Association of polymorphisms in thromboxane A2 receptor and thromboxane A synthase 1 with cerebral infarction in a Korean population

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Thromboxane A2 (TBXA2) is a potent vasoconstrictor in cerebral circulation and is a known contributor to the pathogenesis of cerebral infarction. Thromboxane A2 synthase 1 (TBXAS1) and thromboxane A2 receptors (TBXA2R) are key components in TBXA2 function. We examined whether genetic variants in *TBXA2R* and *TBXAS1* are risk factors for cerebral infarction by genotyping 453 Korean patients with noncardiogenic cerebral infarction and 260 controls. A few, specific polymorphisms in the *TBXA2R* (−3372G>C, +4710T>C and 4839T>C) and *TBXAS1* (+16184G>T, +141931A>T and +177729G>A) genes were chosen and investigated. Logistic regression showed the frequencies of *TBXAS1* +16184G>T and *TBXAS1- ht3* were significantly more frequent in cerebral infarction (P = 0.002, OR = 2.75 and P = 0.01, OR = 1.57, respectively), specifically in small-artery occlusion (SAO) type of cerebral infarction (P = 0.0003 and 0.005, respectively). These results suggest specific *TBXAS1* gene polymorphisms may be a useful marker for development of cerebral infarction, especially SAO type in Korean population. [BMB reports 2009; 42(4): 200-205]

INTRODUCTION

Cerebral infarction is one of the most common causes of death and disability (1) with a complex etiology involving both genetic and modifiable, pre-disposing determinants (2, 3). Identifying the genetic risk for cerebral infarction is important both for risk prediction and for prevention of potential incidents. Although several genes involved in hemostasis, renin angio-

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tensin system and lipid metabolism have been identified as possible susceptibility genes, their associations with cerebral infarction are still controversial (4).

Thromboxane A2 (TBXA2) is a potent vasoconstrictor and stimulator of platelet aggregation with high levels produced in atherosclerosis (5) and during acute stroke (6). The mechanism by which aspirin prevents cerebral infarction is through the potent inhibition of cyclooxygenase-1, thereby blocking the production of TBXA2 (7-9). Thus, TBXA2 is considered an important mediator of cerebral infarction (10). The enzyme that catalyzes its synthesis, thromboxane A2 synthase 1 (TBXAS1), is a key component for TBXA2 function along with the receptor that mediates its action, thromboxane A2 receptor (TBXA2R). TBXA2R is a member of the family of G protein-coupled receptors and performs an essential role in hemostasis by interacting with TBXA2 to induce platelet aggregation. Point mutations in *TBXA2R* can result in a dominantly-inherited bleeding disorder characterized by a defective platelet response to TBXA2 (11). Moreover, a depressed response to vascular injury is commonly observed in mice genetically deficient in *TBXA2R* or treated with a TBXA2R antagonist (12). TBXAS1, a cytochrome P450 enzyme, increases levels of TBXA2 through the conversion of prostaglandin H2. Both TBXA2R and TBXAS1 have been implicated in the physiopathology of numerous diseases, including atherosclerosis, myocardial infarction, stroke and asthma (13-15). However, any impact their genetic polymorphisms may have on cerebral infarction remains unknown. To examine whether polymorphisms in the *TBXA2R* and *TBXAS1* genes have effects on the development of cerebral infarction, we screened for variations in the *TBXA2R* and *TBXAS1* genes in the Korean population.

RESULTS AND DISCUSSION

Clinical demographics (Table 1)

We studied 453 patients with noncardiogenic cerebral in-

farctions, classified into either large-artery atherosclerosis (LAA) (198, 44%) or small-artery occlusion (217, 48%) depending on the proposed pathomechanism. For 38 patients (8%), the classification was 'uncertain'. Patients were also significantly older and included more males than the control population (P < 0.05), which itself did not have cerebral infarctions documented on brain CT or MRI. It is valuable that false controls with silent infarcts be excluded. Risk factors such as hypertension, DM, hypercholesterolemia and smoking were more prevalent in the infarction group. No significant difference in risk factors was observed between the LAA and SAO subgroups (P > 0.05).

Association of polymorphisms in the TBXA2R and TBXAS1 genes with the development of cerebral infarction

Three SNPs in the *TBXA2R* gene ($-3372G>C$, $+4710T>C$, $+4839T>C$), adjusted for age, gender, and risk factors, were analyzed using logistic regression models. Marginal associations of *TBXA2R* $+4839T>C$ and *TBXA2R-Block2-ht1* with the development of cerebral infarction in a recessive model were observed. The minor allele frequencies (MAFs) of *TBXA2R* $+4839T>C$ and *TBXA2R-Block2-ht1* were lower in the cerebral infarction group (0.208) than in controls (0.236, P = 0.04, OR = 0.46, 95% CI = 0.21-0.97 in recessive models; Table 2). However, their relationship was no longer significant after adjusting the p-value for multiple comparisons (P^{corr} = 0.11).

Three SNPs in the *TBXAS1* gene ($+16184G>T$, $+141931A>T$, $+177729G>A$) were also analyzed. Both *TBXAS1* $+16184G>T$ and *TBXAS1-ht3* exhibited significant association with the development of cerebral infarction. In a recessive model, the frequency of *TBXAS1* $+16184G>T$ was significantly higher (0.380) in subjects with cerebral infarction than in controls (0.351, P = 0.002, P^{corr} = 0.006, OR = 2.75, 95% CI = 1.46-5.18). Similarly, *TBXAS1-ht3* occurred more frequently (0.252) in subjects with cerebral infarction than in controls (0.219, P = 0.01, P^{corr} = 0.03, OR= 1.57; Table 2).

A specific haplotype of *TBXA2R* was recently reported as being closely related with cerebral infarctions in individuals of Japanese ethnicity (16). Six SNPs were investigated and two (rs2271875 and rs768963) showed significantly different geno-type distributions in cerebral infarction. These SNPs were not investigated in our study. Among the other four loci in their report, of which none revealed any relationship with cerebral infarction, rs4523 ($+4839T>C$) and rs11085026 ($+4710T>C$) were evaluated in our study to have no association with cerebral infarction. To our knowledge this is the first report describing associations between polymorphisms in the *TBXAS1* gene and cerebral infarction. TBXAS1 produces TBXA2 from prostaglandin H2 during its last generation step (17). Therefore it does not interfere with the generation of other arachidonic acid metabolites such as prostaglandin I2, potent vasodilator and antithrombotics. Based on these theoretical benefits, TBXA2 modulators have been constructed by developing various TBXAS1 inhibitors either individually or in combination with a TBXA2R antagonist. They have demonstrated inhibition of platelet aggregation (18) along with benefits in the acute and chronic stages of cerebral infarction (19). We did not measure TBXA2 or its metabolites in this study because a substantial number of patients had taken anti-platelet medication before blood sampling. To address the possibility the *TBXAS1* $+16184G>T$ and *-ht3* polymorphisms significantly modulate the function of TBXA2 and thereby affect the development of cerebral infarction, further investigation is required.

Subtypes of cerebral infarction

When we examined the association between various SNPs from both *TBXAS1* and *TBXA2R* and subtypes of athero-thrombotic cerebral infarction, no significant differences were observed depending on LAA or SAO (http://www.snpgenetics.com/user/additional_list.asp). However, additional analysis of SAO or LAA development when compared to controls found *TBXAS1* $+16184G>T$ and *TBXAS1-ht3* significantly associated with an increased risk of SAO development. Specifically, the MAFs of *TBXAS1* $+16184G>T$ and *TBXAS1-ht3* were significantly higher (0.390 and 0.261, respectively) in SAO subjects than in controls (0.351 and 0.219, respectively; P=0.005-0.0003, P^{corr} = 0.01-0.0009, OR=1.66-3.89 and P=0.005, P^{corr} = 0.01, OR=1.8, respectively; Table 3).

In this study, we demonstrated the occurrence of the polymorphisms *TBXAS1*+16184G>T and *TBXAS1-ht3* were significantly higher in Korean patients with noncardiogenic cere-

Table 1. Clinical characteristics of study subjects

	Total			Subtypes		
	Infarction (n = 453)	Controls (n = 260)	P value	LAA (n = 198)	SAO (n = 217)	P value
Age (years)	65 ± 12	52 ± 14	<0.0001	66 ± 12	64 ± 12	0.197
Gender (M)	236 (52%)	104 (40%)	0.002	115 (58%)	106 (49%)	0.088
Hypertension	349 (77%)	80 (31%)	<0.0001	148 (75%)	173 (80%)	0.285
DM	170 (38%)	32 (12%)	<0.0001	77 (39%)	78 (36%)	0.869
Hypercholesterolemia	183 (40%)	62 (24%)	<0.0001	80 (40%)	92 (42%)	0.382
Smoking	213 (47%)	84 (32%)	0.001	106 (54%)	95 (44%)	0.093

Table 2. Logistic analysis of *TBXA2R* and *TBXAS1* polymorphisms with risk of infarction, adjusted by age, gender, hypertension, DM, hypercholesterolemia, and smoking, in a Korean population

Gene	Loci	rs#	Position	AA change	Allele	Frequency Infarction (n=453)	Frequency Controls (n=260)	Codominant OR (95%CI)	P	P^corr	Dominant OR (95%CI)	P	P^corr	Recessive OR (95%CI)	P	P^corr
TBXA2R	−3372G>C	rs3786989	intron1	.	C	0.077	0.099	0.89 (0.55-1.44)	0.64	1	0.98 (0.58-1.64)	0.94		0.11 (0.01-1.43)	0.09	0.25
	+4710T>C	rs11085026	exon3	I265I	C	0.291	0.316	0.95 (0.74-1.21)	0.65	1	0.95 (0.65-1.40)	0.8		0.87 (0.53-1.42)	0.56	1
	+4839T>C	rs4523	exon3	Y308Y	C	0.208	0.236	0.89 (0.65-1.22)	0.47	1	1.03 (0.69-1.54)	0.89		0.46 (0.21-0.97)	0.04	0.11
					ht1	0.582	0.601	0.82 (0.56-1.20)	0.31	0.85	0.92 (0.46-1.82)	0.81		0.69 (0.40-1.19)	0.19	0.51
					ht2	0.198	0.184	1.07 (0.67-1.72)	0.77		1.20 (0.69-2.09)	0.52		0.62 (0.18-2.15)	0.45	1
					ht3	0.077	0.063	1.32 (0.61-2.83)	0.48		1.32 (0.61-2.83)	0.48		.	.	.
					ht4	0.067	0.057	1.35 (0.66-2.75)	0.41		1.40 (0.58-3.37)	0.45		2.01 (0.25-16.48)	0.51	.
TBXAS1	+16184C>T	rs2267682	intron1	.	T	0.380	0.351	1.44 (1.07-1.92)	0.02	0.06	1.26 (0.85-1.87)	0.25	0.75	**2.75 (1.46-5.18)**	**0.002**	**0.006**
	+141931A>T	rs6962291	intron9	.	T	0.470	0.474	0.93 (0.71-1.23)	0.61		0.89 (0.58-1.38)	0.61		0.93 (0.58-1.49)	0.77	1
	+177729G>A	rs3735354	exon10	E388K	A	0.015	0.018	0.68 (0.23-1.97)	0.48		0.68 (0.23-1.97)	0.48		.	.	.
					ht1	0.339	0.333	0.94 (0.70-1.26)	0.66		0.89 (0.60-1.31)	0.55		1.02 (0.54-1.92)	0.96	1
					ht2	0.268	0.297	0.75 (0.56-1.02)	0.07	0.21	0.72 (0.49-1.06)	0.09	0.27	0.66 (0.33-1.33)	0.24	0.72
					ht3	0.252	0.219	**1.57 (1.11-2.22)**	**0.01**	**0.03**	1.52 (1.03-2.25)	0.04	0.12	3.60 (1.11-11.73)	0.03	0.09
					ht4	0.126	0.134	1.04 (0.70-1.56)	0.85		1.10 (0.70-1.74)	0.67		0.65 (0.17-2.55)	0.54	1

The effective number of independent marker loci in *TBXA2R* and *TBXAS1* was calculated to correct for multiple testing, using the software SNPSpD (http://genepi.qimr.edu.au/general/daleN/SNPSpD/), which is based on the spectral decomposition (SpD) of matrices of pair-wise LD between SNPs (31). The number of independent marker loci in *TBXA2R* and *TBXAS1* was calculated as 2.7548 and 2.9878, respectively, and this was applied to correct for multiple testing (Pcorr is P value ×2.7548 or ×2.9878, respectively).

Table 3. Logistic analysis for SAO development versus controls, adjusted by age, gender, hypertension, DM, hypercholesterolemia, and smoking status with SNPs and haplotypes of *TBXA2R* and TBXAS1, in a Korean population

Gene	Loci	Allele	Frequency SAO (n=217)	Frequency Controls (n=260)	Codominant OR (95%CI)	P	P^corr	Dominant OR (95%CI)	P	P^corr	Recessive OR (95%CI)	P	P^corr
TBXA2R	−3372G>C	C	0.088	0.099	1.04 (0.59-1.81)	0.9	1	1.18 (0.65-2.14)	0.59	1	.	.	.
	+4710T>C	C	0.309	0.316	0.92 (0.69-1.22)	0.56	1	0.85 (0.54-1.34)	0.48	1	0.92 (0.52-1.62)	0.77	1
	+4839T>C	C	0.231	0.236	1.06 (0.74-1.54)	0.75	1	1.31 (0.81-2.11)	0.27	1	0.56 (0.23-1.36)	0.2	0.55
		ht1	0.560	0.568	0.95 (0.70-1.31)	0.76	1	1.05 (0.59-1.84)	0.88	1	0.87 (0.54-1.40)	0.56	1
		ht2	0.207	0.196	0.99 (0.67-1.46)	0.95	1	1.02 (0.63-1.64)	0.94	1	0.84 (0.30-2.38)	0.74	1
		ht3	0.069	0.068	1.06 (0.55-2.04)	0.86	1	1.06 (0.55-2.04)	0.86	1	.	.	.
		ht4	0.071	0.068	1.10 (0.64-1.89)	0.74	1	1.17 (0.57-2.38)	0.67	1	1.02 (0.24-4.25)	0.98	.
TBXAS1	+16184G>T	T	**0.390**	**0.351**	**1.66 (1.16-2.36)**	**0.005**	**0.01**	1.39 (0.87-2.21)	0.17	0.51	**3.89 (1.86-8.16)**	**0.0003**	**0.0009**
	+141931A>T	T	0.450	0.474	0.84 (0.61-1.17)	0.31	0.93	0.78 (0.47-1.29)	0.34	1	0.82 (0.47-1.46)	0.51	1
	+177729G>A	A	0.022	0.018	0.98 (0.31-3.11)	0.97	1	0.98 (0.31-3.11)	0.97		.	.	.
		ht1	0.313	0.333	0.78 (0.55-1.12)	0.18	0.54	0.74 (0.47-1.16)	0.19	0.57	0.74 (0.34-1.62)	0.46	1
		ht2	0.278	0.297	0.76 (0.53-1.08)	0.13	0.39	0.74 (0.47-1.17)	0.19	0.57	0.61 (0.26-1.39)	0.23	0.69
		ht3	**0.261**	**0.219**	**1.80 (1.19-2.71)**	**0.005**	**0.01**	1.76 (1.11-2.79)	0.02	0.06	4.18 (1.12-15.52)	0.03	0.09
		ht4	0.127	0.134	1.15 (0.71-1.86)	0.58	1	1.28 (0.75-2.20)	0.37	1	0.37 (0.05-2.82)	0.34	1

The effective number of independent marker loci in *TBXA2R* and *TBXAS1* was calculated to correct for multiple testing, using the software SNPSpD (http://genepi.qimr.edu.au/general/daleN/SNPSpD/), which is based on the spectral decomposition (SpD) of matrices of pair-wise LD between SNPs (31). The number of independent marker loci in *TBXA2R* and *TBXAS1* was calculated as 2.7548 and 2.9878, respectively, and this was applied to correct for multiple testing (Pcorr is P value ×2.7548 or ×2.9878, respectively).

bral infarction, independent of potential confounders and other risk factors. Cerebral infarction is a heterogeneous condition classified into numerous subtypes depending on the suggested mechanism (20), the outcome of which, along with the recurrence rate and management plan, varies according to the infarction subtype (21, 22). In addition, we found no significant difference in frequency of the *TBXA2R* and *TBXAS1* SNPs between the two major subtypes of noncardiogenic cerebral infarction, LAA and SAO. However, when compared to controls separately, *TBXS1*+16184G>T and *TBXS1-ht3* were significantly associated with the development of SAO. This suggests *TBXAS1*+16184G>T and *TBXAS1-ht3* tend to relate with the development of SAO more than that of LAA.

SAO in cerebral infarction results from the occlusion of single, small perforating arteries measuring 100-400 μm (23). The most common pathomechanism of its symptomatic lesion is the incidence of a microatheroma consisting of atheromatous plaque with or without complicating thrombosis (24). TBXA2 is a known contributor to atherosclerosis by increasing platelet activation, thrombosis and endothelial dysfunction. The imbalance between increased production of TBXA2 and decreased prostaglandin I2 has further been demonstrated to be related with the microangiopathy in diabetes (25, 26). In addition, the TBXAS inhibitor Ozagrel was found to be effective in increasing blood flow around the infracted area in the acute stage of lacunar infarction (27). These studies seemingly suggest the possible pathogenic role of TBXAS in SAO. Therefore, with previous studies focusing more on the contribution TBXA2 in LAA development (28), further research is necessary to validate the relationship between the polymorphisms *TBXAS1*+16184G>T and *TBXAS1 ht3* and SAO.

In conclusion, we demonstrated the SNPs *TBXAS1*+16184G>T and *TBXAS1-ht3* were associated with non-cardiogenic cerebral infarction, especially SAO type, in individuals of Korean ethnicity. It raises the potential of these SNPs being used as genetic markers for non-cardiogenic cerebral infarction. Further studies are needed to evaluate the function and mechanism of these SNPs in the development of cerebral infarction.

MATERIALS AND METHODS

Subjects
This study was approved by the institutional review board of Soonchunhyang University Bucheon Hospital, Bucheon, Korea. All participants gave written informed consent.

Patients consecutively diagnosed with non-cardiogenic cerebral infarction between November 2004 and November 2006 at Soonchunhyang University Hospital, Korea agreed to participate in the study (n = 453). All patients underwent complete blood cell counts, blood chemistry, lipid profiles, coagulation abnormalities, urinalysis, chest roentgenogram, ECG, brain imaging (computed tomography (CT) in 46 patients or magnetic resonance image (MRI) in 407 patients) and cerebral vas-

cular evaluation (MR or CT angiography). The diagnosis of cerebral infarction was defined as ischemic stroke with signs and symptoms lasting >24 h and having relevant lesions as detected by MRI or CT. Patients with atrial fibrillation on ECG, valvular heart disease, a history of recent myocardial infarction, or evidence of an intracardiac thrombus on an echocardiogram were not included. We classified patients into two distinct subtypes of non-cardiogenic cerebral infarction according to the TOAST classification (20). Patients were labeled as large-artery atherosclerosis (LAA) if a lesion >1.5 cm was present along with >50% relevant arterial stenosis, as determined by vascular evaluation showing cortical impairment or brain stem or cerebellar dysfunction. Small-artery occlusion (SAO) was diagnosed when a lesion <1.5 cm without >50% arterial stenosis was present in addition to traditional clinical lacunar syndrome with no evidence of cortical dysfunction. Any remainders not able to be classified into either group were labeled as 'uncertain'. Blood sampling within a week of stroke onset (3 ± 1.7 days) was performed in 315 patients, while the remaining patients' blood was obtained during the chronic stage of infarction (87 ± 20 days). Based on negative history of stroke and negative findings of brain CT or MRI, a total of 260 control subjects without cerebral infarction were recruited consecutively from a health examination center and outpatient clinic in our hospital during the same period. They underwent neuroimaging in order to evaluate headaches or other non-localizable neurological complaints. Risk factors for cerebral infarction include hypertension, diabetes mellitus (DM), hypercholesterolemia and smoking. Hypertension was defined as a sitting systolic blood pressure >140 mmHg and a diastolic pressure >90 mmHg on three occasions at > 1 day intervals, or the current use of anti-hypertension drugs and history of hypertension. A diagnosis of DM was based on the World Health Organization (WHO) criteria. Hypercholesterolemia was defined as plasma total cholesterol ≥ 240 mg/dl under the adult treatment guidelines by US national cholesterol education program (29), or the current use of a lipid-lowering drug after a confirmed diagnosis of hypercholesterolemia. Smokers were defined as current or former smokers.

Genotyping
All subjects included in this study were Korean. Single-nucleotide polymorphisms (SNPs) in the genes TBXA2R (−3372G>C (rs3786989), +4710T>C (rs11085026) and +4839T>C (rs4523)) and TBXAS1 (+16184G>T (rs2267682), +141931A>T (rs6962291) and +177729G>A (rs3735354)) were characterized in 453 patients with cerebral infarctions alongside 260 controls. The SNPs *TBXA2R* +4710T>C and *TBXA2R* +4839T>C were selected based on previous investigations (30) while *TBXA2R* −3372G>C selected additionally after showing more than a 5% allele frequency in HapMap database. The three *TBXAS1* SNPs, +16184G>T, +141931A>T and +177729G>A, were chosen based on our preliminary study revealing their close relationship with aspirin re-

sponse in asthmatics (unpublished data).

Genotyping polymorphic sites required amplifying primers and probes specifically designed (Applied Biosystems, Foster City, CA); information regarding the probes is available on our Web site (http://www.snp-genetics.com/user/additional_list.asp). The TaqMan assay was performed as described previously (30, 31). One allelic probe was labeled with the FAM dye and the other with the fluorescent VIC dye. PCRs were performed in TaqMan Universal Master mix without UNG (Applied Biosystems) and used PCR primer concentrations of 900 nmol/L and TaqMan MGBprobe concentrations of 200 nmol/L. Reactions were performed in a 384-well format in a 5 μL total reaction volume using 20 ng of genomic DNA. Plates were placed in a thermal cycler (PE 9700; Applied Biosystems) and heated at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles at 95°C for 15 s, 60°C for 1 min and a final soak at 25°C. The TaqMan assay plates were transferred to a Prism 7900HT instrument (Applied Biosystems) to measure the fluorescence intensity in each well of the plate. Fluorescence data files from each plate were analyzed using automated allele-calling software (SDS 2.1).

Statistics

Linkage disequilibrium (LD) was inferred using the Haploview algorithm developed by the Broad Institute, which searches for a spine of strong $|D'|$ and LD coefficient r^2 running from one marker to another (32). Haplotypes of each individual were inferred using the algorithm (PHASE, version 2.0) developed by Stephens et al (33). There were two and four common haplotypes in TBXA2R and TBXAS1, respectively. Subjects harboring missing genotypes were omitted from the analysis of individual SNPs and haplotypes. Logistic regression models were used for calculating odds ratios (95% confidence interval (CI)) and corresponding P values, controlling for age (continuous value), gender (male = 0, female = 1) and the presence of risk factors (positive = 0, negative = 1) as co-variates. Statistical analyses were performed using SAS v9.1 (SAS Institute, Cary, NC) and SPSS v11 software (SPSS Inc., Chicago, IL). P values of < 0.05 were deemed to be statistically significant.

To correct for multiple testing, the effective number of independent marker loci in *TBXA2R* and *TBXAS1* was calculated using the software SNPSpD (http://genepi.qimr.edu.au/general/daleN/SNPSpD/), which is based on the spectral decomposition (SpD) of matrices of pair-wise LD between SNPs (34). The number of independent marker loci in *TBXA2R* and *TBXAS1* was calculated as 2.7548 and 2.9878, respectively. These figures were then applied in order to correct for multiple testing (P_{corr} is P value $\times 2.7548$ or $\times 2.9878$, respectively). Statistical power was calculated using DSS researcher's toolkit (http://www.dssresearch.com/toolkit/spcalc/power_p2.asp), and the average statistical power of this study was 20.1%.

Acknowledgements

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