Original Article
Association between the ADAMT33 variant and carotid artery intima-media thickness in the Chinese Han population

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Abstract: Background: The ADAM33 with a disintegrin domain and a metalloprotease domain attaches an important role in regulating smooth vascular cell migration and proteolysis. In the present study, we investigated the association between ADAM33 variants and carotid artery intima-media thickness (CIMT) in the Chinese Han population. Methods: In a community population (n=620), CIMT was determined using the ultrasound to detect the carotid artery intima-media thickness. We screened the ADAM33 variations using PCR-direct sequencing method and investigated the relationship between ADAM33 variations and CIMT in Chinese Northern Han population. Results: The ADAM33 expression was increased in the atherosclerotic carotid artery from CIMT patients compared with the normal subjects by the immunohistochemical staining. Furthermore, ADAM33 rs514174 was closely related to the increased risk of CIMT patients (OR=1.43, 95% CI: 1.08-1.89, P≤0.05). In addition, the rs514174 TT genotype of ADAM33 was significantly associated with the increased ADAM33 mRNA expression in patients with CIMT (P<0.05). Conclusion: Our study provides the further support for the ADAM33 rs514174 variant as a direct risk factor for CIMT. The ADAM33 rs514174 variant attaches an important role for the early identification occurrence of CIMT.

Keywords: ADAM33, carotid intima-media thickness, carotid plaque, polymorphism

Introduction

Atherosclerosis, leading to the occurrence of coronary artery disease (CAD) and stroke, is a major cause of morbidity and mortality in the industrialized countries [1]. However, the pathogenesis of atherosclerosis is poorly understood and new methods are required to predict the existing atherosclerosis risk at present [2, 3].

During the process of atherosclerosis, the extracellular matrix remodeling attaches an important role in the atherosclerotic plaque development, instability and rupture. Carotid intima-media thickness (CIMT), as a reliable marker can early predicting the occurrence of future atherosclerosis events [1, 4]. From the recent genome-wide association mapping studies, several genetic variants known to be related to the risk of CAD and stroke have also been found to be associated with the occurrence of CIMT risk, suggesting that there may be similar

pathogenic mechanisms between CAD and stroke and susceptibility to CIMT [4, 5].

CIMT is a reliable marker for early atherosclerosis and can predict future cardiovascular events such as CAD and stroke. Moreover, CIMT is usually used as an intermediate phenotype for the atherosclerosis in the clinical studies. CIMT has the significant heritability and several genetic variants related to risk of CAD and stroke have been subsequently reported to be associated with the interindividual variability in the atherosclerotic process of CIMT [6]. Thus, the mechanism that these genetic variants influencing the atherosclerosis process of CIMT may be similar to that which will contribute to alter the susceptibility to CAD and stroke [7].

The ADAMs containing a disintegrin and metalloprotease domain are the transmembrane and secretory proteins belonging to the zinc protease superfamily. ADAM33, as a member of the

ADAMs family locates on chromosome 20 p13 and contains the 22 exons [8]. ADAM33 palys an important role in the inflammation and vasculature regenerative processes, which plays the key roles in the process and development of atherosclerosis. For example, ADAM33 was reported to be expressed in the atherosclerotic plaques and there was a significant association between ADAM33 rs574174 variant and the atherosclerosis severity in a cohort of CAD patients in the Caucasian population [9, 10]. However, the association between the ADAM33 variants and CIMT in carotid plaques patients was unknown in the Chinese Han population.

Therefore, we performed a case-control study to determine the association between the ADAM33 variations and CIMT to investigate whether ADAM33 rs514174 variation was associated with CIMT.

Methods

Study population

620 consecutively recruited subjects aged 50-75 years from the General Hospital of Northern Theater Command outpatients were included in our study. We collected the carotid artery intima-media thickness measurements by the ultrasound, blood samples were collected through the selected cases, and the full clinical data for each individual were gathered by the full-time staff. This study conformed well with the principles outlined in the Declaration of Helsinki. Signed informed consent for participation in the study was obtained from all individuals.

Ultrasound imaging

Carotid artery ultrasound as a valuable data to investigate the value of CIMT was performed using a portable ultrasound machine (HI VISION Preirus, Hitachi Medical Systems, Japan). Optimized images of left and right carotid artery intima-media thickness were selected and frozen at the end of diastollic heart. The CIMT of the common carotid artery was measured by the ultrasonic software at both the near and far walls of the left and right sides in an area free of atherosclerotic plaque. The ultrasound parameters were captured and analyzed using semi-automated software analysis for each carotid region. The instrument and methods used were as previously described [11].

DNA isolation and genotype

We used the modified salt-extraction method to isolate the DNA from the 5 ml whole blood from the 620 subjects. Following the manufacturer's instruction (Tiangen Biotech Co. Ltd., Beijing, China) genomic DNA were collected from the blood leukocyte pellets [12]. The ADAM33 forward and reverse primer such as 5'-CTCTCCAGATGCTGGCATCG-3' and 5'-TGTTT-TAAGGAACATCACA-3' were designed by the Primer 5.0 software. Through the direct PCR sequencing (http://www.sangon.com/) the ADAM33 rs514174 variant was determined. The resulting fragments were separated on the 2% agarose gel stained with ethidium bromide and visualized under ultraviolet light. All the subjects genotypes were retyped twice by the independent investigators who did not know the subjects' identities and phenotypes.

RNA extraction and real-time quantitative PCR (RT-qPCR) amplification

The Ficoll gradient density centrifugation method was used for the peripheral blood mononuclear cell (PBMC) separation. PBMCs were resuspended in RPMI medium with 10% fetal bovine serum. Total RNA was extracted from PBMCs using Trizol reagent (Invitrogen, USA) and reverse-transcribed into cDNA using the appropriate reagents, including random hexamers, Superscript II, and dNTPs (TaKaRa Bio, Japan). Using the ABI Prism 7300 Sequence Detection System (Applied Biosystems) the RT-qPCR was performed in a final volume of 20 µl with the SYBRPremix Ex Taq II (TaKaRa Bio, Japan). ADAM33 forward and reverse primers were the 5'-TCTCATGAGCCCAGCAGCCA-3' and the 5'-CAAGCTGCCTGCAGGTGCTG-3'. β-Actin forward and reverse primers were 5'-AGCGA-GCATCCCCCAAAGTT-3' and 5'-GGGCACGAAGG-CTCATCATT-3'. Furthermore, PCR reactions were duplicated for each sample and mean values were used for the further analysis. We used the relative quantification to evaluate the target expression of ADAM33 gene, and the housekeeping gene expression of β-actin was used as an internal control to normalize the target ADAM33 mRNA expression.

Carotid arterial tissue

10 consecutive patients with the internal carotid stenosis (≥70%) treated with carotid endarterectomy at the General Hospital of Shenyang Military Region. Carotid plaques were removed

Table 1. The Characteristics of subjects between the patients with CIMT and control subjects

Variables	Control Subjects (n=310)	Patients with CIMT (n=310)	P
Mean age (years)	64.7±9.6	65.5±10.3	>0.01
Male/Female	193/117	189/121	Match
BMI (kg/m²)	26.1±2.5	26.8±3.2	>0.01
DM (%)	38 (12.2)	78 (25.1)	<0.01
Hypertension (%)	65 (20.9)	180 (58.1)	<0.01
Smokers (%)	56 (18.1)	152 (49.0)	0.01
TG (mmol/l)	2.11±0.98	2.33±1.27	<0.01
TC (mmol/l)	4.33±1.05	4.75±1.09	<0.01
LDL (mmol/l)	2.28±0.56	2.63±0.52	<0.01
HDL (mmol/l)	1.20±0.28	1.53±0.31	<0.01

HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; TC, total cholesterol; TG, triglyceride. BMI, body mass index; DM, diabetes mellitus. Continuous data are expressed as the mean ± SD. P<0.05 versus control group.

from the patients using the carotid endarterectomy and snap-frozen on dry ice and stored at -80°C. Carotid artery tissues containing the atherosclerotic lesions and apparently normal mural areas were collected from the atherosclerosis patients (6 males, 4 females; aged 56-72 years). 4 samples were from normal controls (individuals who died in traffic accidents, with no history of CAD).

Immunohistochemistry

Formalin-fixed, paraffin-embedded carotid arteries were serially sectioned (4 μm). In order to conduct the antigen retrieval we treated the carotid arteries sections with 0.1 mol/l sodium citrate (pH 6.0) (5 min), then rinsed with PBS. After blocking with the avidin-biotin blocking solution, the tissue sections were incubated overnight at the 4°C with the primary antibodies against ADAM33 (Abcam, Cambridge, UK), SMA (Sigma, USA) and CD68 (Dako, Glostrup, Denmark). Then the carotid arteries tissue sections were incubated with the biotinylated goat anti-rabbit or anti-mouse IgG antibodies (Abcam, Cambridge, UK). Immunoreactivity was detected with DAB staining and carotid arteries sections were counterstained with hematoxylin. Furthermore, the digital images were obtained under a light microscope (Leica, Germany).

Statistical analysis

The statistical data (SPSS version 21.0 software) were collected as the means ± SEM. We used the Student's t test or the Mann-Whitney U test to compare among the different groups, and then multiple groups comparision by one-way ANOVA were assessed. The mRNA expression level between CIMT patients and control subjects were also analysed by ANOVA. The distribution of genotype and allele frequencies between CIMT patients and control groups were analysed by the Hardy-Weinberg equilibrium. The CIMT patients were evaluated by the *P* values, 95% confidence intervals (95% CIs) and odds ratios (ORs). The population size of 620 individuals is sufficient to detect a 2% variance explained by a single variant on CIMT at α=0.05 and power of 80%.

Results

Baseline characteristics

The baseline characteristics of the study participants were shown in **Table 1**. CIMT measurements and DNA for genotyping analysis were available for all the collected subjects. Genotype frequencies were in Hardy-Weinberg equilibrium. There were no significant differences between the patients with CIMT and controls subjects in terms of age or sex. The mean age of the patients with CIMT was 65.5±10.3 years and of control subjects was 66.2±9.6 years. The sex male-to-female ratio was 1.65:1 in patients with CIMT and 1.56:1 in control subjects. Compared with the control subjects, patients with CIMT had higher total cholesterol and higher low-density lipoprotein than control subjects (**Table 1**).

Increased ADAM33 expression in human carotid atherosclerosis tissue

Immunostaining of carotid plaques showed that ADAM33 expression was low in normal carotid tissue and ADAM33 expression was increased in the carotid atherosclerotic plaques. ADAM33 expression was observed in vascular smooth muscle cells (VSMCs), and macrophages of human carotid artery atherosclerotic plaques. ADAM33 was colocalized with a proportion of VSMCs (SMA), and macrophages (CD68) in atherosclerotic carotid artery plaques. Furthermore, the VSMCs expressing ADAM33 were predominantly located near the intima-media border, while macrophages expressing ADAM33 were located in the basement of fibrous plaques (**Figure 1**).
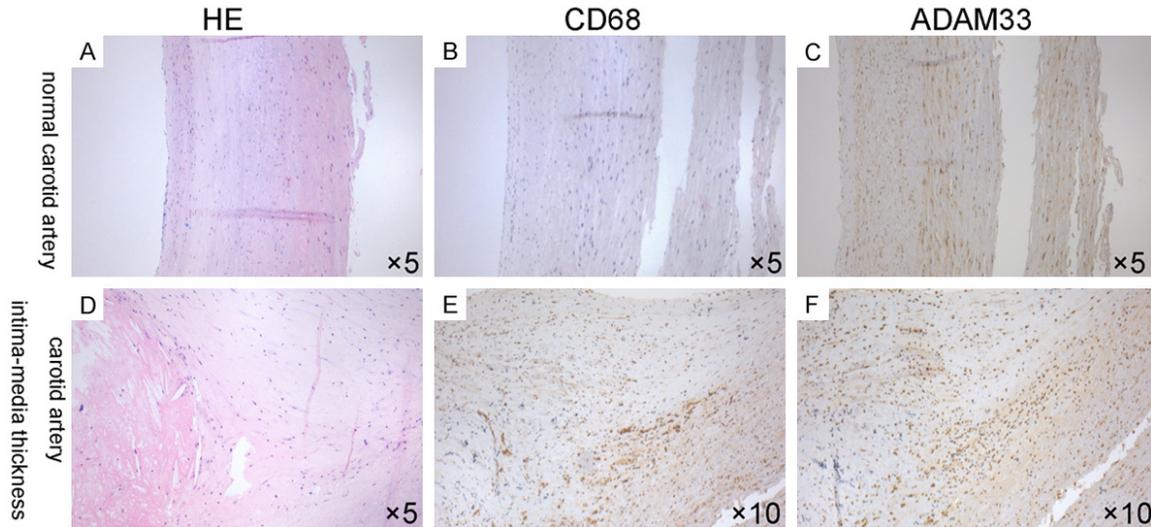
Figure 1. Immunohistochemical staining for ADAM33 and macrophages (CD68) in human carotid arteries of patients treated with carotid endarterectomy. HE Images show the human normal carotid arties (A) and human carotid arteries of patients treated with carotid endarterectomy (D). (B, E) Images showing the presence of macrophages. (C, F) Images showing the ADAM33 expression in the human normal carotid arties and human carotid arteries of patients treated with carotid endarterectomy. (A-C) ×5 magnification. (D-F) ×10 magnification.

Table 2. Frequency of ADAM33 rs514174 genotype and alleles in patients with CIMT and control subjects

Genotype	Control subjects (n=310)	Patients with CIMT (n=310)	OR	95% CI	P
rs514174					
CC	213 (68.7)	190 (61.3)			0.03
CT	86 (27.7)	96 (31.0)			
TT	11 (3.5)	24 (7.7)			
Dominant model CC/CT versus TT	299 (96.4)	286 (92.3)	2.18	1.08-4.37	0.02
Recessive model CC versus CT/TT	97 (31.9)	120 (38.7)	1.24	0.99-1.54	0.03
C allele	512 (82.6)	476 (82.6)	1.43	1.08-1.89	0.01
T allele	108 (17.4)	144 (23.2)			

P values are obtained for the comparison between the patients with carotid artery intima-media thickness and control subjects by χ^2-test; P<0.05.

Association between ADAM33 rs514174 variant and risk of CIMT

CIMT measurements and DNA for genotyping were available from the 620 subjects. There was a significant association between ADAM33 rs514174 variant and CIMT (P=0.01, OR=1.43, 95% CI: 1.08-1.89) (**Table 2**). After the correction for cardiovascular risk factors such as age, sex, body mass index, smoking status, low-density lipoprotein cholesterol and history of arterial hypertension, there was still a statistical significance between the ADAM33 rs514174 variant and CIMT. Individuals with the risk genotype had higher CIMT values than those with the CT or CC genotype (1.41±0.17 and 1.13± 0.14 mm, respectively) (**Figure 2**). Furthermore, increased CIMT risk associated with the rs-514174 genotype and allele was found in the male patients stratified by sex (P<0.05). However, in the female subjects, the association between ADAM33 rs514174 variant and CIMT risk was not statistically significant (**Table 3**).

Plasma ADAM33 mRNA expression in ADAM33 rs514174 variant subsets

Furthermore, we investigated the ADAM33 mRNA expression in PBMCs and explored the association between the ADAM33 mRNA
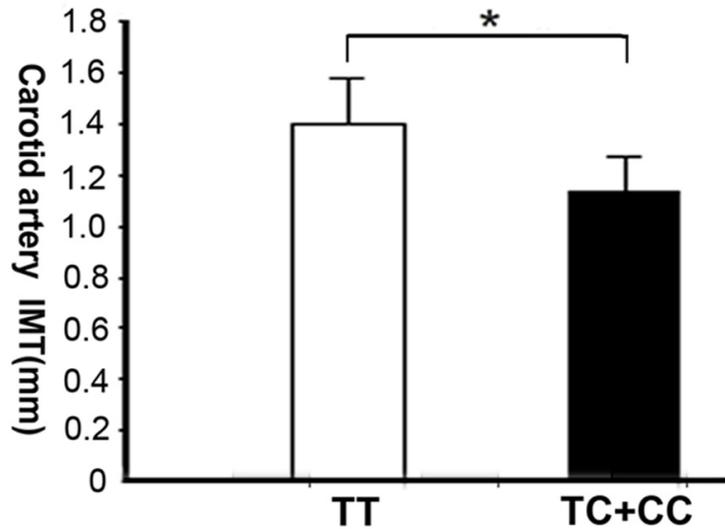
Figure 2. Correlation between carotid artery intima-media thickness and ADAM33 variant rs574174. Carotid artery intima-media thickness data are presented as mean ± SD.

Table 3. Genotype and alleles frequencies of ADAM33 rs514174 in patients with CMIT and control subjects in the female subjects

	Females			
	Control subjects n=117	Patients with CIMT n=121	P value	OR (95%CI)
CC	83 (70.9)	77 (63.6)	0.152	0.718 (0.455-1.132)
CT	28 (23.9)	34 (28.1)		
TT	6 (5.1)	10 (8.3)		
C	194 (82.9)	188 (77.7)	0.449	
T	40 (17.1)	51 (22.3)		

P values are obtained for the comparisons between the patients with CIMT and control subjects by χ^2-test.

domain, transmembrane domain and cytoplasmic domain belongs to the zinc protease superfamily memeber [10, 13, 14]. ADAM33 contains the pro-domain and catalytic domain to regulate VSMC migration, and contains the cytoplasmic domain and EGF-domain to regulate the angiogenesis. ADAM33-null mice does not exhibit the morphological or behavioral abnormalities compared with the wild-type mice, while over-expression ADAM33 mice shows that the ADAM33 protein takes part in the course of atherosclerosis [9, 15-17]. ADAM33 also attaches a crucial part in the endothelial cells adhesion, VSMCs migration and elastic fibers proteolysis in blood vessels, and thus is fundamental for the control of atherosclerotic pathogenensis. Overproduction of ADAM33 may lead to excessive shedding of inflammatory mediators and growth factors, which induces the pathological states such as the proliferation of VSMCs, inflammatory cells migration and endothelial cells adhesion leading to aggravate the cardiovascular atherosclerotic disorders [9, 10].

expression and the ADAM33 rs514174 variant in the CIMT patients. ADAM33 mRNA expression in PBMCs was significantly higher in patients with CIMT than the control subjects (P< 0.01). To investigate whether the ADAM33 rs514174 variant influenced ADAM33 mRNA expression in PBMCs, we examined the relationship between ADAM33 mRNA expression in PBMCs and the ADAM33 rs514174 variant (**Figure 3A**). Patients with the rs514174 TT genotype exhibited the increased ADAM33 mRNA expression in PBMCs when compared with control subjects (P<0.05) (**Figure 3B**).

Discussion

ADAM33 as a membrane-anchored protein containing a pro-domain, catalytic domain, disintegrin domain, cysteine-rich domain, EGF-

CIMT thickening with a carotid artery intima-media thickness greater than 0.9-1 mm as an indicative signal of early atherosclerosis appearing before CAD and stroke formation provides a measure for the early atherosclerosis and vascular remodeling, so the examination of CIMT is particularly important for the early atherosclerotic detection [4, 11]. Genetic evidences have also found the association between the ADAM33 variant and the occurrence of CAD. Our study showed that ADAM33 rs514-174 variant is associated with carotid artery intima-media thickness for the first time in the CIMT patients in Chinese Han population. Our findings provide the further support that ADAM33 rs514174 TT genotype carriers have a higher CIMT than those with C allele carriers among CIMT patients. Thus, ADAM33 appears
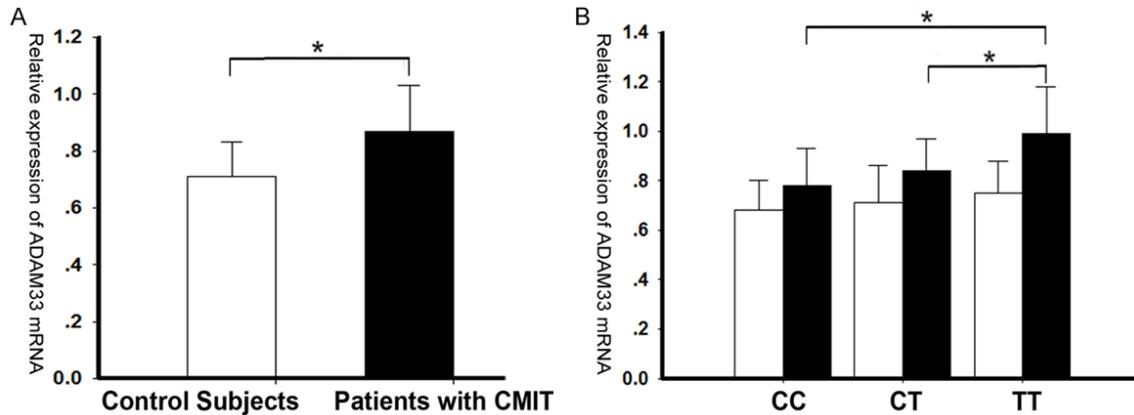
Figure 3. ADAM33 mRNA expression in PBMCs. A. Relative ADAM33 mRNA expression in PBMCs. from patients with CIMT and control subjects. B. Relative ADAM33 mRNA expression in PBMCs from patients with CIMT and control subjects stratified according to the presence of the ADAM33 variant rs574174 allele. Blank and black boxes represent the relative expression of ADAM33 in control subjects and patients with PBMCs, respectively. *, P<0.05.

to be an important candidate gene to explain the early atherosclerosis in CAD and stroke patients. Moreover, the association was more significant in males CIMT patients. However, the association between ADAM33 variants and CIMT requires the further verification with a larger sample population. We also found that ADAM33 was preferentially expressed on SMCs and macrophages in atherosclerotic carotid arteries plaques compared with the control carotid artery, suggesting that ADAM33 may attach an important part in the development of atherosclerotic plaque.

There were some limitations to this study. First, our sample size was large enough, so the conclusions cannot be extended to the whole Chinese Han population. Second, the recruited population was from the northern region of China, thus the results may not be applicable to other ethnic groups, and need to be confirmed in other ethnic groups. Third, larger prospective studies were necessary to fully elucidate the role of ADAM33 in the process of atherosclerosis. Fourth, the mechanisms underlying the associations between the ADAM33 rs514174 variant and CIMT risk require further investigation.

In summary, our study shows a significant association between the ADAM33 rs514174 variant and increased risk of CIMT in the Chinese Han population. Genetic ADAM33 variation is therefore a promising new candidate in the pathogenesis of atherosclerosis. The current study provides the new information about the possible role of ADAM33 in the development of CIMT. Future studies are required to further determine the role of ADAM33 in the pathogenesis of atherosclerosis and to examine its prognostic and therapeutic potential.

Disclosure of conflict of interest

None.

Abbreviations

CIMT, carotid intima-media thickness; CI, confidence interval; OR, odds ratio; PCR, polymerase chain reaction; SNP, single nucleotide polymorphism.

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