The long QT syndrome type 2 (LQT2) is an inheritable life-threatening arrhythmic disorder and one of the most common genetic variants in long QT syndrome. There are some indications for treatment of the patients with LQT2 but it is impossible to completely prevent fatal arrhythmia. To develop novel therapy for the patients with LQT2, it has been desired to generate disease-specific and patient-specific disease model.

Human induced pluripotent stem (iPS) cells are somatic cell-derived pluripotent stem cells with infinite proliferation ability and multipotency. Patient-specific iPS cells can be derived from patient somatic cells, have all genomic information encoded in patient’s genome including mutation and all SNPs, and can be ideal disease models of the patients. To generate disease model for LQT2 by iPS cells, we should firstly generate iPS cells from the patient with LQT2 and confirm the genomic mutation in iPS cells. In this study, we showed the successful generation of iPS cells from a patient with KCNH2 G603D mutation. The patient specific iPS cells properly expressed stem cell markers, such as NANOG and OCT3/4. We also confirmed that the KCNH2 G603D (G1808A) mutation was taken over in patient specific iPS cells. These patient-specific iPS cells may contribute to the future analysis for disease pathogenesis and drug innovation.

Key words: iPS cell, Long QT syndrome, Disease modeling

Introduction

The generation of induced pluripotent stem (iPS) cell is firstly reported in 2006 with great surprise. Human iPS cells are similar to human embryonic stem (ES) cells in terms of proliferation and differentiation ability, and can be generated from adult somatic cells. Now we can easily generate iPS cells from patient’s somatic cells and those iPS cells have all genomic information of the patient genome. Many human diseases are caused by genomic mutation. Disease modeling using human iPS cells is newly emerged research field to analyze genetic human diseases. Actually, there are many fatal genetic diseases without effective therapy. To develop newly effective therapies for those diseases, first of all we have to generate disease models. In the past, there had been solely animal models of human genetic disease, such as specific gene knockout mice, transgenic mice and autochthonous diseased animals. Although those models gave us many valuable information regarding to the mechanisms of human genetic diseases, most crucial problem is that those models are not human. So it is often difficult to model human diseases using experimental animals. In another important point of view, among humans, each individual shows highly rich in genomic diversity in terms of racial differences and single nucleotide polymorphisms (SNPs). So it has been highly expected to generate not only disease-specific models, but also disease-specific and patient-specific disease models. To
generate patient-specific disease models, now we can use iPS cells.

Long QT syndrome is one of the most common fatal cardiac arrhythmic disorders. Recent molecular and electrophysiological examination established the fundamental disease concepts. But there is no definitive therapy invented so far. Here again, one of the most important points in drug discovery is to generate excellent disease models. Current experimental models for arrhythmic disorders mostly depend on animal model and heterologous expression system in human non-cardiomyocytes or non-human cardiomyocytes. The long QT syndrome type 2 (LQT2) is one of the most common genetic variants in long QT syndrome, and accounts for approximately 40% of genotyped patients. LQT2 is caused by mutation of a potassium channel gene, hERG (human ether-a-go-go related gene), now referred to KCNH2. To generate the physiological cardiac action potential in human cardiomyocytes, in addition to inward sodium and calcium currents, several potassium currents are notably involved. The inward-rectifier background current (IK1), the rapidly activating and inactivating transient outward current (Ito), and the ultrarapid (IKur), rapid (IKr), and slow (IKs) components of delayed rectifier currents. Those potassium currents have pivotal roles in electrophysiological homeostasis in human cardiomyocytes and the mutations in potassium current genes result in several human arrhythmic disorders. KCNH2 encodes the α-subunit of the IKr channel, and membrane depolarization induced by strong inward currents produces a sequence of conformation changes within the channel that allows permeation of potassium ions. As a clinical phenotype, LQT2 is likely to result in cardiac events during exercise or emotional stress in more than half cases and during rest or sleep in some cases. More specifically, an auditory stimulus (telephone, alarm clock, ambulance siren, etc) can be a specific trigger in LQT2. β-blocker use significantly reduces the risk of cardiac arrhythmic events in LQT2. And maintenance of the extracellular potassium concentration by long-term oral potassium supplementation is also reported to be effective because it shortens the QT interval in LQT2 patients. Besides those therapies, we cannot fully prevent sudden cardiac death in LQT2 patients. So we have to carry on the drug development for LQT2 by using LQT2 disease model.

In this study we showed the generation of iPS cells from a patient with KCNH2 G603D mutation. These patient-specific iPS cells may contribute to future analysis for disease pathogenesis and drug innovation.

**Materials and Methods**

**Patient consent**

All subjects provided informed consent for blood testing for genetic abnormalities associated with hereditary long QT syndrome. The isolation and use of patient somatic cells was approved by the Ethics Committee of Keio University (approval no. 20-92-5) and the Ethics Committee of Tokyo Medical and Dental University (approval no. 2009-27), and was performed only after the patient and the parent had provided written informed consent.

**Generation of human iPS cell**

Human iPS cells were established from T lymphocytes as described previously. Briefly, peripheral blood mononuclear cells (PBMCs) were separated by the centrifugation of heparinized whole blood sample obtained, using a Ficoll-Paque PREMIUM (GE Healthcare) gradient. The mononuclear cells were seeded on the anti-human CD3 antibody (BD Pharmingen)-coated 6-well plates in 2 mL GT-T502 (KOJIN BIO) medium per well, and incubated for 5 to 7 days until the activated T cells reached 80% to 90% confluent. Activated PBMCs were collected and transferred at 1.5 × 10⁶ cells per well to a fresh anti-CD3 antibody coated 6-well plate, and incubated for an additional 24 hours. Then, the solution which contained sendai virus vectors individually carrying each of OCT3/4, SOX2, KLF4, and c-MYC were added at 10 MOI. After 24 hours of infection, the medium was changed to fresh GT-T502 medium, and the cells were collected and split at 5 × 10⁴ cells into 10 cm-plates pre-seeded with mouse embryonic fibroblasts (MEFs) at more 24 hours after infection. After an additional 24 hours of incubation, the medium was changed to human iPS cell medium supplemented with 4 ng/mL of bFGF. The cells were cultured for another 20 days. On day 25, ES cell-like colonies were dissociated mechanically and transferred to a 24 well plate on the MEF feeder cells.

**iPS cell culture**

Human iPS cells were maintained on irradiated MEF feeder cells in human iPS cell culture medium, consisting of 80% DMEM/F12 (Sigma-Aldrich), 20% KO Serum Replacement (Invitrogen), 4 ng/mL basic fibroblast growth factor (bFGF: WAKO), 2 mmol/L L-glutamine (Invitrogen), 0.1 mmol/L non-essential amino acids (Sigma-Aldrich), 0.1 mmol/L 2-mercaptoethanol, 100 U/mL penicillin, and 100 μg/mL streptomycin.
IPS cell generation with LQT2 mutation

19iPS cell generation with LQT2 mutation (Invitrogen). The human iPS cell medium was changed every 2 days and the cells were passaged using 1 mg/mL collagenase IV (Invitrogen) every 5-7 days. 293FT cells were cultured in DMEM supplemented with 10% FBS (Nichirei Bioscience), 1 × 10⁻⁴ M non-essential amino acids (NEAA; Sigma-Aldrich), 2 mmol/L L-glutamine (Invitrogen), 100 U/mL penicillin, and 100 μg/mL streptomycin (Invitrogen).

Immunocytochemistry

Immunostaining was used to analyze the expression of pluripotency markers. Cells were placed on a 35 mm glass-bottomed dish (IWAKI) before being fixed with 4% paraformaldehyde for 30 min at 4°C. The cells were then rinsed three times with phosphate-buffered saline (PBS) and permeabilized with 0.2% Triton-X 100 in PBS. The cells were then washed and blocked with Immunoblock (DS Pharma) three times for 5 min each time. Samples were incubated overnight at 4°C with each of the primary antibodies: anti-NANOG (1:200 dilution; ab21624; Abcam), anti-OCT3/4 (1:100 dilution; sc-5279; Santa Cruz). Following incubation with primary antibodies, samples were incubated at room temperature for 1 h with the following secondary antibodies: Alexa Fluor 488 chicken anti-mouse IgG (1:200 dilution; A21200; Abcam), anti-OC1T3/4 (1:100 dilution; sc-5279; Santa Cruz), and Alexa Fluor 594 goat anti-rabbit IgG (1:200 dilution; A11037; Invitrogen). After cells had been washed by PBS, samples were mounted using Vectashield Hard Set Mounting Medium with 4,’6- diamidino-2-phenylindole (DAPI) (Vector Laboratories) for nuclear staining. Images were obtained using a ×10 objective lens (NA = 0.45) on a fluorescence microscope (BZ-9000; Keyence).

Genome sequencing

DNA sequencing was used to confirm the presence of the LQT2 mutation in patient-derived iPSCs. Genomic DNA was isolated using a Gentra Puregene Cell Kit (Qiagen) and the region encoding KCNH2, including the mutation, was amplified using polymerase chain reaction (PCR) with the following primer set: 5’-TAGCCTGCATCTGGTACGC-3’ (forward) and 5’-GCCCGCCCCTGGGCACACTCA-3’ (reverse). The PCR product (277 bp) was electrophoresed on a 1% agarose gel and purified using a Wizard SV Gel and PCR Clean-Up System (Promega). The purified PCR product was sequenced with original primers.

Results

Novel KCNH2 mutation

A 10-year-old man was given surgery for funnel chest, without any symptoms. Before operation, routine surface electrocardiogram (ECG) was recorded (Figure 1A). At that time, QT interval prolongation at ECG was firstly pointed out. The patient had no history of previous syncopal episode, palpitation or other cardiac symptoms. But his mother showed repetitive syncopal episodes at rest, triggered by sudden loud noises such as alarm clock and telephone call. Exercise testing shortened the QT interval and epinephrine challenge induced the QT interval prolongation and the form of polymorphic ventricular tachycardia called torsades de pointes. She underwent the genetic test which showed the novel KCNH2 G603D (G1808A) mutation. Therefore he also underwent the genotype analysis which also showed the novel KCNH2 G603D (G1808A) mutation (Figure 1B).

Figure 1. Novel KCNH2 mutation in the patient.

A. Electrocardiogram from the patient during sinus rhythm. B. Sequence analysis of genomic KCNH2 in the patient. The novel KCNH2 G603D (G1808A) mutation.
IPS cell generation from a patient with KCNH2 mutation

To generate IPS cells, we used peripheral blood cells as donor somatic cells from the patient. Separated peripheral mononuclear cells were stimulated by CD3 antibody and IL-2 to activate T lymphocytes. And activated T lymphocytes were reprogrammed by using Sendai virus carrying SOX2, OCT3/4 (also known as POU5F1), KLF4, and MYC. Several clones were generated, expanded and stored. All IPS cell lines showed typical IPS cell morphology and expressed human pluripotency markers (Fig. 2a and b). These IPS cells were moved to petri-dishes and formed embryoid bodies with spontaneous beating, which indicated that these patient-specific IPS cells properly differentiated into beating cardiomyocytes in vitro.

KCNH2 G603D (G1808A)

Sequence analysis of genomic KCNH2 in the patient-specific IPS cell colony. The novel KCNH2 G603D (G1808A) mutation.

KCNH2 mutation in IPS cells derived from a patient with KCNH2 mutation

To confirm that the generated IPS cells have a same mutation as the patient has, the genotype analysis was performed. It revealed the KCNH2 G603D (G1808A) mutation was taken over (Figure 3).

Discussion

In the present study, we successfully generated IPS cells from a patient with the KCNH2 G603D mutation who didn’t exhibit any symptoms but showed prolonged QT interval at ECG. This patient is still young and may exhibit the cardiac symptom in the future. In real clinical setting, it is very important to know whether patients with genetic mutation will develop severe diseases or not. If we can predict the severity in the future disease manifestation, we can easily determine to do those patients, e.g., intensive care, exercise limitation, no medication and so on. So it is valuable to establish patient-specific disease model and develop the systems to evaluate the characteristics of patient-specific diseases. Patient-specific IPS cells may contribute to these concepts.

In terms of disease modeling using IPS cells, LQT2 is firstly noticed because LQT2 is one of the most common genetic variants in long QT syndrome and there is no definitive therapy for LQT2. And drug discovery often failed at the expense of immense cost, due to the side effects related to HERG which is LQT2 associated gene product, following QT prolongation and lethal arrhythmia. First report showed the generation of LQT2 patient-specific IPS cells harboring A614V missense mutation in the KCNH2 gene, which was previously shown to lead to a significant reduction of
IKr which is responsible for LQT2\(^1\). Detailed whole-cell patch-clamp and multi-electrode array (MEA) recordings revealed significant prolongation of the action potential duration in LQT2 iPS cell-derived cardiomyocytes. Voltage-clamp studies confirmed a significant reduction of the cardiac potassium current IKr. LQT2 iPS cell-derived cardiomyocytes also showed marked arrhythmogenicity, characterized by early-after depolarizations (EAD) and triggered arrhythmias. And calcium-channel blockers, \(K_{\text{ATP}}\)-channel openers and late sodium channel blockers ameliorate the disease phenotype in LQT2 iPS cell-derived cardiomyocyte. Second report showed the generation of LQT2 patient-specific iPS cells harboring G1681A missense mutation in the KCNH2 gene, which was also previously shown to lead to a significant reduction of IKr\(^2\). MEA and patch-clamp recording showed prolonged field/action potential duration in LQT2 iPS cell-derived cardiomyocytes. LQT2 iPS cell-derived cardiomyocytes developed EADs when challenged with the E4031 (IKr blocker) and isoprenaline. Action potential duration and EAD were ameliorated by propranolol, nadolol, nicorandil and an activator of hERG, PD118057. The other report showed the generation of LQT2 patient-specific iPS cells harboring R176W missense mutation in the KCNH2 gene\(^3\). The KCNH2 R176W mutation is relatively common variant and was reported to have the frequency of 0.5% in apparently healthy individuals. Although there were some reports showed that this mutation was related to long QT syndrome, the majority of these individuals were completely asymptomatic and unaware of their carrier status, as is the case with this patient. In heterologous expression system, R176W reduced hERG tail current density by \(\sim 75\%\), but upon coexpression with wild type the difference in current densities was nullified. But the action potential duration of LQT2 iPS cell-derived cardiomyocytes was significantly longer than that of control, and IKr density of the LQT2 iPS cell-derived cardiomyocytes was significantly reduced. Consistent with clinical observations, the LQT2 iPS cell-derived cardiomyocytes demonstrated a more pronounced inverse correlation between the beating rate and repolarization time compared with control cells. Additionally, LQT2 iPS cell-derived cardiomyocytes were more sensitive than controls to potentially arrhythmogenic drugs, including sotalol, and demonstrated arrhythmogenic electrical activity.

In this study we chose a patient with a novel mutation in the KCNH2 G603D. Patient showed QT interval prolongation but never showed any symptoms. To treat properly and prevent cardiac lethal arrhythmia, we believe it is valuable to generate experimental methods to predict how susceptible to lethal arrhythmia in various stimulations in those patients. Actually, many genomic variations such as many SNPs in each patient’s genome affect disease manifestation even in the patients with major functional mutation and may be the cause of low penetrance for long QT syndrome\(^4\). So it is difficult to accurately predict disease susceptibility only by genomic information such as patient’s mutation and SNPs. Patient-specific iPS cells have all genomic information encoded in patient’s genome including mutation and all SNPs, and can be ideal disease models for the patients. Actually, each patient shows different disease phenotype and drug response, which is also partly due to patient genomic variation. In terms of personalized medicine, we can also try many notorious and beneficial drugs on patient-specific iPS cell-derived cardiomyocyte and predict disease susceptibility before the patient will use those drugs. To generate patient-specific disease models using iPS cells, we established the patient-specific iPS cells and confirmed the patient-specific iPS cells had the same mutation as the patient.

**Acknowledgments**

The authors thank all the laboratory members for their critical comments and helpful discussions.

**References**

8. Horigome H, Nagashima M, Sumitomo N, et al. Clinical characteristics and genetic background of congenital...


