

A novel mutation in the *SCN5A* gene contributes to arrhythmogenic characteristics of early repolarization syndrome

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Abstract. Several genetic variants have been associated with early repolarization syndrome (ERS). However, the lack of functional validations of the mutant effects has limited the interpretation of genetic tests. In the present study, we identified and characterized a novel sodium channel, voltage gated, type V alpha subunit (*SCN5A*) mutation that was associated with ERS. A 67-year-old male proband suffering from recurrent syncope underwent a documented electrocardiogram (ECG) for polymorphic ventricular tachycardia (VT). It was noted that baseline 12-lead ECG exhibited a predominantly elevated ST-segment which mimicked acute myocardial ischemia in lead V₂-V₆, and the ECG also demonstrated J waves in lead II, III, aVF and V₂-V₆. Using genetic analysis, we noted that the proband carried a novel heterozygous missense mutation of A1055G in the *SCN5A* gene. Whole-cell configuration of patch-clamp analysis revealed that the mutation significantly decreased peak sodium current (I_{Na}) density and shifted the steady-state inactivation curve of I_{Na} to a more negative potential. Confocal imaging suggested that in the mutant channel a defect of protein expression both on the cell membrane and in cytoplasm was present. The present study demonstrated that a novel heterozygous missense mutation of A1055G in *SCN5A* led to 'loss-of function' of the sodium channels, and we suggest that it accounts for the arrhythmogenic characteristics of ERS.

Introduction

The early repolarization (ER) pattern on the electrocardiogram (ECG) is defined as ≥0.1 mV J-point elevation of either notched or slurred morphology in at least 2 contiguous

inferior and/or lateral leads. It was first described as a common ECG variant in healthy human subjects in 1936 by Shipley and Hallaran (1). For a long time, the ECG pattern of ER has been regarded as a benign phenomenon, which is observed predominantly in young healthy men, male athletes, and African Americans (2,3). However, over the past few decades, using evidence from various studies, the pattern of ER has been considered as associated with an increased risk of arrhythmogenic sudden death (4-7). Thus, early repolarization syndrome (ERS), which is also termed inherited J wave syndrome, was proposed in order to describe the ER pattern with arrhythmic phenotypes, which is diagnosed only in patients who have been resuscitated after cardiac arrest, those with documented ventricular fibrillation (VF) or polymorphic ventricular tachycardia (VT), and possibly in the relatives of the syndrome carriers in whom a genetic mutation is documentable (8). There is increasing evidence suggesting that ERS is related to mutations in ion channel genes. Haïssaguerre *et al* have reported that mutations in the adenosine triphosphate (ATP)-sensitive gene potassium channel, inwardly rectifying subfamily J, member 8 (*KCNJ8*) was responsible for idiopathic VF associated with ER (9). Mutations in the cardiac L-type calcium channel, including calcium channel, voltage-dependent, L type, alpha 1C subunit (*CACNA1C*), calcium channel, voltage-dependent, beta 2 subunit (*CACNB2B*) and calcium channel, voltage-dependent, alpha 2/delta subunit 1 (*CACNA2D1*), have also been associated with ERS and sudden cardiac death (10).

In the present study, in a proband with ERS, we identified a novel mutation in the sodium channel, voltage gated, type V alpha subunit (*SCN5A*) gene and investigated the functional consequences of the novel mutation.

Patients and methods

Clinical examination. For the purposes of the present study, the proband and his family members who participated in the study provided written informed consent before genetic and clinical investigations, and the study conformed to the Declaration of Helsinki and local ethics committees. The proband underwent clinical evaluations, including laboratory tests, a 12-lead ECG, echocardiography, chest roentgenogram, magnetic resonance imaging (MRI) and coronary angiography in Fuwai Hospital (Beijing, China).

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Genetic analysis. Genomic DNA was extracted from peripheral blood leukocytes using a TIANamp Blood DNA isolation kit (Tiangen, Beijing, China) according to the manufacturer's instructions. Genetic testing was performed in order to identify mutations in ion channel genes including ATP-binding cassette, sub-family C (CFTR/MRP), member 9 (*ABCC9*), *KCNQ1*, *KCNH2*, *KCND3*, *KCNE1*, *KCNE2*, *KCNJ8*, *CACNA1C*, *CACNB2*, *CACNA2D1*, *SCN1B* and *SCN5A*. All exons of these genes were amplified and screened by direct sequencing. To be considered a mutation, the variant had to have changed or disrupted the open reading frame and be absent in 500 unrelated, healthy individuals (1,000 reference alleles) of similar ethnicity [derived from our own database as previously described (11,23)].

Mutagenesis and transfections. The wild-type (WT)-SCN5A cDNA (GenBank ID: NM198056; Geneway, Beijing, China) was subcloned into a pcDNA3.1 expression vector (Geneway). The missense mutation consistent with the variant detected in the proband (A1055G-SCN5A) was created in the pcDNA3.1 WT-SCN5A plasmid by a site-directed mutagenesis strategy as previously described (12). The mutations were verified by sequencing.

293 cells (from the Cell Resource Center, IBMS, CAMS/PUMC, Beijing, China) were first transiently co-transfected with 0.6 μ g WT or an equal amount of mutant *SCN5A* constructs using Effectene transfection reagent (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Green fluorescent protein (GFP) plasmids (0.2 μ g; Geneway) were then co-transfected for use as a reporter gene. The sodium current (I_{Na}) was recorded after the transfected cells were cultured for 36 h. More than two independent transfection experiments were conducted for further patch-clamp and confocal experiments to confirm the reproducibility of the results.

Functional analysis. In the present study, I_{Na} was measured using a whole-cell configuration of the patch-clamp technique with Axon Patch 700B amplifiers (Axon Instruments, Foster City, CA, USA). All of the patch-clamp experiments were performed at room temperature (20–22°C). The transfected cells were bathed in a solution that contained 140 mmol/l NaCl, 4 mmol/l KCl, 1.8 mmol/l $CaCl_2$, 0.75 mmol/l $MgCl_2$ and 5 mmol/l HEPES, at pH 7.4, which was adjusted with NaOH. The glass pipettes were filled with a solution of 120 mmol/l CsF, 20 mmol/l CsCl, 5 mmol/l ethylene glycol tetraacetic acid and 5 mmol/l HEPES, at pH 7.4, which was adjusted with CsOH. The resistance of pipettes in the bathing solution ranged from 1.5 to 2 M Ω . No leak subtraction was applied during the recording, and the membrane capacitance was measured for each of the cells. Both steady-state inactivation curves and steady-state activation curves were calculated using Boltzmann distribution.

Confocal imaging. 293 cells were fixed with 4% paraformaldehyde for 15 min at room temperature, and they were then permeabilized with 0.2% Triton X-100 for 10 min. Non-specific sites were blocked by incubation with 5% bovine serum albumin (BSA) for 1 h at room temperature. Subsequently, the cells were incubated with mouse monoclonal anti-Nav1.5 IgM primary antibody (1:40 dilution; ab62388) (Abcam, Cambridge, UK) overnight at 4°C. The following day, the cells were incubated with the anti-mouse FITC-conjugated donkey secondary anti-

body (AB10081-100ul; Jackson ImmunoResearch, West Grove, PA, USA, 1:500 dilution) for 30 min in the dark at room temperature. Finally, the coverslips were then mounted using mounting medium containing DAPI (Beyotime Biotechnology Co., Ltd., Shanghai, China). Confocal images were obtained using a confocal laser scanning microscope (Leica TCS SP2, Leica, Leica Microsystems, Wetzlar, Germany) and were analyzed using NIH image software (ImageJ).

Statistical analysis. I_{Na} data for patch-clamp analysis were analyzed using pClamp9 software (Molecular Device). Continuous variables are expressed as the means \pm SE and were compared using the unpaired Student's t-test or one-way ANOVA when appropriate. A P-value <0.05 was considered to indicate a statistically significant difference.

Results

Clinical manifestation and genotyping. The 67-year-old male proband was hospitalized due to recurrent syncope, accompanied by palpitations, which occurred over a period of 1 month prior to hospitalization. Baseline 12-lead ECG exhibited coved ST-segment elevation which mimicked acute myocardial ischemia in lead V_2 – V_6 , and the ECG also revealed J waves in lead II, III, aVF and V_2 – V_6 (Fig. 1A). Polymorphic VT (Fig. 1B and C), which were terminated successfully by direct current cardioversion, were documented on the ECG obtained during his syncope at the emergency department and in the ward during the admission process. Coronary angiography exhibited no stenosis in the coronary arteries. Structural abnormalities were excluded by echocardiography, an MRI and a chest roentgenogram, and electrolyte disturbances were excluded by laboratory tests. His medical history revealed that the proband had suffered from paroxysmal palpitations for two years and that one of his brothers had died from sudden death syndrome (Fig. 2A). According to the clinical characteristics, the proband was diagnosed with ERS and was provided with an implantable cardioverter defibrillator (ICD). The other family members of the proband were asymptomatic, and the son of the proband had a normal ECG pattern.

Genetic analysis revealed that the proband carried a novel heterozygous missense mutation of c.1055 A>G in the *SCN5A* gene (Fig. 2B), which had led to the substitution of a tyrosine by a cysteine in position 352 (Tyr352Cys). The gene encoded the predominant cardiac sodium channel α subunit and the corresponding substitution located in the extracellular loop between segment five and six of domain I in sodium channel α subunit (Fig. 2C). This variant was predicted to have deleterious effect on the protein by the bioinformatics tools SIFT (13). Moreover, the mutation was not detected in the son of the proband, nor in ethnicity-matched 500 healthy unrelated controls.

Electrophysiology. In the present study, whole-cell configuration of the patch-clamp technique was used to record I_{Na} from 293 cells, which were transiently transfected with WT-SCN5A or mutant (A1055G-SCN5A) sodium channels. The mean peak I_{Na} density of A1055G-SCN5A channels (-33.39 ± 14.29 pA/pF, $n=7$) was markedly lower than that of WT-SCN5A channels (-238.68 ± 97.13 pA/pF, $n=7$, $P<0.001$). Additionally, hetero-

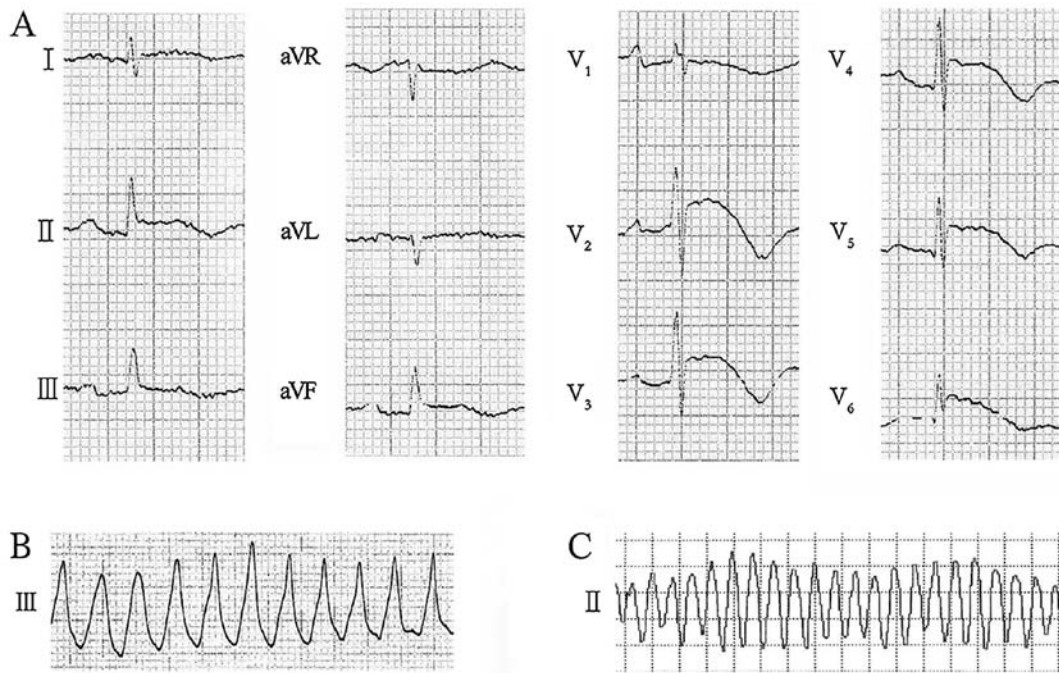


Figure 1. Electrocardiogram (ECG) of the proband. This recorded sinus rhythm and syncopes. (A) ECG during sinus rhythm showed J-waves in lead II, III, aVF and V₂-V₆, and coved ST-segment elevation which mimicked acute myocardial ischemia in lead V₂-V₆; (B) ECG during his syncope recorded at the emergency department showed polymorphic ventricular tachycardia; (C) Torsades de pointes (TdP) recorded during his syncope during admission.

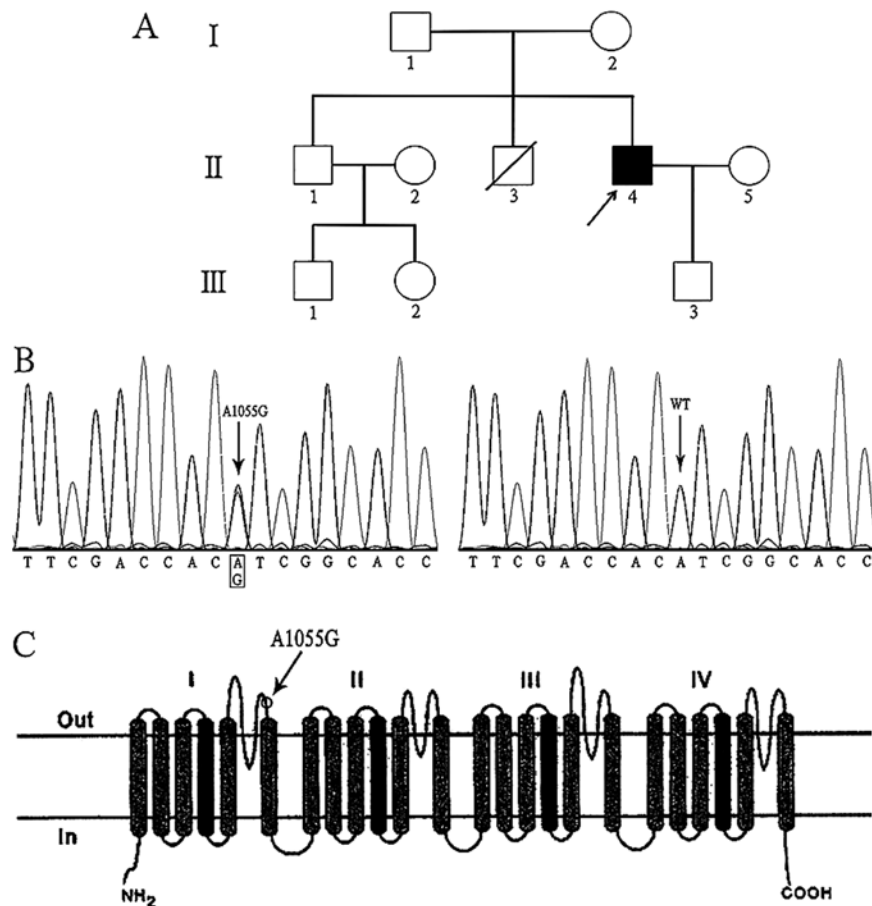


Figure 2. DNA sequence analysis and schematic representation of the α subunit of the sodium channel. (A) Pedigree of the family. Squares and circles represent male and female members, respectively. The solid square (with the arrow) represents the proband carrying a heterozygous A1055G mutation, and II-3 suffered from sudden death. (B) DNA sequence analysis of the proband demonstrated an A to G mutation of the sodium channel, voltage gated, type V alpha subunit (*SCN5A*) gene at position 1055 of the α subunit of the sodium channel. (C) Schematic representation of the α subunit of the sodium channel indicates the location of the mutation.

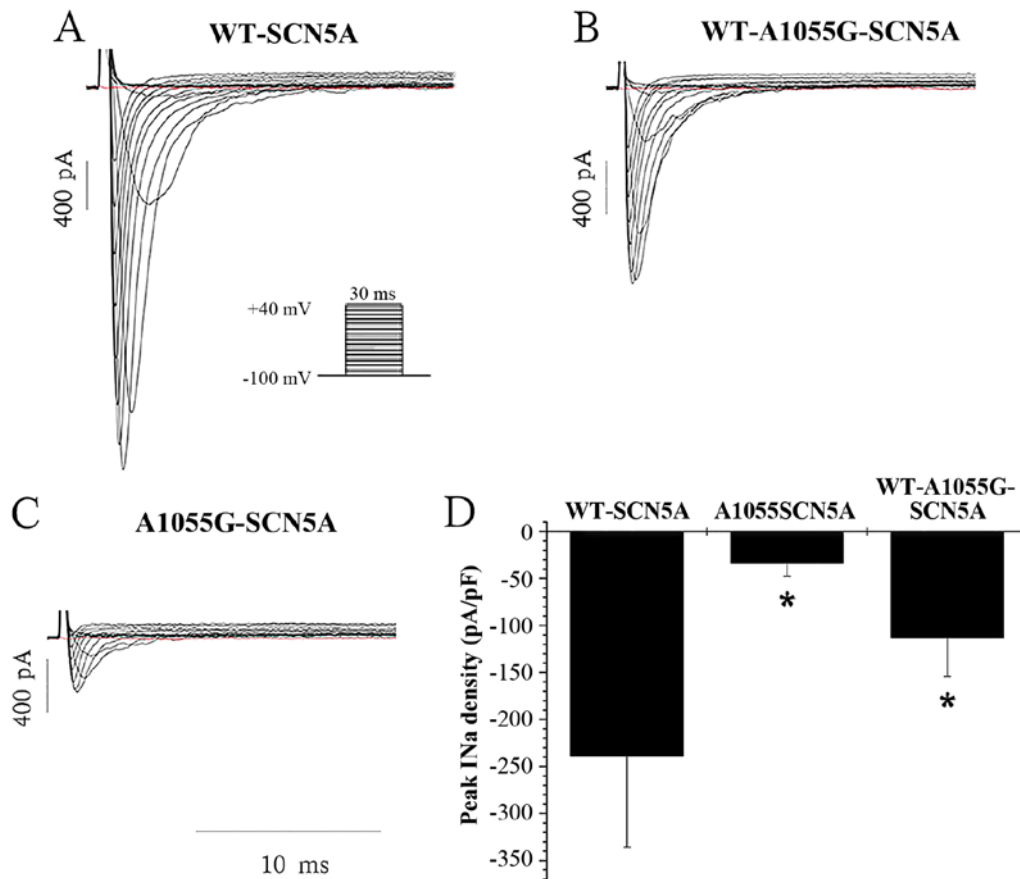


Figure 3. The representative sodium current (I_{Na}) traces recorded from HEK293 cells transfected transiently with (A) wild-type (WT)-sodium channel, voltage gated, type V alpha subunit (SCN5A); (B) WT-A1055G-SCN5A; (C) A1055G-SCN5A; and (D) the peak I_{Na} densities were significantly reduced by the mutation. *Significant difference from the WT, $P < 0.05$.

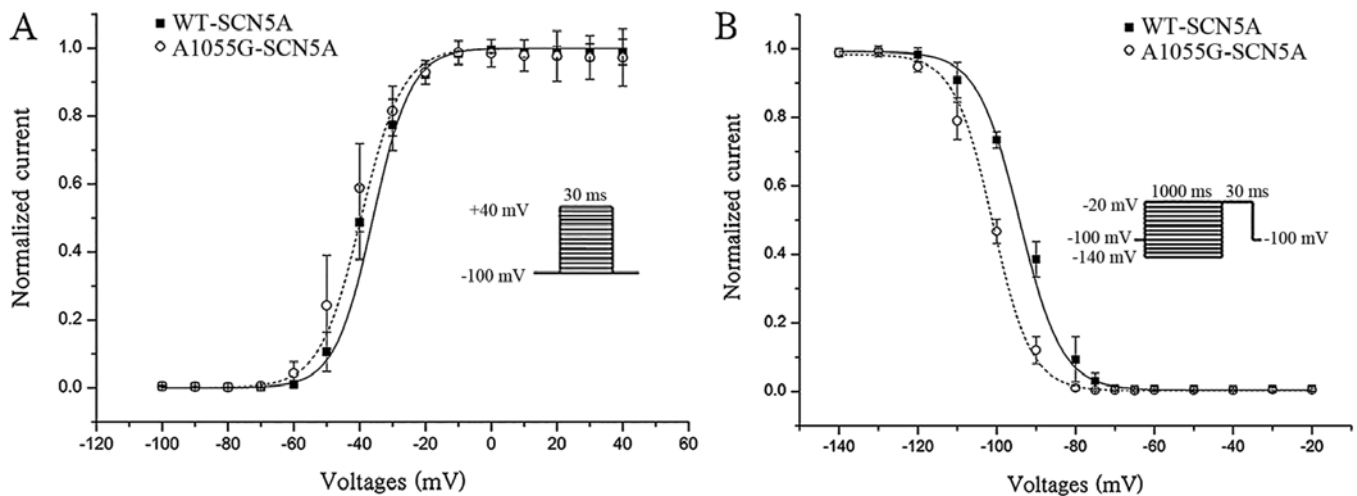


Figure 4. Kinetics of the sodium channels. (A) The steady-state activation curve exhibited no changes between HEK293 cells transfected transiently with wild-type (WT)-sodium channel, voltage gated, type V alpha subunit (SCN5A) and A1055G-SCN5A; (B) The A1055G mutation shifted the steady-state inactivation curve to a more negative potential.

zygous co-expression of WT-SCN5A and A1055G-SCN5A channels (WT-A1055G-SCN5A), which mimic the genotype of the proband, significantly reduced the peak I_{Na} density (-112.69 ± 41.66 pA/pF, $n=6$) to 47% of the WT-SCN5A channels (-238.68 ± 97.13 pA/pF, $n=7$, $P < 0.001$). The changes in peak I_{Na} density are summarized in Fig. 3.

The steady-state activation curve of A1055G-SCN5A channels exhibited no significant changes compared with that of WT-SCN5A channels ($P > 0.05$, Fig. 4A), while the steady-state inactivation curve of A1055G-SCN5A channels was shifted to a more negative potential compared with that of WT-SCN5A channels ($P = 0.003$, Fig. 4B).

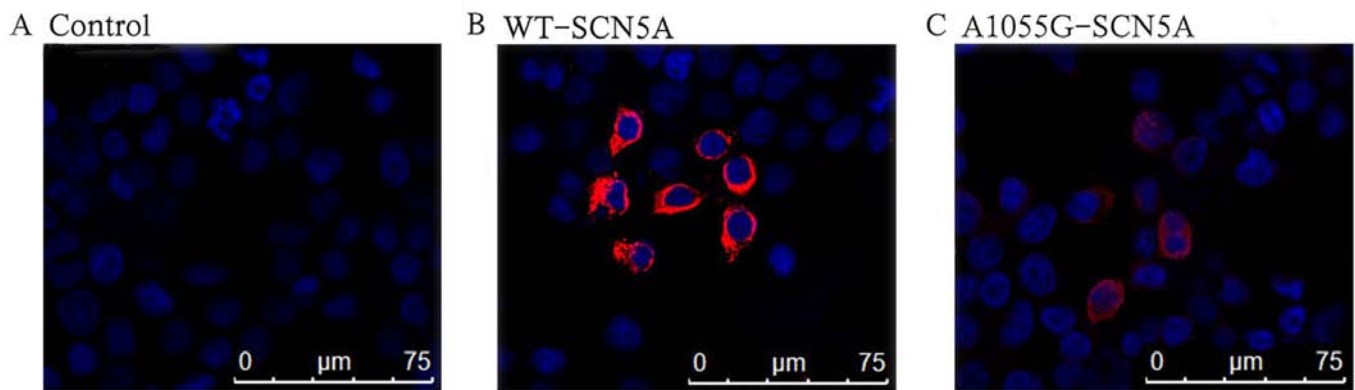


Figure 5. Confocal imaging of the wild-type (WT) and mutant channels. (A) The background fluorescence of the non-transfected (control) cells. Blue fluorescence indicates the nuclei; (B) The peripheral localization of fluorescence in cells transfected with WT-sodium channel, voltage gated, type V alpha subunit (SCN5A); (C) Reduced fluorescence in the cell membrane and cytoplasm was detected in cells transfected with A1055G-SCN5A.

Confocal imaging. In order to understand the mechanisms underlying the marked reduction in I_{Na} , immunocytochemical analysis was performed to explore cellular expression and the localization of the WT and mutant channels. The non-transfected cells had blue background fluorescence (Fig. 5A). As anticipated, the cells transfected with WT-SCN5A channels displayed strong red fluorescence on the cell membranes (Fig. 5B). By contrast, the mutation of A1055G considerably reduced the expression in both the cell membrane and cytoplasm (Fig. 5C).

Discussion

In the present study, we discovered a novel pathogenic variant in *SCN5A* of A1055G, which was correlated with ERS. This heterozygous missense mutation was identified in a male proband suffering from recurrent syncope, in whose ECG we noted an ER pattern in inferior leads and predominantly elevated ST-segment in mass precordia leads (V_2 - V_6). Polymorphic VT was documented, and the functional analysis suggested the mutation had severely deleterious effects on the sodium channel, which we suggest is the pathogenic mechanism for the arrhythmogenic characteristics of ERS.

Moreover, in the baseline 12-lead ECG we noted coved ST-segment elevation in lead V_2 - V_6 , which mimicked type 1 Brugada syndrome (BrS). The ECG diagnostic criteria of type 1 BrS indicate that a coved pattern should be present in lead V_1 - V_2 (14). However, it should be noted that lead V_1 in the ECG of the proband did not exhibit any such coved pattern. Furthermore, according to research by Zorzi *et al*, the ST-segment elevation at J point (ST_J) and at 80 msec after J point (ST_{80}) was useful for distinguishing ER from BrS (15). An upsloping ST-segment configuration ($ST_J/ST_{80} < 1$) had a high diagnostic accuracy for the diagnosis of ER, which presented in the ECG of the proband. Thus, the proband was diagnosed with ERS. However, the absence of a sodium channel blockade test to further confirm the diagnosis was one of limitations.

In 2011, Noseworthy *et al* found that the ER pattern had a heritable basis in the general population (13). ERS has been associated with mutations in six genes, namely *KCNJ8*,

ABCC9, *CACNA1C*, *CACNB2*, *CACNA2D1* and *SCN5A* (16). Medeiros-Domingo *et al* put forward the theory that gain-of-function in the cardiac I_{K-ATP} channel secondary to the missense mutation S422L was the pathogenic mechanism for the phenotypic expression of both BrS and ERS (17). Previously, Hu *et al* found mutations in *ABCC9*, which encoded ATP-binding cassette transporter of I_{K-ATP} (SUR2A) (18). The mutations also caused a gain-of-function in I_{K-ATP} and contributed to both BrS and ERS (18). Loss-of-function mutations were discovered in *CACNA1C*, *CACNB2* and *CACNA2D1*, which respectively encode $\alpha 1$, $\beta 2$ and $\alpha 2\delta$ subunits of the cardiac L-type calcium channel, as well as in *SCN5A* (10,19). Loss-of-function mutations in *SCN5A* are related to a wide range of inherited arrhythmia syndromes, such as BrS, progressive cardiac conduction disease, and sick sinus syndrome (20-22). In one of our previous studies, a mutation of G4297C in *SCN5A* was found to be responsible for ERS. It decreased I_{Na} density due to abnormal translation processes, which were rescued by a synonymous polymorphism of T5457C on the same allele of the gene (23).

In the present study, another novel loss-of-function mutation of A1055G in *SCN5A* was identified in the patient with ERS. After excluding other gene variants (*KCNJ8*, *ABCC9*, *CACNA1C*, *CACNB2* and *CACNA2D1*) related to ERS, a variant of A1055G was speculated to be a causative mutation. The *SCN5A* mutation was considered to be pathologic based on the following reasons: firstly, the variant was heterozygous; secondly, the variant was not detected in ethnicity-matched 500 healthy unrelated controls or in the son of the proband, whose ECG was normal. However, the genetic material and ECG from the living brother of the proband was not available. Additionally, the variant resulted in a substitution of a tyrosine by a cysteine in the extracellular loop between segments five and six of domain I, which located in highly conserved region across mammals. Furthermore, the functional analysis demonstrated remarkably decreased I_{Na} and obviously altered biologic features of sodium channel.

None of the previously identified variations are enough to distinguish benign from malignant ER variants (24). Based on the episodes of life-threatening arrhythmia which occurred in the proband and based on the results of the patch-clamp,

the A1055G mutation studied here is thought to be a variant indicated higher risk of malignant arrhythmia. According to previous research, the estimated absolute risk of sudden cardiac death in subjects with ER pattern was 70/100,000 every year (25). It was recommended that ERS be divided into three subtypes: Type 1, which has an ER pattern distributed predominantly in the lateral precordial leads, was prevalent among healthy male athletes with lower risk of arrhythmias; Type 2, which had an ER pattern distributed predominantly in the inferior or inferolateral leads, was associated with a higher level risk of arrhythmias; Type 3, which had an ER pattern distributed globally in the inferior, lateral and right precordial leads, was associated with the highest level of risk of developing malignant arrhythmias (26). It was reported that mutations in *SCN5A* were associated with Type 3 ERS under baseline conditions or following a sodium block challenge (19). In the present study, the proband who carried a mutation in *SCN5A* was diagnosed with Type 3 ERS according to the manifestation of the ECG, which was in accordance with the information above.

In previous research, the mechanism underlying ERS has been posited to be the increasing action potential (AP) notches at either the left or right ventricular epicardium, which creates a transmural voltage gradient that causes the appearance of J-waves with or without ST segment elevation on the ECG (27,28). In this way, dispersion of repolarization between the epicardium and endocardium as well as within the epicardium was generated, leading to transmural dispersion and a vulnerable window across the ventricular wall, finally resulting in phase 2 reentry that triggers VT or VF (24,28). The imbalanced repolarizing currents caused by either decreased inward currents, such as I_{Na} or I_{Ca} , or increased outward currents, such as I_{to} , I_{K-ATP} , I_{K-ACh} , or other outward currents, give rise to ERS (24,29,30). In the present study, the patch-clamp analysis demonstrated the heterozygous expression of A1055G reduced peak I_{Na} density to 47% of the WT. Possible explanations for this phenomenon are the defects of protein expression demonstrated by the immunocytochemical analysis, which displayed reduced red fluorescence in both the cell membrane and cytoplasm. Moreover, A1055G accelerated the inactivation of the sodium channel, which possibly led to a relatively increased outward current in ER of AP and finally caused arrhythmogenic characteristic in the patient with ERS. The results of functional tests, as well as the proposed mechanisms, are consistent with findings in previous studies (23). Thus, we suggest that both the defects of channel surface expression and altered kinetic features of sodium channels, which resulted from the mutation of A1055G, were critical for ERS in the proband.

In conclusion, in the present study we demonstrated that a novel heterozygous missense mutation A1055G in *SCN5A* led to a loss-of-function in the sodium channels, which likely accounts for the clinical phenotype of the proband and contributed to the arrhythmogenic characteristics of ERS.

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