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ORIGINAL ARTICLE

Purinergic receptor P2Y, G-protein coupled, 2 (P2RY2) gene is associated with cerebral infarction in Japanese subjects

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G-protein-coupled purinergic receptor P2Y2 (P2RY2) has an important role in the process of atherosclerosis related to cerebral infarction (CI). The aim of this study was to investigate the relationship between the P2RY2 gene and CI through a haplotype-based case–control study, including the separate analysis of two gender groups. A total of 237 CI patients and two control groups (control 1, 254; control 2, 255) were genotyped for five single nucleotide polymorphisms (SNPs) in the human P2RY2 gene (rs4944831, rs1783596, rs4944832, rs4382936, rs10898909). Among women, the distribution of the dominant rs4944832 phenotype (GG *vs.* GA+AA) differed significantly between the CI patients and the control 1 group (P=0.043) and between the CI patients and the control 2 group (P=0.029). Logistic regression analysis showed that the GG genotype of rs4944832 was significantly more prevalent in the female CI patients than in the control 1 (P=0.021) and control 2 groups (P=0.005). For all subjects, the overall distribution of the haplotype established by rs1783596-rs4382936-rs10898909 was significantly different between the CI patients and the control 1 group (P=0.027). For all subjects, the frequency of the T-A-G haplotype (rs1783596-rs4382936-rs10898909) was also significantly higher (P=0.031), whereas the frequency of the T-C-G haplotype (rs1783596-rs4382936-rs10898909) was significantly lower (P=0.029) in the CI patients than in the control 1 group. The present results indicate that the T-A-G haplotype of the human P2RY2 gene is a susceptibility haplotype for CI in Japanese subjects, and that the GG genotype is a genetic marker for CI, particularly in Japanese women.
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Keywords: adenosine triphosphate; case–control study; haplotype; P2RY2; single nucleotide polymorphism

INTRODUCTION

Cerebral infarction (CI) is a leading cause of disability and death worldwide. Vascular neurologists have recently put forward a new 'universal' tissue definition of CI.[1] This review proposes that CI be defined as brain or retinal cell death because of prolonged ischemia. It is generally accepted that atherosclerosis of the cerebral vessels induces cerebral ischemia. Some studies[2] have shown that the proportion of causative factors for CI explained by genetic factors may be as large as 20 to 40%, although diet, exercise and smoking remain critical risk factors. A number of polymorphisms in candidate genes are associated with atherosclerosis in certain populations and have been of considerable help in identifying CI susceptibility loci.[3]

Recently, the purinergic system has been shown to have an undisputed and crucial role in the modulation of vascular tone, and to be of similar importance as a regulator of the inflammatory response occurring in atherosclerosis.[4] Purinergic receptors are ubiquitously expressed throughout the human body and are classified as P1 receptors and P2 receptors. P2 receptors include P2X and P2Y receptors, and the latter are seven-membrane span receptors coupled through G proteins. At least eight P2Y receptor subtypes (P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13 and P2Y14) have been identified[5] 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), stimulates the growth of vascular smooth muscle cells (SMCs), endothelial cells and blood cells, and is involved in platelet aggregation and coagulation of regulation and inflammation, all of which are associated with the development of CI.[6]

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Extracellular nucleotides, including adenosine triphosphate (ATP) and uridine triphosphate, that are released from a variety of arterial and blood cells can bind to P2RY2 on the cell surface. Activation of P2RY2 has been shown to induce both the proliferation and migration of vascular SMCs and apoptosis, a process involved in the evolution of atherosclerotic plaques.[7] Some evidence suggests that P2RY2 is implicated in the development of vascular disease.[8] Interestingly, a more recent study found that high shear stress, associated with vascular diseases, can selectively upregulate P2Y2 receptors in perfused arterial SMCs.[9] It has been proposed that upregulation of P2Y receptors is a potential diagnostic indicator for the early stages of atherosclerosis.[10] A direct pathological role of P2RY2 is reinforced by recent evidence[11] showing that the upregulation and activation of P2RY2 in rabbit arteries mediate the intimal hyperplasia that accompanies atherosclerosis. Guns *et al.*[12] found that in the aorta of P2Y2-knockout mice, endothelium-dependent relaxation by ATP was inhibited, showing the role of P2RY2 in endothelial dysfunction. These studies show that the P2RY2 gene has an important role in the process of atherosclerosis, the main cause of CI.

To our knowledge, there have been no earlier studies on the association between the human P2RY2 gene and CI. This study was a retrospective haplotype-based case–control study. The aim was to investigate the association between the human P2RY2 gene and CI by using single nucleotide polymorphisms (SNPs) in conjunction with separate analyses of data pertaining to gender groups.

METHODS

Subjects

The study group consisted of 237 CI patients (mean age, 63.2 ± 12.8 years, m/f ratio=1.69). Subtypes of CI included thrombotic and lacunar stroke. Diagnosis was based on neurological examination and on the findings of computed tomography or magnetic resonance imaging, or both. In addition, all patients had neurological deficit ratings greater than grade 3 on the modified Rankin Scale. The study also enrolled 254 Japanese subjects as the control 1 group (mean age, 77.8 ± 4.2 years, m/f ratio=0.90). All subjects were members of the New Elder Citizen Movement in Japan and resided in the Greater Tokyo Metropolitan Area. Although some of the subjects in the control 1 group had vascular risk factors such as hypertension, hypercholesterolemia and diabetes mellitus, none had a history of CI. All of the subjects in the control 1 group were confirmed to have grade 0 on the modified Rankin Scale of neurological deficits. Individuals with atrial fibrillation were excluded from both the CI and control groups. Participants with cancer or autoimmune disease, including antiphospholipid antibody syndrome, were also excluded.[13] A total of 255 sex-matched healthy individuals (mean age, 51.4 ± 10.0 years, m/f ratio=1.66) were also enrolled as the control 2 group. None of the subjects in the control 2 group had a history of hypertension and CI, and all had systolic blood pressure <130 mm Hg and diastolic blood pressure <85 mm Hg. The patients and two control groups were taking no medications influencing the adenylate cyclase pathway. Informed consent was obtained from each subject in accordance with the protocol approved by the Human Studies Committee of Nihon University.[14] Sample sizes were considered to be appropriate for a case–control study.[15]

Genotyping

The human P2RY2 gene is located on chromosome 11q13.5–q14. 1, spans approximately 18.1 kb, and contains three exons. There are 104 SNPs for the human P2RY2 gene listed 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 $r^2 \geqslant 0.5$. For minor allele frequencies, we used a cutoff level of $\leqslant 0.2$. According to the above criteria, we selected rs4944831 (SNP1, C-12034890-10, registration number by Applied Biosystems), rs1783596 (SNP2, C-8893575-10), rs4944832 (SNP3, C-27987464-10), rs4382936 (SNP4, C-1830488-20) and rs10898909 (SNP5, C-1830487-10) for this study (Figure 1). SNP1 was located in intron 1, whereas SNP2 was located in the coding region of exon 3. SNP3, SNP4 and SNP5 were located in the 3′-flanking region.

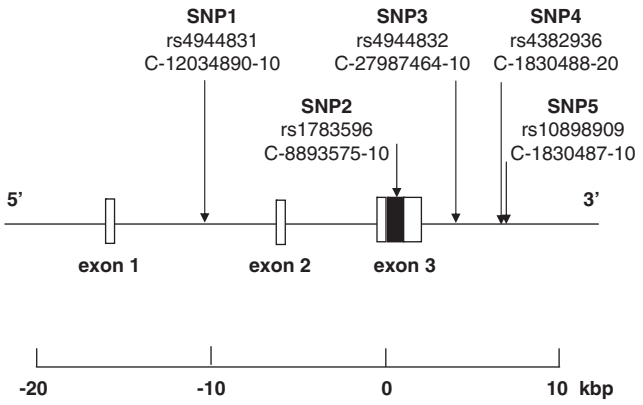

Figure 1 Structure of the human P2RY2 gene. The gene consists of three exons (boxes) separated by two introns. Lines show introns and intergenic regions. Filled box shows the coding region. Arrows indicate the locations of single nucleotide polymorphisms (SNPs). kbp, kilobase pairs.

Blood samples were collected from all participants and genomic DNA was extracted from peripheral blood leukocytes by phenol and chloroform extraction.

Genotyping was performed using the TaqMan SNP Genotyping Assay (Applied Biosystems, Foster City, CA, USA). TaqMan SNP Genotyping Assays were performed by Taq amplification.[16] 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 of 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 nonspecific probe hybridization, and results in low background fluorescence in the 5′-nuclease PCR assay (TaqMan; Applied Biosystems). Cleavage results in increased emission of a reporter dye. Each 5′-nuclease assay requires two unlabeled PCR primers and two allele-specific probes. Each probe is labeled with two reporter dyes at the 5′-end. In this study, VIC and FAM were used as reporter dyes. The primers and probes used in the TaqMan SNP Genotyping Assays (Applied Biosystems) were chosen on the basis of the information available on the Applied Biosystems website (http://www3.appliedbiosystems.com/AB_Home/index.htm).

PCR amplification was performed using 2.5 µl of TaqMan Universal Master Mix, No. AmpErase UNG (2×) (Applied Biosystems) 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).

Each 96-well plate contained 80 DNA samples of an unknown genotype and four reaction mixtures containing reagents but no DNA (control samples). 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). 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 of each scoring method were saved in two separate output files for later comparison.

Biochemical analysis

We measured serum concentrations of creatinine using the standard methods used by the Clinical Laboratory Department of Nihon University Hospital.

Statistical analysis

All continuous variables were expressed as means ± s.d. Differences in continuous variables between the CI patients and controls were analyzed using the Mann–Whitney *U*-test. Differences in categorical variables were analyzed using Fisher's exact test. The Hardy–Weinberg equilibrium was assessed by χ^2 analysis. Differences in the distributions of genotypes and alleles between the CI patients and controls were analyzed 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[17] and the software SNPAlyze version 3.2 (Dynacom, Yokohama, Japan). Pairwise linkage disequilibrium analysis was performed using five SNPs. We used |D′| values greater than 0.5 to assign SNP locations to one haplotype block. SNPs with an r^2 value less than 0.5 were selected as tagged. In the haplotype-based case–control analysis, the frequency distribution of the haplotypes and *P*-values were calculated by χ^2 analysis. In addition, logistic regression analysis was performed to assess the contribution of major risk factors. Statistical significance was established at *P*-values less than 0.05. Statistical analyses were performed using SPSS software for Windows (version 12; SPSS, Chicago, IL, USA).

RESULTS

Table 1 shows the clinical characteristics of the study participants. The mean age of the control 1 group was higher than that of the CI patients. In humans, the use of the so-called 'super controls' has been widely accepted in case–control studies for common diseases that appear later in life.[13] Here, we used a 'super control' group, as healthy elderly subjects have been found to be more suitable than young or middle-aged subjects when determining phenotypes of cerebrovascular diseases related to aging. CI is an age-influenced disease, and the use of a 'super control' group rather than an age-matched control group is therefore better for increasing statistical power in these types of experiments. For the three groups, all subjects, men and women, the following values were significantly higher in the CI patients than in the two control groups: systolic blood pressure, diastolic blood pressure, and prevalence of hypertension and diabetes mellitus. For all subjects and men, serum creatinine and the prevalence of smoking and drinking and hyperlipidemia were significantly higher for the CI patients than for the control 1 group.

Table 2 shows the distributions of genotypes and alleles of the five SNPs. As the elderly subjects in the 'super-control' group may have had protective SNPs, and the patients and the control 1 group were not sex-matched, we added another group that was sex-matched and had a younger age (control 2 group). The genotype distribution of each SNP did not show significant differences from the Hardy–Weinberg equilibrium values in the control 1 and control 2 groups (data not shown). For women, the distribution of the dominant form of rs4944832 (GG *vs.* GA+AA) differed significantly between the CI patients and the control 1 group (P=0.043), and between the CI patients and the control 2 group (P=0.029). There were also significant differences in the allelic distribution of rs4944832 between the female CI patients and the control 1 group (P=0.039), and between the female CI patients and the control 2 group (P=0.017). Dominant and recessive models were defined by their overall frequency among controls. As similar results were seen in the control 2 group, we believe that the present 'super-elderly' subjects had no protective SNPs.

Table 3 shows the results of logistic regression analysis. As the relationship between rs4944832 and CI in women in Table 2 was not clear after using Bonferroni's correction,[18] we examined this relationship by logistic regression analysis to confirm the results. For women, logistic regression was performed using the following parameters: GG genotype of rs4944832, pulse rate, creatinine with or without

Table 1 Characteristics of study participants

	Total				*Men*				*Women*			
	CI patients	*Control 1*	*Control 2*	*P-value 1*	*P-value 2*	*CI patients*	*Control 1*	*Control 2*	*P-value 1*	*P-value 2*	*CI patients*	*Control 1*	*Control 2*	*P-value 1*	*P-value 2*
Number of subjects	237	254	255			149	120	159			88	134	96		
Age (years)	63.2±12.8	77.8±4.2	51.4±10.0	<0.001*	<0.001*	61.0±11.6	78.0±4.6	51.0±6.1	<0.001*	<0.001*	67.0±13.9	77.6±3.8	52.1±14.3	<0.001*	<0.001*
Body mass index (kg m^{-2})	23.3±3.4	22.7±2.8	22.6±3.2	0.098	0.074	23.1±3.0	22.9±2.8	22.9±3.2	0.515	0.523	23.6±4.4	22.6±2.9	22.2±3.3	0.099	0.052
Systolic blood pressure (mm Hg)	151.6±27.3	135.7±16.8	112.0±10.8	<0.001*	<0.001*	150.7±27.4	135.0±16.0	112.5±10.6	<0.001*	<0.001*	153.0±27.1	136.4±17.6	111.3±11.1	<0.001*	<0.001*
Diastolic blood pressure (mm Hg)	88.1±17.1	79.0±11.1	69.1±8.6	<0.001*	<0.001*	89.4±17.7	78.9±10.0	69.9±8.2	<0.001*	<0.001*	85.9±15.9	79.0±12.1	67.7±9.2	0.001*	0.001*
Pulse rate (beats per min)	76.8±14.7	69.8±10.9	73.4±13.0	<0.001*	0.019*	75.3±14.0	68.7±11.6	73.0±14.2	<0.001*	0.226	79.4±15.5	70.9±10.0	74.0±10.9	<0.001*	0.020*
Creatinine (mg per 100 ml)	1.1±0.8	0.8±0.2	0.8±0.2	<0.001*	<0.001*	1.2± 0.9	1.0±0.2	0.9±0.2	0.006*	<0.001*	0.9±0.7	0.8±0.2	0.7±0.1	0.067	0.012*
Hypertension (%)	32	7	0	<0.001*	<0.001*	32	8	0	<0.001*	<0.001*	33	7	0	<0.001*	<0.001*
Hyperlipidemia (%)	26	17	18	0.012*	0.039*	22	9	15	0.004*	0.081	32	23	24	0.152	0.234
Diabetes (%)	14	2	3	<0.001*	<0.001*	13	3	3	0.006*	0.017*	17	1	3	<0.001*	0.002*
Smoking (%)	29	9	28	<0.001*	0.755	40	15	30	<0.001*	0.361	10	3	16	0.141	0.278
Drinking (%)	31	10	38	<0.001*	0.092	42	14	40	0.001*	0.275	13	7	22	0.021*	0.094

Abbreviation: CI, cerebral infarction.
Continuous variables are expressed as mean ± s.d. Categorical variables are expressed as percentages.
P-values 1 was for the comparison between CI and Control 1, whereas *P*-values 2 was for the comparison between CI and Control 2.
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 CI patients and two control groups

		Total					*Men*					*Women*				
		CI patients	*Control 1*	*Control 2*	*P-value 1*	*P-value 2*	*CI patients*	*Control 1*	*Control 2*	*P-value 1*	*P-value 2*	*CI patients*	*Control 1*	*Control 2*	*P-value 1*	*P-value 2*
rs4944831 (SNP1)																
Genotype	T/T	151 (63.7%)	166 (65.4%)	163 (63.9%)	0.783	0.693	97 (65.1%)	80 (66.7%)	104 (65.4%)	0.678	0.527	54 (61.4%)	86 (64.2%)	59 (61.5%)	0.909	0.992
	T/G	75 (31.7%)	74 (29.1%)	76 (29.8%)			45 (30.2%)	32 (26.6%)	43 (27.0%)			30 (34.1%)	42 (31.3%)	33 (34.3%)		
	G/G	11 (4.6%)	14 (5.5%)	16 (6.3%)			7 (4.7%)	8 (6.7%)	12 (7.6%)			4 (4.5%)	6 (4.5%)	4 (4.2%)		
Dominant model	TT	151 (63.7%)	166 (65.4%)	163 (63.9%)	0.704	0.962	97 (65.1%)	80 (66.7%)	104 (65.4%)	0.788	0.955	54 (61.4%)	86 (64.2%)	59 (61.5%)	0.671	0.990
	TG+GG	86 (36.3%)	88 (34.6%)	92 (36.1%)			52 (34.9%)	40 (33.3%)	55 (34.6%)			34 (38.6%)	48 (35.8%)	37 (38.5%)		
Recessive model	GG	11 (4.6%)	14 (5.5%)	16 (6.3%)	0.661	0.427	7 (4.7%)	8 (6.7%)	12 (7.6%)	0.484	0.299	4 (4.5%)	6 (4.5%)	4 (4.2%)	0.981	0.900
	TG+TT	226 (95.4%)	240 (94.5%)	239 (93.7%)			142 (95.3%)	112 (93.3%)	147 (92.4%)			84 (95.5%)	128 (95.5%)	92 (95.8%)		
Allele	T	377 (79.5%)	406 (79.9%)	402 (78.8%)	0.881	0.783	239 (80.2%)	192 (80.0%)	251 (78.9%)	0.954	0.696	138 (78.4%)	214 (79.9%)	151 (78.6%)	0.714	0.956
	G	97 (20.5%)	102 (20.1%)	108 (21.2%)			59 (19.8%)	48 (20.0%)	67 (21.1%)			38 (21.6%)	54 (20.1%)	41 (21.4%)		
rs1783596 (SNP2)																
Genotype	T/T	82 (34.6%)	97 (38.2%)	78 (30.6%)	0.674	0.637	47 (31.5%)	45 (37.5%)	50 (31.5%)	0.452	0.830	35 (39.8%)	52 (38.8%)	28 (29.2%)	0.681	0.181
	T/C	115 (48.5%)	119 (46.8%)	131 (51.4%)			71 (47.7%)	56 (46.7%)	80 (50.3%)			44 (50.0%)	63 (47.0%)	51 (53.1%)		
	C/C	40 (16.9%)	38 (15.0%)	46 (18.0%)			31 (20.8%)	19 (15.8%)	29 (18.2%)			9 (10.2%)	19 (14.2%)	17 (17.7%)		
Dominant model	TT	82 (34.6%)	97 (38.2%)	78 (30.6%)	0.409	0.343	47 (31.5%)	45 (37.5%)	50 (31.5%)	0.306	0.985	35 (39.8%)	52 (38.8%)	28 (29.2%)	0.885	0.130
	TC+CC	155 (65.4%)	157 (61.8%)	177 (69.4%)			102 (68.5%)	75 (62.5%)	109 (68.5%)			53 (60.2%)	82 (61.2%)	68 (70.8%)		
Recessive model	CC	40 (16.9%)	38 (15.0%)	46 (18.0%)	0.562	0.735	31 (20.8%)	19 (15.8%)	29 (18.2%)	0.297	0.570	9 (10.2%)	19 (14.2%)	17 (17.7%)	0.386	0.146
	TC+TT	197 (83.1%)	216 (85.0%)	209 (82.0%)			118 (79.2%)	101 (84.2%)	130 (81.8%)			79 (89.8%)	115 (85.8%)	79 (82.3%)		
Allele	T	279 (58.9%)	313 (61.6%)	287 (56.3%)	0.378	0.412	165 (55.4%)	146 (60.8%)	180 (56.6%)	0.202	0.758	114 (64.8%)	167 (62.3%)	107 (55.7%)	0.599	0.077
	C	195 (41.1%)	195 (38.4%)	223 (43.7%)			133 (44.6%)	94 (39.2%)	138 (43.4%)			62 (35.2%)	101 (37.7%)	85 (44.3%)		
rs4944832 (SNP3)																
Genotype	G/G	105 (44.3%)	108 (42.5%)	101 (39.6%)	0.833	0.472	56 (37.6%)	52 (43.3%)	63 (39.6%)	0.454	0.913	49 (55.7%)	56 (41.8%)	38 (39.6%)	0.098	0.048*
	G/A	106 (44.7%)	114 (44.9%)	119 (46.7%)			71 (47.6%)	48 (40.0%)	72 (45.3%)			35 (39.8%)	66 (49.2%)	47 (48.9%)		
	A/A	26 (11.0%)	32 (12.6%)	35 (13.7%)			22 (14.8%)	20 (16.7%)	24 (15.1%)			4 (4.5%)	12 (9.0%)	11 (11.5%)		
Dominant model	GG	105 (44.3%)	108 (42.5%)	101 (39.6%)	0.690	0.291	56 (37.6%)	52 (43.3%)	63 (39.6%)	0.339	0.714	49 (55.7%)	56 (41.8%)	38 (39.6%)	0.043*	0.029*
	GA+AA	132 (55.7%)	146 (57.5%)	154 (60.4%)			93 (62.4%)	68 (56.7%)	96 (60.4%)			39 (44.3%)	78 (58.2%)	58 (60.4%)		
Recessive model	AA	26 (11.0%)	32 (12.6%)	35 (13.7%)	0.577	0.354	22 (14.8%)	20 (16.7%)	24 (15.1%)	0.669	0.935	4 (4.5%)	12 (9.0%)	11 (11.5%)	0.214	0.087
	GA+GG	211 (89.0%)	222 (87.4%)	220 (86.3%)			127 (85.2%)	100 (83.3%)	135 (84.9%)			84 (95.5%)	122 (91.0%)	85 (88.5%)		
Allele	G	316 (66.7%)	330 (65.0%)	321 (62.9%)	0.573	0.222	183 (61.4%)	152 (63.3%)	198 (62.3%)	0.647	0.827	133 (75.6%)	178 (66.4%)	123 (64.1%)	0.039*	0.017*
	A	158 (33.3%)	178 (35.0%)	189 (37.1%)			115 (38.6%)	88 (36.7%)	120 (37.7%)			43 (24.4%)	90 (33.6%)	69 (35.9%)		
rs4382936 (SNP4)																
Genotype	C/C	125 (52.7%)	143 (56.3%)	133 (52.2%)	0.723	0.989	84 (56.4%)	71 (59.2%)	85 (53.5%)	0.856	0.865	41 (46.6%)	72 (53.7%)	48 (50.0%)	0.574	0.863
	C/A	94 (39.7%)	94 (37.0%)	102 (40.0%)			52 (34.9%)	38 (31.6%)	60 (37.7%)			42 (47.7%)	56 (41.8%)	42 (43.7%)		
	A/A	18 (7.6%)	17 (6.7%)	20 (7.8%)			13 (8.7%)	11 (9.2%)	14 (8.8%)			5 (5.7%)	6 (4.5%)	6 (6.3%)		
Dominant model	CC	125 (52.7%)	143 (56.3%)	133 (52.2%)	0.429	0.897	84 (56.4%)	71 (59.2%)	85 (53.5%)	0.645	0.607	41 (46.6%)	72 (53.7%)	48 (50.0%)	0.298	0.644
	CA+AA	112 (47.3%)	111 (43.7%)	122 (47.8%)			65 (43.6%)	49 (40.8%)	74 (46.5%)			47 (53.4%)	62 (46.3%)	48 (50.0%)		
Recessive model	AA	18 (7.6%)	17 (6.7%)	20 (7.8%)	0.698	0.918	13 (8.7%)	11 (9.2%)	14 (8.8%)	0.899	0.980	5 (5.7%)	6 (4.5%)	6 (6.3%)	0.686	0.871
	CA+CC	219 (92.4%)	237 (93.3%)	235 (92.2%)			136 (91.3%)	109 (90.8%)	145 (91.2%)			83 (94.3%)	128 (95.5%)	90 (93.7%)		
Allele	C	344 (72.6%)	380 (74.8%)	368 (72.2%)	0.428	0.884	220 (73.8%)	180 (75.0%)	230 (72.3%)	0.757	0.675	124 (70.5%)	200 (74.6%)	138 (71.9%)	0.333	0.764
	A	130 (27.4%)	128 (25.2%)	142 (27.8%)			78 (26.2%)	60 (25.0%)	88 (27.7%)			52 (29.5%)	68 (25.4%)	54 (28.1%)		

Table 2 Continued

		Total						Men						Women					
		CI patients		*Control 1*		*Control 2*	*P-value 1*	*P-value 2*	*CI patients*		*Control 1*		*Control 2*	*P-value 1*	*P-value 2*	*CI patients*		*Control 1*		*Control 2*	*P-value 1*	*P-value 2*
rs10898909 (SNP5)																						
Genotype	G/G	70	(29.5%)	71	(28.0%)	82 (32.2%)	0.680	0.767	48	(32.2%)	41	(34.2%)	55 (34.6%)	0.863	0.871	22	(25.0%)	30	(22.4%)	27 (28.1%)	0.797	0.601
	G/A	117	(49.4%)	121	(47.6%)	118 (46.3%)			72	(48.3%)	54	(45.0%)	76 (47.8%)			45	(51.1%)	67	(50.0%)	42 (43.8%)		
	A/A	50	(21.1%)	62	(24.4%)	55 (21.6%)			29	(19.5%)	25	(20.8%)	28 (17.6%)			21	(26.9%)	37	(27.6%)	27 (28.1%)		
Dominant model	GG	70	(29.5%)	71	(28.0%)	82 (32.2%)	0.698	0.530	48	(32.2%)	41	(34.2%)	55 (34.6%)	0.735	0.659	22	(25.0%)	30	(22.4%)	27 (28.1%)	0.653	0.632
	GA+AA	167	(70.5%)	183	(72.0%)	173 (67.8%)			101	(67.8%)	79	(65.8%)	104 (65.4%)			66	(75.0%)	104	(77.6%)	69 (71.9%)		
Recessive model	AA	50	(21.1%)	62	(24.4%)	55 (21.6%)	0.382	0.899	29	(19.5%)	25	(20.8%)	28 (17.6%)	0.780	0.676	21	(26.9%)	37	(27.6%)	27 (28.1%)	0.534	0.511
	GA+GG	187	(78.9%)	192	(75.6%)	200 (78.4%)			120	(80.5%)	95	(79.2%)	131 (82.4%)			67	(76.1%)	97	(72.4%)	69 (71.9%)		
Allele	G	257	(54.2%)	263	(51.8%)	282 (55.3%)	0.443	0.735	168	(56.4%)	136	(56.7%)	186 (58.5%)	0.946	0.596	89	(50.6%)	127	(47.4%)	96 (50.0%)	0.512	0.913
	A	217	(45.8%)	245	(48.2%)	228 (44.7%)			130	(43.6%)	104	(43.3%)	132 (41.5%)			87	(49.4%)	141	(52.6%)	96 (50.0%)		

Abbreviation: CI, cerebral infarction.
P-values 1 was for the comparison between CI and Control 1, whereas *P*-values 2 was for the comparison between CI and Control 2.
P-values for genotype were calculated by Fisher's exact test. *$P<0.05$.

hypertension, diabetes mellitus or drinking frequency, because these confounding factors showed significant differences among women in Table 1. The GG genotype of rs4944832 was found to be significantly higher in female CI patients than in the control 1 group ($P=0.021$) and the control 2 group ($P=0.005$), which further showed the relationship between rs4944832 and CI in women.

Figure 2 shows the patterns of linkage disequilibrium for the P2RY2 gene in the control 1 group, with their $|D'|$ and r^2 values. Four SNPs (SNP2, SNP3, SNP4 and SNP5) were located in one haplotype block, as the $|D'|$ values were beyond 0.25. However, SNP1 was not located in this haplotype block. As the r^2 values for SNP2–SNP3 were greater than 0.5, at least one SNP from SNP2 and SNP3 was suitable for the haplotype-based case–control study. Therefore, given that the minor allele frequency of SNP2 was larger than that of SNP3, we constructed the haplotypes using SNP2, SNP4 and SNP5.

In the haplotype-based case–control study, there were four combinations: SNP2–SNP4, SNP2–SNP5, SNP4–SNP5 and SNP2–SNP4–SNP5 (Table 4). For all subjects, the overall distribution of the haplotype established by SNP2–SNP4–SNP5 was significantly different between the CI patients and the control 1 group ($P=0.027$). For all subjects, the frequency of the T-A-G haplotype (SNP2–SNP4–SNP5) was also significantly higher ($P=0.031$), whereas the frequency of the T-C-G haplotype (SNP2–SNP4–SNP5) was significantly lower ($P=0.029$) for the CI patients than for the control 1 group. For women, the frequency of the T-A-A haplotype (SNP2–SNP4–SNP5) was significantly higher for the CI patients than for the control 1 group ($P=0.012$).

DISCUSSION

In 1994, Parr *et al.*[19] first reported the sequence and functional expression of cDNA-encoding P2RY2 cloned from airway epithelial cells, whereas in 1996, Dasari *et al.*[20] mapped the human P2RY2 gene to chromosome 11q13.5–q14.1 using a PCR analysis of human–rodent hybrid cell line DNAs. Earlier studies in mice have identified important roles for P2RY2 in a variety of processes, including nucleotide-regulated Ca^{2+} signaling in lung fibroblasts and airway epithelial cells, nucleotide-stimulated Cl^- secretion in the trachea and gallbladder, neuronal growth, stimulation of K^+ secretion in the colon and neutrophil chemotaxis.[21] P2RY2 may participate in various physiological responses by the activation of nucleotides. Extracellular ATP or UTP binds to P2RY2, and this combination activates phospholipase C and/or adenylate cyclase.[22] In turn, phospholipase C activation generates inositol 1,4,5-triphosphate, a mediator of Ca^{2+} release from intracellular stores, and diacylglycerol, an activator of protein kinase C, whereas adenylate cyclase generates cyclic adenosine monophosphate, an activator of protein kinase A.

Atherosclerosis related to CI is an immunoinflammatory process that involves complex interactions between the vessel wall and blood components and is thought to be initiated by endothelial dysfunction.[23] From a vascular biology perspective, the processes of cellular adhesion, monocyte and macrophage attachment, and transmigration of immune cells across the endothelium are crucial steps in early atherogenesis and in the later stages of mature plaque rupture.[24] Inflammatory cells express P2RY2 with multiple effects. P2RY2 is expressed on T lymphocytes and macrophages and has been suggested to be important in atherosclerosis.[25] Recent studies have found an important role for P2RY2 in the development of intimal hyperplasia in rabbit carotid arteries.[11] With regard to cerebral vessels, increased stroke risk has been associated with an increased rate of atherosclerosis progression in carotid vessels. Various evidence[26,27] has also shown that P2Y2 receptors in the smooth muscle cell mediate the dilation of

Table 3 Odds ratios and 95% confidence intervals for each risk factor, and GG genotype of rs4944832 associated with cerebral infarction in female subjects

	Control 1				*Control 2*		
Risk factor	*Odd ratios*	*95% confidence interval*	*P-value*	*Risk factor*	*Odd ratios*	*95% confidence interval*	*P-value*
GG genotype	4.185	1.237–14.159	0.021*	GG genotype	3.317	1.423–7.733	0.005*
Pulse rate	1.022	0.969–1.078	0.424	Pulse rate	1.038	1.004–1.073	0.030*
Hypertension	1.235	0.324–4.695	0.757	Hypertension	2.173	0.811–4.824	0.998
Diabetes mellitus	5.917	0.999–34.483	0.050	Diabetes mellitus	4.998	1.119–22.322	0.035*
Drinking	1.331	0.186–9.508	0.776	Creatinine	7.542	0.963–59.063	0.054

*$P<0.05$.

SNP		SNP2	SNP3	SNP4	SNP5
SNP1	I D'I	0.117	0.024	0.108	0.240
	r^2	0.006	0.000	0.009	0.013
	SNP2	I D'I	0.803	0.405	0.746
		r^2	0.558	0.089	0.323
		SNP3	I D'I	0.445	0.901
			r^2	0.124	0.408
			SNP4	I D'I	0.776
				r^2	0.189

ID´I > 0.25
r^2> 0.5

Figure 2 Pairwise linkage disequilibrium (LD) in the human P2RY2 gene, as evaluated by ID'I and r^2 values. Pairwise LD was computed for the five marker pairs that were studied in the human P2RY2 gene. Pairs in LD (ID'I$\geqslant$0.25 or $r^2\geqslant$0.5) are shown as shaded values.

rat cerebral arterioles. Marrelli *et al*.[28] found that P2Y2 receptors have an important role in the dilation of the rat's middle cerebral artery after ischemia reperfusion. These studies indicate that the P2RY2 gene has a close relationship with CI.

On the basis of the results of these animal studies, we planned to assess the association between the P2RY2 gene and CI using a haplotype-based case–control study. In this study, we found that the distribution of the dominant model of rs4944832 (GG *vs*. GA+AA) differed significantly between the female CI patients and the two control groups. The present results indicate that the risk of CI is increased in subjects with the GG genotype of rs4944832, which is associated with CI for Japanese women. We also found a significant difference in the allelic distribution of rs4944832 between the female CI patients and the two control groups. In this study, logistic regression analysis indicated that for women, the GG genotype of

Table 4 Haplotype analysis in CI patients and control 1

				Overall P-value			*Frequency in total*			*Frequency in men*			*Frequency in women*		
Haplotype				*Total*	*Men*	*Women*	*CI patients*	*Control 1*	*P-value*	*CI patients*	*Control 1*	*P-value*	*CI patients*	*Control 1*	*P-value*
	SNP2	SNP4		0.217	0.179	0.662									
H1	T	C					0.468	0.524	0.084	0.447	0.527	0.069	0.510	0.519	0.880
H2	C	C					0.257	0.224	0.227	0.291	0.223	0.079	0.195	0.228	0.387
H3	C	A					0.154	0.160	0.815	0.155	0.168	0.699	0.158	0.149	0.778
H4	T	A					0.120	0.092	0.158	0.106	0.082	0.348	0.138	0.104	0.307
	SNP2		SNP5	0.823	0.279	0.704									
H1	T		A				0.412	0.435	0.454	0.397	0.418	0.627	0.212	0.173	0.308
H2	C		G				0.366	0.337	0.352	0.407	0.377	0.464	0.058	0.076	0.465
H3	T		G				0.176	0.181	0.874	0.157	0.190	0.301	0.436	0.450	0.772
H4	C		A				0.046	0.047	0.951	0.039	0.015	0.109	0.294	0.301	0.879
		SNP4	SNP5	0.767	0.796	0.795									
H1		C	A				0.434	0.455	0.526	0.412	0.401	0.765	0.233	0.241	0.887
H2		C	G				0.292	0.293	0.941	0.326	0.349	0.550	0.472	0.506	0.460
H3		A	G				0.250	0.225	0.327	0.237	0.217	0.553	0.023	0.020	0.981
H4		A	A				0.024	0.027	0.665	0.024	0.033	0.491	0.273	0.233	0.323
	SNP2	SNP4	SNP5	0.027*	0.118	0.076									
H1	T	C	A				0.394	0.408	0.679	0.378	0.385	0.922	0.056	0.091	0.205
H2	C	C	G				0.221	0.180	0.114	0.251	0.214	0.286	0.049	0.075	0.327
H3	C	A	G				0.144	0.157	0.539	0.156	0.163	0.797	0.013	0.001	0.080
H4	T	A	G				0.106	0.068	0.031*	0.081	0.054	0.230	0.011	0.020	0.546
H5	T	C	G				0.071	0.113	0.029*	0.075	0.136	0.115	0.421	0.431	0.857
H6	C	C	A				0.039	0.047	0.584	0.034	0.015	0.221	0.178	0.150	0.450
H7	T	A	A				0.018	0.028	0.258	0.019	0.033	0.339	0.159	0.082	0.012*
H8	C	A	A				0.006	0.000	0.073	0.050	0.000	0.204	0.113	0.151	0.283

Abbreviation: CI, cerebral infarction.
Haplotypes were estimated using SNPAlyze software (version 3.2; Dynacom, Yokohama, Japan).
P-values were calculated by χ^2 analysis. *$P<0.05$.

rs4944832 was significantly more common in the CI patients than in the two control groups. This indicates that the GG genotype is a genetic marker for CI in Japanese women.

Morris *et al*.[29] found that for genes with multiple susceptibilities, analysis based on haplotypes has advantages over analysis based on individual SNPs, particularly when linkage disequilibria between the SNPs are weak. Consequently, in this study, we successfully established haplotypes for the P2RY2 gene from the various combinations of the three selected SNPs. For the present haplotype analysis, although there were significant differences in the frequency of the T-A-A haplotype (SNP2–SNP4–SNP5) between the female CI patients and the control 1 group, we believe that this difference is not particularly important, as the overall distribution of the combination (SNP2–SNP4–SNP5) for women did not significantly differ between the CI patients and the control 1 group (P=0.076). In this study, for all subjects, the frequency of the T-A-G haplotype was significantly higher for the CI patients than for the control 1 group, and the T-A-G haplotype can be regarded as a susceptibility haplotype for CI in Japanese subjects. The frequency of the T-C-G haplotype was significantly lower for the CI patients than for the control 1 group, and the T-C-G haplotype can be regarded as a resistance haplotype for CI in Japanese subjects.

In this study, the GG genotype of rs4944832 showed a gender-specific significant difference (for women only). Although being male is recognized as a risk factor for CI, some basic and clinical studies have shown the opposite results.[30,31] ATP, the ligand for P2RY2, has been shown to increase the production of progesterone and estradiol in human granulosa–luteal cells.[32] Several clinical and experimental studies have suggested that estrogen is not universally neuroprotective in experimental cerebral ischemia [31] and may not be beneficial for ischemic preconditioning in experimental ischemia models.[33] Recent clinical studies have suggested that there are unanticipated and paradoxical effects of estrogen and hormone replacement therapy relative to stroke risk, as it is currently administered to women.[34] One study has suggested that women on hormone replacement therapy have a higher risk of perioperative stroke.[35] Therefore, depending on the ischemic conditions present and the level of ATP, estrogen has the ability to harm ischemically compromised brain tissue.

In conclusion, the T-A-G haplotype of the human P2RY2 gene may be a susceptibility haplotype for CI in Japanese subjects, and the GG genotype could be a genetic marker for CI, particularly in Japanese women. This study also shows that the T-C-G haplotype is a resistance marker for CI in Japanese subjects. Further studies are needed to isolate functional mutations in the P2RY2 gene that modulate the process of atherosclerosis, and to evaluate the function of P2RY2 variants that are involved in the metabolism of sex hormones.

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