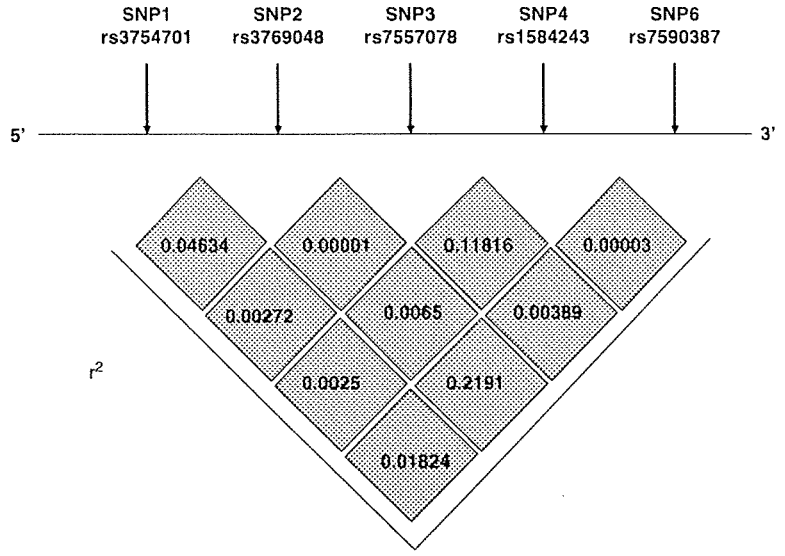

Figure 2 Pair wise linkage disequilibrium in the human RAMP1 gene, as evaluated by r^2.

Table 3 Haplotype-based case–control study analysis in patients with CI and control subjects

RAMP1 polymorphism					*Overall P-value*	*Frequency in total*		P-*value*
					Total	*CI patients*	*Control subjects*	
Haplotypes	SNP2	SNP3	SNP6					
H1	Mn	Mj	Mn		<0.01	0.374	0.308	0.048*
	A	C	C					
H2	Mn	Mj	Mj		<0.01	0.278	0.351	0.029*
	A	C	G					
Haplotypes	SNP2	SNP3	SNP4	SNP6				
H3	Mn	Mj	Mj	Mj	<0.01	0.206	0.265	0.047*
	A	C	C	G				
Haplotypes	SNP2	SNP4	SNP6					
H4	Mn	Mj	Mj		<0.01	0.203	0.266	0.047*
	A	C	G					
Haplotypes	SNP1	SNP2	SNP6					
H5	Mj	Mn	Mn		<0.01	0.140	0.075	0.002*
	T	A	C					

Abbreviations: CI, cerebral infarction; Mj, major; Mn, minor; SNP, single-nucleotide polymorphism.
Haplotypes with frequency 0% were estimated using SNPAlyze software. *P*-value was calculated by χ^2-analysis. *$P<0.01$.

haplotype (established by SNP2–SNP3–SNP6: 0.048) and the T-A-C haplotype (established by SNP1–SNP2–SNP6: 0.0024) were significantly higher for CI patients than for control subjects

Discussion

Receptor activity-modifying protein-1 is involved in the vascular endothelium and mouse bone marrow macrophages.[16,17] The human RAMP-1 gene is located on chromosome 2, at 2p36–2q37.1, spanning approximately 60 kb and containing 3 exons. RAMP1 protein has 148 amino acids with a 26-amino-acid N-terminal signal peptide, an extracellular domain of approximately 90 amino acids, a single transmembrane domain of 20 amino acids and a C-terminal tail of 10 amino acids. RAMPs are required to transport CRLR to the plasma membrane. CRLR, a receptor with seven transmembrane domains, can function as either a CGRP receptor or an adrenomedullin receptor, depending on which members of the RAMP family are expressed. The specificity of the CGRP receptor appears to be

decided by RAMP1, as the N terminus of RAMP1 is essential for CGRP binding.[18]

Receptor activity-modifying protein-1, -2 and -3 comprise the RAMP family. The three RAMPs share only 30% sequence identity and differ in their tissue distributions.[19] RAMP1 protein is involved in terminal glycosylation, maturation and presentation of the CGRP receptor to the cell surface. RAMP1-deficient mice (RAMP1(−/−)) show inflammatory responses and CGRP signalling through CLR/RAMP1 receptors, which have a crucial role in the regulation of proinflammatory cytokine production in dendritic cells.[7] Furthermore, Sundararajan *et al.*[20] reported that the administration of troglitazone or pioglitazone 24 h before PPAR-γ ligands markedly reduces infarction volume and improves neurological function following middle cerebral artery occlusion in rats. It is known that drug-treated animals show reduced inflammation, as evidenced by decreased immunoreactivity for microglial/macrophage markers and reduced interleukin-1β, cyclooxygenase-2 and inducible nitric oxide synthase. Thus, we speculated that variations in RAMP1 SNPs are associated with CI in humans.

It appeared that the discrepancy from the Hardy–Weinberg equilibrium in SNP5 was not related to the mistakes in genotyping, as the genotyping results from the scatter plot by SDS 7700 were clearly discriminated. This phenomenon may have been due to a copy number variant. In this study, we tested five SNPs other than SNP5 in the human RAMP1 gene, and performed a genetic association study in 171 CI patients and 234 age-matched control groups, and there were significant differences in overall distribution of genotypes and alleles for all of the SNPs between the CI and control groups. This analysis had no effect on the results of the case–control association study and haplotype-based case–control study, because all SNPs in this study are useful genomic markers showing large minor allele frequencies. Therefore, the accuracy of data was ensured for only five SNPs. As a consequence, we found that five haplotypes, the A-C-G haplotype (established by SNP2–SNP3–SNP6: $P=0.029$), the A-C-C-G haplotype (established by SNP2–SNP3–SNP4–SNP6: 0.047), the A-C-G haplotype (established by SNP2–SNP4–SNP6: 0.047), the A-C-C haplotype (established by SNP2–SNP3–SNP6: 0.048) and the T-A-C haplotype (established by SNP1–SNP2–SNP6: 0.0024), differed significantly between the CI patients and control subjects. In particular, the T-A-C haplotype was significantly higher in CI patients.

This study is the first to examine correlations between the human RAMP1 gene and CI using SNPs. We found that haplotypes of the RAMP1 gene are useful genetic markers of CI. Future studies will include investigations of SNPs in CRLR, which is another factor in the effects of CGRP on CI. Such studies may clarify the effects of CRLR on adrenomedullin.

Study limitations

This study only aimed at identification of the genetic markers in the RAMP1 gene related to CI. The final goal of the overall study is to isolate susceptibility mutations or polymorphisms in this gene. Some genetic markers may be linked to true susceptibility variants, and the data on these genetic markers may be useful for achieving this final goal.

What is known about this topic?

- Calcitonin gene-related peptide (CGRP) is a neuroimmune modulator related to cardiovascular regulation.[4]
- CGRP signalling is expressed via the CGRP receptor, which is composed of calcitonin receptor-like receptor (CRLR) and receptor activity-modifying protein-1 (RAMP1).[5]

What this study adds?

- The results suggest that the T-A-C haplotype with the combinations of rs3754701–rs3769048–rs7590387 is a genetic marker for cerebral infarction, and that RAMP1 or neighbouring genes are associated with increased susceptibility to cerebral infarction.

Conflict of interest

The authors declare no conflict of interest.

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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.

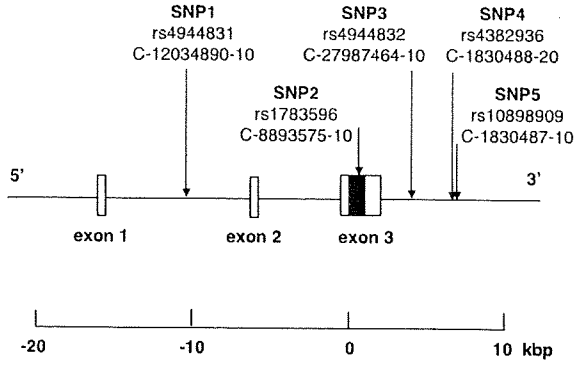

Figure 1 Structure of the human P2RY2 gene. The gene consists of three exons (boxes) separated by two introns. Lines show introns and intergenic regions. Filled box shows the coding region. Arrows indicate the locations of single nucleotide polymorphisms (SNPs). kbp, kilobase pairs.

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	0.406	0.748
	r^2		0.558	0.089	0.323
SNP3	I D'I			0.445	0.801
	r^2			0.124	0.408
SNP4	I D'I				0.776
	r^2				0.189

I D'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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Association of the Purinergic Receptor P2Y, G-Protein Coupled, 2 (*P2RY2*) Gene With Myocardial Infarction in Japanese Men

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Background: Atherosclerosis leads to myocardial infarction (MI) and *P2RY2* plays an important role in this process. The aim of the present study was to investigate the association between human *P2RY2* and MI via a haplotype-based case–control study that additionally analyzed the group by sex.

Methods and Results: The 310 MI patients and 254 controls were genotyped for 5 single-nucleotide polymorphisms (SNPs) of the human *P2RY2* gene (rs4944831, rs1783596, rs4944832, rs4382936, rs10898909). Data were separately analyzed for the total, male, and female subjects. For men, the GA+AA genotype of rs10898909 was significantly higher in MI patients as compared with controls (P=0.040). Logistic regression analysis found a significant difference for the genotype (P=0.016). As compared with controls, the frequencies of the C-A and T-C-A haplotypes were significantly higher (P=0.016, and P=0.045, respectively) in men, whereas the frequencies of the C-G and T-A-A haplotypes were significantly lower (P=0.023, and P=0.025, respectively) in MI patients.

Conclusions: The GA+AA genotype, as well as the C-A and T-C-A haplotypes, of human *P2RY2* could be genetic markers for MI in Japanese men. (*Circ J* 2009; **73:** 2322–2329)

Key Words: Case–control study; Haplotype; *P2RY2*; Single-nucleotide polymorphism

Myocardial infarction (MI), a leading cause of morbidity and mortality worldwide, is a complex multifactorial and polygenic disorder that results from an interaction between the individual's genetic make-up and various environmental factors. Coronary atherosclerosis is the essential pathological change that is noted in most MI patients. Atherosclerosis is an immunoinflammatory process that involves complex interactions between the vessel wall and blood components, and it is thought that this is initiated by endothelial dysfunction.[1] The discovery that in certain populations there are a number of polymorphisms in candidate genes that are associated with atherosclerosis has been of considerable help in understanding the genetics of MI.[2,3] With the development of extensive collections of single-nucleotide polymorphisms (SNPs), there is the possibility that these might be used as markers in genome-wide association mapping studies that could identify the MI susceptibility loci.

Recent evidence suggests that the importance of the purinergic system might be similar to that of the sympathetic and renin–angiotensin-aldosterone systems with regard to the regulation and pathophysiology of the cardiovascular system.[4,5] Purinergic receptors are ubiquitously expressed throughout the human body and have been classified as P1 or P2. The P2 receptors have been further classified into 2 groups, P2X and P2Y, with the latter having 7 membrane-spanning receptors that are coupled via G proteins. Pharmacological and molecular cloning studies have identified at least 8 P2Y receptor subtypes (P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13 and P2Y14).[6] 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 (SMC), endothelial cells and blood cells. It also appears to be involved in platelet aggregation and in the regulation of coagulation and inflammation, all of which are associated with the development of MI.[7]

Extracellular nucleotides, including adenosine triphosphate (ATP) and uridine triphosphate (UTP), which are released from a variety of arterial and blood cells, can bind to the P2Y2 cell surface receptors. Activation of P2RY2 has been shown to induce not only the proliferation and migration of vascular SMC, but also apoptosis, a process that is involved in the evolution of atherosclerotic plaque.[8] In addition, P2RY2 mediates both the vasodilation and vasoconstriction of arteries that may be involved in the vascular

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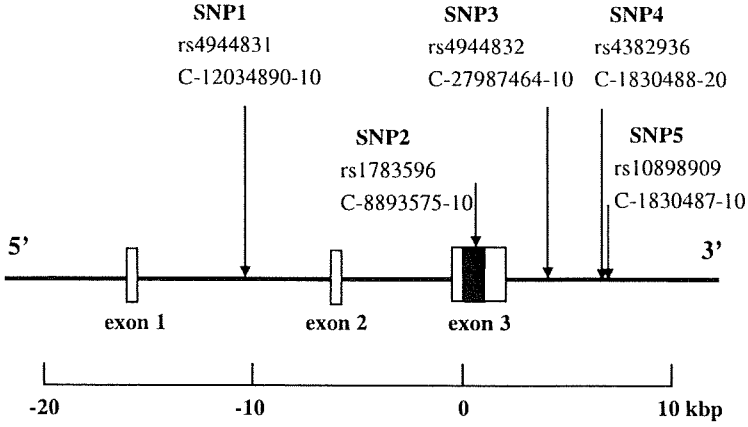

Figure. Structure of the human P2RY2 gene. The gene consists of 3 exons (boxes) separated by 2 introns. The lines show introns and intergenic regions. The filled box shows the coding region. The arrows indicate the single-nucleotide polymorphisms (SNPs).

remodeling that accompanies atherosclerosis. Evidence from both basic research and clinical studies indicates that purinergic signaling plays an important role in the atherosclerotic process. Guns et al found that in the aorta of *P2Y2*-knockout mice, there was inhibition of the ATP-endothelium-dependent relaxation,[9] which substantiates the role of *P2RY2* in endothelial dysfunction. A recent study also reported that upregulation and activation of *P2RY2* in rabbit arteries mediates the intimal hyperplasia that accompanies atherosclerosis,[10] further confirming a direct pathological role for *P2RY2*. Wihlborg et al proposed that UTP is released from the heart during cardiac ischemia and that patients will have higher plasma levels of UTP with MI.[11] All these studies show that *P2RY2* plays an important role in both atherosclerosis and the pathological changes associated with MI.

To the best of our knowledge, there has not been a previous study on the association between human *P2RY2* and MI. Therefore, our aim was to investigate this via a haplotype-based case–control study that used SNPs in conjunction with separate analyses of data with regard to sex.

Methods

Subjects

Subjects diagnosed with MI were recruited at Nihon University Itabashi Hospital and other neighboring hospitals in Tokyo from 1993 to 2008. We enrolled 310 MI patients (mean age, 61.8±9.9 years). All subjects who agreed to participate in the study were evaluated by way of a detailed questionnaire that provided information about coronary risk factors such as smoking habit, the presence of diabetes mellitus or hypertension. MI history was confirmed by the presence of 2 or more of the following: previous incidence of chest pain that was indicative of MI, a post MI follow-up that confirmed creatine kinase plus creatine kinase MB levels that were more than 3-fold higher than the upper reference limit, or the presence of characteristic ECG changes at the time of diagnosis (ST-segment elevation >0.1 mV in at least 2 leads). The study also enrolled 254 Japanese subjects as controls (mean age, 77.8± 4.2 years). All control subjects were members of the New Elder Citizen Movement in Japan and resided in the Greater Tokyo Metropolitan Area. Although some of the control subjects had vascular risk factors, such as hypertension, hypercholesterolemia, and diabetes mellitus, none had any history of MI. The sample size for the current study was in line with the sample size numbers that have been proposed as being appropriate in these types of the case–control studies.[12]

Hypertension was defined as blood pressure ≥160/100 mmHg on repeated measurement and/or the current use of antihypertensive drugs because of a history of arterial hypertension. Hyperlipidemia was defined as plasma total cholesterol >6.5 mmol or plasma triglycerides >2 mmol and/or the current use of lipid-lowering drugs that were being administered because of an established diagnosis of hyperlipidemia. Diabetes mellitus diagnosis was based on the WHO criteria.[13] To be classified as a smoker or drinker, subjects had to be currently smoking tobacco or drinking alcohol during the study period or they had to have a history of smoking and drinking. Informed consent was given by each subject in accordance with the protocol approved by the Human Studies Committee of Nihon University.[14]

Genotyping

The human *P2RY2* gene is located at chromosome 11q13.5–q14.1, spans approximately 18.1 kbp, and contains 3 exons. There are 104 SNPs 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 $r^2 \geq 0.5$. 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.

In accordance with these criteria, we selected rs4944831 (C-12034890-10, registration number of Applied Biosystems, Inc, Foster, CA, USA), rs1783596 (C-8893575-10), rs4944832 (C-27987464-10), rs4382936 (C-1830488-20), and rs10898909 (C-1830487-10) for this gene (**Figure**). SNP1 was located in intron 1, whereas SNP2 was located in the coding regions of exon 3; 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 leukocytes by phenol and chloroform extraction.[15] Genotyping was performed using the TaqMan SNP Genotyping Assay (Applied Biosystems), which uses the Taq amplification method.[16] In the 5' nuclease assay, discrimination occurs during the polymerase chain reaction (PCR) because

Table 1. Characteristics of the Study Participants

	Total			Men			Women		
	MI patients	Controls	P value	MI patients	Controls	P value	MI patients	Controls	P value
No. of subjects	310	254		254	120		56	134	
Age (years)	61.8±9.9	77.8±4.2	<0.001*	61.1±9.3	78.0±4.6	<0.001*	65.3±11.6	77.6±3.8	<0.001*
BMI (kg/m^2)	23.9±3.2	22.7±2.8	<0.001*	24.0±3.1	22.9±2.8	<0.001*	23.1±3.8	22.6±2.9	0.309
Systolic BP (mmHg)	134.5±22.1	135.7±16.8	0.479	134.8±21.2	135.0±16.0	0.909	133.1±26.2	136.4±17.6	0.343
Diastolic BP (mmHg)	80.9±14.7	79.0±11.1	0.110	81.2±14.6	78.9±10.0	0.133	79.0±15.3	79.0±12.1	0.984
Pulse rate (beats/min)	76.8±15.4	69.8±10.9	<0.001*	76.1±13.2	68.7±11.6	<0.001*	80.7±23.5	70.9±10.0	<0.001*
Creatinine (mg/dl)	1.0±0.8	0.8±0.2	<0.001*	1.0±0.8	1.0±0.2	0.211	1.0±1.0	0.8±0.2	0.006*
Hypertension (%)	15	11	0.123	16	9	0.083	14	13	0.766
Hyperlipidemia (%)	40	17	<0.001*	40	9	<0.001*	43	23	0.006*
Diabetes (%)	26	2	<0.001*	26	3	<0.001*	27	1	<0.001*
Smoking (%)	51	9	<0.001*	54	15	<0.001*	34	3	<0.001*
Drinking (%)	27	10	<0.001*	30	14	0.001*	18	7	0.020*

Continuous variables are expressed as mean±standard deviation. Categorical variables are expressed as percentage.
P value of continuous variables was calculated by Mann-Whitney U test. The P value of categorical variables was calculated by Fisher's exact test. *P<0.05.
MI, myocardial infarction; BMI, body mass index; BP, blood pressure.

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 2 unlabeled PCR primers and 2 allele-specific probes. Each probe is labeled with 2 reporter dyes at the 5' end. In the present study, VIC and FAM were used as the reporter dyes. The choice of primers and probes used in the TaqMan SNP Genotyping Assays (Applied Biosystems) was based on information available on the Applied Biosystems website (http://myscience.appliedbiosystems.com).

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 TaqMan SNP Genotyping Assay Mix (40×), primers at a concentration of 331.2 nmol/L each, and probes at a final concentration of 73.6 nmol/L 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 4 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). The plates were read on the SDS 7700 instrument that used the endpoint analysis mode of the SDS version 1.6.3 software package (Applied Biosystems). 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 2 separate output files for later comparison.

Biochemical Analysis

We measured the plasma concentration of creatinine, plasma total cholesterol, plasma triglycerides and plasma glucose using the standard methods of the Clinical Laboratory Department of Nihon University Hospital.

Statistical Analysis

All continuous variables are expressed as means±SD. Differences in continuous variables between the MI patients and the controls were analyzed using the Mann-Whitney U test. Differences in categorical variables were analyzed using Fisher's exact test. Hardy-Weinberg equilibrium was assessed by chi-squared analysis. Differences in the distributions of genotypes and alleles between the MI patients and the controls were analyzed using Fisher's exact test. Based on the genotype data of the genetic variations, we performed a linkage disequilibrium analysis and a haplotype-based case–control analysis, using the expectation maximization algorithm[17] and the software SNPAlyze version 3.2 (Dynacom Co Ltd, Yokohama, Japan). The pairwise linkage disequilibrium analysis was performed using 5 SNPs. We used |D'| values >0.25 to assign SNP locations to 1 haplotype block. SNPs with an r^2 value <0.5 were selected as tagged. In the haplotype-based case–control analysis, haplotypes with a frequency <0.01 were excluded. The P value was calculated by a permutation test. 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 (ver. 12; SPSS, Inc, Chicago, IL, USA).

Results

Table 1 shows the clinical characteristics of the study participants. The mean age of the subjects in the control group was significantly higher than the mean age of those in the MI group. In humans, the use of the so-called "super control" has been widely accepted in case–control studies for common diseases that appear later in life.[18] 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 cardiovascular diseases related to aging. MI is an age-influenced disease, and therefore a "super control" group rather than an age-matched control group is better in this type of experiments with regard to increasing the statistical power.[19,20] For the total, men, and women subject groups, the following values were significantly higher for the MI patients as

Table 2. Genotype and Allele Distributions in Patients and Controls

		Total			Men			Women		
		MI patients	Controls	P value	MI patients	Controls	P value	MI patients	Controls	P value
rs4944831 (SNP1)										
Genotype	T/T	188 (60.6%)	166 (65.4%)		151 (59.4%)	80 (66.7%)		37 (66.1%)	86 (64.2%)	
	T/G	114 (36.8%)	74 (29.1%)	0.049*	97 (38.2%)	32 (26.6%)	0.020*	17 (30.3%)	42 (31.3%)	0.946
	G/G	8 (2.6%)	14 (5.5%)		6 (2.4%)	8 (6.7%)		2 (3.6%)	6 (4.5%)	
Dominant model	TT	188 (60.6%)	166 (65.4%)	0.250	151 (59.4%)	80 (66.7%)	0.180	37 (66.1%)	86 (64.2%)	0.803
	TG+GG	122 (39.4%)	88 (34.6%)		103 (40.6%)	40 (33.3%)		19 (33.9%)	48 (35.8%)	
Recessive model	GG	8 (2.6%)	14 (5.5%)	0.074	6 (2.4%)	8 (6.7%)	0.041*	2 (3.6%)	6 (4.5%)	0.777
	TG+TT	302 (97.4%)	240 (94.5%)		248 (97.6%)	112 (93.3%)		54 (96.4%)	128 (95.5%)	
Allele	T	490 (79.0%)	406 (79.9%)	0.713	399 (78.5%)	192 (80.0%)	0.648	91 (81.3%)	214 (79.9%)	0.755
	G	130 (21.0%)	102 (20.1%)		109 (21.5%)	48 (20.0%)		21 (18.7%)	54 (20.1%)	
rs1783596 (SNP2)										
Genotype	T/T	124 (40.0%)	97 (38.2%)		109 (42.9%)	45 (37.5%)		15 (26.8%)	52 (38.8%)	
	T/C	137 (44.2%)	119 (46.8%)	0.819	106 (41.7%)	56 (46.7%)	0.592	31 (55.3%)	63 (47.0%)	0.283
	C/C	49 (15.8%)	38 (15.0%)		39 (15.4%)	19 (15.8%)		10 (17.9%)	19 (14.2%)	
Dominant model	TT	124 (40.0%)	97 (38.2%)	0.661	109 (42.9%)	45 (37.5%)	0.321	15 (26.8%)	52 (38.8%)	0.114
	TC+CC	186 (60.0%)	157 (61.8%)		145 (57.1%)	75 (62.5%)		41 (73.2%)	82 (61.2%)	
Recessive model	CC	49 (15.8%)	38 (15.0%)	0.782	39 (15.4%)	19 (15.8%)	0.905	10 (17.9%)	19 (14.2%)	0.520
	TC+TT	26 (84.2%)	216 (85.0%)		215 (84.6%)	101 (84.2%)		46 (82.1%)	115 (85.8%)	
Allele	T	385 (62.1%)	313 (61.6%)	0.868	324 (63.8%)	146 (60.8%)	0.436	61 (54.5%)	167 (62.3%)	0.155
	C	235 (37.9%)	195 (38.4%)		184 (36.2%)	94 (39.2%)		51 (45.5%)	101 (37.7%)	
rs4944832 (SNP3)										
Genotype	G/G	148 (47.7%)	108 (42.5%)		124 (48.8%)	52 (43.3%)		24 (42.9%)	56 (41.8%)	
	G/A	132 (42.6%)	114 (44.9%)	0.351	105 (41.3%)	48 (40.0%)	0.156	27 (48.2%)	66 (49.2%)	0.990
	A/A	30 (9.7%)	32 (12.6%)		25 (9.9%)	20 (16.7%)		5 (8.9%)	12 (9.0%)	
Dominant model	GG	148 (47.7%)	108 (42.5%)	0.215	124 (48.8%)	52 (43.3%)	0.321	24 (42.9%)	56 (41.8%)	0.892
	GA+AA	162 (52.3%)	146 (57.5%)		130 (51.2%)	68 (56.7%)		32 (57.1%)	78 (58.2%)	
Recessive model	AA	30 (9.7%)	32 (12.6%)	0.270	25 (9.9%)	20 (16.7%)	0.058	5 (8.9%)	12 (9.0%)	0.995
	GA+GG	280 (90.3%)	222 (87.4%)		229 (90.1%)	100 (83.3%)		51 (91.1%)	122 (91.0%)	
Allele	G	428 (69.0%)	330 (65.0%)	0.147	353 (69.5%)	152 (63.3%)	0.093	75 (67.0%)	178 (66.4%)	0.918
	A	192 (31.0%)	178 (35.0%)		155 (30.5%)	88 (36.7%)		37 (33.0%)	90 (33.6%)	
rs4382936 (SNP4)										
Genotype	C/C	178 (57.4%)	143 (56.3%)		148 (58.3%)	71 (59.2%)		30 (53.6%)	72 (53.7%)	
	C/A	118 (38.1%)	94 (37.0%)	0.528	94 (37.0%)	38 (31.6%)	0.194	24 (42.8%)	56 (41.8%)	0.957
	A/A	14 (4.5%)	17 (6.7%)		12 (4.7%)	11 (9.2%)		2 (3.6%)	6 (4.5%)	
Dominant model	CC	178 (57.4%)	143 (56.3%)	0.789	148 (58.3%)	71 (59.2%)	0.870	30 (53.6%)	72 (53.7%)	0.984
	CA+AA	132 (42.6%)	111 (43.7%)		106 (41.7%)	49 (40.8%)		26 (46.4%)	62 (46.3%)	
Recessive model	AA	14 (4.5%)	17 (6.7%)	0.259	12 (4.7%)	11 (9.2%)	0.095	2 (3.6%)	6 (4.5%)	0.777
	CA+CC	296 (95.5%)	237 (93.3%)		242 (95.3%)	109 (90.8%)		54 (96.4%)	128 (95.5%)	
Allele	C	474 (76.5%)	380 (74.8%)	0.521	390 (76.8%)	180 (75.0%)	0.595	84 (75.0%)	200 (74.6%)	0.939
	A	146 (23.5%)	128 (25.2%)		118 (23.2%)	60 (25.0%)		28 (25.0%)	68 (25.4%)	
rs10898909 (SNP5)										
Genotype	G/G	74 (23.9%)	71 (28.0%)		61 (24.0%)	41 (34.2%)		13 (23.2%)	30 (22.4%)	
	G/A	160 (51.6%)	121 (47.6%)	0.510	127 (50.0%)	54 (45.0%)	0.111	33 (58.9%)	67 (50.0%)	0.345
	A/A	76 (24.5%)	62 (24.4%)		66 (26.0%)	25 (20.8%)		10 (17.9%)	37 (27.6%)	
Dominant model	GG	74 (23.9%)	71 (28.0%)	0.270	61 (24.0%)	41 (34.2%)	0.040*	13 (23.2%)	30 (22.4%)	0.901
	GA+AA	236 (76.1%)	183 (72.0%)		193 (76.0%)	79 (65.8%)		43 (76.8%)	104 (77.6%)	
Recessive model	AA	76 (24.5%)	62 (24.4%)	0.977	66 (26.0%)	25 (20.8%)	0.279	10 (17.9%)	37 (27.6%)	0.155
	GA+GG	234 (75.5%)	192 (75.6%)		188 (74.0%)	95 (79.2%)		46 (82.1%)	97 (72.4%)	
Allele	G	308 (49.7%)	263 (51.8%)	0.484	249 (49.0%)	136 (56.7%)	0.051	59 (52.7%)	127 (47.4%)	0.347
	A	312 (50.3%)	245 (48.2%)		259 (51.0%)	104 (43.3%)		53 (47.3%)	141 (52.6%)	

P value of genotype was calculated by Fisher's exact test. *P<0.05.
Abbreviation see in Table 1.

compared with controls: pulse rate, and the prevalence of hyperlipidemia, diabetes, smoking and drinking. For the total and men subject groups, the body mass index (BMI) was significantly higher for the MI patients than the controls. For the total and women subject groups, serum creatinine was significantly higher for the MI patients as compared with the controls. There were no significant differences noted between the MI patients and the controls for systolic or diastolic blood pressure or the prevalence of hypertension.

Table 3. OR and 95%CI for Each Risk Factor and Genotype of rs4944831 and rs10898909 Associated With MI in Japanese Men

Risk factor	Men		
	OR	95%CI	P value
TG+TT genotype of rs4944831	8.622	0.603–123.280	0.112
BMI	1.140	0.978–1.329	0.094
Pulse rate	1.036	0.993–1.081	0.104
Hyperlipidemia	2.390	0.871–6.560	0.091
Diabetes mellitus	2.574	0.770–8.602	0.125
Smoking	1.914	0.792–4.625	0.149
Drinking	1.928	0.800–4.650	0.144
GA+AA genotype of rs10898909	3.344	1.258–8.929	0.016*
BMI	1.157	0.991–1.350	0.065
Pulse rate	1.033	0.990–1.079	0.132
Hyperlipidemia	2.320	0.846–6.363	0.102
Diabetes mellitus	2.374	0.690–8.170	0.170
Smoking	2.200	0.888–5.455	0.089
Drinking	2.266	0.912–5.630	0.078

*P<0.05.
OR, odds ratios; CI, confidence intervals. Other abbreviations see in Table 1.

Table 4. Pairwise Linkage Disequilibrium for the 5 SNPs in the Controls

		|D'| value				
	SNP	SNP1	SNP2	SNP3	SNP4	SNP5
r^2 value	1		0.117	0.024	0.108	0.240
	2	0.006		0.803*	0.405*	0.746*
	3	0.000	0.558*		0.445*	0.901*
	4	0.009	0.089	0.124		0.776*
	5	0.013	0.323	0.408	0.189	

*|D'|>0.25; r^2>0.5.
SNP, single-nucleotide polymorphism.

Table 2 shows the distributions of the genotypes and alleles of the 5 SNPs. The genotype distribution for each of the SNPs did not exhibit any significant difference from the Hardy-Weinberg equilibrium values found for the controls (data not shown). For the total and men subject groups, the genotype distribution of rs4944831 differed significantly between the MI patients and the controls (P=0.049, and P=0.020, respectively). For men, the distributions of the recessive model of rs4944831 (GG vs TG+TT) and the dominant model of rs10898909 (GG vs GA+AA) differed significantly between the MI patients and the controls (P=0.041, and P=0.040, respectively). Dominant and recessive models were defined based on their frequency among the total controls.

Table 3 presents the results of the logistic regression analysis, which was performed using the following parameters: genotype (TG+TT genotype of rs4944831, and GA+AA genotype of rs10898909, respectively), BMI, pulse rate, with or without hyperlipidemia, diabetes mellitus, smoking, and drinking. The results indicated there was a significant difference for the GA+AA genotype of rs10898909 (P=0.016), but not for the TG+TT genotype of rs4944831, between the male MI patients and the controls.

Table 4 shows the patterns of the linkage disequilibrium in the controls for *P2RY2*, together with their |D'| and r^2 values. Because the |D'| values were beyond 0.25 for 4 of the SNPs (SNP2, SNP3, SNP4 and SNP5), they were considered to be located within 1 haplotype block. However, SNP1 was not located within this haplotype block. Because the r^2 values for SNP2–SNP3 were >0.5, this indicated that only 1 of these SNPs was required in order to perform 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.

Table 5. Haplotype Analysis in Patients and Controls

Haplotype				Overall P value			Frequency in total			Frequency in men			Frequency in women		
				Total	Men	Women	MI patients	Controls	P value	MI patients	Controls	P value	MI patients	Controls	P value
	SNP2	SNP4		0.691	0.635	0.283									
H1	T	C					0.548	0.524	0.418	0.572	0.527	0.267	0.415	0.519	0.114
H2	C	C					0.216	0.224	0.741	0.195	0.223	0.383	0.335	0.228	0.067
H3	C	A					0.163	0.160	0.876	0.167	0.168	0.935	0.121	0.149	0.607
H4	T	A					0.073	0.092	0.294	0.065	0.082	0.451	0.129	0.104	0.669
	SNP2		SNP5	0.905	0.099	0.410									
H1	T		A				0.453	0.435	0.516	0.463	0.418	0.263	0.136	0.173	0.417
H2	C		G				0.329	0.337	0.768	0.315	0.377	0.113	0.065	0.076	0.676
H3	T		G				0.168	0.181	0.536	0.175	0.190	0.552	0.408	0.450	0.477
H4	C		A				0.050	0.047	0.785	0.047	0.015	0.070	0.391	0.301	0.066
		SNP4	SNP5	0.323	0.034*	0.473									
H1		C	A				0.490	0.455	0.256	0.499	0.401	0.016*	0.309	0.241	0.180
H2		C	G				0.275	0.293	0.457	0.268	0.349	0.023*	0.441	0.506	0.200
H3		A	G				0.222	0.225	0.896	0.222	0.217	0.855	0.032	0.020	0.545
H4		A	A				0.013	0.027	0.122	0.010	0.033	0.167	0.218	0.233	0.743
	SNP2	SNP4	SNP5	0.201	0.034*	0.074									
H1	T	C	A				0.453	0.408	0.153	0.464	0.385	0.045*	0.045	0.091	0.167
H2	C	A	G				0.159	0.157	0.944	0.161	0.163	0.982	0.029	0.000	0.008*
H3	C	C	G				0.176	0.180	0.848	0.158	0.214	0.100	0.034	0.076	0.169
H4	T	C	G				0.100	0.113	0.555	0.112	0.136	0.369	0.392	0.429	0.541
H5	T	A	G				0.068	0.068	0.990	0.065	0.054	0.656	0.019	0.021	0.734
H6	C	C	A				0.043	0.047	0.718	0.040	0.015	0.138	0.280	0.150	0.010*
H7	T	A	A				0.000	0.028	0.051	0.000	0.033	0.025*	0.089	0.082	0.786
H8	C	A	A										0.113	0.151	0.459

Haplotypes with a frequency >0.01 were determined using the software SNPAlyze (ver. 3.2; Dynacom Co Ltd, Yokohama, Japan).
P value calculated by permutation test. *P<0.05.
Abbreviation see in Table 1.

In the haplotype-based case–control study, the 4 possible combinations were SNP2–SNP4, SNP2–SNP5, SNP4–SNP5 and SNP2–SNP4–SNP5 (**Table 5**). For men, the overall distributions of the haplotype established by SNP4–SNP5 and SNP2–SNP4–SNP5 were significantly different between the MI patients and the controls (P=0.034, and P=0.034, respectively). For men, the frequencies of the C-A haplotype (SNP4–SNP5) and T-C-A haplotype (SNP2–SNP4–SNP5) were significantly higher for the MI patients as compared with controls (P=0.016, and P=0.045, respectively). In contrast, the frequencies of the C-G haplotype (SNP4–SNP5) and the T-A-A haplotype (SNP2–SNP4–SNP5) were significantly lower for the MI patients as compared with controls (P=0.023, and P=0.025, respectively). For women, the frequencies of the C-A-G haplotype (SNP2–SNP4–SNP5) and C-C-A haplotype (SNP2–SNP4–SNP5) were significantly higher for the MI patients as compared with controls (P=0.008, and P=0.010, respectively).

Discussion

In 1994, Parr et al were the first to report the sequence and functional expression of cDNA that had been cloned from airway epithelial cells and which encoded *P2RY2*.[21] In 1996, Dasari et al were the first to map human *P2RY2* to chromosome 11q13.5–q14.1 by performing a PCR analysis of the human–rodent hybrid cell line DNAs.[22] *P2RY2* is expressed in a variety of areas within the human body and previous studies in mice have identified that it plays important roles in various 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.[23] *P2RY2* may also participate in other types of physiological responses by the activation of nucleotides. Extracellular ATP or UTP binds to P2RY2 and it is this combination that activates phospholipase C (PLC) and/or adenylate cyclase.[24] In turn, PLC activation generates inositol 1,4,5-triphosphate, a mediator of Ca^{2+} release from intracellular stores, and diacylglycerol, which is an activator of protein kinase C. When adenylate cyclase is activated, this can then generate cyclic adenosine monophosphate, which is an activator of protein kinase A.

Recently, *P2RY2* was reported to have an undisputed and crucial role in the modulation of vascular tone, which is of similar importance to that of the regulators of the inflammatory response. Atherosclerosis is the main cause of MI and is now considered to be an inflammatory disease. The most important inflammatory cells for atherosclerosis are the monocytes that differentiate into macrophages or dendritic cells in plaque, and the T-helper and suppressor lymphocytes that coordinate the inflammatory reaction in plaque.[25] Inflammatory cells express P2RY2 with multiple effects. P2RY2 is expressed on both T lymphocytes and macrophages, and it has been suggested that is has an important role in atherosclerosis.[26] Various studies have demonstrated that activation of P2RY2 in endothelial cells causes the expression of VCAM-1, which mediates the adherence of the monocytes to the vascular endothelium[27] and ultimately leads to penetration of the vessel wall where promotion of arterial inflammation associated with atherosclerosis occurs. Rayment et al postulated that the expression of the P2Y2 receptor in the coronary artery of the pig is associated with smooth muscle contraction and their studies indicated that *P2RY2* is a MI candidate causative gene.[28]

To further elucidate the overall mechanism, we planned to assess the association between *P2RY2* and MI by using a haplotype-based case–control study. As shown in **Table 1**, there was no significant difference noted for blood pressure between the MI patients and controls. However, because we did not measure blood pressure until after MI onset, these values may not reflect the patients' actual levels. Unfortunately, we could not determine the true blood pressure levels in the MI patients because of the previous administration of therapeutic medications and the myocardial impairment that was present. It is ethically and clinically unfeasible to even consider stopping and/or delaying the administration of medications in subjects with hypertension and then following them until a MI occurs. Because of this, we were unable to estimate the effect of blood pressure as a confounding factor, making this a limitation of our cross-sectional case–control study.

In this study, there was a significant difference in the genotypic distributions of the recessive model of rs4944831 (GG vs TG+TT) and the dominant model of rs10898909 (GG vs GA+AA) between the male MI patients and the controls. Thus, the present results indicate that Japanese men with the recessive model of rs4944831 and the dominant model of rs10898909 have an increased risk of MI. The present logistic regression results indicate that male MI patients had a significantly higher GA+AA genotype distribution of rs10898909 than the controls, but there was no significant difference between the MI patients and controls for the TG+TT genotype distribution of rs4944831. Although a significant association was noted for the GA+AA genotype, no associations were observed for the other risk factors. The relatively small number of control subjects with diseases, including diabetes and hyperlipidemia, might be partly responsible for these results. As seen in **Table 1**, although our results indicate there was significant difference for the prevalence of diabetes and hyperlipidemia in the male MI patients as compared with controls, the actual number of control subjects with diabetes and hyperlipidemia was very small. Of the 120 controls, 3 (3%) had diabetes and 11 (9%) had hyperlipidemia. Therefore, the possibility exists that the small sample size could lead to confusing results when analyzing the association between the MI cases with diabetes or hyperlipidemia. To further clarify our results, we searched for other previously reported MI susceptibility genes. Because the odds ratios (OR) calculated for these susceptibility genotypes ranged from 1.18 to 2.54, we believe the OR of 3.344 that was determined for the current analysis was quite remarkable. Therefore, our analyses suggest that among the many genetic risk factors, the GA+AA genotype of rs10898909 in human *P2RY2* plays a significant role in the pathogenesis of MI.

Morris et al found that for genes with multiple susceptibilities, analyses based on haplotypes have advantages over analyses based on individual SNPs, particularly when there are weak linkage disequilibria between the SNPs.[29] Consequently, in the present study, we were able to successfully establish haplotypes for *P2RY2* by using different combinations of the 3 selected SNPs. Even though there were significant differences noted for the C-A-G haplotype (SNP2–SNP4–SNP5) and the C-C-A haplotype (SNP2–SNP4–SNP5) for women between the MI patient group and the controls in the present haplotype analysis, these differences may not actually be significant, as the overall distribution of the haplotype (SNP2–SNP4–SNP5) for women was not significantly different between the MI patients and

controls. In the present study, the C-A haplotype (SNP4–SNP5) and T-C-A haplotype (SNP2–SNP4-SNP5) frequencies for men were significantly higher for the MI patients as compared with controls, and thus these may be susceptibility haplotypes for MI in Japanese men. In contrast, the C-G haplotype (SNP4–SNP5) and T-A-A haplotype (SNP2–SNP4–SNP5) frequencies in men were significantly lower for the MI patients as compared with controls, and therefore these may be resistance haplotypes for MI in Japanese men.

Previous case–control studies have identified gene variants that are associated with sex-specific susceptibility to MI.[30] Our present haplotype-based case–control study also demonstrated that there were significant sex-specific differences (for men only). P2RY2 is a G-protein coupled receptor that has a high affinity for ATP and UTP, and it plays a role in a number of responses via the activation of extracellular nucleotides. Foresta et al found that rat Leydig cells possess P2Y2 receptors and that activation of the receptor by extracellular ATP stimulates testosterone secretion.[31] Being male is known to be a classic risk factor for cardiovascular disease, and this significant sex difference is attributed, at least in part, to the negative effects that testosterone has on the cardiovascular system.[32] Testosterone's association with an increased cardiovascular risk is because it adversely affects the plasma lipid and lipoprotein profiles, thrombosis and cardiac hypertrophy. In addition, it is also suspected to have proatherogenic effects.[33] Cavasin et al found that testosterone worsens cardiac function in a mouse model of MI in males, suggesting that the myocardial testosterone receptor may have a harmful effect on the myocardial response to injury.[34]

P2RY2 has a wide tissue distribution, but although it is highly expressed in the endothelium, VSMC, heart, and inflammatory cells, there is little expression in the perivascular sensory nerves. Up until now it has been unknown whether the platelets, red blood cells or the perivascular sympathetic nerves can express P2RY2.[35] However, as it is known that P2RY2 plays an important role in inflammation, and in VSMA proliferation and migration, previous studies have examined whether antagonists to P2RY2 can be used to protect against atherosclerosis.[36] At the present time, unfortunately, there are no blood- or urine-based methods of measuring P2RY2 function in the human body. In contrast, in animals there are in vitro methods that can be used to detect P2RY2 function in dissected organs and tissues, including quantitative real-time PCR, western blot or immunoblotting, and immunohistochemistry. Unfortunately, because we could not obtain informed consent from subjects who were not receiving medication, we were unable to collect any samples that would have made it possible for us to perform functional analyses of this gene.

In conclusion, the present study examined the association between the human *P2RY2* gene and MI. Our results indicate that both the GA+AA genotype of rs10898909 and the C-A and T-C-A haplotypes could be genetic markers for MI. In addition, the C-G and T-A-A haplotypes might be genetic markers for MI resistance in Japanese men. Further studies need to be done in order to isolate the functional mutations of *P2RY2* that modulate the process of atherosclerosis, and to evaluate the function of the *P2RY2* variants that are involved in the metabolism of the sex hormones.

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Haplotype-Based Case–Control Study on Human Apurinic/Apyrimidinic Endonuclease 1/Redox Effector Factor-1 Gene and Essential Hypertension

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BACKGROUND
Oxidative DNA damage is involved in the pathophysiology of essential hypertension (EH), which is a multifactorial disorder. Apurinic/apyrimidinic endonuclease 1/redox effector factor-1 (APE1/REF-1) is an essential endonuclease in the base excision repair pathway of oxidatively damaged DNA, in addition to having reducing properties that promote the binding of redox-sensitive transcription factors. Blood pressure in APE1/REF-1-knockout mice is reported to be significantly higher than in wild-type mice. The aim of this study was to investigate the relationship between EH and the human *APE1/REF-1* gene through a haplotype-based case–control study using single-nucleotide polymorphisms (SNPs).

METHODS
We selected five SNPs in the human *APE1/REF-1* gene (rs1760944, rs3136814, rs17111967, rs3136817, and rs1130409), and performed case–control studies in 265 EH patients and 266 age-matched normotensive (NT) subjects.

RESULTS
rs17111967 was found to show nonheterogeneity among Japanese subjects. There were no significant differences in the overall distribution of genotypes or alleles for each SNP between EH and NT groups. In the overall distribution of the haplotype-based case–control study constructed based on rs1760944, rs3136817, and rs1130409, the frequency of the G-T-T haplotype was significantly higher in the EH group than in the NT group (2.1% vs. 0.0%, $P = 0.001$). Multiple logistic regression analysis also revealed significant differences for the G-T-T haplotype, even after adjustment for confounding factors (OR = 8.600, 95% CI: 1.073–68.951, $P = 0.043$).

CONCLUSIONS
Based on the present results, the G-T-T haplotype appears to be a genetic marker of EH, and the *APE1/REF-1* gene appears to be a susceptibility gene for EH.

Keywords: apurinic/apyrimidinic endonuclease 1/redox effector factor-1; blood pressure; case–control study; essential hypertension; haplotype; hypertension; single-nucleotide polymorphism

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Oxidative stress, which is cellular damage caused by excess generation of reactive oxygen species, has recently been reported to be a factor in various diseases, including cancer, leukemia, cerebral infarction, myocardial infarction, and hypertension.[1,2] DNA damage is one of the biological injuries caused by oxidative stress. It has also been reported that oxidative DNA damage increases in hypertension and cardiovascular diseases.[3]

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Apurinic/apyrimidinic endonuclease 1/redox effector factor-1 (APE1/REF-1) is a protein with multiple roles and that impacts a wide variety of important cellular functions.[4] It is known that APE1/REF-1 has two major functions. On one hand, APE1/REF-1 acts as an apurinic/apyrimidinic endonuclease during the second stage of the DNA base excision repair pathway, which is responsible for the repair of cellular oxidative DNA damage.[5] On the other hand, APE1/REF-1 is also known as redox effector factor-1 (REF-1), which is important for the activation of transcription factors such as activator protein 1 (AP1), p53, and nuclear factor κB.[6,7] The human *APE1/REF-1* gene is located on chromosome 14q11.2–q12 and consists of five exons and four introns spanning 2.64 kilobase pairs,[8] encoding 318 amino acids.[9] The apurinic/apyrimidinic endonuclease activities on APE1/REF-1 reside in the N-terminal region, whereas the redox properties require a conserved C-terminal region.[10]

There have been no studies examining the association between the human *APE1/REF-1* gene and essential hypertension (EH).