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Correlation between carotid atherosclerotic plaque properties and serum levels of lncRNA CCAT2 and miRNA-216b

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Abstract. – OBJECTIVE: The purpose of this study was to uncover the clinical values of serum long non-coding RNA (lncRNA) CCAT2 and miRNA-216b in the properties of carotid atherosclerotic plaques.

PATIENTS AND METHODS: Patients with carotid atherosclerotic plaques were assigned into stable plaque group (n=60) and unstable plaque (n=75) group based on their examination results of cervical contrast-enhanced CT examination. Maximal plaque thickness (MAPT) and intima-media thickness (IMT) in each group were determined. Serum levels of lncRNA CCAT2 and miRNA-216b in patients with carotid atherosclerotic plaques were detected by Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR). Moreover, the correlation between serum levels of CCAT2 and miRNA-216b was analyzed by Pearson's correlation analysis. Besides, potential correlations of serum levels of CCAT2 and miRNA-216b with MAPT and IMT in patients with carotid atherosclerotic plaques were assessed as well.

RESULTS: Results revealed that MAPT and IMT were markedly higher in unstable plaque group than those in stable plaque group. Serum level of CCAT2 was higher in unstable plaque group, while miRNA-216b was lower than those in stable group. A negative correlation was identified between serum levels of CCAT2 and miRNA-216b. In addition, CCAT2 level was positively correlated with IMT and MAPT in patients with carotid atherosclerotic plaques, while miRNA-216b level was negatively correlated with them.

CONCLUSIONS: Detection of serum levels of CCAT2 and miRNA-216b could be applied for predicting the rupture of carotid atherosclerotic plaques. They may be potential biomarkers predicting ischemic stroke.

Key Words:
Carotid atherosclerosis, LncRNA CCAT2, MiRNA-216b, IMT, MAPT.

Introduction

Carotid atherosclerosis is a non-inflammatory pathology in arterial walls, accompanied by intravascular and extracellular lipid accumulation in endovascular cells, thickening of arterial walls and stenosis of arterial lumen[1]. As a part of systemic atherosclerosis, carotid atherosclerosis is considered as the screening window for atherosclerosis. Seriously, the rupture of atherosclerotic plaques will lead to acute thromboembolic diseases, which is the major reason for fatal cardiovascular events.

Long non-coding RNAs (lncRNAs), non-coding RNAs with over 200 nucleotides long, are extensively distributed in organisms. Most lncRNAs are located in the nucleus, and only 15% of them are distributed in the cytoplasm[2]. Functionally, lncRNAs are closely involved in disease development by regulating epigenetics, X chromosome silencing, genomic imprinting, etc. In recent years, vital functions of lncRNAs in carotid atherosclerosis have been identified[3,4]. LncRNA CCAT2 locates on human chromosome 8q24, which is highly expressed in many types of tumors[5,6], and it is involved in the regulation of tumor cell phenotypes. However, the specific function of CCAT2 in carotid atherosclerosis remains unclear.

MiRNAs are small, non-coding RNAs with 22 nucleotides long. They are capable of regulating gene expressions by binding 3'UTR of mRNA, thus degrading them or inhibiting their translation[7,8]. MiRNAs are widely involved in cholesterol metabolism, phenotype transformation of smooth muscle cells, angiogenesis and inflammatory responses in the pathogenic process of atherosclerosis[9]. It is reported that[10] miRNA-216b serves as an anti-tumor role in laryngeal carci-

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noma. MiRNA-216b suppresses proliferative and invasive abilities, and arrests cell cycle progression in nasopharyngeal carcinoma by targeting K-Ras. In this paper, serum levels of CCAT2 and miRNA-216b in patients with carotid atherosclerosis were detected, and their clinical potentials are uncovered to be biomarkers.

Patients and Methods

Patients

A total of 135 patients with carotid atherosclerosis treated in the First Affiliated Hospital of Wenzhou Medical University from April 2016 to December 2018 were retrospectively analyzed, including 75 cases of unstable plaques and 60 cases of stable plaques. Patients complicated with unilateral or bilateral carotid artery occlusion were excluded. In the meantime, 60 healthy subjects undergoing physical examination during the same period were enrolled as controls. Enrolled patients underwent cervical contrast-enhanced CT examination for identifying the stability of plaques. Patients and their families have been fully informed. This investigation was approved by the Ethics Committee of the First Affiliated Hospital of Wenzhou Medical University.

Sample Collection

All subjects were fasting for food for 12 h. Elbow vein blood was collected, centrifuged at 4°C, 3000 r/min for 10 min. The supernatant was further centrifuged at 4°C, 3000 r/min for 10 min. Then, the supernatant serum sample was subpacked in non-RNA enzyme cryogenic vials, and stored at –80°C.

Cervical Contrast-Enhanced CT Examination

Cervical contrast-enhanced CT examination was conducted by 64-slice spiral CT (Philips Brilliance). Then, 80 mL of non-ionic contrast agent iopromide was administrated into the antecubital vein at the flow rate of 5 mL/s, and the site of examination was 2 cm proximal to carotid bifurcation and 1 cm distal to carotid bifurcation. Next, local intima-media thickening of arterial wall $\geq$ 1.3 mm, with or without stenosis of arterial wall or abnormal blood flow signals was identified to be atherosclerotic plaques. Based on the characteristics of the thickest bilateral plaques, they were divided into low, mixed and high echo. Among them, high echo was suggested as stable plaques, and low and mixed echo were considered as unstable plaques.

Determination of IMT and MPAT

On the image of the longitudinal section of the carotid artery, IMT of the carotid artery was the distance between the two high echo lines on the posterior side wall. IMTs on the common carotid artery (2 cm proximal to the carotid bifurcation), bifurcation and internal carotid artery (1 cm distal to the carotid bifurcation) were detected. After that, the average IMT was calculated from bilateral records of 6 IMTs. The total thickness of plaques was calculated by carotid artery imaging, and the average Ct, the minimum Ct, and the maximum Ct (MAPT) were recorded.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

TRIzol method (Invitrogen, Carlsbad, CA, USA) was applied for isolating RNAs from serum samples. Through reverse transcription of RNA, the extracted complementary deoxyribose nucleic acid (cDNA) was used for PCR detection by SYBR Green method (TaKaRa, Otsu, Shiga, Japan). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the internal reference. The primer sequences were listed as follows: CCAT2, forward: 5'-CCCTGGTCAAATTGCTTAACCT-3', reverse: 5'-TTATTCGTCCCTCTGTTTTATGGAT-3'; GAPDH, forward: 5'-GCTCTCTGCTCCTCCTGTTC-3', reverse: 5'-ACGACCAAATCCGTTGACTC-3'; miRNA-216b, forward: 5'-GCCGCGCTAAAGTGCTTA-3', reverse: 5'-CACCAGGGTCCGAGGT-3'; U6, forward: 5'-TGCGGGTGCTCGCTTCGGC-3', reverse: 5'-CCAGTGCAGGGTCCGAGGT-3'.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 20.0 (IBM, Armonk, NY, USA) was used for all statistical analysis. Data were expressed as mean ± SD (standard deviation). The *t*-test was used for analyzing differences between two groups. Pearson's correlation analysis was performed to assess the relationship between two variables. $p<0.05$ indicated the significant difference.

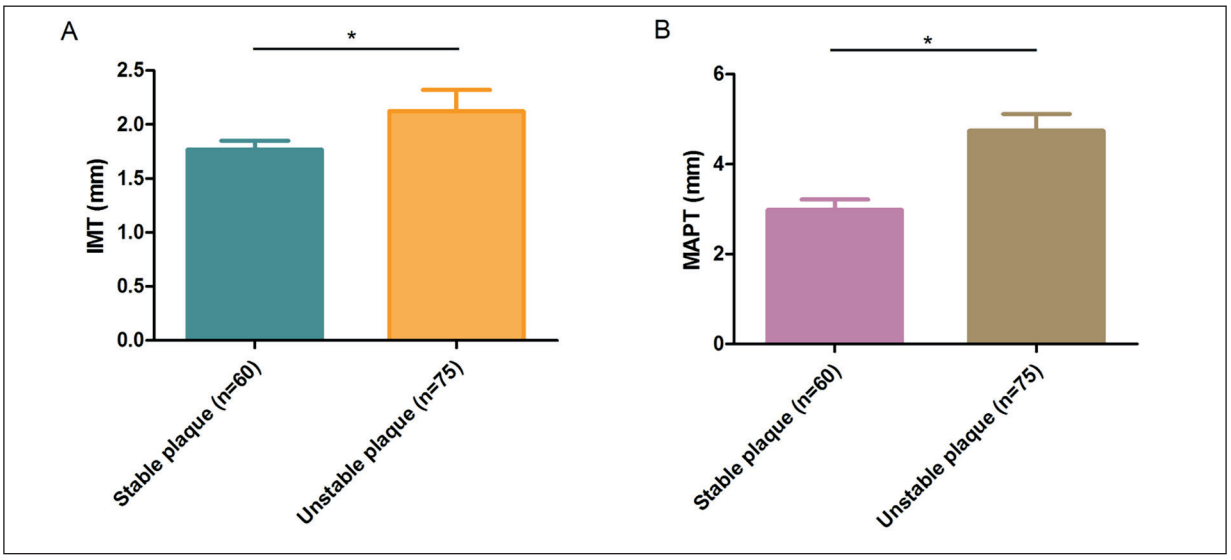

Figure 1. IMT and MAPT in patients with carotid atherosclerosis. IMT (**A**) and MAPT (**B**) in stable plaques (n=60) or unstable plaques (n=75).

Results

IMT and MAPT In Patients With Carotid Atherosclerosis

Based on results of cervical contrast-enhanced CT examination, a total of 60 cases of stable plaques and 75 cases of unstable ones were collected. Compared with stable plaques, IMT (Figure 1A) and MAPT (Figure 1B) were higher in unstable plaques, demonstrating that IMT and MAPT are capable of identifying the stability of carotid atherosclerotic plaques.

Serum Levels of CCAT2 and miRNA-216b In Patients With Carotid Atherosclerotic Plaques

Serum levels of CCAT2 and miRNA-216b were detected in patients with carotid atherosclerotic plaques. Compared with that in controls, CCAT2 was highly expressed in carotid atherosclerotic plaques, especially unstable ones (Figure 2A). Conversely, miRNA-216b level was reduced in unstable plaques than those of stable ones (Figure 2B).

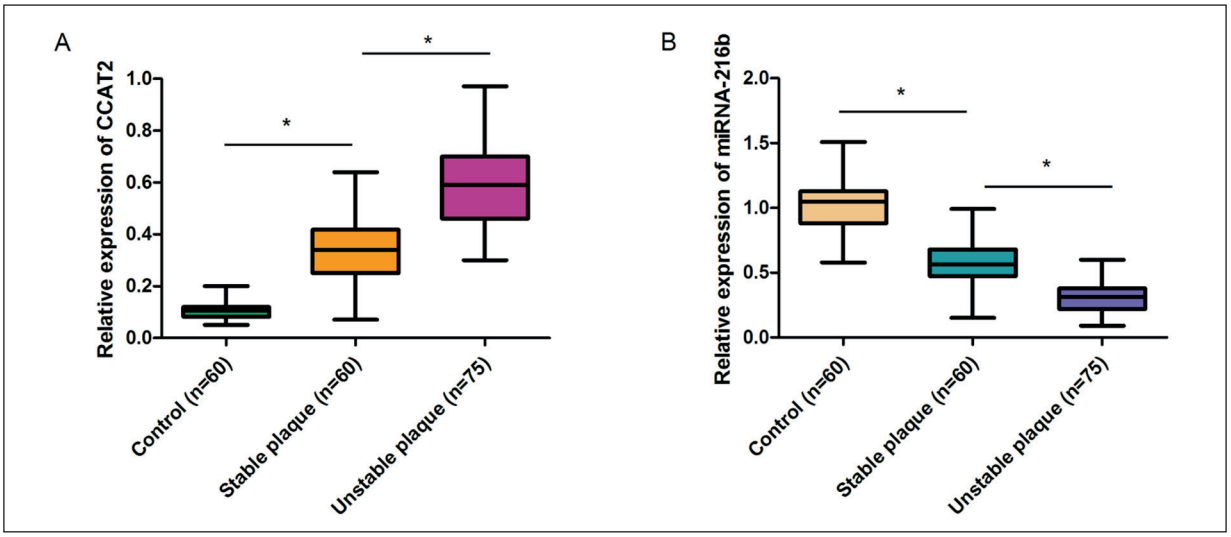

Figure 2. Serum levels of CCAT2 and miRNA-216b in patients with carotid atherosclerotic plaques. Serum levels of CCAT2 (**A**) and miRNA-216b (**B**) in controls (n=60), stable plaques (n=60) or unstable plaques (n=75).

Correlation Between Serum Levels of CCAT2 and MiRNA-216b

Pearson's correlation analysis was conducted to assess the correlation between CCAT2 and miRNA-216b level. It is revealed that serum level of CCAT2 was negatively correlated with that of miRNA-216b in patients with carotid atherosclerotic plaques (r=-0.3441, p<0.001, Figure 3).

Correlations of Serum Levels of CCAT2 and miRNA-216b With IMT and MAPT

Correlations of serum levels of CCAT2 and miRNA-216b with stability of carotid atherosclerotic plaques were evaluated. It was found that CCAT2 level was positively correlated to IMT (Figure 4A) and MAPT (Figure 4B). Conversely, miRNA-216b level was negatively correlated to them (Figure 4C, 4D). It is suggested that high level of CCAT2 and low level of miRNA-216b were risk factors of carotid atherosclerotic plaque rupture.

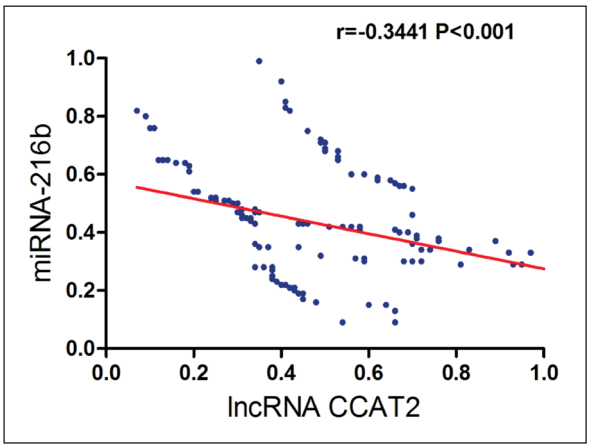

Figure 3. Correlation between serum levels of CCAT2 and miRNA-216b.

Discussion

Atherosclerosis is a chronic vascular disease characterized as inflammation and remodeling of

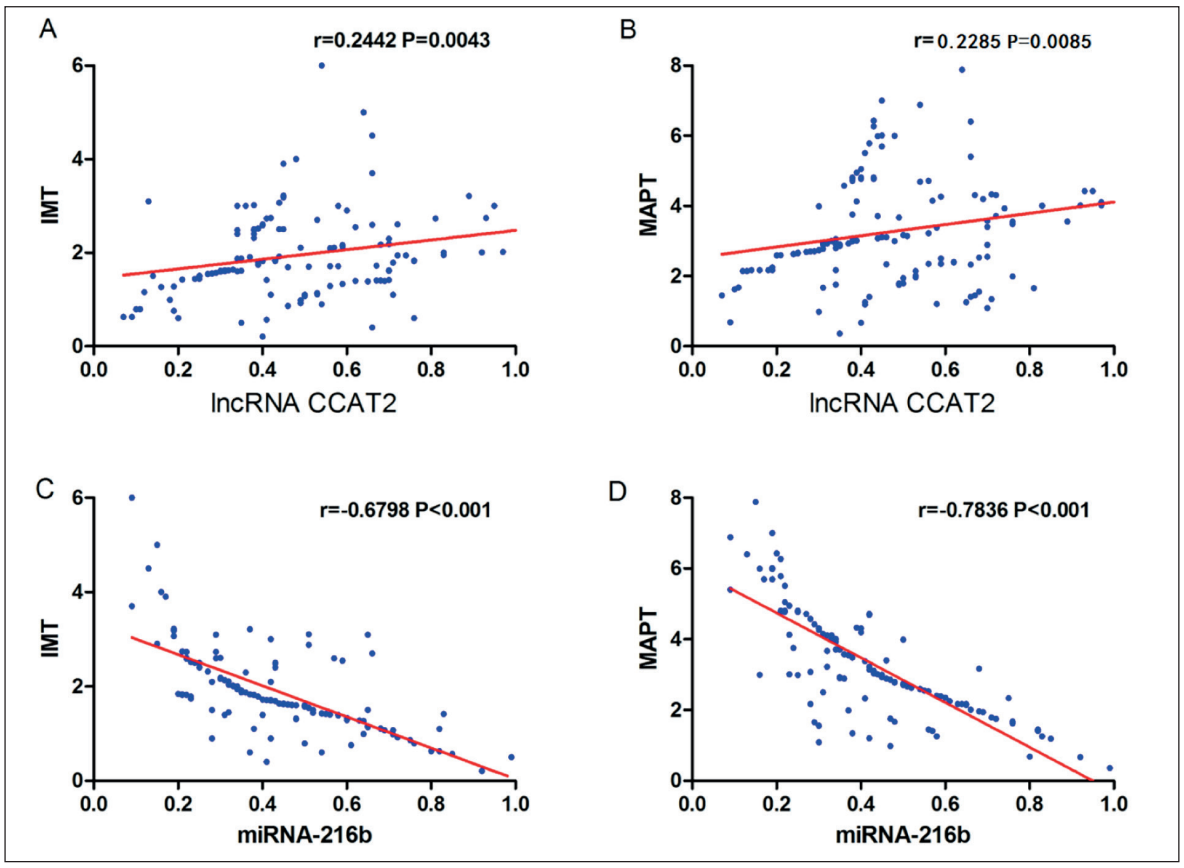

Figure 4. Correlations of serum levels of CCAT2 and miRNA-216b with IMT and MAPT. **A,** Correlation between serum level of CCAT2 and IMT. **B,** Correlation between serum level of CCAT2 and MAPT. **C,** Correlation between serum level of miRNA-216b and IMT. **D,** Correlation between serum level of miRNA-216b and MAPT.

vascular wall. Atherosclerotic plaques are easily formed at the beginning or bifurcation of the carotid artery. Carotid atherosclerosis is usually considered to evaluate ischemic cerebrovascular events. The pathogenesis of atherosclerosis is complicated, involving activated epithelial cells by blood flow disorder and oxidation and fibrosis of vessel walls[11]. Lipid metabolism balance is an important risk factor for atherosclerosis. Accumulation of lipids in the inner wall of the artery contributes to the formation of atherosclerotic plaques. Once plaques are ruptured, acute cardiovascular events could be fatal[12].

LncRNA CCAT2 was initially identified by Ling et al[13]. CCAT2 locates in a highly conserved region enriched with regulatory elements markers. In cervical cancer and hepatocellular carcinoma, CCAT2 is considered as a prognostic hallmark predicting a poor prognosis[5,14]. Analysis of this study manifested that CCAT2 was upregulated in carotid atherosclerotic plaques, especially those unstable plaques.

MiRNA-216b is lowly expressed in tumors as a tumor-suppressor gene[15,16]. Deng et al[10] demonstrated that miRNA-216b is downregulated in nasopharyngeal carcinoma, and overexpression of miRNA-216b can suppress the growth and invasiveness of tumor cells. Liu et al[17] suggested that overexpression of miRNA-216b remarkably inhibits liver cancer cells to proliferate and metastasize. In cervical cancer, lowly expressed miRNA-216b promotes proliferative ability[18]. The findings of this research revealed that miRNA-216b was downregulated in carotid atherosclerotic plaques. MiRNA-216b level was lower in unstable plaques compared with those of stable ones.

The interaction between lncRNAs and miRNAs is of significance during the development of atherosclerosis[19]. Gao et al[20] pointed out that lncRNA H19 is a highly risk factor for coronary heart disease. Let-7 is able to alleviate oxidative stress-induced injury on vascular smooth muscle, which could suppress autophagy and apoptosis of cells[21]. Cao et al[22] uncovered that H19 absorbs let-7 as a miRNA sponge, thus regulating biological functions of vascular smooth muscle. In this paper, serum levels of CCAT2 and miRNA-216b were found to be negatively related in patients with carotid atherosclerotic plaques. Notably, both CCAT2 and miRNA-216b were correlated with IMT and MAPT, suggesting their potentials as indicators predicting carotid atherosclerosis. The novelty of this study was that we investigated for the first time the expression of lncRNA CCAT2 and miR-216b in the serum of patients with atherosclerosis, and the regulatory relationship between the two in the pathogenesis of patients, and also evaluated the formation and rupture of carotid atherosclerotic plaques. These findings laid the foundation for further mechanism research.

Conclusions

The above findings indicated that detection of serum levels of CCAT2 and miRNA-216b could be applied for predicting the rupture of carotid atherosclerotic plaques. They may be potential biomarkers predicting ischemic stroke.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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