Table 2. Genotype distribution in the control, MI, and CI groups.

Variants		Control	MI	P value	CI	P value
		290	313		176	
rs670950	Genotype					
(SNP1)	C/C	248 (85.5%)	250 (79.9%)		143 (81.3%)	
	C/T	41 (14.1%)	56 (17.9%)		29 (16.5%)	
	T/T	1 (0.3%)	7 (2.2%)	0.051	4 (2.3%)	0.109
	Dominant model					
	C/C, C/T	289 (99.7%)	306 (97.8%)		172 (97.7%)	
	T/T	1 (0.3%)	7 (2.2%)	0.043*	4 (2.3%)	0.050
	Recessive model					
	C/C	248 (85.5%)	250 (79.8%)		143 (81.3%)	
	C/T, T/T	42 (14.5%)	63 (20.1%)	0.068	33 (18.8%)	0.224
	Allele					
	C	537 (92.6%)	556 (88.8%)		315 (89.5%)	
	T	43 (7.4%)	70 (11.6%)		37 (10.5%)	
	Odds ratio for MAF		0.636 (0.427–0.947)		0.682 (0.430–1.081)	
rs2306801	Genotype					
(SNP2)	G/G	219 (75.5%)	231 (73.8%)		144 (81.8%)	
	G/A	63 (21.7%)	75 (24.0%)		30 (17.0%)	
	A/A	8 (2.8%)	7 (2.2%)	0.758	2 (1.1%)	0.212
	Dominant model					
	G/G, G/A	282 (97.2%)	306 (97.7%)		174 (98.9%)	
	A/A	8 (2.8%)	7 (2.2%)	0.681	2 (1.1%)	0.241
	Recessive model					
	G/G	219 (75.5%)	231 (73.8%)		144 (81.8%)	
	G/A, A/A	71 (24.5%)	82 (26.2%)	0.629	32 (18.2%)	0.112
	Allele					
	G	501 (86.4%)	537 (85.8%)		318 (90.3%)	
	A	79 (13.6%)	89 (14.2%)		34 (9.7%)	
	Odds ratio for MAF		0.951 (0.686–1.319)		1.475 (0.963–2.258)	
rs2306799	Genotype					
(SNP3)	G/G	202 (69.7%)	242 (77.3%)		135 (76.7%)	
	G/A	80 (27.6%)	69 (22.0%)		36 (20.5%)	
	A/A	8 (2.8%)	2 (0.6%)	0.028*	5 (2.8%)	0.224
	Dominant model					
	G/G, G/A	282 (97.2%)	311 (99.3%)		171 (97.2%)	
	A/A	8 (2.8%)	2 (0.6%)	0.042*	5 (2.8%)	0.958

Table 2 continued. Genotype distribution in the control, MI, and CI groups.

Variants		Control	MI	P value	CI	P value
		290	313		176	
	Recessive model					
	G/G	202(69.7%)	242(77.3%)		135(76.7%)	
	G/A, A/A	88(30.3%)	71(22.7%)	0.033*	41(23.3%)	0.099
	Allele					
	G	484(83.4%)	553(88.3%)		306(86.9%)	
	A	96(16.6%)	73(11.7%)		46(13.1%)	
	Odds ratio for MAF		1.503 (1.082–2.086)		1.319 (0.903–1.928)	
rs347519	Genotype					
(SNP4)	T/T	213(73.4%)	246(78.6%)		137(77.8%)	
	T/C	70(24.1%)	65(20.8%)		34(19.3%)	
	C/C	7(2.4%)	2(0.6%)	0.107	5(2.8%)	0.473
	Dominant model					
	T/T, T/C	283(97.6%)	311(99.3%)		171(97.2%)	
	C/C	7(2.4%)	2(0.6%)	0.073	5(2.8%)	0.778
	Recessive model					
	T/T	213(73.4%)	246(78.6%)		137(77.8%)	
	T/C, C/C	77(26.6%)	67(21.4%)	0.139	39(22.2%)	0.288
	Allele					
	T	496(85.5%)	557(89.0%)		308(87.5%)	
	C	84(14.5%)	69(11.0%)		44(12.5%)	
	Odds ratio for MAF		1.367 (0.972-1.922)		1.185 (0.801–1.754)	
rs3786954	Genotype					
(SNP5)	A/A	210(72.4%)	246(78.6%)		135(76.7%)	
	A/G	71(24.5%)	65(20.8%)		36(20.5%)	
	G/G	9(3.1%)	2(0.6%)	0.035*	5(2.8%)	0.585
	Dominant model					
	A/A, A/G	281(96.9%)	311(99.4%)		171(97.2%)	
	G/G	9(3.1%)	2(0.6%)	0.024*	5(2.8%)	0.872
	Recessive model					
	A/A	210(72.4%)	246(78.6%)		135(76.7%)	
	A/G, G/G	80(27.6%)	67(21.4%)	0.077*	41(23.3%)	0.306
	Allele					
	A	491(84.7%)	557(89.0%)		306 (86.9%)	
	G	89(15.3%)	69(11.0%)		46 (13.1%)	
	Odds ratio for MAF		1.463 (1.045–2.050)		1.206 (0.822–1.770)	

* Significant difference in distribution. MI – myocardial infarction; CI – cerebral infarction; MAF – minor allele frequency. () – 95% confidence interval.

Table 3. Odds ratios and 95% confidence intervals for each risk factor and SNP genotype associated with MI.

SNP1(rs670950)	P value	OR	95% confidence interval	
			Lower limit	Upper limit
BMI	0.000	1.18	1.10	1.26*
Creatinine	0.003	3.16	1.49	6.70*
T-Cho	0.000	0.99	0.98	0.99*
HT	0.264	1.40	0.78	2.52
DM	0.000	21.25	8.77	51.50*
Dominant model (C/C+C/T vs T/T)	0.225	0.24	0.03	2.39
SNP3(rs2306799)				
BMI	0.000	1.18	1.10	1.26*
Creatinine	0.003	3.13	1.49	6.58*
T-Cho	0.000	0.99	0.98	0.99*
HT	0.254	1.40	0.78	2.51
DM	0.000	23.69	9.59	58.53*
Dominant model (G/G+G/A vs A/A)	0.096	7.40	0.70	78.17
SNP5(rs3786954)				
BMI	0.000	1.18	1.10	1.26*
Creatinine	0.003	3.06	1.45	6.44*
T-Cho	0.000	0.99	0.98	0.99*
HT	0.258	1.40	0.78	2.51
DM	0.000	23.81	9.61	58.98*
Dominant model (A/A+A/G vs G/G)	0.065	0.12	0.01	1.14
SNP3(rs2306799)				
BMI	0.000	1.19	1.11	1.27*
Creatinine	0.004	2.99	1.41	6.35*
T-Cho	0.000	0.99	0.98	0.99*
HT	0.195	1.48	0.82	2.66
DM	0.000	24.24	9.91	59.27*
Recessive model (G/G vs G/A+A/A)	0.005	1.96	1.22	3.14*

* Significant difference. BMI – body mass index; T-Cho – total cholesterol; HT – Hypertension; DM – diabetes mellitus; OR – odds ratio.

differences were seen for SNP3 and SNP5 between the MI and control groups. There were also significant differences between the MI and control groups for the dominant or recessive model distributions of SNP1, SNP3, and SNP5. There were no differences noted for any of the SNPs between the CI and control groups.

Multiple logistic regression analysis revealed that the frequency of the G/G genotype (recessive model) of SNP3 in the MI group was significantly higher than in the control group (p=0.005). This result did not change even after adjustment for the confounding factors referred to in Table 1, with the calculated odds ratio determined to be 1.96 (95% confidence interval: 1.22–3.14) (Table 3). There were no significant differences found by the multiple logistic regression analyses performed on the other three genotypes.

Figure 2 shows the pair-wise LD patterns for the KCNN4 gene. SNP1 and SNP2 were not located within the same haplotype block. Data from the International Human Haplotype Map (HapMap) Project (*http://www.hapmap.org/index.html.en*) indicated that SNP1 is included within another LD block and that SNP2 is located in the middle of the former LD block and in the other three SNPs' LD block. SNP3, SNP4, and SNP5 were located within one haplotype block.

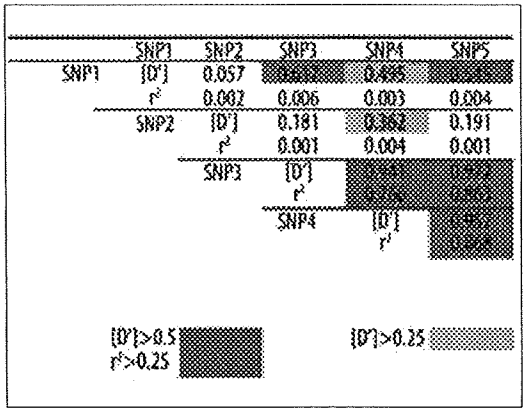

Figure 2. Pair-wise linkage disequilibrium (LD) analysis. Pair-wise LD among the six marker pairs studied in the TP gene was computed along with the LD pairs ($|D'| > 0.5$ or $r^2 > 0.25$), which are shown as the dark gray-shaded values ($|D'| > 0.25$ are shown as light gray-shaded values).

The haplotype-based case-control study was performed using SNP3, SNP4, and SNP5. There was a significant difference in the overall distributions noted for the combination of SNP3-SNP4-SNP5 between the MI and control groups. The G-T-A haplotype (H1) was significantly more frequent in the MI group (88.8%) than in the control group (83.6%). Thus the results suggest that the H1 haplotype might be a susceptibility haplotype for MI. Significant differences in the overall distributions were also shown for the combination of SNP3-SNP4-SNP5 between the CI and control groups. It was assumed that the A-T-A (H3) and A-T-G (H4) haplotypes might be the resistant haplotypes for MI and CI (Table 4). However, since the frequencies of the H3 and H4 haplotypes were very low, careful analysis of the data was required and additional data will need to be collected in the future to definitively prove this relationship.

Furthermore, we have constructed the diplotype for the individuals in each group and performed a multiple logistic regression analysis. After adjustments for BMI, creatinine, total cholesterol, and the incidence of hypertension and di-

Table 4. Haplotype analysis in controls and each study group (MI and CI).

Combination of SNPs	Overall distribution		Distribution of individual haplotypes					
	Chi-square	P value	Haplotype		Frequency		Chi-square	P value
					Control	MI		
SNP3-SNP4-SNP5	19.7	<0.001*	H1	G-T-A	0.836	0.888	6.975	0.008*
			H2	A-C-G	0.136	0.112	1.676	0.196
			H3	A-T-A	0.016	0.000	9.798	0.002#
			H4	A-T-G	0.012	0.000	7.608	0.006#
					Control	CI		
SNP3-SNP4-SNP5	10.2	0.017*	H1	G-T-A	0.836	0.873	2.332	0.127
			H2	A-C-G	0.136	0.127	0.158	0.691
			H3	A-T-A	0.016	0.000	5.498	0.019#
			H4	A-T-G	0.012	0.000	4.267	0.038#

* Shows significant difference for the susceptibility haplotypes of the disease; # shows significant difference for the resistant haplotypes of the disease; MI – myocardial infarction; CI – cerebral infarction.

Table 5. Odds ratios and 95% confidence intervals for each risk factor and haplotype associated with MI.

Risk factor	P value	OR	95% confidence interval	
			Lower limit	Upper limit
BMI	0.000	1.19	1.11	1.28*
Creatinine	0.003	3.03	1.43	6.43*
T-Cho	0.000	0.99	0.98	0.99*
HT	0.199	1.47	0.82	2.65
DM	0.000	24.39	10.00	58.82*
H1 haplotype (homozigote)	0.003	2.03	1.27	3.25*

* Significant difference; BMI – body mass index; T-Cho – total cholesterol; HT – hypertension; DM – diabetes mellitus; OR – odds ratio.

abetes, the frequency of occurrence of the H1 homozygote was found to be significantly higher in the MI patients than in the control group (p=0.003) (Table 5).

Discussion

Atherosclerosis is one of the major causes of MI [24–27]. Atherosclerosis involves multiple processes, including endothelial dysfunction, inflammation, vascular proliferation, and matrix alteration [4]. In addition there is intra-arterial accumulation of varying quantities of intracellular and extracellular lipids, macrophages, T lymphocytes, proteoglycan, collagen, calcium, necrotic debris, and VSMCs [25]. Cheong et al. reported that expression of KCNN4 was associated with growing VSMCs in human neointimal hyperplasia [15].

In this study the frequency of the G/G genotype of SNP3 in the MI group was significantly higher than in the control group, even after adjusting for confounding factors. Furthermore, the H1 haplotype of SNP3-SNP4-SNP5 was significantly more frequent in the MI than in the control groups. These results indicate that there is a susceptibility SNP and haplotype for MI. Based on the diplotype and logistic regression analyses, we succeeded in identifying the susceptibility diplotype (homozygote of H1 haplotype) for MI (Table 5). MI and CI are thought to be multifactorial disorders, which are disorders caused by more than one mutation. Our results indicate that these polymorphisms and haplotypes might very well be genetic markers for MI and that diplotype analysis could be a powerful tool that could be used to isolate these markers. In contrast, no susceptibility markers were found for any of the distributions between the CI and control groups. In our study, the CI group consisted of patients with non-cardioembolic CI, which included atherothrombotic and lacunar infarctions. One possible explanation for our results might be that different mechanisms occur for CI, such as thrombotic infarction or hemodynamically determined infarction [18]. In addition to this, since the CI sample size was smaller than that for MI, there could have been a difference between the CI and the control groups. One of the challenges in the future will be to collect further data to clarify these results definitively. Therefore, the KCNN4 gene polymorphisms are markers of the genetic variant that is associated only with atherosclerotic infarction. Our results provide important insight into the linking of human multifactorial diseases, especially for the genetic variants of MI.

In contrast, there have been some reports that KCNN4 mRNA is not detectable in the human brain [1,13]. Ishii et al. carried out Northern blot analyses and detected KCNN4 mRNA in many peripheral tissues, particularly in the smooth muscle tissues, but never in the brain [1]. The absence of KCNN4 expression in the central nervous system is consistent with other previously published reports [28–30]. However, there are still other reports that differ. Some researchers have used the rat acute subdural hematoma model to show that the edema of injured brain and perilesional tissue reduced water content when KCNN4 blockers were administered [31–33]. Differences in the expression of the KCNN4 gene between the human brain and the brains of other animals might exist and there might also be differences in expression between cerebrovascular and brain tissues. Because there has been no previous research on the association between the KCNN4 gene and CI, further critical and accurate analyses of this area need to be undertaken.

Genetic analyses of complex traits and diseases and population-based gene identification studies are more easily performed with SNPs versus other polymorphisms, such as microsatellite markers. In fact, SNPs with high genomic frequencies are particularly useful for discovering susceptible genes. Moreover, because new SNP alleles arise as mutations at different *loci* and at different points in time, and because they occur in such great abundance compared with genomes, groups of neighboring SNPs may create a haplotypic diversity that can be exploited in direct association studies [34]. Morris et al. found that analyses based on haplotypes have advantages over those based on individual SNPs in genes with multiple susceptibilities, particularly when the linkage disequilibria between the SNPs are weak [35]. We hypothesized that haplotype analysis would be useful for assessing the association between haplotypes and vascular diseases such as MI and CI. Therefore, based on the previous findings, we attempted to establish haplotypes of the KCNN4 gene consisting of three SNPs. In addition to the MI group results, our findings indicated that the overall haplotype distributions for the CI group also differed from the controls (Table 4), even though there were no significant differences observed in a case-control study that used an individual SNP. With regard to the linking of a haplotype, such as SNP3-SNP4-SNP5 in the MI and CI groups, it is assumed that there is a specific mutation involved, and when trying to determine a relationship between a genetic variation and phenotypes, it is theorized that a haplotype-based case-control study provides more information than can be obtained when only using a case-control study that employs a single SNP [36]. In the present study we identified the H3 and H4 haplotypes as the resistant haplotypes for MI and CI. Since these two haplotypes were very rare in the control group, a larger sample size might be needed to replicate and definitively confirm the current results.

There are some limitations associated with the current study. For example, case-control studies sometimes exhibit pseudo-positive results due to sample scales or the selection of the genetic markers. Our results indicated that the KCNN4 genotype and haplotype variations were associated with higher MI risk, even though there was no association noted between the variation and the CI group. Since our results were not always positive, we believe this indicates that our findings are indeed reliable. However, in order to confirm the reliability of the present data, familial linkage studies along with transmission disequilibrium tests need to be performed.

Conclusions

The SNPs and haplotypes in the KCNN4 gene showed that significant differences exist between MI and control patients. The results indicate that these polymorphisms and haplotypes might very well be genetic markers for MI and suggest that KCNN4 or a neighboring gene could possibly act as a susceptibility gene for MI.

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ORIGINAL ARTICLE

The purinergic receptor P2Y, G-protein coupled, 2 (*P2RY2*) gene associated with essential hypertension in Japanese men

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P2RY2 **has an important function in the regulation of blood pressure by activating adenosine triphosphate (ATP). The aim of this study was to investigate the association between the human *P2RY2* gene and essential hypertension (EH) through a haplotype-based case–control study that included two gender groups. The 273 EH patients and 255 age-matched controls were genotyped for five single-nucleotide polymorphisms (SNPs) of the human *P2RY2* gene (rs4944831, rs1783596, rs4944832, rs4382936 and rs10898909). Data were analysed for men and women separately and then as a combined total group. For the total and the men only groups, the genotype distribution of the T allele of rs4944831 and the recessive model (GG vs TG + TT) of rs4944831 differed significantly between the EH patients and controls ($P = 0.028$ and 0.019; $P = 0.009$ and 0.008, respectively). Logistic regression showed that for the total and men groups, the TG + TT genotype of rs4944831 was more prevalent in EH patients than in the controls ($P = 0.026$ and 0.011, respectively). For men, the overall distribution of the haplotype (SNP2-SNP4-SNP5) was significantly different between the EH patients and the controls ($P = 0.006$). As compared with controls, the frequency of the T-A-G haplotype was significantly higher, whereas the T-C-G haplotype was significantly lower for the EH patients ($P = 0.001$ and 0.014, respectively). In conclusion, the present results indicate that rs4944831 and the T-A-G haplotype of the human *P2RY2* gene might be genetic markers for EH in Japanese men.**

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Keywords: *P2RY2*; single-nucleotide polymorphism; haplotype; case–control study; adenosine triphosphate

Introduction

Essential hypertension (EH) is most likely a polygenic disorder that results from the inheritance of a number of susceptibility genes and involves environmental, demographic, vascular and neuroendocrine factors.[1] More than 50 genes have been examined in association studies with hypertension, with this number continuing to grow. A number of polymorphisms in the candidate genes have been associated with differences in blood pressure (BP) level in certain populations and have been of considerable help in understanding the genetics of hypertension.[2] Development of extensive collections of single-nucleotide polymorphisms (SNPs) raises the possibility that these SNPs can be used as markers in genome-wide association mapping studies to identify hypertension susceptibility loci.

Purinergic receptors have been classified as P1 and P2 receptors. At least eight P2 receptor subtypes (P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13 and P2Y14) have been identified[3] in pharmacological and molecular cloning studies. The P2Y2 receptor, which is also referred to as *P2RY2* (official name: purinergic receptor P2Y, G-protein coupled, 2), belongs to the family of G-protein coupled receptors. G-proteins mediate the intracellular effects of many vasoactive and proliferative stimuli. Recently, G-protein signalling was found to be enhanced in cultured cells of various hypertensive subjects.[4] Thus, the purinergic system may be

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similar in importance as the sympathetic and renin–angiotensin–aldosterone (RAA) systems are for cardiovascular regulation and pathophysiology.

P2RY2 is expressed in different regions of the kidney, which includes the vasculature and the glomerulus, in addition to the tubular epithelia and collecting duct system.[5] The epithelial sodium channel (ENaC) in the renal collecting duct is critically involved in the regulation of renal Na^+ reabsorption and K^+ excretion, as well as in the genetic forms of arterial hypertension.[6] Lehrmann *et al.*[7] found that P2PY2 might mediate the adenosine triphosphate (ATP) responses involved in the inhibition of the ENaC-mediated Na^+ reabsorption. Furthermore, a variety of experimental approaches have provided evidence that local ATP release and *P2RY2* stimulation may inhibit water reabsorption in the collecting duct.[8]

Previous studies in mice have identified important functions for *P2RY2* in a variety of processes, including nucleotide-regulated Ca^{2+} signalling in lung fibroblasts and airway epithelial cells,[9] nucleotide-stimulated Cl^- secretion in the trachea and gallbladder,[10] neuronal growth,[11] stimulation of K^+ secretion in the colon[12] and neutrophil chemotaxis.[13] In 2007, Rieg *et al.*[14] reported that salt-resistant hypertension occurs in the P2Y2 receptor (mice homologue of the human *P2RY2* gene) knockout mice because the baroreceptor response to variations in salt intake remains intact. Furthermore, the study showed that the P2Y2 receptor gene has an important function in the regulation of both the BP and the renal reabsorption of Na^+ and fluid in the animal model.

To the best of our knowledge, there have been no previous studies on the association between the human *P2RY2* gene and EH. Therefore, the aim of this study was to investigate the association between the human *P2RY2* gene and EH in Japanese subjects through a haplotype-based case–control study that used SNPs.

Materials and methods

Subjects

Subjects diagnosed with EH were recruited at Nihon University Itabashi Hospital and other neighbouring hospitals in Tokyo from 1993 to 2008. We enrolled 273 EH patients in this study, with a male/female (m/f) ratio of 1.97. EH was diagnosed based on the following criteria: seated systolic BP (SBP) > 160 mm Hg or diastolic BP (DBP) > 100 mm Hg on three occasions within 2 months after the first BP reading. None of the EH patients were receiving antihypertensive medication. Patients diagnosed with secondary hypertension were excluded. A total of 255 normotensive age-matched healthy individuals (m/f ratio = 1.66) were enrolled as controls. None of the controls had a family history of hypertension, and all had an SBP < 130 mm Hg and a DBP < 85 mm Hg. Informed consent was obtained from each subject in accordance with the protocol approved by the Human Studies Committee of Nihon University.[15]

Genotyping

The human *P2RY2* gene is located at chromosome 11q13.5-q14.1, spans approximately 18.1 kilobase pairs and contains three exons. A total of 104 SNPs are listed for the human *P2RY2* gene in the National Center for Biotechnology Information SNP database Build 129 (http://www.ncbi.nlm.nih.gov/SNP). We screened the data for Tag SNPs on the International HapMap Project website (http://www.hapmap.org/index.html.ja) using a cutoff level of $r^2 \geq 0.5$. For the minor allele frequencies, we used a cutoff level of ≤ 0.2. SNPs with relatively high minor allele frequencies have been shown to be very useful as genetic markers for genetic case–control studies.

According to the above criterion, we selected rs4944831 (C-12034890-10, registration number by Applied Biosystems Inc., Foster City, CA, USA), rs1783596 (C-8893575-10), rs4944832 (C-27987464-10), rs4382936 (C-1830488-20) and rs10898909 (C-1830487-10) for this gene (Figure 1). SNP1 was located in intron 1, whereas SNP2 was located in the coding regions of exon 3 of this gene. SNP3, SNP4 and SNP5 were located in the 3′-flanking region of the gene.

Blood samples were collected from all participants, and genomic DNA was extracted from the peripheral blood leucocytes by phenol and chloroform extraction.[16]

Genotyping was performed using the TaqMan SNP Genotyping Assay (Applied Biosystems Inc.). The TaqMan SNP Genotyping Assays were performed using the method of Taq amplification.[17] In the 5′ nuclease assay, discrimination occurs during the polymerase chain reaction (PCR) because of allele-specific fluorogenic probes that, when hybridized to the template, are cleaved by the 5′ nuclease activity

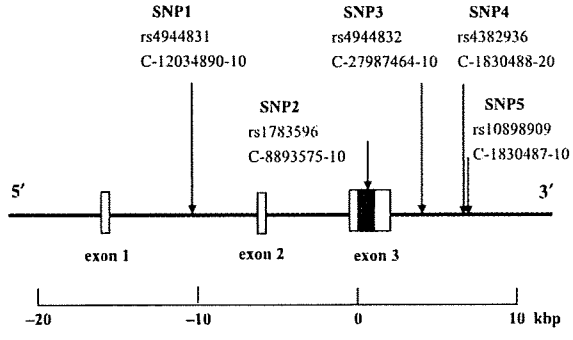

Figure 1 Structure of the human *P2RY2* gene. The gene consists of three exons (boxes) separated by two introns. The lines show introns and intergenic regions. The filled box shows the coding region. The arrows indicate the locations of single-nucleotide polymorphisms (SNPs). kbp, kilo-base pairs.

of the Taq polymerase. The probes contain a 3′ minor groove-binding group that hybridizes to single-stranded targets with greater sequence specificity than ordinary DNA probes. This reduces non-specific probe hybridization, and results in low background fluorescence in the 5′ nuclease PCR assay (TaqMan; Applied Biosystems Inc.). Cleavage results in increased emission of a reporter dye. Each 5′ nuclease assay requires two unlabelled PCR primers and two allele-specific probes. Each probe is labelled with two reporter dyes at the 5′ end. In this study, VIC and FAM were used as the reporter dyes. The primers and probes used in the TaqMan SNP Genotyping Assays (Applied Biosystems Inc.) were chosen based on information available on the Applied Biosystems Inc. website (http://myscience.appliedbiosystems.com).

PCR amplification was performed using 2.5 μl of TaqMan Universal Master Mix, No. AmpErase UNG (2 ×) (Applied Biosystems Inc.) in a 5 μl final reaction volume containing 2 ng DNA, 0.046 μl of TaqMan SNP Genotyping Assay Mix (40 ×), primers at a concentration of 331.2 nmol l^{-1} each, and probes at a final concentration of 73.6 nmol l^{-1} each. Thermal cycling conditions consisted of 95 °C for 10 min and then 50 cycles of 92 °C for 15 s and 60 °C for 1 min in a GeneAmp 9700 system (Applied Biosystems Inc.).

Each 96-well plate contained 80 DNA samples of an unknown genotype and four reaction mixtures containing reagents but no DNA (control). The control samples without DNA are a necessary part of the Sequence Detection System (SDS) 7700 signal processing, as outlined in the TaqMan Allelic Discrimination Guide (Applied Biosystems Inc.). The plates were read on the SDS 7700 instrument with the end-point analysis mode of the SDS version 1.6.3 software package (Applied Biosystems Inc.). The genotypes were determined visually based on the dye-component fluorescent emission data depicted in the X–Y scatter plot of the SDS software. The genotypes were also determined automatically by the signal processing algorithms of the software. The results of each scoring method were saved in two separate output files for later comparison.[18]

Biochemical analysis

We measured the plasma concentration of creatinine and uric acid, using standard methods used by the Clinical Laboratory Department of Nihon University Hospital.[19]

Statistical analysis

All continuous variables were expressed as mean ± s.d. Differences in continuous variables between EH patients and control individuals were analysed using the Mann–Whitney *U*-test. Differences in categorical variables were analysed using a Fisher's exact test. Hardy–Weinberg equilibrium was assessed by χ^2 analysis. Differences in distributions of genotypes and alleles between EH patients and control individuals were analysed using Fisher's exact test. On the basis of the genotype data of the genetic variations, we performed linkage disequilibrium analysis and haplotype-based case–control analysis, using the expectation maximization algorithm[20] and the software SNPAlyze version 3.2 (Dynacom Co, Ltd, Yokohama, Japan).[21] The pairwise linkage disequilibrium analysis was performed using five SNPs. We used $|D'|$ values >0.5 to assign SNP locations to one haplotype block. SNPs with an r^2 value <0.5 were selected as tagged. In the haplotype-based case–control analysis, the frequency distribution of the haplotypes was calculated by a χ^2 analysis. In addition, logistic regression analysis was performed to assess the contribution of the major risk factors. Statistical significance was established at *P*-values <0.05. Statistical analyses were performed using SPSS software for Windows (version 12; SPSS Inc., Chicago, IL, USA).

Results and discussion

Table 1 shows the clinical characteristics of the study participants. For the combined total group, and separated men and women only groups, the following values were significantly higher for the EH patients as compared with the controls: body mass index, SBP, DBP and the incidence of drinking and smoking. For both the total and the men only groups, pulse rate, the incidence of hyperlipidemia and the incidence of diabetes were significantly higher for EH patients as compared with the controls. For men, the uric acid plasma concentration was significantly higher for the EH patients as compared with the controls. There were no significant differences noted for age and serum creatinine between the EH patients and the controls.

Table 2 shows the distribution of the genotypes and alleles for the five SNPs. For each SNP, the genotype distributions were not significantly different from the Hardy–Weinberg equilibrium values (data not shown) in the controls. For both the total and men only groups, the genotype distribution of rs4944831 differed significantly between the EH patients and the controls ($P=0.028$ and 0.019, respectively). The distribution of the recessive model of rs4944831 (GG vs TG + TT) also differed significantly for the total and men only groups between the EH patients and the controls ($P=0.009$ and 0.008, respectively). Dominant and recessive models were defined by the frequencies noted among the total control individuals. There were no significant differences in overall distributions of the frequencies in the alleles for any of the five SNPs between the EH and control groups.

Table 3 shows the results of the logistic regression analysis. Logistic regression was performed using

Table 1 Characteristics of study participants

	Total			*Men*			*Women*		
	EH patients	*Controls*	P-*value*	*EH patients*	*Controls*	P-*value*	*EH patients*	*Controls*	P-*value*
Number of subjects	273	255		181	159		92	96	
Age (years)	50.2 ± 6.3	51.4 ± 10.0	0.093	49.6 ± 6.8	51.0 ± 6.1	0.051	51.3 ± 5.3	52.1 ± 14.3	0.619
Body mass index (kg/m^2)	24.6 ± 3.6	22.6 ± 3.2	<0.001*	24.8 ± 3.3	22.9 ± 3.2	<0.001*	24.3 ± 4.0	22.2 ± 3.3	<0.001*
Systolic blood pressure (mm Hg)	173.9 ± 20.6	112.0 ± 10.8	<0.001*	171.8 ± 19.8	112.5 ± 10.6	<0.001*	178.1 ± 21.6	111.3 ± 11.1	<0.001*
Diastolic blood pressure (mm Hg)	107.1 ± 13.7	69.1 ± 8.6	<0.001*	107.8 ± 14.0	69.9 ± 8.2	<0.001*	105.8 ± 13.0	67.7 ± 9.2	<0.001*
Pulse rate (beats min^{-1})	77.3 ± 14.4	73.4 ± 13.0	0.005*	77.1 ± 14.8	73.0 ± 14.2	0.027*	77.8 ± 13.6	74.0 ± 10.9	0.076
Creatinine (mg per 100 ml)	0.9 ± 0.3	0.8 ± 0.2	0.143	0.9 ± 0.2	0.9 ± 0.2	0.178	0.7 ± 0.2	0.7 ± 0.1	0.799
Uric acid (mg per 100 ml)	5.7 ± 1.6	5.6 ± 4.5	0.813	6.2 ± 1.5	5.8 ± 1.4	0.010*	4.6 ± 1.2	5.3 ± 7.1	0.388
Hyperlipidmia (%)	26	18	0.022*	23	14	0.041*	33	24	0.188
Diabetes (%)	10	3	0.003*	11	3	0.008*	8	3	0.171
Drinking (%)	63	38	<0.001*	76	48	<0.001*	38	22	0.015*
Smoking (%)	53	28	<0.001*	64	35	<0.001*	33	16	0.006*

Abbreviation: EH, essential hypertension.
Continuous variables were expressed as mean ± standard deviation. Categorical variables were expressed as percentage. The *P*-value of the continuous variables was calculated by a Mann–Whitney *U*-test. The *P*-value of the categorical variables was calculated by Fisher's exact test.
*$P<0.05$

the following parameters: TG+TT genotype of rs4944831, body mass index, pulse rate, with or without hyperlipidemia, diabetes mellitus and smoking or drinking. The results indicated that the TG+TT genotype of rs4944831 was significantly higher in the EH patients vs the control individuals for the total and men only groups ($P=0.026$ and 0.011, respectively). At the same time, for the total and men only groups, the body mass index, pulse rate and the use of alcohol still differed significantly between the EH patients and controls. There was also a significant difference noted for smoking between the EH patients and the controls in the men only group.

Table 4 shows patterns of linkage disequilibrium in control individuals for the *P2RY2* gene, with their $|D'|$ and r^2 values. Out of the five SNPs, four (SNP2, SNP3, SNP4 and SNP5) had $|D'|$ values that were beyond 0.5, which means they were all located within one haplotype block. SNP1 was the only exception, as the results indicated that it was located in a different haplotype block. As the r^2 values for SNP2-SNP3 were >0.5, simply having at least one SNP made it possible to perform the haplotype-based case–control study. Therefore, given that the minor allele frequency of SNP2 was larger than that observed for SNP3, we constructed the haplotypes using SNP2, SNP4 and SNP5.

For the haplotype-based case–control study, there were four combinations, which included SNP2-SNP4, SNP2-SNP5, SNP4-SNP5 and SNP2-SNP4-SNP5 (Table 5). For men, the overall distribution of the haplotype established by SNP2, SNP4 and SNP5 was significantly different between the EH patients and the controls ($P=0.006$). For the total group, the frequency of the T-C-G haplotype (SNP2-SNP4-SNP5) was significantly lower for the EH patients as compared with the controls ($P=0.020$). For men, both the frequency of the T-A haplotype (SNP2-SNP4) and T-A-G haplotype (SNP2-SNP4-SNP5) were significantly higher for the EH patients as compared with the controls ($P=0.043$ and 0.014, respectively). In contrast, the frequency of the T-C-G haplotype (SNP2-SNP4-SNP5) was significantly lower for the EH patients as compared with the controls ($P=0.001$).

In 1994, Parr *et al.*[22] were the first to report the sequence and functional expression of the cDNA cloned from the airway epithelial cells that encoded *P2RY2*, which is a protein with properties of the P2U nucleotide receptor. In 1996, Dasari *et al.*[23] were the first to perform PCR analysis of the human-rodent hybrid cell line DNAs, and this led to the mapping of the human *P2RY2* gene to chromosome 11q13.5-q14.1. *P2RY2* belongs to the family of the G-protein coupled receptors. This family has several receptor subtypes with different pharmacological selectivity. *P2RY2* may participate in various types of physiological responses, including secretion, membrane potential, cell proliferation, platelet aggregation, neurotransmission, cardiac function and muscle contraction[24] by activating ATP. ATP is released from cells such as the platelets and co-released with neurotransmitter granules from the autocrine nerves by exocytosis.[25] Extracellular ATP binds to *P2RY2*, with the combination activating phospholipase C and phosphatidylinositol hydrolysis. This can then generate diacylglycerol and inositol 1,4,5-triphosphate, which stimulate protein kinase C and cytosolic calcium (Ca^{2+}) mobilization, respectively.[26]

Guyton[27] proposed that all types of hypertension have impaired renal Na^+ excretion and thus, a rise in the BP stabilizes the Na^+ homeostasis by increasing renal Na^+ excretion through pressure natriuresis. The mechanism for the regulation of ENaC by *P2RY2* proposed by Kunzelmann *et al.*[28] states that under resting conditions, the inner leaflet of the lipid bilayer contains a high concentration of

Table 2 Genotype and allele distributions in patients with EH and in controls

	Total			*Men*			*Women*		
	EH patients	*Controls*	P-*value*	*EH patients*	*Controls*	P-*value*	*EH patients*	*Controls*	P-*value*
rs4944831 (SNP1)									
Genotype									
T/T	176 (64.5%)	163 (63.9%)	0.028*	117 (64.6%)	104 (65.4%)	0.019*	59 (64.1%)	59 (61.5%)	0.725
T/G	92 (33.7%)	76 (29.8%)		61 (33.7%)	43 (27.0%)		31 (33.7%)	33 (34.3%)	
G/G	5 (1.8%)	16 (6.3%)		3 (1.7%)	12 (7.6%)		2 (2.2%)	4 (4.2%)	
Dominant model									
TT	176 (64.5%)	163 (63.9%)	0.896	117 (64.6%)	104 (65.4%)	0.882	59 (64.1%)	59 (61.5%)	0.705
TG+GG	97 (35.5%)	92 (36.1%)		64 (35.4%)	55 (34.6%)		33 (35.9%)	37 (38.5%)	
Recessive model									
GG	5 (1.8%)	16 (6.3%)	0.009*	3 (1.7%)	12 (7.6%)	0.008*	2 (2.2%)	4 (4.2%)	0.437
TG+TT	268 (98.2%)	239 (93.7%)		178 (98.3%)	147 (92.4%)		90 (97.8%)	92 (95.8%)	
Allele									
T	444 (81.3%)	402 (78.8%)	0.310	295 (81.5%)	251 (78.9%)	0.402	149 (81.0%)	151 (78.6%)	0.573
G	102 (18.7%)	108 (21.2%)		67 (18.5%)	67 (21.1%)		35 (19.0%)	41 (21.4%)	
rs1783596 (SNP2)									
Genotype									
T/T	87 (31.9%)	78 (30.6%)	0.948	60 (33.2%)	50 (31.5%)	0.939	27 (29.3%)	28 (29.2%)	0.998
T/C	137 (50.2%)	131 (51.4%)		88 (48.6%)	80 (50.3%)		49 (53.3%)	51 (53.1%)	
C/C	49 (17.9%)	46 (18.0%)		33 (18.2%)	29 (18.2%)		16 (17.4%)	17 (17.7%)	
Dominant model									
TT	87 (31.9%)	78 (30.6%)	0.751	60 (33.2%)	50 (31.5%)	0.738	27 (29.3%)	28 (29.2%)	0.978
TC+CC	186 (68.1%)	177 (69.4%)		121 (66.8%)	109 (68.5%)		65 (70.7%)	68 (70.8%)	
Recessive model									
CC	49 (17.9%)	46 (18.0%)	0.978	33 (18.2%)	29 (18.2%)	0.999	16 (17.4%)	17 (17.7%)	0.954
TC+TT	224 (82.1%)	209 (82.0%)		148 (81.8%)	130 (81.8%)		76 (82.6%)	79 (82.3%)	
Allele									
T	311 (57.0%)	287 (56.3%)	0.822	208 (57.5%)	180 (56.6%)	0.822	103 (56.0%)	107 (55.7%)	0.961
C	235 (43.0%)	223 (43.7%)		154 (42.5%)	138 (43.4%)		81 (44.0%)	85 (44.3%)	
rs4944832 (SNP3)									
Genotype									
G/G	109 (39.9%)	101 (39.6%)	0.700	69 (38.1%)	63 (39.6%)	0.625	40 (43.5%)	38 (39.6%)	0.842
G/A	133 (48.7%)	119 (46.7%)		90 (49.7%)	72 (45.3%)		43 (46.7%)	47 (48.9%)	
A/A	31 (11.4%)	35 (13.7%)		22 (12.2%)	24 (15.1%)		9 (9.8%)	11 (11.5%)	
Dominant model									
GG	109 (39.9%)	101 (39.6%)	0.940	69 (38.1%)	63 (39.6%)	0.777	40 (43.5%)	38 (39.6%)	0.588
GA+AA	164 (60.1%)	154 (60.4%)		112 (61.9%)	96 (60.4%)		52 (56.5%)	58 (60.4%)	
Recessive model									
AA	31 (11.4%)	35 (13.7%)	0.411	22 (12.2%)	24 (15.1%)	0.429	9 (9.8%)	11 (11.5%)	0.710
GA+GG	242 (88.6%)	220 (86.3%)		159 (87.8%)	135 (84.9%)		83 (90.2%)	85 (88.5%)	
Allele									
G	351 (64.3%)	321 (62.9%)	0.650	228 (63.0%)	198 (62.3%)	0.847	123 (66.8%)	123 (64.1%)	0.570
A	195 (35.7%)	189 (37.1%)		134 (37.0%)	120 (37.7%)		61 (33.2%)	69 (35.9%)	
rs4382936 (SNP4)									
Genotype									
C/C	150 (54.9%)	133 (52.2%)	0.806	96 (53.0%)	85 (53.5%)	0.936	54 (58.7%)	48 (50.0%)	0.386
C/A	102 (37.4%)	102 (40.0%)		67 (37.0%)	60 (37.7%)		35 (38.0%)	42 (43.7%)	
A/A	21 (7.7%)	20 (7.8%)		18 (10.0%)	14 (8.8%)		3 (3.3%)	6 (6.3%)	
Dominant model									
CC	150 (54.9%)	133 (52.2%)	0.521	96 (53.0%)	85 (53.5%)	0.938	54 (58.7%)	48 (50.0%)	0.232
CA+AA	123 (45.1%)	122 (47.8%)		85 (47.0%)	74 (46.5%)		38 (41.3%)	48 (50.0%)	
Recessive model									
AA	21 (7.7%)	20 (7.8%)	0.948	18 (10.0%)	14 (8.8%)	0.720	3 (3.3%)	6 (6.3%)	0.337
CA+CC	252 (92.3%)	235 (92.2%)		163 (90.0%)	145 (91.2%)		89 (96.7%)	90 (93.7%)	
Allele									
C	402 (73.6%)	368 (72.2%)	0.591	259 (71.5%)	230 (72.3%)	0.821	143 (77.7%)	138 (71.9%)	0.193
A	144 (26.4%)	142 (27.8%)		103 (28.5%)	88 (27.7%)		41 (22.3%)	54 (28.1%)	
rs10898909 (SNP5)									
Genotype									
G/G	81 (29.7%)	82 (32.2%)	0.757	60 (33.1%)	55 (34.6%)	0.251	21 (22.8%)	27 (28.1%)	0.275
G/A	127 (46.5%)	118 (46.3%)		76 (42.0%)	76 (47.8%)		51 (55.4%)	42 (43.8%)	
A/A	65 (23.8%)	55 (21.6%)		45 (24.9%)	28 (17.6%)		20 (21.7%)	27 (28.1%)	

Table 2 Continued

	Total			*Men*			*Women*		
	EH patients	*Controls*	P-*value*	*EH patients*	*Controls*	P-*value*	*EH patients*	*Controls*	P-*value*
Dominant model									
GG	81 (29.7%)	82 (32.2%)	0.537	60 (33.1%)	55 (34.6%)	0.779	21 (22.8%)	27 (28.1%)	0.405
GA+AA	192 (70.3%)	173 (67.8%)		121 (66.9%)	104 (65.4%)		7 (77.2%)	69 (71.9%)	
Recessive model									
AA	65 (23.8%)	55 (21.6%)	0.539	45 (24.9%)	28 (17.6%)	0.104	20 (21.7%)	27 (28.1%)	0.312
GA+GG	208 (76.2%)	200 (78.4%)		136 (75.1%)	131 (82.4%)		72 (78.3%)	69 (71.9%)	
Allele									
G	289 (52.9%)	282 (55.3%)	0.441	196 (54.1%)	186 (58.5%)	0.254	93 (50.5%)	96 (50.0%)	0.916
A	257 (47.1%)	228 (44.7%)		166 (45.9%)	132 (41.5%)		91 (49.5%)	96 (50.0%)	

Abbreviation: EH, essential hypertension.
The *P*-value of the genotype was calculated by Fisher's exact test. $^*P<0.05$.

Table 3 Odds ratios and 95% confidence intervals for each risk factor and the TG+TT genotype of rs4944831 associated with essential hypertension in the combined total and men only groups

Risk factor	*Total*			*Men*		
	Odd ratios	*95% confidence interval*	P-*value*	*Odd ratios*	*95% confidence interval*	P-*value*
TG+TT genotype	4.868	1.204–19.686	0.026*	16.86	1.934–146.950	0.011*
Body mass index	1.171	1.087–1.261	0.000*	1.134	1.031–1.247	0.010*
Pulse rate	1.038	1.018–1.060	0.000*	1.042	1.015–1.068	0.002*
Hyperlipidemia	0.676	0.387–1.180	0.168	0.730	0.345–1.542	0.409
Diabetes mellitus	0.614	0.224–1.682	0.342	0.413	0.112–1.512	0.181
Smoking	0.671	0.414–1.087	0.105	1.961	1.075–3.584	0.028*
Drinking	1.773	1.075–2.924	0.025*	2.392	1.190–4.808	0.014*

$^*P<0.05$.

Table 4 Pairwise linkage disequilibrium for the five SNPs in control individuals

		|D'| value				
	SNP	*SNP1*	*SNP2*	*SNP3*	*SNP4*	*SNP5*
r^2 value	SNP1		0.066	0.089	0.025	0.254
	SNP2	0.001		0.832	0.549	0.721
	SNP3	0.001	0.325		0.430	0.827
	SNP4	0.000	0.150	0.121		0.779
	SNP5	0.014	0.326	0.409	0.189	

Abbreviation: SNP, single-nucleotide polymorphism.
$|D'|>0.5$.
$r^2>0.5$.

phosphatidylinositol-bisphosphate, with the latter then binding the N-terminus of β-ENaC. Stimulation of *P2RY2* activates phospholipase C, which hydrolyses and lowers the concentration of phosphatidylinositol-bisphosphate with resultant decreases in the ENaC activity achieved by lowering the open probability. Rieg *et al.*[14] indicated in a recent animal study that the *P2RY2* gene was a candidate causative gene of hypertension. The study showed that there was a greater reabsorption of water in the collecting duct and that Na^+ through ENaC could potentially cause an initial induction of isotonic hyperreabsorption in P2Y2 receptor knockout mice, resulting in a salt-resistant hypertension. The aim of our current animal study was to assess the association between the *P2RY2* gene and EH by using a haplotype-based case–control study. Our results showed that the genotypic distribution of the T allele of rs4944831 and the recessive model (GG vs TG + TT) of rs4944831 significantly differed between the EH patients and the controls for both the total and men only groups. The present results also indicated that the risk of EH is increased in subjects with the T allele of rs4944831 and that the recessive model of rs4944831 is associated with EH in Japanese men. The logistic regression analyses indicated that for both the total and the men only groups, the TG + TT genotype distribution of rs4944831 was significantly different between the EH patients and the controls. This suggests that the SNP is most probably associated with gender-specific hypertension. We also showed that body mass index, pulse rate and alcohol consumption were risk factors associated with EH for both the total and men only groups. This study also showed that smoking was a risk factor of EH, especially in men.

Table 5 Haplotype analysis in patients with EH and in the controls

Haplotype				*Overall* P-*value*			*Frequency in total*		P-*value*	*Frequency in men*		P-*value*	*Frequency in women*		P-*value*
				Total	*Men*	*Women*	*EH patients*	*Controls*		*EH patients*	*Controls*		*EH patients*	*Controls*	
	SNP2	SNP4		0.300	0.115	0.295									
H1	T	C					0.481	0.492	0.689	0.464	0.501	0.350	0.520	0.476	0.354
H2	C	C					0.256	0.229	0.307	0.251	0.222	0.391	0.257	0.243	0.812
H3	T	A					0.089	0.071	0.253	0.110	0.065	0.043*	0.040	0.082	0.067
H4	C	A					0.175	0.208	0.161	0.174	0.212	0.225	0.183	0.200	0.746
	SNP2		SNP5	0.864	0.650	0.999									
H1	T		G				0.155	0.170	0.512	0.161	0.190	0.328	0.143	0.135	0.869
H2	C		G				0.374	0.383	0.770	0.380	0.395	0.689	0.363	0.365	0.993
H3	T		A				0.415	0.392	0.471	0.413	0.376	0.325	0.417	0.422	0.947
H4	C		A				0.056	0.055	0.895	0.045	0.039	0.672	0.077	0.078	0.941
		SNP4	SNP5	0.774	0.352	0.475									
H1		C	G				0.288	0.302	0.608	0.282	0.341	0.103	0.299	0.239	0.195
H2		A	G				0.242	0.251	0.728	0.260	0.244	0.667	0.206	0.261	0.217
H3		C	A				0.449	0.420	0.340	0.434	0.382	0.186	0.478	0.480	0.986
H4		A	A				0.022	0.027	0.566	0.025	0.033	0.603	0.017	0.020	0.745
	SNP2	SNP4	SNP5	0.088	0.006*	0.608									
H1	T	C	G				0.078	0.120	0.020*	0.063	0.145	0.001*	0.113	0.076	0.236
H2	C	C	G				0.210	0.181	0.216	0.220	0.194	0.405	0.186	0.163	0.550
H3	T	A	G				0.077	0.050	0.063	0.097	0.046	0.014*	0.029	0.059	0.148
H4	C	A	G				0.165	0.202	0.119	0.162	0.200	0.164	0.177	0.202	0.558
H5	T	C	A				0.401	0.373	0.341	0.399	0.357	0.292	0.406	0.402	0.897
H6	C	C	A				0.047	0.048	0.966	0.034	0.027	0.715	0.072	0.078	0.783
H7	T	A	A				0.014	0.020	0.534	0.015	0.018	0.821	0.012	0.020	0.441
H8	C	A	A				0.008	0.006	0.773	0.010	0.013	0.854	0.005	0.000	1.000

Abbreviation: EH, essential hypertension.
Haplotypes were estimated using software SNPAlyze (version 3.2; Dynacom Co., Ltd., Yokohama, Japan). *P*-value was calculated by χ^2 analysis. *$P<0.05$.

Morris and Kaplan[29] found that for genes with multiple susceptibilities, analyses based on haplotypes have advantages over analyses based on individual SNPs, particularly when linkage disequilibria between the SNPs are weak. Although there were no significant differences in the overall distributions of the frequencies in the alleles for any of the five SNPs between the EH and control groups, we were able to successfully establish haplotypes for the *P2RY2* gene from the different combinations that were created from the three selected SNPs in this study. In the present haplotype analysis, there was a significant difference between the EH patients and the controls with regard to the frequency of the T-C-G haplotype (SNP2-SNP4-SNP5) in the total group and for the T-A haplotype (SNP2-SNP4) in the men only group ($P=0.020$ and 0.043, respectively). However, we believe that these differences are not indicative of the true situation, as the overall distribution of the T-C-G haplotype established by SNP2, SNP4, SNP5 and the T-A haplotype established by SNP2, SNP4 were not significantly different between the EH patients and the controls ($P=0.088$ and 0.115, respectively). In this study, the frequency of the T-A-G haplotype (SNP2-SNP4-SNP5) for men was significantly higher for the EH patients as compared with the controls ($P=0.014$). Therefore, this haplotype can be regarded as being a susceptibility haplotype for EH in Japanese men. In contrast, the frequency of the T-C-G haplotype (SNP2-SNP4-SNP5) was significantly lower for EH patients as compared with the controls ($P=0.001$), and thus can be regarded as a resistance haplotype for EH in Japanese men.

Some case–control studies have identified gene variants associated with gender-specific susceptibility to EH.[30,31] The present haplotype-based case–control study also showed gender-specific significant differences (for men only). *P2RY2* is one of the G-protein coupled receptors that have a high affinity for ATP. *P2RY2* has a function in a number of responses through the activation of ATP. Foresta *et al.*[32] found that rat Leydig cells possess P2-purinergic receptors and that activation of these receptors by extracellular ATP stimulates testosterone secretion. There are some studies[33,34] that have shown that testosterone may have an important function in regulating BP through a complex interaction. Abnormal plasma concentrations of testosterone may result in BP elevation in males. Unfortunately, there are no previous studies that have reported data on the different BP levels that exist between the male and female *P2RY2* knockout mice.

P2RY2 has a wide tissue distribution[35] and it has an important function in the regulation of BP. Although at the present time, there are no methods that can be used to measure the function of *P2RY2* in the human body by simply using blood or urine, methods do exist for detecting the function of *P2RY2 in vitro* by using dissected organs or tissues of animals. These detection methods include quantitative real-time PCR, western blot or immunoblotting, and immunohistochemistry. Unfortunately, because of the ethical aspects of obtaining such samples in humans, we were unable to conduct functional analyses of this gene in this study.

In conclusion, this study examined the association between the human *P2RY2* gene and EH. Our results indicate that rs4944831 and the T-A-G haplotype of the human *P2RY2* gene might be genetic markers for EH, and that the T-C-G haplotype may be a protective genetic marker for EH in Japanese men. Further studies are needed to isolate the functional mutations in the *P2RY2* gene that regulate the BP, and to evaluate the function of the *P2RY2* variants that are involved in the metabolism of sex hormones.

What is known about this topic?

- *P2RY2* has an important function in the regulation of blood pressure and the renal reabsorption of Na^+ through the ENaC channel by the activation of ATP.
- The purinergic system may be similar in importance as the sympathetic and renin–angiotensin–aldosterone systems are for cardiovascular regulation and pathophysiology.

What this study adds?

- rs4944831 and the T-A-G haplotype of the human *P2RY2* gene could be genetic markers for EH, and the T-C-G haplotype might be a protective genetic marker for EH in Japanese men.
- The present haplotype-based case–control study showed gender-specific significant differences (for men only), which may be associated with testosterone secretion.

Conflict of interest

The authors declare no conflict of interest.

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ORIGINAL ARTICLE

Haplotype-based case–control study of receptor (calcitonin) activity-modifying protein-1 gene in cerebral infarction

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Calcitonin gene-related peptide (CGRP) receptor is a complex molecule that consists of calcitonin receptor-like receptor and receptor activity-modifying protein-1 (RAMP1). It was recently reported that RAMP1-deficient mice (RAMP1(−/−)) showed inflammatory responses with a transiently significant increase in serum CGRP levels and proinflammatory cytokines when compared with RAMP1(+/+) mice. The aim of this study was to investigate the relationship between the human RAMP1 gene and cerebral infarction (CI) using single-nucleotide polymorphisms (SNPs) in a Japanese population. We selected six SNPs in the human RAMP1 gene (rs3754701, rs3769048, rs7557078, rs1584243, rs10199956 and rs7590387) and performed a case–control study using each SNP and haplotype in 171 CI patients and 234 controls. There were no significant differences in overall distribution of genotype and allele frequencies of the SNPs between the CI and control groups. However, there was a significant difference in overall distribution between the CI and control groups ($P<0.001$) in the haplotype-based case–control study with the combinations of rs3754701–rs3769048–rs7590387. The T-A-C susceptibility haplotype for CI was significantly more frequent than in the control group ($P=0.0024$). The results suggest that the T-A-C haplotype is a genetic marker for CI, and that RAMP1 or neighbouring genes are associated with increased susceptibility to CI.

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Keywords: RAMP1; single-nucleotide polymorphism; haplotype; case–control study

Introduction

Cerebral infarction (CI) is thought to be a heterogeneous multifactorial disease associated with several environmental factors and genetic variants. Factors such as high blood pressure, cigarette smoking, diabetes mellitus, arterial diseases and atrial fibrillation can lead to the development of CI. Identification of susceptibility genes for CI might enhance the prediction of risk for the disease. In addition, it has been reported that inflammatory reactions are associated with CI, particularly as interleukin-1, interleukin-6, tumour necrosis factor-α, transforming growth factor-β, interleukin-10, interferon-γ and granulocyte-colony-stimulating factor are produced in the acute phase of CI.[1–3]

Calcitonin gene-related peptide (CGRP) is a neuroimmune modulator related to cardiovascular regulation.[4] CGRP signalling is expressed through the CGRP receptor, which is composed of calcitonin receptor-like receptor (CRLR) and receptor activity-modifying protein-1 (RAMP1).[5] Furthermore, αCGRP is known to have the most potent pharmacological vasodilatory activity.[6] Tsujikawa *et al.* reported that RAMP1-deficient mice (RAMP1(−/−)) showed increased inflammatory responses and high serum levels of proinflammatory cytokines when compared with RAMP1(+/+) mice. In addition, αCGRP and βCGRP appear to equally suppress the production of tumour necrosis factor-α and interleukin-12 in bone marrow-derived dendritic cells stimulated with lipopolysaccharide.[7] Um *et al.* reported that the expression of IL-1 in the brain was increased during the early and chronic stage of CI.[8]

The aim of this study was to investigate the relationship between CI and human RAMP 1 gene

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using single-nucleotide polymorphisms (SNPs) and haplotypes in a Japanese population.

Materials and methods

Subjects

Subjects diagnosed with CI were recruited at Nihon University Itabashi Hospital and other neighbouring hospitals in Tokyo between 1993 and 2003. This study included a group of 171 patients who were diagnosed with CI. Control subjects were selected from among the outpatients at our hospital during the same period. The study group consisted of 171 patients (101 men and 70 women; mean age 65.8 ± 13.1 years) diagnosed with CI by computed tomography or magnetic resonance imaging. All patients had neurological deficits that persisted for >1 month. A total of 234 subjects without CI (113 men and 121 women; mean age 77.7 ± 4.1 years) were used as control subjects. Control subjects had vascular risk factors, such as hypertension and hypercholesterolaemia, but no cerebrovascular disease. Diagnosis of diabetes mellitus was based on the World Health Organization (WHO) criteria. Hypercholesterolaemia was defined as plasma total cholesterol >220 mg per 100 ml, or current use of a lipid-lowering drug in addition to a confirmed diagnosis of hypercholesterolaemia. Smokers were defined as current or former smokers, whereas nonsmokers were defined as subjects with no history of previous or current smoking. History of smoking was recorded and current smokers included individuals who had stopped smoking <1 year before enrolment. Daily alcohol intake was assessed by interview. The frequency of drinking during a typical week and the alcohol intake on each occasion were determined and used to calculate the alcohol intake per week, which was then divided by 7 to obtain the mean alcohol intake per day. Subjects were asked to estimate their alcohol intake on the basis of *gou* (180 ml), which is a traditional Japanese drinking unit; 1 *gou* of Japanese Sake contains 20 g of ethanol, while a similar amount (180 ml) of Japanese Shochu contains 50 g of ethanol, a standard bottle of beer (550 ml) contains 22 g of ethanol, 2 single whiskeys (60 ml) contain 20 g of ethanol and 120 ml of wine contains 12 g of ethanol. Both groups were recruited from the northern area of Tokyo, and informed consent was obtained from each individual, as per the protocol approved by the Human Studies Committee of Nihon University.[9]

Genotyping

On the basis of the allelic frequency data for the registered SNPs from the National Center for Biotechnology Information (NCBI) website and from the Applied Biosystems Inc. (Foster City, CA, USA) -Celera Discovery System, we selected SNPs with minor allele frequencies of 20% or greater. This criterion was selected because SNPs with a high minor allele frequency are very useful as genetic markers in genetic association studies. We thus selected six SNPs in the human RAMP1 gene as markers for the genetic association experiment.

The minor allele frequencies for each SNP among the Japanese subjects were >10% (screening estimate from the Celera Company), which indicates that they should all be effective genetic markers. All SNPs were confirmed using the dbSNP on the NCBI website and the Applied Biosystems-Celera Discovery System.

Accession numbers were as follows: rs3754701 (C_27496443_10); rs3769048 (C_9091474_10); rs7557078 (C_2149706_10); rs1584243(C_2149746_10); rs10199956 (C_30171509_10); and rs7590387 (C_26481962_10). All SNPs were located in introns (Figure 1). Blood samples were collected from all participants, and genomic DNA was extracted from peripheral blood leukocytes by phenol and chloroform extraction.[10] Genotyping was performed using the TaqMan SNP Genotyping Assay (Applied Biosystems). TaqMan SNP Genotyping Assays were performed using the method for Taq amplification.[11]

On the 5′-nuclease assay, discrimination occurs during PCR due to allele-specific fluorogenic probes that, when hybridized to the template, are cleaved by the 5′-nuclease activity of Taq polymerase. The probes contain a 3′-minor groove-binding group that

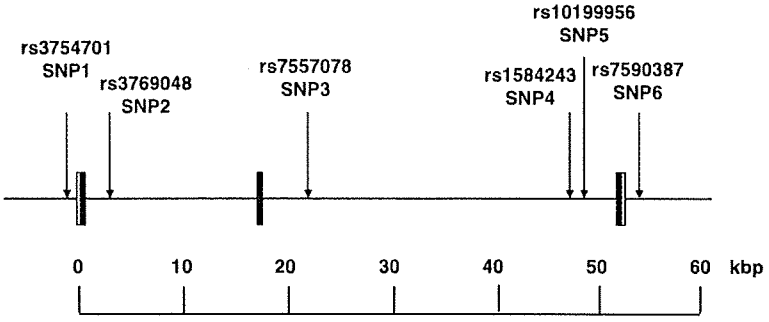

Figure 1 Organization of the gene encoding human RAMP1, and the location of single-nucleotide polymorphisms (SNP) used in this association study. Open and closed boxes indicate untranslated regions and translated regions in exons, respectively. Lines indicate introns.

hybridizes to single-stranded targets with greater sequence specificity than ordinary DNA probes. This reduces nonspecific probe hybridization, and results in low background fluorescence on 5′-nuclease PCR assay (TaqMan, Applied Biosystems). Cleavage results in increased emission of reporter dye. Each 5′-nuclease assay requires two unlabelled PCR primers and two allele-specific probes. Each probe is labelled with two reporter dyes at the 5′-end. In this study, VIC and FAM were used as reporter dyes. Primers and probes used in TaqMan SNP Genotyping Assays (Applied Biosystems) were selected on the basis of the information available at the Applied Biosystems website (http://myscience.appliedbiosystems.com).

PCR amplification was performed using 6 μl of TaqMan Universal Master mixand No AmpErase UNG (2 ×) (Applied Biosystems) in a 12-μl final reaction volume containing 2 ng of DNA, 0.22 μl of TaqMan SNP Genotyping Assay mix (20 × or 40 ×), primers at a concentration of 900 nmol l^{-1} each, and probes at a final concentration of 200 nmol l^{-1} each. Thermal cycling conditions were as follows: 50 °C for 2 min; 95 °C for 10 min; 40 cycles of 95 °C for 15 s; and 62 °C for 1 min. Thermal cycling was performed using the GeneAmp 9700 system (Applied Biosystems Inc.). Each 96-well plate contained 80 DNA samples of an unknown genotype and four reaction mixtures containing reagents but no DNA (control). Control samples without DNA are a necessary part of Sequence Detection System (SDS) 7700 signal processing, as outlined in the TaqMan Allelic Discrimination Guide (Applied Biosystems). Plates were read on the SDS 7700 instrument with the end point analysis mode of the SDS version 1.6.3 software package (Applied Biosystems). Genotypes were determined visually on the basis of the dye-component fluorescent emission data depicted in the X-Y scatter-plot of the SDS software. Genotypes were also determined automatically by the signal processing algorithms of the software. The results for each scoring method were saved in two separate output files for later comparison.[12]

We performed haplotype analysis on five SNPs. On the basis of the genotype data of the four genetic variations, the frequency of each haplotype was estimated using the expectation/maximization algorithm.[13] For linkage disequilibrium analysis and haplotype-based case–control study, SNPAlyze version 3.2 was used (Dynacom Co. Ltd, Yokohama, Japan), which is available at http://www.dynacom.co.jp/products/package/snpalyze/index.html.

Statistical analysis

Data are shown as means ± s.d. Differences between the CI and control groups were assessed by analysis of variance, followed by Fisher's protected least significant difference test. Hardy–Weinberg equilibrium was assessed by χ^2-analysis. When the sizes of the expected values were small (<2.0), genotypes were combined.[14] The overall distribution of the SNP alleles was analyzed by 2 × 2 contingency tables, and the distribution of the SNP genotypes between the CI patients and controls was tested using a two-sided Fisher's exact test and multiple logistic regression analysis. Statistical significance was established at $P<0.05$. The threshold value of the frequencies of the haplotypes included in the analysis was set to $1/2n$ (n is the numbers of subjects in each group), as suggested by Excoffier and Slatkin.[15] All haplotypes below the threshold value were excluded from analysis. Overall distribution of haplotypes was analyzed using 2 × m contingency tables with a value of $P<0.05$ considered to indicate statistical significance. The P-value significance of each haplotype was determined by χ^2-analysis and the permutation method using the software SNPAlyze version 3.2.[14]

Results

The clinical features for CI patients and the control group are shown in Table 1. Systolic blood pressure and diastolic blood pressure, age, pulse rate, serum concentrations of creatinine and frequencies of smoking, drinking and diabetes mellitus were significantly higher in the CI group, as compared with the control group. Body mass index did not significantly differ between the two groups.

The distribution of genotypes and alleles of the six SNPs is shown in Table 2. Because the genotype distribution of SNP5 in the controls was significantly different from the Hardy–Weinberg equilibrium values ($P=0.0007$), we excluded SNP5 from the statistical analysis. Thus, there were no significant differences in overall distributions of frequencies in genotypes and alleles for any of the six SNPs between the CI and control groups.

On linkage disequilibrium analysis, we omitted SNP5 because its genotype distribution in the control group showed significant differences from the Hardy–Weinberg equilibrium values. As most values for |D′| between each pair of SNPs were beyond 0.5, all five SNPs are located in one haplotype block (data not shown), and because the r^2 values of all combinations were below 0.5 (Figure 2), all five SNPs were suitable for conducting a haplotype-based case–control study.

Among the 23 combinations of SNPs, seven showed significant differences in the overall study. Of the 21 combinations, we show the top five haplotypes with the smallest P-values in Table 3. The frequency of the A-C-G haplotype (established by SNP2–SNP3–SNP6: $P=0.029$), the frequency of the A-C-C-G haplotype (established by SNP2–SNP3–SNP4–SNP6: 0.047) and the frequency of the A-C-G haplotype (established by SNP2–SNP4–SNP6: 0.047) were significantly lower for CI patients than for control subjects. The frequencies of the A-C-C

Table 1 Characteristics of study participants

	Total			*Men*			*Women*		
	Control	*CI*	P-*value*	*Control*	*CI*	P-*value*	*Control*	*CI*	P-*value*
Number of subjects	234	171		113	101		121	70	
Age (years)	77.7 ± 4.1	65.8 ± 13.1	<0.0001*	78.0 ± 4.7	63.7 ± 11.9	<0.0001*	77.4 ± 3.5	68.8 ± 14.3	<0.0001*
BMI (kg m^{-2})	22.7 ± 2.8	23.3 ± 3.6	0.1003	22.7 ± 2.8	23.0 ± 3.1	0.5308	22.6 ± 2.8	23.7 ± 4.5	0.0844
SBP (mm Hg)	135.8 ± 17.1	151.8 ± 26.1	<0.0001*	135.8 ± 16.3	149.6 ± 25.5	<0.0001*	135.9 ± 17.9	155.0 ± 26.8	<0.0001*
DBP (mm Hg)	78.2 ± 10.4	86.3 ± 16.0	<0.0001*	79.1 ± 10.3	87.3 ± 16.0	<0.0001*	77.4 ± 10.5	84.9 ± 15.9	<0.0002*
Pulse (beats min^{-1})	69.5 ± 11.0	76.7 ± 14.8	<0.0001*	68.1 ± 11.9	75.8 ± 14.4	<0.0001*	70.7 ± 9.91	78.0 ± 15.5	<0.0002*
Creatinine (mg per 100 ml)	0.84 ± 0.22	1.03 ± 0.65	<0.0001*	0.94 ± 0.21	1.13 ± 0.58	0.0018 *	0.75 ± 0.18	0.90 ± 0.73	0.0353*
Total cholesterol (mg per 100 ml)	218.7 ± 46.1	194.7 ± 52.2	<0.0001*	205.1 ± 32.4	192.8 ± 52.8	0.0423 *	231.2 ± 52.9	197.4 ± 53.1	<0.0001*
Hypercholesterolaemia (%)	18.8	24.9	<0.0001*	9.7	52.3	<0.0001*	27.3	61.4	<0.0001*
Diabetes (%)	0.0	17.0	<0.0001*	0.0	14.9	<0.0001*	0.0	20.0	<0.0001*
Drinking (%)	6.0	30.0	<0.0001*	9.7	42.6	<0.0001*	2.5	11.4	0.0105*
Smoking (%)	3.8	29.8	<0.0001*	7.1	436.0	<0.0001*	0.8	10.0	0.0023*

Abbreviations: BMI, body mass index; CI, cerebral infarction; Control, non-cerebral infarction; DBP, diastolic blood pressure; HDL, high-density lipoprotein; SBP, systolic blood pressure.
Continuous variables are expressed as mean ± s.d. Categorical variables were expressed as percentages. *P*-values of continuous variables were calculated by Mann–Whitney *U*-test. *P* values of categorical variables were calculated by Fisher's exact test. *$P<0.05$.

Table 2 Genotype and allele distributions in normotensives and patients with CI

Number of participants	*Total*			*Men*			*Women*		
	Control 234	*CI* 171	P-*value*	*Control* 113	*CI* 101	P-*value*	*Control* 121	*CI* 70	P-*value*
Variants									
SNP1									
Genotype									
T/T	124 (0.530)	84 (0.491)		56 (0.496)	54 (0.535)		68 (0.562)	30 (0.429)	
A/T	90 (0.385)	72 (0.421)		46 (0.407)	40 (0.396)		44 (0.364)	32 (0.457)	
A/A	20 (0.085)	15 (0.088)	0.733	11 (0.097)	7 (0.069)	0.714	9 (0.074)	8 (0.114)	0.192
T/T and A/T	214 (0.915)	156 (0.912)		102 (0.903)	94 (0.931)		112 (0.926)	62 (0.886)	
A/A	20 (0.085)	15 (0.088)	0.937	11 (0.097)	7 (0.069)	0.458	9 (0.074)	8 (0.114)	0.358
T/T	124 (0.530)	84 (0.491)		56 (0.496)	54 (0.535)		68 (0.562)	30 (0.429)	
A/T and A/A	110 (0.470)	87 (0.509)	0.442	57 (0.504)	47 (0.465)	0.568	53 (0.438)	40 (0.571)	0.076
Allele									
T	338 (0.722)	240 (0.702)		158 (0.699)	148 (0.733)		180 (0.744)	92 (0.657)	
A	130 (0.278)	102 (0.298)	0.525	68 (0.301)	54 (0.267)	0.443	62 (0.256)	48 (0.343)	0.072
SNP2									
Genotype									
G/G	119 (0.509)	84 (0.491)		60 (0.531)	48 (0.475)		59 (0.488)	36 (0.514)	
G/A	88 (0.376)	74 (0.433)		42 (0.372)	44 (0.436)		46 (0.380)	30 (0.429)	
A/A	27 (0.115)	13 (0.076)	0.301	11 (0.097)	9 (0.089)	0.635	16 (0.132)	4 (0.057)	0.258
G/G and G/A	207 (0.885)	158 (0.924)		102 (0.903)	92 (0.911)		105 (0.868)	66 (0.943)	
A/A	27 (0.115)	13 (0.076)	0.190	11 (0.097)	9 (0.089)	0.836	16 (0.132)	4 (0.057)	0.102
G/G	119 (0.509)	84 (0.491)		60 (0.531)	48 (0.475)		59 (0.488)	36 (0.514)	
G/A and A/A	115 (0.491)	87 (0.509)	0.731	53 (0.469)	53 (0.525)	0.416	62 (0.512)	34 (0.486)	0.722
Allele									
G	326 (0.697)	242 (0.708)		162 (0.717)	140 (0.693)		164 (0.678)	102 (0.729)	
A	142 (0.303)	100 (0.292)	0.735	64 (0.283)	62 (0.307)	0.591	78 (0.322)	38 (0.271)	0.297
SNP3									
Genotype									
C/C	212 (0.906)	152 (0.889)		103 (0.912)	86 (0.851)		109 (0.901)	66 (0.943)	
C/A	19 (0.081)	19 (0.111)		9 (0.080)	15 (0.149)		10 (0.083)	4 (0.057)	

Table 2 Continued

Number of participants	*Total*			*Men*			*Women*		
	Control 234	*CI 171*	P-*value*	*Control 113*	*CI 101*	P-*value*	*Control 121*	*CI 70*	P-*value*
A/A	3 (0.013)	0 (0.000)	0.205	1 (0.009)	0 (0.000)	0.186	2 (0.017)	0 (0.000)	0.441
C/C and C/A	231 (0.987)	171 (1.000)		112 (0.991)	101 (1.000)		119 (0.983)	70 (1.000)	
A/A	3 (0.013)	0 (0.000)	0.137	1 (0.009)	0 (0.000)	0.343	2 (0.017)	0 (0.000)	0.280
C/C	212 (0.906)	152 (0.889)		103 (0.912)	86 (0.851)		109 (0.901)	66 (0.943)	
C/A and A/A	22 (0.094)	19 (0.111)	0.573	10 (0.088)	15 (0.149)	0.172	12 (0.099)	4 (0.057)	0.312
Allele									
C	443 (0.947)	323 (0.944)		215 (0.951)	187 (0.926)		228 (0.942)	136 (0.971)	
A	25 (0.053)	19 (0.056)	0.895	11 (0.049)	15 (0.074)	0.269	14 (0.058)	4 (0.029)	0.193
SNP4									
Genotype									
C/C	127 (0.543)	94 (0.550)		63 (0.558)	56 (0.554)		64 (0.529)	38 (0.543)	
C/T	91 (0.389)	61 (0.357)		41 (0.363)	37 (0.366)		50 (0.413)	24 (0.343)	
T/T	16 (0.068)	16 (0.094)	0.584	9 (0.080)	8 (0.079)	0.999	7 (0.058)	8 (0.114)	0.304
C/C and C/T	218 (0.932)	155 (0.906)		104 (0.920)	93 (0.921)		114 (0.942)	62 (0.886)	
T/T	16 (0.068)	16 (0.094)	0.353	9 (0.080)	8 (0.079)	0.991	7 (0.058)	8 (0.114)	0.162
C/C	127 (0.543)	94 (0.550)		63 (0.558)	56 (0.554)		64 (0.529)	38 (0.543)	
C/T and T/T	107 (0.457)	77 (0.450)	0.889	50 (0.442)	45 (0.446)	0.964	57 (0.471)	32 (0.457)	0.853
Allele									
C	345 (0.737)	249 (0.728)		167 (0.739)	149 (0.738)		178 (0.736)	100 (0.714)	
T	123 (0.263)	93 (0.272)	0.772	59 (0.261)	53 (0.262)	0.975	64 (0.264)	40 (0.286)	0.653
SNP5									
Genotype									
G/G	142 (0.607)	83 (0.485)		66 (0.584)	50 (0.495)		76 (0.628)	33 (0.471)	
G/T	68 (0.291)	75 (0.439)		36 (0.319)	44 (0.436)		32 (0.264)	31 (0.443)	
T/T	24 (0.103)	13 (0.076)	0.009	11 (0.097)	7 (0.069)	0.199	13 (0.107)	6 (0.086)	0.041
G/G and G/T	210 (0.897)	158 (0.924)		102 (0.903)	94 (0.931)		108 (0.893)	64 (0.914)	
T/T	24 (0.103)	13 (0.076)	0.360	11 (0.097)	7 (0.069)	0.461	13 (0.107)	6 (0.086)	0.629
G/G	142 (0.607)	83 (0.485)		66 (0.584)	50 (0.495)		76 (0.628)	33 (0.471)	
G/T and T/T	92 (0.393)	88 (0.515)	0.015	47 (0.416)	51 (0.505)	0.192	45 (0.372)	37 (0.529)	0.035
Allele									
G	352 (0.752)	241 (0.705)		168 (0.743)	144 (0.713)		184 (0.760)	97 (0.693)	
T	116 (0.248)	101 (0.295)	0.132	58 (0.257)	58 (0.287)	0.479	58 (0.240)	43 (0.307)	0.150
SNP6									
Genotype									
G/G	94 (0.402)	68 (0.398)		46 (0.407)	43 (0.426)		48 (0.397)	25 (0.357)	
G/C	106 (0.453)	82 (0.480)		54 (0.478)	44 (0.436)		52 (0.430)	38 (0.543)	
C/C	34 (0.145)	21 (0.123)	0.770	13 (0.115)	14 (0.139)	0.784	21 (0.174)	7 (0.100)	0.221
G/G and G/C	200 (0.855)	150 (0.877)		100 (0.885)	87 (0.861)		100 (0.826)	63 (0.900)	
C/C	34 (0.145)	21 (0.123)	0.514	13 (0.115)	14 (0.139)	0.604	21 (0.174)	7 (0.100)	0.166
G/G	94 (0.402)	68 (0.398)		46 (0.407)	43 (0.426)		48 (0.397)	25 (0.357)	
G/C and C/C	140 (0.598)	103 (0.602)	0.935	67 (0.593)	58 (0.574)	0.782	73 (0.603)	45 (0.643)	0.588
Allele									
G	294 (0.628)	218 (0.637)		146 (0.646)	130 (0.644)		148 (0.612)	88 (0.629)	
C	174 (0.372)	124 (0.363)	0.788	80 (0.354)	72 (0.356)	0.958	94 (0.388)	52 (0.371)	0.696

Abbreviations: CI, cerebral infarction; Control, non-cerebral infarction; SNP, single-nucleotide polymorphism.