Figure 3. Irbesartan attenuates the Hypo-S-induced shortening of APD_{90} in guinea pig atrial myocytes. Superimposed traces of action potentials in Iso-S followed by Hypo-S (a), Hypo-S + 1 μM irbesartan (b), or Hypo-S + 50 μM irbesartan (c), respectively. (d) The percentage decrease in APD_{90} after exposure to Hypo-S without and then with irbesartan (1 μM and 50 μM). Although the effects of irbesartan on the shortening of APD_{90} were significant, no statistical difference was observed in the resting membrane potentials. $**p < 0.01$ vs. control.

Table I. Effect of 1 μM irbesartan on deactivation time constants (τ) of I_{Ks} in isosmotic solution (Iso-S) and hyposmotic solution (Hypo-S) at four different test potentials.

Parameters		Deactivation τ (ms) at different test potentials			
	N	−60 mV	−50 mV	−40 mV	−30 mV
Iso-S	27	194.9 ± 10.7	291.0 ± 18.5	426.2 ± 29.7	590.7 ± 43.9
Hypo-S	14	271.9 ± 21.2[b]	398.2 ± 35.7[b]	542.3 ± 52.2[a]	678.2 ± 65.8
(Δ%)		(43.25 ± 5.15)	(41.57 ± 6.62)	(27.34 ± 4.09)	(16.17 ± 4.04)
Hypo-S + 1 μM irbesartan	13	246.3 ± 23.6[a]	357.8 ± 32.7	491.1 ± 46.0	622.3 ± 59.4
(Δ%)		(25.75 ± 7.67)	(23.99 ± 7.12)	(19.94 ± 6.67)	(7.84 ± 5.17)

I_{Ks}: delayed rectifier K^+ current; N: number of cells; Δ%, percentage increase of τ values over Iso-S; data are expressed as the mean ± S.E.M.; $^a p < 0.05$ and $^b p < 0.01$ vs. Iso-S.

Discussion

Cellular electrophysiological studies have indicated that the most important impact of AF on ion channels is the marked reduction in inward $I_{Ca,L}$ currents,[12] which leads to atrial contractile dysfunction and induces increased membrane stretching of outward I_{Ks} currents.[17,21,22,24] Changes in both $I_{Ca,L}$ and I_{Ks} contribute to the atrial APD shortening

and the loss of its physiological rate adaptation, which promotes atrial electrical and structural remodeling and creates a substrate for persistent AF.[12,23]

In the present study, we found that irbesartan attenuated the stretch-induced enhancement of I_{Ks}, suggesting that the drug possesses the ability to improve the pathophysiological conditions precipitating AF. This hypothesis is supported by the recent identification of *KCNQ1* (encoding the α-subunit of the I_{Ks} channel) S140G and R14C mutations in familial AF cases, in which both "gain-of-function" mutations cause an enhancement of I_{Ks}.[25,26] In addition, atrial membrane stretching results in an increase of I_{Ks}-mediated shortening of the atrial APD, which may facilitate the maintenance of AF.[17,27,28] Irbesartan can rescue shortening of the APD that is induced by cell membrane stretching. Thus, the inhibition of extreme APD shortening as a result of atrial membrane stretching is involved in the mechanism underlying irbesartan-mediated AF prevention.

Evidence suggests that the RAS plays a pivotal role in the occurrence and maintenance of AF. Atrial membrane stretching during AF not only activates AT_1R,[29,30] but also induces the secretion of Ang II from cardiomyocytes.[14,15] Madrid and colleagues[31] reported that irbesartan in combination with amiodarone was more effective at preventing the recurrence of AF than amiodarone alone. Several recent clinical reports[6-10] and animal experiments[2,32] have also confirmed the effect of AT_1R blockers on AF. In the present study, therapeutically relevant concentrations of irbesartan attenuated the stretch-induced increase, but not baseline, levels of atrial I_{Ks}, suggesting that the action of the drug on electrical changes is associated with blocking AT_1R. This result is consistent with a previous study[33] that found that irbesartan prevented the arrhythmogenic effect of Ang II by blocking AT_1R in human atrial myocardium. Zankov et al. reported that the selective AT_1R blockers valsartan and candesartan attenuate Ang II- and stretch-induced enhancement of I_{Ks} and shortening of APD, respectively, by activating AT_1R in guinea pig atrial myocytes.[16,17] Based on these previous findings together with the observations of irbesartan in this study, we conclude that the actions of ARBs on electrical changes associated with the dilation or stretch of the atria are involved in the AT_1R blockade and are beneficial for the prevention of acute electrical remodeling during AF.

In the present study, we also found that Hypo-S depolarized resting membrane potentials in guinea pig myocytes. Since the inward rectifier current I_{K1} is responsible for maintaining the resting membrane potential,[23] and irbesartan did not affect the depolarization of the resting membrane potential caused by atrial membrane stretching, we postulate that I_{K1} is not the therapeutic target of AT_1R blockers during AF.[34] This lack of effect on the resting membrane potential was also observed with candesartan in a previous study.[17]

Conclusions

Irbesartan-mediated AT_1R blockade attenuates the electrical changes induced by stretching atrial myocytes. This is likely why AF patients derive a pronounced benefit with ABRs such as irbesartan.

Acknowledgements

Conflict of interest

None declared.

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A Nonsynonymous Polymorphism in *Semaphorin 3A* as a Risk Factor for Human Unexplained Cardiac Arrest with Documented Ventricular Fibrillation

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Abstract

Unexplained cardiac arrest (UCA) with documented ventricular fibrillation (VF) is a major cause of sudden cardiac death. Abnormal sympathetic innervations have been shown to be a trigger of ventricular fibrillation. Further, adequate expression of *SEMA3A* was reported to be critical for normal patterning of cardiac sympathetic innervation. We investigated the relevance of the semaphorin 3A (*SEMA3A*) gene located at chromosome 5 in the etiology of UCA. Eighty-three Japanese patients diagnosed with UCA and 2,958 healthy controls from two different geographic regions in Japan were enrolled. A nonsynonymous polymorphism (I334V, rs138694505A>G) in exon 10 of the *SEMA3A* gene identified through resequencing was significantly associated with UCA (combined P = 0.0004, OR 3.08, 95%CI 1.67–5.7). Overall, 15.7% of UCA patients carried the risk genotype G, whereas only 5.6% did in controls. In patients with *SEMA3A*[I334V], VF predominantly occurred at rest during the night. They showed sinus bradycardia, and their RR intervals on the 12-lead electrocardiography tended to be longer than those in patients without *SEMA3A*[I334V] (1031 ± 111 ms versus 932 ± 182 ms, P = 0.039). Immunofluorescence staining of cardiac biopsy specimens revealed that sympathetic nerves, which are absent in the subendocardial layer in normal hearts, extended to the subendocardial layer only in patients with *SEMA3A*[I334V]. Functional analyses revealed that the axon-repelling and axon-collapsing activities of mutant *SEMA3A*[I334V] genes were significantly weaker than those of wild-type *SEMA3A* genes. A high incidence of *SEMA3A*[I334V] in UCA patients and inappropriate innervation patterning in their hearts implicate involvement of the *SEMA3A* gene in the pathogenesis of UCA.

Introduction

Unexpected sudden death in healthy individuals remains a daunting problem. Unexplained cardiac arrest with documented ventricular fibrillation (UCA) including idiopathic ventricular fibrillation (IVF) is defined as spontaneous VF that is not associated with a known structural or electrical heart disease.

IVF is diagnosed in up to 10% of survivors of out-of-hospital cardiac arrest [1].

Many reports have documented the role of abnormal sympathetic innervations as a trigger of VF [2–6]. Sympathetic innervation of the heart is determined during development by chemoattractive and chemorepulsive factors. Semaphorins, members of a conserved family of both secreted and integral membrane

Author Summary

Unexplained cardiac arrest with documented ventricular fibrillation (UCA) is defined as spontaneous ventricular fibrillation (VF) that is not associated with known structural or electrical heart diseases and is one of the major causes of sudden cardiac death. Identification of the genes responsible for UCA may further increase our understanding of mechanisms of UCA and facilitate more accurate diagnosis and preventive treatment, especially in asymptomatic disease-carrying relatives of the patient. However, molecular mechanisms of UCA have not been fully clarified due to the high mortality rate and difficulty of diagnosis. In this study, UCA patients are shown to have a high incidence of a polymorphism in the Semaphorin 3A gene (rs138694505, *SEMA3A*^{I334V}). The result confirms previous reports that the abnormal sympathetic innervation is a trigger of UCA because *SEMA3A* is crucial for the establishment of normal innervation patterns in the heart. Furthermore, experimental data presented here indicate that *SEMA3A*^{I334V} disrupts the *SEMA3A* function and impairs appropriate innervation patterning. Finally, the study suggests that *SEMA3A*^{I334V} is a risk factor for human UCA and contributes to the etiology of UCA.

proteins, are typical chemorepulsive factors acting on the growth cone as guidance cues to control the establishment of neural connections [7,8]. Recently, *SEMA3A* was shown to form an epicardial-to-endocardial transmural sympathetic innervation pattern in the heart. In addition, disruption of innervation patterning in both *SEMA3A* -deficient and *SEMA3A*-overexpressing mice resulted in sudden death or lethal arrhythmias [9,10].

Identification of the genes responsible for UCA may further increase our understanding of the pathophysiology of UCA and facilitate the diagnosis and prophylactic treatment, especially in asymptomatic, disease-carrying relatives of the proband. In the current study, we investigated the significance of the SEMA3A gene polymorphisms in the etiology of UCA.

Results

Genetic analysis of the *SEMA3A* gene in UCA patients

The subjects were divided into two geographic regions based on their birthplace information, as shown in Figure S1. The characteristics of the two regional groups of UCA patients enrolled in this study are listed in Table 1. There was no significant difference in the clinical characteristics among the two UCA groups. As for control groups, the gender distribution was similar in the two groups, but individuals were older in Eastern Japan as compared to Western Japan (70±9 years vs. 47±16 years).

Only one nonsynonymous polymorphism was identified in exon 10 of the *SEMA3A* gene through resequencing of the coding region. This polymorphism causes an amino acid substitution from isoleucine to valine (I334V, *SEMA3A*^{I334V}) and is identical with the SNP that was recently submitted to dbSNP (rs138694505). There was a significant difference in genotype frequencies between UCA cases and controls in the western Japan (dominant model P = 0.007). This association was replicated in the Eastern Japan (P = 0.008). The Breslow-Day test showed no heterogeneity among the groups, and the overall degree of association by the Mantel-Haenszel test was P = 0.0004 (OR 3.08, 95%CI 1.67–5.70) (Table 2). Collectively, 13 of the 83 UCA patients (15.7%) carried the risk genotype G, whereas only 5.6% did in the controls. The

Table 1. Characteristics of UCA with VF patients.

	Western Japan	Eastern Japan
No. of patients	52	31
Age (years)	43±17	43±14
Male gender	40 (76.9%)	24 (77.4%)
Documented VF	52 (100%)	31 (100%)
History of syncope	9 (17.9%)	9 (29.0%)
History of atrial fibrillation	11 (21.2%)	7 (22.6%)
Family history of sudden cardiac death	11 (21.2%)	6 (19.4%)
Time of VF events		
Daytime (8:00–20:00)	31 (59.6%)	17 (54.8%)
Nighttime (20:00–8:00)	21 (40.4%)	14 (45.2%)
Situation at VF events		
During exercise or physical effort	16 (30.8%)	11 (35.5%)
During sleeping or just after getting up	13 (25.0%)	13 (42.0%)
After meals or drinking	2 (3.8%)	1 (3.2%)
During driving	4 (7.7%)	1 (3.2%)
Relaxed at home	10 (19.2%)	2 (6.5%)
At restroom	2 (3.8%)	0 (0%)
Unknown/Others	9 (17.6%)	3 (9.6%)
Posture at VF events		
During standing	26 (50.0%)	13 (41.9%)
Seated or supine position	41 (46.2%)	17 (54.8%)
Unknown	2 (3.8%)	1 (3.2%)

UCA: unexplained cardiac arrest, VF:ventricular fibrillation, Data was mean±SD.
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SEMA3A^{I334V} carrier frequency appeared to be relatively stable throughout the age classes (Data not shown).

Genotype distribution of *SEMA3A* polymorphisms (rs138694505) among ethnicity

According to the 1000 Genomes Project, regional differences in the *SEMA3A*^{I334V} (rs138694505) frequency are evident among populations. For example, the frequency of the G allele is 2.1% in East Asians (3.93% in Japanese), 1.35% in West Africans, 1.86% in Americans, and 0% in Europeans (Table 3).

Phenotype characterization of UCA patients and clinical findings of UCA patients with and without *SEMA3A*^{I334V} (rs138694505)

Two UCA cases were severe (Figure 1, patients 1 and 2). They suffered from VF at a young age and had a family history of sudden cardiac death. VF attacks recurred on several occasions in these patients. In one patient (Patient 1), VF recurred twice after discharge and was terminated by an implanted cardioverter defibrillator (ICD) shock (Figure 2 upper panel). According to the ICD records, a preceding transient bradycardia was followed by short coupled ectopic ventricular beats, finally leading to VF. Another patient (Patient 2) went into an electrical storm at midnight one day after hospitalization (Figure 2 lower). VF occurred suddenly during sinus bradycardia. She had been suffering repeated epileptic seizures with loss of consciousness from the age of 15. Most patients with *SEMA3A*^{I334V} were found to have sinus bradycardia and sinus node dysfunction by an

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Table 2. SEMA3A polymorphism (SEMA3AI334V: rs138694505) in patients with UCA and controls.

Region	IVF			control			Odds ratio	95%CI	P[a]	P_het[b]
	AA	AG	GG	AA	AG	GG				
Western Japan	45	7	0	1943	102	1	2.9	1.3–6.7	0.007	
	86.5%	13.5%	0.0%	95.0%	5.0%	0.0%				
Eastern Japan	25	6	0	850	60	2	3.3	1.3–8.3	0.008	
	80.6%	19.4%	0.0%	93.2%	6.6%	0.2%				
Combined[c]							3.08	1.67–5.70	0.0004	0.86

SEMA3A: semaphorin 3A, UCA: unexplained cardiac arrest,
[a]P value of chi-square test in dominant model,
[b]Result of Breslow-Day test,
[c]Combined meta-analysis was performed using the Mantel-Haenszel method.
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electrophysiological study. Figure 1 shows the ECGs before the ICD implantation in patients 1, 2 and 3. Because of the sinus bradycardia and in order to prevent a VF recurrence, the ICD was set to the AAI⁺ mode at 60–75 bpm. The number of tests that was performed in the UCA patients is shown in Table S2. The phenotype characterization in each UCA patient with *SEMA3A*[I334V] is shown in Table S3. Patient 3 had persistent AF and patient 13 had chronic AF. Patients 1,2,5,7 and 9 had 1st degree atrioventricular block.

Table 3. Regional differences in rs138694505 G allele frequency among populations.

Population		Genotype count			G allele frequency(%)
		AA	AG	GG	
European	CEU	87	0	0	0
	TSI	98	0	0	0
	GBR	89	0	0	0
	FIN	93	0	0	0
	IBS	14	0	0	0
	subtotal	381	0	0	0
East Asian	CHB	95	2	0	1.03
	JPT	82	7	0	3.93
	CHS	98	1	1	1.50
	subtotal	275	10	1	2.10
West African	YRI	85	3	0	1.70
	LWK	95	2	0	1.03
	subtotal	180	5	0	1.35
American	ASW	57	4	0	3.28
	MXL	64	2	0	1.52
	PUR	52	3	0	2.73
	CLM	60	0	0	0
	subtotal	233	9	0	1.86
Total		1069	24	1	1.19

Allele frequencies were estimated using the 1000 Genome Project dataset.
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In Table 4 we present the clinical, electrocardiographic, and echocardiographic findings between the UCA patients with and without *SEMA3A*[I334V]. VF occurred predominantly at rest and during the night in the patients with *SEMA3A*[I334V]. In contrast, it occurred during exercise and during the day in most patients without *SEMA3A*[I334V] (VF occurred during the night 69.2% vs. 37.1%, $P = 0.032$, VF occurred at rest 69.2% vs. 34.3%, $P = 0.015$).

Some of the patients with *SEMA3A*[I334V] had sinus bradycardia, and their RR intervals on the 12-lead ECG tended to be longer than those without (1031 ± 111 ms vs. 932 ± 182 ms, $P = 0.039$). None of the UCA subjects regularly took β-blockers during their ECG recordings. One patient without Sema3a[I334V] took 100 mg/day of oral amiodarone when recording the ECG. The other cases did not have any anti-arrhythmic agents. Early repolarization (ER) was evident in only two *SEMA3A*[I334V] cases (15.4%), whereas 34 patients (48.6%) without *SEMA3A*[I334V] demonstrated ER ($P = 0.02$). The other 12-lead ECG parameters, signal-averaged ECG, and echocardiographic findings, were similar in the patients with and without *SEMA3A*[I334V].

Screening of the *SEMA3A* region using tag SNPs

To screen the entire SEMA3A gene, 47 tag SNPs were additionally genotyped in the UCA patients from Hiroshima/Nagasaki University and the healthy controls from Hiroshima University (Table S1). All SNPs were successfully genotyped in >98% of the samples. Among them, one SNP, rs1533996, was not polymorphic. The other SNPs were within the Hardy-Weinberg equilibrium (P>0.01) in the controls except for rs13437857 (P = 0.0031) and rs10280701 (P = 0.000086). The p value of the I334V in the population (p = 4.53E-08) was still significant even if a Bonferroni correction for the tag-SNP approach was applied (p = 2.12E-06). None of the 47 tag SNPs were significantly associated with UCA after the Bonferroni correction. The I334V variant showed a moderate linkage disequilibrium only with rs740948 ($r^2 = 0.43$). A haplotype analysis revealed that no haplotype had a stronger association with UCA than the single marker analysis (data not shown).

Sympathetic nerve localization and nerve growth factor (NGF) expression in UCA patients with and without *SEMA3A*[I334V] (rs138694505)

Representative immunofluorescence images for vinculin (a cell surface marker) and anti-tyrosine hydroxylase (TH) in the sympathetic nerves in the subendocardial layer of patients with and without *SEMA3A*[I334V] are shown in Figure 3. Under normal conditions, the TH nerves were reported to exist in the subepicardial layer of cardiomyocytes, not in the subendocardial
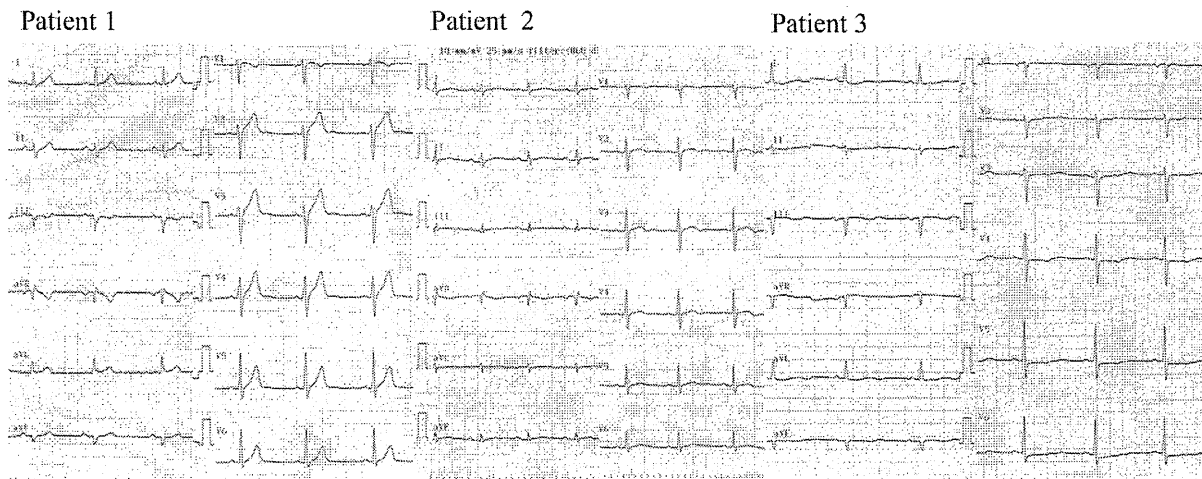
−238−

Patient 1   Patient 2   Patient 3

Figure 1. Twelve-lead ECG of patients with *SEMA3A*[I334V]**.** Twelve-lead ECG of typical patients wth sinus bradycardia (left to right, each patient 1–3) before the ICD implantation.
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layer (9). In patients without $SEMA3A^{I334V}$, no TH nerves were observed in the subendocardial layer, consistent with earlier findings in normal subjects. In patients with $SEMA3A^{I334V}$, in contrast, TH nerves were distributed in the subendocardial layer (right panel, the arrowheads indicate TH positive nerves). This finding was consistently observed in patients with $SEMA3A^{I334V}$ (N = 4) but not without $SEMA3A^{I334V}$ (N = 8), suggesting abnormal sympathetic innervation in the heart of UCA patients with SEMA3A^{I334V}. On the other hand, NGF, a neural attractant factor, was similarly expressed in the subendocardial layer in patients with and without $SEMA3A^{I334V}$ (Figure 4).

Expression and function of SEMA3A and SEMA3A[I334V]

As a result of a DRG repulsion assay, $SEMA3A^{WT}$-expressing cells repelled the DRG axons on the proximal side of the ganglia (Figure 5, left). In contrast, DRG explants were less responsive to $SEMA3A^{I334V}$ (Figure 5, middle).

Figure 6 shows the percentage of collapsed growth cones in the E8 chick embryos incubated with media containing $SEMA3A^{WT}$, $SEMA3A^{I334V}$ and vector only (negative control) at 0.3, 0.1, and 0.03 dilutions of a concentrated media, respectively. At all dilutions, $SEMA3A^{WT}$, and $SEMA3A^{I334V}$ were similarly expressed and secreted (Figure 6). The secreted proteins for both $SEMA3A^{WT}$ and $SEMA3A^{I334V}$ were similar in size (approximately 65 kDa). The growth cone collapse by $SEMA3A^{I334V}$ was less frequent than that of $SEMA3A^{WT}$ at all concentrations. ($SEMA3A^{WT}$ vs. $SEMA3A^{I334V}$: 84.8±1.5% vs. 75.8±1.8% at a dilution of 0.3, $P=0.009$, and 70.2±1.1% vs. 57.2±2.4% at a dilution of 0.1, $P=0.009$; Figure 6, lower).

Discussion

To the best of our knowledge, this is the first report demonstrating that UCA patients have a high incidence of I334V SNP (rs138694505) in the *SEMA3A* located at chromosome 5. Furthermore, new experimental data presented here indicates that $SEMA3A^{I334V}$ disrupts the *SEMA3A* function of inhibiting neural growth and impaired appropriate innervation patterning in the heart. Finally, this study suggested that $SEMA3A^{I334V}$ is a risk factor for human UCA and contributes to the pathogenesis of UCA.

Many studies have reported the relationship between abnormal autonomic nerve activity and lethal ventricular arrhythmias, and in most of them I^{123}-MIBG imaging was used to aid in the detection of sympathetic innervation abnormalities [3–5,11]. However, the molecular mechanisms determining these innervation densities in patients with lethal arrhythmia have not been fully clarified. Elucidation of underlying genetic defects will provide further insight into the pathogenesis of UCA, but identification of the genes involved in UCA is very difficult because of its high mortality rate and subsequent diagnostic difficulties. Unlike other monogenic arrhythmia syndromes (e.g., long QT syndrome, catecholaminergic polymorphic ventricular tachycardia and Brugada syndrome), the diagnosis of UCA cannot be made on the basis of ECG abnormalities prior to the occurrence of VF. In addition, UCA is only diagnosed by excluding any identifiable structural or functional cardiac diseases among the few survivors of VF. One case report indicated that a missense variant of the KCNJ8 gene, a subunit of the K_{ATP} channel, conferred a predisposition to dramatic depolarization changes and ventricular vulnerability [12]. In another report, Alders et al. demonstrated that a haplotype on chromosome 7, which includes the DPP6 gene (associated with potassium channel I_{to} subunits), was the causal gene of IVF [13,14].

Sympathetic innervation of the heart is sculpted during development by chemoattractive factors such as NGF and chemorepulsive factors such as *SEMA3A*. NGF acts through the Trk A and p75 neurotrophin receptors in sympathetic neurons. Lorenz et al. reported heterogeneous ventricular sympathetic innervation, altered β-adrenergic receptor expression, and rhythm instability in mice lacking the p75 neurotrophin receptor within the heart [15]. Ieda et al. [9,10] reported that cardiac innervation patterning is disrupted in *SEMA3A*-deficient and *SEMA3A*-overexpressing mice, leading to lethal arrhythmias and sudden death. On the basis of this background information, we focused on *SEMA3A*, which plays a crucial role in cardiac innervation patterning [7–10,16], as abnormal sympathetic innervations have been demonstrated in patients with UCA. We observed that a polymorphism in exon 10 of the *SEMA3A* gene (i.e., *SEMA3A*[I334V]), located in the semaphorin domain, which plays an essential role in *SEMA3A* [17], was highly prevalent in patients
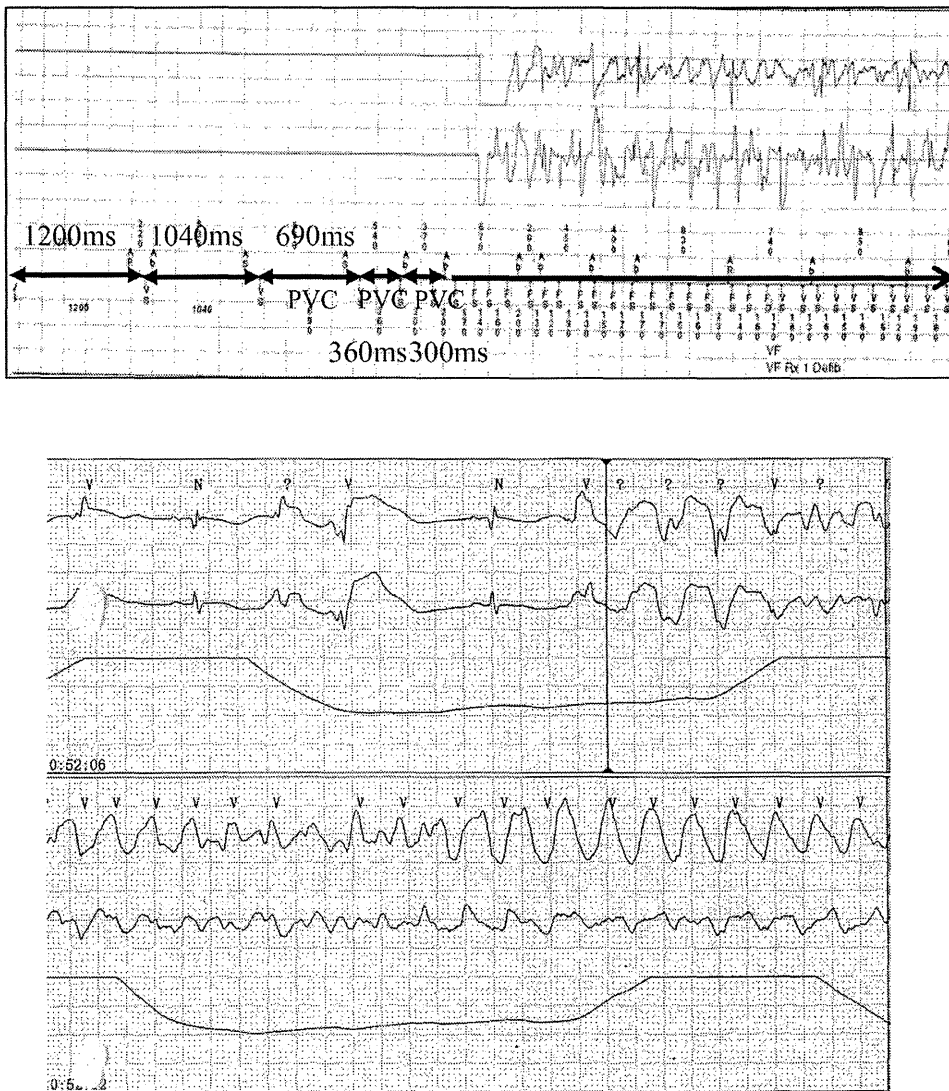
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Figure 2. Ventricular fibrillation in patients with *SEMA3A*^{I334V}. After discharge, VF recurred twice and was terminated by ICD shocks in one male patient (patient 1). According to the ICD records, a preceding transient bradycardia was followed by short coupling ectopic ventricular beats and finally VF occurred (upper). The day after admission to the emergency unit, another female patient (patient 2) went into an electrical storm. VF occurred suddenly during sinus bradycardia (lower).
doi:10.1371/journal.pgen.1003364.g002

with UCA and strongly associated with UCA pathophysiology. To our knowledge, this is the first report that investigates the relevance of functional mutations or polymorphisms in *SEMA3A* with respect to human diseases.

We divided the case and control subjects into two geographical groups based on their birthplace in Japan. Significant results observed in Western Japan were replicated in the Eastern Japan group, and the combined P value and odds ratio calculated by the Mantel-Haenszel test were 0.0004 and 3.08, respectively.

According to publicly available data from the 1000 Genomes Project, the frequency of this risk allele of *SEMA3A* is similar among populations other than Europeans, suggesting that this variant may be relevant to the etiology of UCA across these populations. In our study, the G allele frequency was 2.8% in the controls, which was consistent with that reported in Japanese

(3.9%) and East Asian populations (2.1%) in the 1000 Genomes Project.

Haïssaguerre et al. reported an increased prevalence of ER characterized by J-point elevation among patients with a history of UCA [18]. Antzelevitch et al. classified ER patterns for risk stratification of VF [19]. The genetic basis for ER is slowly coming into better focus. Burashnikow et al. identified loss of function mutations in the $\alpha 1$, $\beta 2$, and $\alpha 2\delta$ subunits of the cardiac L-type calcium channels (CACNA1C, CACNB2, and CACNA2D1) in patients with ER syndrome [20]. Abe et al. reported that ER may be closely associated with depolarization abnormalities and autonomic modulation [21]. In this study, only two UCA cases with *SEMA3A*^{I334V} demonstrated ER. Instead, the characteristics of the cases with *SEMA3A*^{I334V} suffered VF attacks in a relaxed state and presented with sinus bradycardia/sinus node dysfunc-

Table 4. Comparison of the clinical and electrocardiographic findings in UCA patients with and without $SEMA3A^{I334V}$.

	$SEMA3A^{I334V}$: rs138694505 (+)	$SEMA3A^{I334V}$: rs138694505 (−)	
	N = 13	N = 70	
Clinical Data			
Age of VF occurrence (y)	48±17	42±16	P = 0.273
Gender (Male%)	9 (69.2%)	55 (78.5%)	P = 0.462
History of syncope	3 (23.1%)	15 (21.5%)	P = 0.894
History of atrial fibrillation	3 (23.1%)	15 (21.5%)	P = 0.895
Family History of SCD	4 (30.1%)	13 (18.5%)	P = 0.317
VF occurred during night time	9 (69.2%)	26 (37.1%)	P = 0.032*
VF occurred at rest	9 (69.2%)	24 (34.3%)	P = 0.015*
Twelve Lead ECG Findings			
RR (ms)	1031±111	932±182	P = 0.039*
PQ (ms)	180±36	171±30	P = 0.312
QRS (ms)	110±43	97±17	P = 0.498
QTc (ms)	421±35	413±42	P = 0.238
Presence of J wave	2 (15.4%)	34 (48.6%)	P = 0.020*
Signal Averaged ECG Findings			
fQRSd (ms)	128±31	121±24	P = 0.705
RMS 40 (uV)	39±28	30±26	P = 0.511
LAS 40 (ms)	36±7	37±10	P = 0.760
Echocardiographic Findings			
LVDd (mm)	46.5±5.2	48.1±5.7	P = 0.411
IVSTd (mm)	9.0±1.1	9.2±2.0	P = 0.881
EF (%)	66.2±6.9	63.4±7.6	P = 0.242

UCA: unexplained cardiac arrest, VF:ventricular fibrillation, SCD:sudden cardiac death, SEMA3A: Semaphorin 3A, LVDd: left ventricular end diastolic volume, IVSTd: interventricular septum thickness, EF:ejection fraction, fQRSd: filtered QRS duration, RMS 40: root mean square 40 ms, LAS 40: under 40 uV duration, Data are presented as mean ± SD,
*p<0.05 between $SEMA3A^{I334V\ (+)}$ vs $SEMA3A^{I334V(−)}$.
doi:10.1371/journal.pgen.1003364.t004

tion. These findings are consistent with the report by Ieda et al. [9,10] that $SEMA3A^{-/-}$ mice lacked a cardiac sympathetic innervation gradient and exhibited satellite ganglia malformations, which led to marked sinus bradycardia due to sympathetic dysfunction. Some of the UCA cases in our study may have a mild degree of depolarization or repolarization abnormalities, although we could not detect any obvious organic diseases such as cardiomyopathy by diagnostic imaging or manifest conduction disturbances. The other patients did not have any depolarization or repolarization abnormalities. The patients with $SEMA3A^{I334V}$ do not have a homogeneous phenotype and we have to follow up the clinical course of the UCA patients with $SEMA3A^{I334V}$ for a long period.

The frequency of AF was 21.6% and rather high in the UCA subjects of our study for unknown reasons and the frequency was similar in the patients with and without $SEMA3A^{I334V}$. One possible reason was that the episodes of AF after resuscitation were included in the past history of AF.

In our study, immunofluorescence staining of the RV revealed that sympathetic nerves were distributed in the subendocardial layer only in patients with $SEMA3A^{I334V}$. If $SEMA3A$ exists in adequate quantities in the endocardial layer and functions normally, sympathetic nerves extending to the endocardial layer are suppressed. We assumed that in UCA patients with $SEMA3A^{I334V}$, the epicardial-to-endocardial transmural sympathetic innervation patterning had deteriorated.

An $SEMA3A^{WT}$- and $SEMA3A^{I334V}$-concentrated media did not grossly affect the expression, stability, or secretion of the ligand. As for the molecular weight of $SEMA3A$, when it was expressed in HEK293, the full semaphorin domain (65 kDa) was cleaved and detected in a conditioned media [22]. The sizes of the secreted proteins in both $SEMA3A^{WT}$ and $SEMA3A^{I334V}$ were equal and coincident with the semaphorin domain including a dimerization interface and Neurolipin-1 (Nrp-1)-binding residue, and the biological activity was sufficient for the acquisition of a high repulsive activity [22].

The function of repelling the DRG axons was weaker and growth cone collapse was less frequent in $SEMA3A^{I334V}$ than in $SEMA3A^{WT}$. Therefore, one allele of $SEMA3A$ leads to a disruption of the sympathetic innervation of the heart under relevant conditions. These findings were consistent with immunofluorescence observations strongly suggesting that $SEMA3A^{I334V}$ can disrupt the ability of $SEMA3A$ to repel or collapse DRG axons and sensory neuron growth cones under equal conditions of the neural attractant NGF.

Merte et al. reported that a forward genetic screen in mice identified a novel loss of function $SEMA3A^{K108N}$ mutation, which bound to Nrp-1 but failed to repel or collapse DRG axons in vitro [23]. $SEMA3A^{I334V}$ exists in blade 5 of the 7-bladed propeller structure of the semaphorin domain and performs a crucial function in $SEMA3A$. Residues 333–335 in 5S of $SEMA3A$ constitute the dimerization interface. The $SEMA3A$-65K dimerization interface overlaps with sites responsible for the initial high-
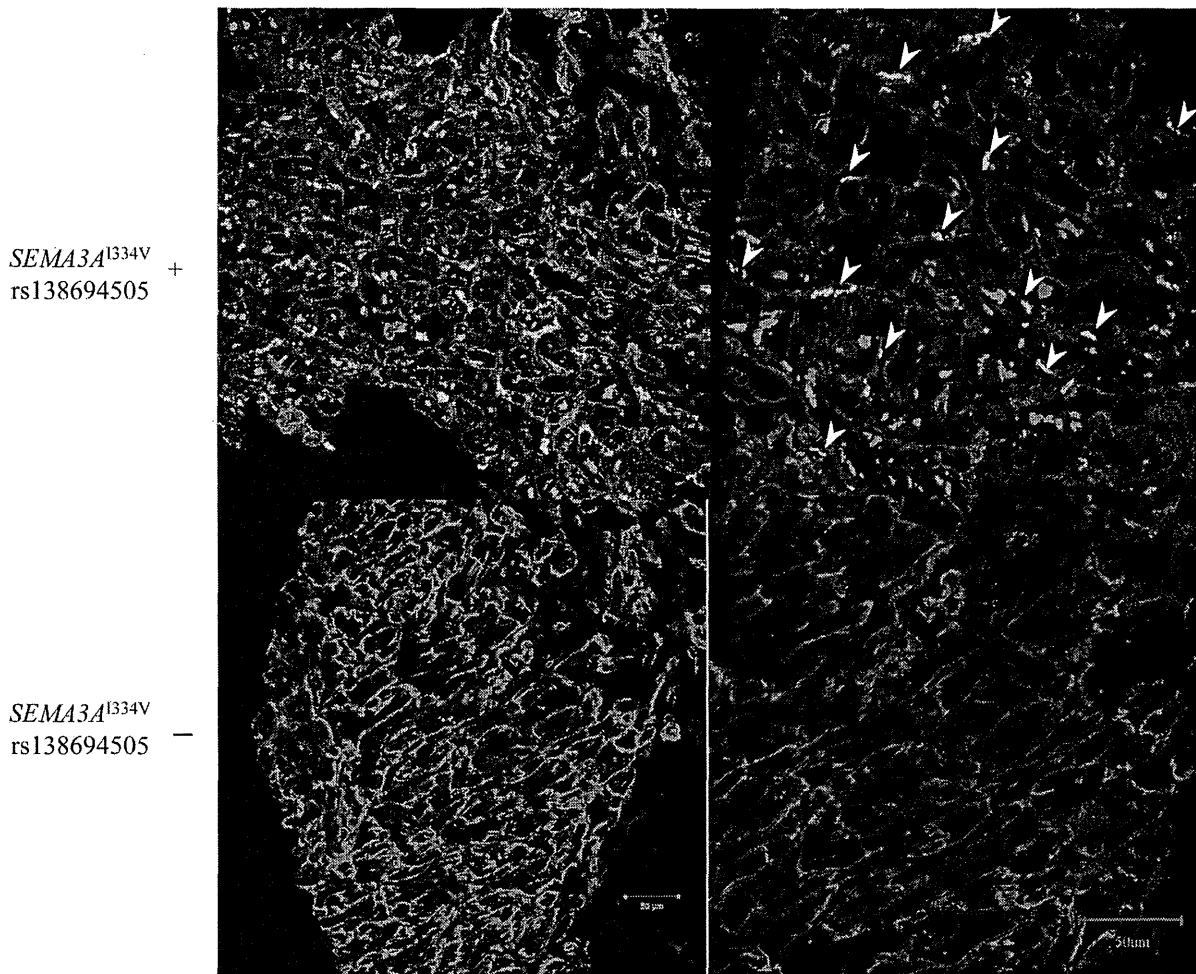
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Figure 3. Immunofluorescence staining for Vinculin and anti-TH in the subendocardial layer of patients with and without $SEMA3A^{I334V}$. Immunofluorescence staining of cardiac biopsy specimens revealed that TH positive nerves, as sympathetic nerves, which are absent in the subendocardial layer in normal hearts, extended to the subendocardial layer only in patients with $SEMA3A^{I334V}$ (red; anti-Vinculin, green; anti-TH). The samples were examined using a confocal microscope and captured with a 20×objective lens in the figures on the left and with a 40× objective lens in the figures on the right. The arrowheads show the TH positive nerves (Upper panels: $SEMA3A^{I334V}$+, Lower panels: $SEMA3A^{I334V}$−).
doi:10.1371/journal.pgen.1003364.g003

affinity binding to the domain of Nrp-1. Binding of *SEMA3A* to Nrp-1 leads to a conformational change in Plexin-A1, which is transmitted to the cytosolic domain [17].

In the association analysis, $SEMA3A^{I334V}$ was highly prevalent in patients with UCA and associated with the UCA pathophysiology. On the other hand, none of the control subjects with $SEMA3A^{I334V}$ had any signs of disease at the time of the study, indicating incomplete penetrance or additional environmental or genetic factors.

Our study had several limitations. First, it was very difficult to congregate many UCA cases and therefore the size of our study population was too small to obtain any robust findings. Secondly, we were not able to study the segregation data in the UCA patients with SEMA3A^{I334} because their families refused screening. A future prospective study with a larger cohort will be required to obtain these data. A further functional study would also be desirable to determine whether any abnormal innervation can be observed in healthy carriers by using autopsy specimens

In conclusion, a polymorphism of $SEMA3A^{I334V}$ diminishes the cardiac sympathetic innervation gradient and partially contributes to the etiology of UCA. This finding is important in elucidating the pathogenesis of UCA.

Materials and Methods

Subjects

We recruited a total of 83 UCA patients (64 male and 19 female, mean age 43±16 years) from Hiroshima University Hospital, Nagasaki University Hospital, Shiga University of Medical Science, and the National Cerebral and Cardiovascular Center. We recruited 2958 controls (1540 male and 1452 female, mean age 54±18 years) from Hiroshima University Hospital, Osaka-Midosuji Rotary Club (Osaka, Japan), Shiga University of Medical Science, and Niigata University Graduate School of Medical and Dental Sciences. All patients and controls in this paper were unrelated Japanese individuals.
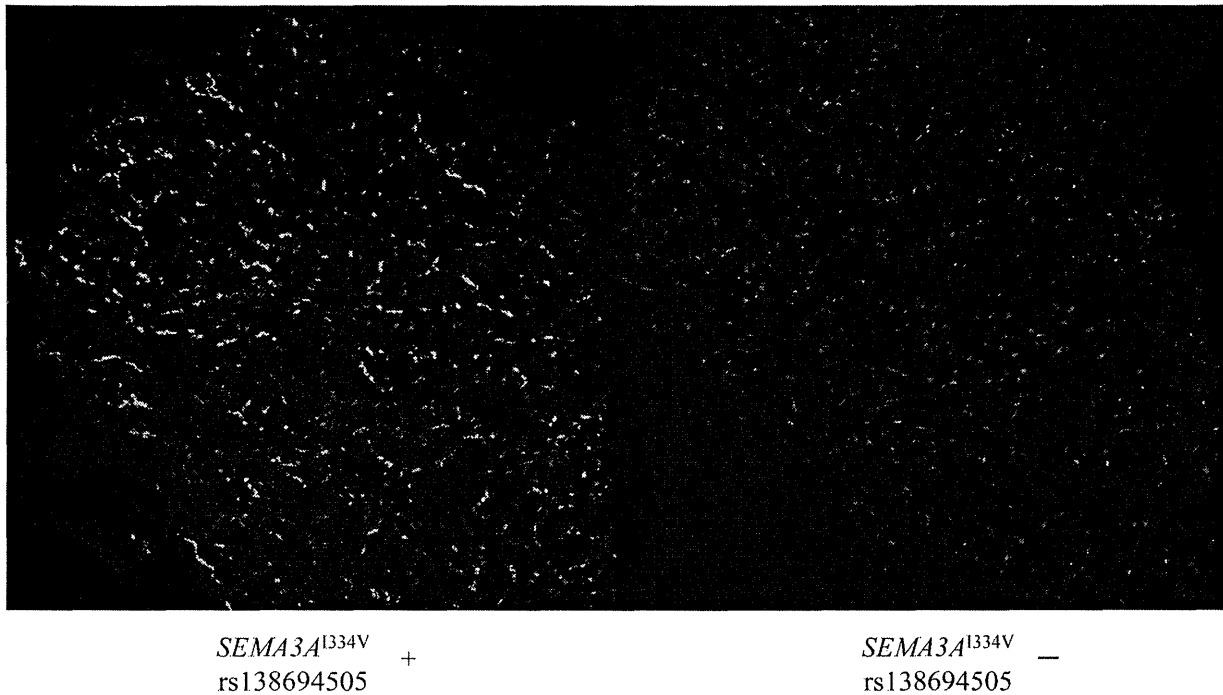
anti-Tyrosine Hydroxylase and NGF

$$SEMA3A^{I334V} \quad +$$
rs138694505

$$SEMA3A^{I334V} \quad -$$
rs138694505

Figure 4. Immunofluorescence staining for Vinculin and NGF in the subendocardial layer of patients with and without $SEMA3A^{I334V}$.
On the other hand, the levels of the NGF, a neural attractant factor, were expressed in the subendocardial layer and are comparable between patients with (left panel) and without (right panel) $SEMA3A^{I334V}$ (red; anti-NGF; green: anti-TH).
doi:10.1371/journal.pgen.1003364.g004

Case and control subjects were collected from various regions of Japan. Although the Japanese population has rather low genetic diversity, it has been shown that population structures may lead to spurious associations [24]. Therefore, to eliminate the possibility of a population stratification, we divided case and control subjects into two groups geographically based on their birthplace information (i.e., Western Japan and Eastern Japan) (Figure S1).

The Institutional Ethics Committee of the Graduate School of Biomedical Science at Hiroshima University approved all procedures involving human tissue usage. Written informed consent was obtained from all subjects prior to participation.

Twelve subjects enrolled in the study were diagnosed and treated at the Hiroshima University Hospital; the other subjects were diagnosed and treated at other affiliated hospitals and their information was provided to us.
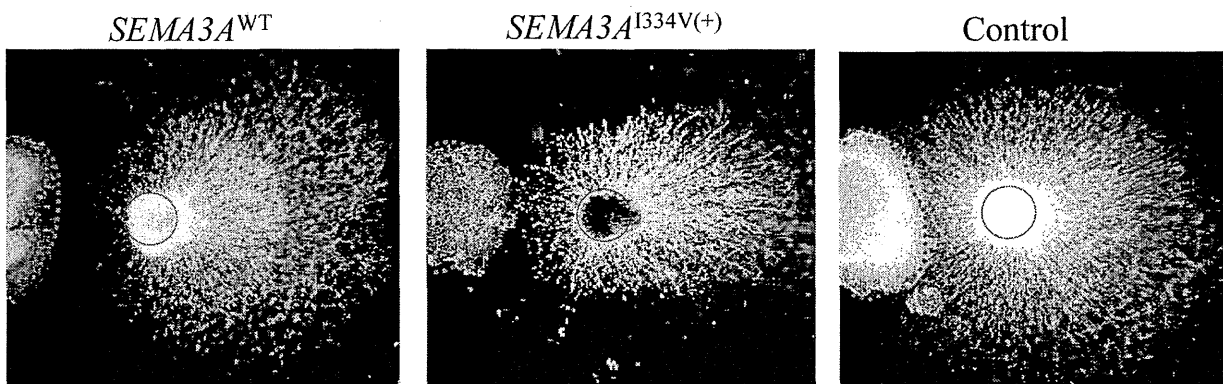
$$SEMA3A^{WT} \qquad SEMA3A^{I334V(+)} \qquad \text{Control}$$

Figure 5. DRG repulsion assay of the $SEMA3A^{WT}$, $SEMA3A^{I334V}$, **or control.** $SEMA3A^{WT}$ expressing cells repelled DRG axons on the proximal side of the ganglia (left). In contrast, DRG explants were less responsive to $SEMA3A^{I334V}$ (middle).
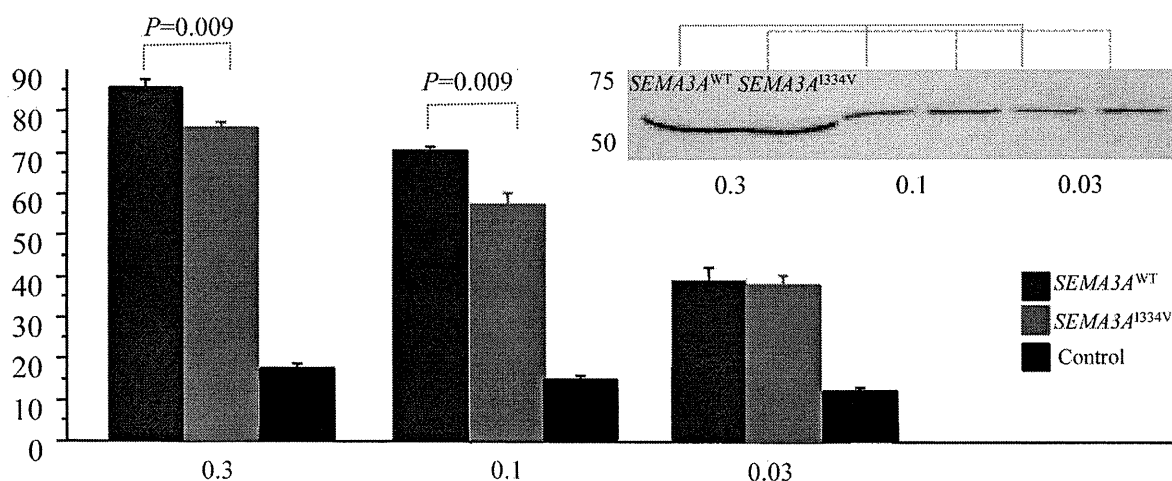doi:10.1371/journal.pgen.1003364.g005

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Figure 6. Growth cone collapse assay of the *SEMA3A*^{WT}, *SEMA3A*^{I334V}, or control. The percent of collapsed growth cones of the E8 chick embryos incubated with medium containing vector only, *SEMA3A*^{WT}, or *SEMA3A*^{I334V} at dilutions (0.03, 0.1, and 0.3) of a concentrated media. All dilutions of the concentrated media of the *SEMA3A* or *SEMA3A*^{I334V} expressed in HEK293T cells were similarly secreted. *SEMA3A*^{WT} and *SEMA3A*^{I334V} led to a collapse of the DRG neuron growth cones in all concentrations, but growth cone collapses by *SEMA3A*^{I334V} (red bar) were significantly less than those by *SEMA3A*^{WT} (blue bar) at the dilutions (0.3, 0.1) of the concentrated media ($P = 0.009$).
doi:10.1371/journal.pgen.1003364.g006

Diagnosis of UCA

We defined UCA as that without structural heart disease and in the absence of signs of an arrhythmia syndrome such as Brugada syndrome, catecholaminergic polymorphic ventricular tachycardia and long QT syndrome. All patients with cardiac arrest underwent a physical examination, 12 lead ECG [25], echocardiography and coronary angiography to rule out any underlying heart disease. Those who met the inclusion criteria were enrolled and underwent additional testing (signal averaged ECG, T wave alternance, cardiac magnetic resonance imaging, computer tomography, provocation tests, cardiac biopsy or an electrophysiological study), if possible. The numbers of further noninvasive or invasive tests against UCA patients varied from institute to institute. Patients with exonic mutations in SCN5A and a positive pilsicainide challenge test were excluded from the sample. Early repolarization (ER) was defined as a QRS slurring or notching of ≥ 0.1 mV in more than two consecutive leads of the 12-lead ECG.

Sequence analysis of *SEMA3A* genomic DNA and genotyping

Peripheral blood was obtained from all the subjects. Genomic DNA was extracted from leukocytes using a QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany) according to the standard protocol. Using Go Taq (Promega, Madison, WI, USA), all coding regions of the *SEMA3A* located at chromosome 5 were amplified by PCR from 2.5-ng genomic DNA using our original primers in 17 UCA patients and 15 healthy controls entered from Hiroshima University. These amplified coding regions were then resequenced using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) to identify mutations and polymorphisms.

Subsequently, SNP genotypes were genotyped in All of the UCA subjects and healthy control subjects using the Invader assay or the TaqMan assay, as described previously [26,27].

Tag SNP selection

The 47 tag SNPs were genotyped only in the UCA patients and the healthy controls entered from Hiroshima University and Nagasaki University. Using the HapMap database (public release

#27, hapmap.ncbi.nlm.nih.gov) and the Haploview program (www.broad.mit.edu/mpg/haploview) and based on selection criteria of $r^2 > 0.8$ and a minor allele frequency of > 0.01 for the Japanese population, tagging-SNPs were selected from the *SEMA3A* region spanning approximately 247 kb, from approximately 5 kb upstream of the transcription start site to 5 kb downstream of the 3' untranslated region.

Plasmid construction

The complete coding region of human *SEMA3A* was amplified from cDNA with forward (tgttagtgttgccatgaggtct) and reverse (gcattcacctgtgttctctgttag) primers. To generate Flag- *SEMA3A*, the coding sequence DYKDDDD was introduced between the codons for G25 and K26 (NM_006080.2). The I334V mutation was introduced by site-directed mutagenesis using the QuickChange (Stratagene, La Jolla, CA, USA). Full-length human wild-type (*SEMA3A*^{WT}) or mutant *SEMA3A* (*SEMA3A*^{I334V}) cDNA was cloned into pcDNA3.1(+) (Invitrogen, Carlsbad, CA, USA).

Immunofluorescence staining of anti-tyrosine hydroxylase (TH), nerve growth factor (NGF), and vinculin

Transverse sections of a septal site of the RV outflow tract were obtained by biopsy from 12 UCA subjects (4 patients with *SEMA3A*^{I334V} and 8 patients without *SEMA3A*^{I334V}). These sections were embedded in an OCT compound (Sakura, Torrance, CA, USA) and frozen with liquid nitrogen. Immunofluorescence staining was performed using the frozen sections with rabbit anti-TH (AB152, Millipore, Billerica, MA, USA) antibodies and mouse anti-vinculin (Sigma-Aldrich, St. Louis, MO, USA) antibodies diluted at concentrations of 1:100 and 1:200, respectively, in 1% BSA/PBS. Alexa 488-conjugated goat anti-rabbit and Alexa 568-conjugated goat anti-mouse antibodies (Invitrogen) were used as secondary antibodies. As for NGF, sheep polyclonal to NGF (ab49205, Abcam, Cambridge, MA, USA) and rabbit anti-TH (ab152, Millipore) were used as primary antibodies at concentrations of 1:100 in 1% BSA/PBS. Alexa568 donkey anti-sheep (A21099) and Alexa488 donkey anti-rabbit (A21206) antibodies were used as secondary antibodies. Nuclei were stained

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with 10 μM of Hoechst 33342 (Molecular probes). Samples were examined using a confocal microscope and captured with a 20× and 40× objective lens on a Zeiss LSM 510 laser scanning microscopy system (Carl Zeiss, Thornwood, NY, USA).

DRG repulsion assay and growth cone collapse assay of *SEMA3A*^{WT} and *SEMA3A*^{I334V}

The DRG were dissected from E8 chick embryos. HEK293T cells were transfected with Flag- *SEMA3A*^{WT} or *SEMA3A*^{I334V} expression vector or equal amounts of empty vector (control) using Gene Juice Transfection Reagent (Novagen, Madison, WI, USA). The DRG and *SEMA3A* -expressing HEK293T cell aggregates were embedded as described previously [28]. Samples were incubated at 37°C in a 5% CO_2 humidified incubator for 48 h and examined using an inverted microscope. For DRG repulsion assays, 10–15 DRG cells were examined, each with Sema3A^{WT}, Sema3A^{I334V}, or a control.

For the purpose of a growth cone collapse assay, the conditioned medium of the *SEMA3A* -expressing HEK293T cells was concentrated [22]. A Western blot analysis was performed using both dilutions of the *SEMA3A*^{WT} and *SEMA3A*^{I334V} concentrated media with anti-FlagM2 (Sigma). Growth cone collapse assays were performed as previously described using chick E8 DRG explants grown on laminin (Invitrogen)- and poly-L-lysine (Sigma)-coated 48-well plates (BD Falcon/353078). The dilution series of the *SEMA3A*^{WT}, *SEMA3A*^{I334V} and vector only concentrates were added to each well and incubated at 37°C in a 5% CO_2 humidified incubator for 30 min. The explants were fixed with 4% paraformaldehyde in 10% sucrose PBS (pH 7.4), and the samples were examined using an inverted microscope [29]. In each dilution series, 5 or 6 growth cone collapse assays were investigated. Each in vitro assay was performed in triplicate.

For quantification, we counted at least 50 growth cones to score on each explant. We assigned each growth cone as either collapsed or not collapsed, and the results were expressed as the percentage of collapsed to all counted growth cones. We compared the percentage of those collapsed between the *SEMA3A*^{WT} and *SEMA3A*^{I334V}.

Statistical analysis

Normally distributed continuous variables are presented as the mean ± SD. Continuous data between the two groups were analyzed using the nonparametric Mann–Whitney U test. For testing the genetic associations in the case–control studies, the chi-square test and Cochran–Armitage trend test were used. Tests for the Hardy–Weinberg equilibrium among the cases and controls were conducted for observed and expected genotype frequencies using an ordinary chi-square test, where a *P*-value of <0.05 was considered statistically significant. For a meta-analysis of 3 individual cases and controls, we used the Mantel-Haenszel test.

Supporting Information

Figure S1   The case and control subjects were divided into two groups geographically based on their birthplace information (i.e., Western Japan and Eastern Japan).
(PDF)

Table S1   Forty-seven tag SNPs of *SEMA3A* were additionally genotyped in the UCA patients and the healthy controls from Hiroshima University. The I334V variant had a moderate linkage disequilibrium only with rs740948 ($r^2 = 0.43$). None of the other SNPs were significantly associated with the UCA after a Bonferroni correction. a: Tagging-SNPs other than I334V were selected based on the selection criteria of an r 2 of >0.8 and minor allele frequency of >0.01in the HapMap-JPT population. b: chi-square test P value in the allele frequency model (uncorrected). c: Hardy-Weinberg equilibrium tests in the control subjects.
(DOCX)

Table S2   The number of tests that we performed for the UCA in VF patients.
(DOCX)

Table S3   Phenotype characterizations in each UCA patient with *SEMA3A*^{I334V}. Patient 3 had persistent AF and patient 13 had chronic AF. Patients 1,2,5,7 and 9 had 1st degree atrioventricular block. Patient 1 had positive late potentials and the fQRSd was increased in a number of patients.
(DOCX)

Author Contributions

Conceived and designed the experiments: YN KC HO ST YK. Performed the experiments: MT HS DM CNH TT KA YH KH SO. Analyzed the data: NO KS YU-M KK CM MF YW HW TA WS MH. Contributed reagents/materials/analysis tools: AS YY KO IW NM AN NE. Wrote the paper: YN HO.

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Original Article

Genetic screening of *KCNJ8* in Japanese patients with J-wave syndromes or idiopathic ventricular fibrillation[☆]

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ABSTRACT

Background: J-point elevation has been demonstrated to be associated with ventricular fibrillation (VF) and has been proposed as a cause of the J-wave syndrome (JWS). A mutation of *KCNJ8*, S422L, was reported as a culprit gene. This study aimed to determine the prevalence of *KCNJ8* mutations in a Japanese population with JWS or idiopathic VF (IVF).
Methods: A total of 230 probands with JWS and IVF underwent genetic screening of *KCNJ8*. To analyze and compare clinical and electrocardiographic characteristics, the probands were divided into 4 groups: Brugada (Br) pattern only, early repolarization (ER) pattern only, Br and ER patterns, and true IVF.
Results: The results of the genetic analysis revealed no S422L or other *KCNJ8* mutations and indicated no significant difference between the groups.
Conclusion: The *KCNJ8* mutation showed no association with JWS or IVF among our Japanese patients.
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1. Introduction

Ventricular fibrillation (VF) is the most malignant arrhythmia that causes sudden cardiac death. VF, which occurs in apparently healthy individuals without structural heart diseases, is regarded as an idiopathic or primary electrical disease. Among patients with idiopathic VF (IVF), an increased prevalence of early repolarization (ER) was observed, which was considered as a benign electrocardiographic (ECG) finding in the past [1]. Recently, the concept of J-wave syndrome (JWS), including the ER and Brugada (Br) syndromes, has emerged [2].

The mutation *KCNJ8*-S422L was first identified as the cause of ER syndrome in a 14-year-old Caucasian girl with VF. An ECG showed a prominent ER pattern in the inferolateral leads [3]. *KCNJ8* encodes an inward rectifier potassium 6.1 protein (Kir6.1),

which is a subunit of cardiac ATP-sensitive K (K_{ATP}) channels. The succeeding cases of the mutation were found in five men and two women with ER or Br syndrome [4,5]. However, whether *KCNJ8* mutations are present among the Asian population remains to be known. Therefore, the present study aimed to investigate the prevalence of *KCNJ8* gene mutation in Japanese patients with JWS or IVF.

2. Method

2.1. Study population

The study population was selected from unrelated families and consisted of 230 Japanese probands with IVF, Br syndrome, or suspected Br syndrome. All the probands underwent genetic analysis between 1996 and 2011 at the Shiga University of Medical Science and Kyoto University Graduate School of Medicine. Probands with structural heart diseases or other inheritable arrhythmic disorders were excluded from the study.

2.2. DNA isolation and mutation analysis

The genetic analysis protocol was approved by the institutional ethics committees of both universities and performed under the respective institutional guidelines. All the patients provided informed
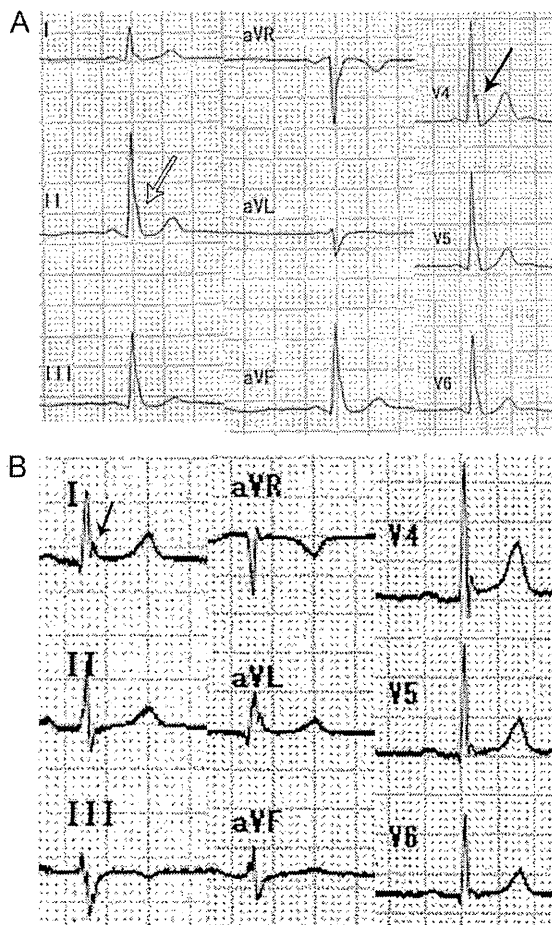
Fig. 1. Twelve-lead ECGs representing an early repolarization (ER) pattern. (A) ER pattern in the inferior and lateral leads. (B) ER pattern in the lateral leads only. Arrows indicate a slurring (opened) or notching (closed) ER morphology.

consent before undergoing the genetic analysis. Genomic DNA was isolated from leukocyte nuclei. We performed genetic screening for KNCJ8, SCN5A, CACNA1C, CACNB2, SCN1B, KCNQ1, KCNH2, KCNE1-3, and KCNE5 using denatured high-performance liquid chromatography (WAVE System Model 3500, Transgenomic, Omaha, NE, USA) [6–8], high-resolution melting (LightCycler®480, Roche Diagnostics GmbH, Mannheim, Germany), and direct sequencing with the ABI PRISM 3130 sequencer (Applied Biosystems, Foster City, CA, USA). The cDNA sequence numbering of KCNJ8 was based on the GenBank reference sequence (NC_000012). We excluded all the probands who had genetic test results positive for all the above-mentioned genes, except KCNJ8.

2.3. Clinical phenotype

We analyzed the following demographic characteristics in 230 patients: sex, age at diagnosis, history of symptoms, and indication for treatment with an implantable cardioverter-defibrillator (ICD). Patients who had a history of syncope or aborted cardiac arrest were considered symptomatic. The presence of Br [9] and ER patterns was examined using a 12-lead ECG. PR intervals, QRS durations, and QT intervals were manually measured in lead V_5. QTc intervals were calculated by correcting for heart rate using the Bazett formula. Patients in whom QTc values in resting ECG were lower than $350\ ms^{1/2}$ or higher than $470\ ms^{1/2}$ (men) and $480\ ms^{1/2}$ (women) were also excluded [10].

2.4. Data analysis

The patients with JWS were stratified into 4 groups according to the following ECG characteristics: Br pattern only [9] (Br group), ER pattern only (ER group), overlapping Br and ER patterns (Br–ER group), or IVF with neither a Br nor ER pattern (true IVF group). Patients whose ECG displayed only a Br-type ST elevation in leads V_1 through V_3 [9] were categorized under the Br-only group, regardless of whether the Br pattern was spontaneous or drug induced. We defined ER as either a slurring or notching J-point elevation of at least 0.1 mV from the baseline in at least 2 conjugate leads (Fig. 1A and B) [11]. Patients who exhibited both Br and ER patterns were assigned in the Br–ER group, whereas those with VF/ventricular tachycardia whose ECG showed no Br or ER pattern were assigned in the true IVF group.

2.5. Statistical analysis

All continuous data were expressed as mean ± standard deviation values. One-way analysis of variance with the Fisher least significant difference test and Games-Howell test was used for multiple comparisons. A $P < 0.05$ was considered statistically significant.

3. Results

3.1. Gene analysis

The gene analysis revealed that neither KCNJ8-S422L nor other KCNJ8 mutations were present in our Japanese JWS and IVF cohorts. Nevertheless, we found a previously reported synonymous variant, p.I37I (c.C457T, rs112604741), in 2 probands, 1 who had VF belonged to the true IVF group and the other, who was asymptomatic, belonged to the Br–ER group. The mutation was absent in 112 healthy controls; however, the frequency of this SNP was reported to be 1.5% in heterozygotes (http://www.ncbi.nlm.nih.gov/snp).

3.2. Clinical characteristics

Table 1 presents the classification of the patients enrolled in this study according to clinical and ECG characteristics. The data of 230 probands (205, men) were included in the analysis. The patients' mean age at diagnosis was 45.1 ± 15.4 years. In terms of ECG characteristics, 135 patients had an ECG showing a Br pattern only; 15 an ER pattern only; and 53 Br and ER patterns. Only 27 patients with VF had no ECG abnormality.

The total clinical phenotypes are presented in Table 1. The symptomatic group included 72 patients who had experienced syncope and 75 who had an aborted cardiac arrest. Among the 230 probands, 88 (38%) had cardioverter-defibrillator implants. In the ECG analysis, the mean heart rate, PR, QRS, and QTc interval were 67.1 ± 13.6 bpm, 176.5 ± 25.8 ms, 94.6 ± 52.0 ms, and 404.2 ± 32.6 $ms^{1/2}$, respectively. Of the 230 patients, 188 (82%) had a Br ECG pattern (Br and Br–ER subgroups); 68 (30%), an ER pattern (ER and Br–ER subgroups); 53, (23%), Br and ER patterns; and 27 (12%), neither a Br nor ER pattern (true IVF).

Table 1 summarizes the clinical and ECG characteristics in the Br, ER, Br–ER, and true IVF groups. Male patients accounted for 89%, 87%, 96%, and 78% of the patients in the groups, respectively. The mean ages were similar in the 4 groups. Most of the ER and true IVF patients had experienced an aborted cardiac arrest compared with the Br and Br–ER patients (93% and 93% vs. 17% and 25%; $P < 0.0001$), indicating a statistically significant difference. The reason for the difference is that most of the patients in

Table 1
Comparison of the clinical and electrocardiographic (ECG) characteristics.

	Total	Br only	ER only	Br–ER	True IVF
n (%)	230	135 (59)	15 (7)	53 (23)	27 (12)
Men	205 (89)	120 (89)	13 (87)	51 (96)	21 (78)
Age (years)	45.1 ± 15.4	45.3 ± 15.5	44.2 ± 14.0	46.3 ± 14.1	42.7 ± 18.5
Symptomatic	139 (60)	66 (49)	15 (100)	31 (59)	27 (100)
Syncope	72 (31)	46 (34)	2 (13)	18 (34)	6 (22)
Aborted cardiac arrest	75 (33)	23 (17)	14 (93)	13 (25)	25 (93)
Treatment ICD	88 (38)	41 (30)	10 (67)	21 (40)	16 (60)
Heart rate (bpm)	67.1 ± 13.6	68.3 ± 14.5	63.8 ± 13.1	65.4 ± 11.7	66.4 ± 13.1
PR interval (ms)	176.5 ± 25.8	176.6 ± 24.2	167.3 ± 24.6	179.8 ± 28.9	174.6 ± 27.9
QRS duration (ms)	94.6 ± 52.0	97.8 ± 65.2	90.0 ± 17.7	88.5 ± 23.1	92.7 ± 17.5
QTc interval ($ms^{1/2}$)	404.2 ± 32.6	401.2 ± 30.9	416.5 ± 55.1	401.8 ± 29.1	417.6 ± 25.4
Men	403.1 ± 33.0	400.1 ± 31.5	417.0 ± 58.1	401.6 ± 28.3	417.0 ± 25.6
Women	412.0 ± 28.2	409.6 ± 24.9	413.2 ± 44.2	405.4 ± 63.2	419.3 ± 27.4

Br, Brugada pattern; ER, early repolarization pattern; IVF, idiopathic ventricular fibrillation.
The values are presented as n (%) and mean ± SD. PR, QRS and QTc were calculated in lead V_5.

the ER and true IVF groups were immediately hospitalized after the first attack, whereas most of the Br patients were receiving medication based on an ECG abnormality during the asymptomatic stage. However, no significant differences were observed in heart rate, PR interval, and QRS duration. Regarding QTc intervals, the probands in the Br subgroup had slightly shorter intervals than those in the true IVF subgroup ($401.2 ± 30.9$ vs. $417.6 ± 25.4 \, ms^{1/2}$, $P=0.052$).

4. Discussion

We found no KCNJ8 mutations in our 230 Japanese JWS and/or IVF probands from unrelated families. JWS and Br syndromes are regarded as primary electrical disorders causing VF that occur in individuals without structural heart disease. Antzelevitch and Yan [12] proposed the concept of JWS, in which the fundamental mechanism of the J-point elevation is caused by an increased transient outward current (I_{to}) due to a decrease in sodium or calcium current or an increase in potassium current. Irrespective of ECG subtypes such as the ER, Br, or Br–ER pattern, increased I_{to} seems to play a central role in generating life-threatening events in patients with JWS [13].

A KCNJ8 mutation, S422L, was identified in 6 probands and 1 family member with JWS [3–5]. The probands consisted of 4 Br syndrome and 2 ER patients. Functional analyses showed that this hotspot mutation increased the activity of cardiac ATP-sensitive K current (I_{K-ATP}), which resulted in the AP notch accentuation in collaboration with epicardial I_{to}, and then caused a larger J-wave and/or ST-segment elevation on the patients' ECGs. In our cohort, however, we failed to identify a KCNJ8 mutation. This finding may be related to the difference in ethnicities between Asian and Western countries.

Recently, 2 novel KCNJ8 mutations, E322del and V346I, were reported in cases of sudden infant death syndrome [14]. In the functional analysis, both mutations displayed a decrease in I_{K-ATP}, which was opposite to the effect of S422L mutation on I_{K-ATP} [4,5]. Therefore, JWS and sudden infant death syndrome seemed to have different underlying mechanisms, though having the same culprit gene, KCNJ8.

Based on our subgrouping according to ECG patterns, men were predominant in the Br, ER, or Br–ER group compared with the true IVF group (Table 1). This sex difference was also noted in previous reports [15–17] and may be partially due to different levels of I_{to} channel expression between sexes [18]. In this regard, we recently reported that 2 novel KCNE5 mutations produced gain-of-function effects on I_{to} [19]. Because KCNE5 is located in

the X-chromosome, it is interesting that all 3 male mutation carriers were symptomatic and 2 of them displayed Br, whereas only 2 of the 8 female carriers were symptomatic, none of whom had a Br pattern.

4.1. Study limitations

The ER or Br ECG pattern is known to fluctuate temporarily. If a patient had an ER pattern, for example, among several ECG records, we stratified the patient in the ER subgroup (Table 1). However, in some cases, only 1 ECG was available; therefore, it was difficult to exclude the possibility of missing specific ECG patterns that might have been detected by multiple ECG recordings. The ER pattern has been reported to be predominant in 5–10% of general populations [20–22]. A total of 112 controls underwent genetic testing for KCNJ8, but their clinical data were not available because of complete anonymity. Some of them may have had an ER ECG pattern. One possible reason why we were not able to identify any KCNJ8 mutation within our cohort is that the number of our patients ($n=230$) might have been relatively small.

5. Conclusion

We identified no KCNJ8 gene mutation in our IVF and JWS patients. Therefore, KCNJ8 seemed to be not a major gene responsible for the JWS or IVF cases in Japan.

Conflict of interest

None

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

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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

A rrhythmogenic 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 etiol-

ogy 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.

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