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研究課題名（和文）心室細動発症におけるTMEM168遺伝子変異解析とトラスレーショナル研究への応用

研究課題名（英文）Identification of TMEM168 protein mutation in familial Brugada syndrome

研究代表者
ＺＡＮＫＯＶ　ＤｉｍｉｔａｒＰ（Zankov, Dimitar Petrov）

国立研究開発法人国立循環器病研究センター・研究所・室長

研究者番号：２０６３１２９５

交付決定額（研究期間全体）：（直接経費）　　3,400,000円

研究成果の概要（和文）：Brugada症候群家系の全エクソン解析の結果、TMEM168遺伝子にp.R539Qのヘテロ変異を同定した。HL-1細胞にTMEM168-R539Qを発現させたところ、著明なNaV1.5の発現低下とNa+電流減少が観察された。Tmem168-R539Qノックインマウスでは、薬物投与にて心室頻拍および伝導障害が誘発された。またこのノックインマウスの心室筋でもNaV1.5の発現低下とNa+電流減少を認めた。これらの変化はNaV1.5へのNedd4-2の結合の増加とユビキチン化に依存していた。本結果は、TMEM168変異がBrugada症候群の不整脈発生に関与していることを示している。

研究成果の学術的意義や社会的意義
This study identified novel genetic variant associated with Brugada Syndrome. TMEM168 protein carrying the mutation is localized in the nuclear membrane and so far has unknown function. The mechanism of modulation of Na+ channel is unusual and will contribute to understanding of arrythmogenesis.

研究成果の概要（英文）：Brugada syndrome (BrS) is an inherited channelopathy identified as a cause of sudden cardiac death. Only 10-15% of Japanese BrS patients are diagnosed genetically. We used whole-exome sequencing to investigate candidate mutations in a BrS family. A heterozygous R539Q mutation was detected in TMEM168 gene of symptomatic individuals. Endogenous and transfected in HL-1 cells TMEM168 wild-type (WT) and mutant showed nuclear membrane localization. A significant decrease in Nav1.5 protein and Na+ current was observed in HL-1 cells expressing mutant TMEM168. Ventricular tachyarrhythmias and conduction disorders were pharmacologically induced in the heterozygous Tmem168 knock-in mice, but not in WT mice. Nav1.5 protein and Na+ current were reduced in ventricular myocytes in Tmem168 knock-in hearts. The impairment was dependent on increased Nedd4-2 binding to Nav1.5 and subsequent ubiquitination. Our results show a link between TMEM168 R539Q mutation and arrhythmogenesis in a family with BrS.

研究分野：Cardiac electrophysiology

キーワード：Inherited arrhythmia　Brugada syndrome　Sodium channel　Ubiquitination

1.研究開始当初の背景

(1) Sudden cardiac death (SCD) is defined as an unexpected death resulting from a cardiovascular condition within 1 hour after the onset of symptoms. SCD remains a significant burden with high all-cause mortality (estimated 15%-20% of all deaths) despite advances in the prevention and treatment of cardiovascular diseases [1].
(2) Brugada syndrome (BrS) is characterized by a typical electrocardiography: ST-segment elevation in right precordial leads and absence of structural heart disease [2]. BrS is endemic in Southeast Asia with a prevalence of 12/10 000 individuals. Four percent of all SCDs and nearly 20% of SCDs in the nonstructural cardiac disease group are caused by BrS. The only effective treatment at present is an implantable cardioverter-defibrillator (ICD). BrS is an autosomal dominant disease with incomplete penetrance and various clinical manifestations. Loss-of-function mutations in the *SCN5A* gene (encoding $Na_V1.5$, an α-subunit of the voltage-gated cardiac Na^+ channel) have been found in 20%-25% of BrS patients. Other genes encoding protein components of the cardiac Na^+, Ca^{++}, and K^+ channels have also been identified. However, only 30%-35% of clinical cases have been genetically diagnosed in Western world; in Japan between 10 and 15% [3].

2.研究の目的

Purpose of this study is to identify the causative genetic variant in a family with inherited Brugada syndrome, characterize functional significance and cellular mechanisms disturbed by the genetic defect.

3.研究の方法

Genomic DNA of 3 symptomatic and 1 clinically healthy individuals was analyzed by whole-exome sequencing, followed by bioinformatics pipeline and online prediction tools. Based on bioinformatics evaluation TMEM168 1616G>A variant was selected for functional analysis. In vivo technique: ECG, echography, genome editing (CRISPR/Cas9) in mice, together with molecular biology, immunostaining, cellular electrophysiology (patch-clamp) in culture cells or mouse cardiac myocytes evaluated the phenotype and cellular pathways altered by the mutation.

4.研究成果

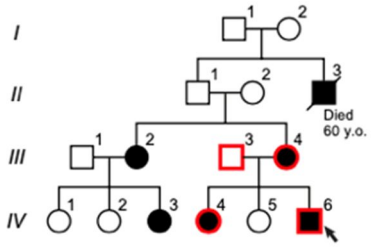

Figure 1

(1) Proband was a 20-year-old man (pedigree in Fig. 1) brought to an emergency hospital. Ventricular fibrillation and ST elevation at the V_1 lead after recovery, suggestive of BrS, were recorded there. ICD was implanted. Mother and sister also had a history of syncope. They underwent electrophysiological study, ventricular fibrillation was induced, and both were implanted with ICD.

(2) Exome sequencing: Whole exome sequencing was performed on gDNA from *III*-3, *III*-4, *IV*-4, and *IV*-6 (pedigree). The three symptomatic individuals did not possess any of the known causative gene mutations of BrS. After analysis (genome analysis tool kit, GATK, pipeline) we identified 15 single-nucleotide variants (SNV), confirmed by Sanger sequencing. The highest bioinformatics-tool score received TMEM168 1616G>A (R539Q) variant, new, and so far, not linked with arrhythmia syndromes missense mutation. The function of TMEM168 is unknown.

(3) Function of TMEM168: In the human heart sample (Fig. 2, arrowheads) and HL-1 cells, TMEM168 was seen at perinuclear region and co-localized with a nuclear membrane intermediate filament lamin A/C, suggesting nuclear membrane localization. We next cloned human *TMEM168* and generated enhanced green fluorescent protein (EGFP)-tagged expression vectors for WT and R539Q mutant *TMEM168*. In patch-clamp experiment, transfection of HL-1 cells with EGFP-TMEM168-R539Q significantly reduced the peak Na^+ current amplitude in contrast to WT-carrying vector. This reduction was in parallel with decrease of Nav1.5 protein in Western blot and Nav1.5-associated fluorescence in immunostained HL-1 cells.

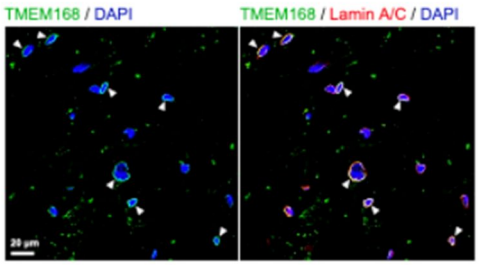

Figure 2

(4) Tmem168 1616G>A knock-in mice: *TMEM168* is highly conserved among mammalian species: the mouse and human proteins are 95% identical (BLAST). We generated *Tmem168* 1616G>A knock-in mice to investigate whether this substitution had the potential to cause fatal ventricular arrhythmia in vivo. Heterozygous *Tmem168* mutation knock-in mice, genetically similar to the symptomatic individuals in the analyzed family, were indistinguishable from littermate WT mice under normal conditions. The cardiac dimensions and func- tion on echocardiography were identical in WT and knock-in mice. ECG recordings at the basal state, did not exhibited any electrical disturbances, neither in WT nor in heterozygous *Tmem168* mutation knock-in mice. To mimic the diagnostic test for BrS in patients ajmaline, a Na^+ channel blocker, was intraperitoneally administered in WT and heterozygous *Tmem168* knock-in mice. Ajmaline application caused remarkable ST- segment elevation in precordial lead (V_1 lead) in the knock-in mice. Ajmaline administration induced ventricular tachyarrhythmias and conduction abnormalities in the knock-in mice, but not in WT mice (Fig. 2).

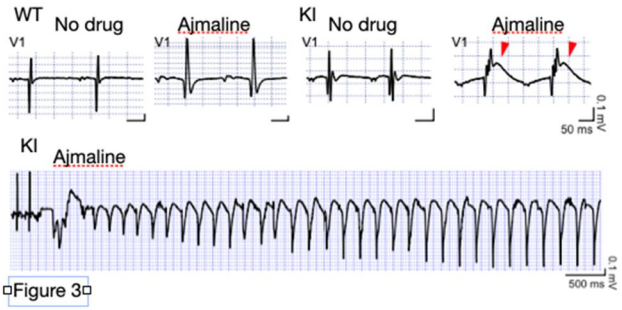

Figure 3

Electrophysiological study using ventricular myocytes isolated from WT and heterozygous *Tmem168* mutation knock-in mice demonstrated the results consistent with those from HL-1 cells. The Na^+ current was significantly reduced in the cardiomyocytes isolated from the knock-in mice, compared with those from WT mice. Both WT and *Tmem168* knock-in cardiomyocytes had very small sustained Na^+ current without significant difference in the amplitude. In addition, the maximum upstroke velocity (dV/dt_{max}) of the action potentials decreased in knock-in cardiomyocytes, while there was no difference in the resting membrane potential. Like in HL-1 cells electrophysiological findings coincided with reduction of cell surface expression of $Na_v1.5$ demonstrated by Western blot and immunohistochemistry.

(5) Understanding the cellular effects of *Tmem168* mutation on Nav1.5: The *Scn5a* mRNA level was evaluated by qPCR in the whole heart, nuclear and cytosolic fractions of the WT and heterozygous knock-in mouse hearts. No significant differences were detected among these samples suggesting posttranscriptional regulation of $Na_v1.5$ expression. It is known that the membrane fraction of $Na_v1.5$ is modulated by ubiquitination, which is mediated by Nedd4-2 E3 ubiquitin ligase binding to $Na_v1.5$. We investigated the association of $Na_v1.5$ with ubiquitin and Nedd4-2 in the WT and heterozygous *Tmem168* mutation knock-in mice. $Na_v1.5$ and its putative degradation products were more highly immunoprecipitated with ubiquitin in the heterozygous knock-in heart than the WT heart. The association between $Na_v1.5$ and Nedd4-2 was also increased in the knock-in heart in the immunoprecipitation assay, despite the reduced $Na_v1.5$ expression level in the

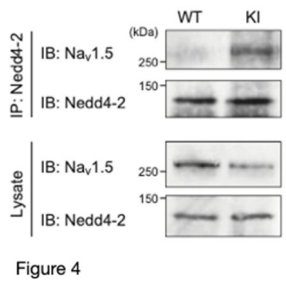

Figure 4

knock-in heart (Fig. 4). Collectively, the ubiquitination and degradation of $Na_v1.5$ were accelerated and the cell surface expression of $Na_v1.5$ was reduced in the *Tmem168* mutation knock-in heart.

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５．主な発表論文等

〔雑誌論文〕　計2件（うち査読付論文　2件／うち国際共著　2件／うちオープンアクセス　2件）

１．著者名 Akio Shimizu, Dimitar P.Zankov, Seiko Ohno, Minoru Horie, Hisakazu Ogita	４．巻 34
２．論文標題 Identification of transmembrane protein 168 mutation in familial Brugada syndrome	５．発行年 2020年
３．雑誌名 The FASEB Journal	６．最初と最後の頁 6399-6417
掲載論文のDOI（デジタルオブジェクト識別子） 10.1096/fj.201902991R	査読の有無 有
オープンアクセス オープンアクセスとしている（また、その予定である）	国際共著 該当する

１．著者名 Yamaha N, Asano Y, Fujita M, Zankov DP, Komuro I, Horie M, Kitakaze M, Takashima S	４．巻 139(18)
２．論文標題 Mutant KCNJ3 and KCNJ5 potassium channels as novel molecular targets in bradyarrhythmias and atrial fibrillation	５．発行年 2019年
３．雑誌名 Circulation	６．最初と最後の頁 2157-2169
掲載論文のDOI（デジタルオブジェクト識別子） 10.1161/CIRCULATIONAHA.118.036761.	査読の有無 有
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〔学会発表〕　計2件（うち招待講演　0件／うち国際学会　1件）

１．発表者名 Dimitar Petrov Zankov, Hisakazu Ogita
２．発表標題 A Novel TMEM168 Gene Mutation in Familial Brugada Syndrome Attenuates Na+ channel Function by Modulating Nav1.5 Expression
３．学会等名 Japanese Circulation Society Meeting
４．発表年 2019年

１．発表者名 Dimitar Petrov Zankov
２．発表標題 Human-specific desmoglein 2 mutations in mice models of arrythmogenic right ventricular cardiomyopathy reproduce patients' phenotype
３．学会等名 European heart rhythm association congress（国際学会）
４．発表年 2021年

〔図書〕　計0件

〔産業財産権〕

〔その他〕

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６．研究組織

	氏名 （ローマ字氏名） （研究者番号）	所属研究機関・部局・職 （機関番号）	備考
研究分担者	扇田　久和 (Ogita Hisakazu) (50379236)	滋賀医科大学・医学部・教授 (14202)	

７．科研費を使用して開催した国際研究集会

〔国際研究集会〕　計0件

８．本研究に関連して実施した国際共同研究の実施状況

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