Haploinsufficiency in Combination With Aging Causes SCN5A-Linked Hereditary Lenègre Disease

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OBJECTIVES The goal of this study was to investigate the genotype-to-phenotype relationship between SCN5A gene mutation and progressive cardiac conduction defect in order to gain insights into the pathophysiological mechanisms of the disease.

BACKGROUND Progressive cardiac conduction defect is a frequent disease commonly attributed to degeneration and fibrosis of the His bundle and its branches. In a French family, we have identified a splicing mutation in the SCN5A gene leading to hereditary progressive cardiac conduction defect.

METHODS We have extended the size of the pedigree and phenotyped and genotyped all family members, and also investigated in vitro the functional consequences of the mutation.

RESULTS Among 65 potentially affected members, 25 individuals were carriers of the IVS.22+2 T→C SCN5A mutation. In relation to aging, gene carriers exhibit various types of conduction defects. P-wave, PR, and QRS duration increased progressively with age in gene carriers and in noncarriers. Whatever the age, conduction parameters were longer in gene carriers. The widening in the QRS complex with aging was more pronounced in gene carriers older than 40 years. Functional studies showed that the IVS.22+2 T→C SCN5A mutation lead to exon 22 skipping and to a complete loss of function of the affected allele, but to a normal trafficking of the mutated gene product.

CONCLUSIONS Our findings demonstrate that hereditary Lenègre disease is caused by a haploinsufficiency mechanism, which in combination with aging leads to progressive alteration in conduction velocity. (J Am Coll Cardiol 2003;41:643–52) © 2003 by the American College of Cardiology Foundation

Progressive cardiac conduction defect is characterized by an age-related alteration in the conduction of the cardiac impulse through the His-Purkinje system. Progressive cardiac conduction defect, also called Lenègre or Lev’s disease, is a frequent affection that remains a major cause of pacemaker implantation (1). It is commonly thought that the disease issues from a slow process developing over decades, affecting the His bundle and its branches (2). Lenègre and Lev hypothesized that it was a primary degenerative disease or an exaggerated aging process of unknown origin with sclerosis selectively affecting the conducting tissue (2,3). As early as the 1970s, familial investigation of patients with chronic bifascicular block (4) or complete atrioventricular (AV) block (5) suggested that genetic factors could be involved in the pathophysiology of the disease. Hereditary cases of bundle branch block have often been reported (6–10). In 1995, a first locus was mapped to 19q13.2-13.3 (11). More recently, we reported a large French family in which a cardiac conduction defect was transmitted as an autosomal dominant trait and was caused by a splicing mutation (IVS.22+2 T→C) in the SCN5A gene coding for the cardiac-specific sodium channel (12). SCN5A is the first identified gene responsible for hereditary Lenègre disease.

We have now extended the size of the pedigree and genotyped and phenotyped all family members. In addition, we have investigated in vitro the functional consequences of the mutation with the objective to gain better insight into the pathophysiology of the disease. Our findings demonstrate that mutation in the SCN5A gene induces a progressive alteration in the conduction of the cardiac impulse in the atria as well as the ventricle. The cardiac conduction defect already present in infancy worsened progressively with age, leading to the typical aspect of the idiopathic progressive conduction block as originally described by Lenègre and by Lev. We also show that hereditary Lenègre disease is caused by a haploinsufficiency mechanism, which in combination with aging leads to progressive alteration of the conduction velocity.

METHODS

Clinical investigation. The study was conducted according to the French guidelines for genetic research and was approved by the ethical committee of Nantes University Hospital. Informed written consent was obtained for each family member who agreed to participate in the study. In this family, members of generation I were 10 brothers and sisters of Patient I-11 (Fig. 1). Overall, we identified a large
family of more than 200 members. Sixty-five potentially affected members who agreed to participate in the study were selected. All 65 members were genotyped by direct sequencing of exon 22 (ABI 377 automated sequencer). Subjects were considered as gene carriers if they were carrying the IVS.22+2 T→C splicing mutation in the SCN5A gene. Clinical investigation included a review of medical history, a physical examination, and a 12-lead electrocardiogram (ECG). Heart rate, PR interval, QRS, QT, QTc duration, and P and QRS axis were measured automatically at rest (Mac Vu Marquette Inc., Milwaukee, Wisconsin). QRS axis was classified as normal when its value was between −30° and +90°. Out of this range it was classified as abnormal. Conductive defects were defined using the conventional classification (13,14). Parietal block was defined as a QRS wider than 115 ms without morphology of left bundle branch block (LBBB) or right bundle branch block (RBBB). PR duration shorter than 210 ms was considered normal. A limitation of standard ECG remains the assessment of the P-wave and QRS duration. To increase the accuracy of these measurements, we performed signal-averaging electrocardiogram for P and QRS complexes using commercially available software (Mac Vu Marquette Inc., Milwaukee, Wisconsin). Measures were performed using 40 to 250 Hz filters. 

**Exon trapping experiments.** A 6 Kb SCN5A genomic fragment containing exon 21 to exon 23 was polymerase chain reaction (PCR) amplified (Expand 20 Kb PCR System; Roche Molecular Diagnostic) using deoxyribonucleic acid from a gene carrier as template. Primers were designed to flank exon 21 and 23 and to introduce a Not I restriction site (Int-20-F: ATAGAATCGCGCCGCTTCAATGTCACCTGTC; Int-23-R: ATAGAATGCAGGCTATTGGGAGGAAGGTC). Polymerase chain reaction products were not digested and subcloned into a pSPL3 exon-trapping vector (Exon Trapping System; Life Technologies). Direct sequencing of recombinant clones was used to identify wild-type (WT) and mutant genomic fragments. Mutated and WT pSPL3-exon 21-23 plasmids were transfected into COS-7 cells, using polyethylenimine (PEI 22kDa). Forty-eight hours post-transfection, cells were harvested and total ribonucleic acid was extracted. Total ribonucleic acid was retrotranscribed with SA2 primer according to manufacturer specifications and PCR amplified using exonic primers (Ex-21-F: GCCTGACTTCCCTCATCGTA; Ex-23-R: CTTGCCCTGTTGTGTCACAGA). Amplification products were run on a 1.7% TBE agarose gel and formally characterized by direct sequencing.

**Functional studies.** Our method to record inward currents in COS-7 SCN5A-transfected cells has previously appeared (15). Briefly, using PEI 22kDa, COS-7 cells were transfected with either WT SCN5A plasmid or a mutated plasmid lacking exon 22 (Δexon 22-SCN5A). Twenty-four hours post-transfection, currents were recorded at room temperature using the whole-cell configuration of the patch-clamp technique. Current measurements were normalized using cell capacitance. The pipette medium contained (mmol/l): NaCl 10, CsCl 64.5, aspartic acid 70.5, HEPES 5, and pH 7.2 with CsOH; the extracellular medium used to record Na+ currents contained (mmol/l): NaCl 145, CsCl 4, CaCl2 1, MgCl2 1, HEPES 5, glucose 5, and pH 7.4 with NaOH. For imaging, cells transfected with GFP-tagged channel plasmids (either WT or exon 22 deleted) were fixed for 15 min with 4% (v/v) paraformaldehyde in phosphate-buffered saline (in mmol/l: Na2HPO4 9.1, NaH2PO4 1.7, NaCl 150, pH adjusted to 7.4 with NaOH). Cells were rinsed twice and the coverslips were mounted in Mowiol. The cells were observed with an epifluorescence microscope using interference blue (fluorescein isothiocyanate) filter and an oil-immersion 100× lens. They were also imaged with a scanning laser confocal microscope using 488 nm illumination and an oil-immersion 100× lens.

**Statistical methods.** Clinical data are expressed as mean value ± SD. The duration of PR, QRS, and P-wave were compared with Student t test. A p value of <0.05 was considered to be significant. Simple and piecewise linear regression analyses were performed for the effect of aging on conduction parameters (16). Model thresholds were tested by the mean of F ratio test. Patch-clamp data are expressed as mean ± SEM. Statistical significance of the observed effects was assessed by means of the t test or two-way analysis of variance when needed.

**RESULTS**

**Clinical phenotype.** Within the 65 living members included in the study, 25 were carrying the splice mutation whereas 40 were noncarriers (Fig. 1). Eight patients had complete RBBB associated with (Patients: III-29, IV-1, IV-16, IV-18) or without (Patients: III-27, III-31, III-32, IV-19) a left posterior hemibloc (LPHB). One had incomplete RBBB (Patient III-20). Two had complete LBBB (II-20, III-28). One had an isolated left anterior hemibloc (LAHB) (Patient III-23). One patient had isolated LPHB (II-2). Eight patients had parietal blocks in the absence (Patients: III-1, III-2, III-7, III-25, IV-20) or presence (Patients: II-3, II-15, II-19) of a left-axis deviation. Ten patients had a first-degree AV block (Patients: II-3, II-19,
II-20, III-2, III-23, III-25, III-27, III-28, III-29, III-32). Four patients (an 11-year-old boy [Patient IV-2] and three women of 36 [Patient III-6], 47 [Patient III-8], and 50 years [Patient III-24]) had an ECG within the normal limits. Examples of abnormal ECG patterns are shown in Figure 2. No member of this family showed ST-segment elevation in the right precordial leads.

Among the affected patients, seven had symptoms in relation with their conduction defect. Syncope occurring at rest was the most common symptom, leading to pacemaker implantation in six patients (Patients: II-3, II-15, II-19, II-20, III-25, III-31). In Patient III-25, for example, an electrophysiologic test was conducted because of syncope and parietal block (QRS: 164 ms). The HV interval was 55 ms at baseline and increased to 120 ms after ajmalin injection (1 mg/kg as an intravenous bolus). In this patient, ajmalin produced no ST-segment elevation in the right precordial leads. When she was admitted for pacemaker implantation, she had a cardiac arrest attributable to complete AV block, necessitating cardiopulmonary resuscitation. All patients equipped with a pacemaker remained free of symptoms.

Mean age of affected and unaffected family members was 48 ± 20 years and 40 ± 18 years, respectively (nonsignificant, NS). On average, their systolic blood pressure was 121 ± 14 mm Hg for the affected and 125 ± 12 mm Hg for the unaffected (NS), whereas the diastolic pressure was 77 ± 9 mm Hg and 73 ± 9 mm Hg, respectively (NS). An echocardiography was performed in every family member older than 40 years. No structural anomalies were evidenced. The ECG parameters of family members are shown in Figure 3. On average, the heart rate was slower among gene carriers (68 ± 10 beats/min) in comparison with noncarriers (75 ± 11 beats/min; p = 0.03). The PR interval was longer in affected members than in the unaffected subjects (206 ± 33 ms vs. 149 ± 22 ms, p < 0.00001). QRS duration was longer in the affected group than in the unaffected group (130 ± 28 ms vs. 95 ± 13 ms, p < 0.00001). The QTc duration was similar in both groups (423 ± 29 ms vs. 415 ± 19 ms). The duration of the averaged and filtered P-wave was longer in the affected than in the unaffected group (143 ± 13 ms vs. 119 ± 13 ms; p < 0.0001). Finally, the averaged and filtered QRS duration was also longer in affected family members (140 ± 20 ms vs. 116 ± 10 ms, p < 0.0001). In the gene-carrier population, there were no differences in the conduction parameters that could be related to gender. Conduction parameters were also compared between noncarriers and an age- and gender-matched population of control patients (n = 122). No significant differences were found, thus suggesting that noncarriers did not carry a latent conduction defect.

Progressive development of the conduction defect. A major feature of the phenotype was the progressive development of cardiac conduction defect in relation to age. Two sets of data showed progressive alteration in the conduction.

Figure 1. Pedigree of the four-generation family with progressive cardiac conduction defect. Filled symbols = patients carrying the mutation or obligate carriers; open symbols = noncarriers. PM = patients with a pacemaker.
In five patients, serial ECGs were obtained over an 11- to 16-year period before the present study (Table 1). In five patients, the QRS duration consistently increased with aging. In four patients, the PR duration behaved similarly. A typical example of the evolution of ECG patterns is shown in Figure 4.

The age of the patients who participated to the study ranged from 15 to 81 years. We plotted conduction parameters in relation to age (Fig. 5). Whatever the age, averaged and filtered P-wave, PR, and QRS duration were longer in affected patients than in unaffected. There was a shift in the regression line for P-wave and PR duration toward higher values in affected patients, whereas the slopes, expressed in ms per year, were comparable in the two groups. The increase in averaged and filtered P-wave and PR duration were 0.37 ms/year versus 0.47 ms/year and 0.88 ms/year versus 0.60 ms/year for unaffected and affected members, respectively. In contrast, the QRS duration evolved differently in relation to age between the two groups. In general, there was a more pronounced QRS lengthening with age in affected than in unaffected patients. In addition, an age-dependent variability in the QRS duration was evidenced in affected patients.

![Figure 2. Examples of electrocardiogram patterns in affected members. Patient II-19 was a 70-year-old woman with parietal block and left-axis deviation (heart rate [HR]: 64 beats/min, P: 144 ms, PR: 215 ms, QRS: 160 ms). A pacemaker was implanted because of several episodes of syncope. Patient III-2 was a 49-year-old man with a parietal block and undetermined axis (HR: 54 beats/min, P: 150 ms, PR: 244 ms, QRS 128 ms). Patient III-31 was a 48-year-old woman with right bundle branch block (HR: 64 beats/min, P: 153 ms, PR: 205 ms, QRS: 172 ms). Sudden widening of QRS complexes and occurrence of 2:1 atrioventricular block during exercise led to pacemaker implantation. Patient II-20 was a 78-year-old woman with left bundle branch block (HR: 59 beats/min, P: 157 ms, PR: 248 ms, QRS: 196 ms). A pacemaker was implanted after two episodes of syncope.](Image)
the affected group. The variance was significantly different before and after the age of 40 (ratio variance test; \( p < 0.001 \)), indicating a threshold effect of age. Therefore, a piecewise linear regression was conducted in the affected group. This variability was not found in the unaffected group, demonstrating a differential evolution of the QRS duration over aging.

When the subgroup of patients younger than 40 years was considered (7 affected and 19 nonaffected), conduction parameters were also prolonged in the affected group (P-wave duration 132 ± 12 ms vs. 115 ± 10 ms, \( p = 0.002 \); PR 179 ± 11 ms vs. 140 ± 20 ms, \( p < 0.00001 \); and QRS duration 116 ± 8 ms vs. 92 ± 14 ms, \( p = 0.0001 \)). These data demonstrate that a conduction anomaly was already present early in life in the absence of specific conduction defects, which were never observed before 40 years.

**Functional consequences of the IVS.22+2 T→C splice mutation.** Given that SCN5A expression is cardiac specific (17) and no cardiac tissue was available from affected individuals, the abnormal transcripts resulting from the +2 donor splice mutation were characterized in vitro. Mutations in a consensus splice site should result in abnormal splicing. The abnormal transcript is predicted to an in-frame skipping of exon 22 and an impaired gene product missing the voltage-sensitive DIIIIS4 segment. To further support this assumption, we conducted exon-trapping experiments of the mutated gene. As illustrated in Figure 6, transcripts obtained with the mutated construct showed exon 22-deletion, as demonstrated by direct sequencing.

Wild-type and exon 22-deleted SCN5A cardiac sodium channels were then expressed in COS-7 cells. Illustrative current traces are shown in Figure 7. In cells transfected with the WT SCN5A plasmid, the peak inward current density was \(-59.3 \pm 10.5 \text{ pA/pF} \) (test potential at \(-20 \text{ mV}; n = 8 \)). The Na\(^+\) channel beta-1 subunit has been shown to increase SCN5A expression in Xenopus oocytes (18). In COS-7 cells expressing WT SCN5A plus beta-1 subunit, an increase in peak-current density was also observed \((-112.9 \pm 24.2 \text{ pA/pF}; n = 12 \) ). In contrast, in 10 experimental cells transfected with the Δexon 22-SCN5A mutant alone and in nine additional cells co-transfected with Δexon 22-SCN5A plus beta-1 subunit, no transient inward current was recorded in response to depolarization. In order to investigate the consequences of mutation on protein maturation, we expressed WT and Δexon22-SCN5A-GFP fusion proteins in mammalian cells. Figure 7, E, shows pictures of representative transfected cells observed with either conventional epifluorescence or laser confocal microscopy. These data suggest that the mutated Δexon22 SCN5A protein was correctly processed to the cell membrane.

**Table 1.** Serial Electrocardiogram Parameters in Five Affected Family Members

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<th>Patient #</th>
<th>Year</th>
<th>PR (ms)</th>
<th>QRS (ms)</th>
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<th>PR (ms)</th>
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<tr>
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<td>160</td>
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<tr>
<td>II-3</td>
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<td>200</td>
<td>160</td>
<td>1998</td>
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<td>170</td>
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Mean 212 146 225 172.4
DISCUSSION

The present study shows that the pathophysiology of the hereditary Lenègre disease related to *SCN5A* combines haploinsufficiency of the cardiac Na⁺ channel gene and an additional mechanism of unknown nature altering cardiac conduction in relation to aging. The present data also suggest that this latter mechanism may be a physiologic process, because progressive lengthening of conduction times also occurs (although less markedly) in noncarriers from the same pedigree. In idiopathic (acquired) Lenègre disease, chronic cardiac conduction defect progressively develops over decades, leading to complete AV block. In the pedigree reported here, the progressive development of the

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**Patient III-31**

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<tr>
<th>Year</th>
<th>DI</th>
<th>DII</th>
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**Figure 4.** Serial electrocardiograms performed in Patient III-31 showing progressive development of conduction defect. Electrocardiograms recorded in 1982, 1998, and 2000 show progressive increase in QRS duration (QRS: 130, 140, and 172 ms).
conduction defects was comparable to that observed in idiopathic Lenègre disease. Several investigators (1,2) have dismissed the ischemic hypothesis, and acquired Lenègre disease is regarded as a degenerative process in relation to age. On the basis of histologic studies, Lenègre and Lev hypothesized that progressive AV block was caused by progressive fibrosis of the His bundle and its branches (2,3). However, fibrosis of the conduction network is also observed in the elderly in the absence of conduction defect (19). Thus, we postulate that hereditary Lenègre disease results from a genetic defect of the Na\(^+\)/H\(^+\) channel that exacerbates the natural age-related progressive conduction slowing caused by fibrosis or an alternative process.

In the present family, young affected patients had an ECG within normal limits. Using selective criteria, that is, the presence of specific conduction defects, complete penetrance is late during aging. However, even in young patients, P, PR, and QRS duration were longer compared with the unaffected group. More accurate criteria for conduction parameters in relation to age are needed to identifying affected patients in their younger age. A 50% reduction in the number of functional sodium channels should result in a consistent reduction in the speed of propagation of the cardiac impulse and hence in a prolongation of the conduction parameters. Why was conduction so slightly affected in young patients? There are several possibilities to consider. It may be that the young human heart does not need all the Na\(^+\)/H\(^+\) channels for impulse propagation, and that 50% of the channels still provide enough charge carriers for (almost) normal conduction. It would be interesting to know whether these patients have only 50% of INa and whether they have a reduced upstroke velocity of their action potential, especially under conditions of impedance loading. A compensatory mechanism could very well explain the phenotype. For example, it may be interesting to know whether coupling is normal or whether the decrease in INa is compensated with a change in coupling. Some of these important questions could be addressed in an animal model with targeted disruption of the SCN5A gene. Such a model has been established, most recently in the mouse (20). Myocytes from adult heterozygous SCN5A/+ mice show a 50% reduction in their inward Na\(^+\) current amplitude. In these mice, the heart rate was slowed by about 9% (10% in our patients; Fig. 3), the PR interval increased by 35% (38% in our patients), the P-wave duration increased by 27% (vs. 20% in our patients) and the QT duration was unchanged (as in our patients). Although the electrophysiology characteristics of the mouse heart markedly differ from those of the human heart (21), the phenotype of the SCN5A/+ mouse is in agreement with our conclusion that the hereditary Lenègre disease is caused by an haploinsufficiency mechanism. It will be of major interest to determine whether the hearts from SCN5A/+ mice undergo age-related changes in their conduction parameters.

Figure 5. Evolution with aging of conduction parameters in affected (filled symbols) and unaffected subjects (open symbols). (A) Averaged and filtered P-wave duration. (B) PR duration. (C) QRS duration. In A and B, data were fitted with a linear regression analysis. In C, assessment of the residuals showed that the linear model was poorly adapted to fit the relation between QRS duration and aging in affected members. The variance was significantly different before and after the age of 40 (ratio variance test; p < 0.001). This was indicative of a threshold effect of age. Two linear regression analyses were thus performed before and after the age of 40.

Mutations in the SCN5A gene have been associated with LAHB, and LPHB) were present in the family as in the classical description of the idiopathic Lev-Lenègre disease.
different clinical entities, including long QT syndrome 3 (22), Brugada syndrome (23), and hereditary Lenègre disease (12). Brugada syndrome, a syndrome with unique ECG anomalies (RBBB and ST-segment elevation in the precordial leads) and a risk of ventricular fibrillation, can also be caused by a 50% reduction in SCN5A functional expression (24). We have shown that conduction anomalies are also typical of Brugada syndrome related to SCN5A but not of Brugada syndrome related to other genes (25). Comparison of conduction parameters between the 25 carriers of the Δexon 22-SCN5A mutant and a group of 29 SCN5A-related Brugada patients from our own series (13 families) showed no obvious difference, except for the QRS complex, which may be slightly wider in the Δexon 22-SCN5A population (130 ± 28 ms vs. 114 ± 24 ms; p < 0.03). Thus, reduction in the SCN5A-related Na\(^+\) current causes conduction anomalies in the Brugada syndrome as in hereditary Lenègre disease. However, none of the 25 gene carriers from the present pedigree presented abnormal ST-segment elevation. Then, why does haploinsufficiency lead to Brugada syndrome in some families and to hereditary Lenègre disease in others? We have most recently reported a large family in which the same G1406R SCN5A missense mutation causes either Brugada syndrome or hereditary Lenègre disease, depending on the family member (16). Similar to the splice mutation reported here, the G1406R mutation located between the DIII-S5 and DIII-S6 domain of the Na\(^+\) channel showed no detectable Na\(^+\) current in expression studies in spite of normal protein trafficking. This observation suggests that the specific phenotype depends not only on the SCN5A mutation but also on the balance between the other ion channels, and that so-called modifier genes could interfere with the phenotype. Most interestingly, a second Brugada syndrome locus (3p22-25) (26) has been recently identified very close to the 3p21 locus containing SCN5A. Although the identity of the gene at 3p22-25 is not yet known, it certainly interferes with the electrophysiologic function of the heart. It is thus possible that minor differences (polymorphisms) in that gene could modulate the consequence of a SCN5A mutation (no recombination event occurred between locus 3p22-25 and 3p21 in any patient carrying the IVS.22 +2 T→C mutation). Further confirmation of that hypothesis will await identification of the gene contained within 3p22-25.

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Figure 7. Functional experiments of the mutated gene product. Whole-cell patch-clamp recordings of wild-type SCN5A (A and B) and Δexon 22–SCN5A (C and D) in the absence (A and C) or presence (B and D) of the beta-1 regulatory subunit. Holding potential: −100 mV. Depolarizing steps from −60 mV to +30 mV in 10 mV increments. Vertical bar = 0.5 nA. Horizontal bar = 5 ms. (E) Membrane trafficking of GFP-tagged wild-type (WT) or exon 22 deleted (Δexon 22) proteins. Top panels = conventional microscopy; lower panels = confocal microscopy imaging. Scaling is 10 microns.

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