Figure 1 also illustrates the positions of the 22 variations. For subsequent analysis of *SCN5A* haplotype structures we assigned 5 LD blocks, so that the closely associated SNPs ($\rho^2 > 0.20$) could be grouped together. Blocks 1, 2 and 3 included only a single SNP, c.87G>A (p.Ala29Ala), c.482+184A>G, and c.703+130G>A, respectively, because these SNPs showed little LD ($\rho^2 < 0.20$) with the other variations. Block 4, spanning 40 kb (from introns 9 to 21), included 9 variations (c.1140+98A>G, c.1141–3C>A, c.1339–24G>A, c.1519–68C>T, c.1673A>G; p.His558Arg, c.3269C>T; p.Pro1090Leu, c.3578G>A; p.Arg1193Gln, c.3667–89dupA, c.3840+73G>A). The remaining 10 variations (c.4299+53T>C, c.4299+116G>A, c.4542+86A>G, c.4813+164C>G, c.4813+215T>C, c.4813+262A>C, c.5457T>C; p.Asp1819Asp, c.5963T>G; p.Leu1988Arg, c.6174A>G, c.6255T>C) were assigned to block 5, ranging from intron 24 to exon 28 (7 kb).

Haplotypes and their Associations with Arrhythmogenesis

First, the haplotype frequencies for the blocks were evaluated. For blocks 1 to 3, the haplotype frequencies were the same as the allele frequencies of the single SNPs. Therefore, significant differences in haplotype (allele) frequencies were found only in block 3 (c.703+130G>A) as described above. In block 4, eight common haplotypes were inferred with frequencies over 1% (**1a*–**1e*, **2a*, **3a* and **4a*), accounting for 97% of all the observed haplotypes (Table 4a). In block 5, five common haplotypes with frequencies over 1% (**1a*–**1d*, and **2a*) accounted for 99% of all the inferred haplotypes (Table 4b). The frequency of the block 5 **2* haplotype (**2a* and **2b*) bearing c.5963T>G (p.Leu1988Arg) was about eight times higher in the controls than in the patients. For the other haplotypes in blocks 4 and 5, however, no significant differences in haplotype frequencies between the patients and controls were obtained.

Next, the combinations of in-block haplotypes (inter-block haplotypes; e.g., block1 **1a* - block2 **1a* – block3 **1a* – block4 **1a* – block5 **1a*) were assessed. However, there were too many inter-block haplotypes, each having low frequencies, to obtain statistical significance by comparing them between the patients and controls. Nonetheless, the haplotypes harbouring the SNPs c.703+130G>A or c.5963T>G (p.Leu1988Arg), which showed significant differences in allele frequencies between the patients and controls, showed unique linkages beyond the blocks. The **2* haplotypes (**2a* and **2b*) in block 5 were always associated with the **2a* haplotype in block 4, which harbours 3 linked SNPs, including the nonsynonymous SNP c.1673A>G (p.His558Arg). The SNP c.703+130G>A in block 3 was mostly associated with **1b* in block 5, which harboured three SNPs including c.4299+53T>C. Thus, in spite of recombination between LD blocks, some block haplotype combinations were sustained. To assess the association between these inter-block haplotypes and risk of arrhythmias, we then applied the permutation and model-free analysis and estimation haplotype (PM+EM+) methods using the two haplotype-tagging SNPs c.1673A>G (p.His558Arg) and c.5963T>G (p.Leu1988Arg) or c.703+130G>A and c.4299+53T>C. (Table 5).

When the combinations between c.1673A>G (p.His558Arg) and c.5963T>G (p.Leu1988Arg) were analyzed, 3 haplotypic combinations were inferred in both of the two groups. The haplotype AG (558His-1988Arg) was completely absent in both groups, and the haplotype GG (558Arg-1988Arg) was present less frequently in the patients compared to the healthy controls. The global permutation test indicated that there was a significant difference in distribution of the haplotypes between the patients and controls ($\chi^2 = 7.42$, $p = 0.0260$). In accordance with this result, the frequency of haplotype GG (558Arg-1988Arg) was significantly lower in the patients than in the controls ($p = 0.018$).

As for the 4 haplotypes estimated from c.703+130G>A and c.4299+53T>C, haplotype AT was about 3 times as frequent in the patients as in the controls. However, the global difference between the patients and controls had a borderline significance ($\chi^2 = 8.64$, $p = 0.0550$) by the global permutation test.

Discussion

Since the defect in *SCN5A* was first reported to be a cause of LQT-3 (Wang *et al.* 1995), a variety of genetic alterations in *SCN5A* have been suggested to influence the pathophysiology of cardiac arrhythmias and/or pharmacological sensitivities to antiarrhythmic drugs. In this study we comprehensively searched for

Table 4 Haplotypes of blocks 4 and 5 and their frequencies in *SCN5A* for Japanese arrhythmic patients and healthy controls

a) block 4 Exon/Intron	Intron 9	Intron 9	Intron 10	Intron 11	Exon 12	Exon 18	Exon 20	Intron 20	Intron 21			
Position (NT_022517.16)	g.38573687	g.38573267	g.38572048	g.38571267	g.38571045	g.38546571	g.38542501	g.38533787	g.38533452			
Position (NM_198056.1)	c.1140 + 98	c.1141 − 3	c.1339 − 24	c.1519 − 68	c.1673	c.3269	3578	c.3667 − 89	c.3840 + 73			
Nucleotide change	A>G	C>A	G>A	C>T	A>G	C>T	G>A	dupA	G>A			
Amino acid change					His558Arg	Pro1090Leu	Arg1193Gln					
Haplotypes[a]										Frequency (%)		
										All subjects	Patients	Controls
*1 *1a										68.22	69.57	68.50
*1b									■	4.11	3.88	3.92
*1c								■		3.80	4.45	2.94
*1d	■									3.73	3.54	3.40
*1e				■				■		1.94	1.78	1.82
*1f				■						0.29	0.32	0.26
*2 *2a		■	■		■					7.41	5.36	8.58
*2b		■	■		■			■		0.47	0.26	0.71
*2c			■		■					0.40	0.30	0.45
*3 *3a							■			6.06	6.04	5.53
*4 *4a						■			■	2.09	2.59	1.56
*4b						■				0.29	0.32	0.25
*5 *5a										0.28	0.70	0.02
Others with frequencies less than 0.25 %		■	■		■	■			■	0.91	0.89	2.06

b) block 5 Exon/Intron	Intron 24	Intron 26	Intron 27	Intron 27	Intron 27	Exon 28	Exon 28	Exon 28 (3′-UTR)	Exon 28 (3′-UTR)			
Position (NT_022517.16)	g.38524294	g.38522686	g.38521231	g.38521180	g.38521133	g.38518031	g.38517525	g.38517314	g.38517233			
Position (NM_198056.1)	c.4299 + 53	c.4542 + 86	c.4813 + 164	c.4813 + 215	c.4813 + 262	5457	5963	c.6174	c.6255			
Nucleotide change	T>C	A>G	C>G	T>C	A>C	T>C	T>G	A>G	T>C			
Amino acid change						Asp1819Asp (silent)	Leu1988Arg					
Haplotypes[a]										Frequency (%)		
										All subjects	Patients	Controls
*1 *1a										48.85	50.30	47.82
*1b	■						■	■		26.99	26.51	27.35
*1c		■	■	■	■	■		■		11.31	11.45	11.21
*1d						■		■		10.95	11.14	10.80
*1e						■				0.25	0.30	0.22
*2 *2a							■		■	1.26	0.30	1.94
*2b							■			0.25	0.00	0.43
Others with frequencies less than 0.25 %)										0.14	0.00	0.23

[a]The wild-type haplotype was designated as *1, and the nonsynonymous SNP-bearing haplotypes were numerically defined. The subtypes were named with small alphabetical letters in the order of their frequencies.
White cells indicate major allele, and ■ indicate minor allele.
The haplotypes found in two or more chromosomes (frequencies>0.25%) were shown.

Table 5 Haplotype frequencies of two haplotype-tagging SNPs and their associations with the risk of arrhythmias

c.1673A>G (p.His558Arg) and c.5963T>G (p.Leu1988Arg)

	Selected locus		Haplotype frequency (%)			
Haplotype	c.1673A>G (p.His558Arg)	c.5963T>G (p.Leu1988Arg)	All subjects	Patients	Controls	Statistics
AT	A (His558)	T (Leu1988)	90.95	92.77	89.66	N.S.
GT	G (Arg558)	T (Leu1988)	7.54	6.93	7.97	N.S.
AG	A (His558)	G (Arg1988)	0.00	0.00	0.00	-
GG	G (Arg558)	G (Arg1988)	1.51	0.30	2.37	$p = 0.0181$
			Global permutation test[a]	$\chi^2 = 7.42$, $p = 0.0260$		

c.703+130G>A and c.4299 + 53T>C

	Selected locus		Haplotype frequency (%)		
Haplotype	c.703+130G>A	c.4299+53T>C	All subjects	Patients	Controls
GT	G	T	70.55	69.89	71.06
AT	A	T	2.32	3.60	1.35
GC	G	C	18.65	16.25	20.31
AC	A	C	8.49	10.25	7.27
			Global permutation test[a]	$\chi^2 = 8.64$, $p = 0.0550$	

[a]The empirical p values were obtained after 1000 permutations.

genetic variations in *SCN5A*. A total of 69 variations were detected in 166 unrelated Japanese arrhythmic patients and 232 healthy controls. Among them, 54 variations were novel and 15 were previously identified.

The fifteen reported variations include 4 nonsynonymous SNPs (p.His558Arg, p.Pro1090Leu, p.Arg1193Gln, and p.Val1951Leu). p.His558Arg has been extensively studied in various ethnic groups. Yang *et al.* (2002) have compared the frequency of p.His558Arg in patients diagnosed with the drug-associated LQT syndrome with those in three control populations: patients tolerating QT-prolonging drugs, and populations in Tennessee and accross the United States. p.His558Arg was detected at similar allele frequencies of 0.18–0.24 in all populations. As for the Japanese population, its frequencies were reported to be 0.132 for 56 healthy controls and 0.08 for 50 individuals by Takahata *et al.* (2003) and Iwasa *et al.* (2000), respectively. In our study, the allele frequency of p.His558Arg in arrhythmic patients (0.072) was slightly lower than that in the healthy controls (0.103), although a significant difference was not obtained ($p = 0.1307$). p.Pro1090Leu is a well-known nonsynonymous SNP localized in the linker between DII and DIII. The frequency of p.Pro1090Leu was 12/334 (0.036) and 11/464 (0.024) for the patients and the controls, respectively, and comparable to that reported in the Japanese (0.04) by Iwasa *et al.* (2000). Vatta *et al.* (2002) reported that p.Arg1193Gln accelerated the fast inactivation of the Na^+ channel *in vitro*. In our study, p.Arg1193Gln showed similar allele frequencies of 0.063 both in patients and healthy controls. From these results, it is likely that these three common SNPs are not directly involved in arrhythmogenesis in the Japanese. p.Val1951Leu, found at low frequencies in both patients (0.003) and healthy controls (0.004), was also detected at a similar frequency (0.005) by Iwasa *et al.* (2000). Although this SNP was reported in patients with BrS by Priori *et al.* (2002) and Mok *et al.* (2004), p.Val1951Leu alone is unlikely to be directly related with the pathogenesis of BrS.

Six novel nonsynonymous SNPs (p.Phe532Cys, p.Arg689His, p.Pro701Leu, p.His1200Tyr, p.Val 1667Ile, and p.Arg1739Gln) were found separately in six arrhythmic patients. When flanking amino acid sequences of these SNPs were aligned with related

sodium channel sequences (Supplementary Figure 1, online), the 6 amino acid residues of SCN5A, 532Phe, 689Arg, 701Pro, 1200His, 1667Val, and 1739Arg, were found top be highly conserved among the different Na^+ channel α subunit isoforms. Amino acid 532Phe, 689Arg, and 701Pro are localized in the linker between DI and DII, where the mutations associated with inherited arrhythmia syndrome such as p.Gly514Cys, p.Leu567Gln and p.Leu619Phe have already been reported (Tan *et al.* 2003). 1200His is located in the linker between DII and DIII, where the BrS-related variation p.Arg1193Gln (Vatta *et al.* 2002) is localized. 1667Val in DIVS5 and 1739Arg in the P loop of DIV form a pore structure together with DIVS6, where sodium channel blocking drugs are thought to bind. p.Arg1739Gln is a mutation located next to p.Gly1740Arg that was identified in a patient with BrS by Priori *et al.* (2002). Since these 4 novel nonsynonymous SNPs were not detected in 232 healthy controls, it is possible that they are related to cardiac arrhythmia pathogenesis, although these patients were not diagnosed with LQT or BrS. Some patients who carry these novel nonsynonymous SNPs simultaneously have p.His558Arg, p.Pro1090Leu or p.Val1951Leu (Table 3). At present it is not clear whether 1739Gln and 558Arg, 701Leu and 1951Leu or 1667Ile and 1090Leu are on the same chromosome. If they are, the interactions between the substituted residues may affect the biological properties of the Na^+ channels.

As for the five other novel nonsynonymous SNPs detected in healthy controls p.Glu428Lys is localized in the linker between DI and DII, p.Ala1148Thr and p.Ala1186Thr in the linker between DII and DIII, and p.Arg1913Cys and p.Ala1932Val in the C-terminal intracellular loop. 428Glu, 1148Ala and 1913Arg are well conserved, while 1186Ala and 1932Ala are not conserved (Supplementary Figure 1, online). Because the healthy individuals who carry these SNPs failed to show symptoms of arrhythmias, these SNPs are unlikely to affect Na^+ channel function. However, it has been recently suggested that some variations in *SCN5A* may increase the risk of acquired long QT syndrome triggered by drug administration in healthy individuals, who are clinically asymptomatic and have normal QT intervals under normal circumstances. Therefore, the possibility cannot be excluded that these five novel nonsynonymous SNPs in healthy controls influence the susceptibility to cardiac ion channel blockade and QT prolongation. We have begun analyzing the electrophysiological properties of the novel variations using heterologous expression systems. These studies will elucidate the importance of these variable sites in Na^+ channel function.

Another novel nonsynonymous SNP, p.Leu1988Arg, was heterozygous in eleven healthy subjects and one patient, and the allele frequency of p.Leu1988Arg was significantly lower in the patients than in the controls by Fisher's extract test ($p = 0.018$). Since all twelve subjects with p.Leu1988Arg also have p.His558Arg (Table 3), weak LD ($r^2 = 0.15$) was shown between p.His558Arg and p.Leu1988Arg (Figure 2). Haplotype analysis using the 2 SNPs, c.1673A>G (p.His558Arg) and c.5963T>G (p.Leu1988Arg), also showed that the frequency of the haplotype GG (558Arg–1988Arg) was significantly lower in the patients than in the healthy controls. These results suggest that the haplotype GG has been positively selected because of its protective effect against arrhythmias.

Although physiological characterization of Na^+ channels containing both p.His558Arg and p.Leu1988Arg are necessary to elucidate the negative association of the haplotype GG (558Arg-1988Arg) with cardiac arrhythmias, some underlying mechanisms could be speculated. 1988Leu is localized in the C-terminal intracellular loop but is not conserved among different Na^+ channel isoforms (Supplementary Figure 1, online). However, Cormier *et al.* (2002) reported that truncation of the distal region of the C-terminus (1921Leu stop mutant) reduced peak currents without affecting channel gating, by whole cell patch clamp recordings. Thus, it cannot be excluded that 1988Leu may be involved in regulating the density of functional Na^+ channels in the surface membrane. On the other hand, it has been postulated that p.His558Arg modulates functional changes of the Na^+ channel caused by other variations, and plays a role in intragenic complementation, although this common variation itself does not alter the voltage dependence of activation and inactivation kinetics of wild-type channels. For instance, Ye *et al.* (2003) showed that p.His558Arg restored the trafficking defect caused by the LQT-3 variation, p.Met1766Leu. Viswanathan *et al.* (2003) reported that p.His558Arg could attenuate

the abnormal gating effect caused by the proximal variation, p.Thr512Ile, *in vitro*. The favourable channel modulation by 1988Arg in conjunction with 558Arg for protection against arrhythmias might result in the positive selection of the GG (558Arg–1988Arg) haplotype in the controls. Alternatively, 558Arg could restore any functional deterioration caused by 1988Arg. If the intragenic complementation by 558Arg acts on 1988Arg, the rare frequency of the AG (558His–1988Arg) haplotype, and the preference of the GG (558Arg–1988Arg) haplotype, in the controls is reasonable. Another possible explanation is that other variations, which reside in the GG (558Arg-1988Arg) haplotype, such as c.6255T>C in the 3′-UTR, might change the stability of the mRNA leading to a protective effect on arrhythmias. In this regard Yang *et al.* (2004) demonstrated that −92C>A located in the promoter of *SCN5A* increased luciferase activity in neonatal cardiac myocytes. They proposed that this polymorphism might represent the first example of an allele that could protect against serious arrhythmias.

Comparison of the allele frequencies between patients and controls clearly suggests that c.703 + 130G>A is associated with an increased risk of arrhythmias, although the functional role of this SNP remains unknown. Because the haplotype AT in patients was 3 times as common as in the controls, we could not rule out the possibility that c.703+130G>A is linked with an unidentified variation that influences susceptibility to cardiac arrhythmias. In the dbSNP database of NCBI many SNPs were reported in introns 5 and 6, proximal to c.703+130G>A, including rs6797133, rs6786119, rs6776383, rs6791081 and rs6793943. Further analyses are needed to reveal a haplotype containing c.703+130G>A that increases the risk for arrhythmias.

In conclusion, 69 genetic variations, including 54 novel ones, were detected in *SCN5A*. Eleven novel missense variations were found in eleven different individuals, of which 6 were found in arrhythmic patients and 5 were in healthy controls. Another novel missense variation (p.Leu1988Arg) was found in the patients at a significantly lower frequency than in the healthy controls ($p<0.05$). Furthermore, the frequency of a novel intronic SNP, c.703+130G>A, was significantly higher in the patients than in the controls. The analysis of LD and haplotype structures of *SCN5A* revealed the possibility that the haplotype harbouring p.Leu1988Arg and p.His558Arg is associated with protection against arrhythmias. These results indicate that some genetic variations and haplotypes of *SCN5A* are positively or negatively associated with cardiac rhythm disturbance in Japanese. These findings provide fundamental information necessary to further elucidate the effects of genetic variations of *SCN5A* on channel function and cardiac rhythm.

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Supplementary Material

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