Rare Nonsynonymous Variants in Lipid Metabolism Related Genes in Coronary Artery Disease

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Abstract

Background: CAD (Coronary Artery Disease) is a complex disease that influenced by environment and genetic factors. In this study, we aim to investigate the relationship between rare nonsynonymous variants in lipid metabolism related genes and CAD in Chinese Han population.

Methods: A total of 252 samples were recruited in this study, including 120 CAD cases and 132 normal health controls. Rare variants were detected via NGS based targeted sequencing. Pathogenicity prediction were performed with SIFT and Polyphen-2.

Results: The present study identified 33 nonsynonymous rare variants including two novel variants located in ANGPTL4 (G47E) and SCARB1 (L233F) gene, respectively. Association analysis showed CAD patients carried more nonsynonymous variants in all mutation sets, but did not reach statistically significant.

Conclusions: Targeted sequencing was a powerful tool to uncover rare variants in coronary artery disease. Clinical relevance of rare variants in CAD etiology needs to be investigated in future larger sample sizes.

Introduction

Coronary artery disease (CAD) is a common chronic inflammatory disease which also remains the leading cause of death worldwide [1]. It was estimated that 700,000 people died from CAD in China every year [2]. In addition to conventional risk factors such as hypertension, dyslipidemia, diabetes, obesity and smoking, genetic factors also play an important role in CAD pathogenesis. To reduce the occurrence of CAD, it is important to find the biomarkers that responsible for CAD etiology [3].

Genome-wide association studies have identified many variants that associated with CAD [4-7]. GWAS studies focus on common variants and these susceptibility variants are always located within intronic or intergenic regions with relatively small effect. Rare variants that might also associated with CAD are generally missed.

Rare variants are genetic variations with frequency less than 1% and sometimes much lower [8, 9]. Nonsynonymous variants are predicted to change the amino acid sequence of protein, which include missense variants (a single amino acid substitution), nonsense variants (create a premature stop codon) and frameshift variants (alter the reading frame of a protein). Nonsynonymous variants could affect protein function and significantly contribute to the etiology of complex diseases [10]. However, those variants are likely to be under strong negative selection and may be missed by whole genome association mapping for identify genes in complex disease [11].

Due to the increase of throughput and decrease in costs, NGS(Next Generation Sequencing) based technology has been wildly used in human disease researches. Extensive research using exome sequencing identified rare variants responsible for Mendelian diseases. Targeted sequencing is a rapid and cost-effective way to detect known and novel variants in selected sets of genes or genomic regions, and proven to be an efficient technique for screening variants in complex disease [12, 13]. It has been shown that targeted sequencing of a subset of genes generates results with identical quality to Sanger sequencing [14].

Lipid disorder is one of the most important risk factors for CAD. In this study, we conduct targeted sequencing of 12 genes that involved in lipoprotein metabolism to investigate the relationship between rare variants and coronary heart disease. We aim to find nonsynonymous variants that confer susceptibility to CAD in Chinese Han population, therefore, shed light on the exploration of CAD pathogenesis.

Materials And Methods

Study population

A total of 120 CAD patients and 132 non-CAD controls were recruited from Renji Hospital between 2016 and 2020. Individuals with incomplete information were excluded. All the participants were unrelated Chinese Han individuals. This study was approved by the Medical Ethics Committee of Renji Hospital and compliant with the principles set forth by the Declaration of Helsinki. The diagnostic criteria for CAD cases were defined as followings: at least one of the major segments of coronary arteries (right coronary artery, left circumflex, or left anterior descending arteries) with more than or equal to 50% organic stenosis based on coronary angiography. All unaffected controls were determined to be free of CAD. 5ml peripheral blood sample was collected from each subject.

Targeted sequencing

Genomic DNA was extracted using TianGen DNA extraction kit (TianGen Ltd, Beijing, China) following standard protocol. DNA concentration and quality was measured using NanoDrop spectrophotometer (Thermo Scientific, USA). All purified DNA were stored at -80°C.50ng DNA was used for PCR amplification. PCR primers were designed using Oligo 6.0 and synthesized by Shanghai Free Biotechnology Co., Ltd (Shanghai, China). Coding regions of target gene were captured by multiplex PCR and followed by adaptor adding. The final panel consisted of 203 amplicons with and average size of 250 bp. Paired-end sequencing (2X150) was performed with Illumina NovaSeq sequencing instruments (Novogene, Beijing, China).

Variant analysis

Nonsynonymous exonic variants were called by BWA and SAM tools according to the following quality control criteria: (1) at lease 50X coverage; (2) Q-score >30; (3) at least 40% variant frequency. Variations that absent or with a minor allele frequency <0.01 in public database (dbSNP; Exome Aggregation Consortium; 1000 Genomes Project) were regarded as rare variants. Pathogenicity prediction of missense mutation were performed with SIFT

(http://provean.jcvi.org/index.php) and Polyphen-2 (http://genetics.bwh.harvard.edu/pph2/index.shtml) [15, 16]. Association between APOA5 mutation carrier status and CAD were performed using the Mann–Whitney U test.

Results

We generated a multiplex PCR panel to capture the coding region of 12 lipid metabolism related genes (ANGPTL3, ANGPTL4, APOA1, APOA5, APOC1, APOC3, CETP, LDLR, LIPC, LPL, PCSK9 and SCARB1) (table 1). Targeted sequencing was performed on 120 unrelated CAD patients and 132 unrelated health controls. Rare nonsynonymous variants with frequency <1% were selected for further analysis.

Table 1: Selected 12 genes of NGS custom gene pane

gene	location	Ref seq no.	No. of coding exons	Transcript length (bp)	Protein length (aa)
ANGPTL3	1p31.3	NM_014495.4	7	2926	460
ANGPTL4	19p13.2	NM_139314.3	7	1872	406
APOA1	11q23.3	NM_000039.3	4	899	267
APOA5	11q23.3	NM_001371904.1	3	1881	366
APOC1	19q13.32	NM_001645.5	4	514	83
APOC3	11q23.3	NM_000040.3	4	535	99
CETP	16q13	NM_000078.3	16	1691	493
LDLR	19p13.2	NM_000527.5	18	5173	860
LIPC	15q21.3	NM_000236.3	9	2559	499
LPL	8p21.3	NM_000237.3	10	3565	475
PCSK9	1p32.3	NM_174936.4	12	3637	692
SCARB1	12q24.31	NM_005505.5	13	3405	509

A total of 33 coding non-synonymous variants passing quality control filter were discovered in 12 gene regions, including 32 missense variants and one 7 bp duplication variant (table 2). All of them were heterozygous mutations. Two novel variants were discovered in this study which were not observed in Exome Aggregation Consortium (ExAC) and dbSNP database. One single nucleotide variant in ANGPTL4 gene that introduce a missense mutation at position 47, resulting in the amino mutation G47E (GGA-GAA, located in the first exon of ANGPTL4, at nucleotide 8,364,461 on chromosome 19). The other single nucleotide variant in SCARB1 gene that introduce a missense mutation at position 233, resulting in the amino mutation L233F (CTC-TTC, located in the fifth exon of SCARB1, at nucleotide 124,811,899 on chromosome 12). We also identified novel genotypes at 4 existing SNVs in dbSNP database. Two novel variants and four novel genotypes were validated by bi-directionally Sanger sequencing and demonstrated 100% concordance (figure 1).

Mutation pathogenicity analysis were performed using SIFT and Polyphen-2. 12 variants were predicted to be deleterious by SIFT and 18 were predicted to be possibly damaging or probably damaging by PolyPhen-2. 10 variants were predicted to be tolerated by SIFT and benign by PolyPhen-2 indicating that these variants results in truncated protein but does not imply pathogenic. 8 variants were predicted to be deleterious/damaging in both programs. 22 variants were predicted to be damaging or deleterious in at least one program.

Table2: rare nonsynonymous variants identified in this study

Gene	chr	pos	Nucleotide	AA	novel[a]	Effect	dbSNP	freq(case/con)	SIFT	sift_class	
ANGPTL3	1	62598787	T/C	I/T		missense variant	rs201826477	0/1	0.01	deleterious	0
ANGPTL3	1	62598796	T/C	I/T		missense variant	rs112068132	1/1	0	deleterious	0
ANGPTL4	19	8364461	G/A	G/E	yes	missense variant		1/0	0.003	deleterious	1
ANGPTL4	19	8371453	G/A	V/I		missense variant	rs200918932	1/0	0.1	tolerated	0
ANGPTL4	19	8373780	A/G	Q/R		missense variant	rs756440132	0/1	0.3	tolerated	0
APOA1	11	116837116	G/C	Q/E		missense variant	rs1254205437[a]	1/0	0.11	tolerated	0
APOA1	11	116837080	C/A	V/L		missense variant	rs201148448	1/1	0.07	tolerated	0
APOA1	11	116837053	C/T	G/S		missense variant	rs28931574	0/1	0.08	tolerated	0
APOA1	11	116836173	G/T	R/S		missense variant	rs1591330063[a]	1/0	0.649	tolerated	0
APOA5	11	116791670	C/A	G/V		missense variant	rs548745995	1/0	0.19	tolerated	0
APOA5	11	116790166	G/C	L/V		missense variant	rs556600766[a]	1/0	0.005	deleterious	0
APOC1	19	44916230	CTTGGAT/ CTTGGATCTTGGAT	ALD/ ALDLGX		frameshift variant	rs767630355	0/1	-	-	-
APOC1	19	44916292	G/A	R/H		missense variant	rs369438021	1/0	0.6	tolerated	0
CETP	16	56962013	C/G	L/V		missense variant	rs1460617147[a]	0/1	0.294	tolerated	0
CETP	16	56969935	G/A	R/Q		missense variant	rs184615182	1/0	0.37	tolerated	0
LDLR	19	11105250	G/A	R/H		missense variant	rs201102461	0/1	0.04	deleterious	0
LDLR	19	11105408	G/A	D/N		missense variant	rs200727689	1/0	0.03	deleterious	1
LDLR	19	11105492	C/G	P/A		missense variant	rs1013147010	0/1	0.194	tolerated	0
LDLR	19	11105505	T/G	F/C		missense variant	rs879254586	1/0	0.18	tolerated	0
LDLR	19	11116900	C/T	H/Y		missense variant	rs730882109	1/0	0	deleterious	1
LIPC	15	58563522	G/A	S/N		missense variant	rs1015457944	0/1	0.26	tolerated	0
LIPC	15	58563665	C/T	R/C		missense variant	rs573340043	1/0	0.06	tolerated	0
LPL	8	19951811	G/A	A/T		missense variant	rs145657341	1/0	0.04	deleterious	1
LPL	8	19954327	G/A	R/H		missense variant	rs750750025	0/1	0.15	tolerated	0
LPL	8	19955927	G/A	A/T		missense variant	rs1800011	1/0	0	deleterious	0
PCSK9	1	55046626	C/T	A/V		missense variant	rs770592607	0/1	0.33	tolerated	0
PCSK9	1	55052650	G/A	A/T		missense variant splice region variant	rs768795323	1/0	0.03	deleterious	0
PCSK9	1	55052698	G/A	G/S		missense variant	rs149489325	0/1	0.04	deleterious	0

PCSK9	1	55052739	C/A	S/R		missense variant	rs768846693	1/0	0.01	deleterious	0
PCSK9	1	55061420	C/T	P/L		missense variant	rs72646525	1/0	0.19	tolerated	0
SCARB1	12	124863717	C/T	G/S		missense variant	rs4238001	0/1	0.1	tolerated	0
SCARB1	12	124811911	T/C	S/G		missense variant	rs10396213	1/1	0.09	tolerated	0
SCARB1	12	124811899	C/T	L/F	yes	missense variant		1/0	0.147	tolerated	0

a: novel genotype in dbSNP database

Table 3: Association of rare non-synonymous variants identified in this study with risk of CAD

Mutation set	N cases./controls	case	controls	Freq case	Freq con	OR	P
All	120/132	21	15	17.50%	11.36%	1.65	0.16
deleterious(SIFT) [a]	120/132	9	4	7.50%	3.03%	2.59	0.11
damaging(PolyPhen-2) [b]	120/132	10	8	8.33%	6.06%	1.41	0.48
SIFT or PolyPhen [c]	120/132	13	10	10.83%	7.58%	1.48	0.37
SIFT and PolyPhen [d]	120/132	6	2	5.00%	1.52%	3.42	0.22

a: annotated as "deleterious" or "damaging" by SIFT

b: annotated as "possibly damaging" or "probably damaging" by PolyPhen-2

c: annotated as "deleterious" or "damaging" by SIFT, or as "possibly damaging" or "probably damaging" by PolyPhen-2

d: annotated as "deleterious" or "damaging" by SIFT, and as "possibly damaging" or "probably damaging" by PolyPhen-2

We investigated the relationship between rare nonsynonymous variants and risk of CAD (table 3). 23 subjects carried variants that predicted to be damaging or deleterious in at least one program. 13 of them were identified in patient group and 10 of them were found in control group. 8 subjects carried variants that predicted to be damaging and deleterious in both programs, while 6 were identified in patient group and 2 in control group, respectively. 13 subjects carried variants that predicted to be deleterious by SIFT, while 9 were identified in patient group and 4 in control group, respectively. 18 subjects carried variants that predicted to be damaging by PolyPhen-2, while 10 were identified in patient group and 8 in control group, respectively. Patient group shown higher frequency of variants carriage status in all mutation sets. However, none of them reach statistically significant.

Discussion

Exome sequencing has been proven to be a powerful tool to uncover novel causal mutation of Mendelian diseases [17]. Recently, large-scale efforts have applied exome sequencing to study rare variants in complex disease [18]. Through targeted sequencing of coding region of SCARB1, Zanoni et al. showed that P376L carriers have a significantly higher HDL-C level and an increased risk of coronary heart disease [19]. Four rare variants in the coding region of apolipoprotein C3 (APOC3) that disrupt APOC3 function were found to be associated with lower plasma triglyceride levels and have a reduced risk of coronary heart disease [20]. Dewey et al. showed that carrying inactivating mutations in ANGPTL4 had lower levels of triglycerides and a lower risk of coronary artery disease compared with noncarriers [21]. Compound heterozygotes for two distinct nonsense mutations in ANGPTL3 resulted in decreased plasma LDL cholesterol levels and familial combined hypolipidemia [22]. Rare alleles at LDLR and APOA5 confer risk for early onset myocardial infarction [23]. Rare nonsynonymous variants can facilitate the exploration of disease pathogenesis, and provide supportive evidence for putative drug targets for novel therapies.

NGS based targeted sequencing of known disease genes and important candidate genes could identify not only disease causing variants but also variants of uncertain significance, which can be challenging for genetic counselling. In the present study, 33 nonsynonymous rare variants were identified using targeted sequencing. Two novel variants were identified in CAD cohort. One of them was a variant that introduces a missense mutation in ANGPTL4 (G47E), predicted to be deleterious by SIFT and probably damaging by PolyPhen-2. The other one was a variant that introduces a missense mutation in SCARB1 (L233F), predicted to be tolerated by SIFT and possibly damaging by PolyPhen-2. Three variants (rs200727689 in LDLR, rs730882109 in LDLR and rs768795323 in PCSK9) have been reported to be pathogenic or likely-pathogenic in ClinVar database, and all of them were identified in CAD cohort and linked to familial hypercholesterolemia in ClinVar database.

Rare variants having a population prevalence of <1% and may not be statistically associated with diseases of interest even in large samples. It was predicted that 27–29% of nonsynonymous mutations are neutral or nearly neutral, 30–42% are moderately deleterious, and the remainder are highly deleterious or lethal [11]. Our results show CAD patients carried more heterozygous nonsynonymous variants in all mutation sets, but the difference did not reach statistically significant. Larger sample sizes and functional researches are needed to clarify the impact of these variants.

In summary, we described a novel targeted NGS panel including 12 lipid metabolism genes. This panel is highly accurate to identify rare variants. This study suggested that targeted sequencing approaches can be used to discover rare mutations that contribute to the etiology of CAD risk, and may lead to the discovery of novel pharmaceutical targets for disease prevention and treatment. However, there are several limitations of this study. (1) This assay was designed to detect single nucleotide variants and small indels, and larger indels or structural rearrangements will be missed. (2) Whether these variants alter CAD risk remain unclear due to statistically underpowered. Larger sample size is needed to increase statistical power. (3) It is difficult to determine the pathogenicity of novel variants by computational methods alone. Functional testing may help to clarify the impact of these variants. (4) As an aging-related disease, subjects in control group might develop CAD in the future and lead to a misclassification bias [24]. Further studies are needed to validate these findings and explore these variations as potential pathogenic mutations for CAD.

Conclusions

A total of 252 samples were recruited in this study, including 120 CAD cases and 132 normal health controls. The present study identified 33 nonsynonymous rare variants including two novel variants located in ANGPTL4 (G47E) and SCARB1 (L233F) gene, respectively. Association analysis showed CAD patients carried more nonsynonymous variants in all mutation sets, but did not reach statistically significant.

Targeted sequencing was a powerful tool to uncover rare variants in coronary artery disease. Clinical relevance of rare variants in CAD etiology needs to be investigated in future larger sample sizes.

Abbreviations

CAD (Coronary Artery Disease)

NGS(Next Generation Sequencing)

Declarations

Conflict of interest statement

The authors declare that they have no competing interests.

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Authors' contributions

Wei Li, Yongyi Wang and Ritai Huang performed the annotation analysis, produced figures, and participated in drafting the manuscript. Feng Lian,Weijun Wang and Genxing Xu provided the data interpretation and offered the study advice. Song Xue designed and supervised the project, generated results, provided data interpretation. All authors read, revised, and approved the final manuscript.

Availability of data and materials

All data generated or analysed during this study are included in this published article [and its supplementary information files].

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Ethics approval and consent to participate

This study was approved by Shanghai Jiaotong University School of Medicine, Renji Hospital Ethics Committee(KY2021-159-B). Informed Consents were signed by all participants.

Consent for publication

All authors agree to publish all raw data in the article and use that data compliantly.

Competing interests

The authors declare that they have no competing interests.

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Figures

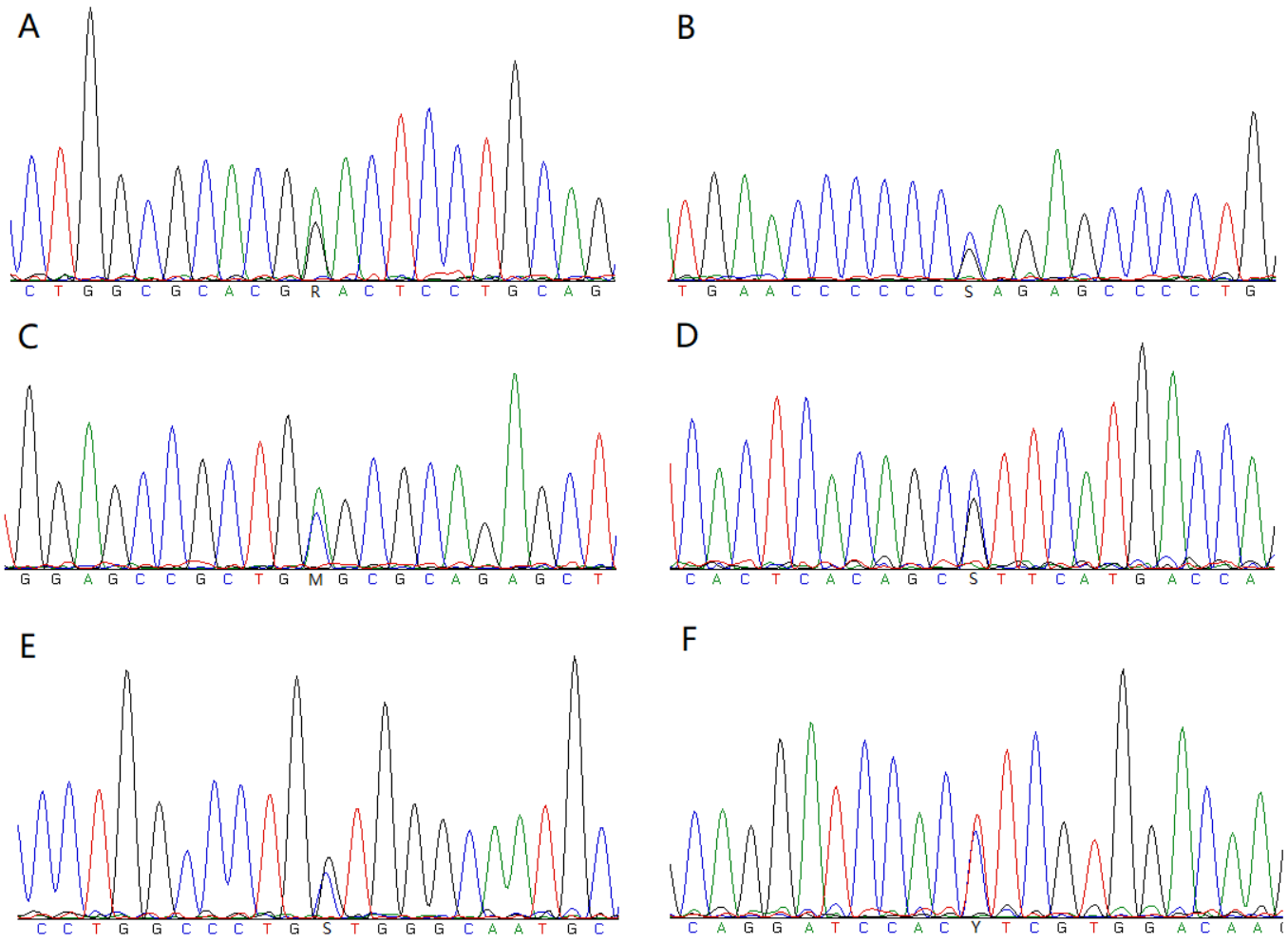

Figure 1

Sanger capillary electrophoresis sequencing results of 6 novel variants/genotype. A: ANGPTL4 G47E; B: APOA1 rs1254205437 G/C; C: APOA1 rs1591330063 G/T; D: APOA5 rs556600766 G/C; E: CETP rs1460617147 C/G; F: SCARB1 L233F.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementarymaterials1Sangersequencingresults.zip
- Supplementarytable1Primersequences.xlsx
- Supplementarytable2Originalanalysisresultsoftargetedsequencing.xlsx