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Original Article

Copy number variation of the Lipoprotein(a) (*LPA*) gene is associated with coronary artery disease in a southern Han Chinese population

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Abstract: Copy number variations (CNVs), genomic duplication or deletion events occurring at larger than 1 kb scale, contribute to the complex diseases substantially. Lipoprotein(a) [Lp(a)] is a major inherited risk factor for atherosclerosis and coronary artery disease (CAD). We investigated the association between a CNV of the Lp(a) (*LPA)* gene and CAD. The case-control study included 271 CAD patients and 207 controls diagnosed by coronary angiography. A taqman real-time fluorescence PCR based technique was developed according to the $2 \times 2^{-\Delta\Delta Ct \pm SD}$ calculation method. We detected *LPA* CNVs with a range of 1, 2 and 3. The 1 copy number carriers had a significantly reduced risk of CAD compared with those with 2 copy number after adjusting for the confounding factors ($P < 0.001$, OR = 0.38, 95% CI 0.23-0.64). Further stratified analyses suggested a significant protective effect of the 1 copy number in the elderly population ($P = 0.008$), females ($P = 0.007$) as well as in populations with non-hyperlipidemia ($P = 0.003$), hypertension ($P = 0.001$), non-smoking ($P < 0.001$) and high Lp(a) (≥ 0.3 g/L) levels ($P = 0.001$). The 1 copy number of the *LPA* gene may be an independent protective factor of CAD in a southern Han Chinese population, particularly in females and the elderly.

Keywords: Lipoprotein(a), copy number variation, coronary artery disease

Introduction

Coronary artery disease (CAD) is a common complex trait with a wide range of clinical manifestations, from asymptomatic to stable coronary disease and acute coronary syndrome [1, 2]. To date, studies have identified the principal factors and genes that cause susceptibility or provide protection to CAD [3-6]. High serum Lipoprotein(a) (Lp(a) ≥ 0.3 g/L) has been recognized as an independent risk factor for CAD and stroke [7-9]. The mechanism of Lp(a) atherogenicity likely involves both its ability to interfere with plasminogen activation and atherogenic potential as a lipoprotein particle after receptor-mediated uptake. Lp(a) has a complex structure consisting of a low-density lipoprotein-like moiety which is covalently attached to the unique glycoprotein apoLipoprotein(a) (apo(a)), which have size heterogeneity. Apo(a) size polymorphism is a result of the variability in the *LPA* (MIM #152200) genomic sequence and as an indication in determining the circulating Lp(a) levels.

LPA, located on chromosome 6q25-q26, is highly polymorphic and it genetically determines the circulating Lp(a) level. Several functional polymorphisms in *LPA* have been identified. A C93T variation (rs1853021) in the promoter region of *LPA* is associated with a reduced risk of peripheral arterial disease and a low Lp(a) level in an Italian population [10]. The pentanucleotide TTTTA repeat (PNTR) polymorphism located at the 5' untranslated region of the *LPA* gene has been suggested to account for 10% to 14% of the variation in the circulating Lp(a) levels [11] and the risk of CAD in Caucasians [12]. Recently, genome-wide association studies (GWAS) have indicated that the copy number variations (CNVs) may explain some of the missing heritability for complex

traits [13]. CNV is defined as a DNA segment that is 1 kb or larger and is present at variable copy numbers in comparison to the reference genome [14, 15]. Many of these structural variations are segmental duplications or deletions and may underlie, in part, the inter-individual differences in susceptibility to complex diseases [16-18].

Contradictory results have been reported regarding the association of the *LPA* gene CNVs with CAD in Caucasians according to published GWAS [19, 20], identifying the *LPA* CNVs associated with CAD may have clinical implications for the differences in disease prevalence observed between different populations. We hypothesize that the *LPA* gene CNVs might be another causal role in the pathological process of CAD. Our current study aimed to examine whether the CNVs is present in *LPA* gene itself and the possible association of the *LPA* gene CNVs with the risk of CAD in a southern Han Chinese population.

Materials and methods

Ethics statement

This study was approved by the ethics committee of Ruijin Hospital, Shanghai Jiao Tong University School of Medicine. The authors followed the guidelines of the World's Association Declaration of Helsinki. All participants signed an informed consent form for the study.

Study population

All participants were from a population that is primarily of Southern Han Chinese ethnicity and were recruited from the same demographic region between November 2010 and May 2013 in the Ruijin Hospital in Shanghai. The study group was comprised of 271 CAD patients (mean age 63.8 ± 8.6 yr and 58.3% male), who were admitted to the intensive coronary care unit and had angiographically documented stenosis of at least 70% in at least one proximal epicardial coronary artery. The control group included 207 unrelated individuals with normal coronary arteries (NCA) as determined by angiography (mean age 64.2 ± 8.2 yr and 48.8% male).

Exclusion criteria for the study included the following: age below 18 years old or above 80 years old, persistent CCS class IV angina, a positive stress test, heart failure or cardiogenic shock, known history of cancer or chronic-immune-mediated or autoimmune diseases, or the current use of an immunosuppressive, and any other disorders likely to interact with the variables under investigation.

Data collection

Data on socio-demographic characteristics, lifestyle factors and medical histories was collected using an interviewer-administered questionnaire. The questionnaire included details on personal history, presence of diseases, drug intake and smoking habits. Body mass index (BMI) was calculated by dividing the individual's body mass by their height squared. In order to guarantee precise data, two trained cardiologists independently reviewed all the medical histories to estimate the cardiovascular risk factors. Diabetes mellitus (DM) was defined as fasting glucose greater than 7 mmol/L or currently receiving anti-diabetic medication. Hypertension (HTN) was defined as having a blood pressure greater than 140/90 mmHg or currently on anti-hypertension medication. Stroke was defined and classified according to the Trial of Org 10172 in Acute Stroke Treatment (TOAST) criteria [21]. Smoking status was defined as non-smokers, ex-smokers or current smokers. Hyperlipidaemia (HLP) was defined according to the National Cholesterol Education Program Adult Treatment Panel III Guidelines (NCEP ATIII) [22], including hypercholesterolemia, hypertriglyceridemia and high low-density cholesterol (LDL-C).

Determination of LPA copy number

Genomic DNA was isolated from peripheral white blood cells as described previously [23]. Because there are a large number of repetitive sequences in the *LPA* gene, it is hard to choose appropriate sequences to represent the entire gene CNVs. We detected that the target sequences within exon45 had a highly specificity using BLAST search (http://www.ncbi.nlm.nih.gov/BLAST/). In theory, the *LPA* gene CNVs can be detected indirectly by calculating the CNVs of the target sequences. A Taqman real-time fluorescence quantitative PCR assay, specifically for amplification of genomic *LPA*, was established by using a specific set of amplification primers (forward 5'-cttggattgaggg-

aatgatgaga-3'; reverse 5'-ccttacccacgtttcagcttcta-3') and the probe (5'-FAMTAGGTTGATGCTTCACTCTGTCTCCC-TAMRA-3'). Quantitative *LPA* amplification data was normalized to the internal reference gene, albumin (*ALB*, [MIM#103600]), which was co-amplified simultaneously in a single-tube biplex assay [24] (forward primer: 5'-gctgtcatctcttgtgggctgt-3'; reverse primer: 5'-actcatgggagctgctggttc-3'; probe: 5'-VICCCTGTCATGCCCACACAAATCTCTCCC-TAMRA-3'). The primers and probe for the *LPA* gene were designed using Primer Express software version 3 (Applied Biosystems). To ensure specificity and to avoid polymorphic sites of the primer and probe sequences, a BLAST search was performed (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). For the *ALB* gene, the primers and probes used were published elsewhere [25]. Primers were purchased from Sangon (Shanghai, China) and probes were obtained from Takara (Dalian, China). Real-time qualitative PCR (RT-qPCR) was performed using the ABI prism 7900 sequence-detection system (CA, USA).

The following reagents were used for amplification in a 25 μL reaction volume: 10 μL of DNA (approximately 20 ng), 300 nM of each primer, 10 μL of 2 × Master Mix, 250 nM of probe and sterile water adjusted for final volume. The thermal cycling was initiated with 2 min incubation at 50°C, followed by a denaturation step.

The *LPA* CNVs calls were performed by two scientists independently. All samples within each run were assayed in triplicate and averaged to determine the copy number. The mean concordance was 99.3%. Each 96-well plate run contained one negative control and two quality control samples. In each assay, a standard curve was run and recorded. In order to amplify the *LPA* and *ALB* genes in a one-tube biplex assay, limiting primer conditions were identified to avoid competition of the two reactions [26, 27]. Both the standard curve method and the comparative threshold cycle method were used for quantification, as described elsewhere [25, 28], with few modifications. The cycle number at which the fluorescence reached a fixed threshold, termed the threshold cycle (Ct), was determined (Ct is proportional to the amount of initial target sequence). A set of 4 tenfold dilutions equivalent to 1×10^2 to 1×10^5 copies/reactions of the same calibrator DNA was prepared to generate a standard curve of Ct value against the log [DNA] on each PCR plate for the *ALB* gene (known to have two normal copies per diploid genome) and the *LPA* gene. Copy number is calculated using the formula $2 \times 2^{-\Delta\Delta Ct \pm SD}$, where $\Delta\Delta Ct = (Ct\ ALB_{calibrator} - Ct\ LPA_{calibrator}) - (Ct\ ALB_{sample} - Ct\ LPA_{sample})$. The normalized haploid *LPA* gene copy number is expected to N = 2, as compared to N = 1 for a haploid genotype and N > 2 for a homozygous gene duplication. All the RT-qPCR assays were performed in a blinded manner without knowledge of the case-control status of the subjects.

DNA sequencing

To obtain specific sequence data for an 89 bp fragment within exon 45, genomic DNA was PCR amplified using primers specific to the *LPA* gene and were then sequenced on an Applied Biosystems 3100 capillary sequencer using a Big-Dye chemistry reaction protocol (CA, USA). No SNP has been reported to be located within this region according to published databases (http://www.ncbi.nlm.nih.gov/projects/SNP/). The size of the *ALB* gene PCR product was 72 bp. The sequences of the amplification and nested sequencing primers are available on request.

Statistical analyses

Data were analyzed using the SPSS 20.0 statistical software (SPSS, Chicago, IL, USA). Continuous variables are presented as mean ± standard deviation (SD) or median with 5th and 95th percentiles; whereas, categorical data is summarized as percentages. The Pearson χ^2 test or Fisher's exact test was used to compare the differences in categorical clinical variables between CAD patients and controls. The continuous variables were compared using the unpaired student's *t*-test. The odds ratios (ORs) and 95% confidence intervals (CIs) were calculated using a stepwise logistic regression incorporating the environmental confounding factors to estimate the association between the *LPA* CNVs and the risk of CAD. The ORs were evaluated for 1 copy number and 3 copy number genotypes compared to the normal 2 copy number genotype. A two-tailed *P* value < 0.05 was considered statistically significant.

Table 1. Baseline characteristics of cases and controls

Characteristics	CAD (N = 271)	NCA (N = 207)	*P* value[a]
Male, %	58.3	48.8	0.04
Age, y	63.8 ± 8.6	64.2 ± 8.2	0.59
BMI, kg/m^2	24.0 ± 3.1	24.4 ± 3.4	0.18
Hypertension, %	67.5	66.2	0.77
Hypercholesterolemia, %	30.6	47.2	0.00
Stroke history, %	16.6	13.0	0.30
Diabetes, %	25.5	10.6	0.00
Smoking, %	36.5	19.2	0.00
Lp(a) ≥ 0.3 g/L, %	39.9	29.9	0.03

BMI: body mass index. Age and BMI are given as mean ± SD. [a]: The unpaired t-test was used for age and BMI and the X^2 test was used for other categorical characteristics.

Results

Clinical characteristics

The clinical and demographic features of CAD patients and controls are shown in **Table 1**. There were no significant differences of age, BMI, HTN or stroke history between the CAD patients and controls. There was a significantly higher proportion of males in the CAD group than in the control group (P = 0.04). In addition, there was also an increased prevalence of DM ($P < 10^{-3}$), smoking ($P < 10^{-3}$) and high Lp(a) (≥ 0.3 g/L, P = 0.03) in the CAD group. However, the CAD group had a significantly lower proportion of HLP ($P < 10^{-3}$) than the NCA group.

LPA CNVs influence on CAD

We detected *LPA* CNVs with a range of 1, 2 and 3. The details of the copy number frequencies of the CAD patients and controls are shown in **Table 2**. The 2 copy number of the *LPA* gene is the most common, with a frequency of 62.4% in the CAD group and 55.1% in the NCA group. One copy number depicts a one copy deletion in the *LPA* gene, while 3 copy number depicts a one copy duplication relative to the common 2 copy number. There were significant differences in the *LPA* CNV distributions between the CAD and NCA groups (P = 0.002). The 1 copy number carriers had a reduced risk of CAD relative to those with 2 copy number (P = 0.02, OR = 0.64, 95% CI 0.44-0.93). After adjusting for the confounding factors such as gender, age, BMI, HTN, DM, HLP, stroke history, smoking habit and Lp(a) (with categories ≥ 0.3 g/L and < 0.3 g/L), the magnitude of the association was dramatically reinforced in patients with 1 copy number, conferring a significant protective effect on CAD ($P < 10^{-3}$, OR = 0.38, 95% CI 0.23-0.64). Using the 2 copy number genotype as a reference, a non-significant relationship was observed between the 3 copy number of the *LPA* gene and the increase of CAD risk (P = 0.07, OR = 4.93, 95% CI 0.91-26.86), following adjustment for the above confounding factors.

When data was stratified by the age and gender, a distinct association was observed between the 1 copy number of the *LPA* gene and a reduced risk of CAD in the population with patients > 65 years old (P = 0.01, OR = 0.35, 95% CI 0.16-0.76) and a marginal association was observed in the population with patients ≤ 65 years old (P = 0.05, OR = 0.47, 95% CI 0.22-0.98). Risk reduction given by the 1 copy number of the *LPA* gene in females (P = 0.01, OR = 0.29, 95% CI 0.12-0.71), was significantly different from that in the male population (P = 0.06, OR = 0.52, 95% CI 0.26-1.03).

Further subgroup analysis defined by cardiovascular risk factors suggested that a heterogeneous association between the 1 copy number of the *LPA* gene and CAD, showing a significant protective effect in populations with non-HLP ($P < 10^{-3}$, OR = 0.36, 95% CI 0.18-0.71), HTN ($P < 10^{-3}$, OR = 0.32, 95% CI 0.17-0.62) and non-smoking ($P < 10^{-3}$, OR = 0.28, 95% CI 0.14-0.56). As for DM, the 1 copy number was significantly associated with a reduced risk of CAD in both the DM patients (P = 0.03, OR = 0.19, 95% CI 0.04-0.88) and non-DM patients ($P < 10^{-3}$, OR = 0.42, 95% CI 0.24-0.74).

A direct association between the Lp(a) levels and the *LPA* CNVs in the Southern Han Chinese population was not detected (P = 0.126, data not shown). To further evaluate the influence of Lp(a) on the association between the *LPA* CNVs and CAD, subjects were classified into groups referring either high or low Lp(a) levels. A cutoff value of 0.3 g/L was used [29]. Interestingly, the 1 copy number carriers had a 77% reduced risk of CAD relative to the 2 copy number carriers in the high Lp(a) (≥ 0.3 g/L) population ($P < 10^{-3}$, 95% CI 0.10-0.55), but not in the low Lp(a) (< 0.3 g/L) population (P = 0.06, OR = 0.52, 95% CI 0.26-1.02) (**Table 3**).

Table 2. The distribution of *LPA* CNVs in cases and controls and the risk prediction of CAD

LPA CNVs	CAD, n (%)	NCA, n (%)	P_{χ^2}[a]	Crude			Adjusted[b]		
				OR	95% CI	P	OR	95% CI	P
1	85 (31.4)	90 (43.5)	0.002	0.64	0.44-0.93	0.02	0.38	0.23-0.64	0.00
2	169 (62.4)	114 (55.1)		1.00 (reference)			1.00 (reference)		
3	17 (6.3)	3 (1.4)		3.82	1.10-13.34	0.04	4.93	0.91-26.86	0.07

[a]: *P* values were calculated by the χ^2 test for differences in LPA CNVs between patients and controls. [b]: ORs adjusted for age, gender, BMI, diabetes, hypertension, stroke history, smoking habit, Lp(a) and hyperlipidaemia.

Table 3. Stratified analyses of the *LPA* 1 copy number with the risk of CAD in cases and controls

Group	Crude			Adjusted[a]		
	OR	95% CI	P	OR	95% CI	P
Age						
> 65	0.67	0.39-1.15	0.15	0.35	0.16-0.76	0.01
≤ 65	0.61	0.36-1.03	0.07	0.47	0.22-0.98	0.05
Gender						
Male	0.77	0.46-1.29	0.32	0.52	0.26-1.03	0.06
Female	0.48	0.27-0.85	0.01	0.29	0.12-0.71	0.01
Lp(a)						
≥ 0.3 g/L	0.38	0.19-0.73	0.00	0.23	0.10-0.55	0.00
< 0.3 g/L	0.84	0.51-1.36	0.84	0.52	0.26-1.02	0.06
Hyperlipidaemia						
Yes	0.68	0.36-1.29	0.24	0.51	0.22-1.18	0.12
No	0.62	0.38-1.01	0.06	0.36	0.18-0.71	0.00
Diabetes						
Yes	0.59	0.22-1.57	0.29	0.19	0.04-0.88	0.03
No	0.65	0.43-0.99	0.04	0.42	0.24-0.74	0.00
Hypertension						
Yes	0.58	0.37-0.92	0.02	0.32	0.17-0.62	0.00
No	0.77	0.39-1.52	0.45	0.65	0.25-1.70	0.38
Smoking						
Yes	0.63	0.29-1.36	0.24	0.73	0.28-1.88	0.51
No	0.56	0.35-0.89	0.02	0.28	0.14-0.56	0.00

[a]: ORs adjusted for age, gender, BMI, diabetes, hypertension, stroke history, smoking habit, Lp(a) and hyperlipaemia.

Discussion

In the current study, we detected a significant association between the *LPA* gene deletion and a reduced risk of CAD in a Southern Han Chinese population. The relationship was heterogeneous when data was classified by age, gender and other cardiovascular factors. To our knowledge, this is the first case-control study that examined the *LPA* CNVs and risk of CAD in a Chinese population and provides evidence that *LPA* gene segmental deletions may be an independent protective factor of CAD when considering other CAD risk factors.

The region that harbors the *LPA* gene on chromosome 6 is remarkably variable and commonly polymorphic in the CNV maps demonstrated by Redon et al. [30] and Wong et al. [31]. However, the association between the *LPA* CNVs and cardiovascular diseases remains uncertain due to conflicting data shown in a range of independent studies. A recent GWAS revealed a cluster of genes *SLC22A3-LPAL2-LPA* on chromosome 6q25-26 that are strongly associated with CAD. The investigators identified that the *LPA* gene is highly variable; however, several CNVs are not well characterized by DNA arrays used in this Caucasian European population [19]. Another GWAS identified the variants at the *LPA* locus (rs10455872 and rs3798220) that were significantly associated with CAD and had a reduced copy number of the *LPA* gene that was strongly associated with an increased level of Lp(a) in a European population [20]. Although the candidate gene approach cannot replace GWAS in unraveling the genetic architecture of complex diseases, there may still be a need for using a candidate gene approach, especially when looking at variations with low frequencies [32]. Furthermore, most of these GWAS were conducted in Caucasians, thus revealing a need for research in other human populations.

Our finding confirmed the association of the *LPA* CNVs with CAD in a Southern Han Chinese population. The CAD patients and controls involved in our study were diagnosed using coronary angiography, which minimizes the misclassification of study participants as much as possible. Controls were randomly selected from the same geographic region as CAD patients. This further stratified analysis suggests that the protective effect of the *LPA* 1

copy number is noteworthy in the elderly population and in females. Additionally, an interaction between the *LPA* CNVs, CAD and other cardiovascular risk factors was detected. The 1 copy number of *LPA* gene may reduce the risk of CAD in populations with HTN or without HLP and non-smoking. We also detected that the 3 copy number of *LPA* gene prone to an association with an increased risk of CAD. However, this estimate is not statistically significant, most likely due to the low frequency of high copy numbers in the Chinese population. A larger well-designed study is required to confirm the robustness of our study.

We failed to find a direct correlation between the circulating Lp(a) levels and the *LPA* CNVs in our study. This may be explained by the fact that the effect of the *LPA* CNVs may not be strong enough to determinate the circulating Lp(a) level but may influence the function of Lp(a). Other segmental variations in the *LPA* gene, such as the kringle IV-type 2 repeats and the cis-acting sequences, have been documented to determine the main variations in circulating Lp(a) levels [33]. We found that the 1 copy number of the *LPA* gene is associated with a reduced risk of CAD in the high Lp(a) population. CNVs influence gene expression, phenotypic variation and adaptation by disrupting gene functions and altering gene dosage; however, the mechanism of these genetic alterations and their impact on disease susceptibility remains unclear [30]. Previous studies [34-36] have demonstrated that Lp(a) may act as a preferential acceptor that tightly binds oxidized phospholipid transferred from tissues or from other lipoproteins, which means oxidized phospholipids are preferentially associated with Lp(a). This process may accelerate the development of atherosclerosis [37]. It is possible that the low copy number of the *LPA* gene modifies the binding affinity to glycosaminoglycans and fibronectin. Low copy number of the *LPA* gene may also reduce the impairment of fibrinolytic activity [29] and binding to pro-inflammatory oxidized phospholipids and prevent the progress of atherosclerosis [38]. Further studies are required to demonstrate a synergistic mechanism between a low copy number of *LPA* and other lipid fractions in the development of atherosclerosis.

Our study should be viewed with limitations in mind. First, our study was designed using a retrospective method, which precludes further comments on the cause-effect relationship. The controls may not have been selected independent of exposure, resulting in an increased possibility of selection bias. A population-based study design is superior and may provide a good representation of the potential allele frequency. Second, we only studied the *LPA* CNVs and did not analysis the genomic variations within the *LPA* gene, including the repeats of plasminogen-like kringle IV domains [39] and (TTTTA) n=1 kb at position -1400 [40], which determine the molecular property and circulating levels of Lp(a). Third, it has been acknowledged that Lp(a) has a distinct interaction with other lipid fractions in the pathology of CAD and it is recommended to investigate the genetic variations in other lipid fractions when estimating the association of the *LPA* CNVs and CAD. Fourth, our study subjects were limited to Chinese ancestry. It is unclear whether the result can be replicated in other racial groups and further validation is needed. Finally, although we developed a Taqman real-time fluorescence PCR based technique to quantitate the *LPA* CNVs, it is still necessary to confirm the contribution of the *LPA* CNVs to CAD using different approaches. A recent large GWAS typed 19,000 individuals into distinct copy-number classes at 3.432 polymorphic CNVs using a purpose-designed array. However, the common CNVs typed are unlikely to contribute greatly to the genetic basis of common human diseases [41]. It is recommended that considerable care in interpreting putative CNV associations combined with independent replication on a different experimental platform.

In conclusion, our study found that the 1 copy number of the *LPA* gene was associated with a reduced risk of CAD in a southern Han Chinese population. We provided evidence that the *LPA* CNVs moderately contribute to the prevention of CAD and might be a new biomarker for susceptibility of cardiovascular diseases, particularly in females or the elderly. Our findings complement previous reports on the *LPA* structure variety [39, 42, 43], and further studies are needed to investigate the function of the *LPA* deletion and its underlying biological mechanism.

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Disclosure of conflict of interest

None.

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