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研究課題名（和文）心室細動発症に関連する遺伝子変異のゲノムワイド探索とその分子機序の解明

研究課題名（英文）Genome-wide analysis to discover the gene mutation that relates to ventricular fibrillation and its molecular mechanism

研究代表者

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研究成果の概要（和文）：心室細動を家族性に発症している家系の血液サンプルを用いて、次世代シーケンサーによるゲノムワイド解析で原因遺伝子変異を探索したところ、発症者特異的にTMEM168遺伝子にこれまでに知られていない遺伝子変異を見出した。この遺伝子変異により、Naチャネル機能が抑制されており、心筋Naチャネル構成分子であるNav1.5発現量が低下していた。この原因遺伝子変異をノックインした遺伝子改変マウスで、心室頻拍、心室細動が誘発された。

研究成果の概要（英文）：Ventricular fibrillations are critical arrhythmias that cause sudden cardiac death. Thus, it is essentially important to investigate the underlying mechanisms of the arrhythmias. In this project, we conducted exome sequencing analysis to identify novel culprit gene (s) in a family with inherited fatal ventricular arrhythmias, and found heterozygous variant (1616G>A; p. R539Q) in the TMEM168 gene. TMEM168 localized in the nuclear membrane. Variant TMEM168 ectopically expressed in HL-1 cardiomyocytes reduced Na+ current density in the patch-clamp recording, and attenuated the endogenous expression of Nav1.5, a Na+ channel subunit. We newly generated Tmem168 1616G>A knock-in mice. Pharmacological stimulations provoked ventricular tachycardias/fibrillations and conduction disorders in the knock-in mice. Analogous to HL-1 cells, Nav1.5 expression was reduced in the knock-in heart. Presented results associate the novel gene variant of TMEM168 with arrhythmogenicity.

研究分野：　循環器学、生化学

キーワード：　不整脈　ゲノム解析　遺伝子異常

１．研究開始当初の背景

Sudden cardiac death (SCD) is unresolved all-cause mortality burden to the human society despite the advances of clinical cardiology. Usual cause (>50%) is coronary artery disease, but epidemiological studies point at inherited arrhythmias (channelopathies) to reaching up to 30% of SCD cases in the population below 40 years of age [Deo et al., *Circulation*. 2012]. Contemporary progress of molecular medicine and sequencing technologies has opened a new era allowing genetic diagnosis and identification of arrhythmogenic mechanisms in familial channelopathies.

In 1992, a new electrocardiographic (ECG) and clinical syndrome was proposed consisting of right bundle branch block, ST-segment elevation on ECG, which caused SCD without structural heart disease [Brugada et al., *J Am Coll Cardiol*. 1992]. In 1996, the term Brugada syndrome (BrS) was coined. BrS is endemic in Southeast Asia with prevalence of 12/10,000 individuals, but affects 1 to 5/10,000 in Western countries with clear male sex predominance. Clinical presentation of BrS is variable at diagnosis ranging from no symptoms to SCD or syncope due to ventricular fibrillation (Vf). BrS is responsible for 4% of all SCD and nearly 20% of SCD in non-structural cardiac disease group. The only effective treatment at present is implantable cardioverter-defibrillator (ICD) device. BrS is autosomal dominant disease with incomplete penetrance and various expressivity implying the existence of a pool of individuals with clinically silent form. It was found that 20-25% of the BrS patients have loss-of-function mutations in the *SCN5A* gene, encoding α-subunit of cardiac Na^+ channel. A number of mutations in other genes that encode proteins of macromolecular complexes of cardiac Na^+ channel, calcium, or potassium channels have been identified. However, genetically diagnosed patients in BrS are only 30-35% of clinical cases [Juang J-MJ & Horie M. *J Arrhythm*. 2016]. Thus, continuous efforts to identify the gene mutation related to BrS is important, and the identification critically contribute to the development of a new therapy preventing Vf and SCD caused by BrS.

２．研究の目的

(1) Exome-wide analysis of genomic DNA obtained from symptomatic and healthy individuals in a family with inherited BrS.

(2) Identification of culprit genetic variant associated with the arrhythmia.

(3) Discovery of the role of detected protein in the heart electrophysiology.

(4) generation and characterization of knock-in mice harboring identified genetic defect.

３．研究の方法

(1) Subjects. Pedigree of the investigated BrS family is shown in Figure 1. Genomic DNA was extracted from the individuals outlined in a red color. Symptomatic individuals are shown in grey. The proband is indicated by an arrow.

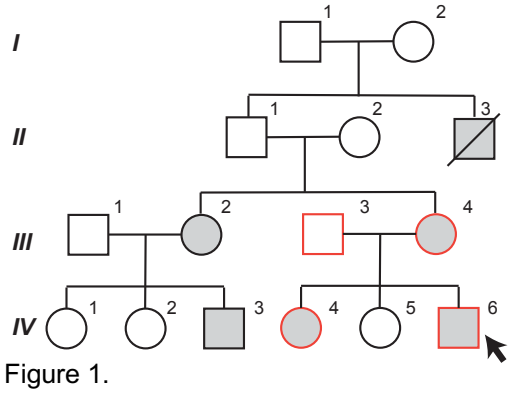

Figure 1.

(2) Exome sequencing and bioinformatics. Genomic DNA library preparation was conducted according to "SureSelect^{XT} Target Enrichment System for Illumina Paired-End Sequencing Library" protocol. For the exome capture, Agilent SureSelect^{XT} Human All Exon 50Mb Kit V4 (Agilent Technologies, USA) was used. Paired-end sequencing was performed on Illumina Genome Analyzer IIx (Illumina, Inc., USA) producing 71 bp reads. Bioinformatics pipeline followed the reported procedures based on the Genome Analysis Tool Kit [DePristo MA, et al. *Nat Genet*. 2011]. Identified variant TMEM168 1616 G>A was validated by Sanger sequencing.

(3) Cloning of TMEM168 gene. Coding sequence of human TMEM168 was cloned from HEK293 cDNA by PCR amplification (Phusion High-Fidelity DNA Polymerase, Finnzymes, Finland). The cloned sequence was compared to the NCBI reference NM_001287497.1. TMEM168 (wild type, WT, and variant) was subcloned into pEGFP-N1 and pFLAG-CMV1 expression vectors.

(4) Cell culture, transient transfection, and immunochemistry. COS-7 cells were cultured following the standard protocol. HL-1 cardiomyocytes were cultured as

originally described [Claycomb WC, et al. *Proc Natl Acad Sci USA*. 1998]. TMEM168 vectors were transfected into cultured cells by Lipofectamin 2000 Reagent (Invitrogen, USA) following the manufacturer's instructions. For immunocytochemistry, the cells were seeded on coverslips, fixed with 4% paraformaldehyde, and blocked with 1% bovine serum albumin (BSA). Primary antibodies were applied in the BSA blocking solution overnight followed by 1 h incubation with fluorescent dye-labeled secondary antibodies, and mounted on slides with anti-fading agent (Vectashield, Vector Laboratories, USA). Confocal images were taken by C1si Laser Scanning Microscope (Nikon, Japan). Cryosections of mice hearts were processed as above for immunohistochemistry.

(5) Electrophysiological recordings. HL-1 cardiomyocytes were voltage-clamped using the standard whole-cell patch-clamp technique with EPC-10 amplifier (HEKA, Germany). Na^+ current was elicited at room temperature (25°C) from holding potential -140 mV by 50 ms depolarizing voltage commands between -100 and +40 mV with 5-mV increment. Bath solution contained (in mmol/l): NaCl 145, KCl 4, $CaCl_2$ 1.8, $MgCl_2$ 1, HEPES 10, Glucose 10, pH 7.35 (NaOH). Patch-clamp pipettes (borosilicate glass, Narishige, Japan) had resistance of ~1.0 MΩ when filled with pipette solution containing (in mmol/l): NaF 10, CsF 110, CsCl 20, EGTA 10, HEPES 10, Na_2-ATP 3, pH 7.35 (CsOH). Voltage dependence of Na^+ current activation was studied by building average conductance/voltage (G/V) relation. Value of G for every voltage was calculated as: $G = I / (V - V_{rev})$, where I is Na^+ current amplitude and V_{rev} is reversal potential for Na^+. Mean values of G/V relation and inactivation curve were fitted to the Boltzmann equation. Single HL-1 cell membrane capacitance (C_m) was calculated based on the capacitive transients during 20 ms voltage-clamp steps (±5 mV). Electrophysiological data were analyzed by Igor Pro 7 software (Wavemetrics, USA).

(6) Protein samples and Western blot (WB). HL-1 cells were transfected with EGFP-tagged TMEM168 WT or TMEM168 R539Q mutant. After 48 h, EGFP-positive cells were separated by FACS in the FACSAria (Becton, Dickinson, and Co., USA) and protein samples were prepared in RIPA buffer. Protein samples from mice hearts were prepared in lysis buffer containing (in mmol/l) sucrose 320, Tris-HCl (pH 7.5) 20, EDTA 2. WBs were conducted following the standard procedure.

(7) Quantitative PCR. HL-1 cells were transfected with TMEM168 vectors and separated by FACS as explained above. Total RNA was extracted from the EGFP-positive cells using TRIzol Reagent (Invitrogen). cDNA was obtained by reverse transcription PCR (ReverTra Ace™ qPCR RT Master Mix, TOYOBO, Japan) with random primer. For qPCR the samples were prepared using Light Cycler 480 SYBR Green I Master (Roche, Germany) and amplified in Roche Light Cycler 480 with the primer targeting mouse Scn5a. C_T was normalized to the ß-actin in the same sample.

(8) Tmem168 1616 G>A knock-in mice and ECG recordings. Knock-in mice were generated by the CRISPR/Cas9 genome editing technology and the G>A substitution was verified by Sanger sequencing. ECG (lead I) was recorded by LEG-1000 device (Nihon Kohden, Japan) using needle electrodes. Mice were anesthetized by intraperitoneal (i.p.) pentobarbital 30 mg/kg and electrodes are attached to the forepaws. Noradrenaline 2 mg/kg is delivered by i.p. injection. 30 min later a second i.p. injection delivered caffeine 125 mg/kg [Frasier CR, et al. *Proc Natl Acad Sci USA*. 2016]. ECG was recorded continuously for 45-60 min after the addition of caffeine.

(9) Statistics. Student's t-test and one-way ANOVA were used for comparison of numerical data. A value of $p < 0.05$ was considered statistically significant.

４．研究成果

(1) Exome sequencing and TMEM168 variant identification. An average of ~5.5 G unique bases were generated and aligned to the targeted genome regions (exons) giving the 40x or more coverage of ~71% of all targeted bases. 47 - 48k variants (novel variants ~3k) per sample were detected with calculated Ti/Tv ratio of 2.6. We selected novel, heterozygous, nonsynonymous variants common for the three symptomatic individuals but missing in the normal one and thus created list of 18 SNPs and 2 indels. We applied the following criteria for selecting the primary candidate gene: expression in the heart, prediction of functional consequences to the protein, available information in the protein databases. The best score after this evaluation had the variant TMEM168 1616 G>A (p.R539Q). TMEM168 is highly

expressed in the heart and R to Q substitution is predicted to be damaging for the protein function. The molecule has 11 putative transmembrane domains and R^{539} is positioned between 10^{th} and 11^{th} ones.

(2) Cellular physiology of TMEM168: localization, expression, and modulation of ion channels. Confocal images of COS7 cells transfected with EGFP-tagged WT or TMEM168 R539Q mutant demonstrated that both TMEM168 proteins are visible around the nucleus and adjacent regions. To confirm the presence of TMEM168 proteins in the nuclear membrane, COS7 cells were transfected with EGFP-tagged proteins and immunostained to detect lamin A, a nuclear membrane intermediate filament. Co-localization of TMEM168 and lamin A was evident for both WT and TMEM168 mutant. Confocal images also demonstrated that endogenous Tmem168 occupies the same nuclear area in cardiac ventricle of WT mice and HL-1 cells.

Interaction of TMEM168 with cellular electrophysiology was evaluated in transfected HL-1 cells: TMEM168 R539Q mutant significantly reduced normalized peak Na^+ current amplitude ($p < 0.05$ between -70 and +10 mV) in comparison with the HL-1 cells transfected with TMEM168 WT (Figure 2). To evaluate the effect of TMEM168 on Na^+ channel expression in HL-1 cells, we separated transfected cells and prepared protein samples. Comparison of $Na_v1.5$ band density in WB detected reduction of Na^+ channel α-subunit in HL-1 cells expressing TMEM168 R539Q mutant. Moreover, total RNA was extracted from transfected HL-1 cells (after FACS selection) to evaluate transcription of the α-subunit of Na^+ channel. The result of qPCR indicated that TMEM168 did not affect transcription of the α-subunit.

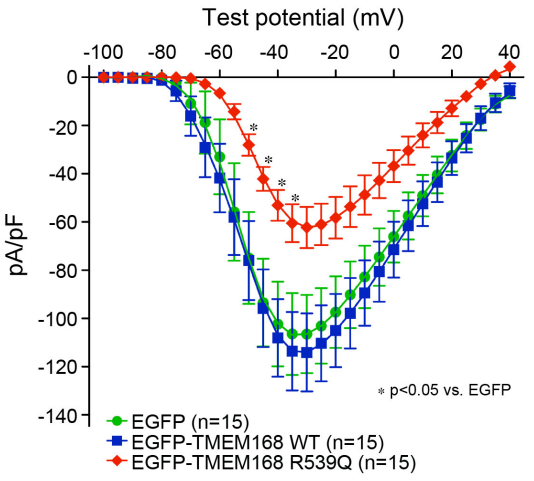

Figure 2.

(3) Phenotype of Tmem168 1616 G>A knock-in mice. Human and mouse Tmem168 proteins are 95% identical. Therefore, it is expected that 1616 G>A substitution will induce similar effect on the protein in mice. We generated knock-in mice incorporating Tmem168 1616G>A substitution in order to investigate cardiac phenotype *in vivo*. WT and knock-in mice were anesthetized, and ECG was recorded at basic state and during consecutively delivered norepinephrine and caffeine to challenge cardiac electrical stability as reported earlier. At basic condition (only effect of pentobarbital 30 mg/kg), neither WT nor knock-in mice had electrical disturbances in the ECG. Norepinephrine alone (2 mg/kg) did not produce arrhythmias, although significantly increased heart rate in both WT and knock-in mice was observed, compared with the condition in the absence of pharmacological stimulation. Addition of caffeine (120 mg/kg) provoked tachyarrhythmias in 14 of the 23 knock-in mice, but not in WT ones (Figure 3). We observed premature ventricular complexes, ventricular tachycardias (the above ECG record) with different duration and morphology, and episodes of supraventricular tachycardia with P waves. Two knock-in mice had conduction abnormalities. Norepinephrine and the combination of two compounds significantly reduced QT and corrected QT intervals (QTc, Bazett's formula).

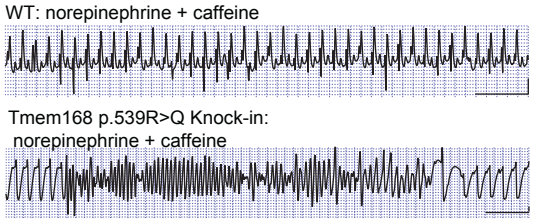

Figure 3.

Quantification of $Na_v1.5$ in protein samples from the hearts of WT and knock-in mice by WB as well as immunohistochemistry of cardiac sections revealed, similar to HL-1 cells, reduction of $Na_v1.5$ and $Na_v1.5$ fluorescence intensity in the hearts of knock-in animals.

５．主な発表論文等
（研究代表者、研究分担者及び連携研究者には下線）

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〔その他〕
ホームページ等
http://www.shiga-med.ac.jp/~hqbioch2/

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