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H．知的財産権の出願・登録状況

なし

1．特許取得

なし

2．実用新案登録

なし

3．その他

なし

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分担研究報告書

疾患特異的ヒトiPS細胞を用いた遺伝性不整脈疾患の解析
—カテコラミン誘発性多形性心室頻拍—

分担研究者　牧山　武　　京都大学大学院医学研究科循環器内科学　助教

研究要旨：カテコラミン誘発性多形性心室頻拍（CPVT）は、運動や情動などのカテコラミン刺激によって、心室頻拍・細動による突然死を引き起こす遺伝性不整脈疾患である。我々は、リアノジン受容体（RyR2）遺伝子異常が同定されているCPVT患者より、ヒト人工多能性幹（iPS）細胞を作製し分化心筋の解析を行った。電気的ペーシング下にCa transient測定を行ったところ、CPVT患者由来分化心筋では、健常人由来分化心筋に比べて、カテコラミン負荷後に拡張期細胞内Ca増加（diastolic Ca wave）を生じる細胞が有意に多かった。本研究にて、CPVTの病態を細胞レベルで一部再現でき、今後、薬効評価などの疾患モデルとしての有用性が期待される。

A．研究目的

カテコラミン誘発性多形性心室頻拍（CPVT）は、運動や情動などのカテコラミン刺激によって、心室頻拍・細動による突然死を引き起こす遺伝性不整脈疾患である。原因遺伝子として約50-60%に筋小胞体からのCa放出に関わるリアノジン受容体（RyR2）遺伝子異常が検出される。今回、CPVTの病態解明を目的とし、患者よりヒト人工多能性幹（induced pluripotent stem: iPS）細胞を作製し分化心筋の解析を行った。

B．研究方法

運動時の失神既往、二方向性心室頻拍を認め、RyR2遺伝子異常（p.I4587V）が検出されているCPVT患者において、皮膚を採取し、皮膚線維芽細胞を樹立した。ヒトiPS細胞の作製は、高橋、山中らの方法（Cell 131: 861-872）を用い、レトロウイルスにて以下の4遺伝子（OCT3/4、SOX2、KLF4、c-MYC）を導入し、iPS細胞を得た。心筋分化は胚様体形成法（Yang et al. Nature 2008）にて行った。心筋分化後、3か月の分化心筋を酵素処理後、単一細胞になるようにdishに接着させた。Ca imaging dyeとしてFluo-8を用い、単一心筋細胞のCa transientを計測した（AQUACOSMOS, 浜松フォトニクス）。計測は、15secずつ、rate 30, 60/分にて電気的ペーシングを行い、イソプロテレノール100nM負荷後、同様に電気的ペーシングを行い記録した。

（倫理面への配慮）

本研究は、京都大学医学部の倫理委員会にて承認済みである。

C．研究結果

単一分化心筋細胞のCa transient測定波形を示す。図1は、健常人iPS細胞由来分化心筋（201B7）の結果であるが、頻拍ペーシング下、イソプロテレノール負荷にても拡張期細胞内Ca増加（diastolic Ca wave）を認めなかった。

図1　健常人iPS細胞由来分化心筋のCa transient記録　（赤矢印はペーシングトリガー）

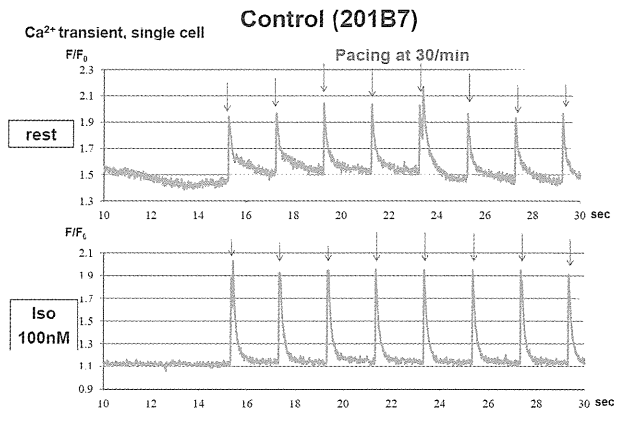
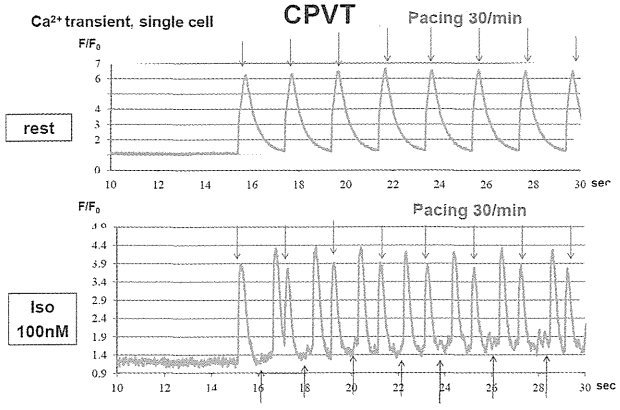


図2にCPVT-iPS細胞由来心筋細胞のCa transient代表波形を示す。下段の青矢印のようにイソプロテレノール負荷後、拡張期細胞内Ca増加（diastolic Ca wave）を認め、それに伴うtriggerd activityも観察された。

図２　CPVT-iPS細胞由来分化心筋のCa transient記録　（赤矢印はペーシングトリガー、黒矢印はdiastolic Ca wave）



統計解析にて、CPVT-iPS細胞由来分化心筋では、イソプロテレノール負荷後、diastolic Ca waveを認める細胞が有意に多かった。（図３）

（CPVT 55%（n＝31）v.s. コントロール22%（n＝36），pacing rate 30/min, p＝0.02, CPVT 50%（n＝34）v.s. コントロール19%（n＝43），pacing rate 60/min, p＝0.01）

図３　イソプロテレノール負荷後、diastolic Ca waveを認める頻度

D．考察

本研究にて、RyR2遺伝子異常を持つCPVT患者から疾患特異的ヒトiPS細胞を作製した。Ca diastolic waveは、CPVT model miceにてもみられる現象であり、CPVTの心筋細胞レベルのphenotypeが再現できていると考えられた。

現在、本モデルにおいて、各抗不整脈の薬効評価を進めている。

E．結論

本研究にてCPVT患者iPS細胞由来分化心筋にて細胞レベルの病態を一部再現できた。本モデルはさらなる病態解明や薬効評価に役立つと期待される。

F．健康危険情報

なし。

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H．知的財産権の出願・登録状況（予定を含む）

1．特許取得

なし

2．実用新案登録

なし

3．その他

なし

Ⅲ．研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

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Ⅳ. 研究成果の刊行物・別刷

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Age-Dependent Clinical and Genetic Characteristics in Japanese Patients With Arrhythmogenic Right Ventricular Cardiomyopathy/Dysplasia

Seiko Ohno, MD, PhD; Iori Nagaoka, MD, PhD; Megumi Fukuyama, MD; Hiromi Kimura, MD, PhD; Hideki Itoh, MD, PhD; Takeru Makiyama, MD, PhD; Akihiko Shimizu, MD, PhD; Minoru Horie, MD, PhD

Background: Arrhythmogenic right ventricular cardiomyopathy/dysplasia (ARVC/D) is a heart muscle disease caused by desmosomal gene mutations, and presents as ventricular tachycardia and sudden cardiac death. Although the mean age at onset or diagnosis of ARVC/D are reported to be around the 30–40 s, the age-dependent clinical and genetic differences remain unknown.

Methods and Results: A total of 35 consecutive Japanese probands (23 male) who were clinically diagnosed with ARVC/D were enrolled in the present study, and genetic analysis of *PKP2*, *DSP*, *DSG2*, and *DSC2* was done. The mean age at the first symptom and at diagnosis was 38.6±14.8 years and 40.5±17.7 years, respectively. Probands in whom the onset was cardiopulmonary arrest were significantly younger (22.3±15.3 years) than those with arrhythmia (41.1±13.2 years) or congestive heart failure (45.7±8.5 years). On genetic screening, 19 mutation carriers were identified. Although there was no age dependence for each gene mutation carrier, carriers with *PKP2* premature stop codon developed the disease at a significantly younger age than other mutation carriers.

Conclusions: The initial clinical manifestations in some young probands were very severe, and *PKP2* mutations with a premature stop codon would be associated with disease onset at a younger age.

Key Words: Arrhythmia; Cardiac arrest; Cardiomyopathy; Genes

Arrhythmogenic right ventricular cardiomyopathy/dysplasia (ARVC/D) is a disease characterized by right ventricular dysfunction and malignant arrhythmia.[1] Recent advances in molecular genetics have clarified the genetic background of ARVC/D. To date, 10 different genes have been reported to cause ARVC/D.[2–9] The majority of ARVC/D-causing genes encode desmosomal proteins: *PKP2*, encoding plakophilin 2; *DSP*, desmoplakin; *DSG2*, desmoglein 2; *DSC2*, desmocolin 2; and *JUP*, junctional plakoglobin. The most common gene variant identified in ARVC/D patients is reportedly *PKP2*, in approximately 25% of ARVC/D patients.[3]

In Japan, we first reported an ARVC/D patient with *PKP2* mutation,[10] and, recently, 4 of 8 ARVC/D patients were reported to have desmosomal gene mutations.[11] In other Asian countries, although *PKP2* mutations were identified only in China,[12–14] no other desmosomal gene mutation has been reported. Therefore, further examination of the ARVC/D etiology in Asian ethnicities is required.

Reportedly, ARVC/D patients become symptomatic at around 40 years old.[15,16] More recently, however, clinical features of pediatric ARVC/D patients with desmosomal gene mutations have been reported.[17] Most patients in that study were family members of those diagnosed with ARVC/D, and only patients with mutations were included. Moreover, there has been little discussion of sporadic cases in young patients, regarding the early detection or prevention of sudden death. The age-dependent clinical/genetic differences remain to be studied in terms of ARVC/D, including mutation-negative or sporadic cases.

In this study, we screened mutations in 4 desmosomal genes in 35 Japanese probands diagnosed with ARVC/D and then analyzed clinical and mutational characteristics, especially with regard to age.

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Department of Cardiovascular and Respiratory Medicine, Shiga University of Medical Science, Otsu (S.O., I.N., M.F., H.K., H.I., M.H.); Department of Cardiovascular Medicine, Kyoto University Graduate School of Medicine, Kyoto (S.O., T.M.); and Faculty of Health Sciences, Yamaguchi University Graduate School of Medicine, Ube (A.S.), Japan

Mailing address: Minoru Horie, MD, PhD, Department of Cardiovascular and Respiratory Medicine, Shiga University of Medical Science, Seta-Tsukinowa-cho, Otsu 520-2192, Japan. E-mail: horie@belle.shiga-med.ac.jp

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Table 1. Subject Clinical Characteristics and Diagnosis

Family no.	Sex	Age at onset (years)	Age at diagnosis (years)	RV function and structure	Task force criteria					Diagnosis		
					Tissue	Repolarization	Depolarization/ Conduction	Arrhythmia	Family history	Major criteria	Minor criteria	Category
1	M	29	30	A			I	I	M	2	2	Definite
2	M	48	49	A	I			I		1	2	Definite
3	M	16	16	A			A	I	M	3	1	Definite
4	F	36	36	A			A	I		2	1	Definite
5	M	51	51		I		A	A		2	1	Definite
6	F	–	15	A		I		I	M	2	2	Definite
7	M	40	64	A	A		I			2	1	Definite
8	F	15	15	A				A	M	3	0	Definite
9	M	44	49	A		I		A	M	3	1	Definite
10	F	47	47	A		I		A	M	3	1	Definite
11	M	71	72	A			I	I	M	2	2	Definite
12	M	42	45	A		A		A		3	0	Definite
13	M	40	40	A			I	I	M	2	2	Definite
14	M	41	41	A	A		I	I	M	3	2	Definite
15	F	–	16					I	M	1	1	Borderline
16	M	5	5	A				A		2	0	Definite
17	M	17	17	A		A		I	M	3	1	Definite
18	M	34	38			A	I	I		1	2	Definite
19	F	25	25			A		I		1	1	Borderline
20	F	50	63		I			I		0	2	Possible
21	M	43	43	A				A		2	0	Definite
22	M	30	30	A			I			1	1	Borderline
23	M	26	32				A	I		1	1	Borderline
24	F	47	47	A			I	A		2	1	Definite
25	M	58	70	A				I	A	2	1	Definite
26	F	17	18				I	I		0	2	Possible
27	M	48	56	A				A	M	3	0	Definite
28	F	42	43		A	A		A	M	4	0	Definite
29	M	25	25	A		A	A	I	M	4	1	Definite
30	F	55	63	A		A		A		3	0	Definite
31	M	55	56			I	I	A	M	2	2	Definite
32	M	49	50	I			I	I	M	1	3	Definite
33	M	27	34			A		A	M	3	0	Definite
34	M	54	62	A			I	I	M	2	2	Definite
35	F	44	53	A			I	I	M	2	2	Definite

A, major criteria; I, minor criteria; M, mutation positive; RV, right ventricular.

Methods

Subjects

The subjects consisted of 35 probands (23 male) clinically diagnosed with or suspected to have ARVC/D from unrelated families, and 16 family members from 8 families. Each underwent detailed clinical and cardiovascular examinations for diagnosis, including an electrocardiogram (ECG), echocardiography, magnetic resonance imaging, and Holter ECG. Some patients underwent RV angiography, myocardial biopsy, and signal-averaged electrocardiography. They were classified into 3 diagnostic categories of ARVC/D: definite, probable, and possible according to the 2010 diagnostic criteria.[18] They were referred consecutively to either of the present laboratories for genetic evaluation. All subjects submitted written informed consent in accordance with the guidelines approved by each institutional review board.

Genotyping

Genomic DNA was isolated from venous blood lymphocytes, as previously described.[19] Using polymerase chain reaction analysis and direct DNA sequencing, we performed a comprehensive open reading frame/splice site mutational analysis of 4 major ARVC/D susceptibility genes: *PKP2*, encoding plakophilin 2; *DSP*, encoding desmoplakin; *DSG2*, encoding desmoglein 2; and *DSC2*, encoding desmocolin 2. The cDNA sequences of *PKP2*, *DSP*, *DSG2*, and *DSC2* were based on the GenBank reference sequences NM_004572.3, NM_004415.2, NM_001943.3, and NM_004949.3, respectively. We did not screen for *JUP* (encoding junction plakoglobin). In addition to desmosomal genes, we screened for *LMNA*[20] in the present probands, and identified a missense mutation in 1 patient. In this study, we excluded the patient with a *LMNA* mutation from the analysis. All new putative pathogenic variants were examined in 200 reference alleles derived from unrelated

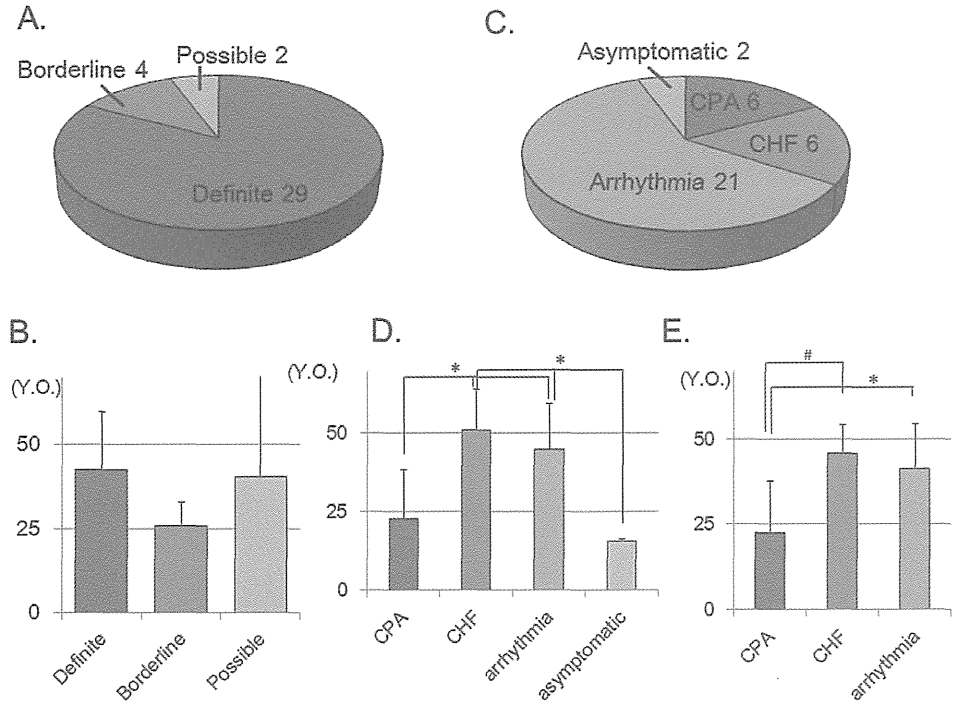

Figure 1. Characteristics of the probands. Number of the probands according to (**A**) diagnostic criteria and (**C**) first symptom. (**B**,**D**,**E**) Mean age at diagnosis according to (**B**) diagnostic category or (**D**) first symptom; and (**E**) at onset according to first symptom. CHF, congestive heart failure; CPA, cardiopulmonary arrest. *P<0.05, #P<0.01.

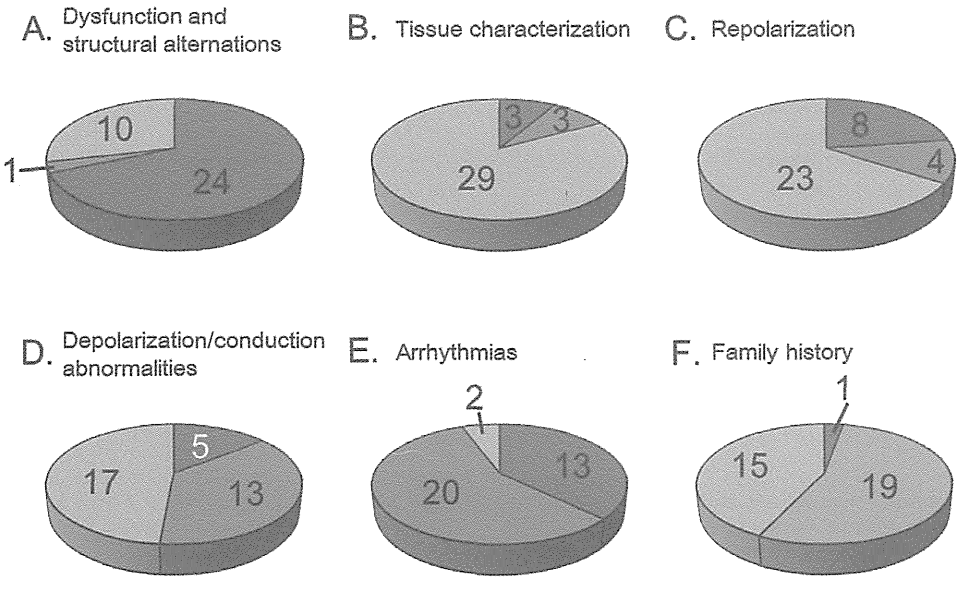

Figure 2. Number of the patients who fulfilled the criteria for arrhythmogenic right ventricular cardiomyopathy/dysplasia. Red, major criteria; blue, minor criteria; orange, none; light green, gene mutation carriers in major criteria.

Table 2. Desmosomal Gene Mutations Identified in the Present Study

No.	Sex	Age at diagnosis (years)	Category	PKP2		DSP		DSG2		DSC2	
				Codon	Amino acids	Codon	Amino acids	Codon	Amino acids	Codon	Amino acids
1	M	30	Definite	1725–1728dupGATG	R577DfsX5						
2	M	49	Definite								
3	M	16	Definite	1132 C>T	Q378X						
4	F	36	Definite								
5	M	51	Definite								
6	F	15	Definite							394 C>T	R132C
										582 C>G	N194K
										607 C>T	R203C
7	M	64	Definite								
8	F	15	Definite			8269 G>C	D2757H				
9	M	49	Definite			2360 A>G	Y787C				
10	F	47	Definite	2150 C>T	P717L†						
11	M	72	Definite	953 A>C	H318P†						
12	M	45	Definite								
13	M	40	Definite			4741 A>G (H)	K1581E† (H)	1592 T>G	F531C		
14	M	41	Definite	976 G>A (H)	A326T† (H)	593 A>C (H)	Q198P (H)				
15	F	16	Borderline			8455 A>C	M2819L†				
16	M	5	Definite								
17	M	17	Definite	1725–1728dupGATG	R577DfsX5						
18	M	38	Definite								
19	F	25	Borderline								
20	F	63	Possible								
21	M	43	Definite								
22	M	30	Borderline								
23	M	32	Borderline								
24	F	47	Definite								
25	M	70	Definite								
26	F	18	Possible					2780 C>T	P927L#		
27	M	56	Definite	875–890 del	L266QfsX104						
28	F	43	Definite					1481 A>C	D494A† (H)		
29	M	25	Definite	2119 C>T	Q707X						
30	F	63	Definite								
31	M	56	Definite					1481 A>C	D494A†		
32	M	50	Definite			4741 A>G	K1581E†				
33	M	34	Definite	2095 C>T	Q699X			2780 C>T	P927L#		
34	M	62	Definite			1203 G>T	K401N				
35	F	53	Definite	1725–1728dupGATG	R577DfsX5						

†Reported in NCBI SNP database; #identified in the present control cohort. H, homozygous mutation.

healthy Japanese controls.

Age Analysis

The present probands were divided into 2 groups according to age at diagnosis: ≤40 years old (younger group, n=16) and >40 years old (older group, n=19). We also compared the clinical and genetic characteristics between the 2 groups.

Statistical Analysis

All continuous variables are reported as mean±SD. Differences between continuous variables were evaluated using the Wilcoxon rank sum test for 2 groups and 1-way ANOVA for ≥3 groups. Categorical variables were analyzed using chi-square test (for counts ≥5) or Fisher exact test (for counts <5) for 2 groups and Kruskal-Wallis ANOVA rank test for >2 groups. Significance was considered at $P<0.05$.

Results

Clinical Features

Clinical subject characteristics are summarized in **Table 1**. According to the 2010 diagnostic ARVC/D criteria,[18] 29 probands