miRNA polymorphisms (*miR-146a*, *miR-149*, *miR-196a2* and *miR-499*) are associated with the risk of coronary artery disease

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Abstract. Small non-coding microRNAs (miRNAs) are not only important for heart and vascular development but are also important in cardiovascular pathophysiology and diseases, such as ischemia and atherosclerosis-related diseases. However, the effect of *miR-146a*, *miR-149*, *miR-196a2* and *miR-499* polymorphisms on coronary artery disease (CAD) susceptibility remain unknown. The aim of the present study was to examine the genotype frequencies of *miR-146a*, *miR-149*, *miR-196a2* and *miR-499* polymorphisms in patients with CAD, and assess their clinical applications for diagnosing and monitoring CAD. Using polymerase chain reaction-amplified DNA, microRNA polymorphisms were analyzed in 522 patients with CAD and 535 control subjects. The *miR-149* rs2292832 C>T and *miR-196a2* rs11614913 T>C polymorphisms were shown to be significantly associated with CAD prevalence. In subgroup analyses according to disease severity, the *miR-146a* rs2910164GG genotype was significantly associated with CAD risk in the stent ≥2 group. In addition, *miR-146a*G/*-149*T/*-196a2*C/*-499* G allele combination was significantly associated with CAD prevalence (G-T-C-G and G-C-C-G of *miR-146a*/*-149*/*-196a2*/*-499*). The combination genotypes of *miR-146a*GG/149TC+CC and *miR-149*CC/196a2TC were significantly associated with CAD incidence. In subgroup analyses, *miR-146a* rs2910164 C>G increased the risk of developing CAD in non-smoking, hypertensive and nondiabetic subgroups. Furthermore, *miR-149* rs2292832 C>T and *miR-196a2* rs11614913 T>C was shown to increase CAD risk in females and patients aged >63 years old. The *miR-149*T allele, *miR-196a2*C allele and *miR-146a*G/*-149*T/*-196a2*C/*-499* G allele combination were associated with CAD pathogenesis. The combined effects of environmental factor and genotype combination of miRNA polymorphisms may contribute to CAD prevalence.

Introduction

Cardiovascular disease is the single most prevalent health problem. It is associated with the highest rates of mortality and morbidity worldwide, accounting for >14% of all fatalities, and is predicted to remain so until 2030 (1). Coronary artery disease (CAD) is the most common type of cardiovascular disease, in which a plaque builds up inside the coronary arteries that can lead to a complete blockage of blood flow to the heart, resulting in a heart attack. Moreover, plaque build up narrows coronary arteries, which results in decreased blood flow to the heart that can cause chest pain (angina), shortness of breath, or other symptoms. Previous epidemiological studies have identified the role of several modifiable and non-modifiable risk factors in the pathogenesis and prognosis of CAD, including age, gender, smoking, obesity, diet, life style, and genetic factors (2).

One of the major challenges in cardiovascular disease is the identification of reliable clinical biomarkers that can be routinely measured in blood plasma. MicroRNAs (miRNAs), hold promise as novel biomarkers for clinical diagnosis and can be found in a number of bodily fluids, including blood, urine, saliva, plasma and serum. They are protected from degradation in the circulation through association with lipids, proteins or microparticles, rendering them an attractive disease biomarker candidate (3). miRNAs are short non-coding RNA sequences that regulate the expression of multiple target genes, predominantly by binding to the 3'-untranslated region of mRNA transcripts, resulting either in translational inhibition

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or mRNA degradation (4). In the cardiovascular system, miRNAs are not only important for heart and vascular development but are also essential in cardiovascular pathophysiology and cardiovascular diseases, such as arrhythmia, ischemia and coronary atherogenesis (5). miRNAs have been increasingly implicated in the control of various biological processes, including cell differentiation, cell proliferation, cell growth and apoptosis, and numerous pathological processes, such as cancer, Alzheimer's disease and cardiovascular disease (3). Polymorphic miRNA-mediated gene regulation and mutations in the corresponding sequence space (machinery, miRNA precursors and the target sites) are likely to make a significant contribution to phenotypic variation, including the susceptibility to diseases, such as cancer and cardiovascular disease (6). A single nucleotide polymorphism (SNP) is a DNA sequence variation of a single nucleotide, adenine (A), thymine (T), cytosine (C) or guanine (G), on genomic DNA. An SNP in an miRNA sequence may alter miRNA expression and/or maturation and have been shown to be associated with the progression of CAD. *miR-146a*G>C, *miR-149*C>T, *miR-196a2*T>C and *miR-499*A>G polymorphisms have been reported to be associated with lung, breast, thyroid, colon and gastric cancer (7). Recently, four well-known miRNA polymorphisms in pre-miRNA sequences [*miR-146a*C>G (rs2910164; chromosome 5, 159912418), *miR-149*T>C (rs2292832; chromosome 2, 241395503), *miR-196a2*T>C (rs11614913; chromosome 12, 54385599) and *miR-499*A>G (rs3746444; chromosome 20, 33578251)] have been investigated in a variety of diseases and were found to contribute to pathogenesis (8).

In particular, significantly increased levels of miR-146a (6.25-fold) compared with controls and patients with the wild type variant were associated with the CC genotype in patients with CAD (P<0.0001) (9). hsa-miR-149 may be involved in congenital heart disease by regulating methylenetetrahydrofolate reductase (MTHFR), but which can be altered by regulation of MTHFR, because the binding site has an rs4846049 polymorphism in the MTHFR 3'-untranslated region (10). In addition, it has been suggested that the plasma *miR-499* concentration may be a biomarker of myocardial infarction in humans (11,12). Further follow-up case-control studies, identified two SNPs (rs11614913 and rs3746444) in hsa-miR-196a2 and hsa-miR-499 that are associated with an increased risk of developing cancer (13-15), congenital heart disease (16) and dilated cardiomyopathy. Thus, it was hypothesized that SNPs in these miRNAs may also contribute to susceptibility to and unfavorable prognosis of CAD.

According to recent data, the *miR-146a*G, *miR-149*T, *miR-196a2*C and *miR-499* G alleles are possible genetic predisposing factors in various diseases (17-19). Moreover, these four miRNAs have been shown to affect vascular damage responses, such as those in abortion, cancer, ischemic stroke and cardiovascular disease (8,17,18,20,21). The *miR-146a*, *-149*, *-196a2* and *-499* alleles are closely associated with the regulation of tumor necrosis factor-α (TNF-α), MTHFR, Annexin A1 (ANXA1), and C-reactive protein (CRP), respectively (8). Furthermore, in the circulatory system, these miRNA targets are important in thrombosis and inflammatory signaling pathways. Recent advances in genetic research have systematically identified and analyzed human polymorphisms in miRNAs and/or miRNA target sites (22,23). However, the majority of these studies focus on SNPs in the target sites and their effects on disease-related miRNAs (8,22-24). One of these studies has mentioned the interplay effects between miRNAs' SNP and target gene SNPs in disease (24). There are currently no data regarding the role of miRNA polymorphisms in CAD pathogenesis. Therefore, the present study aimed to investigate a miRNA-miRNA synergistic effect associated with CAD by genetic association analyses of these four well known miRNA variants and CAD patients, with or without percutaneous coronary intervention (PCI) according to disease severity.

Materials and methods

Study population. The study subjects were recruited from the South Korean provinces of Seoul and Kyeonggi-do between 2006 and 2015 from the Department of Cardiology at the CHA Bundang Medical Center in Seongnam, South Korea. The study was approved by the Institutional Review Board of CHA Bundang Medical Center (Seongnam, Korea). In total, 522 patients with CAD were referred from the Department of Cardiology at CHA Bundang Medical Center, CHA University. All patients who presented with stable coronary artery disease or acute coronary syndromes (including unstable angina with or without ST-segment elevation) and at least one coronary lesion with >50% stenosis in a vessel with a diameter of 2.25-4.00 mm between 2006 and 2015, were screened for eligibility. No restrictions were placed on the total number of treated lesions, which vessels were treated, lesion length, or the number of stents implanted. Exclusion criteria were history of acute myocardial infarction and life expectancy <1 year. All patients underwent coronary angiography and electrocardiography. Diagnoses were made by coronary angiography, and were confirmed by at least 1 independent experienced cardiologist.

In total, 535 gender- and age (±5 years)-matched control subjects from patients presented at the Department of Cardiology at the CHA Bundang Medical Center (Seongnam, Korea) during the same period for health examinations, including biochemical testing, electrocardiograms, and coronary computed tomography scans. Exclusion criteria were the same as those used in the patient group, as well as a recent history of anginal symptoms. Hypertension was defined as systolic pressure >140 mmHg and diastolic pressure >90 mmHg on >1 occasion and included patients currently taking hypertensive medications. Diabetes mellitus was defined as a fasting plasma glucose level >126 mg/dl (7.0 mmol/l) and included patients taking diabetic medications. Smoking refers to patients who currently smoke. Hyperlipidemia was defined as a high fasting serum total cholesterol (TC) level (≥240 mg/dl) or an antihyperlipidemic agent treatment history.

Genetic analyses. DNA was extracted from peripheral blood leukocytes using the G-dex II Genomic DNA Extraction kit (iNtRON Biotechnology, Inc., Seongnam, Korea), according to the manufacturer's instructions. Polymerase chain reaction (PCR) restriction fragment length polymorphism assays to analyze the *miR-146a*C>G, *miR-196a2*T>C and *miR-499*A>G polymorphisms. Genotyping of the *miR-149*T>C polymorphism was determined using quantita-

tive PCR (RG-3000, Corbett Research, Mortlake, Australia) for allelic discrimination. Primers and TaqMan probes were designed using Primer Express Software (version 2.0; Thermo Fisher Scientific, Inc., Waltham, MA, USA), and synthesized and supplied by Applied Biosystems (Foster City, CA, USA). The reporter dyes used were 5-carboxyfluorescein (FAM) and 2',7'-dimethoxy-4',5'-dichloro-6-carboxyfluorescein (JOE). The primer sequences for amplification are as follows: forward: 5'-CAT GGG TTG TGT CAG TGT CAG AGC T-3' and reverse: 5'-TGC CTT CTG TCT CCA GTC TTC CAA-3' for *miR-146a*C>G; forward: 5'-CTG GCT CCG TGT CTT CAC TC-3' and reverse: 5'-CAA CTC GCC CAG CCG-3' for *miR-149*T>C; forward: 5'-CCC CTT CCC TTC TCC TCC AGA TA-3' and reverse: 5'-CGA AAA CCG ACT GAT GTA ACT CCG-3' for *miR-196a2*T>C; and forward: 5'-CAA AGT CTT CAC TTC CCT GCC A-3' and reverse: 5'-GAT GTT TAA CTC CTC TCC ACG TGA TC-3' for *miR-499*A>G. The selected probes were 5'-FAM-TGG GGC AGC CGG AAC AAC-TAMRA-3' (C allele detecting probe) and 5'-JOE-TGG GGC AGC TGG AAC AAC-TAMRA-3' (T allele detecting probe) for *miR-149*T>C. Underlined bases in the primers above are mismatches with the complementary sequence. The *miR-146*aC>G and *miR-196a2*T>C polymorphisms were digested by *Sac*I and *Msp*I, respectively, for 16 h at 37˚C (New England BioLabs, Beverly, MA, USA). The *miR-499*A>G polymorphism was digested with *Bcl*I for 16 h at 50˚C (New England Biolabs). The PCR conditions were as follows: Initial denaturation at 94˚C for 5 min/denaturation at 94˚C for 30 sec; annealing at 58˚C for 30 sec, extension at 72˚C for 30 sec with 32 amplification cycles/final extension at 72˚C for 5 min for the *miR-146a*G>C, *miR-196a2*C>T and *miR-499*C>T polymorphisms. The *miR-149*T>C was designed for real-time quantitative PCR (RT-qPCR): Initial denaturation at 95˚C for 15 min/denaturation at 95˚C for 30 sec/annealing at 58˚C for 50 sec with 50 amplification cycles. The reaction product (12 μl) was run on a 3.0% ethidium bromide-stained agarose gel and directly visualized under ultraviolet illumination. Approximately 10% of the PCR assays were randomly repeated for each of the miRNA polymorphisms and the results were checked for concordance by DNA sequencing using an automatic sequencer (ABI 3730x l DNA analyzer; Applied Biosystems). The concordance of the quality control samples was 100%.

Statistical analysis. To estimate the relative risk of the various genotypes for CAD, the odds ratio (OR) and 95% confidence interval (CI) were calculated. Case and control groups were compared using Student's t-test for continuous variables, and the χ^2 test for categorical variables. For multivariate analyses, logistic regression analyses were used to adjust for possible confounders, including age, gender, hypertension, Diabetes mellitus, hyperlipidemia and smoking. P<0.05 was considered to indicate a statistically significant difference. Multiple hypotheses testing was performed using the Benjamini-Hochberg method to control for false discovery rate (FDR) in the logistic regression analysis. Calculating the FDR is a way to address the problems associated with multiple comparisons, and FDR provides a measure of the expected proportion of false-positives among data. Analyses were performed using GraphPad Prism 4.0 (GraphPad Software, Inc., San Diego, CA, USA), StatsDirect Statistical Software Version 2.4.4 (StatsDirect Ltd., Altrincham, UK), and MedCalc (Version 7.4 for Windows; MedCalc, Ostend, Belgium). The multifactor dimensionality reduction (MDR) method was proposed by Ritchie *et al* (25), and implemented by Hahn *et al* (26). The MDR method has been described in detail previously (25,26). Briefly, the MDR is comprised of 2 steps. The best combination of multifactors is initially selected and the genotype combinations are classified into high and low-risk groups (26). Interaction analyses were performed in the open-source MDR software package (v.2.0) available from www.epistasis.org. Using MDR analyses, all possible allele combinations were constrcted for gene-gene interactions. HAPSTAT software was used to estimate the frequencies of allele combinations for the polymorphisms selected by MDR analysis with strong synergistic effects. HAPSTAT allows testing of the haplotype (or allele combination) effects by maximizing the likelihood (from the observed data) that properly accounts for phase uncertainty and study design. Current versions of the HAPSTAT software (v.3.0) are available from www.bios.unc.edu/~lin/hapstat/.

Results

Characteristics of the study population. Baseline characteristics of the CAD patients and controls are shown in Table I. No significant differences in the age and gender distribution were identified between the patients with CAD and controls, suggesting that our frequency-matching on age and gender was satisfactory. The average body mass index (BMI) of CAD patients was significantly higher than that of the controls (P<0.0001). In addition, the serum total cholesterol (TC) and TG levels were significantly higher and those of high density lipoprotein-cholesterol (HDL-C) were significantly lower in the patients with CAD compared with the controls (P=0.022, P=0.004 and P=0.001, respectively); however, no difference was identified in the level of serum low density lipoprotein-cholesterol (LDL-C) between the two groups (P=0.116). For the disease history, 135 (25.9%) patients had a history of Diabetes mellitus (DM), which was significantly higher compared with controls (P=0.0003), however, no significant difference was identified in the hypertension history between CAD patients and controls. Furthermore, no significant difference was identified in smoking (P=0.165), the hyperlipidemia history (P=0.226), plasma homocysteine (HCY) level (P=0.315), serum folate level (P=0.288) and serum vitamin B12 level (P=0.097) were shown between CAD patients and controls.

miR-149 and miR-196a2 polymorphisms are significantly correlated *with CAD*. *miR-146a*C>G, *-149*T>C, *-196a2*T>C, and *-499*A>G polymorphisms were investigated and their genotype distributions in CAD patients and control subjects were determined (Table II). The adjusted odds ratio (AOR) from logistic regression analyses with respect to age, gender, hypertension, diabetes mellitus and hyperlipidemia was calculated. The miRNA genotype frequencies of controls were consistent with Hardy-Weinberg equilibrium. The *miR-149* rs2292832C>T polymorphism was significantly different between patients with CAD and control subjects (TT vs. TC: COR, 1.312, 95% CI, 1.018-1.692; AOR, 1.336, 95% CI,

Table I. Baseline characteristics between controls and patients with CAD.

Characteristic	Controls	CAD patients	P-value
N	535	522	
Age (years, mean ± SD)	60.68±11.59	60.75±11.61	0.932
Male, n (%)	267 (50.0)	261 (50.0)	0.972
Hypertension, n (%)	243 (45.4)	227 (43.5)	0.736
SBP (mmHg, mean ± SD)	131.65±17.00	128.46±22.76	**0.010**
DBP (mmHg, mean ± SD)	80.64±11.68	79.09±13.63	**0.048**
Diabetes millitus, n (%)	79 (14.8)	135 (25.9)	**0.0003**
FBS (mg/dl)	112.46±37.37	140.90±62.24	**<0.0001**
BMI ≥25 kg/m^2, n (%)	124 (23.2)	263 (50.4)	**<0.0001**
TG (mg/dl, mean ± SD)	140.86±86.07	158.79±110.12	**0.004**
LDL-C (mg/dl, mean ± SD)	116.97±40.17	112.08±39.52	0.116
HDL-C (mg/dl, mean ± SD)	46.72±14.73	43.65±11.10	**0.001**
Smokers (%)	150 (28.0)	176 (33.7)	0.165
Hyperlipidemia (%)	122 (22.8)	142 (27.0)	0.226
HCY (μmol/l, mean ± SD)	9.83±3.97	10.12±5.27	0.315
Folate (nmol/l, mean ± SD)	8.99±8.01	8.38±9.15	0.288
Vitamin B12 (pg/ml, mean ± SD)	753.09±707.34	906.47±1327.12	0.097
Total cholesterol (mg/dl, mean ± SD)	192.38±43.25	186.03±45.69	**0.022**

P-values were calculated by a two-sided t-test for continuous variables and χ^2 test for categorical variables. Bold indicates significant values. CAD, coronary artery disease; SD, standard deviation; BP, blood pressure, FBS, fasting blood sugar; HTN, hypertension; DM, Diabetes mellitus; BMI, body mass index; TG, triglycerides; LDL/HDL-C, low/high density lipoprotein-cholesterol; HCY, homocysteine.

1.031-1.731). The *miR-196a2* rs11614913 T>C polymorphism was significantly different between CAD patients and control subjects (TT vs. TC: COR, 0.736, 95% CI, 0.558-0.971; TT vs. TC+CC: COR, 0.768, 95% CI, 0.592-0.996). However, *miR-146a* rs2910164C>G, and *miR-499* rs3746444A>G polymorphisms were not significantly different between CAD patients and control subjects (Table II).

Genotype frequencies of the miRNA polymorphisms between CAD patients with/without PCI. The *miR-146a*C>G, *-149*T>C, *-196a2*T>C, and *-499*A>G polymorphisms in CAD patients with or without PCI and in control subjects was determined. The incidence of the *miR-146a* rs2910164 C>G polymorphism was significantly different between patients with CAD with a stent and control subjects (CC+CG vs. GG: COR, 1.499, 95% CI, 1.036-2.169; CC+CG vs. GG: AOR, 1.473, 95% CI, 1.005-2.159), and between CAD patients without stent and control subjects (CC vs. GG: COR, 0.531, 95% CI, 0.282-0.998, AOR, 0.517, 95% CI, 0.267-1.000; CC+CG vs. GG: COR, 0.495, 95% CI, 0.272-0.900; CC+CG vs. GG: AOR, 0.479, 95% CI, 0.258-0.891). The *miR-149* rs2292832 C>T polymorphism was significantly different between CAD patients with a stent and control subjects (TT vs. TC: COR, 1.392, 95% CI, 1.042-1.861, AOR, 1.381, 95% CI, 1.023-1.864; TT vs. TC+CC: COR, 1.338, 95% CI, 1.015-1.765). However, the *miR-196a2* rs11614913 T>C was significantly different between patients with CAD without a stent and control subjects (TT vs. TC: COR, 0.678, 95% CI, 0.471-0.975, TT vs. CC: COR, 0.510, 95% CI, 0.308-0.844, AOR, 0.523, 95% CI, 0.313-0.875; TT vs. TC+CC: COR, 0.630, 95% CI, 0.446-0.890, AOR, 0.647, 95% CI, 0.455-0.919; TT+TC vs. CC: AOR, 0.621, 95% CI, 0.388-0.993). The *miR-499* rs3746444 A>G polymorphisms were not identified to be significantly different between patients with CAD with or without a stent and control subjects (Table III).

Analysis of the number of stents after PCI according to disease severity. To examine whether the effect of each polymorphism is related to the number of implanted stents for a given patient, the patients with stents were group divided into two subgroups (=1 and ≥2) according to disease severity. In subgroup analyses, the *miR-146a* rs2910164 C>G polymorphism was significantly associated with increased disease severity in the stent ≥2 group (CC vs. GG: COR, 1.875, 95% CI, 1.013-3.468; CC+CG vs. GG: COR, 1.796; 95% CI, 1.043-3.094). The *miR-149* rs2292832 C>T showed significant association with stent=1 group (TT vs. CC: COR, 1.399, 95% CI, 1.011-1.937; TT vs. TC+CC: COR, 1.366, 95% CI, 1.001-1.863). However, *miR-196a2* rs11614913 T>C, and *miR-499* rs3746444 A>G polymorphisms were not observed to be significantly different between the 2 subgroups (=1 and ≥2) and control subjects (Table IV).

Subgroup analyses. To determine the additional clinical significance, stratified analyses according to age, gender, hypertension, diabetes mellitus, hyperlipidemia and smoking status. In the stratified analyses, it was demonstrated that the *miR-146a* rs2910164 C>G polymorphism showed significant increases in the incidence of CAD compared with the CC genotype in the hypertension (CC+CG vs. GG: AOR, 1.933, 95% CI, 1.133-3.298), nondiabetic (CC+CG vs. GG: AOR,

Table II. Genotype frequencies of miRNA polymorphisms between patients with CAD and control subjects.

Genotype	Controls (n=535)	CAD (n=522)	COR (95% CI)	P-value	AOR (95% CI)	P-value
miR-146a rs2910164 C>G						
CC	202 (37.8)	203 (38.9)	1.000 (reference)		1.000 (reference)	
CG	260 (48.6)	242 (46.4)	0.926 (0.713-1.204)	0.566	0.930 (0.711-1.216)	0.594
GG	73 (13.6)	77 (14.8)	1.050 (0.722-1.527)	0.800	0.995 (0.676-1.464)	0.978
Dominant (CC vs. CG+GG)			0.953 (0.744-1.222)	0.705	0.941 (0.730-1.214)	0.641
Recessive (CC+CG vs. GG)			1.095 (0.775-1.547)	0.607	1.058 (0.743-1.508)	0.755
HWE-P	0.460	0.724				
miR-149 rs2292832 C>T						
TT	263 (49.2)	227 (43.5)	1.000 (reference)		1.000 (reference)	
TC	219 (40.9)	248 (47.5)	1.312 (1.018-1.692)	**0.036**	1.336 (1.031-1.731)	**0.029**
CC	53 (9.9)	47 (9.0)	1.027 (0.668-1.581)	0.902	1.027 (0.659-1.601)	0.905
Dominant (TT vs. TC+CC)			1.257 (0.986-1.601)	0.065	1.276 (0.996-1.634)	0.054
Recessive (TT+TC vs. CC)			0.900 (0.596-1.356)	0.616	0.890 (0.585-1.356)	0.589
HWE-P	0.456	0.073				
miR-196a2 rs11614913 T>C						
TT	153 (28.6)	179 (34.3)	1.000 (reference)		1.000 (reference)	
TC	274 (51.2)	236 (45.2)	0.736 (0.558-0.971)	**0.030**	0.786 (0.591-1.044)	0.096
CC	108 (20.2)	107 (20.5)	0.847 (0.601-1.194)	0.343	0.858 (0.606-1.217)	0.391
Dominant (TT vs. TC+CC)			0.768 (0.592-0.996)	**0.046**	0.801 (0.614-1.046)	0.103
Recessive (TT+TC vs. CC)			1.019 (0.756-1.375)	0.900	0.965 (0.710-1.311)	0.820
HWE-P	0.465	0.078				
miR-499 rs3746444 A>G						
AA	354 (66.2)	358 (68.6)	1.000 (reference)		1.000 (reference)	
AG	168 (31.4)	155 (29.7)	0.912 (0.701-1.187)	0.494	0.910 (0.695-1.192)	0.495
GG	13 (2.4)	9 (1.7)	0.685 (0.289-1.622)	0.389	0.733 (0.297-1.813)	0.502
Dominant (AA vs. AG+GG)			0.896 (0.693-1.159)	0.403	0.895 (0.687-1.165)	0.410
Recessive (AA+AG vs. GG)			0.705 (0.299-1.662)	0.424	0.747 (0.304-1.837)	0.526
HWE-P	0.182	0.091				

Adjusted for age, gender, hypertension, diabetes mellitus and hyperlipidemia. Bold indicates significant values. CAD, coronary artery disease; COR, crude odds ratio; CI, confidence interval, AOR, adjusted odds ratio.

Table III. Genotype frequencies of miRNA polymorphisms between CAD patients and control subjects.

Genotype	Controls (n=535)	Stent (n=329)	COR (95% CI)	P-value	AOR (95% CI)	P-value	Non-stent (n=193)	COR (95% CI)	P-value	AOR (95% CI)	P-value
miR-146a rs2910164C>G											
CC	202 (37.8)	130 (39.5)	1.000 (reference)		1.000 (reference)		73 (37.8)	1.000 (reference)		1.000 (reference)	
CG	260 (48.6)	136 (41.3)	0.813 (0.601-1.100)	0.179	0.803 (0.585-1.102)	0.174	106 (54.9)	1.128 (0.795-1.601)	0.500	1.158 (0.811-1.653)	0.420
GG	73 (13.6)	63 (19.2)	1.341 (0.897-2.006)	0.153	1.293 (0.852-1.963)	0.228	14 (7.3)	0.531 (0.282-0.998)	**0.049**	0.517 (0.267-1.000)	**0.050**
Dominant (CC vs. CG+GG)			0.929 (0.701-1.231)	0.606	0.909 (0.678-1.218)	0.521		0.997 (0.710-1.400)	0.987	1.015 (0.719-1.435)	0.931
Recessive (CC+CG vs. GG)			1.499 (1.036-2.169)	**0.032**	1.473 (1.005-2.159)	**0.047**		0.495 (0.272-0.900)	**0.021**	0.479 (0.258-0.891)	**0.020**
HWE-P	0.460	0.013					0.003				
miR-149 rs2292832C>T											
TT	263 (49.2)	138 (42.0)	1.000 (reference)		1.000 (reference)		89 (46.1)	1.000 (reference)		1.000 (reference)	
TC	219 (40.9)	160 (48.6)	1.392 (1.042-1.861)	**0.025**	1.381 (1.023-1.864)	**0.035**	88 (45.6)	1.187 (0.841-1.677)	0.329	1.257 (0.885-1.785)	0.202
CC	53 (9.9)	31 (9.4)	1.115 (0.684-1.817)	0.663	1.084 (0.648-1.813)	0.758	16 (8.3)	0.892 (0.485-1.640)	0.713	0.944 (0.510-1.746)	0.854
Dominant (TT vs. TC+CC)			1.338 (1.015-1.765)	**0.039**	1.327 (0.996-1.769)	0.053		1.130 (0.812-1.572)	0.468	1.204 (0.860-1.685)	0.279
Recessive (TT+TC vs. CC)			0.946 (0.594-1.508)	0.816	0.923 (0.570-1.496)	0.746		0.822 (0.458-1.476)	0.512	0.873 (0.483-1.577)	0.652
HWE-P	0.456	0.112					0.373				

Table III. Continued.

Genotype	Controls (n=535)	Stent (n=329)	COR (95% CI)	P-value	AOR (95% CI)	P-value	Non-stent (n=193)	COR (95% CI)	P-value	AOR (95% CI)	P-value
miR-196a2 rs11614913T>C											
TT	153 (28.6)	104 (31.6)	1.000 (reference)		1.000 (reference)		75 (38.9)	1.000 (reference)		1.000 (reference)	
TC	274 (51.2)	145 (44.1)	0.779 (0.565-1.072)	0.126	0.874 (0.625-1.223)	0.433	91 (47.1)	0.678 (0.471-0.975)	**0.036**	0.706 (0.487-1.024)	0.067
CC	108 (20.2)	80 (24.3)	1.090 (0.744-1.596)	0.659	1.146 (0.773-1.699)	0.498	27 (14.0)	0.510 (0.308-0.844)	**0.009**	0.523 (0.313-0.875)	**0.014**
Dominant (TT vs. TC+CC)			0.867 (0.643-1.168)	0.347	0.948 (0.694-1.293)	0.734		0.630 (0.446-0.890)	**0.009**	0.647 (0.455-0.919)	**0.015**
Recessive (TT+TC vs. CC)			1.270 (0.915-1.765)	0.154	1.213 (0.862-1.706)	0.268		0.643 (0.407-1.017)	0.059	0.621 (0.388-0.993)	**0.047**
HWE-P	0.465	0.039					0.943				
miR-499 rs3746444A>G											
AA	354 (66.2)	228 (69.3)	1.000 (reference)		1.000 (reference)		130 (67.4)	1.000 (reference)		1.000 (reference)	
AG	168 (31.4)	95 (28.9)	0.878 (0.649-1.187)	0.398	0.871 (0.637-1.192)	0.389	60 (31.1)	0.973 (0.681-1.390)	0.878	0.946 (0.657-1.362)	0.764
GG	13 (2.4)	6 (1.8)	0.717 (0.269-1.912)	0.506	0.941 (0.346-2.559)	0.905	3 (1.5)	0.628 (0.176-2.241)	0.474	0.444 (0.097-2.029)	0.295
Dominant (AA vs. AG+GG)			0.866 (0.645-1.164)	0.340	0.874 (0.644-1.187)	0.389		0.948 (0.668-1.345)	0.764	0.907 (0.633-1.299)	0.594
Recessive (AA+AG vs. GG)			0.746 (0.281-1.982)	0.557	0.998 (0.369-2.699)	0.997		0.634 (0.179-2.249)	0.481	0.439 (0.097-1.988)	0.285
HWE-P	0.182	0.274					0.180				

Adjusted for age, gender, hypertension, diabetes mellitus, and hyperlipidemia. Bold indicates significant values. CAD, coronary artery disease; miR, microRNA; COR, crude odds ratio; AOR, adjusted odds ratio; CI, confidence interval; HWE-P, Hardy-Weinberg equilibrium P-value.

Table IV. Genotype frequencies of miRNA polymorphisms between CAD patients and control subjects.

Genotype	Controls (n=535)	Stent=1 (n=234)	COR (95% CI)	P-value	AOR (95% CI)	P-value	Stent ≥2 (n=95)	COR (95% CI)	P-value	AOR (95% CI)	P-value
miR-146a rs2910164C>G											
CC	202 (37.8)	93 (39.7)	1.000 (reference)		1.000 (reference)		31 (32.6)	1.000 (reference)		1.000 (reference)	
CG	260 (48.6)	99 (42.3)	0.730 (0.521-1.023)	0.067	0.722 (0.508-1.025)	0.068	43 (45.3)	1.078 (0.656-1.772)	0.768	1.049 (0.624-1.765)	0.857
GG	73 (13.6)	42 (17.9)	1.174 (0.749-1.840)	0.484	1.088 (0.682-1.734)	0.724	21 (22.1)	**1.875 (1.013-3.468)**	**0.045**	1.825 (0.966-3.448)	0.064
Dominant (CC vs. CG+GG)			0.827 (0.605-1.131)	0.235	0.807 (0.584-1.116)	0.195		1.252 (0.788-1.990)	0.341	1.217 (0.755-1.962)	0.421
Recessive (CC+CG vs. GG)			1.384 (0.914-2.097)	0.125	1.341 (0.872-2.060)	0.181		**1.796 (1.043-3.094)**	**0.035**	**1.748 (0.996-3.070)**	**0.052**
miR-149 rs2292832C>T											
TT	263 (49.2)	97 (41.5)	1.000 (reference)		1.000 (reference)		41 (43.2)	1.000 (reference)		1.000 (reference)	
TC	219 (40.9)	114 (48.7)	**1.399 (1.011-1.937)**	**0.043**	1.375 (0.985-1.920)	0.062	47 (49.5)	1.377 (0.873-2.171)	0.169	1.318 (0.822-2.114)	0.251
CC	53 (9.9)	23 (9.8)	1.228 (0.719-2.097)	0.453	1.170 (0.669-2.048)	0.582	7 (7.4)	0.847 (0.361-1.990)	0.704	0.832 (0.341-2.032)	0.687
Dominant (TT vs. TC+CC)			**1.366 (1.001-1.863)**	**0.049**	1.339 (0.973-1.844)	0.074		1.274 (0.820-1.977)	0.282	1.242 (0.788-1.957)	0.350
Recessive (TT+TC vs. CC)			1.039 (0.625-1.729)	0.882	1.003 (0.593-1.696)	0.991		0.723 (0.319-1.643)	0.439	0.739 (0.319-1.713)	0.481

Table IV. Continued.

Genotype	Controls (n=535)	Stent=1 (n=234)	COR (95% CI)	P-value	AOR (95% CI)	P-value	Stent ≥2 (n=95)	COR (95% CI)	P-value	AOR (95% CI)	P-value
miR-196a2 rs11614913T>C											
TT	153 (28.6)	74 (31.6)	1.000 (reference)		1.000 (reference)		30 (31.6)	1.000 (reference)		1.000 (reference)	
TC	274 (51.2)	105 (44.9)	0.792 (0.554-1.133)	0.202	0.897 (0.618-1.302)	0.568	40 (42.1)	0.745 (0.446-1.244)	0.260	0.846 (0.493-1.451)	0.543
CC	108 (20.2)	55 (23.5)	1.053 (0.687-1.614)	0.813	1.120 (0.720-1.741)	0.615	25 (26.3)	1.181 (0.658-2.119)	0.578	1.212 (0.663-2.218)	0.533
Dominant (TT vs. TC+CC)			0.866 (0.621-1.209)	0.398	0.957 (0.677-1.351)	0.801		0.868 (0.542-1.391)	0.556	0.955 (0.583-1.565)	0.855
Recessive (TT+TC vs. CC)			1.215 (0.840-1.756)	0.301	1.166 (0.797-1.706)	0.429		1.412 (0.854-2.335)	0.179	1.292 (0.768-2.173)	0.335
miR-499 rs3746444A>G											
AA	354 (66.2)	164 (70.1)	1.000 (reference)		1.000 (reference)		64 (67.4)	1.000 (reference)		1.000 (reference)	
AG	168 (31.4)	65 (27.8)	0.835 (0.594-1.175)	0.300	0.845 (0.594-1.203)	0.350	30 (31.6)	0.988 (0.617-1.582)	0.959	0.991 (0.609-1.611)	0.970
GG	13 (2.4)	5 (2.1)	0.830 (0.291-2.368)	0.728	1.097 (0.378-3.188)	0.865	1 (1.1)	0.426 (0.055-3.310)	0.414	0.568 (0.072-4.514)	0.593
Dominant (AA vs. AG+GG)			0.835 (0.599-1.164)	0.287	0.856 (0.607-1.206)	0.373		0.947 (0.595-1.508)	0.820	0.968 (0.599-1.564)	0.895
Recessive (AA+AG vs. GG)			0.877 (0.309-2.488)	0.805	1.137 (0.394-3.283)	0.812		0.427 (0.055-3.304)	0.415	0.597 (0.076-4.704)	0.624

Adjusted for age, gender, hypertension, diabetes mellitus, and hyperlipidemia. Bold indicates significant values. CAD, coronary artery disease; miR, microRNA; COR, crude odds ratio; AOR, adjusted odds ratio; CI, confidence interval.

1.619, 95% CI, 1.047-2.504), and non-smoking subgroups (CC vs. GG: AOR=1.860, 95% CI, 1.099-3.147). Furthermore, the *miR-149* rs2292832 C>T polymorphism showed significant increases in the incidence of CAD compared with the TT genotype in the older age (age ≥63) (TT vs. TC: AOR, 1.516, 97% CI, 1.041-2.209), female (TT vs. TC: AOR, 1.947, 95% CI, 1.208-3.138; TT vs. TC+CC: AOR, 1.976, 95% CI, 1.244-3.138) and nonsmoking subgroups (TT vs. TC: AOR, 1.604, 95% CI, 1.102-2.333; TT vs. TC+CC: AOR, 1.549, 95% CI, 1.078-2.225; Table V). For CAD, the *miR-196a2* rs11614913T>C genotype was associated with a significantly increased risk of CAD in the older subgroup (age ≥63) (TT vs. TC: AOR, 0.656, 95% CI, 0.435-0.988), and female subgroup (TT+TC vs. CC: AOR, 1.860, 95% CI, 1.097-3.154; Table VI).

Gene-gene interaction analyses using the MDR method. The possible allele combinations of *miR-146a*, *miR-149*, *miR-196a2*, and *miR-499* were constructed to analyze gene-gene interactions (Table VII). As a result, *miR-146a/149*, *miR-146a/499*, *miR-146a/149/196a2*, *miR-146a/149/499*, *miR-146a/196a2/499*, *miR-149/196a2/499*, and *miR-146a/149/196a2/499* models were selected by the MDR method. Several allele combination frequencies were significantly different between patients with CAD and controls. When patients with CAD were compared with controls, *miR-146a/149* (C-T vs. G-C:OR, 1.459, 95% CI, 1.075-1.981), *miR-146a/499* (C-A vs. C-G:OR, 0.707, 95% CI, 0.507-0.985), *miR-146a/149/196a2* (C-T-T vs. G-C-T:OR, 1.713, 95% CI, 1.136-2.584), *miR-146a/149/499* (C-T-A vs. C-T-G:OR, 0.559, 95% CI, 0.370-0.844), *miR-146a/196a2/499* (C-T-A vs. C-T-G:OR, 0.595, 95% CI, 0.367-0.966), *miR-149/196a2/499* (T-T-A vs. T-T-G: OR, 0.603, 95% CI, 0.387-0.940), and *miR-146a/149/196a2/499* (C-T-T-A vs. G-T-C-A: OR, 0.684, 95% CI, 0.475-0.983; G-T-C-G: OR, 43.55, 95% CI, 2.575-736.512; G-C-T-G: OR, 3.437, 95% CI, 1.378-8.572; G-C-C-G: OR, 0.048, 95% CI, 0.003-0.817) were significantly associated with disease prevalence (Table VII).

Allele combinations of miR-146a, -149, -196a2 and -499 polymorphisms with synergistic effects. To investigate the genes without environmental interaction, the combined effects between miRNA polymorphisms and the prevalence of CAD was analyzed. The AOR from the logistic regression analyses with respect to the age, gender, hypertension, diabetes mellitus and hyperlipidemia was calculated. There were significant combined gene (*miR-146a/149*) effects when the environmental influence was excluded in CAD risk. (CG/TT: AOR, 0.677, 95% CI, 0.473-0.970; GG/TC: AOR, 1.683, 95% CI, 1.013-2.797 ; GG/CC: AOR, 4.200, 95% CI, 1.082-16.306 ; GG/TC+CC: AOR, 1.939, 95% CI, 1.206-3.118), *miR-149/196a2* (CC/TC: AOR, 0.436, 95% CI, 0.198-0.961; Table VIII).

Discussion

Circulating miRNAs have many of the essential characteristics to be a good biomarker of noninvasive measurability. For example, a high degree of sensitivity and specificity, which allows the early detection of pathological states, including the time-related changes during the course of disease, a long half-life within the sample, and rapid and cost-effective laboratory detection (22). miRNAs are important in a number of physiological and pathological processes, including tumorigenesis, proliferation, metabolism, immune function and epigenetics (27-30). Emerging evidence has indicated that circulating miRNAs may be biomarkers for cardiovascular diseases, including essential hypertension, heart failure, Diabetes mellitus, stroke, coronary artery disease, acute myocardial infarction and acute pulmonary embolism (15,31-34). To the best of our knowledge, this is the first study to provide evidence that four miRNA polymorphisms are involved in the predisposition to CAD with or without prior PCI. In this study, the *miR-146aC>G* (rs2910164), *miR-149T>C* (rs2292832), *miR-196a2T>C* (rs11614913), and *miR-499A>G* (rs3746444) polymorphisms were investigated in CAD patients with or without PCI. Furthermore, the results also demonstrated that *miR-149T>C* (rs2292832) and *miR-196a2T>C* (rs11614913) polymorphisms were significantly associated with the development of CAD in females and patients over the age of 63 years old. The results also suggest that the *miR-146a*C>G (rs2910164) polymorphism was significantly associated with hypertension, whereas there was no correlation of the smoking and diabetes mellitus groups with an increased risk of developing CAD. Moreover, the severity of CAD was positively correlated with the number of stents implanted: Patients with CAD that had undergone ≥2 stent treatments showed an increased CAD incidence compared with patients having one stent treatment for the miR-146a rs2910164 C>G polymorphism (Table IV). The precise mechanisms of miRNA-mediated gene expression and maturation are largely unknown; however, studies have suggested several mechanisms, including genetic and epigenetic mechanisms (DNA methylation, histone modification and non-coding RNAs) (28,35). In addition, small variations in the quantity of miRNAs may have an effect on thousands of target mRNAs and result in diverse functional consequences. The most common genetic variations, such as SNPs, in miRNA sequences may also be functional and therefore may represent ideal candidate biomarkers for cancer and cardiovascular diseases, including CAD.

miR-146a, *-149*, *-196a2* and *-499* can regulate TNF-α, methylenetetrahydrofolate reductase, ANXA1 and CRP, respectively (10,36,37). According to previously published studies, TNF-α, methylenetetrahydrofolate reductase, ANXA1, and CRP were well-known risk factors for cerebral ischemia (38-40). TNF-α is associated with increased plasminogen activator inhibitor-1 protein levels (30); methylenetetrahydrofolate reductase dysfunction is associated with plasma total HCY accumulation (29); ANXA1 is connected to decreased TNF-α levels (41); and CRP can elevate blood pressure, body mass index, insulin resistance and TG levels (42). In addition, CRP and TNF-α are simultaneously activated in stress conditions (43). These data suggest that TNF-α, ANXA1 and CRP may be closely linked. In fact, MDR analyses in the present study indicated genetic interactions between *miR-146a/-196a2/-499* and *miR-146a/-196a2*. There have been limited studies regarding the functions of miRNA polymorphisms. The *miR-146a*G and *miR-196a2*T allele were shown to be associated with decreased mature miRNA levels (44,45). *miR-149T*>C and *miR-499A*>G were located in a pre-miRNA structure, not the mature miRNA form. However, these polymorphisms were affected by miRNA biogenesis. In addition, there was no mature miRNA expres-

Table V. Stratified effects of miRNA polymorphisms on CAD risk.

	miR-146a rs2910164C>G				*miR-149* rs2292832C>T			
	GG		Recessive (CC+CG vs. GG)		TC		Dominant (TT vs. TC+CC)	
Variable	AOR (95% CI)	P-value	AOR (95% CI)	P-value	AOR (95% CI)	P-value	AOR (95% CI)	P-value
Age (years)								
<63	0.712 (0.411-1.231)	0.224	0.805 (0.489-1.325)	0.393	1.160 (0.805-1.671)	0.426	1.111 (0.784-1.573)	0.555
≥63	1.421 (0.805-2.510)	0.226	1.405 (0.834-2.365)	0.201	**1.516 (1.041-2.209)**	**0.030**	1.415 (0.987-2.029)	0.059
Gender								
Male	1.154 (0.667-1.999)	0.609	1.383 (0.845-2.262)	0.197	1.021 (0.682-1.528)	0.920	0.942 (0.640-1.386)	0.760
Female	1.477 (0.752-2.901)	0.257	1.576 (0.838-2.963)	0.158	**1.947 (1.208-3.138)**	**0.006**	**1.976 (1.244-3.138)**	**0.004**
Hypertension								
Negative	0.945 (0.510-1.749)	0.856	1.086 (0.614-1.919)	0.777	1.408 (0.906-2.189)	0.129	1.225 (0.802-1.871)	0.347
Positive	1.701 (0.942-3.070)	0.078	**1.933 (1.133-3.298)**	**0.016**	1.437 (0.946-2.183)	0.089	1.480 (0.994-2.205)	0.054
Diabetes mellitus								
Negative	1.389 (0.863-2.237)	0.176	**1.619 (1.047-2.504)**	**0.030**	1.395 (0.987-1.971)	0.059	1.305 (0.936-1.819)	0.117
Positive	0.978 (0.403-2.372)	0.961	1.033 (0.458-2.331)	0.937	1.134 (0.598-2.152)	0.699	1.218 (0.663-2.237)	0.526
Hyperlipidemia								
Negative	1.245 (0.762-2.033)	0.382	1.496 (0.952-2.350)	0.081	1.415 (0.993-2.015)	0.054	1.339 (0.954-1.877)	0.091
Positive	1.390 (0.605-3.194)	0.438	1.398 (0.658-2.970)	0.384	1.207 (0.661-2.202)	0.540	1.172 (0.662-2.074)	0.586
Smoking status								
Nonsmokers	**1.860 (1.099-3.147)**	**0.021**	**1.924 (1.194-3.101)**	**0.007**	**1.604 (1.102-2.333)**	**0.014**	**1.549 (1.078-2.225)**	**0.018**
Smokers	0.584 (0.276-1.234)	0.159	0.858 (0.440-1.673)	0.652	1.117 (0.654-1.907)	0.686	1.050 (0.634-1.739)	0.849

Adjusted for age, gender, hypertension, diabetes mellitus and hyperlipidemia. Bold indicates significant values. CAD, coronary artery disease; miR, microRNA; AOR, adjusted odds ratio; CI, confidence interval.

Table VI. Stratified effects of miRNA polymorphisms on CAD risk.

	miR-196a2 rs11614913 T>C				*miR-499* rs3746444 A>G			
	TC		Recessive (TT+TC vs. CC)		TC		Recessive (AA+AG vs. GG)	
Variable	AOR (95% CI)	P-value	AOR (95% CI)	P-value	AOR (95% CI)	P-value	AOR (95% CI)	P-value
Age (years)								
<63	0.970 (0.649-1.450)	0.883	0.931 (0.604-1.436)	0.747	1.617 (0.421-6.220)	0.484	1.672 (0.437-6.389)	0.453
≥63	**0.656 (0.435-0.988)**	**0.044**	1.038 (0.666-1.620)	0.868	0.391 (0.102-1.500)	0.171	0.393 (0.103-1.498)	0.171
Gender								
Male	0.872 (0.555-1.372)	0.554	0.844 (0.529-1.348)	0.478	1.657 (0.478-5.741)	0.426	1.844 (0.535-6.361)	0.333
Female	0.967 (0.567-1.651)	0.903	**1.860 (1.097-3.154)**	**0.021**	0.547 (0.063-4.713)	0.583	0.595 (0.070-5.097)	0.636
Hypertension								
Negative	0.908 (0.551-1.496)	0.705	1.492 (0.894-2.489)	0.126	0.895 (0.259-3.092)	0.861	0.937 (0.275-3.201)	0.918
Positive	0.862 (0.544-1.366)	0.526	1.047 (0.659-1.664)	0.845	1.171 (0.187-7.347)	0.866	1.230 (0.199-7.589)	0.824
Diabetes mellitus								
Negative	0.847 (0.571-1.257)	0.409	1.116 (0.749-1.664)	0.590	1.098 (0.396-3.049)	0.857	1.167 (0.421-3.234)	0.766
Positive	1.067 (0.535-2.125)	0.855	1.482 (0.711-3.091)	0.294	N/A	0.994	N/A	0.994
Hyperlipidemia								
Negative	0.910 (0.610-1.358)	0.645	1.357 (0.913-2.017)	0.131	0.740 (0.224-2.444)	0.622	0.839 (0.256-2.752)	0.772
Positive	0.787 (0.410-1.509)	0.471	0.871 (0.430-1.764)	0.702	1.997 (0.251-15.907)	0.514	2.149 (0.277-16.666)	0.464
Smoking status								
Smokers	0.738 (0.487-1.120)	0.153	1.456 (0.940-2.257)	0.093	1.230 (0.397-3.805)	0.720	1.319 (0.431-4.042)	0.627
Nonsmokers	1.317 (0.722-2.402)	0.369	0.884 (0.498-1.568)	0.673	0.512 (0.048-5.450)	0.579	0.522 (0.049-5.572)	0.591

Adjusted for age, gender, hypertension, diabetes mellitus and hyperlipidemia. Bold indicates significant values. CAD, coronary artery disease; miR, microRNA; AOR, adjusted odds ratio; CI, confidence interval.

Table VII. Frequencies of *miR-146a*, *miR-149*, *miR-196a2* and *miR-499* haplotypes in patients with CAD and in controls.

Haplotype	Overall	Control	CAD	OR (95% CI)	P-value
miR-146a/149					**0.018**
C-T	0.4227	0.4240	0.4195	1.000 (reference)	
G-C	0.1258	0.1072	0.1551	**1.459 (1.075-1.981)**	
miR-146a/499					**0.042**
C-A	0.4997	0.4934	0.5087	1.000 (reference)	
C-G	0.1137	0.1272	0.0931	**0.707 (0.507-0.985)**	
miR-146a/149/196a2					**0.011**
C-T-T	0.2461	0.2480	0.2427	1.000 (reference)	
G-C-T	0.0699	0.0541	0.0910	**1.713 (1.136-2.584)**	
miR-146a/149/499					**0.006**
C-T-A	0.3441	0.3323	0.3616	1.000 (reference)	
C-T-G	0.0789	0.0926	0.0561	**0.559 (0.370-0.844)**	
miR-146a/196a2/499					**0.038**
C-T-A	0.2830	0.2848	0.2815	1.000 (reference)	
C-T-G	0.0559	0.0673	0.0392	**0.595 (0.367-0.966)**	
miR-149/196a2/499					**0.026**
T-T-A	0.3088	0.3052	0.3170	1.000 (reference)	
T-T-G	0.0685	0.0778	0.0490	**0.603 (0.387-0.940)**	
miR-146a/149/196a2/499					
C-T-T-A	0.2007	0.1956	0.2107	1.000 (reference)	
G-T-C-A	0.1141	0.1341	0.0992	**0.684 (0.475-0.983)**	**0.045**
G-T-C-G	0.0158	0.0000	0.0212	**43.55 (2.575-736.512)**	**<0.0001**
G-C-T-G	0.0121	0.0068	0.0250	**3.437 (1.378-8.572)**	**0.008**
G-C-C-G	0.0069	0.0142	0.0000	**0.048 (0.003-0.817)**	**0.001**

Insignificant data were removed from table. Bold indicates significant values. miR, microRNA; CAD, coronary artery disease;OR, odds ratio; CI, confidence interval.

Table VIII. Genotype combination of microRNA polymorphisms.

Combined genotype	Controls (n=535)	CAD (n=329)	AOR (95% CI)	P-value
miR-146a/149				
CG/TT	129 (24.1)	57 (17.3)	**0.677 (0.473-0.970)**	**0.033**
GG/TC	35 (6.5)	35 (10.6)	**1.683 (1.013-2.797)**	**0.044**
GG/CC	3 (0.6)	8 (2.4)	**4.200 (1.082-16.31)**	**0.038**
GG/TC+CC	3 (0.6)	43 (13.1)	**1.939 (1.206-3.118)**	**0.006**
miR-149/196a2				
CC/TC	30 (5.6)	9 (2.7)	**0.436 (0.198-0.961)**	**0.039**

Insignificant data were removed from table. Adjusted for age, gender, hypertension, diabetes mellitus and hyperlipidemia. Bold indicates significant values. CAD, coronary artery disease; miR, microRNA; AOR, adjusted odds ratio; CI, confidence interval.

sion, according to *miR-149*T>C and *miR-499*A>G. However, *miR-499*AA is associated with decreased plasma CRP concentrations (46). Based on these results, it is hypothesized that *miR-146a*C, *miR-196a2*T and *miR-499*A alleles have protective roles in vascular pathogenesis through inhibition of TNF-α and CRP levels. However, it was not possible to measure miRNA, TNF-α and CRP levels in the present study.

Recent advances in genetic research have systematically identified and analyzed human polymorphisms in miRNAs and/or miRNA target sites (2,23). However, the majority of these studies focused on SNPs in the target sites and their effects on disease-related miRNAs, while only a few studies have reported the understanding the synergistic regulation of miRNAs and their potential targeted SNPs cooperative

effects contributing to disease progression. With the rapid identification of disease-related miRNAs, there is a requirement to determine their functional relationships contributing to diseases at a systems biology level.

Data from the present study indicate that specific combinations of miRNA haplotypes are correlated with the incidence of CAD. For example, the G-T-C-G polymorphism of *miR-146a/149/196a2/499* correlates the most strongly with CAD (OR, 43.55, 95% CI, 2.575-736.5, P<0.0001), followed by the G-C-C-G (OR, 0.048, 95% CI, 0.003-0.817, P=0.001) haplotype. The C-T-G polymorphism in *miR-146a/149/499* exhibited the strongest correlation with CAD incidence among all three miRNA haplotypes (OR, 0.559, 95% CI, 0.370-0.844, P=0.006). Thus, the specific combination of miRNA polymorphisms appear to provide synergistic effects.

There are several limitations of the present study. It is not yet clear which genetic polymorphisms predict the phenotypes associated with CAD and disease severity. The present study population comprised of only Korean individuals, and these results require validation in other ethnic groups. This was a hospital-based case-control study that had a relatively small sample size. However, the recruitment of >1,000 individuals from an ethnically homogeneous population (Koreans have a low degree of interracial marriage) is enough to give reliable data.

In conclusion, the *miR-146a*C>G and *miR-149*T>C polymorphisms were associated with an increased risk of CAD in the Korean population. The present study marks the first report of an association between stroke and SBI and miRNA polymorphisms *(miR-146a*C>G, *-149*T>C, *-196a2*T>C and *-499*A>G) in the Korean population. Therefore, additional studies of other racial and ethnic populations regarding the biological functions of miRNA are required to fully understand the role of miRNA polymorphisms in CAD risk.

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