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A Case-Control Study of the Relationship Between *SLC22A3-LPAL2-LPA* Gene Cluster Polymorphism and Coronary Artery Disease in the Han Chinese Population

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Abstract

Background: Mutations in the solute carrier family 22 member 3 (SLC22A3), lipoprotein (a)-like 2 (LPAL2), and the lipoprotein (a) (LPA) gene cluster, which encodes apolipoprotein (a) [apo (a)] of the lipoprotein (a) [Lp (a)] lipoprotein particle, have been suggested to contribute to the risk of coronary artery disease (CAD), but the precise variants of this gene cluster have not yet been identified in Chinese populations.

Objectives: We sought to investigate the association between *SLC22A3-LPAL2-LPA* gene cluster polymorphisms and the risk of CAD in the Han Chinese population.

Patients and Methods: We recruited 551 CAD patients and 544 healthy controls for this case-control study. Four SNPs (rs9346816, rs2221750, rs3127596, and rs9364559) were genotyped in real time using the MassARRAY system (Sequenom; USA) in the *SLC22A3-LPAL2-LPA* gene cluster. All subjects were Chinese and of Han descent, and were recruited from the First Hospital of Jilin University based on convenience sampling from June 2009 to September 2012.

Results: The frequency of the minor allele G (34.8%) in rs9364559 was significantly higher in the CAD patients than in the healthy controls (29.4%) (P = 0.006). There was genotypic association between rs9364559 and CAD (P = 0.022), and these results still remained significant after adjustment for the conventional CAD risk factors through forward logistic regression analysis (P = 0.020, P = 0.019). Haplotype analyses from different blocks indicated that 11 haplotypes were associated with the risk of CAD. Seven haplotypes were associated with a reduced risk of CAD, whereas four haplotypes were associated with an increased risk of CAD.

Conclusions: Rs9364559 in the *LPA* gene may contribute to the risk of CAD in the Han Chinese population; haplotypes which contain rs9346816-G were all associated with an increased risk of CAD in this study.

Keywords: Coronary Artery Disease, Lipoprotein (a), Polymorphism, Genetic Association Studies

1. Background

Coronary artery disease (CAD) is a complex condition, and both genetic and environmental factors may contribute to the pathogenesis of the disease (1). Accumulating evidence from genome-wide association (GWA) studies has identified many novel susceptibility loci for CAD (2-6). However, the effects of these loci on the risk of developing CAD in certain populations is still unknown, and the use of case-control studies of single-nucleotide polymorphisms (SNPs) may help to clarify the genetic contribution to CAD development.

Lp (a) is an LDL-like particle that consists of an apo (a) moiety and one molecule of apolipoprotein B100, which is linked to apo (a) via a disulfide bond (7). In the general population, elevated Lp (a) levels have been well recognized as an independent risk factor for CAD (8-11). Plasma Lp (a) concentration is primarily affected by apo (a) gene polymorphism, which accounts for 91% of the variation (12). A recent GWA study suggested that apo (a) genetic variations may be associated with variable Lp (a) levels in the serum (13). In 2009, another GWA study showed that a cluster of genes - *SLC22A3-LPAL2-LPA* - on chromosome 6q26-27 was strongly associated with the risk of CAD, although the investigators were unable to identify the precise variants at this locus (14).

The *LPA* gene encodes the apo (a) of the Lp (a) lipoprotein particle, and is considered to be associated with Lp (a) levels (10, 15). *LPA* includes a well-characterized 5.6 kilobase (kb), which is a pair copy-number variant that encodes a kringle (IV) domain (16, 17). Higher copy numbers for this domain were found to be associated with lower serum Lp (a) levels (18), presumably due to impaired secretion of the larger protein product (19). However, previous studies sug-

gested that other genetic variants at the *LPA* locus may also affect serum Lp (a) levels (20).

Recently, many studies have explored the association between the *SLC22A3-LPAL2-LPA* gene cluster and the risk of CAD. Tregouet et al. identified the *SLC22A3-LPAL2-LPA* gene cluster as a strong susceptibility locus for CAD through a genome-wide haplotype association (GWHA) study (14). Koch et al. demonstrated that the gene cluster was a strongly susceptive locus for myocardial infarction (MI) in Europeans (21). However, the research of Qi et al. did not confirm the association between haplotypes in this gene cluster and non-fatal acute MI in Hispanics (22). In 2012, Lv et al. explored the association between four SNPs in the *SLC22A3-LPAL2-LPA* gene cluster and CAD in a large Han Chinese sample (23). However, the results from this study revealed that there were no allelic, genotypic, or haplotype associations between rs2048327, rs3127599, rs7767084, and rs10755578 in the *SLC22A3-LPAL2-LPA* gene cluster and CAD (23). Therefore, studying the relationship between the gene polymorphisms of the *SLC22A3-LPAL2-LPA* gene cluster and CAD is important because it could be a potential genetic marker for CAD, and it could therefore help us to detect CAD earlier among the Chinese population.

2. Objectives

The objective of this study was to investigate a possible association between four SNPs of the *SLC22A3-LPAL2-LPA* gene cluster and CAD in a case-control study of the Han Chinese population.

3. Patients and Methods

3.1. Patients

Five-hundred-fifty-one CAD patients (case group: 295 males and 256 females) and 544 healthy controls (control group: 264 males and 280 females) were included in the present genetic study. All subjects were Chinese and of Han descent. CAD patients were recruited from those hospitalized in the cardiology department of the first hospital of Jilin University, a government hospital, from June 2009 to September 2012. Healthy controls were recruited from those having physical examinations in the first hospital of Jilin University at the same time. CAD patients were all diagnosed via coronary computed tomographic (8) angiography (SIEMNS Somatom Definition AS + 128 row spiral CT) by at least two well-trained physicians. CAD was defined as at consisting of least 50% stenosis in any major coronary artery. Patients with non-atherosclerotic vascular diseases, congenital heart disease, cardiomyopathy, valvular disease, renal or hepatic disease, or cancer were all excluded. The healthy controls were randomly selected through a screening process, and the initial results were validated through electrocardiogram, chest X-ray examination, and serum biochemical analysis. The healthy controls featured an absence of any personal or family history of related illness, or any suspected reasons for having CAD. All of the subjects provided written informed consent for the study, which was approved by the ethics committee of Jilin University, Changchun, China (2008-462).

3.2. Clinical Measurements

The blood pressures of the subjects were measured using the HEM-712C Omron blood pressure monitor, and duplicate readings were obtained after the subjects were in resting condition for at least 10 minutes. Height, weight, and body mass index (BMI) measurements were obtained with the Omron HBF-362. Serum lipids and blood glucose were measured using the Hitachi 7180 automatic biochemical analyzer. Hypercholesterolemia was defined as a serum total cholesterol level of 200 mg/dL or more. Habitual smoking was defined as daily use of > 10 cigarettes (24). All equipment was calibrated according to the manufacturer's protocol at the start of the day before measurements were performed by the same observer throughout the study.

3.3. SNP Selection, DNA Extraction, and Genotyping

Tag SNPs were chosen from the genotyped SNPs in the Han Chinese population (HCB) of the HapMap project (the Phase I database). The candidate's SNPs were restricted to minor allele frequencies larger than 15%. Genomic DNA used for polymerase chain reaction (PCR) amplification was extracted from a whole blood sample using a DNA extraction kit (Takara, China) according to the manufacturer's instructions. Primers for amplification were designed with AssayDesigner 3.1 software. The sequences of primers for amplification of the four loci in the *SLC22A3* and *LPA* genes are listed in Table 1. SNP genotyping was conducted using the MassARRAY system (Sequenom) by means of the matrix-assisted laser desorption ionization time of flight mass spectrometry method (MALDI-TOF) according to the manufacturer's instructions (Bio Miao Biological technology (Beijing) Co., Ltd). Genotype calling was performed in real-time with MassARRAY RT software version 3.0.0.4 and analyzed using the MassARRAY Typer software version 3.4 (Sequenom). As quality controls, 5% - 10% of the samples were genotyped in duplicate, and no inconsistencies were observed. Controls distributed in each 384-well plate were also checked for consistency. Cluster plots were made from the low and the high mass allele signals.

Table 1. Amplification and Extension Primers Sequences of the Four Loci in the *LPA* and *SLC22A3* Genes

SNPs	Forward Primer (5' - 3')	Reverse Primer (5' - 3')	Extension Primer (5' - 3')
rs9346816	ACGTTGGATGCTCACTTAATCCCTACCCAC	ACGTTGGATGCTCTGTACCTTCCTTTCCTC	GAAAATCCTTTCCTCCTCTAAAAAAAAC
rs2221750	ACGTTGGATGTTTCTCCCAGAGCCTGCTTC	ACGTTGGATGCTCTTGCTGTGCAGTATTGG	GGGGAAGTATTGGAAATACTGCTCATA
rs3127596	ACGTTGGATGTATGGGATGCCATCCTTCTC	ACGTTGGATGAAGCACTGCAGATGCTTGAG	AGTAATATGCTCATAAGTTCCC
rs9364559	ACGTTGGATGTTTTGTCCATGTACCTGCCC	ACGTTGGATGAGGAGAGGAAGAGCAAAAGC	ATGAGAATTAGGAAGTAAACAGAC

3.4. Statistical Analysis

Data were presented as percentages for the categorical variables, and means ± standard deviations (SDs) for the continuous variables. Differences between the categorical variables for the groups were tested with a Pearson χ^2 test. The differences between the continuous variables for the cases and controls were analyzed through an independent t-test or Mann-Whitney U test. All statistical analyses mentioned above were performed with SPSS 16.0 (SPSS Inc.; Chicago; USA). The Hardy-Weinberg equilibrium for the genotype-c distributions of the SNPs was tested by the chi-square (χ^2) goodness-of-fit test. The Haploview 4.2 (http://www.broadinstitute.org/haploview/haploview; Daly Lab at the borad institute Cambridge, MA 02141, USA) was applied to estimate the linkage disequilibrium (LD) measures (D' and r^2) between paired SNPs. Allelic, genotypic, and haplotype analyses were performed with SHEsis software (http://analysis.bio-x.cn; Bio-X institutes of Shanghai Jiao Tong University; China). The results are expressed as odds ratios (OR) and 95% confidence intervals (CI). $P < 0.05$ was considered to be of statistical significance, and all statistical tests were two sided. Forward logistic regression analysis was performed with adjustments for age, sex, and the presence of diabetes, hypertension, or smoking to evaluate if the polymorphism could predict the increased risk of CAD.

4. Results

4.1. Characteristics of the Participants

The characteristics of the included subjects in both of the groups are listed in Table 2. There was no significant difference in mean ages, gender, or serum triglyceride levels between the case and control groups. However, compared with the healthy controls, the CAD patients were more likely to be smokers, and more likely to have comorbidities of hypertension as well as diabetes mellitus (DM). Additionally, the CAD group had higher levels of serum total cholesterol.

4.2. Allele and Genotype Analysis

Tag SNPs rs9346816 and rs2221750 of SLC22A3, and rs3127596 and rs9364559 of LPA, were genotyped, all of which were located within the introns of the above genes. The results of the goodness-of-fit test showed that the genotypic distributions of rs9346816, rs2221750, rs3127596, and rs9364559 did not deviate from the Hardy-Weinberg equilibrium in both the case and control groups ($P > 0.05$). The distributions of the alleles and genotypes of the four SNPs among the subjects are presented in Tables 3 and 4, respectively. For rs2221750 and rs9346816, G was the major allele, but for rs3127596 and rs9364559, A was the major allele. Analysis with SHEsis software indicated that rs9364559 was associated with CAD ($\chi^2 = 7.411$, $P = 0.006$) and remained significant even after adjustment for the conventional CAD risk factors through forward logistic regression analysis ($\chi^2 = 5.381$, $P = 0.020$). The frequency of the minor allele G in rs9364559 was significantly higher in CAD patients than among the healthy controls (Table 3). However, rs3127596, rs2221750, and rs9346816 were not associated with CAD. In contrast, as shown in Table 4, the test revealed that the genotypic association between rs9364559 and CAD remained significant ($\chi^2 = 7.616$, $P = 0.022$) even after adjustment for the conventional CAD risk factors through forward logistic regression analysis ($\chi^2 = 5.538$, $P = 0.019$), but there was not a statistically significant association between the other three loci and CAD.

4.3. Linkage Disequilibrium Analysis

The linkage disequilibrium between rs3127596 and rs9364559 was relatively high (D' = 0.919, $r^2 = 0.071$). However, the D' values for the other SNPs ranged from 0.251 to 0.712, which did not support significant linkage disequilibrium among these haplotypes.

4.4. Haplotype Association Analysis

The frequencies of the haplotypes from different blocks were estimated (Table 5). All haplotypes with a frequency larger than 3% were included in the subsequent analysis. The results showed that subjects with AGAA (rs9346816, rs2221750, rs3127596, and rs9364559),

Table 2. Baseline Characteristics of the Patients in the Case and Control Groups[a]

Variable	Case Group (n = 551)	Control Group (n = 544)	P Value
Age, y	62.07 ± 11.08	60.94 ± 13.70	0.155
Sex	53.5	48.5	0.097
Smoking	40.5	31.1	0.001
Drinking	23.0	19.1	0.111
Hypertension	42.1	19.3	0.000
Diabetes mellitus	35.9	22.0	0.000
TC, mmol/L	5.04 ± 1.05	4.61 ± 1.25	0.000
TG mmol/L	1.74 ± 1.21	1.74 ± 1.22	0.973
Lp (a), mg/dL	23.73 ± 15.65	22.62 ± 12.32	0.282
BMI, kg/m^2	24.20 ± 2.70	23.93 ± 3.37	0.253
SBP, mmHg	146.26 ± 32.10	135.41 ± 23.51	0.000
DBP, mmHg	87.48 ± 17.50	85.19 ± 12.37	0.026
HR, b/min	79.11 ± 16.23	73.58 ± 15.11	0.006

[a]Values are expressed as mean ± SD or %.

Table 3. SNPs Loci Allelic Frequency Distribution and the Relationship With Coronary Artery Disease

Gene/SNPs	Controls		Cases		χ^2	P Value
	A	G	A	G		
SLC22A3						
rs9346816	461 (0.437)	595 (0.563)	423 (0.401)	633 (0.599)	2.809	0.094
rs2221750	238 (0.231)	792 (0.769)	223 (0.216)	809 (0.784)	0.667	0.414
LPA						
rs3127596	911 (0.850)	161 (0.150)	897 (0.841)	169 (0.159)	0.286	0.593
rs9364559	768 (0.706)	320 (0.294)	718 (0.652)	384 (0.348)	5.381[a]	0.020[a]

[a]Adjustment for the presence of smoking, hypertension, diabetes mellitus, TC, SBP, DBP, and HR through logistic regression analysis.

Table 4. SNPs Loci Genotype Frequency Distribution and the Relationship With Coronary Artery Disease

Gene/SNPs	Controls			Cases			χ^2	P Value
	AA	GA	GG	AA	GA	GG		
SLC22A3								
rs9346816	107 (0.203)	247 (0.468)	174 (0.330)	91 (0.172)	241 (0.456)	196 (0.371)	2.675	0.263
rs2221750	23 (0.045)	192 (0.373)	300 (0.583)	24 (0.047)	175 (0.339)	317 (0.614)	1.276	0.528
LPA								
rs3127596	388 (0.724)	135 (0.252)	13 (0.024)	379 (0.711)	139 (0.261)	15 (0.028)	0.298	0.861
rs9364559	275 (0.506)	218 (0.401)	51 (0.094)	234 (0.425)	250 (0.454)	67 (0.122)	5.538[a]	0.019[a]

[a]Adjustment for the presence of smoking, hypertension, diabetes mellitus, TC, SBP, DBP, and HR through logistic regression analysis.

AAA (rs9346816, rs312759, and rs9364559), AGA (rs9346816, rs2221750, and rs9364559), AGA (rs9346816, rs2221750, and rs3127596), AG (rs9346816 and rs2221750), AA (rs9346816 and rs3127596), and AA (rs9346816 and rs9364559) were as-

sociated with a significantly reduced risk of CAD (P = 0.011, OR = 0.776; P = 0.025, OR = 0.806; P = 0.003, OR = 0.750; P = 0.031, OR = 0.819; P = 0.018, OR = 0.806; P = 0.030, OR = 0.824; P = 0.015, OR = 0.796), while those with GGAA (rs9346816, rs2221750, rs3127596, and rs9364559), GGA (rs9346816, rs2221750 and rs9364559), GGA (rs9346816, rs2221750, and rs3127596), and GG (rs9346816 and rs2221750) were associated with a significantly increased risk of CAD (P = 0.031, OR = 1.340; P = 0.009, OR = 1.347; P = 0.012, OR = 1.272; P = 0.002, OR = 1.323).

4.5. Association between Genotype and Serum Lp (a) Level

We further analyzed the association between the genotypes of the four SNPs and the serum Lp (a) levels in the selected members of the Han Chinese population (Table 6). There were no significant differences in Lp (a) levels among the different genotypes of the four SNPs (for rs9346816 P = 0.472; for rs2221750 P = 0.275; for rs3127596 P = 0.094; and for rs9364559 P = 0.080).

5. Discussion

In our study, we genotyped four SNPs of the *SLC22A3-LPAL2-LPA* gene cluster and evaluated the association between these SNPs and CAD in selected members of the Han Chinese population. We found that rs9364559 in the *LPA* gene is associated with CAD in this particular population. Moreover, 11 haplotypes formed by different blocks were associated with a risk of CAD in this population. Among them, seven haplotypes were associated with a reduced risk of CAD, and four haplotypes were associated with an increased risk of CAD. Additionally, we further analyzed the association between the genotypes of four SNPs and serum Lp (a) levels. However, there were no significant differences in Lp (a) levels among the different genotypes. The above results suggest that rs9364559 in *LPA* may be involved in the pathogenesis of CAD, and the *SLC22A3-LPAL2-LPA* gene cluster is strongly associated with CAD in the Han Chinese population. However, the precise mechanism of rs9364559 involved in the pathogenesis of CAD is still unknown.

In a previous study, the cluster of genes - SLC22A3, LPAL2, and *LPA* - on chromosome 6q26-27, which encodes the apo (a) component of the Lp (a) lipoprotein particle, have been demonstrated to be strongly associated with CAD. Tregouet et al. identified the *SLC22A3-LPAL2-LPA* gene cluster as a risk cluster, and the haplotypes CTTG and CCTC formed by rs2048327, rs3127599, rs7767084, and rs10755578 as risk factors for CAD, in six Caucasian populations (14). Later, Koch et al. examined the genetic variations located in LPA (the apo (a) gene) with TaqMan assays in a sample of 2136 CAD cases and 1211 controls of European decent, and the results showed that the minor alleles of rs3798220 and rs10455872 were associated with an increased risk of CAD (rs3798220-C: P = 0.00080; rs10455872-G: P < 0.00001). However, in the haplotype analysis, none of the nine polymorphisms seemed to be related with the disease (21).

Apo (a) is the characteristic protein component of Lp (a), a low-density lipoprotein-like plasma lipoprotein particle (25, 26). Elevated levels of plasma Lp (a) have been confirmed to be associated with an increased risk of cardiovascular disease (10, 27-31), and the results of some recent studies have suggested that the role of an SNP cluster at the *LPA* locus may be capable of predicting Lp (a) levels. Size-polymorphism of apo (a) has been shown to be a major predictor of Lp (a) levels, but non-size polymorphism in the *LPA* gene may also affect Lp (a) levels (32, 33). Rs3798220, located in the protease-like domain of apo (a), and rs10455872, which was mapped on to intron 25, have repeatedly been reported to be associated with an increased Lp (a) level. The cluster of genes - SLC22A3, LPAL2, and *LPA* - on chromosome 6q26-27, which encode the apo (a) component of the Lp (a) lipoprotein particle, have been found to be strongly associated with CAD risk. In this study, it was shown that rs9364559 in the *LPA* gene may affect the risk of CAD. In the haplotype analysis, 11 haplotypes in the *SLC22A3-LPAL2-LPA* gene cluster were associated with the risk of CAD in the study population. Compared with these haplotypes, haplotypes containing rs9346816-G were all associated with an increased risk of CAD, while other haplotypes appear to be associated with protection from CAD. Although, there is no difference between the two groups for the distributions of rs9346816 alleles and genotypes, the sites with other loci present at the same time may increase the risk of CAD.

It is important to note that in our study, the Lp (a) levels among the different genotypes of the four SNPs in the *SLC22A3-LPAL2-LPA* gene cluster were not significantly different. Although rs9364559 in the *LPA* gene was associated with CAD, no significant difference in the Lp (a) levels was seen among the subgroups. Therefore, our results indicate that elevated plasma Lp (a) levels are not the only way in which CAD can be affected.

At the same time, we are aware of several limitations in our study. The small number of samples may be the main limiting factor, which adversely affects the statistical power and generalizability to the overall Chinese population. In this study, we did not examine the gene expression levels of *SLC22A3* and *LPA* between the controls and the patients. Therefore, further research conducted with a larger sample size and for different ethnicities is necessary.

In conclusion, our case-control study indicated that rs9364559 in the *LPA* gene may affect the risk of CAD in the Han Chinese population. Haplotypes containing

Table 5. Association Analysis Between the Haplotypes Made up of the Four SNPs and CAD[a]

Haplotype	Case	Control	χ^2	P Value	OR	95%CI
rs9346816, rs2221750, rs3127596, rs9364559						
AGAA	268.65 (0.263)	323.34 (0.319)	6.535	0.011	0.776	0.639 ~ 0.943
GGAA	141.61 (0.139)	110.91 (0.109)	4.659	0.031	1.340	1.027 ~ 1.749
rs9346816, rs312759, rs9364559						
AAA	286.04 (0.271)	337.30 (0.320)	5.033	0.025	0.806	0.667 ~ 0.973
rs9346816, rs2221750, rs9364559						
AGA	267.49 (0.261)	326.10 (0.321)	8.579	0.003	0.750	0.619 ~ 0.910
GGA	212.93 (0.208)	166.50 (0.164)	6.771	0.009	1.347	1.076 ~ 1.687
rs9346816, rs2221750, rs3127596						
AGA	364.87 (0.357)	412.71 (0.407)	4.669	0.031	0.819	0.683 ~ 0.982
GGA	363.64 (0.356)	311.69 (0.307)	6.349	0.012	1.272	1.055 ~ 1.533
rs9346816, rs2221750						
AG	367.26 (0.359)	416.18 (0.410)	5.601	0.018	0.806	0.674 ~ 0.964
GG	434.74 (0.425)	363.82 (0.358)	9.456	0.002	1.323	1.106 ~ 1.581
rs9346816, rs3127596						
AA	387.88 (0.368)	436.51 (0.414)	4.712	0.030	0.824	0.691 ~ 0.981
rs9346816, rs9364559						
AA	310.36 (0.294)	362.71 (0.343)	5.976	0.015	0.796	0.662 ~ 0.956

[a] Frequency < 0.03 in both the control and case groups has been dropped.

Table 6. The Relationship Between SNPs Loci Genotypes and Lp (a) Levels

SNP	Lp (a), mg/dL			P Value
	AA	GA	GG	
rs9346816	21.70 ± 16.83	19.07 ± 11.25	21.32 ± 14.90	0.472
rs2221750	26.94 ±10.06	19.59 ± 8.76	20.69 ± 13.64	0.275
rs3127596	18.86 ± 14.47	23.59 ± 12.6	19.96 ± 5.07	0.094
rs9364559	21.53 ± 17.73	18.90 ± 12.23	22.00 ± 9.63	0.080

rs9346816-G were all associated with an increased risk of CAD. Therefore, our study also indicated that the *SLC22A3-LPAL2-LPA* gene cluster is strongly associated with CAD in the Han Chinese population, which is consistent with the findings from previous studies.

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Footnote

Authours' Contribution: Zi-Kai Song conducted the molecular genetic studies, participated in the sequence alignment, and drafted the manuscript. Hong-Yan Cao and Hai-Di Wu conducted the immunoassays and participated in the sequence alignment. Zi-Kai Song and Li-Ting Zhou participated in the design of the study and performed the statistical analysis. Ling Qin conceived of the study, participated in its design and coordination, and helped to draft the manuscript. All authors read and approved of the final manuscript.

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