Original Article

http://dx.doi.org/10.4070/kcj.2012.42.6.406
Print ISSN 1738-5520 • On-line ISSN 1738-5555

kcj
Korean Circulation Journal

Rho-Associated Kinase 2 Polymorphism in Patients With Vasospastic Angina

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Background and Objectives: Recent studies indicate that in response to vasoconstrictor stimuli, the small GTPase RhoA and its downstream effector, Rho-associated kinase 2 (ROCK)/Rho-kinase, are associated with hypercontraction of the vascular smooth muscle of coronary arteries through augmentation of myosin light chain phosphorylation and Ca^{2+} sensitization. Expression of ROCK/Rho-kinase mRNA was significantly increased and up-regulated in the spastic coronary artery in a porcine model, and a specific inhibitor of ROCK/Rho-kinase inhibited coronary artery spasm in humans. We therefore explored the role of ROCK2 polymorphisms in the pathogenesis of vasospastic angina (VA).

Subjects and Methods: We studied 106 patients with VA who exhibited spontaneous or provoked coronary spasm during coronary angiography and compared the prevalence of ROCK2 polymorphisms between this group of patients with VA and controls whose angiograms were normal, and in whom the ergonovine test did not cause spasm (n=107). Five single nucleotide polymorphisms (SNPs) of the ROCK2 gene were selected. SNPs were genotyped by high-resolution melting. Linkage disequilibrium and haplotype analyses were performed using the SHEsis program.

Results: The prevalence of genotypes of the 5 interesting SNPs in patients with VA was not different from that in the control group. In haplotype analysis, the haplotype G-T-C-T-G (in order of rs978906, rs2271621, rs2230774, rs1515210, and rs3771106) was significantly associated with a decreased risk of VA (p=0.007).

Conclusion: The haplotype G-T-C-T-G in the ROCK2 gene had a protective effect against VA, suggesting the involvement of ROCK2 in VA pathogenesis. **(Korean Circ J 2012;42:406-413)**

KEY WORDS: Coronary vasospasm; Rho-associated kinase 2; Polymorphsm, genetic; Haplotypes.

Introduction

Although the prevalence of vasospastic angina (VA) appears to be on the decline throughout the world, probably secondary to widespread use of calcium antagonists, the actual prevalence of coronary spasm has not decreased, even in the era of calcium channel blockers.[1)] Resting chest pain in a relatively young patient during the early morning hours should raise the suspicion of VA, particularly when occurring during sobriety. Although the role of coronary vasoconstriction was originally discovered in "variant angina", there is convincing evidence that various types of coronary constriction play a role in major ischemic heart disease. Moreover, despite treatment with calcium channel blockers and nitrates, recurrent episodes of angina attack in patients with VA are frequently observed, whereas sudden cardiac death or non-fatal myocardial infarction are rare.[2)] Therefore, it is very important to clarify the exact mechanisms of coronary artery spasm to aid in the development of novel and fundamental therapeutic targets. To this end, patients with VA are known to have defective endothelial function due to reduced nitric oxide

Received: September 26, 2011
Revision Received: December 10, 2011
Accepted: January 4, 2012
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• The authors have no financial conflicts of interest.

bioavailability. Moreover, levels of markers of oxidative stress and of plasma C-reactive protein are elevated. Smoking, polymorphisms of the endothelial nitric oxide synthetase (eNOS) gene, and low-grade inflammation have been regarded as the most important risk factors for VA. The recent body of evidence indicates that RhoA and its downstream effector, Rho-associated kinase 2 (ROCK)/Rho-kinase, are associated with hypercontraction of vascular smooth muscle of the coronary artery and regulation of eNOS activity. Thus, endothelial dysfunction through abnormalities of eNOS and enhanced contractility of vascular smooth muscle in coronary artery segments are considered important underlying mechanisms of VA.[3] However, the role of ROCK2 polymorphisms in VA pathogenesis remains undefined. We therefore explored the role of ROCK2 polymorphisms in the pathogenesis of VA.

Subjects and Methods

Study subjects

Study patients and controls consisted of consenting individuals who underwent coronary angiography and ergonovine challenge. Included subjects were of unrestricted age and gender and provided written informed consent for a blood draw to be used for deoxyribonucleic acid (DNA) extraction and in studies approved by the hospital's institutional review board. The study group consisted of 106 Korean patients with VA (91 men; mean age, 56 years; range, 36 to 79 years) who did not have significant coronary artery disease by coronary angiography and had a positive ergonovine challenge. The control group consisting of 107 Korean patients (47 men; mean age, 55 years; range 18 to 83 years) whose coronary angiogram was normal with an atypical symptom and a negative ergonovine challenge. None of the patients had significant coronary artery disease.

Cardiac catheterization and ergonovine provocation test

Except for sublingual nitroglycerin, all antianginal mediations, including calcium channel blockers, nitrates, and beta-blockers, were discontinued for at least 3 days before coronary angiography. The wrist of the patient's right hand was fixed slightly extended to the table. The skin overlying the radial artery was anesthetized by local infiltration using 1 mL of 2% lidocaine. After radial artery puncture, a 5 Fr transradial catheter was inserted according to the Seldinger technique. No vasodilators for the prevention of radial artery spasm in the conventional radial approach were injected. Diagnostic coronary angiography was performed in all patients by the Judkins technique. After coronary angiography was performed, graded doses (50, 100, and 200 μg) of ergonovine were injected intravenously in succession at 3 minutes intervals until coronary spasm was induced or maximal dose was attained. Coronary angiograms were obtained after each dose. Finally, angiograms were obtained in several projections after the injection of 2-5 mg of nitrate into both the left and right coronary arteries, and the organic coronary artery lesion was evaluated. Twelve-lead electrocardiography (ECG) and arterial blood pressure were continuously monitored during the study. A 12-lead ECG was recorded at each stage. For the prevention of delayed spasm, all subjects were given 10 mg of short-acting nifedipine sublingually after completing the test.

A positive ergonovine provocation test was defined as a total occlusion of a site of significant organic narrowing, or as >75% narrowing of a segment of coronary artery that was initially considered normal, and the presence of symptoms or signs (e.g., ST-segment depression, ST-segment elevation) comparable to myocardial isch-

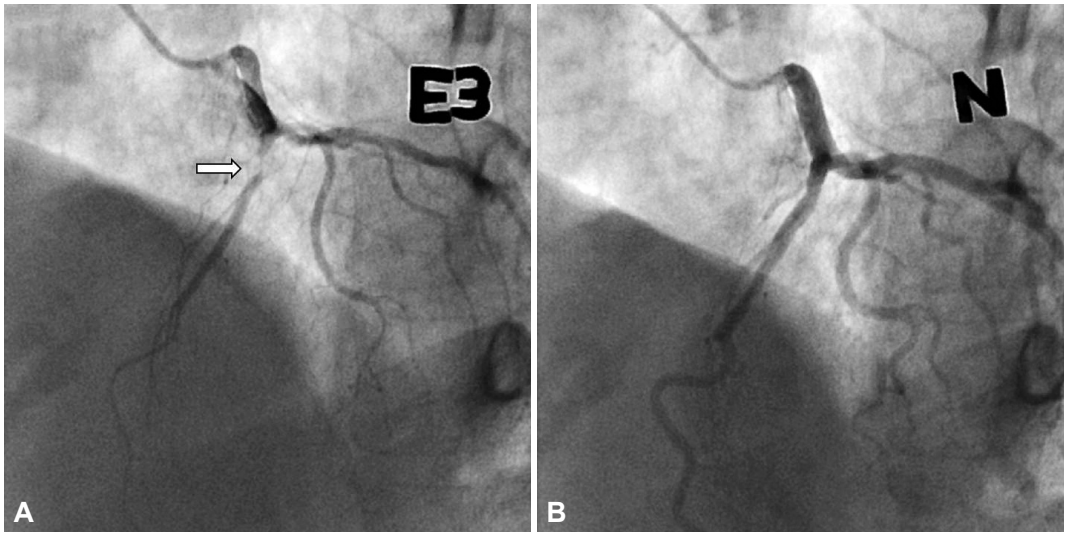

Fig. 1. Representative example of ergonovine provocation testing during diagnostic coronary angiography in a 49-year-old man with vasospastic angina. Intravenous injection of ergonovine (E3) provoked subtotal occlusion of the mid-portion of the left anterior descending artery (arrow) (A), and the angiogram after injection of nitroglycerin showed near normal left coronary artery and relief of total occlusion (B).

http://dx.doi.org/10.4070/kcj.2012.42.6.406

emia, with narrowed segments then recovering, either spontaneously or after induction by intracoronary nitrate, with restoration of the symptoms or signs to the resting condition (Fig. 1).[4]

Catheter-induced spasm was defined as focal spasm localized to the segment of artery adjacent to the catheter tip. Diffuse insignificant narrowing and catheter-induced spasm were regarded as a negative provocation result. Significant coronary stenosis was defined as >75% stenosis of luminal diameter of the right, left anterior descending or left circumflex coronary artery and the major branches, or as >50% stenosis of the left main trunk.

Polymorphism selection

Five single nucleotide polymorphisms (SNPs) were selected for genotyping based on the following criteria: 1) functionality priority, based on their potential functions {nonsynonymous SNPs > splicing-site SNPs > synonymous SNPs > 5' untranslated-region (UTR) SNPs > 3' UTR SNPs > intronic SNPs}; 2) frequency of minor alleles ≥20% among Asians; and 3) spacing of SNPs being relatively even across the gene region (Table 1).

Genomic deoxyribonucleic acid extraction

Approximately 20 mL of either arterial blood at the time of coronary angiography or venous blood immediately after consenting to this study were withdrawn and collected in ethylenediaminetetraacetic acid-containing tubes. Genomic DNA was isolated from peripheral blood leukocytes by using the QuickGene SP kit (FUJIFILM Corporation, Tokyo, Japan), and stored at -20°C until performing genotyping.

Genotyping

Genotyping of SNPs was performed by High-Resolution melting (HRM) analysis.[5-7] All primers used for HRM genotyping were designed using Primer 3 software.[8] A set of primers (5'-TATATGCAA CTCTTCCAGAC-3' and 5'-CCTTTAACTCTTCTCAATCC-3') was used to amplify the 88-bp fragment for the analysis of the exon 33 (rs978906) polymorphism. For the intron 29 (rs2271621) polymorphism, polymerase chain reaction (PCR) primers were 5'-ACATCTGTCTGTGTA ACTGG-3' and 5'-CCTTGGGTGATATT TTATTC-3'. The PCR product was 81-bp long. For the exon 10 (rs2230774) polymorphism, 2 primers (5'-TTAAGTGACTCTCCATCTTG-3'and 5'-CGTACTTC ATTTTTCCTTG-3') were used to amplify the 62-bp fragment. For the intron 5 (rs1515219) polymorphism, PCR primers were 5'-AGTTTATACCAGGTTGTTCC-3' and 5'-ATAAGCTAGGGTTTAAAGGA-3'. The PCR product was 89-bp long. For the intron 3 (rs3771106) polymorphism, a set of primers (5'-TAGTTGAAATGAGAGAGTTG-3'and 5'-GGAGAACATTTCTAA CAACC-3') was used to amplify the 89-bp fragment. PCR and HRM were performed with the Rotor Gene 6000 (QIAGEN Inc., Germantown, MD, USA). For the PCR reaction, each 10 μL PCR mixture contained 0.4 μL of 10 μM primers mix, 3.6 μL RNase-free water, 5 μL of 2 X HRM PCR master mix (QIAGEN Inc., Germantown, MD, USA), and 1 μL extracted genomic DNA. The PCR program included a 95°C activation step for 5 minutes, followed by 40 cycles of 95°C for 10 seconds and 56°C for 30 seconds, and amplification was monitored by measurement of EvaGreen green fluorescence. Following the last cycle, the instrument generated a HRM curve by ramping from 65°C to 85°C at 0.1°C per second with continuous fluorescence acquisition (Fig. 2).

Table 1. SNPs information

dbSNP accession number	Region	Alleles*	Function
rs978906	Exon 33	A/G	3'UTR
rs2271621	Intron 29	T/G	Intron
rs2230774	Exon 10	C/A	Missense
rs1515219	Intron 5	C/T	Intron
rs3771106	Intron 3	G/A	Intron

*Major/minor allele. SNP: single-nucleotide polymorphism, UTR: untranslated-region

Statistical analysis

Continuous variables are expressed as mean±standard deviation and categorical variables as numbers and percentages. Two-tailed Student's t-tests were used for the comparison of mean values, and categorical variables were compared using chi-square tests {Statistical Package for the Social Sciences (SPSS) 12.0 for Windows, SPSS Inc., Chicago, IL, USA}. Analyses of linkage disequilibrium (LD) and haplotype construction were assessed using the SHEsis software platform (http://analysis.bio-x.cn/myAnalysis.php). The SHEsis program used a partition ligation-combination-subdivision EM algorithm in estimation of haplotype reconstruction and frequency. The associations were tested on most likely haplotypes.[9][10] A value of $p<0.05$ was considered statistically significant.

Results

Characteristics of study participants

Clinical and biological characteristics for the 213 included patients are presented in Table 2. Ejection fraction was measured by echocardiography. The incidence of coronary risk factors was compared between the control and VA groups. The prevalence of men and of smokers was significantly higher in the VA group than in the control group ($p<0.001$); however, there were no significant differences between the 2 groups for the other coronary risk factors, including age, hypertension, diabetes mellitus, body mass index, and levels of total cholesterol, triglycerides, high density lipoprotein-cholesterol, and low density lipoprotein-cholesterol.

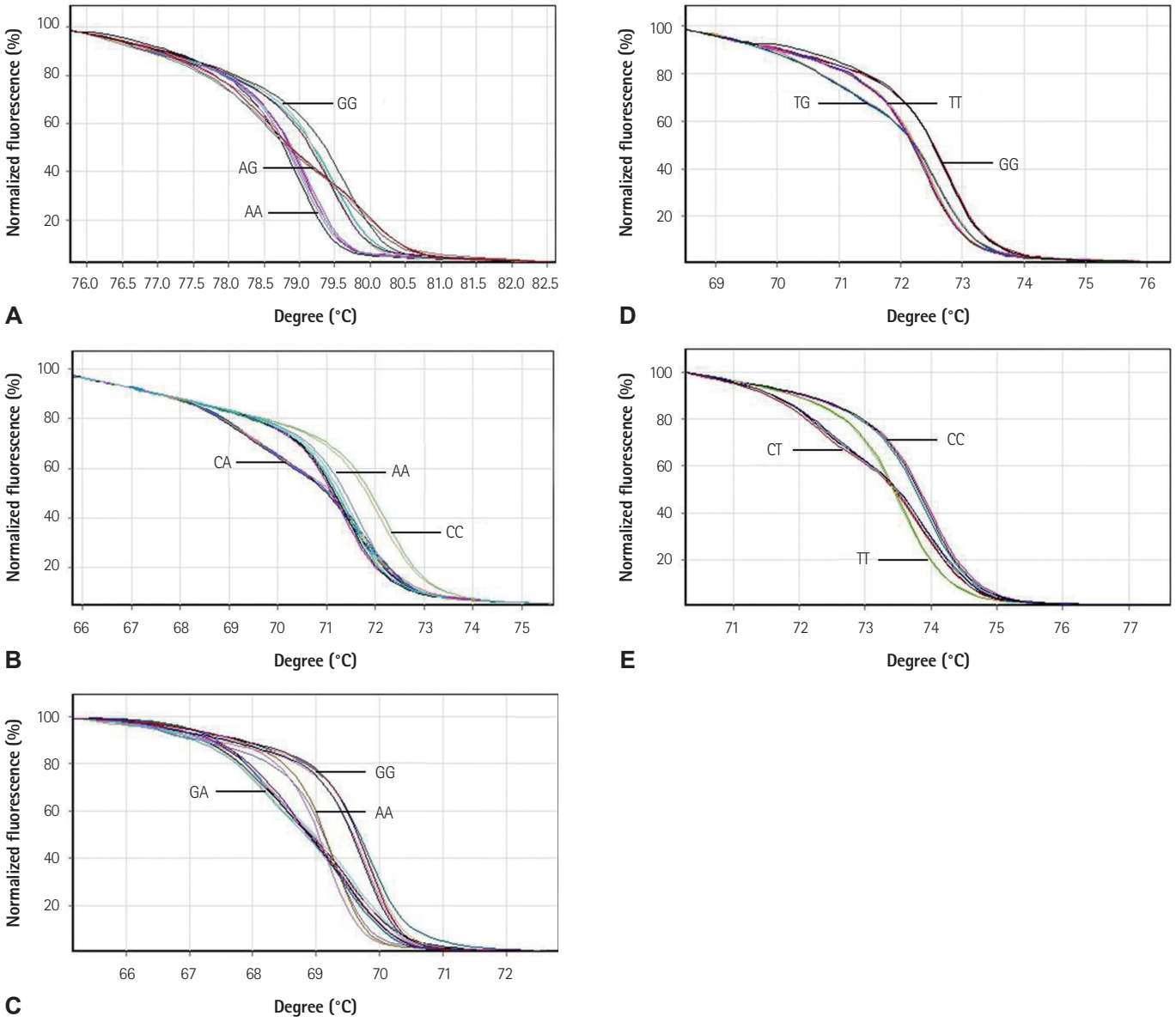

Fig. 2. Normalized high resolution melting (HRM) curves of the 3 possible genotypes for the 5 single-nucleotide polymorphisms of the ROCK2 gene. Triplicate HRM data were captured for the ROCK2 gene in SNP genotyping. Each category of SNP genotype can be readily discriminated prior to thermal shifting normalization. A: rs978906. B: rs2271621. C: rs2230774. D: rs1515219. E: rs3771106. SNP: single nucleotide polymorphism.

Association between genotype distributions and vasospastic angina

The genotype prevalence in the 2 study groups are presented in Table 3. The genotype prevalence in the 2 groups studied agreed with those predicted by Hardy-Weinberg equilibrium on the basis of allele prevalence. The genotype prevalences of all 5 interesting SNPs (rs978906, rs2271621, rs2230774, rs1515219, and rs3771106) of ROCK2 in the VA group were not significantly different from those in the control group. When additive, dominant, and recessive effects of the mutant ROCK2 allele were analyzed, there were no significant differences between the 2 groups (Table 4).

Linkage analysis and haplotype construction of the studied single nucleotide polymorphisms

Haplotypes were derived from the VA group, the control group, and the combined VA-control cohort. The analysis results assessed by the SHEsis program demonstrated that, rs2271621, rs2230774, rs1515219, and rs3771106 polymorphisms had strong LD (D' value >0.8) (Fig. 3) (Table 5).[11] Because no complete LD (D'=1) was detected among the 5 ROCK2 polymorphisms, all of them were included in haplotype analysis. A total of 6 haplotypes whose frequency was >0.3% were obtained, and a significant difference was found between the VA and control groups for the haplotype G-T-C-T-G (p=0.007) (Table 6).

http://dx.doi.org/10.4070/kcj.2012.42.6.406

Table 2. Clinical and biological characteristics of vasospastic angina and control groups

	VA (n=106)	Control (n=107)	p
Age (year)	55.8±9.6	55.2±12.1	0.663
Male/Female, n (ratio)	91/15 (6.1)	47/60 (0.8)	<0.001
Cigarette smoking status (current-, ex-) (%)	70 (66.0)	37 (34.6)	<0.001
Hypertension (%)	83 (78.3)	73 (68.2)	0.097
Diabetes mellitus (%)	7 (6.6)	9 (8.4)	0.617
Dyslipidemia (%)	76 (71.7)	71 (66.4)	0.399
Total cholesterol (mg/dL)	169.5±37.9	168.5±31.6	0.840
Triglyceride (mg/dL)	143.4±102.3	139.1±88.9	0.742
HDL-C (mg/dL)	45.9±12.2	47.6±11.0	0.298
LDL-C (mg/dL)	101.5±34.3	105.6±31.5	0.368
Body mass index (kg/m^2)	24.9±2.8	25.7±3.4	0.057
EF (%)	63.6±7.7	64.2±6.7	0.512

CAD: coronary artery disease, Dyslipidemia: total cholesterol level >200 mg/dL, LDL-C level >130 mg/dL, HDL-C level <30 mg/dL, or triglyceride level >150 mg/dL. EF: ejection fraction, HDL-C: high density lipoprotein-cholesterol, LDL-C: low density lipoprotein-cholesterol, VA: vasospastic angina

Table 3. Prevalence of ROCK2 genotypes in patients with vasospastic angina (n=106) and controls (n=107)

SNPs	Genotype	VA n (%)	Control n (%)	p
rs978906				0.198
	AA	32 (30.2)	26 (24.3)	
	AG	55 (51.9)	51 (47.7)	
	GG	19 (17.9)	30 (28.0)	
rs2271621				0.326
	TT	31 (29.2)	26 (24.3)	
	GT	56 (52.8)	53 (49.5)	
	GG	19 (17.9)	28 (26.2)	
rs2230774				0.424
	AA	25 (23.6)	22 (20.6)	
	AC	54 (50.9)	49 (45.8)	
	CC	27 (25.5)	36 (33.6)	
rs1515219				0.581
	CC	25 (23.6)	32 (29.9)	
	CT	55 (51.9)	51 (47.7)	
	TT	26 (24.5)	24 (22.4)	
rs3771106				0.188
	GG	25 (23.6)	37 (34.6)	
	GA	55 (51.9)	50 (46.7)	
	AA	26 (24.5)	20 (18.7)	

SNP: single nucleotide polymorphism, VA: vasospastic angina

Discussion

This study investigated the association of 5 SNPs (rs978906, rs 2271621, rs2230774, rs1515219, and rs3771106) within the ROCK2 gene, in a case controlled cohort of patients with VA and of Korean race. No association was seen between genotype at any individual SNP and clinical phenotype, suggesting that these SNPs are not associated with VA itself. However, LD analysis by the using SHEsis program[9][10] revealed 4 SNPs to be in strong LD with D' >0.8, allowing the construction of haplotype blocks. Gabriel et al.[11] provided the first evidence that, with the exception of hotspots of high recombination, the human genome is characterized by a block structure with sequences of SNPs that are highly correlated with each other in blocks of LD. In our study, subsequent haplotype analysis revealed the presence of one protective haplotype. Therefore, we provide evidence for an association of ROCK2 gene polymorphisms with the risk of VA and a protective effect of the haplotype G-T-C-T-G on the occurrence of VA, suggesting that the ROCK2 gene might be involved in the pathogenesis of VA in the Korean race. To our knowledge, this is the first haplotype-based association study demonstrating the combined effect of ROCK2 gene polymorphisms on VA, although we do not know the reasons why the individual SNPs were not associated with VA.

Small GTP-binding proteins (G proteins), such as those from the Rho, Ras, Rab, Sarl/Arf, and Ran families, act as molecular "on-off" switches that control multiple intracellular signaling pathways.[12][13] Among them, ROCK/Rho-kinases were the first characterized RhoA effectors and are considered as important regulators of cell growth, migration, and apoptosis via control of actin skeletal assembly.[14] ROCK2 is located on chromosome 12 and contains 1388 amino acids.[15] Although ROCK1 and ROCK2 are ubiquitously expressed, ROCK2 is highly expressed in the brain and the heart, whereas ROCK1 is preferentially expressed in the lung, liver, spleen, kidney, and testis.[16]

Recent evidence indicates that RhoA and its downstream effector, ROCK/Rho-kinases, inhibit myosin light chain phosphatase (MLCP), leading to augmentation of myosin light chain phosphorylation and Ca^{2+} sensitization in response to vasoconstrictor stimuli.[17] Shimokawa[18] demonstrated that the expression of ROCK/Rho-kinase messenger RNA was significantly increased and upregulated in the spastic rather than in the control segment in a porcine model. They also showed that Fasudil, a specific inhibitor of ROCK/Rho-kinase, inhibited coronary artery spasms in animals[19] and humans.[20] These results indicate that ROCK/Rho-kinase plays a key role in induction of vascular smooth muscle hypercontraction by inhibition of MLCP through phosphorylation of the myosin binding subunit.[21] In addition, recent studies have shown that eNOS is regulated by the ROCK/Rho-kinase pathway. Inhibition of RhoA geranylgeranylation by statins decreases membrane GTP bound active RhoA and subsequent ROCK activity, leading to upregulation and activation of eNOS;[22]

Table 4. Additive, dominant, and recessive effects of mutant ROCK2 alleles in patients with vasospastic angina (n=106) and controls (n=107)

SNP ID	Model	Genotype	VA (%)	Control (%)	OR (95% CI)	p
rs978906	Additive	AA	32 (30.2)	26 (24.3)	1.0	
		AG	55 (51.9)	51 (47.7)	-0.876 (0.461-1.667)	0.687
		GG	19 (17.9)	30 (28.0)	-0.515 (0.237-1.115)	0.092
	Dominant	AA	32 (30.2)	26 (24.3)	1.0	
		AG+GG	74 (69.8)	81 (75.7)	-0.742(0.405-1.361)	0.335
	Recessive	AA+AG	87 (82.1)	77 (72.0)	1.0	
		GG	17 (17.9)	30 (28.0)	-0.561 (0.292-1.075)	0.082
rs2271621	Additive	TT	31 (29.2)	26 (24.3)	1.0	
		TG	56 (52.8)	53 (49.5)	-0.886 (0.466-1.685)	0.712
		GG	19 (17.9)	28 (26.2)	-0.569 (0.26-1.244)	0.158
	Dominant	TT	31 (29.2)	26 (24.3)	1.0	
		TG+GG	75 (70.8)	81 (75.7)	-0.777 (0.423-1.427)	0.415
	Recessive	TT+TG	87 (82.1)	79 (73.8)	1.0	
		GG	19 (17.9)	28 (26.2)	-0.616 (0.319-1.189)	0.149
rs2230774	Additive	CC	27 (25.5)	36 (33.6)	1.0	
		CA	54 (50.9)	49 (45.8)	1.469 (0.782-2.762)	0.232
		AA	25 (23.6)	22 (20.6)	1.515 (0.709-3.239)	0.284
	Dominant	CC	27 (25.5)	36 (33.6)	1.0	
		CA+AA	79 (74.5)	71 (66.4)	1.484 (0.820-2.685)	0.192
	Recessive	CC+CA	81 (76.4)	85 (79.4)	1.0	
		AA	25 (23.6)	22 (20.6)	1.192 (0.623-2.281)	0.595
rs1515219	Additive	CC	25 (23.6)	32 (29.9)	1.0	
		CT	55 (51.9)	51 (47.7)	1.380 (0.723-2.637)	0.329
		TT	26 (24.5)	24 (22.4)	1.387 (0.647-2.973)	0.401
	Dominant	CC	25 (23.6)	32 (29.9)	1.0	
		CT+TT	81 (76.4)	75 (70.1)	0.382 (0.751-2.545)	0.298
	Recessive	CC+CT	80 (75.5)	83 (77.6)	1.0	
		TT	26 (24.5)	24 (22.4)	1.124 (0.596-2.119)	0.718
rs3771106	Additive	GG	25 (23.6)	37 (34.6)	1.0	
		GA	55 (51.9)	50 (46.7)	1.628 (0.862-3.074)	0.133
		AA	26 (24.5)	20 (18.7)	1.924 (0.888-4.167)	0.097
	Dominant	GG	25 (23.6)	37 (34.6)	1.0	
		GA+AA	81 (76.4)	70 (65.4)	1.713 (0.940-3.120)	0.079
	Recessive	GG+GA	80 (75.5)	87 (81.3)	1.0	
		AA	26 (24.5)	20 (18.7)	1.414 (0.733-2.728)	0.302

SNP: single nucleotide polymorphism, VA: vasospastic angina, OR: odds ratio, CI: confidence interval

furthermore, direct inhibition of the ROCK/Rho-kinase pathway has been shown to increase eNOS expression.[23] Therefore, ROCK/Rho-kinase is closely associated with endothelial nitric oxide activity. Considering the role of ROCK2 in the regulation of vascular smooth muscle cell contraction and nitric oxide production pathways, one could speculate that a decreased ROCK2 expression and/or activity may reduce vascular smooth muscle contractility and increase nitric oxide production, and consequently, protect against coronary vasospasm. Our data suggest that the haplotype block by SNPs rs978906, rs2271621, rs2230774, rs1515219, and rs3771106 may contain functional sequence variant(s) that reduce the level of ROCK2 expression and/or its activity. However, this hypothesis must be experimentally confirmed.

Data on polymorphisms of ROCK/Rho-kinases in patients with VA is sparse. To the author's knowledge, there is only one report (abstract) on a missense mutation (G930T) in the catalytic domain of

http://dx.doi.org/10.4070/kcj.2012.42.6.406

ROCK2 in association with enhanced Rho-kinase activity. In that study, the prevalence of the mutation was higher in patients with VA than in controls, and was also higher in Japanese patients with ischemic heart disease than in white patients.[24]

A number of mechanisms and precipitating factors may play a role in the pathogenesis of VA. These include endothelial dysfunction, vascular smooth muscle hypersensitivity, increased autonomic tone, increased oxidative stress, decreased magnesium, low-grade inflammation, and genetic susceptibility.[25] Although epidemiological studies have shown that cigarette smoking is a major risk factor for VA,[26] and consistently there was a higher prevalence of smokers in the VA group of our study, cigarette smoking was not an independent factor for VA after adjustment for other conventional risk factors such as age, gender, dyslipidemia, hypertension, and diabetes. To determine an association between cigarette smoking and other study variables, multiple logistic regression analysis was performed on individuals from the VA and control groups. Male gender was considered as a confounding factor and was significantly associated with smoking (odds ratio 31.392) after adjustment for other variables. Interestingly, we could not find any relationship among conventional risk factors, except male gender, for coronary atherosclerosis, namely dyslipidemia, hypertension, age and diabetes, and coronary vasospasm. This finding suggests that the pathogenesis of VA might involve multiple factors or factors that interact with each other, and be different from that of coronary atherosclerosis.

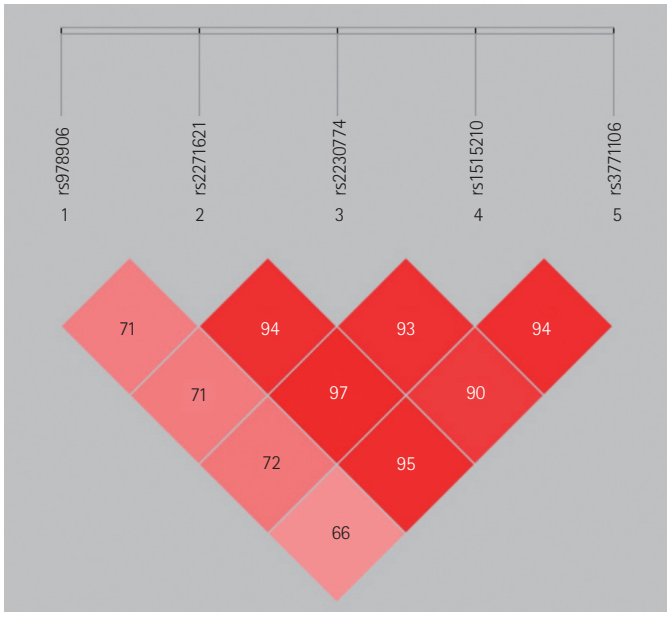

Fig. 3. Linkage disequilibrium status of the 5 single-nucleotide polymorphisms (SNPs) of the ROCK2 gene. In this plot, each square represents a pair-wise comparison between 2 SNPs and the respective D′ is given within each square. Darker square colors of red indicate higher values of D′, up to a maximum of 1.

Table 5. D' values of pair-wise linkage disequilibrium between 5 single-nucleotide polymorphisms indentified in ROCK2 gene

D'	rs2271621	rs2230774	rs1515219	rs3771106
rs978906	0.712	0.716	0.728	0.662
rs2271621	-	0.942	0.979	0.954
rs2230774	-	-	0.939	0.903
rs1515219	-	-	-	0.949

Our study has several limitations. First, the sample size was small, despite enrollment of individuals for >2 years. However, because Hardy-Weinberg equilibrium was satisfied for all groups in this study, selection bias for evaluating the genetic association seems unlikely. Second, we failed to demonstrate whether the SNPs are related to functional alterations of Rho-kinase. Third, we cannot exclude the possibility of having missed non-coding functional variants or rare coding variants because we studied only 5 interesting polymorphisms of ROCK2. Thus, more extensive studies, including whole-genome analysis, are required to confirm the results.

Although the RhoA/Rho-kinase pathway is considered to be an important coronary vasomotor regulator, and inhibition of this pathway improved cardiovascular outcomes, the extent to which this pathway contributes to the pathogenesis of VA is unknown. The exact mechanisms by which the activity of ROCK/Rho-kinase is increased or decreased, and which stage in the RhoA/Rho-kinase pathway is the most important, remain to be elucidated.

In conclusion, there were no genotypic associations between 5 SNPs in the ROCK2 gene and VA cases. However, the haplotype G-T-

Table 6. Haplotype analysis results of vasospastic angina and control patients

Haplotypes	VA frequency	Control frequency	p	OR (95% CI)
A-G-C-C-G	0.392	0.415	0.529	0.881 (0.593-1.308)
A-T-A-T-A	0.071	0.028	0.050	2.608 (0.991-6.864)
A-T-C-C-G	0.052	0.023	0.128	2.258 (0.770-6.621)
G-G-C-C-G	0.033	0.070	0.077	0.445 (0.177-1.116)
G-T-A-T-A	0.401	0.449	0.503	1.146 (0.769-1.708)
G-T-C-T-G	0.000	0.034	0.007	0.000 (0.000-0.000)*

Loci chosen for the haplotype analysis (in order of rs978906, rs2271621, rs2230774, rs1515210, and rs3771106). All frequencies <0.03 were ignored in analysis. *Odds ratio and CI showed zeros because of a zero incidence in VA population. VA: vasospastic angina, CI: confidence interval

C-T-G in ROCK2 gene had a protective effect against VA in the Korean population, suggesting the involvement of ROCK2 in VA pathogenesis.

References

1. Sueda S, Kohno H, Fukuda H, Uraoka T. Did the widespread use of long-acting calcium antagonists decrease the occurrence of variant angina? *Chest* 2003;124:2074-8.
2. Yoo SY, Shin DH, Jeong JI, et al. Long-term prognosis and clinical characteristics of patients with variant angina. *Korean Cir J* 2008;38:651-8.
3. Yasue H, Nakagawa H, Itoh T, Harada E, Mizuno Y. Coronary artery spasm: clinical features, diagnosis, pathogenesis, and treatment. *J Cardiol* 2008;51:2-17.
4. Chahine RA, Raizner AE, Ishimori T, Luchi RJ, McIntosh HD. The incidence and clinical implications of coronary artery spasm. *Circulation* 1975;52:972-8.
5. Liew M, Pryor R, Palais R, et al. Genotyping of single-nucleotide polymorphisms by high-resolution melting of small amplicons. *Clin Chem* 2004;50:1156-64.
6. Reed GH, Wittwer CT. Sensitivity and specificity of single-nucleotide polymorphism scanning by high-resolution melting analysis. *Clin Chem* 2004;50:1748-54.
7. Wittwer CT, Reed GH, Gundry CN, Vandersteen JG, Pryor RJ. High-resolution genotyping by amplicon melting analysis using LCGreen. *Clin Chem* 2003;49(6 Pt 1):853-60.
8. Rozen S, Skaletsky H. Primer3 on the WWW for general users and for biologist programmers. *Methods Mol Biol* 2000;132:365-86.
9. Shi YY, He L. SHEsis, a powerful software platform for analyses of linkage disequilibrium, haplotype construction, and genetic association at polymorphism loci. *Cell Res* 2005;15:97-8.
10. Li Z, Zhang Z, He Z, et al. A partition-ligation-combination-subdivision EM algorithm for haplotype inference with multiallelic markers: update of the SHEsis (http://analysis.bio-x.cn). *Cell Res* 2009;19:519-23.
11. Gabriel SB, Schaffner SF, Nguyen H, et al. The structure of haplotype blocks in the human genome. *Science* 2002;296:2225-9.
12. Fukata Y, Amano M, Kaibuchi K. Rho-Rho-kinase pathway in smooth muscle contraction and cytoskeletal reorganization of non-muscle cells. *Trends Pharmacol Sci* 2001;22:32-9.
13. Takai Y, Sasaki T, Matozaki T. Small GTP-binding proteins. *Physiol Rev* 2001;81:153-208.
14. Noma K, Oyama N, Liao JK. Physiological role of ROCKs in the cardiovascular system. *Am J Physiol Cell Physiol* 2006;290:C661-8.
15. Riento K, Ridley AJ. Rocks: multifunctional kinases in cell behaviour. *Nat Rev Mol Cell Biol* 2003;4:446-56.
16. Nakagawa O, Fujisawa K, Ishizaki T, Saito Y, Nakao K, Narumiya S. ROCK-I and ROCK-II, two isoforms of Rho-associated coiled-coil forming protein serine/threonine kinase in mice. *FEBS Lett* 1996;392:189-93.
17. Shimokawa H. Rho-kinase as a novel therapeutic target in treatment of cardiovascular diseases. *J Cardiovasc Pharmacol* 2002;39:319-27.
18. Shimokawa H. Cellular and molecular mechanisms of coronary artery spasm: lessons from animal models. *Jpn Circ J* 2000;64:1-12.
19. Kandabashi T, Shimokawa H, Miyata K, et al. Inhibition of myosin phosphatase by upregulated rho-kinase plays a key role for coronary artery spasm in a porcine model with interleukin-1beta. *Circulation* 2000;101:1319-23.
20. Masumoto A, Mohri M, Shimokawa H, Urakami L, Usui M, Takeshita A. Suppression of coronary artery spasm by the Rho-kinase inhibitor fasudil in patients with vasospastic angina. *Circulation* 2002;105:1545-7.
21. Somlyo AP, Somlyo AV. Signal transduction and regulation in smooth muscle. *Nature* 1994;372:231-6.
22. Laufs U, Liao JK. Post-transcriptional regulation of endothelial nitric oxide synthase mRNA stability by Rho GTPase. *J Biol Chem* 1998;273:24266-71.
23. Wolfrum S, Dendorfer A, Rikitake Y, et al. Inhibition of Rho-kinase leads to rapid activation of phosphatidylinositol 3-kinase/protein kinase Akt and cardiovascular protection. *Arterioscler Thromb Vasc Biol* 2004;24:1842-7.
24. Kamiunten H, Koike J, Mashiba J, Shimokawa H, Takeshita A. A comprehensive analysis of a novel missense mutation in Rho-kinase that causes coronary vasospasm in the Japanese. *Circ J* 2004;68(suppl I):211. Abstract.
25. Yoo SY, Kim JY. Recent insights into the mechanisms of vasospastic angina. *Korean Circ J* 2009;39:505-11.
26. Sugiishi M, Takatsu F. Cigarette smoking is a major risk factor for coronary spasm. *Circulation* 1993;87:76-9.

http://dx.doi.org/10.4070/kcj.2012.42.6.406