

Potential Association Between Frequent Nonsynonymous Variant of *NPPA* and Cardioembolic Stroke

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Atrial natriuretic peptide (ANP, also known as *NPPA*) and brain natriuretic peptide (BNP, also known as *NPPB*) have been determined as genetic factors for several diseases, including stroke and myocardial infarction, in human and rat models. To investigate the potential association between polymorphisms of the *NPPA* gene and stroke in a Korean population, nine single-nucleotide polymorphisms (SNPs) of *NPPA* and *NPPB* genes were genotyped in a total of 941 Korean subjects, including 674 stroke patients (109 hemorrhagic and 565 ischemic) and 267 unaffected controls. Genotype comparisons of the targeted alleles revealed that there were no significant associations between stroke patients and control subjects, or among hemorrhagic, ischemic, and control groups. However, in logistic analysis for Trial of Org 10172 in Acute Stroke Treatment (TOAST) classification of ischemic stroke, nonsynonymous rs5065 (STOP152Arg) and rs5067 in 3'UTR of *NPPA*, which were in complete linkage disequilibrium, showed significant associations with cardioembolic stroke. These two SNPs showed higher frequencies in cardioembolic stroke patients than those in controls and ischemic patients with small-vessel occlusion ($p=0.002$, adjusted $p=0.02$). It was also found that *NPPA* rs5065C allele in all of the Korean subjects existed as heterozygous compared with Caucasian and African populations. Although further replications in larger cardioembolic stroke subjects are required, our preliminary findings suggest that the nonsynonymous rs5065C of the *NPPA* gene, which could produce a new or dysfunctional transcript, is possibly associated with cardioembolism.

Introduction

STROKES OCCUR WHEN blood supply to a part of brain is interrupted or severely reduced with developing loss of brain functions. Strokes can be classified into two major categories: ischemic and hemorrhagic. Ischemia is caused by interruption of the blood supply, while hemorrhage is caused by rupture of a blood vessel or an abnormal vascular structure. About 80% of strokes are due to ischemia, whereas the remainders are due to hemorrhage. Furthermore, stroke is a complex neurological disorder that most likely results from complicated interactions between genetics, lifestyle, and environment. Family, twin, and population studies have shown significant genetic contributions to disease occurrence (Liao *et al.*, 1997; Rastenyte *et al.*, 1998; Hademenos *et al.*, 2001).

A genome-wide screen using F2 cohort from mating between stroke-prone spontaneously hypertensive rat and stroke-resistant spontaneously hypertensive rat revealed *NPPA* and *NPPB* genes, which are located at chromosome 1p36.21 and 1p36.2, respectively, as blood pressure-

independent genetic factors predisposing to a complex form of stroke (Rubattu *et al.*, 1996; Jeffs *et al.*, 1997). Natriuretic peptides, as polypeptide hormones primarily secreted from the heart, play important roles in the balance of electrolytes, water, and fat, and also in the regulation of cardiac hemodynamics through their cardiovascular, renal, and neural effects (Rubattu *et al.*, 2008; Potter *et al.*, 2009). In addition, the expression, regulation, and therapeutic applications of natriuretic peptides during development and diseases have been demonstrated (Houben *et al.*, 2005; Houweling *et al.*, 2005; Potter *et al.*, 2009). Moreover, polymorphisms of *NPPA* and *NPPB* genes, which encode atrial natriuretic peptide (ANP, also known as *NPPA*) and brain natriuretic peptide (BNP, also known as *NPPB*), respectively, have been shown to be associated with vascular diseases, including stroke, blood pressure, and hypertension (Rubattu *et al.*, 2004; Conen *et al.*, 2009; Newton-Cheh *et al.*, 2009).

Based on disease etiology and clinical features, the Trial of Org 10172 in Acute Stroke Treatment (TOAST) subclassified ischemic stroke into five subtypes as follows: large-artery atherosclerosis (LAA), small-vessel occlusion (SVO),

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cardioembolism (CE), stroke of other determined etiology (SOE), and stroke of undetermined etiology (SUE) (Adams *et al.*, 1993). The interrater reliability of TOAST classification was also assessed recently (Meschia *et al.*, 2006), providing its efficiency in strategies for treatment and management of stroke recurrence. LAA, which approximately accounts for 10%–25% of ischemic strokes, is characterized by significant stenoses of cerebral cortical artery or brain artery. SVO (15%–25%) is one of the traditional clinical lacunar syndromes that are due to occlusion of a single penetrating artery, without evidence of cerebral cortical dysfunction. CE (15%–35%) patients have arterial occlusions derived from an embolus in the heart, and the condition accounts for about 20% of ischemic strokes. Since SUE includes patients with no or more than two causes of stroke, disease occurrence is occasionally high depending on the evaluation (Yip *et al.*, 1997).

Materials and Methods

Study subjects

This study included 267 unaffected controls and 674 stroke subjects recruited for about 3 years (between 2006 and 2009) and diagnosed from three oriental medical hospitals of multicenter. Patient subjects consisted of 109 hemorrhagic cases and 565 ischemic patients. Computed tomography (CT) and magnetic resonance imaging (MRI) evaluations of all participants were included for diagnosis. Written informed consent was obtained from all participants, and the study protocol was approved by the Institutional Review Board of each hospital. Patients with subdural hemorrhage, epidural hemorrhage, and stroke from injuries (e.g., traffic accident and fall) were excluded from this study. Ischemic patients were additionally determined according to the TOAST classification based on clinical features and CT/MRI evaluation. Genomic DNAs and clinical data of healthy controls were obtained from the center for genome science of Korea National Institute of Health.

Genotyping

Genomic DNA was extracted from peripheral blood lymphocytes using Exgene blood SV kit (GeneAll), according to the manufacturer's protocol. We mainly targeted the nonsynonymous single-nucleotide polymorphism (SNPs) of the *NPPB* and *NPPA* genes with heterozygosity above 0.01. SNPs in promoter region of the *NPPA* gene were also included due to the significant effect on *NPPA* expression. The rs198389 of the *NPPB* gene was added because this SNP was recently reported to be associated with elevating plasma BNP levels (Takeishi *et al.*, 2007). Genotyping was performed with 20 ng of genomic DNA by TaqMan assay in the ABI prism 7900HT sequence detection system (Applied Biosystems). Genotyping data were obtained using the ABI-PRISM sequence detection system software version 2.3. The genotype quality score for keeping data was set at 0.25. As a result of this process, 674 stroke patients and 267 normal controls were successfully genotyped.

Data analysis and statistics

Blood test and clinical data of patients in each group and controls were analyzed using STATA/SE 10.1 for windows (StataCorp LP). Hardy–Weinberg equilibrium for individual

TABLE 1. CLINICAL PROFILES OF STUDY SUBJECTS

Clinical profiles	Strokes			TOAST classification of ischemic stroke					Normal controls	p ^a
	Total	Ischemic	Hemorrhagic	LAA	SVO	CE	SOE	SUE		
Number of subjects (n)	674	565	109	123	395	33	7	7	267	
Sex (male/female)	379/295	317/248	62/47	63/60	235/160	13/20	3/4	3/4	115/152	
Age (year)	65.3 ± 12.2	59.3 ± 13.1	66.5 ± 11.7	65.3 ± 12.9	66.9 ± 10.9	69.5 ± 10.1	50.1 ± 25.9	63.6 ± 7.3	49.8 ± 7.9	<0.001
Total cholesterol (mg/dL)	180.9 ± 41.4	182.7 ± 41.7	171.8 ± 38.6	177.6 ± 51.0	184.5 ± 38.7	180.8 ± 40.5	178.3 ± 46.0	182.3 ± 23.5	184.3 ± 33.6	0.242
Triglyceride (mg/dL)	149.8 ± 90.2	150.1 ± 91.9	147.7 ± 79.1	149.6 ± 93.4	153.2 ± 92.1	119.3 ± 73.3	140.6 ± 145.7	130.0 ± 36.6	144.7 ± 91.8	0.444
High-density lipoprotein cholesterol (mg/dL)	39.3 ± 12.1	39.4 ± 11.9	38.4 ± 13.4	37.3 ± 10.9	39.9 ± 12.1	38.8 ± 11.0	48.1 ± 15.3	47.3 ± 14.5	45.2 ± 10.3	<0.001
Na (mM)	140.1 ± 4.9	140.2 ± 5.1	139.5 ± 3.6	139.0 ± 8.6	140.5 ± 3.5	140.5 ± 2.7	141.0 ± 5.7	142.4 ± 3.0	142.5 ± 2.2	<0.001
K (mM)	4.0 ± 0.5	4.0 ± 0.5	4.0 ± 0.5	3.9 ± 0.5	4.0 ± 0.5	4.0 ± 0.6	4.0 ± 0.0	4.1 ± 0.4	4.5 ± 0.5	<0.001
Cl (mM)	104.2 ± 6.8	104.5 ± 7.2	102.9 ± 4.1	103.1 ± 13.6	105.0 ± 3.8	104.1 ± 4.3	104.1 ± 4.0	105.0 ± 2.2	103.2 ± 2.4	0.001
History of hypertension (%)	62	62.4	60	62.4	60.9	80.7	28.6	71.4	10.9	<0.001
History of diabetes mellitus (%)	28	31.4	9.6	30.1	32.1	30.3	14.3	42.9	1.5	<0.001

^ap-Value was compared between total strokes and normal controls.

LAA, large-artery atherosclerosis; SVO, small-vessel occlusion; CE, cardioembolism; SOE, stroke of other determined etiology; SUE, stroke of undetermined etiology; TOAST, Trial of Org 10172 in Acute Stroke Treatment.

polymorphism was evaluated to check the data quality and genotype error using chi-square test, which compared the observed numbers of each genotype with those expected for the population with one degree of freedom. Patients with SOE and SUE were excluded from association analysis due to the small number of subjects. Monomorphic rs5227 and rs35640285 of the *NPPB* gene were also excluded from association analysis. Associations of the genetic variants were assessed by comparing allele frequencies in each case and control, or between each group using the multivariable logistic analysis by STATA/SE 10.1 for windows. Dominant, additive, and recessive models adjusted for age, sex, and histories of hypertension and diabetes mellitus as covariables were tested. *p*-Values <0.05 were taken as statistically significant. Haploview v4.1 software was used to determine linkage disequilibrium (LD) of SNPs in the *NPPA* and *NPPB* genes. Lewontin's *D'* ($|D'|$) and the LD coefficient r^2 between all pairs of biallelic loci were examined (Barrett *et al.*, 2005). Statistical power of single associations was calculated using the Power for Genetic Association Analyses (PGA) software (Menashe *et al.*, 2008).

Results

Study subjects

Clinical and demographic characteristics of 674 Korean patients with stroke and 267 unaffected controls who participated in this study are presented in Table 1. The average

high-density lipoprotein cholesterol, Na⁺, and K⁺ in total stroke patients were significantly decreased compared with those of normal controls, whereas histories of hypertension and diabetes mellitus were higher in cases compared with those of controls as expected (*p*<0.0001, Table 1). In subtypes of ischemic stroke, the proportion of males was higher than females in SVO, while lower in CE, about a 1.5-fold, respectively. Also, the level of triglyceride in CE group was decreased compared with that of other groups (Table 1).

Association studies in strokes and normal controls

Monomorphic *NPPB* rs5227 and rs35640285, which were identified in Korean population, were excluded from analysis. All alleles genotyped in this study were in Hardy-Weinberg equilibrium (*p*>0.05). Among seven polymorphic variants of *NPPB* and *NPPA*, rs198373 and rs198372 as well as rs5065 and rs5067 showed complete LD ($r^2=1$), and rs198389 and rs61757273 were observed to have tight LD upon construction of haplotype block (Fig. 1A). To investigate the associations between SNPs and stroke, logistic analysis adjusted for age, sex, and histories of hypertension and diabetes mellitus as covariables was performed. The minor allele frequency (MAF) of rs198373 and rs198372 in the promoter region of the *NPPA* gene was about 3.5-fold higher in patients with stroke than in controls, but no significant association was found in logistic analysis (*p*>0.05, Table 2). However, in additional comparison among

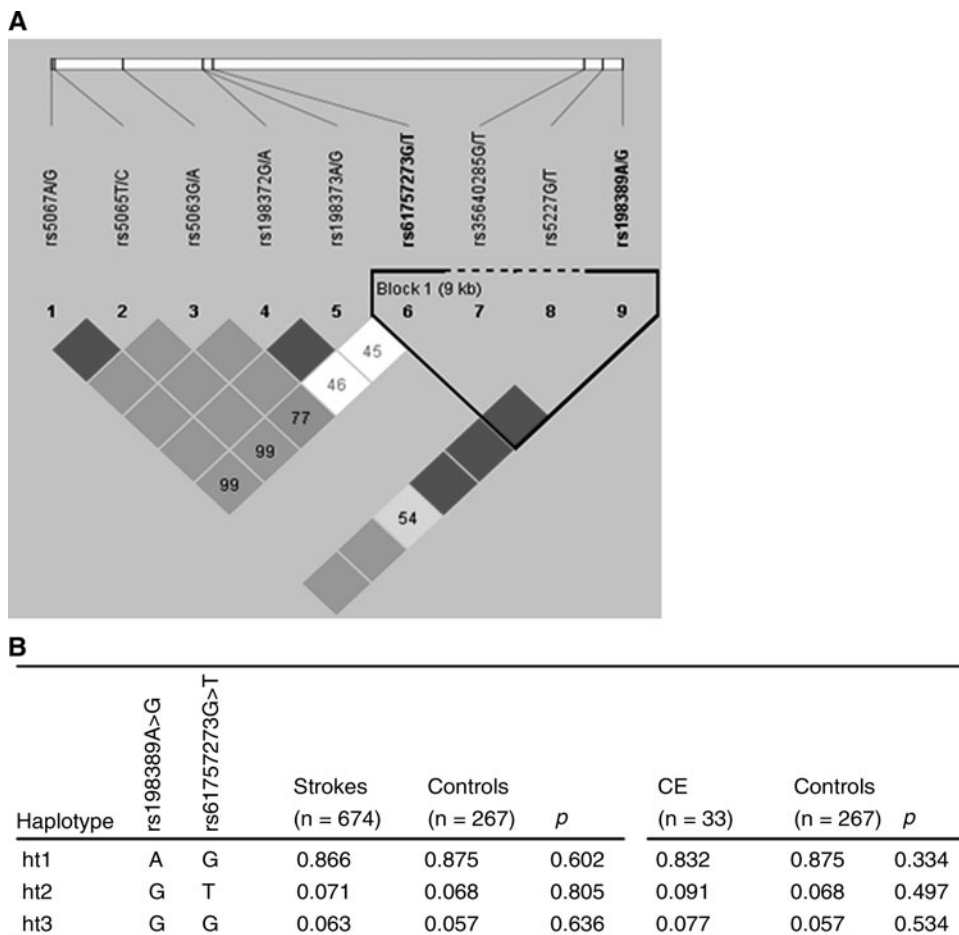


FIG. 1. Haplotypes and LD of the *NPPA* gene. **(A)** Linkage disequilibrium (LD) and haplotype block of nine single-nucleotide polymorphisms in the *NPPB* and *NPPA* genes. LD plot was prepared using Haploview. Colors are used to display pairwise LD as follows: red, $LOD \geq 2$ and $D' = 1$; shades of pink/red, $LOD \geq 2$ and $D' < 1$; white, $LOD < 2$ and $D' < 1$. The numbers indicate pairwise r^2 values shown as a percentage. **(B)** Haplotypes composed of rs198389 in *NPPB* and rs61757273 in *NPPA*, and its associations with stroke and cardioembolic stroke (CE).

TABLE 2. LOGISTIC ANALYSES FOR THE *NPPB* AND *NPPA* POLYMORPHISMS IN TOTAL STROKES AND NORMAL CONTROLS

Gene	SNP ID	Variation	Position	Amino acid change	Heterozygosity ^a	MAF		OR (95% CI)	p-Value	Statistical power (%)
						Strokes (n=674)	Control (n=267)			
<i>NPPB</i>	rs198389	A/G	Promoter		0.523	0.132	0.125	1.2 (0.8–1.8)	0.367	100
	rs5227	G/T	Exon 1	Arg25Leu	0.363	0.000	0.000	—	—	100
	rs35640285	G/T	Exon 2	Val94Phe	0.025	0.000	0.000	—	—	100
<i>NPPA</i>	rs61757273	G/T	Promoter		0.066	0.071	0.068	1.3 (0.8–2.2)	0.313	100
	rs198373	A/G	Promoter		0.069	0.007	0.002	8.1 (0.8–78.2)	0.072	31.68
	rs198372	G/A	Promoter		0.133	0.007	0.002	8.1 (0.8–78.6)	0.071	31.68
	rs5063	G/A	Exon 1	Val32Met	0.133	0.094	0.099	1.1 (0.7–1.7)	0.625	100
	rs5065	T/C	Exon 3	STOP152Arg	0.253	0.007	0.006	1.3 (0.2–7.4)	0.779	73.02
	rs5067	A/G	3'UTR		0.223	0.007	0.006	1.3 (0.2–7.4)	0.785	73.02

p-Values of additive model were obtained by logistic analysis adjusted for age, sex, and histories of hypertension and diabetes mellitus as covariables.

^aHeterozygosity is indicated from Build130 of National Center for Biotechnology Information (NCBI).

MAF, minor allele frequency; UTR, untranslated region; OR, odds ratio; CI, confidence interval; SNP, single-nucleotide polymorphism.

hemorrhagic and ischemic strokes and controls, nominal significances between these two SNPs (rs198373 and rs198372) and ischemic stroke when compared with unaffected controls ($p=0.032$, Supplementary Table S1; Supplementary Data are available online at www.liebertonline.com/dna) have been detected. In the case of association analysis with haplotypes, no significant signals were observed (Fig. 1B). These results indicate that genomic variants of *NPPB* and *NPPA* have no critical effects on the development of stroke itself.

STOP152Arg variant of the *NPPA* gene was associated with cardioembolic stroke

Since TOAST classification is also important for etiology and treatment of stroke, further associations of subtypes of ischemic stroke were analyzed. Patients with SOE and SUE were excluded from association analysis due to the small number of subjects. Interestingly, the minor alleles of rs5065 nonsynonymous variant and rs5067 in 3'-untranslated region (3'UTR) of the *NPPA* gene were significantly frequent in

cardioembolic stroke patients as compared with normal controls (adjusted $p=0.002$, Table 3) and SVO patients (adjusted $p=0.020$, Table 4). In addition, rs198373 and rs198372 of *NPPA* generated nominal evidence of association with SVO compared with those of normal controls (adjusted $p<0.05$, Table 3). Except for the relationship between CE and SVO, no additional associations were observed among other comparisons of subtypes of TOAST classification (Supplementary Table S2). Therefore, these results provide a possibility that two *NPPA* SNPs, the nonsynonymous rs5065 and 3'UTR rs5067, which are in complete LD, could be susceptibility factors for cardioembolic stroke, rather than stroke itself, at least in a Korean population. However, further association analysis for haplotypes showed no significant association with stroke and CE ($p>0.05$, Fig. 1B).

Discussion

Blood pressure-lowering properties of natriuretic hormones have been implicated in response to increased wall stresses. Moreover, mice lacking *NPPA* demonstrated an

TABLE 3. ASSOCIATIONS AMONG CARDIOEMBOLISM, LARGE-ARTERY ATHEROSCLEROSIS, SMALL-VESSEL OCCLUSION AND NORMAL CONTROLS

Gene	SNP	CE (n=33) vs. Control (n=267)				LAA (n=123) vs. Control (n=267)			SVO (n=395) vs. Control (n=267)			
		MAF		p-Value	Statistical power (%)	MAF		p-Value	MAF		p-Value	Statistical power (%)
		CE	Controls			LAA	Controls		SVO	Controls		
<i>NPPB</i>	rs198389A/G	0.156	0.125	0.822	45.46	0.135	0.125	0.564	0.133	0.125	0.373	100
	rs5227G/T	0.000	0.000	—	—	0.000	0.000	—	0.000	0.000	—	—
	rs35640285G/T	0.000	0.000	—	—	0.000	0.000	—	0.000	0.000	—	—
<i>NPPA</i>	rs61757273G/T	0.091	0.068	0.415	30.06	0.095	0.068	0.147	0.065	0.068	0.169	99.99
	rs198373A/G	0.000	0.002	—	—	0.000	0.002	—	0.010	0.002	0.041	18.56
	rs198372G/A	0.000	0.002	—	—	0.000	0.002	—	0.010	0.002	0.040	18.56
	rs5063G/A	0.076	0.099	0.200	30.12	0.111	0.099	0.438	0.090	0.099	0.647	99.99
	rs5065T/C	0.030	0.006	0.002	7.29	0.008	0.006	0.669	0.004	0.006	0.952	25.55
	rs5067A/G	0.030	0.006	0.002	7.29	0.008	0.006	0.674	0.004	0.006	0.945	25.55

Bold value indicates the statistical significance of $P<0.05$.

p-Values of additive model were obtained by logistic analysis adjusted for age, sex, and histories of hypertension and diabetes mellitus as covariables.

TABLE 4. ASSOCIATIONS BETWEEN POLYMORPHISMS OF NPPA AND ISCHEMIC STROKE WITH CARDIOEMBOLISM

Gene	SNP	Functional position	MAF			p-Value			MAF			p-Value			Statistical power (%)
			CE (n = 33)	Control (n = 267)	SVO (n = 395)	Unadjusted	Adjusted ^a	Statistical power (%)	CE (n = 33)	SVO (n = 395)	Unadjusted	Adjusted ^a			
													CE (n = 33)	Control (n = 267)	
NPPB	rs198389A > G	Promoter	0.156	0.125	0.133	0.488	0.822	45.46	0.156	0.133	0.596	0.444	66.11		
	rs5227G > T	Arg25Leu	0.000	0.000	0.000	—	—	—	0.000	0.000	—	—	—		
	rs35640285G > T	Val94Phe	0.000	0.000	0.000	—	—	—	0.000	0.000	—	—	—		
NPPA	rs61757273G > T	Promoter	0.091	0.068	0.065	0.497	0.415	30.06	0.091	0.065	0.414	0.296	40.97		
	rs198373A > G	Promoter	0.000	0.002	0.010	—	—	—	0.000	0.010	—	—	—		
	rs198372G > A	Promoter	0.000	0.002	0.010	—	—	—	0.000	0.010	—	—	—		
	rs5063G > A	Val32Met	0.076	0.099	0.090	0.549	0.200	30.12	0.076	0.090	0.690	0.802	45.15		
	rs5065T > C	STOP152Arg	0.030	0.006	0.004	0.039	0.002	7.29	0.030	0.004	0.023	0.020	7.32		
	rs5067A > G	3'UTR	0.030	0.006	0.004	0.039	0.002	7.29	0.030	0.004	0.023	0.020	7.32		

Bold value indicates the statistical significance of P < 0.05.

p-Values of additive model were obtained by logistic analysis.

^aAdjusted for age, sex, and histories of hypertension and diabetes mellitus as covariates.

elevated blood pressure, whereas transgenic mice over-expressing NPPA or NPPB showed substantially decreased levels of blood pressure (Steinhilper *et al.*, 1990; Ogawa *et al.*, 1994; John *et al.*, 1995; Potter *et al.*, 2009). Genetic polymorphisms of the NPPA gene, such as rs5068 in 3'UTR and rs5065 in exon, have been implicated in the risk of diseases, including hypertension and stroke (Rubattu *et al.*, 2004; Newton-Cheh *et al.*, 2009). Recently, natriuretic peptides have received attention as a cardiovascular risk factor and its potential therapeutic application has been a subject of investigation (Schmitt *et al.*, 2003; Rubattu *et al.*, 2008). In addition, a pharmacogenetic association between the structural variant inducing STOP152Arg in the NPPA gene and modification of antihypertensive medication effects has also been described in patients with hypertension (Lynch *et al.*, 2008). From the results, rs5065 T or C allele carriers showed different cardiovascular disease outcomes depending on the classes of antihypertensive drugs.

Compared with the occurrence of even homozygous rare alleles at rs5065T/C of the NPPA gene in Caucasian and African populations with 2%–18% frequency, only heterozygous genotype was observed from all 941 Koreans in this study, thereby resulting in a low frequency of the allele. This low frequency was also observed in a Chinese ethnic group (Wang *et al.*, 2009). Moreover, the frequency of this C allele approaches about 40% in Africans as estimated in dbSNP database. When comparing incidences of stroke, African population also showed a higher incidence rate than whites (Gross *et al.*, 1984), suggesting that STOP152Arg or at least NPPA gene polymorphisms might be important factors in the etiology of vascular diseases. In addition, although in rat NPPA, the C-terminal region of the molecule was demonstrated to be essential for high-affinity binding to substrates, such as insulin and insulin-degrading enzyme (Muller *et al.*, 1991). Considering that rs5065T/C induces amino acid change from stop codon to arginine and is associated with stroke, blood pressure/hypertension, and even asthma (Rubattu *et al.*, 2004; Conen *et al.*, 2007; Lima *et al.*, 2008), population difference and/or phenotype of NPPA rs5065 might also have important roles in several diseases or functions of the protein. On the other hand, there were confusing representations of the MAF of NPPB rs5227 in dbSNP for populations with two different sets of HapMap results. Based on our results from 943 Korean subjects, however, this SNP is monomorphic in most populations, except for a low MAF in Africans (about 0.067). In addition, rs35640285 has been discovered as a nonpolymorphic site in a Korean population, whereas this SNP is about 1% prevalent in Caucasians. Although there are minor differences in MAF among populations, these two SNPs are not likely to have effects on the expression of the gene, or to be associated with diseases due to its low MAF.

Indeed, the full-length cDNA (BC005893.1) with the rs5065C variant was cloned from the prostate (Strausberg *et al.*, 2002). Although the pathophysiological role of the variant has not been uncovered, its association with high plasma ANP and BNP levels in heart failure patients has been reported (Vassalle *et al.*, 2007). In addition, another transcript (OTTHUMT00000006853 from Ensembl database) for NPPA peptide has been identified. Considering that the rs5065C variant plays an important role on the endothelial dysfunction and vasorelaxation to develop stroke and that

ANP and BNP have cardiovascular responses (Rubattu *et al.*, 2004; Potter *et al.*, 2009), it is also possible that newly translated natriuretic peptide from the rs5065C variant could be associated with cardioembolic stroke (Fig. 2). In addition, this putative peptide, which probably has unknown function or dysfunction, could play a role in the development of the cardiovascular diseases (Rubattu *et al.*, 2008).

A potential strength of this study is the recruitment of patients from multicenter. Furthermore, all of the patients were evaluated with CT and MRI for more accurate diagnosis. However, possible study limitations include the small number of CE patients due to the specificity of recruitment from oriental medical hospitals rather than from emergency of Western-oriented hospitals and the self-reported history of hypertension and diabetes mellitus, which was included for multivariable analysis. Considering the periodic medical examination and the recent concern in health, however, the self-reported histories from subjects are credible references compared with those from direct diagnosis. On the other hand, despite a few CE cases with rs5065 and rs5067 variants, recent genome-wide association studies using common SNPs with MAF over 5% have showed that only a limited amount of the heritable component has been identified in diseases pathogenesis, suggesting that rare variants may confer a substantial risk for complex diseases (Cirulli and Goldstein, 2010). Finally, there were no matches of age between cases and controls. Therefore, further replication studies including larger number of CE patients matched with similar age group will provide a more confident association with increased statistical powers. In addition, cardio-embolism-related clinical information, such as atrial fibrillation and cardiac valvular diseases, is suggested to be included in future replication studies.

This study found nominal associations of rs198373 and rs198372 in promoter region of the *NPPA* gene with ischemic stroke when compared with unaffected controls ($p=0.032$, Supplementary Table S1). However, even though these two SNPs of SVO subgroup showed higher MAFs than CE and LAA subgroups, no statistically significant associations were found (Supplementary Table S2), with no literature evidence for the higher prevalence of *NPPA* variations in LAA pa-

tients. Nevertheless, we do not rule out the potential associations of rs198373 and rs198372 with ischemic stroke; therefore, we additionally performed *in silico* analysis using the Signal Scan program (www.bimas.cit.nih.gov/molbio/signal/) to investigate whether these polymorphisms could be putative binding sites for regulators. Intriguingly, the AACCAAT and CCAAT sequences including the "C" allele of rs198372G/A (or C/T) were predicted as elements for the binding of several regulatory factors (ACF, alpha-CBF, etc.), whereas the ATCAATA sequence including the "T" allele of the SNP was observed to be an element only for the binding of Pit-1 factor (Supplementary Table S3). This suggests that the rs198372 variation could affect the expression of *NPPA*, leading to a possible role in the risk of ischemic stroke.

In this study, lower blood Na⁺ and K⁺ levels in total stroke patients were found compared with those of normal controls. *NPPA* has been known to be involved in the homeostatic control of Na⁺ and K⁺ in the body (Pedersen *et al.*, 1999). More recently, higher potassium intake has been found to be associated with lower risks of stroke, coronary heart disease, and total cardiovascular disease (D'Elia *et al.*, 2011), suggesting that lower concentration of potassium might be related to vascular diseases. In the case of sodium, considering that heart failure is a common cause to hyponatremia, which is characterized by decrease in serum Na⁺ concentration (Kazory, 2010), further studies of the association between abnormality on natriuretic peptides and the blood Na⁺ level in patients with vascular diseases are needed. Another intriguing finding in this study is that the percentage of hypertension history is higher in patients with CE than in those with LAA and SVO (Table 1). Despite inclusion of several populations and no further stratification into LAA and SVO, another report also showed a small increase of the hypertension history in cardioembolic stroke subjects compared with noncardioembolic stroke subgroup (Viehman *et al.*, 2007). Although further studies are required, one potential explanation is that the higher percentage of hypertension history in patients with cardioembolic stroke could be related to atrial fibrillation (Krahn *et al.*, 1995; Giacalone *et al.*, 2010), with the potential involvement of *NPPA* variations.

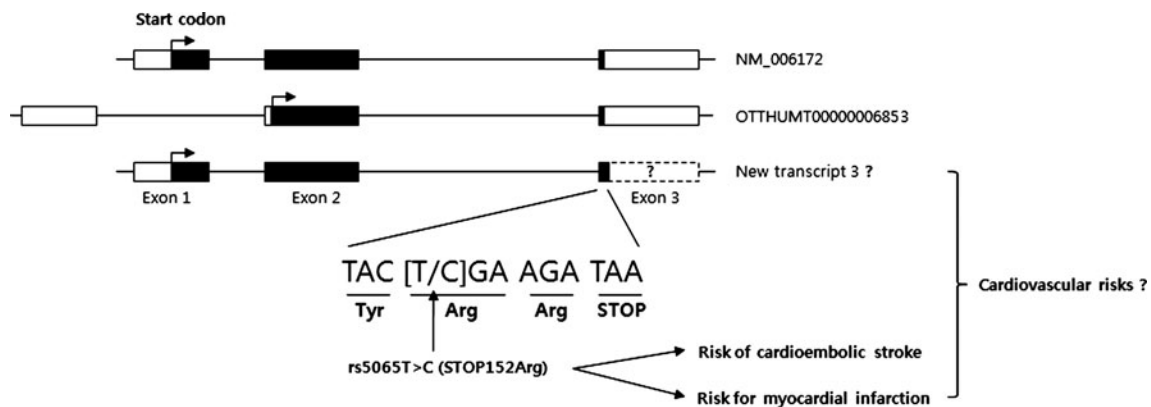


FIG. 2. Schematic representation of human *NPPA* transcripts and the proposed effects of a new transcript potentially derived from STOP152Arg. The coding exons are represented by black blocks, and 5'UTR and 3'UTR by white blocks. The nonsynonymous variant rs5065 (STOP152Arg) has been suggested as a potential risk of cardioembolic stroke in this study as well as a risk of myocardial infarction in another study (Gruchala *et al.*, 2003), whereas further studies are required to elucidate the effects of new transcript potentially derived from STOP152Arg on cardiovascular diseases (Rubattu *et al.*, 2008).

Taken together, NPPA has been known to modulate arterial and cardiopulmonary baroreceptor-mediated blood outflow, leading to the baroreflex control of the circulation (Luchner and Schunkert, 2004). Synthetic analogs of NPPA have also been investigated as a potential therapy for the treatment of heart failure (Potter *et al.*, 2009). Although further functional and replication studies in a large cohort with cardioembolic stroke are required, our preliminary findings may provide supporting information to the disease etiology, suggesting that genetic variations of the *NPPA* gene might be susceptibility factors for cardioembolic stroke through potential alternative transcript and/or transcriptional stability by rs5065 (STOP152Arg) and rs5067 (3'UTR), respectively.

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Disclosure Statement

No competing financial interests exist.

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