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Citation	Heart Rhythm, 2(10), 1128-1134 <a href="https://doi.org/10.1016/j.hrthm.2005.06.032">https://doi.org/10.1016/j.hrthm.2005.06.032</a>
Issue Date	2005-10
Doc URL	<a href="http://hdl.handle.net/2115/16956">http://hdl.handle.net/2115/16956</a>
Type	article (author version)
File Information	H RHYTHM2-10.pdf



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# **Congenital Atrial Standstill Associated with Coinheritance of a Novel SCN5A Mutation and Connexin 40 Polymorphisms**

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## ABSTRACT

**OBJECTIVES:** The purpose of the study was to determine the clinical and biophysical characteristics of a novel *SCN5A* mutation identified in a family with atrial standstill.

**BACKGROUND:** Congenital atrial standstill has been linked to *SCN5A*. Incomplete penetrance observed in atrial standstill has been attributed in part to the digenic inheritance of polymorphisms in the atrial-specific gap junction connexin 40 (Cx40) in conjunction with an *SCN5A* mutation.

**METHODS:** Family members of an apparently sporadic case of atrial standstill underwent genetic screening of *SCN5A* and atrial-specific genes including Cx40. Biophysical properties of the wild type (WT) and the mutant *SCN5A* channels in a heterologous expression system were studied using the whole-cell patch clamp technique.

**RESULTS:** A novel *SCN5A* mutation, L212P, was identified in the proband (11 years old) and his father. The father was in normal sinus rhythm while the proband had no P waves in his surface ECG and his right atrium could not be captured by pacing. The recombinant L212P Na channel showed a large hyperpolarizing shift in both the voltage-dependence of activation (WT:  $-48.1 \pm 0.9$  mV, L212P:  $-63.5 \pm 1.5$  mV,  $p < 0.001$ ) and inactivation (WT:  $-86.6 \pm 0.9$  mV, L212P:  $-95.6 \pm 0.8$  mV,  $p < 0.001$ ), and delayed recovery from inactivation. Further screenings for genetic variations that might mitigate L212P dysfunction revealed that the proband, but not his father, carries Cx40 polymorphisms inherited from his asymptomatic mother.

**CONCLUSIONS:** These results suggest that genetic defects in *SCN5A* most likely underlie atrial standstill and the coinheritance of Cx40 polymorphisms may be a possible genetic factor that modifies the clinical manifestation of this inherited arrhythmia.

Key words: Atrial standstill, *SCN5A*, connexin 40, incomplete penetrance, polymorphism, SNP, sudden death

List of abbreviations used in the manuscript:

Cx40: Connexin 40, WT: wild-type, AS: atrial standstill, LQT3: type 3 long QT syndrome, SNP: single nucleotide polymorphism, h $\beta$ 1: human Na channel  $\beta$ 1 subunit, SSS: sick sinus syndrome, SA: sinoatrial, TTX: tetrodotoxin

## INTRODUCTION

Atrial standstill (AS) is a rare arrhythmia characterized by bradycardia, absence of P waves, and junctional escape rhythm. Several distinct electrophysiological abnormalities have been recognized in AS, with both persistent and transient forms, and total and partial forms<sup>1</sup>. Familial AS without underlying clinical disorders is extremely rare, and AS episodes often appear to be sporadic, which has hampered identification of responsible genes and the pathophysiology underlying the atria-specific nature of this arrhythmia.

Two mutations have been associated with AS so far in the gene encoding the cardiac Na channel  $\alpha$  subunit *SCN5A*, which is responsible for dominant disorders of inherited arrhythmias including type 3 long QT syndrome (LQT3)<sup>2</sup>, Brugada syndrome<sup>3</sup>, and cardiac conduction system disease<sup>4</sup>. We previously identified a missense mutation R367H in a transient AS case complicated with Brugada syndrome<sup>5</sup>, and the recombinant channel showed total loss of Na current when expressed heterologously. In contrast, another *SCN5A* mutation, D1275N, found in a small Dutch kindred with progressive AS, showed relatively benign biophysical abnormalities<sup>6</sup>. Moreover, some mutation carriers in this pedigree exhibit a normal electrocardiogram, and further screening of atria-specific genes has demonstrated that the affected individuals exclusively carry both D1275N mutation and homozygous polymorphisms in the regulatory region of connexin 40 (Cx40), supporting an argument favoring digenic inheritance. Cx40 is a gap junction protein predominantly expressed in the atrium and the specialized conduction system, and the single nucleotide polymorphisms (SNPs) result in reduced Cx40 expression *in vitro*<sup>6</sup>. Furthermore, these SNPs have been clinically associated with enhanced atrial vulnerability and increased risk of atrial fibrillation<sup>7</sup>. These results suggest that the D1275N allele alone may not be sufficient to manifest a

severe clinical phenotype in AS, and the Cx40 SNPs may cause a genetic predisposition which in turn disrupts the spatial distribution of Cx40 influencing atrial conduction and excitability.

Here we present an AS family with a novel *SCN5A* mutation, L212P. The proband showed progressive atrial dysfunction leading to atrial standstill. In contrast to the relatively benign biophysical abnormalities in D1275N, L212P showed severe dysfunction when expressed heterologously. In addition to L212P, the proband carries the maternal Cx40 SNPs allele in the regulatory regions. In contrast, the proband's father, who carries the L212P allele but not Cx40 SNPs, is phenotypically normal. Genetic defects in the *SCN5A* allele most likely underlie the arrhythmic substrate, but the coinherited Cx40 SNPs may confer a severe and atria-specific clinical phenotype in this AS pedigree.

## METHODS

### *Patients*

A 3-year-old Japanese boy was admitted to a local hospital because of severe bradycardia. He had never had syncope or dizziness, but he was diagnosed as sick sinus syndrome with paroxysmal atrial fibrillation by Holter ECG recordings. A histological specimen from the right ventricular endocardium performed at the age of 5 was normal. At the age of 10, AS with 6.65 sec of the maximum RR interval was evident, and he has been treated with cilostazol 100 mg/day to prevent severe bradycardia<sup>8</sup>. He was admitted to the Hokkaido University Hospital for further examination including DNA diagnostics. Two siblings of the proband's paternal grandfather had died suddenly in their first decade of life, but the rest of

the family members are asymptomatic and phenotypically normal, and no one has received cardiac pacemaker implantation or had a history of cardiac surgery.

### **Molecular genetics**

Informed consent was obtained from study participants according to the guidelines of the medical ethics committee of Hokkaido University. Genomic DNA was extracted from peripheral blood leukocytes by a standard method. PCR was used to amplify the coding region and flanking intron sequence of *SCN5A*, as previously described<sup>9</sup>. Exon 1 of Cx40 was amplified as previously reported<sup>6</sup>, and the exon 2 that covers the entire coding region of Cx40 was amplified by using a primer set (Cx40-F2: 5'-TGGAATCCCAGAACATGATAGA-3', and Cx40-R2: 5'-TCAGTTCAGAACGGAACAGTCT-3') using LA Taq polymerase (Takara Bio). HCN4 gene was amplified as previously described<sup>10</sup>. Amplified PCR products were analyzed by direct sequencing using an ABI Prism 310 genetic analyzer (Applied Biosystems).

Genotype and allelic frequencies of the Cx40 SNPs in general Japanese population were determined by TaqMan technology using genomic DNAs of 99 healthy unrelated individuals. For the SNP at nucleotide -44 (SNP1), a 181 bp fragment of the Cx40 Exon 1 was amplified from genomic DNA using a primer set (SNP1-F:

5'-AGCAAAGACTCTAAGAGCACAAAAGAG-3' and SNP1-R:  
5'-TTCTCCAAC TGCCCACGC-3'). And fluorescent probes were designed to target WT allele (5'-VIC-TATCTGTCGCCCTTCC-Q-3') and SNP1 allele (5'-FAM-TATCTGTCGCCCTTCC-Q-3'). Similarly, a 170 bp fragment was amplified for the SNP at nucleotide +77 (SNP2) using a primer set (SNP2-F:  
5'-GCGTGGGCAGTTGGAGAA-3' and SNP2-R:  
5'-TTTATCCAGCCTAGAATCCAAGCT-3'), and fluorescent probes were designed to

target WT allele (5'-FAM-CCTGGACTTCCTTC-Q-3') and SNP2 allele (5'-VIC-CCTGGACTTCCTTC-Q-3'). Primer and probe design was performed by using Primer Express software (Applied Biosystems), and sequence homogeneity was confirmed by comparison to all available sequences on the UCSC database by using BLAST. PCR was performed in 3 µl reaction mixtures containing 1.5 µl of TaqMan universal PCR master mix (Applied Biosystems), 450 nM concentrations of each primer, 100 nM WT probe, 100 nM SNP probe, and 3 ng of sample DNA. Thermal cycling was performed on the GeneAmp PCR System 9700 (Applied Biosystems) consisted of 2 min at 50°C and 10 min at 95°C for AmpliTaq Gold activation, followed by 40 cycles of 92°C for 15 sec and 58°C (for SNP1) or 60°C (for SNP2) for 1 min. Fluorescence intensity of each well of the plate was read by Prism 7900HT (Applied Biosystems) and the data were analyzed by the SDS 2.0 allele calling software (Applied Biosystems).

#### ***Site-directed mutagenesis***

Wild type (WT) human heart sodium channel  $\alpha$  subunit (hNav1.5) cDNA was subcloned into the pCDNA3.1 plasmid (Invitrogen). Site-directed mutagenesis was performed using Quick-Change site-directed mutagenesis kit (Stratagene). Correct assembly of the mutant was verified by restriction analysis and the mutated regions generated by PCR were sequenced completely to identify clones without polymerase errors. The human cell line tsA-201 was transiently transfected with either WT or L212P plasmid using standard calcium phosphate method in combination with a bicistronic plasmid (pCD8-IRES-h $\beta_1$ ) encoding CD8 and the human Na channel  $\beta_1$  subunit (h $\beta_1$ ) to visually identify cells expressing heterologous h $\beta_1$  with Dynabeads M-450 CD8 (Dynal)<sup>11</sup>. Electrophysiological measurements were performed 24-72 hours after transfection. Functional expression studies were performed on multiple independent recombinants.

### ***Electrophysiology and data analysis***

Na currents were recorded using the whole-cell patch clamp technique. Electrode resistance ranged from 0.8 to 1.5 MΩ. Data acquisition was carried out using an Axopatch 200B patch clamp amplifier and pCLAMP8 software (Axon Instruments). Currents were filtered at 5 kHz (-3dB, four-pole Bessel filter) and digitized using an analog-to-digital interface (Digidata 1322A, Axon Instruments). Experiments were carried out at room temperature (20-22°C). Capacitance and series resistance were adjusted (75-85%) to obtain minimal contribution of the capacitive transients. The holding potential was -120 mV. The bath solution contained (in mM): 145 NaCl, 4 KCl, 1.8 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 10 HEPES; and 10 glucose, pH 7.35 (adjusted with NaOH). The pipette solution (intracellular solution) contained (in mM): 10 NaF, 110 CsF, 20 CsCl, 10 EGTA, and 10 HEPES, pH 7.35 (adjusted with CsOH).

The data were analyzed by using Clampfit (Axon Instruments) and SigmaPlot (SPSS Inc.). Curve fitting was made using a non-linear least squares minimization method. The time course of inactivation was fit with a two exponential function:  $I/I_{max} = A_0 + A_f \times \exp(-t/\tau_f) + A_s \times \exp(-t/\tau_s)$ , where  $A_0$  is a constant value,  $A_f$  and  $A_s$  are the fractions of fast and slow inactivating components,  $\tau_f$  and  $\tau_s$  are the time constants of fast and slow inactivating components, respectively. Steady-state availability for inactivation and conductance-voltage relationship were measured with standard pulse protocols as shown in the inset. Curves were fit with the Boltzmann equation,  $I/I_{max} = [1 + \exp((V - V_{1/2})/k)]^{-1}$  to determine the membrane potential for half maximal inactivation or activation ( $V_{1/2}$ ) and the slope factor  $k$ . Data for WT and L212P are shown with open circles and closed squares, respectively. Recovery from inactivation were measured by a double pulse protocol shown in the inset and was analyzed

by fitting data to two exponentials:  $I/I_{\max} = A_f \times [1 - \exp(-t/\tau_f)] + A_s \times [1 - \exp(-t/\tau_s)]$ .

Results are presented as means  $\pm$  standard error, and statistical comparisons were made using the unpaired Student's *t*-test. Statistical significance was assumed for  $p < 0.05$ .

## RESULTS

### **1. Clinical characteristics**

The proband was diagnosed as sick sinus syndrome complicated with paroxysmal atrial fibrillation at the age of 3. Atrial electrical activity partially remained at this time, but was progressively disrupted until the time that total atrial inexcitability was evident at the age of 11. A permanent pacemaker was not implanted because he remains asymptomatic, and cilostazol was effective in preventing severe bradycardia. The ECG on admission showed the absence of P waves and a junctional rhythm (HR=40 bpm) with ventricular escape beats (Fig 1A). The duration of the QRS complex and QTc interval were normal. A Holter ECG recording after terminating cilostazol exhibited a junctional rhythm with a maximum RR interval of 5.42 sec (Fig 1B). Organic heart diseases were excluded by chest X-ray, echocardiography, and magnetic resonance imaging. Intracardiac recordings performed in the absence of drugs by standard procedures showed a junctional rhythm with ventricular escape beats (Fig 1C). No atrial activity was observed and the His-ventricular interval was slightly prolonged (56 ms). AS was verified because of the absence of electrical activity in the atrial electrograms or a P wave during pacing at the maximum output of the stimulator (Fig 1D). Low frequency deflections in the His bundle recordings preceding the His deflections in Fig 1C and 1D may represent the residual activities at which atrial capture cannot be achieved. Neither programmed electrical stimulation for ventricular arrhythmias nor provocation test

using class IC drugs was performed.

## **2. Genetic screening**

Genetic screening was performed on three cardiac ion channel genes that have been previously associated with AS, atrial fibrillation, or sick sinus syndrome: the cardiac Na channel *SCN5A*<sup>6,12</sup>, a component of I<sub>f</sub> current (hyperpolarization-activated cyclic nucleotide gated, or HCN4)<sup>10</sup>, and Cx40<sup>6</sup>. No mutation was found in HCN4, or in the coding region of Cx40 (exon 2). A T-to-C transition was found at the exon 6 of *SCN5A* leading to amino acid substitution of proline for leucine 212 (L212P, Fig 2A) located at the extracellular loop connecting the transmembrane segments 3 and 4 of domain 1 of the cardiac Na channel, Nav1.5 (Fig 2B). This sequence variation was not found in 400 normal chromosomes, consistent with a disease-related mutation. Genomic sequencing of family members showed that the father is a silent L212P mutation carrier (Fig 2C). No arrhythmia has been documented in the paternal grandfather who died of gastric cancer; however, his two brothers died suddenly during the first decade of life. It is possible that these two individuals were also the L212P carriers and developed lethal arrhythmias. A 12-lead ECG of the proband's father showed normal sinus rhythm with non-specific ST depression in the inferior leads (Fig 2D). Genetic screening of the entire coding region of *SCN5A* revealed no other mutations or polymorphisms except for a synonymous SNP (D1819D, heterozygous) in the proband and his father. ECGs of the proband's mother and maternal grandmother were normal.

The genotype of the Cx40 SNPs was determined using genomic DNA of healthy unrelated Japanese individuals. Two Cx40 SNPs in the promoter region and exon 1 showed complete linkage disequilibrium: all subjects with allele G at position -44 had allele A at +71,

and vice versa. The genotype frequencies were, common: -44GG/+71AA (62/99, 62.6%), heterozygous: -44GA/+71AG (32/99, 32.3%), and homozygous SNPs: -44AA/+71GG (5/99; 5.1%). The allelic frequencies of wild type and polymorphisms of Cx40 were 78.8% and 21.2%, respectively. These values were nearly identical to the ones previously reported<sup>7</sup>, suggesting minimal ethnic variability in the genetic frequencies of these SNPs. Heterozygous SNPs were found in the proband, his mother and maternal grandmother. The proband's father showed a common genotype (Fig 2C). Homozygous SNPs were not found in the family.

### **3. Functional characterization of the L212P mutant Na channel**

Fig 3A illustrates representative whole-cell current traces from cells expressing WT or L212P Na channels in the presence of co-expressed hβ1. L212P showed a robust current with a peak current density comparable to WT. The macroscopic current decay fit with a biexponential function showed that the time constants and the fractions of slow and fast components were comparable. The persistent Na current, a characteristic of most mutant Na channels responsible for LQT3<sup>13</sup>, was not evident in cells expressing L212P. The voltage-dependence of steady-state inactivation of L212P showed a significant hyperpolarizing shift by an average of -9 mV ( $V_{1/2}$ : WT= -86.6±0.9 mV, n=17; L212P: -95.6±0.8 mV, n=11; p<0.001) (Fig 3B). Similarly, the voltage-dependence of activation of L212P showed a large negative shift by an average of -15 mV ( $V_{1/2}$ : WT= -48.1±0.9 mV; n=19, L212P: -63.5±1.5 mV; n=11 p<0.001). The slope factor for inactivation was significantly larger in L212P than WT ( $k_{inact}$ : WT= 7.22±0.18 mV, L212P=6.62±0.16 mV, p<0.05) but similar for activation ( $k_{act}$ : WT= -5.8±0.2 mV, L212P= -5.2±0.4 mV; NS) (Fig 3B). Recovery from inactivation was assessed by a standard double-pulse protocol using a recovery potential of -120 mV. The time constants of fast ( $\tau_f$ ) or slow recovery components ( $\tau_s$ ) were significantly larger in L212P (n=9) than WT (n=11) ( $\tau_f$ : WT=10.8±2.2 ms;

$L212P=20.9\pm2.0$  ms,  $p<0.001$ ;  $\tau_s$ : WT= $159\pm31$  ms,  $L212P=253\pm18$  ms,  $p<0.01$ ), and the fraction of the slow component ( $A_s$ ) was significantly larger in L212P (WT:  $0.16\pm0.02$ ; L212P:  $0.58\pm0.03$ ;  $p<0.05$ ), indicating that the recovery from inactivation was significantly slower in L212P (Fig 3C).

## DISCUSSION

The cardiac Na channel  $\alpha$  subunit, encoded by *SCN5A*, is responsible for the generation and propagation of action potentials in the atrium, ventricle and specialized conduction system. Mutations in *SCN5A* have been identified in hereditary ventricular arrhythmias including LQT3<sup>2</sup>, Brugada syndrome<sup>3</sup>, and cardiac conduction system disease<sup>4</sup>. Biophysical studies of Na channel function have shown that mutations that evoke LQT3 cause excessive Na current (gain-of-function), delaying repolarization and causing early after depolarizations resulting in Torsades de Pointes<sup>13</sup>, whereas Brugada syndrome mutations reduce Na current (loss-of-function), causing premature epicardial repolarization and phase 2 reentry leading to ventricular tachycardia or fibrillation<sup>14</sup>. *SCN5A* mutations have also been identified in supraventricular arrhythmias such as congenital AV block<sup>15</sup>, sick sinus syndrome (SSS)<sup>12</sup>, and AS<sup>5,6</sup>, but the mechanisms are less well defined as compared with those of ventricular arrhythmias. The present study describes clinical, genetic and biophysical features of the novel *SCN5A* mutation, L212P, found in a family with congenital progressive AS.

The heterologously expressed L212P mutant channel exhibited profound gating abnormalities in both activation and inactivation. However, primary dysfunction of the sinoatrial (SA) node due to the *SCN5A* mutation is not the plausible pathogenesis of congenital AS, because the action potential in the SA node is largely calcium channel dependent and is not associated with tetrodotoxin (TTX)-sensitive Na current<sup>16</sup>, while *SCN5A*

encodes TTX-insensitive Na current. SA node dysfunction due to the failure of the impulses to propagate into the adjacent atrial myocardium, referred to as exit block, is the most likely mechanism for congenital AS. The large hyperpolarizing shift (15 mV) of activation in the L212P channel reduces the threshold potential for activation, leading to increased Na current, and the hyperpolarizing shift (9 mV) of steady-state inactivation and delayed recovery from inactivation reduces Na channel availability and slows cardiac impulse conduction.

Consequently, L212P evokes gating effects suggestive of both LQT3 and Brugada syndromes, although the L212P carriers do not exhibit the electrocardiographic features of these syndromes. Such opposing gating properties have been previously shown in an *SCN5A* mutation, G514C, responsible for isolated cardiac conduction disease<sup>17</sup>. The G514C channel exhibits a depolarizing shift in both activation and inactivation. It is assumed that the balance of the opposing gating abnormalities as demonstrated in G514C, under the influence of additional factors involved in impulse propagation, may elicit exit block in the SA node. This exit block may progress to total AS in the L212P carrier without evoking electrophysiological manifestations of Brugada syndrome or LQT3. Furthermore, biophysical abnormalities of L212P are more severe than those of D1275N which showed small hyperpolarizing shift (-3.6 mV) in activation only in the absence of co-expressed β1 subunit<sup>6</sup>. Functional severity of the Na channel mutation may be, at least in part, attributable to the fact that the symptomatic individuals in the Dutch family exclusively carry both D1275N and the homozygous Cx40 SNPs, whereas the proband of our case exhibited AS with L212P and heterozygous Cx40 SNPs.

Dissociation between genotype and phenotype is occasionally observed in inherited diseases; mutation carriers could exhibit a normal phenotype and remain asymptomatic. This phenomenon, referred to as incomplete penetrance, has been well characterized in congenital

LQTS<sup>18</sup>. The current study and recent reports clearly show that incomplete penetrance is commonly observed in all three AS pedigrees reported so far<sup>5,6</sup>, and it may account for the fact that familial AS is extremely rare and AS often appears to be sporadic. Furthermore, these observations suggest that *SCN5A* mutations alone are not sufficient to confer a severe AS phenotype; the clinical consequence of the mutations is determined in individual-specific or family branch-specific manners, rather than a mutation-specific manner, probably influenced by some environmental factors or genetic modifiers associated with atrial conduction. Several lines of evidence implicate the genetic variations of *SCN5A* or Cx40 accompanying the *SCN5A* mutations. Benson *et al.* reported six heterozygous compound *SCN5A* mutations in three congenital SSS families with recessive inheritance<sup>12</sup>. Affected individuals of one pedigree, who exhibit progressive atrial standstill, exclusively carried heterozygous compound *SCN5A* mutations of both G1408R and P1298L. Family members carrying only one of these alleles showed a normal ECG or only first-degree atrioventricular conduction block, despite the fact that G1408R results in a non-functional channel. These observations suggest that a single *SCN5A* mutant allele, even a non-functional allele, may not be sufficient to confer a severe clinical phenotype, and an additional *SCN5A* mutation on the other allele may have a significant impact on the electrophysiological manifestations.

Action potential in cardiac tissue is greatly affected by both membrane excitability due to Na channel availability and gap junction coupling. Cardiac Na channel Nav1.5 is localized in the intercalated disks between neighboring myocytes<sup>19</sup>, and interplay between these molecules has significant relevance under pathological conditions in which one pathological change may compensate for another<sup>20</sup>. Cx40 is a gap junction protein predominantly expressed in the atrium and specialized conduction system, and Cx40-deficient mice showed significantly prolonged P wave duration, PQ interval, and QRS duration<sup>21</sup>. Interplay between

the Na channel and Cx40 is also suggested in heterozygous *SCN5A* knockout mice. These mice show progressive cardiac conduction disturbances with age and myocardial fibrosis, and exhibit a significant decrease in Cx40 expression in the atria, together with P wave prolongation<sup>22</sup>. In the Dutch AS pedigree, affected individuals exclusively carry both the *SCN5A* mutation (D1275N) and homozygous SNPs in the regulatory region of Cx40 which reduces Cx40 expression *in vitro*<sup>6</sup>. Furthermore, these CX40 SNPs have been clinically associated with enhanced atrial vulnerability and increased risk of atrial fibrillation<sup>7</sup>. The prevalence of Cx40 SNPs in the general population is not rare and the Cx40 promoter activity of homozygous SNP is less than 50% of WT<sup>6</sup>, suggesting that the heterozygous Cx40 gives rise to intermediate promoter activity, and the clinical manifestation of Cx40 SNPs alone may not be apparent. Nevertheless, the likelihood of the chance occurrence of Cx40 SNPs and *SCN5A* mutation is rare, and it may explain the phenotypic difference between two L212P carriers in the AS family, and the extremely low prevalence of this arrhythmia. We believe that genetic defects in *SCN5A* most likely underlie AS, but additional genetic factors that modulate atrial electrical coupling are relevant to the clinical manifestation of this inherited arrhythmia. Further genetic and functional studies are required to confirm this mechanism.

#### ***Study limitation***

The size of the family in the present study was quite small and clinical or genetic information was available from only several members in this pedigree. Extensive efforts to gather more genomic and electrophysiological data in AS patients are required to determine the relevance of *SCN5A* and Cx40 polymorphisms in this rare inherited arrhythmia. Experiments using genetically engineered animals or mathematical models are required to elucidate the direct effects of Cx40 polymorphisms on Na channel function.

## ACKNOWLEDGMENTS

The authors are indebted to Dr. Alfred L. George Jr. for critical reading of the manuscript and providing comments. The authors thank K. Morisaki, Y. Matsuo, and H. Ito for technical assistance. This study was supported by research grants (NM) 15090711 from the Ministry of Education, Culture, Sports, Science and Technology, Japan, and from the Japan Research Foundation for Clinical Pharmacology and the Takeda Science Foundation, and a Research Grant from Netherlands Heart Foundation M96.001 (WAG).

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## FIGURE LEGENDS

**Fig 1. Electrocardiographic findings of the proband**

- A. Twelve-lead ECG of the proband. Tracings of leads I-III and V1 are shown. Junctional rhythm (HR 40) with a ventricular escape (second QRS). Note the absence of P waves.
- B. Holter ECG recording after terminating cilostazol. AS with a maximum RR interval of 5.42 sec was documented during sleep.
- C. Tracings of intracardiac recordings. Junctional rhythm and ventricular escape beats (second and fourth QRS). HRA, representing high right atrium, shows far-field V signals but not atrial activities. Low frequency deflections in the His bundle electrogram (HIS) preceding the His deflection may represent the residual atrial activities although there are no areas in the right atrium that was captured that by pacing with maximum output (D). The His-ventricular interval was slightly prolonged (56 ms). RVA represents right ventricular apex.
- D. Atrial pacing with the maximum output of the stimulator (9.9 V, 1.0 ms) failed to capture the atria. STIM represents atrial pacing artifacts.

**Fig 2. Results of DNA sequencing, location of the mutation, and the pedigree**

- A. Sequence electropherogram for exon 6 of *SCN5A* of the proband. The arrow indicates heterozygous missense mutation of proline (CCG) for leucine-212 (CTG).
- B. Predicted topology of the cardiac Na channel Nav1.5 is illustrated with the location of three mutations associated with AS: L212P (this report), R367H<sup>5</sup>, and D1275N<sup>6</sup>. Transmembrane segments S1-S6 in each domain are shown with numbers and boxes.
- C. Pedigree of the AS family. Open symbols indicate unaffected or undetermined

individuals. Closed and shaded symbols indicate genetically affected individuals with or without symptoms, respectively. The genotypes below each person's symbol are, from top to bottom, as follows: *SCN5A*-L212P status (WT and MT indicate wild type and heterozygous L212P, respectively), Cx40 -44 bp SNP (common GG, and heterozygous GA), and Cx40 +71 bp SNP (common AA and heterozygous AG). The proband has inherited the L212P allele from his father and the Cx40 SNPs allele from his mother. SCD represents sudden cardiac death.

- D. Twelve-lead ECG of the proband's father (asymptomatic mutation carrier) shows normal sinus rhythm. None-specific ST depression was evident in the inferior leads.

### **Fig 3. Biophysical properties of L212P mutant Na channel**

- A. Representative whole-cell current traces of WT and L212P Na channels expressed in tsA-201 cells in the presence of co-expressed human  $\beta 1$  subunit. Currents were elicited by 20 ms test pulses (from -90 to +80 mV, 10 mV step) from the holding potential of -120 mV. Currents of the representative traces were normalized with the peak currents to illustrate the time course of current decay of each channel. Scale bars indicate 1 nA and 5 ms.
- B. Steady-state availability for inactivation and conductance-voltage relationship was measured with standard pulse protocols as shown in the inset. Normalized peak current and conductance were fit with the Boltzmann equation (see Methods). Both inactivation and activation curves were significantly shifted in the negative direction in L212P (n=17) compared to WT (n=11).
- C. Recovery from inactivation was measured by the standard double pulse protocol as depicted in the inset. Fractional recovery was fit with the double exponential equation (see Methods). Recovery was significantly slower in L212P (n=9) than WT

(n=11).

## REFERENCES

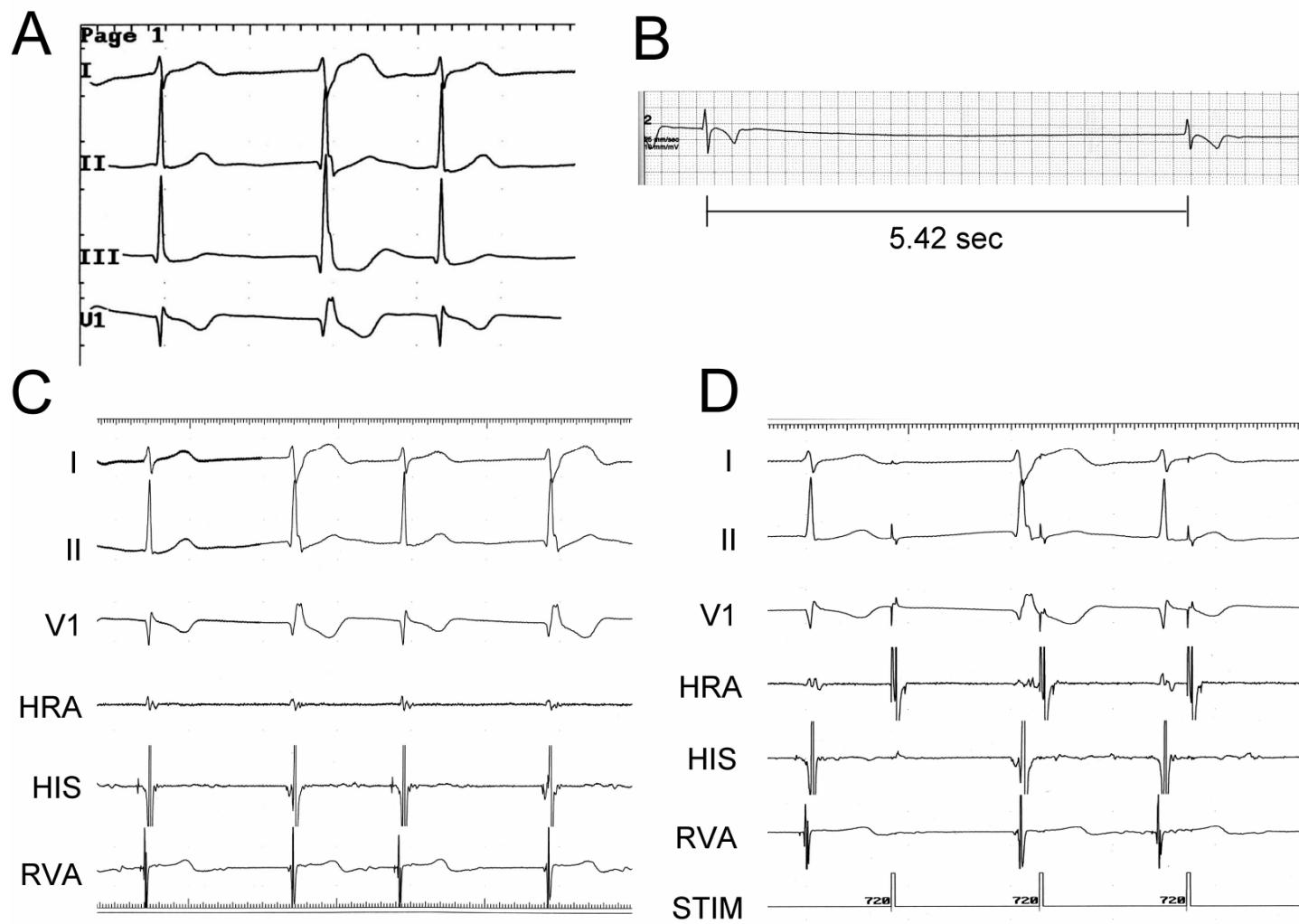
1. Talwar KK, Dev V, Chopra P, Dave TH, Radhakrishnan S. Persistent atrial standstill--clinical, electrophysiological, and morphological study. *Pacing Clin Electrophysiol* 1991;14:1274-1280.
2. Wang Q, Shen J, Splawski I, Atkinson D, Li Z, Robinson JL, Moss AJ, Towbin JA, Keating MT. SCN5A mutations associated with an inherited cardiac arrhythmia, long QT syndrome. *Cell* 1995;80:805-811.
3. Chen Q, Kirsch GE, Zhang D, Brugada R, Brugada J, Brugada P, Potenza D, Moya A, Borggrefe M, Breithardt G, Ortiz-Lopez R, Wang Z, Antzelevitch C, O'Brien RE, Schulze-Bahr E, Keating MT, Towbin JA, Wang Q. Genetic basis and molecular mechanism for idiopathic ventricular fibrillation. *Nature* 1998;392:293-296.
4. Schott JJ, Alshinawi C, Kyndt F, Probst V, Hoornje TM, Hulsbeek M, Wilde AA, Escande D, Mannens MM, Le Marec H. Cardiac conduction defects associate with mutations in SCN5A. *Nat Genet* 1999;23:20-21.
5. Takehara N, Makita N, Kawabe J, Sato N, Kawamura Y, Kitabatake A, Kikuchi K. A cardiac sodium channel mutation identified in Brugada syndrome associated with atrial standstill. *J Intern Med* 2004;255:137-142.
6. Groenewegen WA, Firouzi M, Bezzina CR, Vliex S, van Langen IM, Sandkuijl L, Smits JP, Hulsbeek M, Rook MB, Jongsma HJ, Wilde AA. A cardiac sodium channel mutation cosegregates with a rare connexin40 genotype in familial atrial standstill. *Circ Res* 2003;92:14-22.
7. Firouzi M, Ramanna H, Kok B, Jongsma HJ, Koeleman BPC, Doevedans PA, Groenewegen WA, Hauer RNW. Association of human connexin40 gene polymorphisms with atrial vulnerability as a risk factor for idiopathic atrial fibrillation.

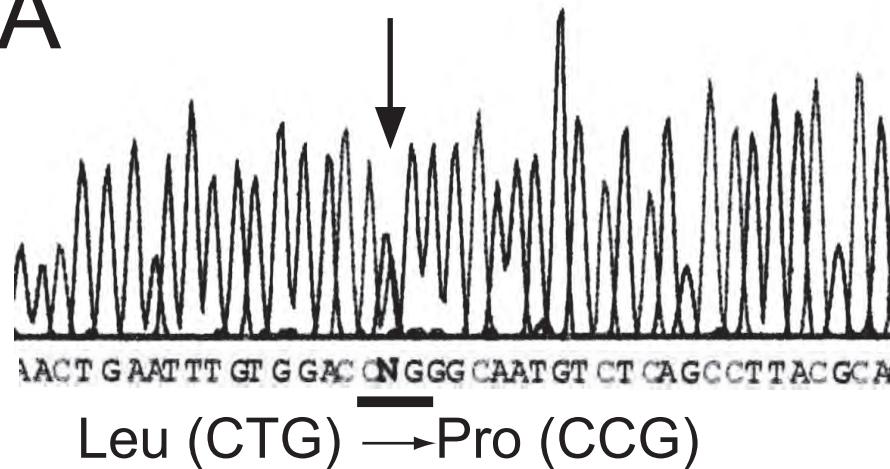
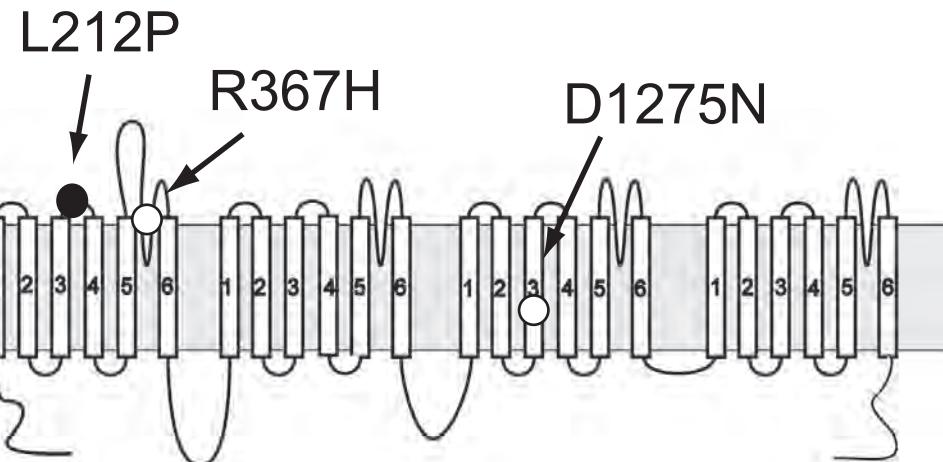
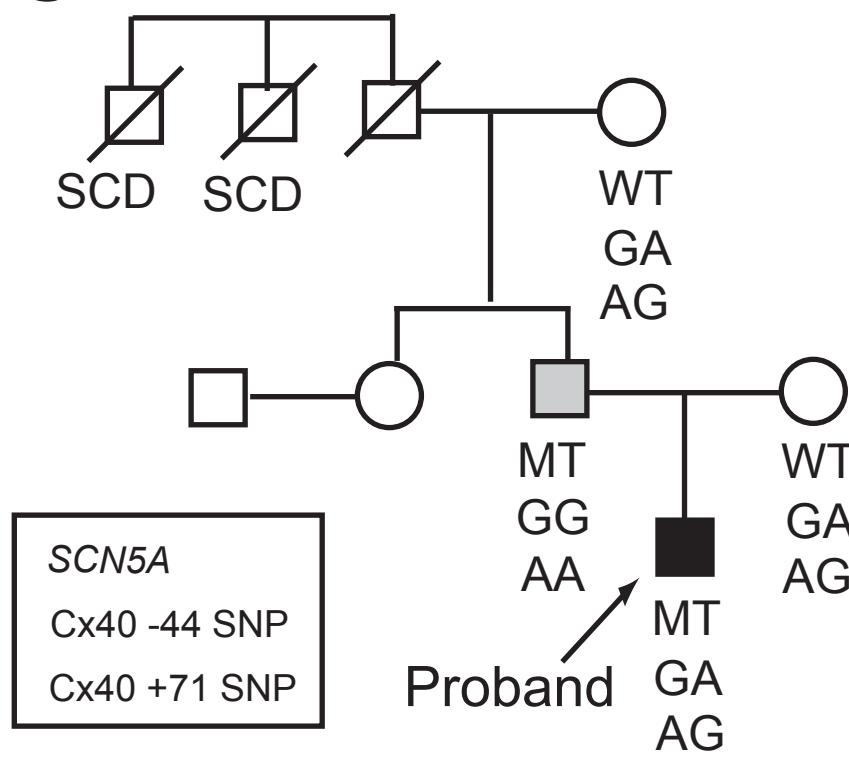
Circ Res 2004;95:e29-33.

8. Atarashi H, Endoh Y, Saitoh H, Kishida H, Hayakawa H. Chronotropic effects of cilostazol, a new antithrombotic agent, in patients with bradyarrhythmias. *J Cardiovasc Pharmacol* 1998;31:534-539.
9. Wang Q, Li Z, Shen J, Keating MT. Genomic organization of the human SCN5A gene encoding the cardiac sodium channel. *Genomics* 1996;34:9-16.
10. Schulze-Bahr E, Neu A, Friederich P, Kaupp UB, Breithardt G, Pongs O, Isbrandt D. Pacemaker channel dysfunction in a patient with sinus node disease. *J Clin Invest* 2003;111:1537-1545.
11. Makita N, Horie M, Nakamura T, Ai T, Sasaki K, Yokoi H, Sakurai M, Sakuma I, Otani H, Sawa H, Kitabatake A. Drug-induced long-QT syndrome associated with a subclinical SCN5A mutation. *Circulation* 2002;106:1269-1274.
12. Benson DW, Wang DW, Dyment M, Knilans TK, Fish FA, Strieper MJ, Rhodes TH, George AL, Jr. Congenital sick sinus syndrome caused by recessive mutations in the cardiac sodium channel gene (SCN5A). *J Clin Invest* 2003;112:1019-1028.
13. Bennett PB, Yazawa K, Makita N, George AL, Jr. Molecular mechanism for an inherited cardiac arrhythmia. *Nature* 1995;376:683-685.
14. Antzelevitch C. The Brugada syndrome: diagnostic criteria and cellular mechanisms. *Eur Heart J* 2001;22:356-363.
15. Wang DW, Viswanathan PC, Balser JR, George AL, Jr., Benson DW. Clinical, genetic, and biophysical characterization of SCN5A mutations associated with atrioventricular conduction block. *Circulation* 2002;105:341-346.
16. Baruscotti M, DiFrancesco D, Robinson RB. A TTX-sensitive inward sodium current contributes to spontaneous activity in newborn rabbit sino-atrial node cells. *J Physiol* 1996;492:21-30.

17. Tan HL, Bink-Boelkens MT, Bezzina CR, Viswanathan PC, Beaufort-Krol GC, van Tintelen PJ, van den Berg MP, Wilde AA, Balser JR. A sodium-channel mutation causes isolated cardiac conduction disease. *Nature* 2001;409:1043-1047.
18. Priori SG, Napolitano C, Schwartz PJ. Low penetrance in the long-QT syndrome: clinical impact. *Circulation* 1999;99:529-533.
19. Kucera JP, Rohr S, Rudy Y. Localization of sodium channels in intercalated disks modulates cardiac conduction. *Circ Res* 2002;91:1176-1182.
20. Shaw RM, Rudy Y. Ionic mechanisms of propagation in cardiac tissue : Roles of the sodium and L-type calcium currents during reduced excitability and decreased gap junction coupling. *Circ Res* 1997;81:727-741.
21. Kirchhoff S, Nelles E, Hagendorff A, Kruger O, Traub O, Willecke K. Reduced cardiac conduction velocity and predisposition to arrhythmias in connexin40-deficient mice. *Curr Biol* 1998;8:299-302.
22. Royer A, van Veen TAB, Le Bouter S, Marionneau C, Griol-Charhbili V, Leoni A-L, Steenman M, van Rijen HVM, Demolombe S, Goddard CA, Richer C, Escoubet B, Jarry-Guichard T, Colledge WH, Gros D, de Bakker JMT, Grace AA, Escande D, Charpentier F. Mouse model of SCN5A-linked hereditary Lenegre's disease. Age-related conduction slowing and myocardial fibrosis. *Circulation* 2005;111:1738-1746.

Fig 1, Makita *et al.*



**A****B****C****D**