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Novel Mutation in the α-Myosin Heavy Chain Gene

Is Associated with Sick Sinus Syndrome

Running title: Ishikawa et al.; MYH6 mutations in familial SSS

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**ABSTRACT**

Background: Recent genome-wide association studies have demonstrated an association between *MYH6*, the gene encoding α-myosin heavy chain (α-MHC), and sinus node function in the general population. Moreover, a rare *MYH6* variant, R721W, predisposing susceptibility to sick sinus syndrome (SSS) has been identified. However, the existence of disease-causing *MYH6* mutations for familial SSS and their underlying mechanisms remain unknown.

Methods and Results: We screened nine genotype-negative probands with SSS families for mutations in *MYH6*, and identified an in-frame 3-bp deletion predicted to delete one residue (delE933) at the highly conserved coiled-coil structure within the binding motif to myosin-binding protein C (MyBP-C) in one patient. Co-immunoprecipitation analysis revealed enhanced binding of delE933 α-MHC to MyBP-C. Irregular fluorescent speckles retained in the cytoplasm with substantially disrupted sarcomere striation were observed in neonatal rat cardiomyocytes transfected with α-MHC mutants carrying delE933 or R721W. In addition to the sarcomere impairments, delE933 α-MHC exhibited electrophysiological abnormalities both *in vitro* and *in vivo*. The atrial cardiomyocyte cell line HL-1 stably expressing delE933 α-MHC showed a significantly slower conduction velocity on multi-electrode array than those of wild-
type α-MHC or control plasmid transfected cells. Furthermore, targeted morpholino

knock-down of MYH6 in zebrafish significantly reduced the heart rate, which was

rescued by co-expressed wild-type human α-MHC but not by delE933 α-MHC.

Conclusions: The novel MYH6 mutation delE933 causes both structural damage of the

sarcomere and functional impairments on atrial action propagation. This report

reinforces the relevance of MYH6 for sinus node function and identifies a novel

pathophysiology underlying familial SSS.

Key words: Sick sinus syndrome, myosin heavy chain, genetics, sinus node, MYH6
Sick sinus syndrome (SSS) is a common arrhythmia often associated with aging, structural heart diseases, or surgical injury, but can also occur in a familial form. Several studies have demonstrated genetic mutations in both sporadic and familial cases of SSS. Affected ion channel or ion channel-associated genes identified to date include sodium channel, Nav1.5 (SCN5A), ankyrin-B (ANK2), and hyperpolarization-activated channel (HCN4). Mutations in HCN4 result in sinus node dysfunction caused by a reduction of the pacemaker current, while SCN5A mutations lead to conduction delay within the sinus node or exit block.

\(\textit{MYH6}\) and \(\textit{MYH7}\) encode the homologous MHC isoforms \(\alpha\)-MHC and \(\beta\)-MHC, respectively, in cardiomyocytes, which play pivotal roles in the organization of sarcomeric structures and muscle contraction. \(\textit{MYH7}\) is predominantly expressed in the adult ventricle, whereas \(\textit{MYH6}\) is mainly expressed in the fetal heart and adult atrium. \(\textit{MYH7}\) is a well-established causative gene with over 300 mutations responsible for hypertrophic cardiomyopathy (HCM) and dilated cardiomyopathy (DCM), while more limited \(\textit{MYH6}\) mutations have been reported in cardiomyopathy and congenital heart disease such as atrial septal defect. On the other hand, recent genome-wide association studies (GWAS) demonstrated that a common nonsynonymous variant A1101V in \(\textit{MYH6}\) was associated with an increased resting
heart rate,\textsuperscript{17-19} while another rare non-synonymous variant (resulting in R721W) was
associated with a high risk of SSS.\textsuperscript{20} Moreover, heterozygous zebrafish carrying the
\textit{MYH6} mutation N695K (\textit{MYH6}\textsuperscript{hu/423/+}) displayed partial atrial contractile defects.\textsuperscript{21}
Based on these observations, it is conceivable that some \textit{MYH6} variations impair the
sarcomere structure and/or function of the atrium, which in turn would cause
electrophysiological abnormalities and sinus node dysfunction. However, it remains to
be elucidated whether 1) \textit{MYH6} is the causative gene for familial SSS, and 2) the
genetic variations of \textit{MYH6} associated with SSS confer pacemaker dysfunction through
structural damage of the sarcomere of the atrial muscle surrounding the sinus node
and/or by functional impairment of the pacemaker channel or sodium channel. The
present study identified a novel \textit{MYH6} mutation in one SSS proband, and investigated
the means by which this could confer sinus node dysfunction.

\section*{METHODS}

\textbf{Genetic Screening of \textit{MYH6} Mutations}

We previously performed genetic screening of mutations in \textit{SCN5A} and \textit{HCN4} in
15 probands afflicted with familial SSS, and found six distinct \textit{SCN5A} mutations.\textsuperscript{22} In
this study, we enrolled nine SSS families out of this cohort, which were free from
SCN5A or HCN4 mutations. Age at diagnosis of the probands (3 male and 6 female) ranged from 3 to 65 years old (44.6 ± 21.8 years old; mean ± SD).

Genomic DNA was extracted from peripheral blood of each subject using standard methods. Coding regions of MYH6 were amplified by PCR using exon-flanking intronic primers (Supplemental Table S1). Direct DNA sequencing was performed using ABI 3130 genetic analyzers (Life Technologies, Carlsbad, CA). Mutations were validated by the analysis of unrelated 400 healthy Japanese individuals and dbSNP, 1,000 Genome Project, Exome Variant Server, and Human Genetic Variation Database (HGVD, Japanese variation database, http://www.genome.med.kyoto-u.ac.jp/SnpDB/). All probands and family members who participated in the study gave their written informed consent in accordance with the Declaration of Helsinki. The research protocol was approved by the Ethics Review Committee of Nagasaki University, and the Ethics Review Committee of Medical Research Institute, Tokyo Medical and Dental University.

Alignment of Amino Acid Sequences and Structural Prediction of α-MHC

Amino acid sequence of human α-MHC was aligned using NCBI HomoloGene program with those of other species, and the phylogenetic conservations were testified.
among human MHC isoforms (the GenBank accession number of each gene is listed in Supplemental tables S2 and S3). Alterations of the coiled-coil structure of the α-MHC were predicted in silico by using SWISS-MODEL (http://swissmodel.expasy.org/), and visualized by a software RasTop (http://www.geneinfinity.org/rastop/).

6 Plasmids and cRNA preparation

A 5.8 kb cDNA fragment of human MYH6 was obtained by reverse transcription-PCR from human heart RNA using a primer pair MYH6-F-EcoRV and MYH6-R-SalI (Table S1), and was cloned into pEGFP-C1 (Takara Bio, Shiga, Japan) to make green fluorescent protein (GFP)-tagged MYH6 plasmid (pEGFP-MYH6). Mutant MYH6 plasmids of R721W (c.2161C>T) and delE933 (c.2797_2799delGAG) were constructed using an overlap-extension PCR strategy.

To assess the binding affinity of the mutant S2 region of α-MHC to MyBP-C on the basis of the previous report, cDNAs corresponding to the binding regions for human α-MHC (S2 region; aa. 884-965 of NP_002462) and human MyBP-C (C1C2 region; aa. 256-363 of NP_000247) were amplified and cloned into the c-myc-tag plasmid pCMV-Tag3B (Takara Bio) (pCMV3B-MYH6-S2) and the pEGFP-C1 (pEGFP-MYBPC3-C1C2), respectively. All constructs were sequenced to ensure that no errors were
introduced.

For the zebrafish experiments, WT and mutant MYH6 cDNA fragments were respectively cloned into pIRES2-EGFP vector (Takara Bio) (pIRES2-EGFP-MYH6) and pCS2+ vector\(^2\) (pCS2-MYH6) by using specific primer pairs. (Table S1) cRNAs of human MYH6 were synthesized using the mMessage mMachine \textit{in vitro} transcription kit (Life Technologies) and purified as described previously.\(^2\) Purified mutant cRNAs were sequenced by the University of Utah sequencing core facility.

\section*{Co-immunoprecipitation assay}

HeLa cells were co-transfected with pEGFP-MYBPC3-C1C2 and pCMV3B-MYH6-S2 using Transfectin lipid reagent (BioRad, Hercules, CA). After 48 hrs of the transfection, cells were lysed in TNE buffer (1% Nonidet P-40, 1 mM EDTA, 150 mM NaCl, and 10 mM Tris-HCl, pH 7.8) containing Protease Inhibitor Cocktail. Total cellular lysate was obtained by centrifugation at 13,000 xg for 5 min, and its protein concentration was measured by BCA protein assay (Thermo Fisher Scientific, Waltham, MA). Co-immunoprecipitation (co-IP) assay was performed using equal amount of cellular lysate with goat anti-myc polyclonal antibody (Sigma-Aldrich, St. Louis, MO) using the Catch and Release version 2.0 reversible immunoprecipitation system.
(Millipore, Billerica, MA). Immunoprecipitates were separated on a 9% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. After blocking with 5% skim milk in PBS, membranes were incubated with primary anti-GFP monoclonal antibody (1:100, Santa Cruz Biotechnology, Dallas, TX) overnight at 4°C and rabbit-anti mouse IgG HRP-conjugated antibody (Dako, Grostrup, Denmark) for 1hr at RT. Signals were visualized by Immobilon Western Chemiluminescent HRP Substrate (Millipore) and Luminescent Image Analyzer LAS-3000 mini (Fujifilm, Tokyo, Japan).

**Immunofluorescence study**

Immunohistological study was performed using neonatal rat ventricular cardiomyocytes prepared from one-day-old Sprague-Dawley rats as described previously.\(^2^5\) Briefly, neonatal rat ventricular cardiomyocytes (4x10^4) were transfected with WT or mutant pEGFP-\(MYH6\) plasmid with Lipofectamine LTX. Twenty-four hrs later, the cells were fixed with 100% ethanol, stained by primary mouse anti-\(\alpha\)-actinin antibody (1:100, Sigma-Aldrich) overnight at 4°C, and visualized with secondary Alexa Fluor 568 goat anti-mouse IgG antibody (1:500, Life Technologies). The fluorescent images were analyzed using LSM510 laser-scanning confocal microscope with a 63x oil immersion objective lens (Carl-Zeiss Microscopy, Jena, Germany).
All care and treatment of animals were in accordance with the guidelines for the Care and Use of Laboratory Animals published by the National Institute of Health (NIH Publication, eighth edition 2011), and subjected to prior approval by the animal protection authorities of Nagasaki University and Tokyo Medical and Dental University.

**Action Potential Propagation Velocity Measurements in HL-1 Cells Stably Expressing Human MYH6**

The mouse atrial cardiomyocyte cell line HL-1 (4x10^5), gift from Dr. Claycomb, was cultured as previously described.26 Cells were transfected with 2 µg of linearized pIRES2-EGFP-MYH6 plasmids of WT or delE933, or pIRES2-EGFP plasmid and 4 µl of Lipofectamine LTX (Life Technologies) according to the manufacturer’s instructions. Forty eight hours after transfection, cells cultured in the presence of 400 µg/ml G418 (Life Technologies) for 4 weeks to establish stable cell lines. Stable HL-1 cells (1x10^5 cells) expressing WT-MYH6, delE933-MYH6, or mock pIRES2-EGFP were plated on 8x8 planner multi-electrode arrays (array size 1 mm x 1 mm; electrode diameter 50 µm; Alpha MED Scientific Inc., Osaka, Japan) precoated with gelatin and fibronectin (Sigma-Aldrich). Seventy two hrs later, a single stimulus of 10 µA was applied on a designated point to initiate spontaneous beating spontaneous,
and electrical field potentials were recorded for one minute. Action potential propagation velocity was calculated by averaging the velocities between the stimulation point and the remaining 63 points. Cell numbers on the array were counted after recordings with detaching them from the arrays with Trypsin-EDTA. These procedures were repeated four times for each line.

**In Vivo Evaluation of Overexpressed MYH6 in Zebrafish**

Transgenic zebrafish (cmlc2:GFP, *Danio rerio*) embryos were used to functionally characterize the zebrafish *myh6* and human *MYH6* variant. *MYH6* ATG-blocking morpholino antisense oligonucleotide (myh6 ATG-MO) was designed to target *myh6* (supplemental table S1). Myh6 ATG-MO (0.5-1 ng/embryo) was injected alone, or co-injected with WT or delE933 *MYH6* cRNA (0.4 ng/embryo) at the 1- to 2-cell stage. After the injection, embryos were maintained in embryo water at 28°C and staged according to age and morphological criteria. Cardiac phenotypes were screened using fluorescent microscopy at 48 hour-post-fertilization (hpf). Heart rate and rhythm were recorded. Videos obtained from the embryos were analyzed using Image J (National Institutes of Health) to determine the heart rate and the duration of cardiac pauses.
Statistical Analyses

Results are presented as means ± SE otherwise stated, and statistical comparisons were made by using one-way analysis of variance (ANOVA) followed by Bonferroni adjustment to estimate the significance of differences between the mean values of all pairwise. Statistical significance was assumed for $P<0.05$. 
RESULTS

Case Presentation

Genetic screening of MYH6 mutations in nine probands with familial SSS identified a novel mutation in a 62-year-old Japanese woman. She attended the hospital because of several episodes of presyncope with which she had been afflicted for 5 years. Her 12-lead electrocardiogram (ECG) showed sinus bradycardia (heart rate 42 bpm) with unusual P wave axis and junctional escape beat (Figure 1A), and Holter ECG revealed sinus arrest with maximum RR interval of 5.59 s (Figure 1B). She had no history of other arrhythmias including atrial fibrillation. Echocardiography revealed mild dilatation of the left ventricle (LV) and right atrium, but there were no obvious signs of cardiomyopathy, congenital heart disease, or cardiac dysfunction (LV internal diameter in diastole: 57 mm, LV posterior wall in diastole: 6 mm, interventricular septal wall in diastole: 6 mm, LV ejection fraction: 63%) (Figure 1C). A pacemaker was implanted following the diagnosis of SSS. Her deceased mother also had a pacemaker implanted because of SSS during the 7th decade of her life.

Identification of the Novel MYH6 Mutation delE933

The novel mutation identified in the proband was an in-frame 3-bp deletion,
c.2797_2799delGAG, located in exon 22 of MYH6. This was predicted to delete one residue within the glutamic acid triplet at aa.931-933 of α-MHC (delE933) (Figure 2A). This triplet is located in the S2 segment of α-MHC, a crucial structure required for binding to MyBP-C and for regional phosphorylation of MyBP-C, thereby facilitating a flexible link between thin and thick filaments. The S2 hinge region is highly conserved among α-MHC from different species as well as between other MHC isoforms (Figure 2B).

The proband has no siblings or offspring, and DNA was not available from her deceased mother. The delE933 mutation was not identified in 800 MYH6 alleles from healthy Japanese controls or in the public genetic variation databases of dbSNP, 1,000 Genomes, Exome Variant Server, and HGVD. The common variation A1101V was not found in the proband, while three out of eight other probands in our cohort were heterozygous for A1101V. The rare MYH6 variation R721W (c.C2161T), associated with SSS in Icelanders, was not found in our familial SSS cohort. No other disease-related mutations were identified in SCN5A, HCN4, SCN3B, KCNJ3, KCNJ5 or GJA5 in our familial SSS cohort. Polymorphisms identified in MYH6 are listed in the Supplemental Table S4.
The delE933-MYH6 Mutation Disrupts Sarcomere Structures

The S2 segment is a coiled-coil domain of α-MHC composed of a motif of heptad repeats of amino acids. SWISS-MODEL simulation predicted that the delE933 mutation would cause local disruption of the coiled-coil structure (Figure 3A).

Immunoprecipitation studies using a recombinant MyBP-C C1-C2 protein, and WT and delE933α-MHC S2 region proteins expressed in HeLa cells showed that the binding ability of α-MHC with MyBP-C was substantially enhanced by the delE933 mutation (Figure 3B).

Because structural damage of sarcomere have been reported in association with MYH6 mutations responsible for atrial septal defect, we next explored whether the MYH6 variation R721W as well as delE933 disrupted integrity of sarcomere structures.

To investigate the functional consequences of MYH6 mutations on the atrial sarcomere structure, we used a heterologous expression system in cultured rat cardiomyocytes in which the predominant ventricle MHC isoform is α-MHC. Neonatal rat ventricular cardiomyocytes were transiently transfected with a GFP-tagged MYH6 WT, delE933 or R721W plasmids. Confocal microscopy analysis revealed comparable GFP intensities following transfection of all three MYH6 plasmids (Figure 3C, a-c), indicating that the expression levels and stability of heterologously expressed α-MHC proteins were
similar. Endogenous sarcomeric α-actinin expression at the Z-disc indicated the sarcomere integrity of transfected myocardial cells (Figure 3C, d-f). Cells expressing WT-MYH6 displayed a striated staining pattern, indicating that heterologous α-MHC was correctly integrated into the sarcomere. However, both MYH6 mutants, delE933 and R721W, exhibited a substantially disrupted α-actinin staining pattern and perinuclear aggregation of α-MHC, suggesting that structural damage to the sarcomere had occurred in cells expressing MYH6 variants predisposing to sinus node dysfunction.

Atrial HL-1 Cells Stably Expressing the delE933-MYH6 Showed Impaired Electrical Propagation

A recent GWAS showed that the Iceland-specific MYH6 variant was significantly associated with atrial fibrillation,20 we hypothesized that the functional defects caused by mutated MYH6 may impact on action potential propagation in the atrium surrounding the sinus node, leading to SSS manifestation. We cultured the mouse atrial cardiomyocyte cell line HL-1 stably expressing WT or mutant MYH6 on 64-well electrode arrays, and analyzed electrical propagation velocities (Figure 4A). The propagation velocity was unchanged between WT-MYH6 and control mock-transfected cells, but cells expressing delE933-MYH6 exhibited a significantly slower propagation
velocity. (Control: 3.6 ± 0.6 mm/s, WT: 3.8 ± 1.2 mm/s, delE933: 2.9 ± 0.8 mm/s,
n=252 for each line, \( P<0.001 \) for WT vs delE933) (Figure 4B). Cell numbers of each array were comparable. (\( P=0.49 \)) (Supplement Table S5). These data suggest that mutant MYH6 delE933 impairs cell-to-cell action potential propagation in the atrial myocardium.

The delE933-MYH6 Failed to Rescue the Heart Rate Reduction in Zebrafish with Morpholino myh6 Knockdown

To determine whether the human MYH6 orthologue myh6 could influence heart rate control in zebrafish, we performed targeted myh6 knock-down experiments with ATG-MO. Zebrafish cardiac phenotypes, including heart rate and cardiac rhythm, were assessed at 48 hpf. The myh6 morphants exhibited atrial dilatation (Figure 4C), which is consistent with a previous report using decreased functional myh6 transcript.\(^{27}\) Myh6 morphants also showed a significantly slower heart rate than uninjected embryos (myh6-MO: 137.7 ± 2.2 bpm, n=28; uninjected 150.2 ± 1.6 bpm, n=25; \( P<0.001 \)) (Figure 4D). Cardiac asystole was not observed in uninjected embryos or morphants. As shown in Figure 4D, co-injection of WT human MYH6 cRNA rescued the bradycardia (148.7 ± 1.4 bpm, n=26; vs myh6-MO \( P<0.001 \)), suggesting that the human MYH6 compensated
for the loss of the zebrafish orthologue. By contrast, human MYH6 carrying the delE933 mutation failed to rescue the bradycardia (142.3 ± 2.5 bpm, n=24). Human MYH6 RNA was detected by RT-PCR in embryos at 24 and 48 hr after injection (Supplemental Figue), suggesting that the delE933 mutation of MYH6 is responsible for sinus node dysfunction.

DISCUSSION

A growing body of evidence from GWAS has demonstrated an association of MYH6 with sinus node function.17-20 A common nonsynonymous single-nucleotide polymorphism of MYH6 (A1101V) was previously shown to be a genetic modifier for resting heart rate and PR interval,18 and this was further replicated in a large meta-analysis including subjects of European ancestry from both the United States and Europe.17, 19 A combination of A1101V with other loci controlling heart rate further reduced the risk of SSS and pacemaker implantation, implicating a heritable quantitative trait.17 By contrast, the rare MYH6 variation R721W, unique to Icelanders, predisposes individuals to SSS and pacemaker implantation.20 These studies clearly demonstrate that MYH6 is a genetic modifier of sinus node function, but the mechanisms of this have been unclear, and it was uncertain whether MYH6 could be a
In this study, we identified a novel MYH6 mutation, delE933, in one SSS individual among nine probands of our familial SSS cohort. We found that the mutant delE933-MYH6 slowed down action potential propagation when heterologously expressed in the atrial myocardial cell line HL-1 (Figure 4A). Moreover, knockdown of endogenous MYH6 leading to reduced heart rate in zebrafish could be compensated for by the co-expression of WT-MYH6 but not by delE933-MYH6 (Figure 4B). To our knowledge, this is the first experimental evidence demonstrating that MYH6 variations can influence heart rate and action potential propagation. However, limited information is available to delineate the functional link between sarcomere components and sinus node function, and it remains unknown whether α-MHC directly affects pacemaker function or if its actions are mediated through undefined mechanisms.

The delE933 mutation is located in the coiled-coil structure of the α-MHC S2 region, a binding motif for MyBP-C, so is predicted to alter the tertiary structure and the cross-linking affinity between two sarcomere components (Figure 2A and 2B). Although the final consequences of such structural and functional modifications are unknown, a previous study that the MyBP-C mutation E334K, responsible for HCM, impaired the ubiquitin-proteasome system, leading to an accumulation of cardiac ion
channels at the sarcomere and electrophysiological dysfunction. Increased protein levels were also observed for several other cardiac ion channels including Kv1.5, Nav1.5, HCN4, Cav3.2, Cav1.2, SERCA, RYR2, and NCX1, which play major roles in controlling normal pacemaker function and atrial conductivity. Based on these findings, we speculate that an abnormal association between delE933-α-MHC and MyBP-C might modulate the expression of cardiac ion channels that impact pacemaker function, which in turn would lead to the development of SSS.

In the present study, overexpression of the SSS-susceptible α-MHC mutants of R721W and delE933 in neonatal rat ventricular cardiomyocytes impaired sarcomere structures. However, it is unknown whether MYH6 mutations preferentially impair the sinus node function, or if they might eventually cause further extensive electrical damage manifesting as atrial fibrillation. Because the patient did not undergo electrophysiological studies or cardiac biopsy, further information regarding the spatial distribution and heterogeneity of pathological damages and electrophysiological abnormalities in the atrium is not available. Nevertheless, the T wave inversion of ECG and mild dilatation of the right atrium and LV (Figure 1A, B) are in accordance with the observation that targeted myh6 knock-down in zebrafish induced the atrial dilatation associated with negative chronotropic effects, indicating that MYH6 mutations may
directly cause substantial damage and electrical disorder to the myocardium in the atrium. This idea is further supported by the finding that heterozygous zebrafish expressing the MYH6 mutation N695K (MYH6<sup>hu4/23</sup>) exhibited loss of atrial contractility with residual beating restricted to the region near the atrioventricular junction and sinus venosus. These observations strongly suggest that the final consequences of MYH6 mutations in humans might also exhibit considerable heterogeneity with respect to the structural and electrophysiological properties of the atrium and sinus node. Furthermore, these structural abnormalities of atrial sarcomere may extend to more severe conduction dysfunctions such as atrial fibrillation or contractile failure, depending on the functional severity caused by each mutation. The slower conduction velocity observed in the HL-1 cells stably expressing delE933-MYH6 in the present study suggests the possible involvement of MYH6 in conduction dysfunction. This idea is supported by a recent GWAS in which correlation studies of the MYH6 variant R721W exhibited a significantly higher association with atrial fibrillation both before (odds ratio: 2.39, p=0.00010) and after (odds ratio: 2.03, p=0.015) exclusion of known SSS cases. It is of note that the R719W of the ventricular β−myosin heavy chain (β-MHC) gene MYH7, homologous to the R721W-MYH6, is responsible for a malignant hypertrophic cardiomyopathy frequently
associated with conduction abnormalities,\textsuperscript{35} suggesting that $\alpha$-MHC and $\beta$-MHC may share some pathophysiological mechanisms affecting cardiac action potential propagation.

SSS commonly occurs in older individual in the absence of accompanying heart diseases but comprises a variety of electrophysiological abnormalities in sinus node impulse formation and propagation. Although less common, SSS also shows familial inheritance and implicated causative genes include those encoding cardiac ion channels such as \textit{SCN5A}. We recently found that familial SSS probands carrying \textit{SCN5A} mutations showed a significantly earlier disease onset and a strong male predominance, whereas non-familial SSS had a disease onset of over 70 years for both sexes, which were affected equally.\textsuperscript{22} The affected members of the SSS family in the present study were both women, aged over 60 years, suggesting that familial SSS with \textit{MYH6} mutations might constitute an SSS subgroup distinct from that caused by \textit{SCN5A} mutations. This may suggest the existence of a new disease entity of inherited arrhythmias attributable to mutations in genes encoding sarcomere proteins other than cardiac ion channel or ion channel-associated genes.

\textbf{Limitation of the study}
Lack of information about the genotype-phenotype cosegregation of delE933-

*MYH6* is the major limitation of this study from the standpoint of human genetics.

Although bioinformatics evaluations as well as *in vitro* and *in vivo* studies have suggested pathophysiological significance of the rare *MYH6* variation delE933, it still does not exclude the possibility that the proband manifested SSS attributable to factors such as aging rather than the *MYH6* mutation. In order to demonstrate the causality between SSS and *MYH6*, more extensive genetic screenings in patients with familial SSS to find novel *MYH6* mutations are required. Furthermore, it remains to be elucidated how the impaired sarcomere structures and conduction velocity elicited by delE933-*MYH6* ultimately result in the sinus node dysfunction. Electrophysiological studies using induced pluripotent stem cell-derived cardiomyocytes from *MYH6* mutation carriers, as well as basic evaluations of *MYH6* using genetically-engineered animals are also warranted.

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Disclosures

None
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Figure Legends

Figure 1. ECG and echocardiography of the SSS proband

(A) ECG recordings of the proband (age 62 years) displayed sinus bradycardia (42 bpm) with unusual P wave axis and junctional escape beat (last beat in V4-V6). T waves in I-III, aVF, and V4-6 were inverted. (B) Holter ECG showed sinus arrest with a maximum RR interval of 5.59 s. (C) Echocardiography revealed mild dilatation of left ventricle (LV) and right atrium without obvious evidence for cardiomyopathy, congenital heart disease, or cardiac dysfunction. (LV internal diameter: 57mm, LV posterior and interventricular wall thickness: each 6mm, LV ejection fraction: 63%)

Figure 2. Genetic and protein information of the MYH6 mutations

(A) An electropherogram of exon 22 of MYH6 of the proband. Boxes indicate the codons of triplicate glutamic acids E931-E933 of the WT allele, and an in-frame deletion of GAG resulting in delE933. (B) Protein structures of α-MHC and its binding partner MyBP-C. α-MHC consists of S1 motor, S2 hinge, and light meromyosin (LMM) regions. The S2 hinge region interacts with the region of MyBP-C between the 1st (C1)
and 2nd globular structure (C2). Locations of the two MYH6 mutations, a rare variant R721W identified in Icelanders, and delE933 (this study), are shown with red dots.

Protein sequence alignment shows that the MyBP-C binding site (residues 884-965) are highly conserved among α-MHCs from different species, and the glutamic acid triplet is perfectly conserved among different species and different MHC isoforms of cardiac (MYH6, MYH7), skeletal muscle (MYH1, MYH2, MYH3, MYH4, MYH8, MYH13, MYH15) and a non-muscle type (MYH14).

Figure 3. In silico prediction and in vitro functional evaluation of delE933-MYH6

(A) Ribbon representation of three-dimensional structure of the S2 region in human α-MHC predicted and visualized by SWISS-MODEL and RasTop, respectively. The coiled-coil structure is partially disrupted at the truncated amino acid E933 (arrowhead).

(B) Co-immunoprecipitation study of the S2 region of α-MHC and C1C2 region of cardiac MyBP-C. The S2 fragment of delE933 shows increased binding to the C1C2 fragment of MyBP-C. A non-specific double band was often observed on the input of a mock pEGFP-C1 plasmid (third column). (C) Fluorescence images of neonatal rat ventricular cardiomyocytes transiently expressing WT, delE933, or R721W MYH6 fused to GFP. WT α-MHC shows a striated pattern of GFP together with the proper striated
sarcomeric pattern of α-actinin (a and d). α-MHC with mutations of R721W and
delE933 show brightly fluorescent speckles without well-organized sarcomere structure
(b and c). The α-actinin images show a misaligned and disrupted pattern of myofibrils (e
and f), indicating sarcomere disintegration. Scale bar: 10 µm

Figure 4. Electrophysiological phenotypes of delE933-MYH6

(A) Representative activation isochronal maps of HL-1 cells stably expressing WT or
delE933 MYH6, and control HL-1 cells. An electrical provocation with 10 µA was input
on the pointed electrode (arrow). (B) Averaged conduction velocity calculated from the
time elapsed for the impulse to reach all remaining electrodes (n=252 for each
recording). (C) Representative fluorescent diastolic images of embryonic zebrafish
hearts at 48 hpf: (a) uninjected control, (b) myh6-MO only, (c) myh6-MO co-injected
with human WT MYH6 cRNA, and (d) myh6-MO co-injected with human delE933
MYH6 cRNA. The atria of the myh6 MO morphant in the presence or absence of co-
injected human MYH6 cRNAs (b-d), were slightly dilated compared with the uninjected
control (a). The ventricle and cardiac looping pattern of the morphants, with or without
MYH6 cRNAs, were similar to that of the control. Scale bars: 100 µm (D) Heart rate
recordings from zebrafish (a-d). Data are shown as box and whisker plots with
minimum, maximum, median, 25th, and 75th quartiles bars. Number in parentheses represents the number of zebrafish in each group.
WHAT IS KNOWN

- Sick sinus syndrome (SSS) is often associated with aging and structural heart diseases, but it may occur in a familial form.
- Recent genome-wide association studies uncovered MYH6 encoding atrial myosin heavy chain as a susceptibility gene for heart rate and SSS, however its underlying mechanisms and the existence of causative mutations for SSS remain unknown.
- Here, we report a novel MYH6 mutation delE933 in an SSS patient who has a family history of SSS.

WHAT THE STUDY ADDS

- When expressed in cardiomyocytes, delE933-MYH6 impaired the atrial action potential propagation and disrupted sarcomere integrity consistent with the R721W-MYH6, a high risk genetic predisposition for SSS demonstrated in Icelanders.
- Our data reinforces the relevance of MYH6 of sinus node function and suggested that structural damages of the sarcomere and functional impairments on atrial action potential propagation may underlie familial SSS with MYH6 mutations.
Figure 1, Ishikawa et al.
**Glutamic acid triplet (E931-E933)**

WT allele

delE933 allele

**Human**

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**MyBP-C**

**α-MHC**

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<th>S2 (hinge)</th>
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<td><strong>R721W</strong> (Icelander SSS)</td>
<td>binding</td>
<td>delE933 (SSS)</td>
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**MyBP-C binding**

**MYH6**

**MYH7**

**MYH1**

**MYH2**

**MYH3**

**MYH4**

**MYH8**

**MYH13**

**MYH15**

**MYH14**

**Figure 2, Ishikawa et al**
A

WT  delE933

B

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<th>α-MHC-S2:</th>
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C

WT  delE933  R721W

α-MHC  α-actinin  merge
Figure 4, Ishikawa et al

A

Control

WT

delE933

B

![Bar chart showing velocity in mm/sec for Control, WT, and delE933.]

P<0.001

P<0.001

C

uninjected control

myh6-MO

myh6-MO + human WT MYH6

myh6-MO + human delE933 MYH6

D

![Box plot showing heart rate (bpm) for Uninjected, myh6-MO, myh6-MO + WT-MYH6, and myh6-MO + delE933-MYH6.]

P=0.032

P<0.001

P<0.001

Heart rate (bpm)

110 120 130 140 150 160 170

(25) (28) (26) (24)