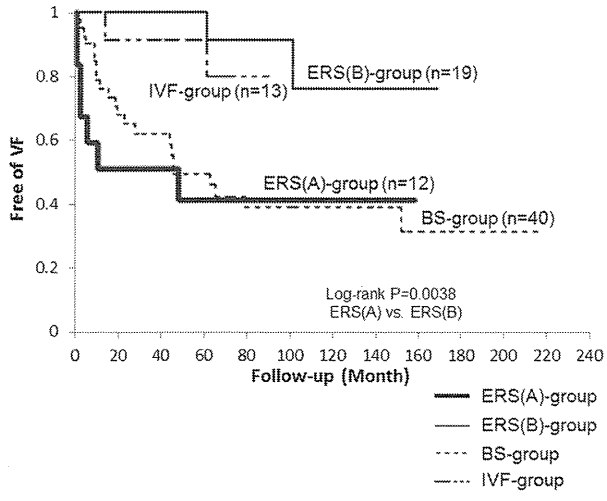

Figure 3 Kaplan-Meier Analysis of Documented VF

Kaplan-Meier analysis of lethal arrhythmic events (documented ventricular fibrillation [VF]) during follow-up according to the clinical subgroups—inferolateral early repolarization combined with non-type 1 anterior early repolarization (ERS[A]-group), pure inferolateral early repolarization without anterior early repolarization (ERS[B]-group), Brugada syndrome (BS)-group, and idiopathic ventricular fibrillation (IVF)-group—in patients with a prior VF.

were attenuated with appearance of S waves and slight prolongation of QRS interval in all patients of the ERS(B)-group (Fig. 2); conversely, the J waves were augmented in various leads of the inferior (Fig. 1B), high lateral (Fig. 1C), and anterior leads (Figs. 1A and 1B) in 9 of 12 patients of the ERS(A)-group (Table 1).

The VF inducibility in the ERS(A)-group was similar to that in the BS-group (ERS[A]: 50%; BS: 81%, p = 0.13). Mutations of *SCN5A* were identified in 8 of 24 patients in the BS-group but in no patients in the ERS group.

Clinical outcome. Mean follow-up period for ERS(A), ERS(B), BS, and IVF groups was 90 ± 57, 76 ± 46, 104 ± 63, and 82 ± 50 months, respectively. Seventy-eight of 84 patients received an ICD. One patient in the ERS(B)-group, 2 patients in the BS-group, and 3 patients in the IVF-group were followed without ICD implantation. No patients died during the follow-up period.

Ventricular fibrillation recurred in 7 of the 12 (58%) patients in the ERS(A)-group and in 22 of the 40 (55%) patients in the BS-group. These groups had significantly higher recurrence rates of VF compared with the ERS(B)-group (2 of 19 [11%]) and IVF-group (2 of 13 [15%]) (ERS[A] vs. ERS[B]: p = 0.012; and BS vs. ERS[B]: p = 0.002). Electrical storm was also significantly higher in the ERS(A)-group (5 of 12 [42%]) and BS-group (10 of 40 [25%]), compared with the ERS(B)-group (0 of 19 [0%]) and IVF-group (0 of 13 [0%]) (ERS[A] vs. ERS[B]: p = 0.0047; and BS vs. ERS[B]: p = 0.022). Among 7 patients in the ERS(A)-group with VF recurrences, 6 had J waves in the high lateral lead. One patient with a VF recurrence in the ERS(B)-group showed J waves in the extensive lead.

Kaplan-Meier curves of the 4 groups are illustrated in Figure 3. Patients in the ERS(A) and BS groups exhibited significantly higher arrhythmic events than those in the ERS(B)-group (log-rank, p = 0.0038). Patients in ERS(B) and IVF groups without known disease showed a more favorable prognosis. Figure 4 illustrates Kaplan-Meier analyses

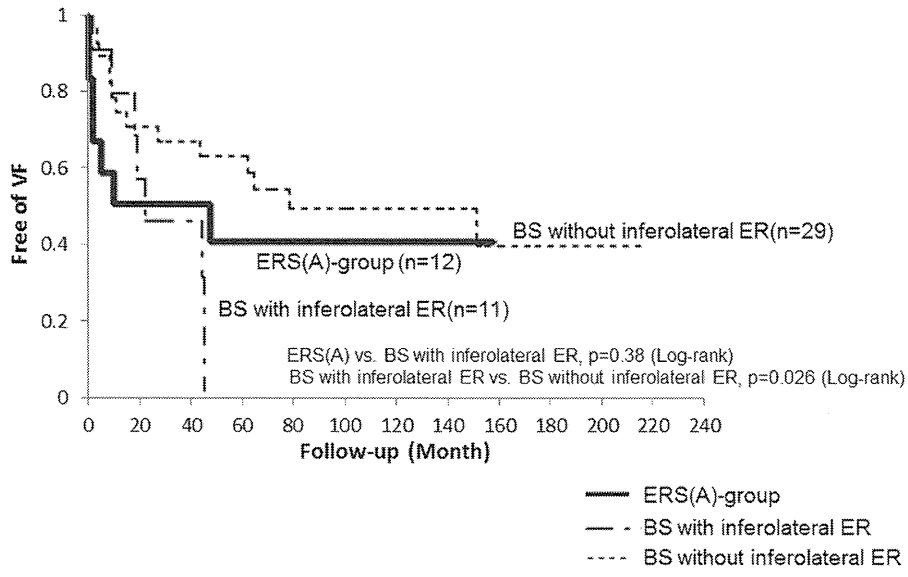

Figure 4 Kaplan-Meier Analyses of Lethal Arrhythmic Events

Kaplan-Meier analyses of lethal arrhythmic events during follow-up according to the clinical subgroups—ERS(A)-group, BS with inferolateral ER group, and BS without inferolateral ER group—in patients with a prior VF. Abbreviations as in Figure 3.

for time free of VF for the ERS(A)-group, BS with inferolateral ER group, and BS without inferolateral ER group. Eleven patients (28%) in the BS-group had J waves in inferolateral leads. Eight of them (73%) had recurrence of VF, and 3 (27%) of them had VF storm in the follow-up. Although BS with inferolateral ER group tended to develop VF recurrences, there was no statistically significant difference between the ERS(A)-group and BS with inferolateral ER group (log-rank, $p = 0.38$). Patients in the BS with inferolateral ER group showed significantly worse outcome compared with those in the BS without inferolateral ER group (log-rank, $p = 0.026$), consistent with a previous report (7).

Medical treatment during follow-up. Quinidine (9) was started after the initial VF episode in 2 patients in the BS-group and in 1 patient in the ERS(A)-group without recurrent VF attack. None of the patients in the ERS(B)-group and IVF-group took antiarrhythmic drugs.

After the second VF attack or electrical storm, bepridil—a multichannel blocker with a transient outward potassium current-blocking effect (10,11)—was administered in 3 patients in the ERS(A)-group; even so, 2 of the 3 developed VF. Two patients treated with cilostazol, a phosphodiesterase III inhibitor that augments $I_{Ca\text{-}L}$ current (10), 1 patient treated with quinidine, and 1 patient treated with denopamine, an oral $\alpha+\beta$–adrenergic stimulant (10), in the ERS(A)-group had no other recurrences. Among the 22 patients in the BS-group with VF recurrences, 3 patients treated with quinidine, 2 with bepridil, 1 with denopamine, and 1 with cilostazol were followed without subsequent VF recurrences.

Discussion

Main findings. Inferolateral ERS consisted of 2 subtypes with heterogeneous clinical profiles. The ERS(A)-group patients were composed of patients with NT1-AER, who comprised 39% of the ERS patients, had clinical profiles similar to BS with high recurrence rates of VF and electrical storm and tended to show J waves in the high lateral leads. In contrast, ERS(B)-group patients, who had a predominant J-wave distribution in the inferior or extensive lead, showed different clinical profiles, such as VF episodes in an awake state and few VF recurrences as observed in IVF patients.

Characteristics of patients with inferolateral ERS. In this patient population, the first VF occurred while sleeping in 32% (10 of 31), VF was induced by programmed electrical stimulation in 38% (6 of 16), and VF recurred in 29% (9 of 31) of all ERS patients; this was in agreement with previous reports by Haïssaguerre et al. (1,12), in which VF attack occurred during sleep, VF inducibility by electrophysiological study, and VF recurrence was observed in 19%, 34%, and 40% of patients, respectively. In addition, that some ERS patients with VF recurrences in the ERS(A)-group responded to quinidine, cilostazol, and denopamine—which were reported to be effective in patients with BS (9–11)—was also consistent with their report. This means that the ERS patients in this study had very similar clinical characteristics to the ERS cohort in the study by Haïssaguerre et al. (1,12).

So far, ERS and BS patients have been considered to share a similar genetic background and to represent a continuous spectrum of phenotypic expression termed J-wave syndrome, which is thought to be mediated by repolarization abnormality due to the transient outward current in the ventricular myocardium (13). However, some clinical manifestations reportedly differ between ERS and BS, whereby VF occurs less frequently at night, originates mainly from the LV, and is inducible to a lesser degree by programmed electrical stimulation in the inferolateral ERS (1,4,5,7,12). Besides, several findings contradicting the repolarization abnormality have been reported. Kawata et al. (14) and Roten et al. (15) separately indicated that sodium channel blockers, pilsicainide and ajmaline, showed a different response in patients with inferolateral J waves (i.e., these drugs led to attenuation or disappearance of J waves), contrary to augmentation of those in Brugada patients. Another was the report by Abe et al. (16), who suggested that circadian changes of signal-averaged ECG parameters in patients with inferolateral J waves implied an association with depolarization abnormalities.

These clinical features are inconsistent with the theoretical basis that ERS should resemble BS, and have caused confusion and difficulty in understanding ERS. This study showed that ERS is a mixture of different ER subtypes—1 (ERS[A]-group) with features similar to BS, and the other (ERS[B]-group) without Brugada features—and that the coexistence of anterior ER determines the clinical characteristics that are partially similar to BS and an unfavorable prognosis in inferolateral ERS.

Prognosis of patients with ERS(A) and ERS(B). With regard to the outcome of patients with clinical features consistent with NT1-AER, Kamakura et al. (7) had already reported in a Japanese Brugada registry with standard 12-lead ECG that patients with a history of VF, no inferolateral ER, and non-type 1 ST-segment elevation even after drug provocation test show poor prognosis, just as In patients with type 1 BS. They also indicated that patients with inferolateral ER and type 1 Brugada ECG have a poorer outcome. In this study, we demonstrated that VF patients with inferolateral ER and NT1-AER also show poor outcome, even though none of the patients in the previous study (7) were included in the ERS group of this study. However, we would like to point out that approximately one-half of NT1-AER could not be detected on the standard ECG (Figs. 1A to 1C) but were only detected on ECGs during high costal recording or after drug provocation test. Besides, the ECGs showed nonspecific ER morphology, just as saddleback ST-segment elevation or upward/downward notching. In this sense, this NT1-AER might be designated a latent ER that is far from previous understanding of the anterior ER, supposedly showing coved-pattern ECG in the right precordial lead (8,13).

Few VF recurrences and VF attacks during activity in patients with pure inferolateral ER (ERS[B]-group) is another

novel finding in this study. These clinical characteristics of the ERS(B)-group were very similar to that of the IVF-group, which excluded VF caused by already-known diseases or anterior ER. Thus far, the precise clinical characteristics of this kind of IVF have not been fully understood, although Haïssaguerre et al. (1) reported that the outcome of IVF patients was better, compared with patients with inferolateral ERS.

Relationship between prognosis of patients and localization of J waves. Antzelevitch and Yan (13) proposed the classification of inferolateral ERS into 3 subtypes, depending on the location of J waves, and suggested that there was a risk of life-threatening arrhythmias as ER grade increases stepwise from 1 to 3, although they did not clearly define ECG morphology of anterior J waves. Tikkanen et al. (2) reported that ER pattern in the inferior lead was associated with the risk for VF. They also stressed the significance of a horizontal or descending ST-segment after J waves as an indicator of poor prognosis in patients with inferolateral ER (17). In our study, J waves were preferentially located in the inferior and extensive leads in the ERS(B)-group, with few VF recurrences in the long-term follow up. Furthermore, no significant difference in VF recurrence was observed between patients with horizontal/descending ST-segment (6 of 17 [35%]) and those with ascending ST-segment elevation (3 of 14 [21%]) in the ERS group ($p = 0.46$). Sixty percent of patients in the ERS(A)-group in whose ECGs J waves were noted in the high lateral leads (I and aVL) had recurrence of VF. This means that VF is likely to recur in patients with anterior and high lateral J waves, irrespective of ST-segment characteristics and the number of leads with J waves, although initial arrhythmic events mainly occur in ER patients with inferior or extensive-global J waves. The close relationship between anterior ER and high lateral J waves can be explained by the anatomical proximity of precordial or upper precordial leads to the lead positions of the left upper chest area where ECG morphology resembles that of I and aVL leads (18).

Mechanism of inferolateral J waves. The mechanism of inferolateral J waves remains unclear and still controversial. There are 2 hypotheses with regard to the origin of J-wave and ST-segment elevation in BS: the repolarization hypothesis and the depolarization hypothesis. In the repolarization hypothesis these are explained by Ito-mediated transmural dispersion of repolarization between the endocardium and epicardium in the RV or LV (13). In the depolarization hypothesis, ST-segment elevation followed by T-wave inversion in the right precordial leads is explained by a potential gradient between the RV outflow tract and RV, which is caused by a significant conduction delay in the RV outflow tract (19). Therefore, the mechanism of ERS(B) does not fit the depolarization hypothesis, because there is no significant ST change in leads V_1 to V_3, although both hypotheses can be applied to the mechanism of ERS(A).

In this study, J waves in 9 of 12 ERS(A) patients were augmented in the anterior or inferolateral leads by sodium channel blockers, and 10 showed saddleback ST-segment elevation in the anterior lead. In contrast, most J waves in ERS(B) patients were attenuated or disappeared along with QRS prolongation when sodium channel blocking agents were given. The positive J-wave response to sodium channel blockers in ERS(A) patients with frequent VF episodes in the parasympathomimetic status is thought to indicate a repolarization abnormality associated with a depolarization abnormality. By contrast, the negative J-wave response to sodium channel blockers in patients in the ERS(B)-group does not seem to indicate the presence of significant transmural dispersion of repolarization in the ventricle, which is represented by the inferolateral leads. In addition, Nademanee et al. (20) recently reported that the catheter ablation of RV or LV Purkinje network eliminated J waves and VF in selected patients with inferolateral ERS. Taken together, J waves in ERS(B) patients seem to be an expression of a depolarization abnormality in some ventricular areas on the basis of a mechanism unexplained by the previous hypotheses, although subsequent ST-segment elevation is explainable by transmural dispersion of repolarization.

Clinical implications. This study demonstrated that inferolateral ERS consisted of heterogeneous ER subtypes with and without NT1-AER. The existence of anterior ER and J waves in the high lateral lead can be useful markers for risk stratification of ERS. In contrast, VF attack during activity or pure inferolateral ER might be predictive of a favorable outcome in ERS patients with VF. A systematic search of the anterior ER with drug challenge test and high costal ECG recording is considered requisite in patients with ERS.

Study limitations. This was a single-center study using retrospective analysis. The small number of patients might limit the interpretation of the results, although it should be pointed out that the number of patients in each group is comparable to that of previous multicenter studies. We had no ERS patients with a coved-AER consisting of coved ST-segment elevation and a flat T-wave, which has already been reported to be linked to various genetic mutations (8,13), although it is possible that such ER might develop type 1 ECG in standard or high costal recordings during follow-up. Further prospective multicenter studies with larger numbers of patients will be needed to confirm these results.

Conclusions

Inferolateral ERS can be divided into 2 heterogeneous groups or subtypes: a pure inferolateral ER group; and a group with inferolateral ER plus non-type 1 anterior ER. Anterior ER seems to be a key predictor of poor outcome in patients with inferolateral ERS.

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Key Words: Brugada syndrome ▪ early repolarization ▪ J-wave ▪ ventricular fibrillation.

PLOS | GENETICS

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.

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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

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.
doi:10.1371/journal.pgen.1003364.t002

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.
doi:10.1371/journal.pgen.1003364.t003

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

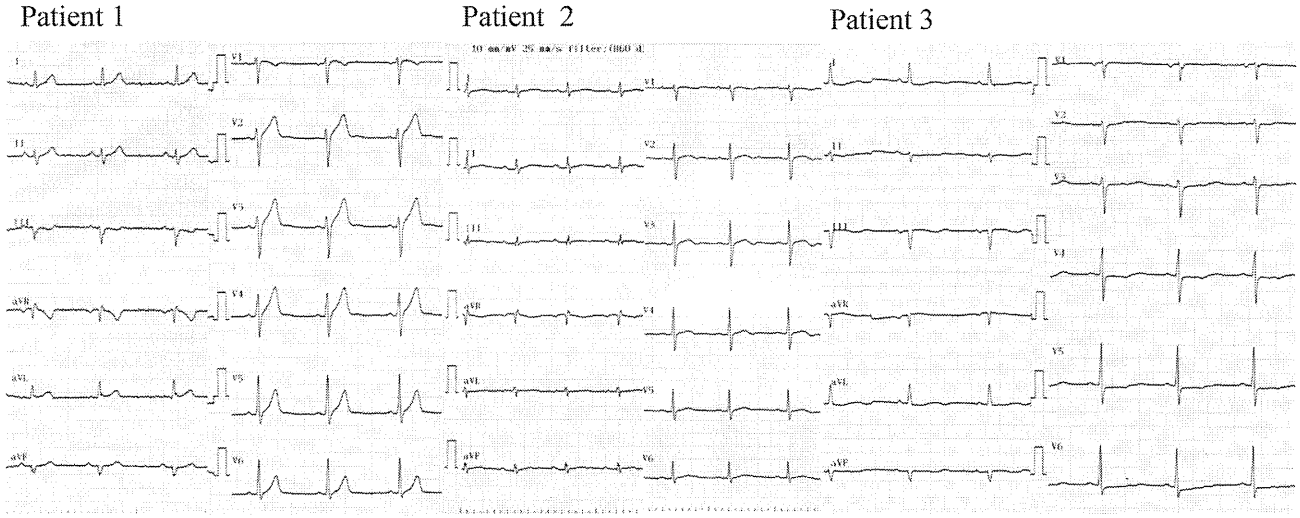


Figure 1. Twelve-lead ECG of patients with *SEMA3A*^{I334V}. Twelve-lead ECG of typical patients wth sinus bradycardia (left to right, each patient 1–3) before the ICD implantation.
doi:10.1371/journal.pgen.1003364.g001

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).

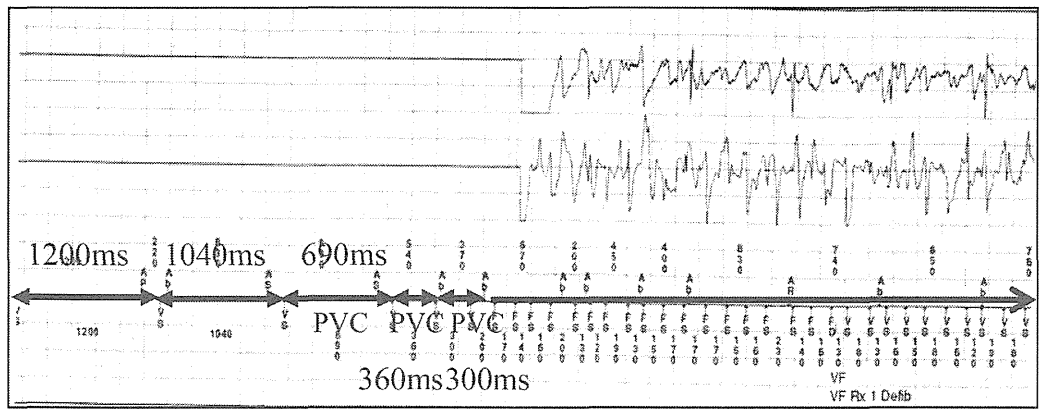
Figure 6 shows the percentage of collapsed growth cones in the E8 chick embryos incubated with media containing *SEMA3A*^{WT}, *SEMA3A*^{I334V} and vector only (negative control) at 0.3, 0.1, and 0.03 dilutions of a concentrated media, respectively. At all dilutions, *SEMA3A*^{WT}, and *SEMA3A*^{I334V} 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



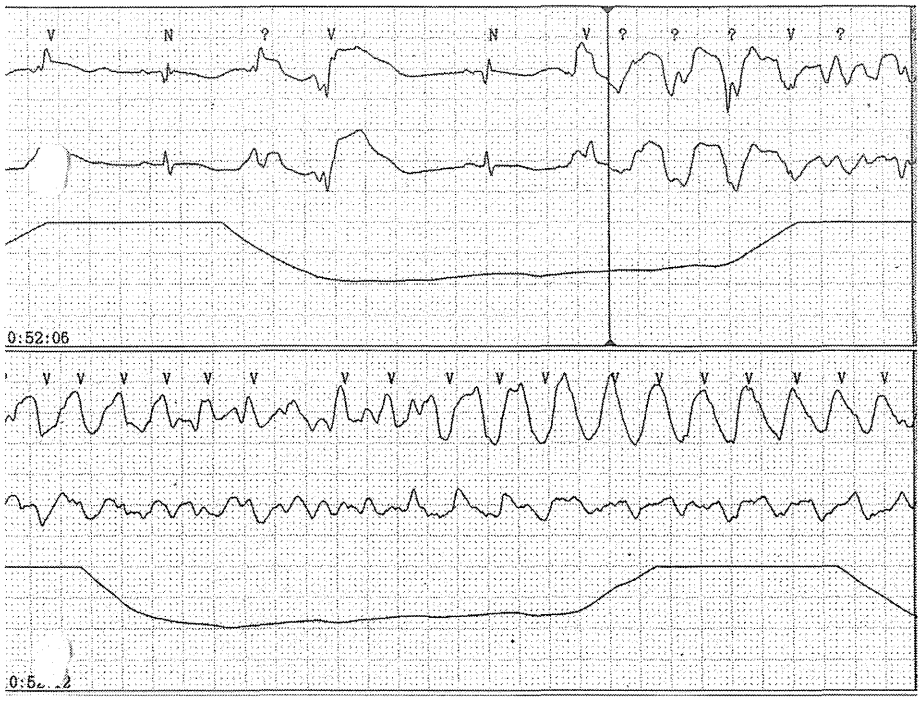


Figure 2. Ventricular fibrillation in patients with *SEMA3A*^{I334V}. After discharge, VF recurred twice and was terminated by ICD shocks in one male patient (patient 1). According to the ICD records, a preceding transient bradycardia was followed by short coupling ectopic ventricular beats and finally 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).
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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 cardiomiopathy 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-

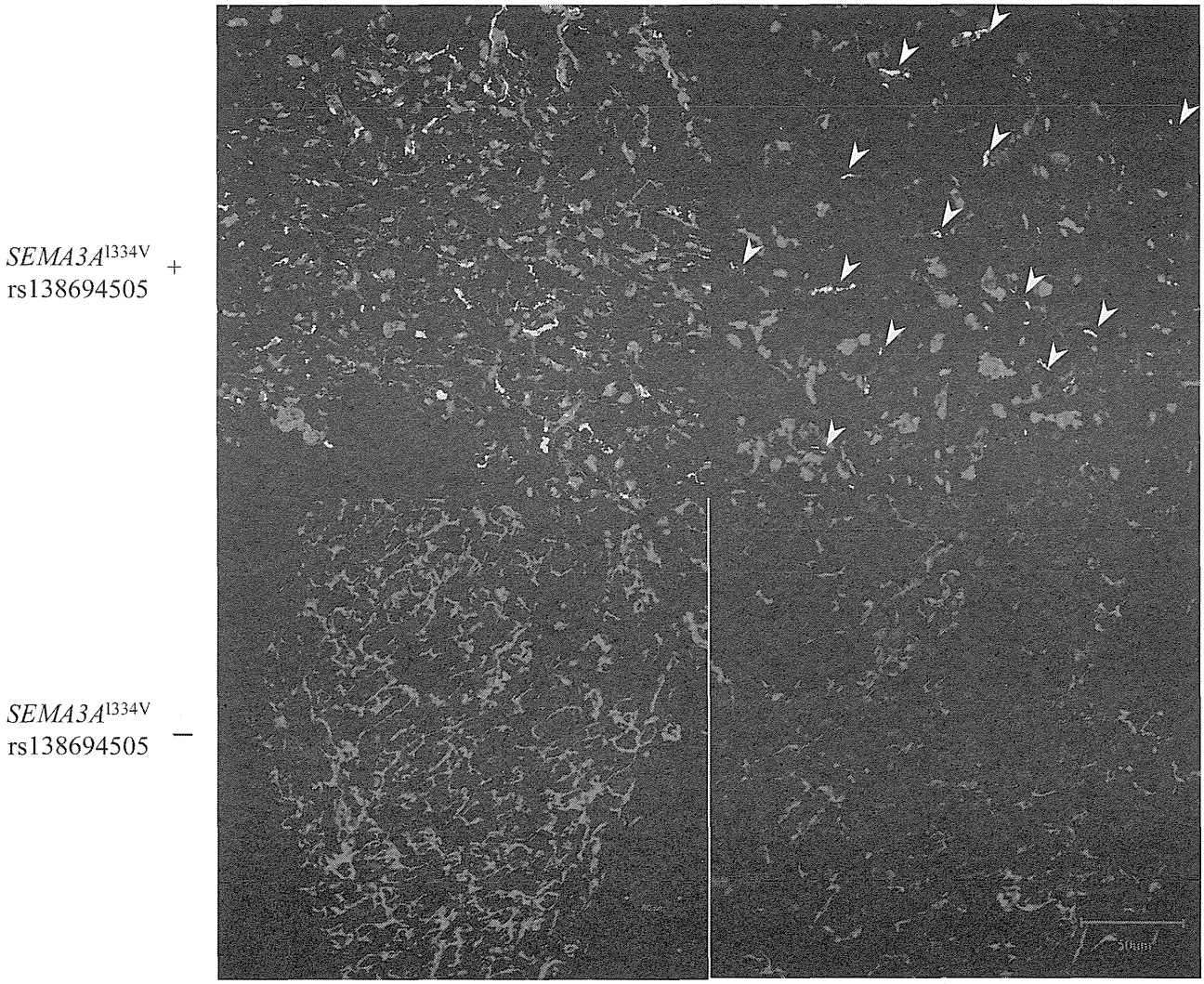

Figure 3. Immunofluorescence staining for Vinculin and anti-TH in the subendocardial layer of patients with and without *SEMA3A*^{I334V}. Immunofluorescence staining of cardiac biopsy specimens revealed that TH positive nerves, as sympathetic nerves, which are absent in the subendocardial layer in normal 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.

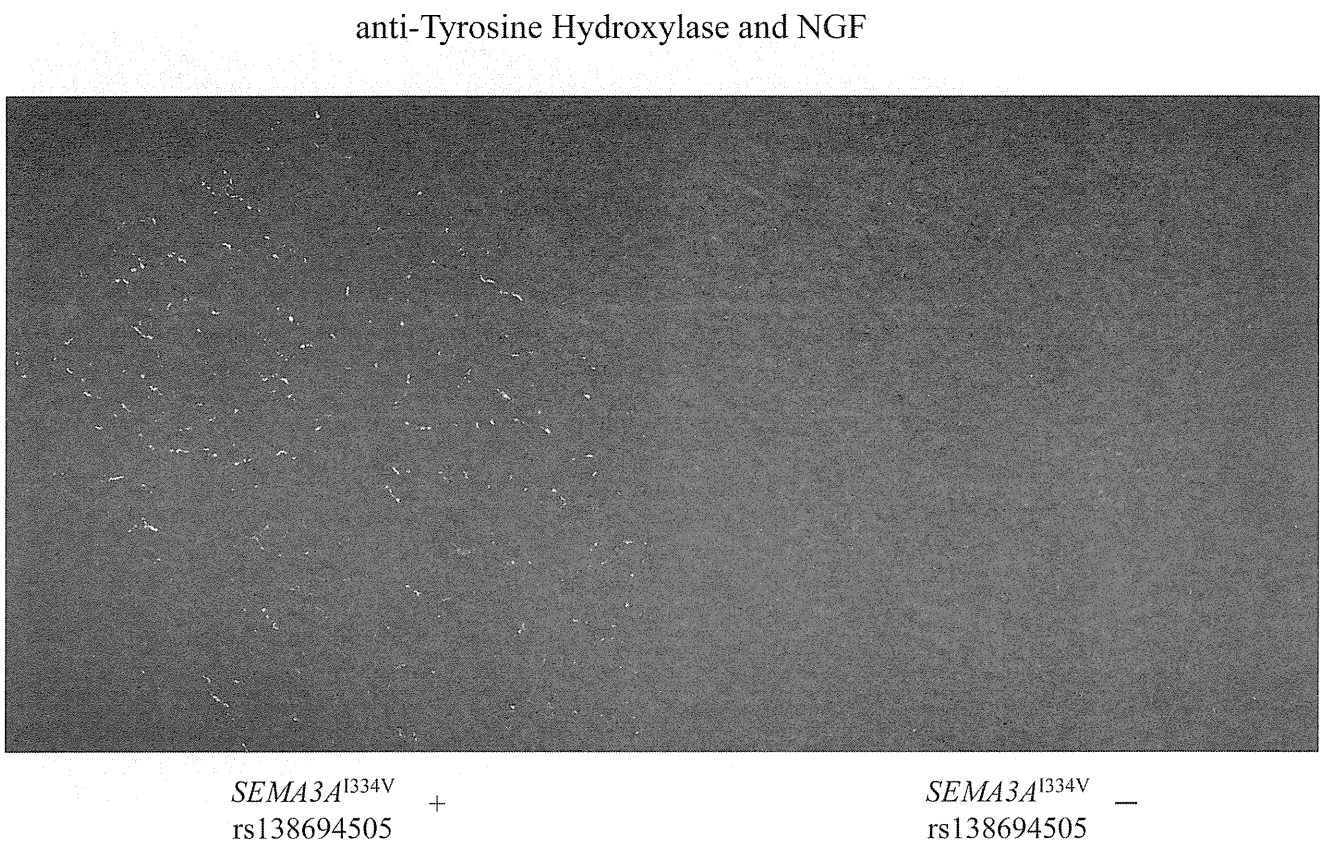

Figure 4. Immunofluorescence staining for Vinculin and NGF in the subendocardial layer of patients with and without *SEMA3A*^{I334V}. On the other hand, the levels of the NGF, a neural attractant factor, were expressed in the subendocardial layer and are comparable between patients with (left panel) 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.

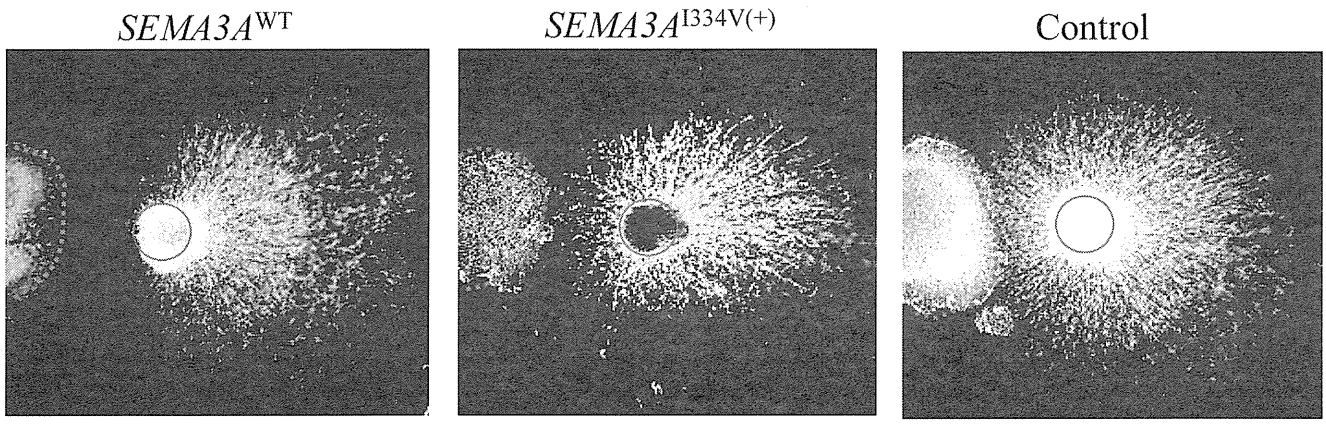

Figure 5. DRG repulsion assay of the *SEMA3A*^{WT}, *SEMA3A*^{I334V}, or control. *SEMA3A*^{WT} expressing cells repelled DRG axons on the proximal side of the ganglia (left). In contrast, DRG explants were less responsive to *SEMA3A*^{I334V} (middle).
doi:10.1371/journal.pgen.1003364.g005

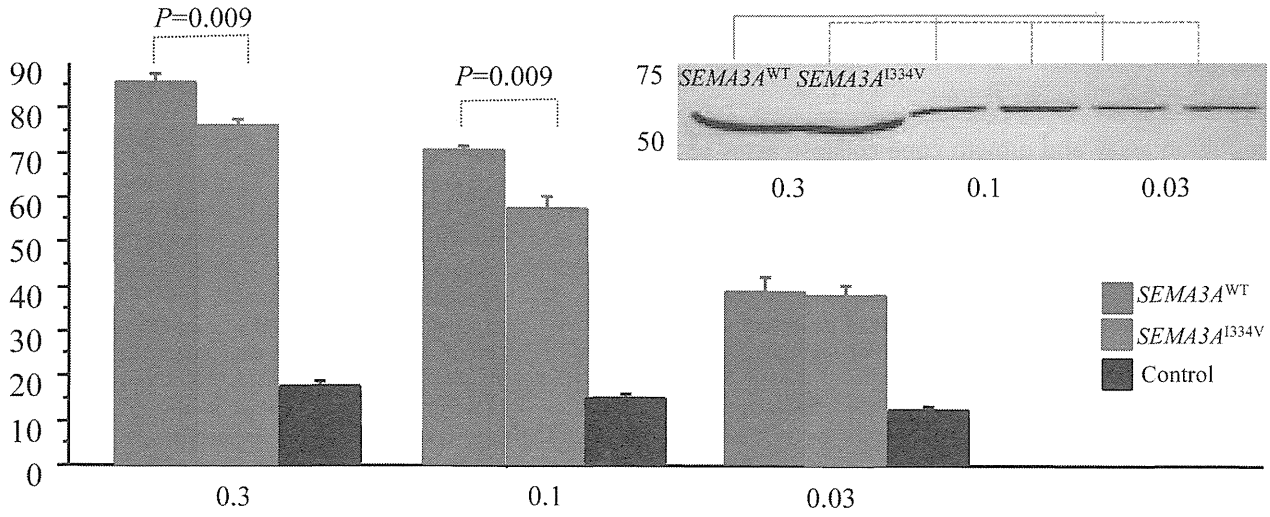

Figure 6. Growth cone collapse assay of the *SEMA3A*^{WT}, *SEMA3A*^{I334V}, or control. The percent of collapsed growth cones of the E8 chick embryos incubated with medium containing vector only, *SEMA3A*^{WT}, or *SEMA3A*^{I334V} at dilutions (0.03, 0.1, and 0.3) of a concentrated media. All dilutions 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 $\geq$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

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)

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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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Prognostic significance of early repolarization in inferolateral leads in Brugada patients with documented ventricular fibrillation: A novel risk factor for Brugada syndrome with ventricular fibrillation

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BACKGROUND Little is known about the clinical and prognostic impact of early repolarization (ER) on patients with Brugada syndrome (BrS), especially those with documented ventricular fibrillation (VF).

OBJECTIVE To investigate the prevalence and prognostic significance of ER in inferolateral leads in patients with BrS and documented VF.

METHODS We investigated 10 different 12-lead electrocardiograms (ECGs) recorded on different days to identify the presence of ER, which was defined as J-point elevation ≥0.1 mV in inferior (II, III, aVF) or lateral leads (I, aVL, V_4–V_6), in 49 individuals (46 men; age 46 ± 13 years) with a type 1 ECG of BrS and previous history of VF.

RESULTS ER was observed persistently (in all ECGs) in 15 patients (31%; P group), intermittently (in at least one but not in all ECGs) in 16 patients (33%; I group), and not observed in 18 patients (37%; N group), yielding an overall ER incidence of 63% (31/49). During the follow-up period (7.7 years), recurrence of VF was documented in all 15 patients (100%) in the P group, and less in 12 patients (75%) in the I group and in 8 patients (44%) in the N group. The P group showed a worse prognosis than N group (P = .0001) by Kaplan-Meier analysis. Either persistent or intermittent ER in an inferolateral lead was an independent predictor of fatal arrhythmic events (hazard ratio 4.88, 95% confidence interval 2.02–12.7, P = .0004; and hazard ratio 2.50, 95% confidence interval 1.03–6.43, P = .043, respectively).

CONCLUSION The prevalence of ER in inferolateral leads was high and an especially persistent form of ER was associated with a worse outcome in BrS patients with documented VF.

KEYWORDS Early repolarization; J wave; Idiopathic ventricular fibrillation; Brugada syndrome; Sudden death

ABBREVIATIONS **BrS** = Brugada syndrome; **ECG** = electrocardiogram; **ER** = early repolarization; **ICD** = implantable cardioverter-defibrillator; **IVF** = idiopathic ventricular fibrillation; **PES** = programmed electrical stimulation; **RV** = right ventricle; **SCD** = sudden cardiac death; **VF** = ventricular fibrillation

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Introduction

Early repolarization (ER) or J wave is often found in the general population and had been considered a benign electrocardiographic (ECG) finding. Its prevalence has been estimated in 1% to 5% of healthy adults.[1–3] Recently, several reports have suggested the association of idiopathic ventricular fibrillation (IVF) with ER in the inferior and/or lateral leads of the ECG.[3–11] ER is more common among patients with IVF than among healthy control subjects, and IVF patients with ER had a worse prognosis than patients without ER.[4]

Brugada syndrome (BrS)[12] is characterized by ST-segment and J-point elevation in the right precordial leads V_1–V_3 and is considered to have a high propensity for sudden cardiac death (SCD).[13,14] Experimental studies have shown that a similar mechanism of repolarization abnormality initiating VF underlies both IVF with ER/J wave and BrS.[15] Some researchers

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consider IVF presenting prominent ER in the inferior or lateral leads as a variant of BrS,[6,9] and inferolateral ER also can be present in patients with BrS.[16–19] However, little is known about the clinical and prognostic impact of ER on patients with BrS, especially those with documented ventricular fibrillation (VF). The aim of this study was to investigate the prevalence and prognostic significance of ER in the inferolateral leads among BrS patients with documented VF and implantable cardioverter-defibrillator (ICD) implantation for secondary prevention.

Methods

Patient characteristics

Forty-nine BrS patients with documented VF and ICD implantation (46 men, age range 22–73 years, mean age 46 ± 13 years) from two Japanese institutes (National Cerebral and Cardiovascular Center and Okayama Univ Graduate School of Medicine) were studied. All subjects had a history of VF or aborted SCD and were diagnosed as having BrS if they met the following inclusion criteria: (1) proband, (2) amplitude of QRS-ST junction ≥0.2 mV (2 mm) with coved-type ST-segment elevation in at least 2 of the 3 right precordial leads (V_1–V_3) at baseline or after provocation with a Class I antiarrhythmic drug, (3) normal findings on physical examination, and (4) no abnormality in either right or left ventricular morphology and/or function demonstrated by chest radiography and echocardiography. Laboratory tests were performed to exclude metabolic or electrolyte abnormalities at the time of ECG diagnosis of BrS. Screening test for SCN5A mutations was performed in 37 individuals, and electrophysiologic study was performed in 43 subjects. Programmed electrical stimulation (PES), with a maximum of 3 ventricular extrastimuli, was delivered from 2 right ventricular (RV) sites (RV apex and RV outflow tract). Premature beats were started in late diastole; the shortest coupling interval of the extrastimulus was limited to 180 ms for safety. Stimulation was performed at twice diastolic threshold. Induced VF was defined as any ventricular tachyarrhythmias lasting >15 seconds, causing syncope, circulatory collapse, or requiring defibrillation. Among the 49 patients, 37 showed spontaneous Brugada-type ECG. In the remaining 12 patients, sodium channel blocker infusion test unmasked Brugada-type ECG.

Drug challenge test

Drug provocation test of sodium channel blocker was performed in patients without spontaneous Brugada-type ECG. Intravenous sodium channel blocker pilsicainide (1 mg/kg, maximum 50 mg, 5 mg/min), flecainide (2 mg/kg, maximum 100 mg, 10 mg/min), or disopyramide (2 mg/kg maximum 100 mg, 10 mg/min) was used.[20–24] The test result was considered positive if a type 1 Brugada ECG appeared in more than 1 right precordial lead (V_1–V_3).[16]

ECG

Twelve-lead ECG were recorded at a paper speed of 25 mm/s with amplification of 10 mm/mV in all patients. According to previous study, ER or J-point elevation was defined as an elevation of the QRS-ST junction (J point) in at least two inferior (II, III, aVF) and/or lateral (I, aVL, V_4–V_6) leads (Figures 1–3).[4] ER amplitude had to be at least 1 mm (0.1 mV) above the baseline level, either as QRS slurring (smooth transition from QRS to the ST segment) or notching (positive J deflection inscribed on the S wave).[4,6,11] In general, ER amplitude varies day by day, and determining the definite ER amplitude in all patients is difficult. Moreover, in some patients ER appears clearly in 1 ECG but disappears in another ECG. To solve this problem, we investigated ten 12-lead ECGs recorded on different days for all patients. All ECGs were interpreted by 2 independent cardiologists who were blinded to the clinical result.

ST-segment patterns after the J wave also were analyzed using Tikkanen criteria.[25,26] Horizontal/descending type was defined as ≤0.1-mV elevation of the ST segment within 100 ms after the J point. The concave/rapidly ascending ST segment was defined as >0.1-mV elevation of the ST segment within 100 ms after the J point or a persistently elevated ST segment >0.1 mV throughout the ST segment. Patients were counted as having horizontal ST segment when the ST segment was ascending type in some leads and horizontal type in others.

Classification of groups

Forty-nine patients were categorized into 3 groups based on the stability of ER in the inferior and/or lateral leads. Patients

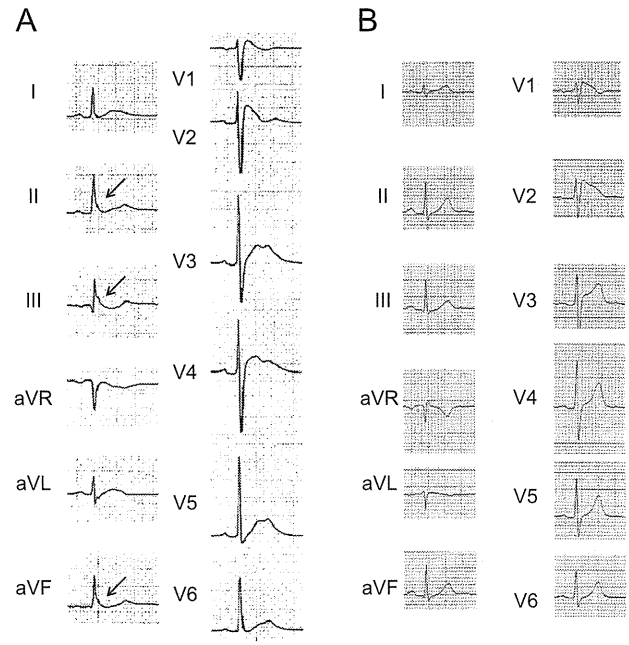

Figure 1 **A:** Twelve-lead ECGs in a patient with Brugada syndrome and early repolarization (ER) in the inferior leads. *Arrows* indicate ER. **B:** Twelve-leads ECG in a patient with Brugada syndrome but without ER repolarization in the inferolateral leads.

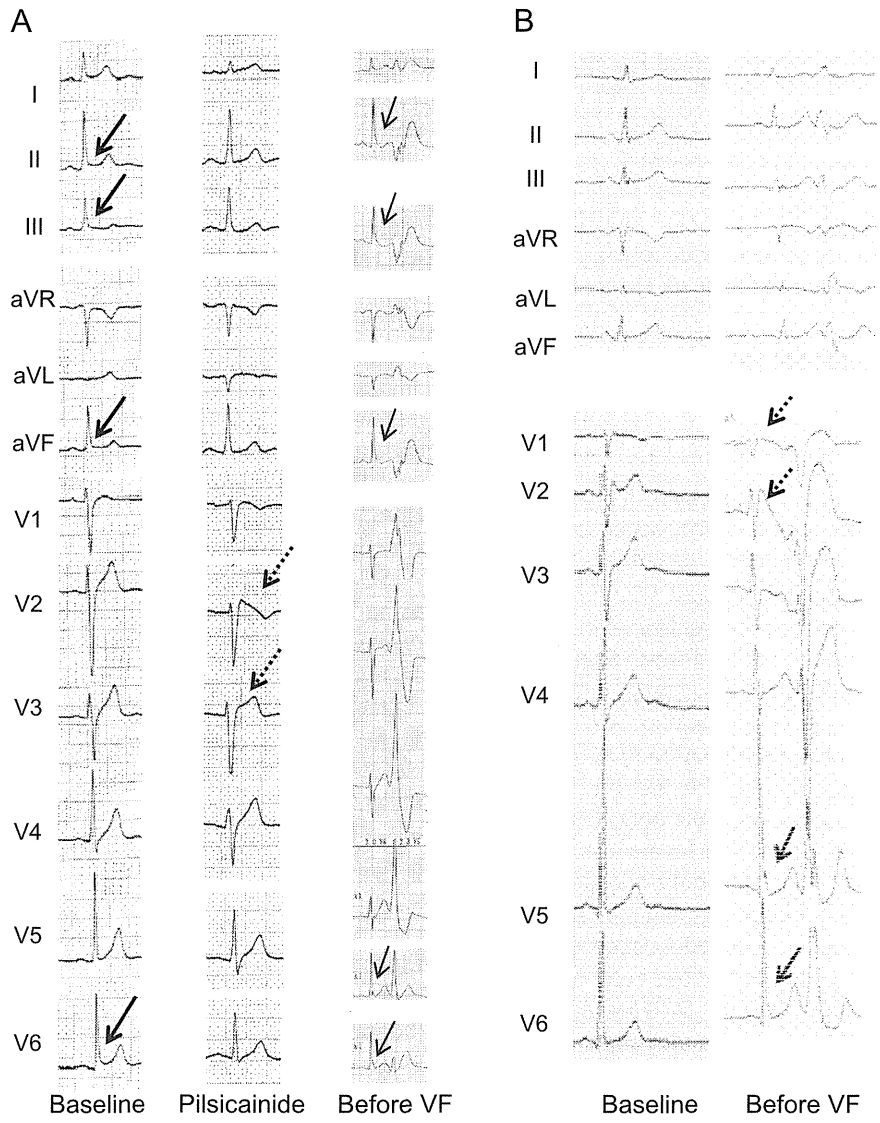

Figure 2 **A:** Twelve-lead ECGs recorded at baseline, during pilsicainide stress test, and recorded just before an episode of ventricular fibrillation (VF) in a Brugada patient with early repolarization (ER) in inferolateral leads. Baseline ECG showed small ER in leads II, III, aVF, and V_6 (*arrows*). During the pilsicainide stress test, despite unmaking of type 1 ST elevation in the right precordial leads (*dotted arrows*), no enhancement or even decrease of ER was observed. On the other hand, ER was clearly accentuated just before an episode of VF compared with baseline (*arrows*), whereas ST elevation in right precordial lead was not observed. **B:** Twelve-lead ECGs recorded at baseline and just before an episode of VF in a Brugada patient with ER in lateral leads. Baseline ECG showed no apparent ER. Just before an episode of VF, accentuation of ER was observed in leads V_5 and V_6 (*arrows*) and was followed by a ventricular premature beat; a similar beat triggered VF a few minutes later. In this patient, ST elevations in right precordial leads (V_2 and V_3, *dotted arrows*) and ER in lateral leads (V_5 and V_6, *arrows*) were observed simultaneously.

with persistent ER (in all ECGs; P group), patients with intermittent ER (in at least one but not all ECGs; I group), and patients without ER (not observed in any ECGs; N group). The prognosis and occurrence of VF were compared among these 3 groups.

Therapy and follow-up

All case subjects received an ICD implantation that provided accurate information on VF recurrence. The subjects visited the hospital routinely every 4 to 6 months for clinical review and device interrogation or whenever patients experienced symptoms relating to ventricular arrhythmia or device discharge at the outpatient clinic. Life-threatening arrhythmic events during follow-up periods include an occurrence of SCD and documented ventricular tachycardia/VF confirmed by intracardiac ECG of their ICD. Electrical storm was defined as 3 or more episodes of VF per day recorded by the memory of the ICD. All patients were followed up at the outpatient clinic of the National Cerebral and Cardiovascular Center (n = 27) or the Okayama University Graduate School of Medicine (n = 22).

Statistical analysis

Continuous variables are expressed as mean ± SD and were compared using the Student *t* test or analysis of variance for normally distributed variables. Fisher exact tests, when

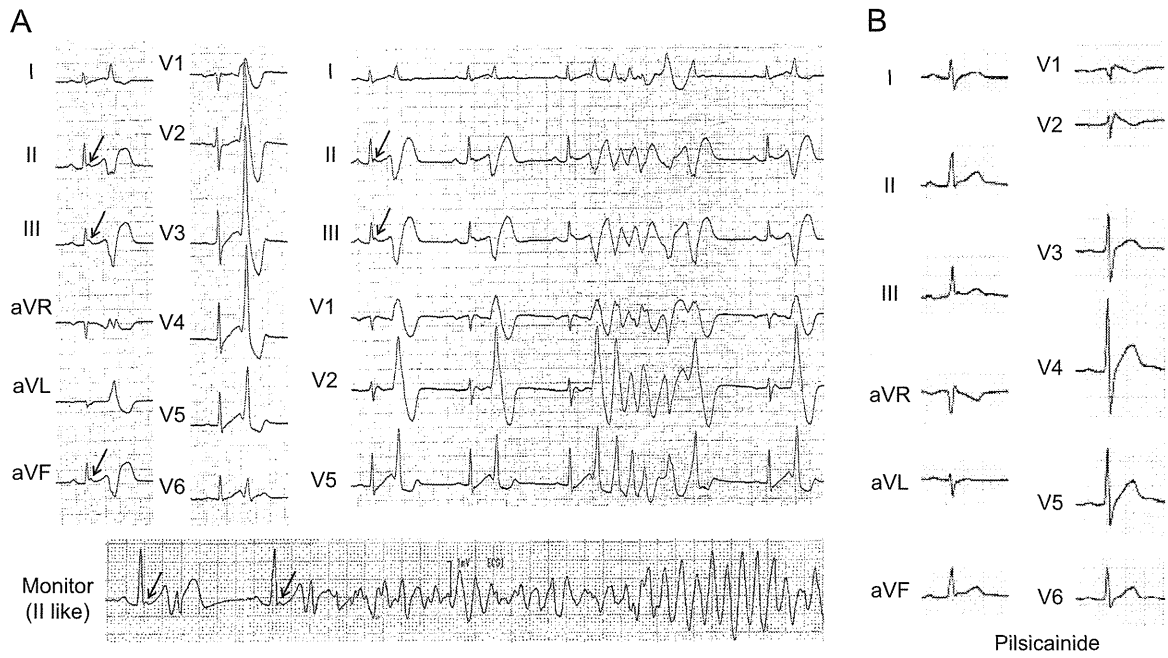

Figure 3 **A, left:** Twelve-lead ECGs in a Brugada patient with early repolarization (ER) in inferior leads II, III, and aVF. A premature ventricular contraction (PVC) with a right bundle branch block morphology and superior axis was observed, indicating that the PVC originated from the left ventricular inferior wall. **Right:** Similar morphology of PVC triggered nonsustained polymorphic ventricular tachycardia. A few seconds later, ventricular fibrillation was recorded on monitor ECG (**bottom panel**). **B:** In this patient, ST elevation in right precordial lead was induced by pilsicainide infusion.

appropriate, were used to compare differences between categorical variables. Survival curves were plotted using Kaplan-Meier methods and analyzed by log-rank test. Event analysis during the follow-up period was performed using the Cox proportional hazards regression model. In the Cox proportional hazards regression model, 95% confidence (CI) limits for the hazard ratio are profile likelihood limits. $P < .05$ was considered significant.

The study was approved by the medical ethical review committees of National Cerebral and Cardiovascular Center. Written informed consent was obtained from all individuals.

Some patients were included in our previous studies: 14 of 49 patients in one study[27] and 19 of 49 patients in another.[18]

Results

Prevalence of inferolateral ER

The inferolateral ER was observed persistently (in all ECGs) in 15 patients (P group), intermittently in 16 patients (I group), and not observed in 18 patients (N group). The prevalence of ER in BrS with a history of VF in the present study was 63% (31/49 patients). Among the 31 patients with the inferolateral ER, 11 showed ER in inferior leads, 11 patients in lateral leads, and 9 patients in both inferior and lateral leads, respectively. As shown in Figures 1, 2, and 3, manifestation of inferolateral ER did not always relate to ST elevation in the right precordial leads.

Clinical, ECG, electrophysiologic, and genetic characteristics

Table 1 compares the clinical, ECG, electrophysiologic, and genetic characteristics and recurrent events during the follow-up period across the P, I, and N groups. Mean age of the 49 patients was 46 years (median 48 years, range 22–73 years), and 46 patients (94%) were male. Thirty-seven of the 49 patients (76%) showed spontaneous ST elevation in the right precordial leads. Family history of SCD occurring before the age of 50 years and SCN5A mutation were observed in 16% and 19%, respectively. PES induced VF in 72% of the patients. There were no significant differences with respect to age, gender, spontaneous ST elevation in right precordial leads, family history (SCD <50 years old), maximum altitude of ER in inferior or lateral leads, inducibility of VF by PES during electrophysiologic study, and SCN5A mutation among the 3 groups.

Prognosis and univariate analysis of clinical variables and inferolateral ER

Mean follow-up period for the entire study population was 93.8 ± 45.6 months (Table 1). Follow-up periods were similar among the 3 groups (P group: 88.7 ± 55.2; I group: 96.5 ± 41.9; N group: 95.6 ± 46.3 months). Thirty-five patients (71%) had recurrence of VF, and 18 patients (37%) experienced electrical storm of VF during the follow-up period. Kaplan-Meier analyses of recurrent VF was performed during follow-up, depending on the presence of spontaneous type 1 ST elevation in right precordial lead, family history of SCD, and existence of inferolateral ER. In Kaplan-Meier survival analysis, time to the first event was shorter in the ER group patients (including both P and I groups) than in the N group patients ($P = .02$). There was no significant difference in prognosis by spontaneous type 1 ST elevation or family history of SCD. Figure 4 shows the

Table 1 Comparison of clinical, electrocardiographic, electrophysiologic, and genetic characteristics and recurrence events during follow-up period across the 3 groups

	All	Persistent	Intermittent	No ER	*P* value
No.	49	15	16	18	Three groups
Age (years)	46.0 ± 12.7	42.6 ± 12.9	43.8 ± 14.8	50.7 ± 9.6	.10
Male gender	94% (46/49)	87% (13/15)	94% (15/16)	100% (18/18)	.19
Spontaneous ST elevation	76% (37/49)	73% (11/15)	75% (12/16)	78% (14/18)	1
ER with horizontal/descending ST	51% (16/31)	60% (9/15)	44% (7/16)	NA	1*
Family history of SCD	16% (8/49)	20% (3/15)	13% (2/16)	17% (3/18)	.89
Maximum amplitude of ER in inferolateral leads (mV)	0.228 ± 0.122	0.265 ± 0.150	0.193 ± 0.080	NA	.25*
Inducibility of VF during electrophysiologic study	72% (31/43)	62% (8/13)	85% (11/13)	71% (12/17)	.40
SCN5A	19% (7/37)	33% (3/9)	18% (2/11)	12% (2/17)	.35
Follow-up period (months)	93.8 ± 45.6	88.7 ± 52.2	96.5 ± 41.9	95.6 ± 46.3	.80
Recurrence of VF	71% (35/49)	100% (15/15)	75% (12/16)	44% (8/18)	.0019
Recurrence of electrical storm	37% (18/49)	47% (7/15)	50% (8/16)	17% (3/18)	.087

Analysis of variance for continuous variables between multiple groups. Fisher exact test for categorical variables. T test for continuous variables between two groups.

ER = early repolarization; SCD = sudden cardiac death; VF = ventricular fibrillation.

*Persistent vs intermittent.

Kaplan-Meier analysis of VF recurrence in each ER subgroup. Patients in the P group had a significantly worse prognosis than did those in the N group ($P = .0001$). The I group also had a tendency for worse prognosis than the N group, but this did not reach statistical significance. Figure 5 shows the Kaplan-Meier analysis of the time to first electrical storm after ICD implantation. The P group showed significantly worse results than the N group ($P = .045$). Table 2 lists the results of univariate analysis of several variables. Univariate analysis revealed that the existence of inferolateral ER (either persistent or intermittent) and ER with horizontal/descending ST segment were correlates for worse prognosis. Multivariate analysis was not performed because those 2 parameters are not independent variables to each other. No other factors were associated with patient prognosis. We then compared prognosis according to the localization of ER: inferior, lateral, and both inferior and lateral. There was no significant difference in the frequency of VF recurrence among the 3 groups according to localization of ER.

Discussion

BrS is characterized by a high incidence of life-threatening ventricular tachyarrhythmias and is responsible for a number of SCD in young healthy people. Patients with a history of syncope, especially those with a previous history of cardiac arrest or VF, are at increased risk for subsequent arrhythmic events compared with asymptomatic individuals.[27–33] Prognosis of BrS varies widely from benign to malignant among

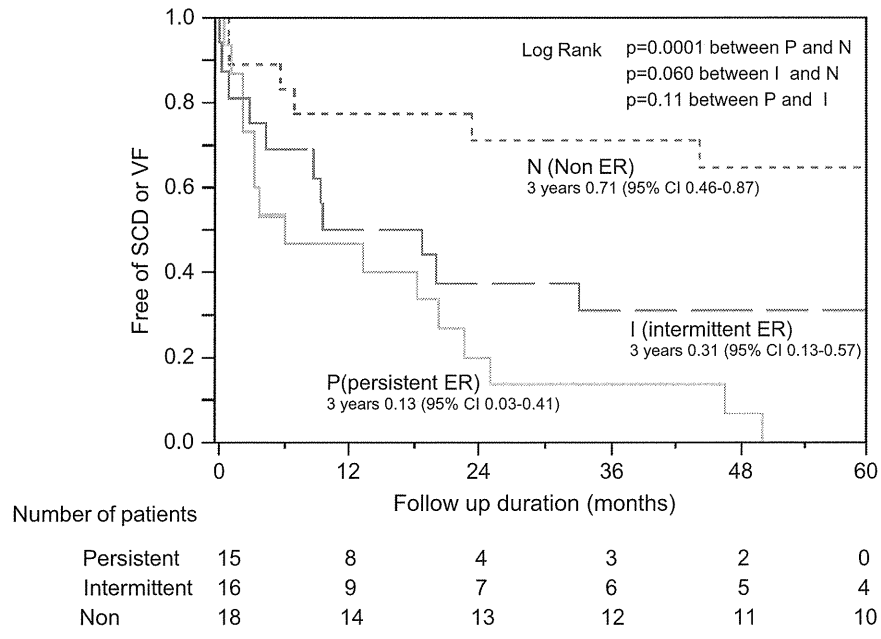

Figure 4 Kaplan-Meier analysis of recurrence of ventricular fibrillation (VF) or sudden cardiac death (SCD) during follow-up depending on frequency of early repolarization (ER) in inferolateral leads (persistent ER vs intermittent ER vs Non ER).