Genetic analysis of sick sinus syndrome in a family harboring compound *CACNA1C* and *TTN* mutations

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Abstract. Sick sinus syndrome (SSS) is a sinus node dysfunction characterized by severe sinus bradycardia. SSS results in insufficient blood supply to the brain, heart, kidneys, and other organs and is associated with the increased risk of sudden cardiac death. Bradyarrhythmia appears in the absence of any associated cardiac pathology and displays a genetic legacy. The present study identified a family with primary manifestation of sinus bradycardia (five individuals) along with early repolarization (four individuals) and atrial fibrillation (one individual). Targeted exome sequencing was used to screen exons and adjacent splice sites of 61 inherited arrhythmia-associated genes, to detect pathogenic genes and variant sites in the proband. Family members were sequenced by Sanger sequencing and protein functions predicted by Polyphen-2 software. A total of three rare variants were identified in the family, including two missense variants in calcium voltage-gated channel subunit alpha1 C *(CACNA1C)* (gi:193788541, NM_001129843), c.1786G>A (p.V596M) and c.5344G>A (p.A1782T), and one missense variant in titin *(TTN)* c.49415G>A (p.R16472H) (gi:291045222, NM_003319). The variants p.V596M and p.R16472H were predicted to be deleterious and resulted in alterations in the amino acid type and sequence of the polypeptide chain, which may partially or completely inactivate the encoded protein. The comparison of literature, gene database, and pedigree phenotype analysis suggests that p.V596M or p.R16472H variants are pathogenic. The complex overlapping variants at three loci lead to a more severe phenotype in the proband, and may increase the susceptibility of individuals to atrial fibrillation. The simultaneous occurrence of V596M and R16472H may increase the severity of early repolarization. Various family members may have carried heterozygous mutants of p.A1782T and p.R16472H due to genetic heterogeneity, however did not exhibit clinical signs of cardiac electrophysiological alterations, potentially attributable to the low vagal tone. To the best of the author's knowledge, this is the first study to suggest the involvement of the novel missense CACNA1C c.1786G>A and TTN c.49415G>A variants in the inheritance of symptomatic bradycardia and development of SSS.

Introduction

Sick sinus syndrome (SSS) is caused by lesions in the sinoatrial node and its adjacent tissues, resulting in sinoatrial node pacemaker function and/or sinoatrial conduction dysfunction. SSS includes conditions such as severe sinus bradycardia, sinus arrest, sinoatrial block, and bradycardia-tachycardia syndrome (flutter, atrial fibrillation, and paroxysmal supraventricular tachycardia) (1,2). SSS may lead to insufficient blood supply to the heart, brain, kidney, and other organs, and cause sudden cardiac death. Symptomatic SSS requires the implantation of an electronic pacemaker. SSS accounts for about half of all pacemaker implantations in the United States (3). Given the low survival rate (less than 10%) (4), the identification of patients with high-risk SSS is of importance for the prevention of sudden cardiac death.

Although a variety of pathological factors leading to sinus node or cardiac nerve dysfunction may cause SSS, a positive correlation was observed between SSS and age (5). Increasing evidences suggest a distinct genetic background in SSS, including some genetic mutations in genes encoding cytoskeletal proteins and ion channels that may result in familial sick sinus syndrome (FSSS). The L-type voltage-gated calcium channel (I-caL) is one of the significant ion channels involved in the automatic cell depolarization in sinoatrial node. Loss of I-cal in some hereditary SSS is of great significance. For instance, the deletion of L-calcium currents in CaV1.3 knockout mice (CaV1.3$^{-/-}$) may contribute to reduced heart rate and sinus arrhythmia (6). The presence of CaV1.3-mediated I-caL dysfunction may clinically lead to sinus node dysfunction and deafness

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syndrome (SANDD). Individuals affected with SANDD present with bradycardia, profound deafness, and dysfunction of atrio-ventricular conduction (7). However, no study has reported the association between sinus bradycardia or SSS and the variant of calcium voltage-gated channel subunit alpha1 C (*CACNA1C*).

The early repolarization (ER) pattern displays two or more continuous wall or side wall leads with a J point elevation above 0.1 mV in the standard 12-lead electrocardiogram (ECG). The ST elevation is above 0.1 mV and exhibits concave upward or oblique type elevation. ER is often inherited and several studies have shown its relationship with malignant arrhythmias and sudden death (8-10). It is known that ER syndrome is a polygenic inherited disease, similar to hereditary sinus brady-cardia, caused by mutations in cardiomyocyte genes encoding ion channels. The most common pathogenic genes are encoded by cardiac electrical ion channels, including calcium channels (CACNA1C, CACNB2B, and CACNA2D1) (11), ATP-sensitive potassium channels (KCNJ8) (12), and sodium channel α subunit 5 (SCN5A) (13). Functional studies have revealed its association with 'enhanced function' or 'loss of function' of ion channels. A change of 1-5% was reported in the incidence of ER in the general population (14) and is most commonly seen in young men (15).

We applied next generation sequencing (NGS) for the iden-tification of a genetically arrhythmic family of SSS and ER characterized by complex bradycardia to further understand the interaction between the complex genotype and phenotype in this family.

Materials and methods

Subjects. The proband of this family is a 76-year-old male patient from Fujian with Han nationality and clinical manifes-tation of recurrent palpitation, flustered, and bradycardia with irregularity. Multiple electrocardiographic examinations indi-cated that the patient had slow atrial fibrillation (the slowest heart rate 29 beats per min). According to the family data provided by the proband, medical records, routine physical examination, ECG, and cardiac ultrasound examination were performed for other 11 family members. In addition, blood, urine, biochemical kit, troponin, pro-BNP, etc., were analyzed and the family genetic map was recorded. The study was approved by the medical ethics committee of the hospital and all subjects signed the informed consent.

Genomic DNA extraction. Genomic DNA was extracted with Tiangen blood genomic DNA extraction kit (DP348) as per the manufacturer's instructions.

Illumina sequencing (16-18) and bioinformatic analysis (19). DNA samples of the proband were detected by Nanodrop 2000 and over 3 µg sample was used for DNA fragmentation. DNA fragments were interrupted to about 250 bp by Covaris method, followed by their recovery. The fragments were subjected to the end-repair reaction and the sequence-specific attachment (adapter) was linked with the end-repair products. The product was amplified and purified based on the universal primer-binding site on the adapter. For the capture and enrich-ment of the target, multiple gene fragments, including target genes *CACNA1C* and *TTN*, were enriched by DNA capture chip

with multiple genes and the enrichment products were sequenced by Illumina (Illumina, Inc., San Diego, CA, USA) (5). For data analysis, the original file was subjected to base reading to obtain double terminal sequence of 90 bp reads. For the removal of the low-quality and polluted reads, the adapter sequence was removed and the purification data analyzed by sequence alignment. Soap software (SOAPdenovo V2.04; SOAP3/GPU V0.01beta; SOAPaligner/soap2 V2.20; SOAPsplice V1.1; SOAPsnp V1.03; SOAPindel V1.0; SOAPsv V1.02) was used to analyze copy number, single nucleotide polymorphism (SNP), and insertion/deletion (INDEL). Annotations were used to screen suspected pathogenic variants. The polymorphism phenotyping version 2 (Polyphen2) software was applied (genetices.bwh. harvard.edu/pph2/) for the prediction of protein functions. The process was completed by Shenzhen Huada Genomics Institute in accordance with its operating standards.

Sanger sequencing. Variants were confirmed using Sanger DNA sequencing. The primers targeting the target sequence were designed using Primer Premier 5 software and synthe-sized by Thermo Fisher Scientific, Inc. (Waltham, MA, USA). *CACNA1C* gene sequence was derived from GenBank (NM_001129843) and the target sequence was 230 bp. The primers used were as follows: Forward, GCTCGGATCTCA TCCCTCTC and reverse, GACGCATCTGAGCACGGA. Another target sequence of *CACNA1C* was 260 bp long and the specific primers used were as follows: Forward, TTCACC CCGAGCAGCTAC and reverse, TCCACTGTCTCCTGA GGGTT. *TTN* gene sequence was obtained from GenBank (NM_003319) and the target sequence was 270 bp. The primers used were as follows: Forward, CATTGTCAAGAA CAAGAGAGGTGAAAC and reverse, CGTATCTGTGCT ATTAATAAAGCTGGAGT. Amplification of polymerase chain reaction (PCR) products: The reaction was carried out in a final volume of 25 µl and comprised 2.5 µl of 10X Ex Taq buffer, 2 µl dNTP (2.5 mmol/l), 3 µl of each of forward and reverse primer (3 mmol/l), 1 µl DNA template, 0.2 µl Ex Taq, and 18.3 µl water. PCR products were amplified on PCR machine (PTC-200, PCR; Bio-Rad Laboratories, Inc., Hercules, CA, USA) under following conditions: Initial denaturation at 94˚C for 5 min, 35 cycles of denaturation at 94˚C for 40 sec, annealing at 58-62˚C for 40 sec, extension at 72˚C for 60 sec, followed by the final extension at 72˚C for 10 min. Purification and sequencing of PCR products: PCR products were obtained from Omega corporation E.Z.N.A.™ Gel Extraction kit. Sequencing was performed according to the standard procedure of BigDye Terminator v1.1 kit PCR products. Sequencing results were obtained by comparing DNAMAN version 5.2.2 with the normal sequence.

Results

Clinical report. There are 20 members of the family, with 5 diseases (Fig. 1). A 76-year-old male (II-1; the proband) patient presented with a 40-year history of palpitation, chest tightness, and stress sweating and was repeatedly admitted to our hospital. The patient had an episode of syncope 8 years ago following progressive palpitation, fatigue, and dizziness. He received no treatment, although his ECG suggested bradycardia and atrial fibrillation. In 2013, a 24-h Holter ECG monitoring
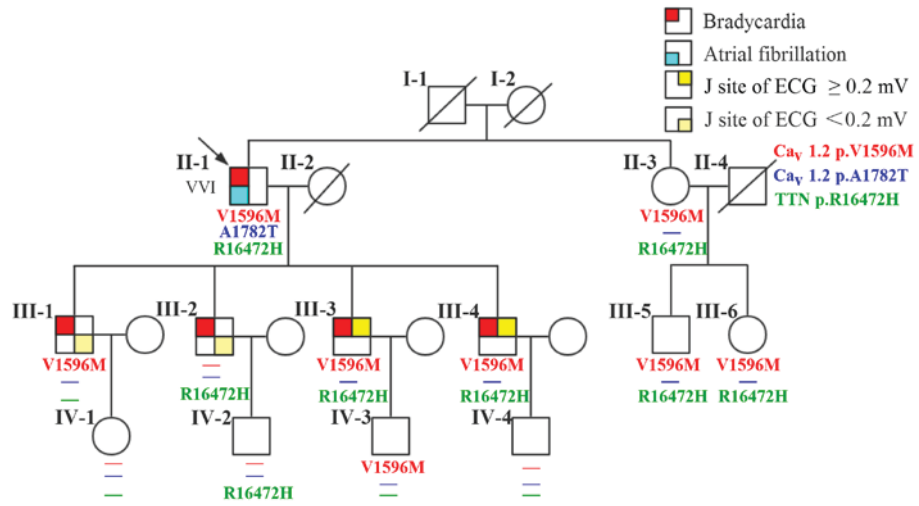
Figure 1. A pedigree, phenotype, and genotype of the family. Males and females are indicated by squares and circles, respectively. Proband is indicated by an arrow. Deceased individuals are indicated by diagonal lines. Phenotypes presented by the family members are depicted by a red top-left square for bradycardia; blue bottom-left square for atrial fibrillation; top-right square and bottom-right square (yellow) represent J-site of ECG ≥0.2 mV and <0.2 mV, respectively. VVI refers to the installation of pacemaker. ECG, 12-lead electrocardiogram.
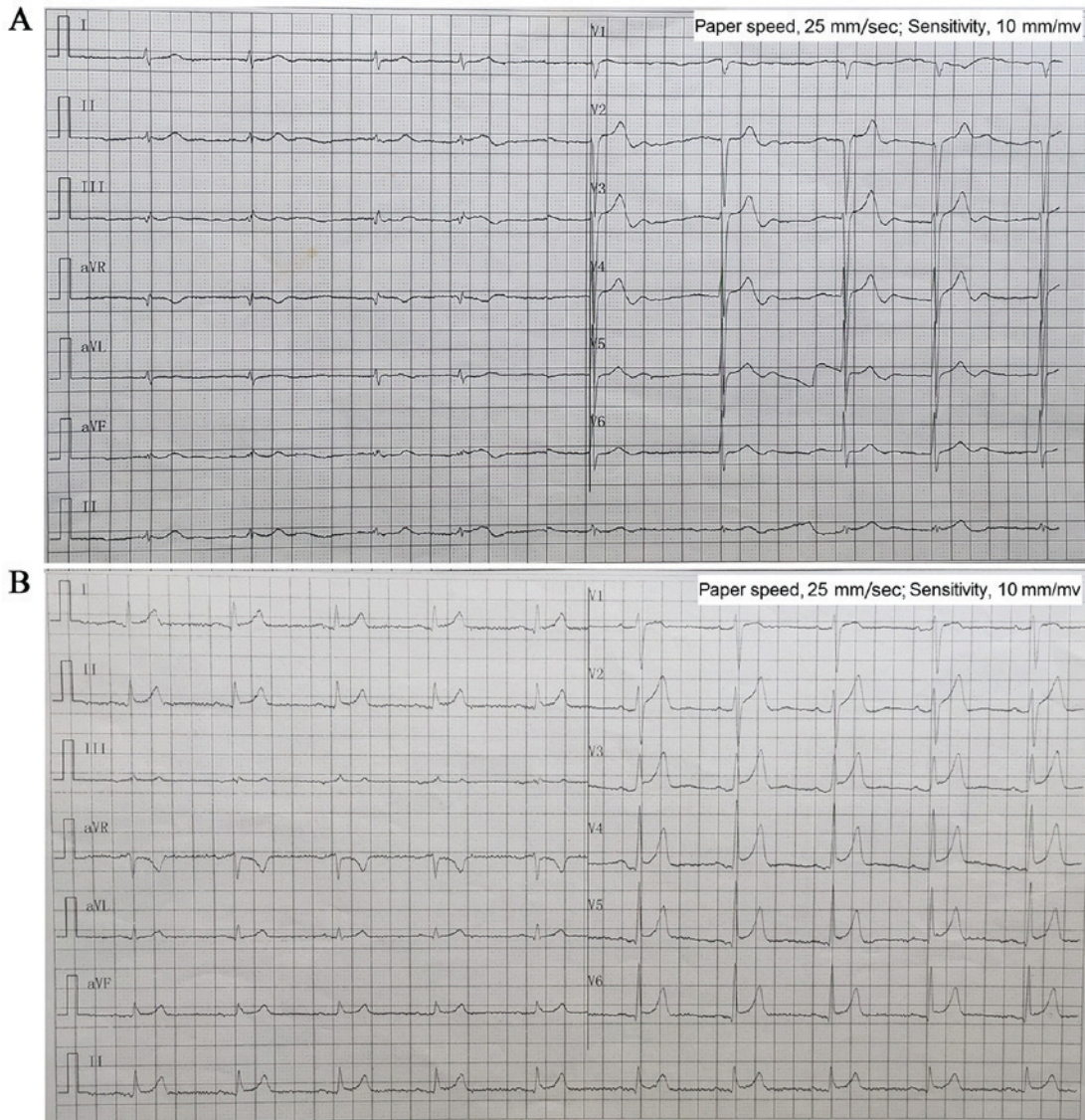
Figure 2. Electrocardiographic data. (A) Proband II-1 before installing pacemaker ECG showed bradycardia and atrial fibrillation (ventricular rate 50 bpm). (B) III-4 ECG showed sinus bradycardia (57 bpm) and early repolarization syndrome (ST elevation in V1-6 was upward concave, J-point elevation ≥0.2 mV). ECG, 12-lead electrocardiogram.

Table I. Clinical data of the family members.

ID	Age (years)	Sex	Onset age (years)	Symptoms	ECG/HOLTER	Echocardiogram
II-1[a]	76	Male	Twenties	Palpitation, chest tightness, stress sweating, emotional, and syncope	Bradycardia, atrial fibrillation	Mitral, tricuspid, and aortic valve regurgitation
II-3	73	Female	Twenties	Palpitation, chest tightness, stress sweating	Normal	Normal
III-1	52	Male	Twenties	Palpitation	Bradycardia, J-point elevation <0.2 mV	Normal
III-2	51	Male	Twenties	Palpitation	Bradycardia, J-point elevation <0.2 mV	Normal
III-3	49	Male	Twenties	Palpitation, chest tightness, stress sweating, and emotional	Bradycardia, J-point elevation ≥0.2 mV	Normal
III-4	43	Male	Twenties	Palpitation, chest tightness, stress sweating, and emotional	Bradycardia, J-point elevation ≥0.2 mV	Normal
III-5	48	Male	NA	Normal	Normal	Normal
III-6	44	Female	NA	Normal	Normal	Normal
IV-1	23	Female	NA	Normal	Normal	Normal
IV-2	22	Male	NA	Normal	Normal	Normal
IV-3	21	Male	NA	Normal	Normal	Normal
IV-4	9	Male	NA	Normal	Normal	Normal

[a]Proband. NA, not available; ECG, 12-lead electrocardiogram.

(Fig. 2A) confirmed bradycardia (average heart rate, 49 bpm; range, 29-89 bpm) and atrial fibrillation. The echocardiogram showed moderate mitral regurgitation, tricuspid regurgitation, and mild aortic valve regurgitation. He was diagnosed with SSS and received a permanent pacemaker implantation. The proband took no drugs such as digitalis, β-blocker, or calcium blocker to slow down his heart rate. Prior to the diagnosis, he had hypertension and an incidence of cerebral infarction. Evaluation of the family history revealed that all his four children (III-1, III-2, III-3, and III-4; all male) presented with bradycardia with an average heart rate <50 bpm; two of them (III-3 and III-4) showed early onset (since their childhood) of symptoms similar to their father's, and the other two (III-1 and III-2) have palpitation when they are tired or emotional swings. ECG (Fig. 2B) diagnosed III-3 and III-4 with ER syndrome (ST elevation in V1-V6 was upward concave, the J-point elevation ≥0.2 mV) and III-1 and III-2 with ER pattern (ST elevation in V4-6 was upward concave, the J-point elevation <0.2 mV).

The proband's parents (I-1 and I-2) had passed away; his mother (I-2) reported symptoms of paroxysmal arrhythmia before her death, but the exact diagnosis was unknown. The proband's four grandchildren (IV-1, IV-2, IV-3, IV-4; aged 10-24 years) and younger sister (II-2) and her two children (III-5 and III-6) presented with no cardiac symptoms and all had normal ECG (Table I). No other cutaneous, retinal, neurologic, and somatic abnormalities were reported or observed in this family.

Genetic analysis. Twelve members in this family were enrolled into the genetic analysis study. First, we conducted a targeted exome sequencing on the proband for 61 genes, which are related with arrhythmia. The enriched and purified targeted regions were sequenced on an Illumina HiSeq 2000 sequencer (Illumina) for 90-bp reads; the mean read depth was 243X and >97% bases were >30X. A total of 123 variants (including point variant and insertion/deletion) were detected, and mutant polymorphism (SNP) loci greater than 1% were filtered out through 1000 Genomes MAF (population frequency information from 1000 genomes project). Values of dbSNP and the remaining nine variants were found in clinvar database, excluding synonymous and intron variants (out of exon over 2 bp), to obtain three variants (Table II). No rare nonsynonymous variants were found except heterozygous c.1786G>A (rs768034509) in *CACNA1C*, c.5344G>A (rs750078053) in *CACNA1C*, and c.49415G>A (rs561977468) in *TTN*, predicting valine to methionine substitution at the amino acid 596 (p. V596M) in CACNA1C (Fig. 3A), alanine to threonine substitution at the amino acid 1782 (p. A1782T) in CACNA1C (Fig. 3C), and arginine to histidine substitution at the amino acid 16472 (p. R16472H) in TTN (Fig. 3E). In addition, c.3979-8delC was present in the intron of MYH6, and c.68-5C>T was present in the intron region of TNNT2. Three synonymous variants were detected on *TTN* (p.Ala3897=p.Tyr6529=p.Gly12282) (Table II). These variants were in the non-coding region, suggestive of the absence of any effect of this family with sinus bradycardia on ER.

These three mutant sites (V596M and A1782T on *CACNA1C* and R16472H on *TTN*) are heterozygous variants. The frequency of the variant in 1000 genomes project was 0, while clinvar database had no relevant report at these sites (http://www.ncbi.nlm.nih.gov/clinvar/?term=CACNA1C [gene] and http://www.ncbi.nlm.nih.gov/clinvar/?term=TTN [gene]). V596M variant in *CACNA1C* was predicted to be damaging with a score of 1.000

Table II. Nine mutations found in Clinvar database of a propositus in a family with hereditary complex sinus bradycardia (MAF ≤0.01).

Genes	RefSeq	Nucleic acid alternation	Amino acid alternation	Mutation location	Zygosity	Chr:location	RS-ID	MAF	Mutation type
CACNA1C	NM_001129843	c.1786G>A	p.Val596Met	EX13	Het	chr12:2676851	rs768034509	0	Missense
CACNA1C	NM_001129843	c.5344G>A	p.Ala1782Thr	EX42	Het	chr12:2788862	rs750078053	0	Missense
MYH6	NM_002471	c.3979-8delC	-	IN28	Het	chr14:23858272	rs193922652	0	-
TNNC1	NM_003280	c.108C>A	p.Ile36Ile	EX3	Het	chr3:52486216	rs202000367	0.0027	Synonymous
TNNT2	NM_000364	c.68-5C>T	-	IN4	Het	chr1:201338978	rs540630390	0	-
TTN	NM_003319	c.11691G>T	p.Ala3897Ala	EX45	Het	chr2:179605180	rs746578	0	Synonymous
TTN	NM_003319	c.19587C>T	p.Tyr6529Tyr	EX79	Het	chr2:179483495	rs397517587	0	Synonymous
TTN	NM_003319	c.36846C>A	p.Gly12282Gly	EX135	Het	chr2:179451897	-	0	Synonymous
TTN	NM_003319	c.49415G>A	p.Arg16472His	EX154	Het	chr2:179434249	rs561977468	0	Missense

Removing the mutated sites of which the MAF was over 1% through dbSNP 1000 Genomes (population frequency information from the 1000 Genomes Project). CACNA1C, calcium voltage-gated channel subunit alpha1 C; TTN, titin.

(sensitivity, 0.00; specificity, 1.00) by PolyPhen-2 software. The variant A1782T was predicted to be benign, with a score of 0.010 (sensitivity, 0.96; specificity, 0.77). We speculate that these two variants are likely to affect the function of CACNA1C-encoded ion channels. R16472H variant in TTN was predicted to be probably damaging with a score of 0.743 (sensitivity, 0.85; specificity, 0.92). These three variant sites are highly conserved among many species (Fig. 4).

Genetic analysis showed that none of the eleven family members carried c.5344G>A variant in CACNA1C gene. On the other hand, c.1786G>A in CACNA1C gene and c.49415G>A in TTN gene were detected in five family members, including the proband's two sons III-3 and III-4, and sister II-3 and her two children III-5 and III-6. Other six family members only had either of them.

Discussion

Cardiomyopathy and ion channel dysfunction are two major causes for inherited cardiac arrhythmias (20). In this study, we identified two novel missense variants in CACNA1C gene (calcium channel gene) and one novel missense variant in TTN gene (cardiomyocyte gene) in a family with symptomatic bradycardia, which eventually progressed into SSS in the proband.

CACNA1C gene encodes an alpha-1 subunit of a voltage-dependent calcium channel. I-caL plays a key role in the generation of spontaneous action potential in pacemaker (e.g., sinoatrial node) cells through the conduction of inward calcium currents. Any dysfunction in I-caL leads to significantly attenuated automaticity of these cells (21). Variants in CACNA1C gene have been associated with cardiac diseases such as Long-QT syndrome (22), Timothy syndrome (23), and Brugada syndrome (24). However, none has been reported in SSS. This is the first study to report missense variants of CACNA1C gene in patients with SSS. The two SNPs in CACNA1C gene detected in this family result in two amino acid replacements, localized at the third transmembrane segment of domain II (DIIS3) (p.596V>M) and carboxyl (C)-terminus (p.1782A>T) of CACNA1C, respectively. However, only p.596V>M variant was thought to be damaging, while p.1782A>T variant was neutral. Regardless, the proband was the only carrier of p.1782A>T variant; therefore, it is unlikely that this variant contributes to the inheritance of bradycardia and ER in the family. Although the proband is also the only one diagnosed with SSS, this diagnosis is unlikely to be associated with p.1782A>T variant but more of a reflection of his disease progression. A previous study showed that the expression of CACNA1C in the sinoatrial node decreases with aging (25); this observation may explain the deterioration of cardiac symptoms in the proband as he aged. It should be alerted that the cardiac symptoms displayed by the proband's sons may eventually develop into SSS. Furthermore, the proband's four sons exhibited ER, an ECG pattern that was unobserved in the proband. This observation may be due to different manifestations of the disease at different stages. We estimate that p.596V>M variant in CACNA1C may partly explain ER observed in the proband's three sons because CACNA1C gene has been identified as a susceptibility gene for ER syndrome (11).

A variant in the gene encoding for the SCN5A, c.2365G>A (p.789V>I), was a disease-causing missense variant for
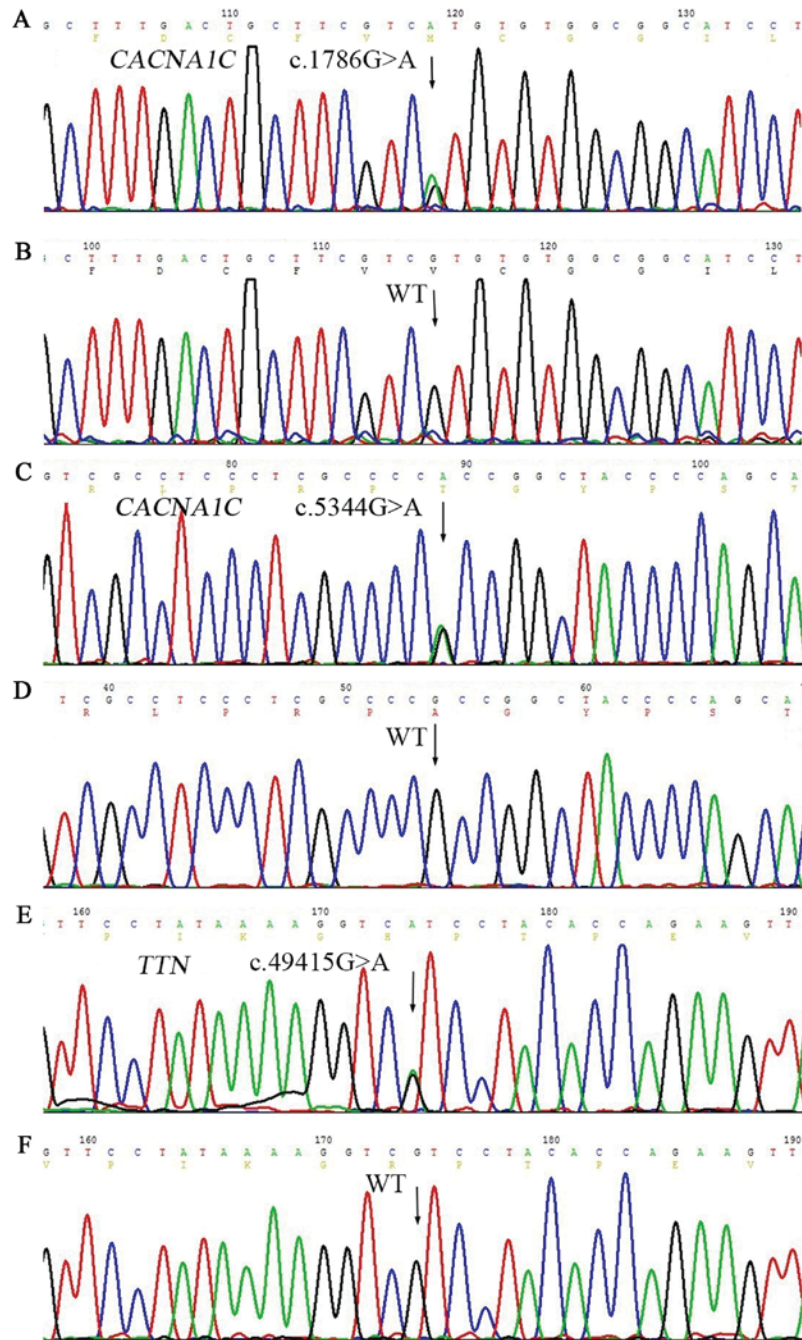
Figure 3. Genetic analysis revealed three novel heterozygous variations of *CACNA1C* and *TTN* gene in the family. (A) Missense variant c.1786G>A in *CACNA1C* gene. (C) Missense variant c.5344G>A in *CACNA1C* gene. (E) Missense variant c.49415G>A in *TTN* gene. The arrows indicate the comparison between the mutant site and normal wild-type. WT corresponds to (B), (D), and (F), respectively. *CACNA1C*, calcium voltage-gated channel subunit alpha1 C; TTN, titin; WT, wild-type.

Brugada syndrome (26) and identified as a paralogue variant for *CACNA1C* variant c.1786G>A (p.596V>M). According to the Paralogue Annotation theory (www.cardiodb.org/paralogue_annotation/), known disease-causing paralogue variants (e.g., *SCN5A* variant c.2365G>A in this case) may be transferred to equivalent amino acids in the protein of interest (e.g., *CACNA1C* variant c.1786G>A in this case) to identify residues likely to be intolerant to the variation (27). Furthermore, *CACNA1C* variant c.1786 G allele, similar to *SCN5A* variant c.2365 G allele, may be intolerant to genetic variant; therefore, G>A variant at this position is very likely to induce arrhythmias.

The gene *TTN* encodes for titin (connectin), the largest known protein, which spans half of a sarcomere and plays critical roles in cardiac (and skeletal) muscle function. Mutations in *TTN* gene have been associated with cardiac diseases such as dilated cardiomyopathy (28), familial hypertrophic cardiomyopathy (29), early-onset myopathy with fatal cardiomyopathy (30), and proximal myopathy with early respiratory muscle involvement (31). The variant c.49415G>A in *TTN* gene detected in this family results in an amino acid replacement, which localizes at an immunoglobulin (Ig)-like and Fn3 domains super-repeat segment in the A-band region. This region was identified as a hot spot for inherited dilated

Figure 4. Amino acid alignment shows conservation among species of (A) valine ("V") at position 1596 in CACNA1C, (B) alanine ("A") at position 1782 in CACNA1C (NP_001123315.1) and (C) arginine ("R") at position 16472 in TTN (NP_003310.4), respectively. CACNA1C, calcium voltage-gated channel subunit alpha1 C; TTN, titin.

cardiomyopathy, a major cause of heart failure and premature death (32); however, the study only included truncation mutations. The missense variant c.49415G>A (p.16472R>H) in this region is likely to be damaging, although it may not directly cause the malfunction of sinoatrial node. A recent study showed that patients with arrhythmogenic right ventricular cardiomyopathy and variants in *TTN* gene were more likely to experience supraventricular arrhythmia such as atrial fibrillation than those without *TTN* variant (33). Likewise, the presence of *TTN* c.49415G>A variant may exacerbate cardiac symptoms accompanied by bradycardia in the proband (atrial fibrillation) and his two sons III3 and III4 (more severe ER).

Genetic mutations in genes encoding ankyrin-B (*ANK2*) (34), hyperpolarization-activated channel (*HCN4*) (35), and *SCN5A* (36) have been associated with sinoatrial dysfunction. However, we failed to observe any clinically meaningful variants in these genes. We cannot rule out genes that were not covered by our genetic analyses and the contribution of their variants to disease development. First, six family members carried both *CACNA1C* c.1786G>A and *TTN* c.49415G>A variants, but three of them (II3, III5, and III6) failed to show any

cardiac symptoms observed in the proband and his two sons III3 and III4. Second, the proband's son III2 carried only *TTN* c.49415G>A variant, which is unlikely to cause bradycardia alone, but he showed symptoms of bradycardia. However, the discrepancies between phenotypes and genotypes are very common in genetics; for instance, phenotypes may not always manifest in all individuals carrying the same genetic variants (incomplete penetrance), and the type and severity of phenotypes vary between genotype-positive individuals (variable expressivity) (37). Factors such as age, sex, and nutrition may also contribute to different manifestations of genetic diseases. In rare diseases such as SSS, finding disease-causing variants by looking non-exhaustedly in a genome-wide range is almost impossible; hence, it is more realistic to focus on target genes to identify disease-causing variants based on the knowledge from databases and previous studies despite the limitation that the target gene list may always be incomplete.

In conclusion, our study suggests the involvement of the novel missense *CACNA1C* c.1786G>A and *TTN* c.49415G>A variants in the inheritance of symptomatic bradycardia and development of SSS. Future studies are required to understand the etiology at the molecular level.

Acknowledgements

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