zebrafish embryo was 431 ± 73 ms, which is comparable to that of humans between 360 and 440 ms. We measured the QT intervals using the tangent method and calculated the QTc with Fridericia's formula for the zebrafish embryo ECG. These approaches are typically used for measurement of ECGs in humans. The QTc durations in the four groups matched expectations and reflected the severity of the disease state. Tricaine was not used in the evaluation of the repolarization phenotype using microscopy, since tricaine might act as a cardiodepressant. However, a slight amount of tricaine was added during ECG recordings to immobilize the larval zebrafish during experimental manipulation. Therefore, electrophysiological data were affected by tricaine to some extent.

Our previous study using *Xenopus* oocyte HES showed that E637K did not express any functional channels [7]. Co-expression of wild type (WT) and E637K elicited only about 30% of the control peak tail current that was expected from expression of WT alone. Kinetic analyses revealed that E637K/WT co-expression decelerated the rate of channel activation, enhanced steady-state inactivation, and altered the K^+ permeability relative to Na^+ in the expressed currents. We speculate that E637 in the vicinity of the pore to lysine may cause conformational and geometric changes of the channel pore, resulting in the alteration in the voltage dependence of channel inactivation and ionic selectivity. Other reports showed an E637K mutation also caused a hERG trafficking defect [21, 22]. Taken together, these data indicate that E637K mutation results in changes in channel properties that can be attributed to multiple impairments including abnormal defective protein trafficking, gating/kinetics, and altered permeability [23]. Several studies showed specific trafficking defective LQT2 mutations that were rescued by lowering the incubation temperature, incubating with I_{Kr} channel blocking drugs, or incubating with a SERCA channel inhibitor such as thapsigargin. Previous work showed that thapsigargin did not rescue the dominant-negative effect of E637K Kv11.1 channel function [21].

There are limitations in this study. First, electrophysiological aspects of zebrafish cardiomyocytes seemed similar to those of humans. However, there are critical differences in the anatomy (single atrium and ventricle) and regeneration ability of zebrafish cardiomyocytes compared to those of humans. Second, the abnormal repolarization (VA and AVB), as observed via microscopic evaluation in larval zebrafish, was observed by 40% in the MO + WT KCNH2 cRNA group, though the repolarization phenotype may not be completely recovered. We conducted these experiments on the basis of previously described methods [11]. A previous study showed that the percent of embryos manifesting abnormal repolarization in the MO + WT KCNH2 cRNA group was similar (about 45%) to that of our study. In addition, we measured larval zebrafish ECGs in the normal

conduction phenotype, which showed that the QTc intervals of the MO + WT KCNH2 cRNA group were comparable to those of the mm MO group (403 ± 25 and 431 ± 73 ms, respectively) and were significantly shorter than those of the MO alone group (525 ± 69 ms, $p < 0.05$). Therefore, we believe that a line of rescue experiments with WT KCNH2 cRNA is sufficient to demonstrate that the MO antisense oligo experiment was reliable. Additionally, only one *KCNH2* mutation (E637K) was evaluated. Further studies are needed to confirm the utility of this new method employing other *KCNH2* mutations. Finally, we did not measure action potential duration (APD) to confirm that both 2:1 AVB and VA represent repolarization phenotypes in larval zebrafish. However, Jou et al. previously evaluated the association between APD and 2:1 AVB, and showed that APD was prolonged significantly in the 2:1 AVB group compared to the normal conduction group. Therefore, a validation study was considered to be unnecessary here.

Conclusions

These results demonstrate the functional abnormality of the E637K mutation in vivo. Functional analysis of *KCNH2* mutation using zebrafish embryos is useful for clear understanding of abnormal repolarization caused by this mutation and can compensate for the limitations of more conventional electrophysiological assessment.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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