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Haplotype-based case study of human CYP4A11 gene and cerebral infarction in Japanese subject

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Abstract *Objective* CYP4A11 is an enzyme that converts arachidonic acid to 20-hydroxyeicosatetraenoic acid, which is involved in regulation of vascular tone in the brain. Recent evidence indicates that the polymorphism of the CYP genes is associated with cerebral infarction (CI). The aim of the present study was to assess the association between the human CYP4A11 gene and CI using a haplotype-based case-control study divided by gender. *Methods* Three SNPs of the human CYP4A11 gene (rs2269231, rs1126742, and rs9333025) were selected and genotyped for 174 CI patients and 293 controls. The data were assessed for three separate groups: total subjects, men and women. *Results* In men, the genotype distribution of rs9333025 significantly differed between the CI patients and control subjects ($P = 0.047$). The distribution of the dominant model of rs9333025 (GG vs. GA + AA) significantly differed between both the total and the men groups ($P = 0.033$, $P = 0.028$, respectively). Logistic regression analysis adjusted for the history of hypertension and diabetes mellitus also showed that the GG genotype was significantly more frequent in the CI patients than in the controls, both for the total and men groups ($P < 0.001$, $P = 0.008$, respectively). The overall distribution of the haplotypes constructed with the 3 SNPs showed significant differences between the CI and the control in total group ($P = 0.049$). The T-C-G haplotype was significantly more frequent in control subjects than in the CI patients in the total group ($P = 0.020$). *Conclusions* The GG genotype of rs9333025 could be a genetic marker for CI in Japanese men. In addition, the T-C-G haplotype might also be a protective marker for CI in Japanese.

Keywords CYP4A11 · Single-nucleotide polymorphism · Haplotype · Case-control study

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Introduction

Cerebral infarction (CI) is a multifactorial disease and a major cause of death and disability throughout the world. In addition to conventional risk factors such as hypertension, diabetes mellitus, and smoking, evidence from animal, clinical, and epidemiological studies have repeatedly supported a genetic contribution to CI susceptibility (e.g., angiotensin-converting enzyme gene, apolipoprotein E gene, beta fibrinogen gene) [1–5]. Cytochrome p450 (CYP), a superfamily of cysteinato-heme enzymes, is responsible for not only the metabolism of xenobiotics but also a host of endobiotics, such as arachidonic acid (AA). CYP-derived AA metabolites play a role in the modulation of cerebrovascular physiology and pathology [6, 7]. Polymorphisms of the CYP genes are also involved in the pathogenesis of stroke, for example, the T6325C genotype of CYP1A1 has been shown to modulate stroke risk in

hypertensive patients [8], CYP2C8 and CYP2C9 decreases expression by cocaine (psychostimulant drugs abuse is associated with an increased risk of stroke) [9], CYP2C11 increases expression in transient ischemic attack (TIA) [10], and polymorphisms in the 5′-flanking region of the PGIS gene (CYP8A) are associated with CI [11]. The CYP4A11 subfamily acts mainly as an enzyme that converts arachidonic acid to 20-hydroxyeicosatetraenoic acid (20-HETE), which plays an important role in the regulation of vascular tone in the brain, kidney, heart, and splanchnic beds [12–14]. Recent research has shown that 20-HETE augments the myogenic constriction of cerebral blood vessels and is a potent constrictor of cerebral arteries. When 20-HETE synthesis is inhibited in the rat model of ischemic stroke, there is reduction of the infarct size [7, 15, 16]. Polymorphisms within the coding or promoter regions of the CYP4A11 gene can affect the metabolism of arachidonic acid, with a net result of an altered 20-HETE generating capacity. Given the potential importance of 20-HETE in the cerebral vascular tone, the aim of the present haplotype-based case-control study was to assess the association between the human CYP4A11 gene and CI.

Subjects and methods

Subjects

The study group consisted of 174 patients (mean age, 66.1 ± 12.8 years) diagnosed with CI. The diagnosis was based on a neurologic examination and the findings of computed tomography (CT) or magnetic resonance imaging (MRI) or both. In addition, all patients had a neurologic deficit rating greater than grade 3 on the modified Rankin Scale. The study also enrolled 293 Japanese subjects as controls (mean age, 77.8 ± 4.0 years). All of the control subjects were members of the New Elder Citizen Movement in Japan residing in the Greater Tokyo Metropolitan Area, and they all had vascular risk factors such as hypertension, hypercholesterolemia, or diabetes mellitus but no history of CI. All controls were confirmed to have grade 0 on the modified Rankin Scale. Individuals with atrial fibrillation were excluded from both the CI and non-CI groups. Participants with cancer or autoimmune disease, including antiphospholipid antibody syndrome, were also excluded. Informed consent was obtained from each subject according to a protocol approved by the Human Studies Committee of Nihon University [17].

Genotyping

We selected 3 SNPs in the human CYP4A11 gene as markers for assessment of the genetic association. The minor allele frequency of each SNP was >10%, indicating that these SNPs should all be effective genetic markers. We designated these SNPs as SNP1, SNP2, and SNP3, in order of increasing distance from the 5′ end of the gene (Fig. 1). All 3 SNPs were confirmed using dbSNP at the NCBI website and the Applied Biosystems-Celera Discovery System (Foster City, CA). The respective accession numbers of SNP1, SNP2, and SNP3 were rs2269231 (C_15876257_10), rs1126742, and rs9333025 (C_29846881_10) (Fig. 1). SNP2, which is located in exon 11, involves a thymidine-to-cytosine substitution (T8590C) that results in a nonsynonymous phenylalanine-to-serine (F-to-S) substitution at amino acid residue 434 of CYP4A11 [18]. SNP1 and SNP3 are located in the introns.

Blood samples were collected from all participants, and genomic DNA was extracted from the peripheral blood leukocytes by extraction with phenol and chloroform [19]. Genotyping was performed using the TaqMan® SNP Genotyping Assay (Applied Biosystems Inc., Foster City, CA, USA). The TaqMan® SNP Genotyping Assays were performed using the method of Taq amplification. 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 (MGB) 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®, ABI). Cleavage results in the increased emission of a reporter dye. Each 5′ nuclease assay requires two unlabeled PCR primers and two allele-specific probes. In the present study, at the 5′ end, all the probes were labeled with two reporter dyes, VIC and FAM. The primers and probes used in the TaqMan® SNP Genotyping Assays (ABI) were chosen based on the information available at the ABI website (http://myscience.appliedbiosystems.com).

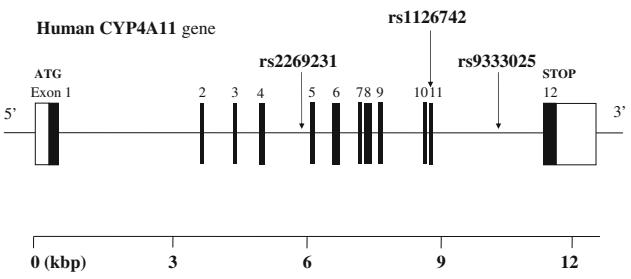

Fig. 1 The structure of the human CYP4A11 gene. This consists of 12 exons separated by 11 introns. Boxes indicate exons, and lines indicate introns and intergenic regions. Filled boxes indicate coding regions. Arrows mark the locations of polymorphisms

PCR amplification was performed using 6 μl of TaqMan® Universal Master Mix, No AmpErase® UNG (2×) (ABI) in a 12 μl final reaction volume containing 2 ng of DNA, 0.22 μl of TaqMan® SNP Genotyping Assay Mix (20× or 40×), primers at a concentration of 900 nmol/l each, and probes at a final concentration of 200 nmol/l each. Thermal cycling conditions were as follows: 50°C for 2 min; 95°C for 10 min; 40 cycles of 95°C for 15 s; and 62°C for 1 min. Thermal cycling was performed using the GeneAmp 9700™ system.

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 (ABI). The plates were read on the SDS 7700 instrument with the end-point analysis mode of the SDS version 1.6.3 software package (ABI). The genotypes were determined visually based on the dye-component fluorescent emission data depicted in the X–Y scatter-plot of the SDS software. The genotypes were also determined automatically by the signal processing algorithms of the software. The results of each scoring method were saved in two separate output files for later comparison.

Biochemical analysis

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

Statistical analysis

All continuous variables were expressed as mean ± SD. Differences in continuous variables between CI patients and control subjects 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 x^2 analysis. Differences in distributions of genotypes and alleles between CI patients and control subjects were analyzed using Fisher's exact test. Based on the genotype data of the genetic variations, we performed linkage disequilibrium (LD) analysis and haplotype-based case-control analysis, using the expectation maximization (EM) algorithm and the software SNPAlyze version 3.2 (Dynacom Co., Ltd., Yokohama, Japan). The pairwise LD analysis was performed using 3 SNP pairs. We used |D′| values of >0.5 to assign SNP locations to 1 haplotype block. SNPs with an r^2 value of <0.5 were selected as tagged. In the haplotype-based case-control analysis, haplotypes with a frequency of <0.01 were excluded. The frequency distribution of the haplotypes was calculated by chi-square analysis. In addition, logistic regression analysis was performed to assess the contribution of confounding factors. Statistical significance was established at $P < 0.05$. Statistical analyses were performed using SPSS software for Windows, version 12 (SPSS Inc., Chicago, IL).

Results

Table 1 shows the clinical characteristics of the study participants. The mean age was significantly higher in the control than in the CI group. In humans, use of the so-called super control has been widely accepted in case-control studies for common diseases that appear later in life [20]. For the total, men, and women subjects, the following values were significantly higher for the CI patients when compared to the control subjects: SBP, DBP, pulse rate, and the prevalence of hypertension and diabetes mellitus. In the controls, the plasma total cholesterol concentration was significantly higher than that seen in the experimental groups. For the total group and the men subjects, serum creatinine was significantly higher for the CI patients as compared to the controls. For the subjects in the men group, the prevalence of hyperlipidemia was significantly different between the CI patients and control subjects. Although the prevalence of smoking was significantly higher in the CI patients than for the control subjects in the total group, when divided by gender, the prevalence of smoking was not significantly different. There were no significant differences in the BMI and the prevalence of drinking between the CI patients and the control subjects.

Table 2 shows the distribution of the genotypes and alleles of the 3 SNPs. The genotype distribution for each of the SNPs in the control group showed good agreement with the Hardy-Weinberg equilibrium values (data not shown). For the men group, the genotype distribution of SNP3 differed significantly between the CI patients and the control subjects ($P = 0.047$). For the total and men groups, the distribution of the dominant model of SNP3 (GG versus GA + AA) differed significantly between the CI patients and the control subjects ($P = 0.033$, $P = 0.028$, respectively). Logistic regression analysis adjusted for the history of hypertension and diabetes mellitus also showed that for the total and men groups, the GG genotype of SNP3 was significantly more frequent in CI patients than in controls ($P < 0.001$, $P = 0.008$, respectively). Dominance and recessiveness of the models were defined by their frequency among the total subjects.

Table 3 shows patterns of linkage disequilibrium in the CYP4A11 gene, with their |D′| and r^2 values. All 3 SNPs are located in 1 haplotype block and, thus, are suitable for a

Table 1 Characteristics of study participants

Number	Total			Men			Women		
	CI patients 174	Control subjects 293	*P*-value	CI patients 104	Control subjects 143	*P*-value	CI patients 70	Control subjects 150	*P*-value
Age (years)	66.1 ± 12.8	77.8 ± 4.0	<0.001*	64.0 ± 11.9	77.8 ± 4.2	<0.001*	69.3 ± 13.5	77.8 ± 3.8	<0.001*
BMI (kg/m^2)	23.0 ± 4.2	22.6 ± 2.8	0.378	23.1 ± 3.0	22.9 ± 2.7	0.779	22.7 ± 5.9	22.3 ± 2.8	0.560
SBP (mmHg)	151.3 ± 25.6	135.7 ± 16.3	<0.001*	149.2 ± 25.3	135.8 ± 15.1	<0.001*	154.4 ± 26.0	135.6 ± 17.4	<0.001*
DBP (mmHg)	86.1 ± 16.0	78.5 ± 11.0	<0.001*	86.9 ± 16.0	78.7 ± 9.9	<0.001*	84.9 ± 16.1	78.4 ± 11.9	0.001*
Pulse (beats/min)	76.8 ± 14.8	70.2 ± 11.1	<0.001*	75.6 ± 14.4	69.0 ± 11.7	<0.001*	78.5 ± 15.4	71.4 ± 10.4	<0.001*
Creatinine (mg/dl)	1.0 ± 0.4	0.9 ± 0.2	<0.001*	1.1 ± 0.4	1.0 ± 0.2	0.002*	0.8 ± 0.3	0.8 ± 0.2	0.109
Total cholesterol (mg/dl)	195.7 ± 50.2	217.9 ± 43.6	<0.001*	191.9 ± 51.8	205.3 ± 31.8	0.016*	201.4 ± 47.5	230.3 ± 49.7	<0.001*
Hypertension (%)	29	8	<0.001*	29	9	<0.001*	29	6	<0.001*
Diabetes (%)	16	2	<0.001*	14	4	0.004*	19	1	<0.001*
Hyperlipidmia	26	20	0.124	22	10	0.012*	31	29	0.676
Smoking (%)	46	48	0.044*	64	51	0.148	18	10	0.284
Drinking (%)	41	40	0.948	58	50	0.366	16	28	0.159

BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; CI, cerebral infarction

Continuous variables were expressed as mean ± standard deviation. Categorical variables were expressed as percentage

The *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. * $P < 0.05$

haplotype-based case-control study. In the haplotype-based case-control analysis, six possible haplotypes (H1 to H6) were predicted for the 3 SNPs in the total and women groups, while six possible haplotypes (H1 to H5, H7) were predicted in the men group (Table 4). For the total group, the overall distribution of the haplotypes predicted by the 3 SNPs showed a significant difference between the CI patients and control subjects ($P = 0.049$). Also for the total group, the T-C-G haplotype was significantly more frequent in the control subjects versus the CI patients ($P = 0.020$).

Discussion

Endogenous CYP metabolites, such as epoxyeicosatrienoic acids (EETs), hydroxyeicosatetraenoic acids (HETEs), prostacyclin (PGI_2), aldosterone, and sex hormones, have been demonstrated to be involved in hypertension, coronary artery disease (CAD), stroke, and other cardiovascular disease (CVD) [21]. Arachidonic acid (AA) is metabolized by the cytochrome P450 4A (CYP4A) enzymes in the cerebral arteries to produce 20-hydroxyeicosatetraenoic acid (20-HETE), which serves as an endogenous vasoconstrictor that activates protein kinase C (PKC). Subsequently PKC depolarizes cerebral vascular smooth muscle cells (VSMC) by inhibiting the opening of the large conductance KCa channel, thereby enhancing Ca^{2+} influx through the voltage sensitive Ca^{2+} channels [15, 22, 23]. Recent observations that elevations in transmural pressure increase 20-HETE levels [7, 15] and that CYP inhibitors attenuate the pressure-induced constriction of the cerebral arteries suggest that 20-HETE augments myogenic constriction of the cerebral blood vessels and plays a role in the autoregulation of the cerebral blood flow (CBF) and the myogenic response of the cerebral arteries [7]. 20-HETE may also contribute to the development of vasospasm following subarachnoid hemorrhage (SAH) [24, 25]. It has been shown that 20-HETE synthesis inhibitor can prevent a fall in CBF following SAH and reduce the infarct size in the rat model of ischemic stroke [16]. It is interesting to note that the CYP4A genes co-localize with the genes responsible for the large infarct volumes in the spontaneously hypertensive stroke-prone rat (SHRSP), which is an experimental model of stroke characterized by a high frequency of spontaneous stroke, as well as an increased sensitivity to experimentally induced focal cerebral ischemia [26]. We hypothesized that variability of the CYP4A11 gene might affect the CI risk. In this association study, we used a "super control" group, as healthy elderly subjects have been found to be more suitable than young or middle-aged subjects when it comes to determining phenotypes of cardiovascular and cerebrovascular diseases related to aging. CI is an age-influenced disease, and therefore, use of a "super control" group rather than an age-matched control group is better in these types of experiments. In this study, the level of total cholesterol and the number of subjects with hyperlipidemia were significantly higher in the control

Table 2 Genotype and allele distributions in patients with CI and control subjects

			Total			Men			Women		
			CI patients	Control subjects	*P*-value	CI patients	Control subjects	*P*-value	CI patients	Control subjects	*P*-value
rs2269231 (SNP1)	Genotype	A/A	41	73	0.468	27	32	0.321	14	41	0.500
		A/T	85	154		46	77		39	77	
		T/T	48	66		31	34		17	32	
	Dominant model	AA	41	73	0.742	27	32	0.514	14	41	0.242
		AT+TT	133	220		77	111		56	109	
	Recessive model	TT	48	66	0.218	31	34	0.288	17	32	0.624
		AT+AA	126	227		73	109		53	118	
	Allele	A	167	300	0.343	100	141	0.788	67	159	0.315
		T	181	286		108	145		73	141	
rs1126742 (SNP2)	Genotype	T/T	106	190	0.602	61	92	0.541	45	98	0.987
		T/C	63	93		41	47		22	46	
		C/C	5	10		2	4		3	6	
	Dominant model	TT	106	190	0.394	61	92	0.364	45	98	0.879
		TC+CC	68	103		43	51		25	52	
	Recessive model	CC	5	10	0.265	2	4	0.660	3	6	0.921
		TC+TT	169	183		102	139		67	144	
	Allele	T	275	473	0.531	163	231	0.512	112	242	0.870
		C	73	113		45	55		28	58	
rs9333025 (SNP3)	Genotype	G/G	105	147	0.094	67	72	0.047*	38	75	0.671
		G/A	53	116		26	57		27	59	
		A/A	16	30		11	14		5	16	
	Dominant model	GG	105	147	0.033*	67	72	0.028*	38	75	0.554
		GA+AA	69	146		37	71		32	75	
	Recessive model	AA	16	30	0.715	11	14	0.840	5	16	0.407
		GA+GG	158	263		93	129		65	134	
	Allele	G	263	410	0.065	160	201	0.100	103	209	0.401
		A	85	176		48	85		37	91	

CI, cerebral infarction

The *P*-value of the genotype was calculated by Fisher's exact test. * $P < 0.05$

Table 3 Pairwise linkage disequilibrium for the three single nucleotide polymorphisms

	SNP	|D′|						
		CI patients			SNP	Control subjects		
		SNP1	SNP2	SNP3		SNP1	SNP2	SNP3
r^2	SNP1		**0.848**	**0.736**	SNP1		**0.743**	**0.607**
	SNP2	0.207		**0.734**	SNP2	0.126		**0.762**
	SNP3	0.190	0.046		SNP3	0.151	0.059	

CI, cerebral infarction

Bold values correspond to $|D'| > 0.5$, $r^2 > 0.5$

subjects, which is the reason why many of the control subjects diagnosed with hyperlipidemia were treated with diet therapy without the administration of antihyperlipidemic drugs.

In the present study, the results showed that for the men, the GG genotype of SNP3 was observed significantly more frequently in the CI patients than in the controls. This indicates that the risk of CI is increased in men with the GG genotype of SNP3. When the logistic regression analysis was adjusted for confounders, the same results were observed. Men are generally at a greater risk for cardiovascular than age-matched, premenopausal women. In addition, it is well documented that the incidence of stroke is higher in men than in women for all age classes [27, 28]. Although the effects of androgen on the cerebral vasculature are still poorly defined, androgen has a close relationship with thrombosis, a disease that is caused by an increase in endothelial TXA_2 synthesis [29], which leads to a potentiation of platelet aggregation and an increase in collagen and other fibrous protein in the arterial vascular tissue [30]. While the human CYP4A11 enzyme does not

Table 4 Haplotype analysis in patients with CI and control subjects

Haplotype	CYP4A11 polymorphism			Overall *P* value			Frequency in total		*P*-value	Frequency in men		*P*-value	Frequency in women		*P*-value
	SNP1	SNP2	SNP3	Total	Men	Women	CI patients	Control subjects		CI patients	Control subjects		CI patients	Control subjects	
				0.049*	0.192	0.3772									
H1	Mj	Mj	Mj				0.075	0.095	0.320	0.084	0.107	0.351	0.060	0.084	0.361
	A	T	G												
H2	Mn	Mj	Mj				0.487	0.433	0.099	0.479	0.433	0.285	0.499	0.426	0.152
	T	T	G												
H3	Mj	Mn	Mj				0.200	0.170	0.267	0.214	0.168	0.215	0.183	0.171	0.747
	A	C	G												
H4	Mj	Mj	Mn				0.213	0.248	0.198	0.187	0.217	0.475	0.243	0.278	0.433
	A	T	A												
H5	Mn	Mj	Mn				0.025	0.037	0.276	0.036	0.059	0.228	0.000	0.021	0.096
	T	T	A												
H6	Mn	Mn	Mj				0.000	0.016	0.020*	0.000	0.000	–	0.016	0.020	0.697
	T	C	G												
H7	Mn	Mn	Mn				0.000	0.000	–	0.000	0.015	0.084	0.000	0.000	–
	T	C	A												

CI, cerebral infarction; Mj, major allele; Mn, minor allele. Mj and Mn indicate a haplotype with major and minor frequencies, respectively

Haplotype with frequency >0.01 was estimated by SNPAlyze software

P value was calculated by chi-square analysis. * $P < 0.05$

exist in mice, the cyp4a14 and cyp4a10 enzymes in mice have a role similar to the human CYP4A11. The human CYP4A11 has been shown to be 72.69% identical with the murine cyp4a14, and 73.02% identical with murine cyp4a10 [31]. In male cyp4a14–/– or cyp4a10–/– mice, there are higher blood pressures seen as compared to the female cyp4a14–/– or cyp4a10–/– mice, respectively [32, 33]. In addition, male cyp4a14–/– mice exhibit an increased plasma androgen and their hypertensive phenotype has been shown to be androgen sensitive [32]. Furthermore, in aromatase (cyp19) knockout mice, a state of androgen dominance is seen in which these mice are highly sensitive to cerebral ischemia related to elevated androgen, with the symptoms completely disappearing upon estrogen treatment [34]. When the previous findings and our present results are taken together, we can speculate that SNP3 may be associated with the sex hormone-mediated activity of the CYP4A11 enzyme. Unfortunately, since we did not obtain subject consent for the purpose of collecting blood samples to determine plasma androgen or estrogen concentrations, we were not able to perform any data analyses related to our speculations.

Morris et al. found that for genes with multiple susceptibilities, analyses based on haplotypes have advantages over analyses based on individual SNPs, particularly when linkage disequilibria between SNPs is weak [35]. Based on such findings, we hypothesized that a haplotype analysis would be useful in the assessment of the association between the haplotypes and CI. Consequently, in the present study, the T-C-G haplotype constructed by the 3 SNPs for the total group was found significantly more frequent in the control subjects compared to that seen in CI patients. Thus, in the total group, the T-C-G haplotype is thought to be a resistance haplotype. In the men group, the T-C-G haplotype could not be found because its frequency was too low. In the women group, there was no difference noted between CI patients and control subjects for the T-C-G haplotype. It was unclear why there was no significant difference noted for the women group in the current study, although this could perhaps be due to the number of subjects in each group after the groups were divided according to gender.

Genetic variations of single risk factors do not significantly influence the overall risk for CI. The impact of a single polymorphism on a complex disease such as CI may be influenced by patient and genetic marker selections, ethnic backgrounds, and sample size, among other factors. Since case-control studies can sometimes produce pseudo-positive results, our collective results that represent a cross-sectional sample of the survivors of cerebrovascular events can therefore only be applied to survivors of CI.

In conclusion, the GG genotype of rs9333025 could be a genetic marker for CI in Japanese men. In addition, the T-C-G haplotype could be a protective marker for CI in Japanese. Further studies are needed to isolate the functional mutations in the CYP4A11 gene that are related to CI.

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