Common and Rare Variants in SCN10A Modulate the Risk of Atrial Fibrillation

Running title: Jabbari et al.; Atrial fibrillation and SCN10A

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Abstract:

Background – Genome-wide association studies (GWAS) have shown that the common single nucleotide polymorphism (SNP) rs6800541 located in SCN10A, encoding the voltage-gated Na,1.8 sodium channel, is associated with PR-interval prolongation and atrial fibrillation (AF). SNP rs6800541 is in high linkage disequilibrium with the non-synonymous variant in SCN10A, rs6795970 (V1073A, r²=0.933). We aim to determine whether common and rare SCN10A variants are associated with early onset lone AF.

Methods and Results – The SCN10A gene was sequenced in 225 lone AF patients. In an association study of the common variant V1073A, we included 515 AF patients, and two control cohorts of 730 individuals free of AF and 6,161 individuals randomly sampled. Functional characterization of two common and two rare variants was performed by whole-cell patch-clamping. In the lone AF cohort, nine rare missense variants and one splice site donor variant were detected. Interestingly, AF patients were found to have lower minor allele frequency of the V1073A variant than controls (odds ratio = 0.74 [0.65-0.86]; p=2.3x10⁻⁴). Functional characterization revealed that both of the common variants, V1073A and L1092P, induced a gain-of-channel function, while the rare missense variants, V94G and R1588Q, resulted in a loss-of-channel function.

Conclusions – We report that the common variant V1073A is associated with decreased susceptibility to AF. In functional studies the two common variants gave rise to a gain-of-function. The rare variants found in lone AF patients showed loss-of-function, indicating that these variants increase susceptibility to AF. Hence, our study suggests that SCN10A variations are involved in the genesis of AF.

Key words: atrial fibrillation arrhythmia, genetic polymorphism, electrophysiology, genotyping, Genome Wide Association Study, lone atrial fibrillation, SCN10A, Voltage Gated Sodium Channel Alpha Subunit Nav1.8, rs6795970, functional characterization
Introduction

Atrial fibrillation (AF), which is the most commonly sustained cardiac arrhythmia, is a global health problem accounting for increasing morbidity, mortality, and healthcare costs.1–3 Identifying and understanding the genetic basis of AF and/or association of genomic regions with AF will provide valuable insight into the pathogenesis of AF, and potentially improve the risk stratification and therapeutic options.

Genome wide association studies (GWAS) have identified 10 loci in the human genome that are associated with AF.4 Thus, common genetic variants play a role in the development of this multifactorial disease.5 Several studies have shown PR-interval prolongation on an electrocardiogram to be an independent risk factor for developing AF.6–8 Five independent GWAS publications have shown that genetic variants in SCN10A influence the PR-interval duration.9–13 Pfeufer et al. showed that five out of nine PR-associated loci from GWAS increased the risk of AF.10 They found that the single nucleotide polymorphism (SNP) rs6800541, which is located in an intron of SCN10A, had the strongest association with PR-interval duration and one of the strongest associations with AF among nine other GWAS hits. This SNP is in high and moderate linkage disequilibrium with two common nonsynonymous SNPs in SCN10A: rs6795970 (V1073A) and rs12632942 (L1092P), respectively.10 The substantial arrhythmogenic potential of genetic variants in SCN10A is underscored by the fact that another SNP (rs10428132) in this gene was the top hit in a GWAS on Brugada syndrome; a condition strongly associated with AF.14,15 Moreover, very recently, a phenome-wide study associated SCN10A, through rs6795970, directly with AF.16 This, however, contradicts with the findings by Holm et al. who did not report an association of rs6795970 with AF.11
SCN10A encodes the voltage-gated sodium channel, Na\textsubscript{v}1.8. This channel is the predominant tetrodotoxin-resistant sodium channel in primary sensory neurons, with particularly high levels of expression in nociceptive neurons, where it plays a key role in peripheral pain processing.\textsuperscript{17,18} Expression has also been shown in vagal, but not in sympathetic fibers.\textsuperscript{19,20}

Recently, a number of studies have indicated that SCN10A mRNA is present in both human and mouse heart and that this channel is involved in the cardiac I\textsubscript{Na} current.\textsuperscript{9,21–23} Yang et al. demonstrated higher expression of Na\textsubscript{v}1.8 transcripts in mouse atria compared to ventricle.\textsuperscript{21} Facer et al. detected Na\textsubscript{v}1.8 protein in both atrial myocytes and nerve fibers in the myocardium.\textsuperscript{24} Using genetic lineage tracing, others have shown that Na\textsubscript{v}1.8 is expressed in aortic bodies and the nerves around blood vessels of the heart.\textsuperscript{20} Interestingly, Verkerk et al. found that Na\textsubscript{v}1.8 is highly expressed in intracardiac neurons.\textsuperscript{22} In summary, these studies suggest that Na\textsubscript{v}1.8 is expressed in both cardiac myocytes and intracardiac neurons.

The recent notion that Na\textsubscript{v}1.8 might be important for cardiac electrophysiological properties raises the possibility that altered function of this gene may be coupled with cardiac arrhythmias.\textsuperscript{25} Thus, in the present study, we investigated whether the common SCN10A variant rs6795970 is associated with AF and thereby would be the variant carrying the effect of the GWAS hit. In addition, we screened 225 lone AF patients for SCN10A variants, and characterized the two rarest variants together with the two common variants functionally using patch-clamp electrophysiology.

**Methods**

**Study Subjects**

Patient records with the ICD-10 diagnose code I48.9 (atrial fibrillation and flutter) were collected and read. Only 225 patients with “lone AF” and onset of disease before age of 40 years were
recruited. Lone AF was defined as AF in absence of clinical or echocardiographic findings of cardiovascular disease, hypertension requiring medical therapy, metabolic- or pulmonary diseases. For the genotyping of the rs6795970 SNP, we recruited a cohort of 358 Scandinavian lone AF patients with onset of AF before the age of 50 (83% male gender, median age of AF onset 34.5 years [interquartile range 28-39 years]) and a cohort of 157 unselected AF patients (68% male gender, median age of 66 years [interquartile range 32-86 years]) (Online Appendix). Blood samples, ECG and clinical data were collected from all participating subjects. The study conforms to the principles outlined in the Declaration of Helsinki and was approved by the local scientific ethics committees, and all patients provided written informed consent.

**Control Population**

A total of 730 healthy subjects (52% males, median 66 years [interquartile range 52-76 years]) from two control cohorts (control groups I and II) were included in this study (Online Appendix). Control group I (complete sequencing of SCN10A) consisted of 216 unrelated healthy Danish blood donors with a normal ECG and without any cardiac symptoms. Control group II comprised 514 ethnically matched, middle-aged men and women without a history of AF or other manifestations of cardiovascular disease; however, with a high prevalence of risk factors for AF. Control groups I and II were previously described in detail.26,27 These control groups were used in the genotyping of rs6795970 using a Taqman assay. To increase the statistical power of our association study, we also used a third control cohort (control group III), comprising 6,161 individuals randomly selected from a Danish cohort study (Inter99, LuCamp).28 Although this control cohort could only provide data on rs6795970, due to the exome-chip which was used in the Inter99 study. This control group is assumed to represent the general population.
SCN10A Screening

The method is available in an Appendix.

SNP Genotyping

We genotyped rs6795970, encoding V1073A, in 515 AF patients of which 358 were lone AF patients. For comparison, in addition to control groups I and II (n_{Total}=730), we also used the data from a European-American population (n=4,300) from the Exome Sequencing Project (ESP). Genotyping of control groups I and II was performed as previously described. Furthermore, we also used the exome-chip data on the rs6795970 SNP from control group III.

Molecular Biology and In vitro Electrophysiology

Introduction of variants, cell culturing and patch-clamping of transiently transfected Neuro2A cells were performed as previously described. A detailed description is available in Online Appendix.

Bioinformatics and Statistical Analysis

All variants were reviewed in publicly available SNP databases (dbSNP, and ESP6500). We used 4 in silico tools to predict whether the variants were disease causing. The MAF in the 2 case cohorts were compared one by one with the 3 control cohorts using the Chi-square test. Similarly, we performed a pooled analysis where MAF in the 2 case groups were compared with a pooled MAF from our largest control population and ESP. Data are presented as mean ± standard error of mean (SEM) unless otherwise noted. Kolmogorov-Smirnov test was applied to confirm Gaussian distribution. Two-tailed Student’s t-test, one-way or two-way ANOVA combined with a Bonferroni post hoc test, or Chi-square tests, were used as appropriate to test for significant differences. A value of \( P < 0.05 \) was considered statistically significant. Further description is available at the Online Appendix.
Results

Genetic screening

We included 225 unrelated Danish patients with onset of disease before the age of 40 years for full genetic screening (clinical data listed in Online Appendix). The individual clinical characteristics of the patients with the rare SCN10A variations are listed in Table 1. In Figure 1 the positions of rare and common missense variants found in SCN10A in lone AF are illustrated in the Na$_v$1.8 protein topology. We identified nine rare missense variants (R14L; V94G; Y158D; R814H; E825D; I999L; R1268Q; C1523Y; R1588Q) and one splice site donor variant (rs75991777) (in exon 4 at second position T>C, Table 2). These variants were neither present in our in-house control group (n = 432 alleles), nor have any been previously reported in conjunction with AF. However, except for I999L and rs75991777, all variants were identified in the Exome Sequencing Project database (n=6,503) with minor allele frequency (MAF) less than 0.5% in the European-American population. rs75991777 has been reported in the dbSNP database from the 1000 Genomes Project with a MAF of 0.1%. The amino acid residues altered in the rare variants were found to be highly conserved across eukaryotic species, except for R814H and E825D, which differed in rat and mouse (data not shown). In our co-segregation analysis, we were able to screen the family members of the patients with I999L, C1523Y and rs75991777 variants. None of the family members diagnosed with AF carried the variant identified in the probands. Family members of remaining geno-positive patients were not available. PolyPhen2 prediction software predicted 78% (7 out of 9) of the rare variants to have a functional effect on protein function (Table 2). By using the Giudicessi et al. agreement of ≥3 in silico tools on these rare variants, it was predicted that 60% of the variants were damaging (Table 2).
Genotyping of V1073A

The result of the SNP genotyping is listed in Table 3. We were able to genotype the SNP rs6795970 in SCN10A in 515 AF patients (358 lone AF and 157 unselected AF patients) with a total call rate of 98.5%. The MAF of rs6795970 in all AF cases was 31.8% compared to 37.8% in 6,161 Danish exomes (Odds Ratio (OR) = 0.78, 95% Confidence Interval (CI) [0.68-0.90]; p=3.9x10^{-4}). The same result was found in a meta-analysis in which we added 4,300 European-American exomes from the ESP6500 database to the 6,161 Danish exomes (OR = 0.74 [0.65-0.86]; p=2.3x10^{-5}, Table 3). When analyzing only the lone AF patients, the same strong association was found. They were also less likely to carry the G allele compared to 6,161 Danish exomes (OR = 0.79, 95% CI [0.66-0.93]; p=0.003) and to the meta-analysis group (6,161 Danes + 4,300 European-Americans from ESP) (OR = 0.75 [0.64-0.89]; p=4.6x10^{-4}). These results indicate a protective effect of rs6795970 against AF (Table 3).

Clinical Features

Nine out of 11 of the AF patients harboring an SCN10A variant had paroxysmal AF and several of these patients also had other arrhythmias (Table 1). The R14L variant was identified in a patient with paroxysmal AF with onset of AF at age 31 and AV-nodal re-entry tachycardia (AVNRT). The V94G variant was found in two patients with paroxysmal and persistent AF with an onset of disease at age 28 and 27, respectively. The missense variants Y158D and R814H were identified in a patient with persistent AF with onset at age 31, who had several radiofrequency ablation (RFA) procedures for AF. The paroxysmal AF patient with onset of disease at age 35 had a splice-site donor variant at exon 4. This patient had normal coronary angiography with atrial flutter, AVNRT, inducible ventricular tachycardia and implantable cardioverter-defibrillator. Furthermore, this patient had a family history of SCD and AF. The
Missense variant E825D was identified in a paroxysmal AF patient with very early onset of disease at age 18. This patient also had AVNRT and several RAF procedures performed. The patient carrying the variant I999L had onset of paroxysmal AF at the age of 35 and also presented incomplete right bundle branch block. This patient also carries the variant L10P in SCN3B, as previously reported. The R1268Q variant were identified in two AF patients with onset of disease at age 23 (also had atrial flutter type II) and 31 (also had Incomplete Right Bundle Branch Block (IRBBB) and several DC conversions). The patient with the rare variant C1523Y was diagnosed with paroxysmal AF at age 30, and in another paroxysmal AF patient with onset of disease at age 28, we identified the variant R1588Q. This latter patient had an RFA procedure for AVNRT. Interestingly, four of the rare variant carriers (≈40%) have AVNRT, in addition to AF, suggesting that the AV-node, and perhaps the autonomic nerve system, could play an important role in the genesis of AF in these patients.

**Electrophysiology**

The electrophysiological properties of Na,1.8 wild type and four variants were investigated by whole-cell patch-clamping of Neuro-2A cells (Figure 2, and 3). We chose to analyze the V94G found in two unrelated patients and R1588Q variants based on the lowest variant frequency in the background population (not present in 2,000 non-AF Danish exomes, data not shown), thereby reducing the risk of investigating a random finding. At the time of variant selection for functional studies, the two variants were not found in the Exome Sequencing database (n=5,400), but later appeared in one exome for each variant when 1,100 additional subjects were included in the database (n=6,500). We also investigated the two non-synonymous common variants V1073A and L1092P. Figure 2A illustrates representative whole-cell currents from the Neuro-2A cells expressing wild type and variant Na,1.8 channels. Na,1.8 channels are activated...
by depolarizing potentials more positive than -15 mV, with a fast activating current peaking at +15 mV (Figure 2B). A part of the current is rapidly inactivated, while a long lasting current component of approximately 10% of the peak current level persists (Figure 2A).

The V94G-Na\textsubscript{1.8} channel does not conduct any current. The R1588Q variant showed peak current amplitude similar to wild type channels, however, it had a faster time to peak, together with a more than 6 mV negative shift in the steady-state inactivation ($V_{\text{h,WT}}$ -68.0±1.8 mV, $V_{\text{h,R1588Q}}$ -74.4±2.5 mV, Figure 3B). As -74.4 mV is close to the resting membrane potential of both atrial cardiomyocytes and neurons, this shift would be expected to play a major role in the channel availability, reducing the number of available channels as compared to wild type Na\textsubscript{1.8}. The combined electrophysiological characterization of R1588Q would therefore be expected to result in a loss-of-function phenotype.

Compared with wild type Na\textsubscript{1.8} (WT, -29.9±4.1 pA/pF), the two common variants expressed larger peak currents (V1073A, -50.6±7.0 pA/pF; L1092P, -61.4±8.5 pA/pF). While the steady-state inactivation properties for V1073A and L1092P were not altered, the steady-state activation properties were shifted to more positive potentials for these two common variants ($V_{\text{h,WT}}$ 1.6±1.3 mV, $V_{\text{h,V1073A}}$ 7.1±1.3 mV, $V_{\text{h,L1092P}}$ 6.4±1.5 mV)(Figure 3A). For V1073A, the time-dependent recovery from inactivation was decelerated (Figure 3C) and the time-to-peak accelerated (Table 4). Both common variants have a slower current decay at a number of different potentials (Figure 3E and 3F). Interestingly, the absolute sustained current level was increased for both V1073A and L1092P (WT, 2.4±0.3 pA/pF; V1073A, 3.9±0.5 pA/pF; L1092P, 4.2±0.7 pA/pF) (Figure 3D). For both WT and the two common variants the sustained current level is 7-8% of the peak current at +15 mV. Hence, the increase in the absolute sustained current level of the variants is probably due to the overall increased activity of these variant
channels. Together, we found a depolarized shift in voltage-dependence of steady-state activation on V1073A and L1092P and decelerated recovery from inactivation on the V1073A variant. However, these two common variants also induced dramatically larger peak-current amplitude, a slower current decay phase from inactivation, and a pronounced larger persistent current. Hence, the combined electrophysiological changes of these two common variants would result in gain-of-function phenotypes.

Discussion

In the present study, we found 10 rare missense SCN10A variants in 225 lone AF patients in which the minor allele frequencies were less than 0.5% in the ESP. Furthermore, we showed that the common non-synonymous variant V1073A (rs6795970) decreased the risk of AF. Functional characterization of the two rarest variants (with the lowest MAF) found in lone AF revealed reduced activity of Na+, while conversely, the common variant V1073A (rs6795970), associated with a decreased risk of AF, was found to increase activity of the channel.

The GWAS-hit, rs6800541, which is in close proximity to SCN10A, has been associated with PR-interval duration. A sub-analysis indicated that this variant also seems to decrease the odds ratio for AF (OR=0.92, p=1.4x10⁻⁰³). Others have tested the association of rs6795970, which is in high linkage with rs6800541, with AF and showed the same trend, however; the association was not statistically significant (OR=0.97, p=0.29). In the present study, we found that rs6795970 protects against both lone AF (OR=0.75, p=4.6x10⁻⁰⁴) and AF in a mixed cohort of patients (OR=0.74, p=2.3x10⁻⁰⁵, Table 3). Functional studies of V1073A revealed a number of altered biophysical parameters, with the greatest one being increased peak and sustained current as well as a slowing of fast inactivation. We therefore suggest that this variant has a gain-of-function phenotype, which seems to be protective against AF.
In lone AF patients, we identified 9 rare variants in SCN10A with MAF less than 0.5% in the ESP and one splice site donor variant in exon 4 (Table 1). We performed electrophysiological patch-clamp studies on the two rarest variants, V94G and R1588Q, which initially were found to be novel. Later, both variants were reported in ESP6500, but it should be noted that this is a nonselective database, where AF patients are not excluded. Since voltage-gated sodium channels are inactivated at voltage potentials close to the resting membrane potential, a negative shift in the steady-state inactivation, as seen for R1588Q, will result in a decreased availability of the channels. The V94G variant did not conduct any current. Hence, both tested variants found in lone AF patients have a loss-of-function phenotype. Since the AF protective variant V1073A had a gain-of-function phenotype, these data indicate that the loss-of-function variants V94G and R1588Q increase the susceptibility for AF. The I999L variant is a novel variant; however, since the patient also has a SCNB3 variant suspected to be disease causing, we did not include functional data of this variant.32

Currently, the role of Na,1.8 in cardiac electrophysiology remains unclear. If the primary role of Na,1.8 channels is in the cardiomyocytes, a loss-of-function phenotype could give rise to AF, analogous to what has been observed for Na,1.5 channels, where decreased Na,1.5 current has been associated with AF via augmented propensity for conduction delay with a subsequent increased risk of re-entrant arrhythmias.33,29 However, of note, both loss-of-function and gain-of-function variations in SCN5A have been reported in several studies of AF.33,29,34,35 Another possibility is that the Na,1.8 peak current does not have a major impact, as it is quantitatively much smaller than the Na,1.5 peak current. But, since the sustained (late) Na,1.8 current is 20-50 fold higher than the corresponding Na,1.5 late current, this depolarizing current could have a significant impact on the action potential duration and refractory period, and thereby protect
against AF.36

Given the sparse expression of Na,v1.8 channels in cardiac myocytes, the association of 
SCN10A variants to AF could be mediated through neuronal input. One study has suggested that 
rs6795970 in SCN10A may modulate the ventricular heart rate response during AF through a 
modulation of the AV-node.37 The AV-node is highly innervated by parasympathetic nerve 
fibers where Na,v1.8 is expressed.38–40 Recognition of disease mechanisms overlap between the 
AF and AV-nodal re-entry tachycardia and the fact that AVNRT may be the triggering factor for 
AF has been reported in several studies.41–44 Consistent with this notion, several of our 
paroxysmal AF patients with the rare variants have AVNRT (Table 1).

In AF patients, there has been substantial evidence of sympathetic tone-dependent AF.45 
Changes in autonomic tone, also known as sympathovagal imbalance, are important triggers in 
some forms of paroxysmal AF and also in the generation and maintenance of persistent AF.40–42 
With the expression of Na,v1.8 channels in vagal fibers and their absence in sympathetic fibers, it 
is possible that the observed effects on Na,v1.8 function of the different variants alters the 
sympathovagal balance.19,20,22

We were able to examine 3 families for co-segregation of SCN10A variants identified in 
the respective probands (Table 1), but none of the family members diagnosed with AF carried 
the variant of the proband. It is, however, not surprising that a monogenetic segregation pattern is 
absent since only a few reports have shown familial co-segregation of rare variants in AF.46 In 
line with our study, it has been suggested that variation in the SCN5A, is not the main cause of 
familial AF.35 Whether a person with a rare variant develops AF probably depends on both the 
genetic background and environmental factors. Thus, the rare loss-of-function variants found in 
our study should most likely be regarded as important modifiers for the genesis of AF.
Perspective

In summary, this study reveals a correlation between variations in SCN10A and AF. The results thereby support the notion of SCN10A being important in cardiophysiology as genetic variations now have been found to be implicated in cardiac conduction, Brugada syndrome, and AF. The fact that SCN10A variations could play a promoting role in lone AF, as well as other types of AF, highlights the importance of further studies on the cellular and electrophysiological factors involved in the development of AF. Hence, our results further contribute to understanding the complexity of cardiac electrophysiology and suggest that SCN10A genotyping in the future could improve risk prediction.

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Conflict of Interest Disclosures: Javad Jabbari is employed at LEO Pharma A/S. Anders G. Holst and Bo Liang are employed at Novo Nordisk A/S, Morten Grunnet at Lundbeck A/S.

References:


**Table 1:** Clinical characteristics of the lone AF patients with rare variants (MAF < 0.5% in EA in ESP6500)

<table>
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<tr>
<th>Patients</th>
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<th>rs ID</th>
<th>Pos. of variants</th>
<th>ECG description</th>
<th>P-wave (ms)</th>
<th>PR interval (ms)</th>
<th>QRS interval (ms)</th>
<th>QTc (ms)</th>
<th>HR (bpm)</th>
<th>Type of AF</th>
<th>Onset of AF</th>
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<td>SR, J-wave in II, III, aVF</td>
<td>119</td>
<td>154</td>
<td>96</td>
<td>398</td>
<td>54</td>
<td>Paroxysmal</td>
<td>30</td>
<td>Family history of AF</td>
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<tr>
<td>11</td>
<td>E27</td>
<td>unknown</td>
<td>R1588Q</td>
<td>SR</td>
<td>110</td>
<td>156</td>
<td>96</td>
<td>419</td>
<td>55</td>
<td>Paroxysmal</td>
<td>28</td>
<td>RFA for AVNRT</td>
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</tbody>
</table>

Positions and clinical characteristics of the patients with rare variants with minor allele frequencies (MAF) less than 0.5% in the European-American (EA) in the Exome Sequencing Project (n=6503, ESP6500) server. SR; Sinus rhythm, SVPC; Supraventricular premature complexes, IRBBB; Incomplete right bundle branch block, AF; Atrial fibrillation, AVNRT; AV-nodal re-entry tachycardia, RFA; Radiofrequency ablation, ICD; Implantable cardioverter-defibrillator, VT; Ventricular Tachycardia
Table 2: Genetic variations in SCN10A in lone AF patients

<table>
<thead>
<tr>
<th>Exon</th>
<th>rs ID</th>
<th>GVS Function</th>
<th>Amino Acid Pos.</th>
<th>eDNA Pos.</th>
<th>ESP6500</th>
<th>PolyPhen-2 Prediction</th>
<th>SIFT</th>
<th>Grantham</th>
<th>Conservation</th>
<th>Agreement ≥3</th>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>EA Allele #</td>
<td>AA Allele #</td>
<td>All Allele #</td>
<td>MAF (%) (EA/AA/All)</td>
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<td>1</td>
<td>rs141207048</td>
<td>missense</td>
<td>R14L</td>
<td>41</td>
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<td>Damaging</td>
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<td>1</td>
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<td>R15R</td>
<td>45</td>
<td>A=92/G=8508 A=15/G=4391 A=107/G=12899</td>
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<td>2</td>
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<td>missense</td>
<td>V94G</td>
<td>281</td>
<td>C=1/A=8599 C=0/A=4406 C=1/A=13005</td>
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<td>Damaging</td>
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<td>Y158D</td>
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<td>I206M</td>
<td>618</td>
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<td>E428E</td>
<td>1284</td>
<td>T=2158/C=6442 T=474/C=3932 T=2632/C=10374</td>
<td>25.09/10.76/20.24</td>
<td>-</td>
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<td>S509P</td>
<td>1525</td>
<td>G=241/A=8359 G=925/A=3481 G=1166/A=11840</td>
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<td>2475</td>
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<td>2850</td>
<td>C=557/T=8043 C=362/T=4044 C=919/T=12087</td>
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<tr>
<td>16</td>
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<td>J962V</td>
<td>2884</td>
<td>C=2253/T=6347 C=482/T=3924 C=2735/T=10271</td>
<td>26.20/10.94/21.03</td>
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<td>G979G</td>
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<td>A=2248/G=6352 A=224/G=4182 A=2472/G=10534</td>
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<td>Tolerated</td>
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<td>-</td>
<td>-</td>
<td>-</td>
<td>Conserved</td>
<td>-</td>
</tr>
</tbody>
</table>

Positions of the variants found in lune AF cohort. The frequency and MAF of the alleles are reported from ESP6500 exome server. PolyPhen-2 prediction reports the possible impact of an amino acid substitution on protein structure and function based on Polymorphism Phenotyping-2 (PolyPhen-2) program. D: Disease Causing; B: Benign; EA: European American; AA: African American; ESP 6500 exomes: Exome Variant Server (chromosomes 1-22, and X); MAF (%): (EA/AA/All): the minor-allele frequency in percent listed in the order of European American (EA), African American (AA) and all populations (All). (delimited by /)
Table 3: Association of rs6795970 frequencies with AF

<table>
<thead>
<tr>
<th>Control cohorts MAF (%)</th>
<th>553 (37.9)</th>
<th>4606 (37.4)</th>
<th>8030 (38.4)</th>
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<tbody>
<tr>
<td>Cases vs. Control I and II (n=730)</td>
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<td></td>
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<tr>
<td>AF cohorts MAF (%)</td>
<td>OR [95% CI]</td>
<td>p-value</td>
<td>OR [95% CI]</td>
</tr>
<tr>
<td>All AF Cases n = 508</td>
<td>0.77 [0.64-0.91]</td>
<td>0.002</td>
<td>0.78 [0.68-0.90]</td>
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<tr>
<td>Lone AF Cases n = 354</td>
<td>0.77 [0.63-0.93]</td>
<td>0.007</td>
<td>0.79 [0.66-0.93]</td>
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</tbody>
</table>

The allele frequencies are presented with Odds ratios (OR) and the p-values for their association with AF. ESP: Exome Sequencing Project, EA: European Americans, AF: Atrial Fibrillation, MAF: Minor Allele Frequency. The Chi-squared tests were used.
Table 4: Electrophysiological characterization of SCN10A variants

<table>
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<tr>
<th></th>
<th>WT</th>
<th>V1073A</th>
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<th>R1588Q</th>
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<td>(n=15)</td>
<td>(n=14)</td>
<td>(n=13)</td>
<td>(n=13)</td>
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</tr>
<tr>
<td>Peak current at 15 mV (pA/pF)</td>
<td>-29.9±4.1</td>
<td>-50.6±7*</td>
<td>-61.4±8.5**</td>
<td>-32.0±6.5</td>
</tr>
<tr>
<td>Steady-state inactivation</td>
<td>(n=16)</td>
<td>(n=13)</td>
<td>(n=14)</td>
<td>(n=12)</td>
</tr>
<tr>
<td>V1/2 (mV)</td>
<td>-68.0±1.8</td>
<td>-67.6±2.1</td>
<td>-65.9±1.4</td>
<td>-74.4±2.5*</td>
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<tr>
<td>K</td>
<td>9.8±1.2</td>
<td>9.7±1.6</td>
<td>9.6±0.5</td>
<td>11.6±0.8</td>
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<tr>
<td>Steady-state activation</td>
<td>(n=19)</td>
<td>(n=14)</td>
<td>(n=12)</td>
<td>(n=14)</td>
</tr>
<tr>
<td>V1/2 (mV)</td>
<td>1.6±1.3</td>
<td>7.1±1.3*</td>
<td>6.4±1.5*</td>
<td>5.5±2.4</td>
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<tr>
<td>K</td>
<td>8.1±0.4</td>
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<td>7.8±0.4</td>
<td>9.2±0.9</td>
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<td>Time course of recovery from inactivation at -85mV (ms)</td>
<td>(n=18)</td>
<td>(n=14)</td>
<td>(n=13)</td>
<td>(n=15)</td>
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<tr>
<td></td>
<td>33.6±5.6</td>
<td>73.6±14.5*</td>
<td>46.2±5.4</td>
<td>45.5±8.7</td>
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<tr>
<td>Time to Peak Current at 15mV (ms)</td>
<td>(n=11)</td>
<td>(n=12)</td>
<td>(n=13)</td>
<td>(n=11)</td>
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<tr>
<td></td>
<td>1.8±0.2</td>
<td>1.3±0.1*</td>
<td>1.5±0.1</td>
<td>1.3±0.1*</td>
</tr>
<tr>
<td>Late INa at 15 mV (pA/pF)</td>
<td>-2.4±0.3</td>
<td>-3.9±0.5*</td>
<td>-4.2±0.7*</td>
<td>-3.3±0.8</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SEM. Two-way ANOVA combined with Bonferroni post-test was used to test for significant differences of Peak current at 15 mV. The other parameters were tested by Two-tailed Student’s t-test. *p<0.05 and **p<0.01 versus wild-type (WT). N: the numbers of cells measured; T: time constant; V1/2: midpoint potential; k: slope factor.
Figure Legends:

Figure 1. Nav1.8 topology. Positions of rare and common variants found in SCN10A in lone AF are indicated on the Nav1.8 protein.

Figure 2. Current recordings of SCN10A variants. A) Representative whole-cell current traces of Nav1.8 wild-type (WT) and mutant channels. Currents were recorded following a voltage step protocol with 5 mV increments from -70 mV to +50 mV, preceded with a -100 mV step. B) Current-voltage (I-V) relationship. The current is normalized to cell capacitance to provide a measure of Na+ current density. C) Peak current density at 15mV, Two-way ANOVA with Bonferroni post-tests was applied to test for significant differences. Mean ± SEM values are presented in Table 4. B, C) n=13-15 for each group. Asterisks indicate the voltages at which the parameters were statistically different versus WT *p<0.05, **p<0.01 and ***p<0.001.

Figure 3. Activation and inactivation properties of SCN10A variants. A) Steady-state activation curves. Activation properties were determined from I-V relationships by normalizing peak INa to driving force and maximal INa, and plotting normalized conductance vs. Vm. B) Steady-state inactivation curves. Protocol is shown in insert. Boltzmann curves were fitted to both steady-state activation and inactivation data. C) Time course of recovery from inactivation following a pre-potential protocol (insert) was fitted to a one-exponential equation: I/Imax =y0+A x exp(-t/τ), t is the time from the beginning of the test pulse, A and τ=fractional amplitude and time constant, respectively. D) Late (sustained) sodium current was normalized to cell size. E,F,G) Voltage dependence of inactivation time constants. The decaying phase of whole-cell current
traces (as in Figure 2A) was fitted with 2 exponential equation: $I/I_{\text{max}} = A_f \times \exp(-t/\tau_f) + A_s \times \exp(-t/\tau_s)$. Lower and upper bundles of symbols indicate fast ($\tau_f$) and slow ($\tau_s$) time constant values respectively, $n=8-11$ for each group. A-D) Averaged values and the numbers of cells measured are presented in Table 4. Asterisks indicate the voltages at which the parameters were statistically different versus WT (*$p<0.05$).
**Na_v 1.8**

- Rare missense variants with minor allele frequency less than 0.5% in European American population in Exome Sequencing Project
- Common missense variants with minor allele frequency more than 0.5% in European American population in Exome Sequencing Project
Common and Rare Variants in SCN10A Modulate the Risk of Atrial Fibrillation
Javad Jabbari, Morten S. Olesen, Lei Yuan, Jonas B. Nielsen, Bo Liang, Vincenzo Macri, Ingrid E. Christophersen, Nikolaj Nielsen, Ahmad Sajadieh, Patrick T. Ellinor, Morten Grunnet, Stig Haunsø, Anders G. Holst, Jesper H. Svendsen and Thomas Jespersen

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Methods

Study Subjects

From eight hospitals in the region of Copenhagen, patient records with the ICD-10 diagnose code I48.9 (atrial fibrillation and flutter) were collected and read. Clinical characteristics of the patients are shown in Table S1.

Table S1. Clinical data of patient and control populations

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<th>Lone AF</th>
<th>AF</th>
<th>Control cohorts I &amp; II</th>
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</tr>
<tr>
<td>Median age of AF onset, y (IQR)</td>
<td>34.5 (28-39)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Median age, y (IQR)</td>
<td>-</td>
<td>66.2 (32-86)</td>
<td>66 (52-76)</td>
</tr>
<tr>
<td>Male gender</td>
<td>297 (83%)</td>
<td>108 (68%)</td>
<td>385 (52%)</td>
</tr>
<tr>
<td>BMI</td>
<td>26</td>
<td>27.1</td>
<td>26</td>
</tr>
<tr>
<td>AF type</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Paroxysmal</td>
<td>224 (62.6%)</td>
<td>19 (12%)</td>
<td>-</td>
</tr>
<tr>
<td>Persistent</td>
<td>113 (31.6%)</td>
<td>93 (58.9%)</td>
<td>-</td>
</tr>
<tr>
<td>Permanent</td>
<td>21 (5.9%)</td>
<td>46 (29.1%)</td>
<td>-</td>
</tr>
<tr>
<td>First degree relatives with AF</td>
<td>127 (35.5%)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Hypertension</td>
<td>-</td>
<td>70 (44.3%)</td>
<td>430 (58%)</td>
</tr>
<tr>
<td>Diabetes</td>
<td>-</td>
<td>17 (10.8%)</td>
<td>30 (4%)</td>
</tr>
</tbody>
</table>

BMI is given as mean values. AF, atrial fibrillation; BMI, body mass index (calculated as weight [kg]/height2 [m2]; IQR, interquartile range.
**SCN10A Screening**

Genomic DNA was isolated from blood samples using the ReliaPrep™ Blood gDNA Miniprep System (Promega, USA). Using intronic primers the entire coding sequence and splice junctions of SCN10A [NM_006514.2] were amplified and analyzed using high-resolution melting curve analysis (Light Scanner, Idaho Technology, Salt Lake City, UT, USA). The control cohorts and publicly available databases (Exome sequencing project database (n=6,503, ESP6500 and dbSNP) were screened for all rare variants identified in the patients. In probands with non-synonymous variants, bidirectional sequencing of genes previously associated with AF was performed.

All primers were designed with M13 tail sequences. Fragments with different melting curves than wild-type DNA were directly sequenced using Big Dye chemistry (DNA analyzer 3730, Applied Biosystems, Foster City, CA, USA). All identified non-synonymous variants were validated by resequencing in an independent polymerase chain reaction.

In probands with non-synonymous variants bidirectional sequencing of SCN1-3B (NM_001037.4, NM_004588, NM_018400.3), SCN5A (NM_00035), KCNQ1 (NM_000218.2), KCNH2 (NM_000238), KCNN3 (NM_002249.5), KCNN2 (NM_021614.2), KCNA5 (NM_002234.2), KCNE1/2/3/5 (NM_001127668, NM_172201, NM_005472.4, NM_012282.2), KCNJ2/3/5 (NM_000891.2, NM_002239.3, NM_000890.3), ANP (NM_006172.3), Cx40/43 (NM_005266.5, NM_000165.3) and LMNA (NM_005572) was performed.

**SNP genotyping**

The DNA was extracted from whole blood, using the ReliaPrep™ Blood gDNA Miniprep System (Promega, USA). The SNP genotype for rs6795970 was determined using fluorescence-based real-time polymerase chain reaction (PCR) (ABI PRISM 7900 Sequence Detection
System; Applied Biosystems, Foster City, CA) and a predeveloped TaqMan assay (Applied Biosystems). An allelic discrimination run was performed allowing for discrimination between the allele compositions of each sample. For genotype and allele frequencies please see Table S2.

Table S2. The Genotype and Allele Frequencies of rs6795970.

<table>
<thead>
<tr>
<th>Genotype Frequency (%)</th>
<th>Allele Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minor Allele Homozygote</td>
<td>Heterozygote</td>
</tr>
<tr>
<td>rs6795970</td>
<td>AA</td>
</tr>
<tr>
<td>Total AF cases n = 508</td>
<td>58</td>
</tr>
<tr>
<td>Lone AF cases n = 354</td>
<td>(11,4)</td>
</tr>
<tr>
<td>Controls Holter n=514</td>
<td>68</td>
</tr>
<tr>
<td>Controls n=216</td>
<td>(13,2)</td>
</tr>
<tr>
<td>Total Controls n=730</td>
<td>95</td>
</tr>
<tr>
<td>Danish Control n=6161</td>
<td>(13,0)</td>
</tr>
<tr>
<td>Controls ESP n = 4300</td>
<td>676</td>
</tr>
<tr>
<td>Controls Danish Cohort+ESP</td>
<td>(15,7)</td>
</tr>
</tbody>
</table>

The genotype distributions and allele frequencies of rs6795970 in different cohorts are presented.
Bioinformatics and Statistical Analysis

We used 4 in silico tools to predict whether the variants were disease causing (PolyPhen-2, SIFT, Grantham Prediction and Conservation). Co-segregation analyses of the variants within the family members were also done. The variants were checked for whether they were located in conserved genomic sequence of the family members of hSCN1A–hSCN10A. Species alignment across eukaryotic species was performed.

Cloning of Sodium Channel Subunits

For the site directed mutagenesis, PCR with the variant selective primers was performed with the following PCR parameters: 1) 96°C/2 min; 2) 96°C/30 sec; 3) 55°C/15 sec; 4) 72°C/7 min; 5) 72°C/10 min, with the total number of cycles (Step 2-4) being 20. The PCR reaction was subsequently digested with DpnI (Fermentas, Denmark) and transformed into and amplified in SURE cells (Agilent Technologies, Denmark).

Cell Culture and transfection

The neuroblastoma cell line, Neuro-2A cells (ATCC, USA), were maintained in 90% Dulbecco’s Modified Eagles Medium supplemented with 10% fetal calf serum and 1% penicillin/streptomycin in a 5% CO₂ incubator at 37°C. Neuro-2A cells were trypsinized, diluted in culture medium, and grown in 35-mm dishes. When grown to 30–50% confluence, cells were transiently co-transfected with 1 μg wild-type (WT) or mutant pc-hSCN10A and 0.2 μg of pcDNA3-EGFP as a reporter gene, using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer’s instructions.

Molecular Cloning of Sodium Channel

The genetic variations in hSCN10A (GenBank Acc. No. [NM_006514.2]), were introduced into the pCMV6-AC-GFP-hSCN10A plasmid (Origene, USA), via site-directed mutagenesis using a
full plasmid overlap PCR strategy (PfuTurbo DNA Polymerase, Stratagene, Denmark). The variants V94G (GTG->GGG), V1073A (GTC->GCC), L1092P (CTA->CCA), and R1588Q (CGA->CAA) were introduced by overlapping oligonucleotides designed in Vector NTI Advanced 10 (Invitrogen, Denmark) and constructed by Eurofins MWG Operon (Germany). All of the plasmid constructs were verified by complete DNA sequencing of the \textit{hSCN10A} cDNA and flanking regions (Macrogen Inc., Seoul, Republic of Korea).

\textit{In vitro} Electrophysiology

Patch-clamp experiments were performed 2–3 days after transfection. Whole-cell currents were measured at room temperature (20–22 °C). The internal pipette solution consisted of (in mM) CsCl 60, Cesium aspartate 70, CaCl$_2$ 1, MgCl$_2$1, HEPES 10, EGTA 11, MgATP 5 (pH 7.2 with CsOH); the external solution consisted of (in mM): NaCl 130, CsCl 5, CaCl$_2$ 2, MgCl$_2$ 1.2, HEPES 10, glucose 5 (pH 7.4 with CsOH). Measurements were made with Pulse software and using an EPC-9 amplifier (HEKA Elektronik, Germany). Borosilicate glass pipettes were pulled on a DPZ-Universal puller (Zeit Instrument, Germany). The pipettes had a resistance of 1.5–2.5 MΩ when filled with intracellular solution. The series resistances recorded in the whole-cell configuration were 2–5 MΩ and were compensated (80 %). As the Neuro-2A cells endogenously express TTX sensitive sodium currents, cells where incubated with 300 nM TTX prior to measurement. Recordings were performed between minute 5 and 10 after obtaining a whole cell configuration. Electrophysiological data were analyzed using Igor Pro (Wavemetrics, USA) and GraphPad Prism (GraphPad Software Inc., USA).
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