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SMAD7 methylation as a novel marker in atherosclerosis

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ABSTRACT

Atherosclerosis is a complicated process comprising inflammation, accumulation of collagen matrix and aberrant DNA methylation. SMAD7 is known to play an important role in fibrosis and inflammation. In recent years, increasing research has concentrated on the connection between DNA methylation and atherosclerosis. The current study was designed to investigate methylation status of some specific gene with a focus on *SMAD7* in atherosclerosis and elucidate their relationship. We found that SMAD7 expression was decreased and its promoter region was markedly methylated in atherosclerotic plaques when compared with normal artery walls. Using MALDI-TOF MS, increased DNA methylation levels of *SMAD7* promoter at CpG unit 5.8.15.16 were found in peripheral blood of atherosclerosis patients relative to matched normal controls, respectively. Correlation analysis revealed that mean DNA methylation levels of *SMAD7* promoter of CpG unit 5.8.15.16 were positively associated with homocysteine levels ($r = 0.724$, $p < .001$) and carotid plaque scores($r = 0.790$, $p < .001$). *SMAD7* promoter is hyper-methylated both in human atherosclerotic plaques and atherosclerosis patients, which is positively associated with homocysteine levels and carotid plaque scores. Thus, methylated *SMAD7* may be a novel predicted marker and therapeutics target for atherosclerosis.

1. Introduction

Atherosclerosis is a common health problem and a considerable cause of cardiovascular morbidity and mortality, which is a complicated process comprising inflammation, collagen matrix accumulation and aberrant DNA methylation [1–3]. In the post-genomic era, more and more attention is paid to the relationship between DNA methylation and atherosclerosis [4]. Homocysteine (Hcy), which is an important independent risk factor of atherosclerosis [5], is involved in the methionine cycle and transfer methylation courses, thus interfering with DNA methylation and regulating gene expression [6]. The disturbance of DNA methylation has been observed in the Hcy-induced atherosclerosis, which shows hypo-methylated global DNA but hyper methylation of some specific genes [7–10].

SMAD7, a downstream inhibitory SMAD in TGF-β signaling, participates in lots of biological processes, including tumorigenesis, fibrosis and inflammation [11,12]. *SMAD7* can be epigenetically modified via DNA methylation and reduced SMAD7 expression was found in hepatic myofibroblasts and rat fibrotic livers attributed to hyper methylation of its promoter [13]. It has been demonstrated that enhanced TGF-β signaling led to significantly larger

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atherosclerotic lesions that contained collagen-rich caps in SMAD7-deficient mice [14]. Study also revealed that expression of *SMAD7* in peripheral blood mononuclear cells was decreased in patients with coronary heart disease [15].

Therefore, in the present study, we test the hypothesis that *SMAD7* promoter is hyper-methylated in atherosclerosis and there is relationship between atherosclerosis and *SMAD7* methylation induced by hyperhomocysteinemia.

2. Materials and methods

2.1. Study population and sample collection

Seventy-one persons were recruited from the First Affiliated Hospital of Guangxi University of Science and Technology between September 2015 and October 2016, including 45 atherosclerosis cases (33 males and 12 females, 68.2 ± 1.5 years) and 26 normal controls (19 males and 7 females, 67.2 ± 1.8 years) who were age- and gender-matched. The atherosclerosis patients had atherosclerotic plaques confirmed by carotid ultrasound and they were excluded some other diseases such as cancer, connective tissue disease, endocrine and metabolic disorders, serious liver disease and renal failure. Normal controls came from health checked individuals without thickening of carotid artery intima-media and formation of atherosclerotic plaques verified by carotid ultrasound. This research was approved by the ethics committee of the hospital and conforms to the Declaration of Helsinki. Informed consent was collected from all subjects enrolled in the project. Blood samples were obtained by the venous puncture using tubes coated with EDTA or not in all subjects maintaining a fasted state in the morning. Peripheral whole blood samples were stored at −80 °C for DNA extraction. Serum was prepared for testing the levels of total homocysteine(tHcy). In addition, paraffin-embeded atherosclerotic coronary artery or aortic tissues from autopsy (4 males and 1 female, ranging in ages from 37 to 67) were collected from the Department of Pathology, School of Medicine, Guangxi University of Science and Technology, according to the guidelines approved by the ethics committee.

2.2. Carotid ultrasound

Bilateral carotid arteries including internal carotid arteries, carotid bulbs and common carotid arteries were measured using B-mode ultrasound (Logiq S7, GE Healthcare, America) with a 5.0–8.3 MHz linear probe. Checking indexes contained carotid intima-media thickness (IMT) and atherosclerotic plaques score. Normal IMT was restricted as less than 1.0 mm and IMT which was more than 1.1 mm exhibiting a focal abnormal wall thickness was defined as carotid plaque. Carotid plaque score was defined as the sum of the maximal thicknesses rather than lengths of all plaques in bilateral carotid arteries in the scanning area [16].

2.3. Homocysteine assay

The levels of tHcy in the serum of the participants were detected by cyclic enzyme method using Homocysteine Assay Kit (Maccura, China), according to the manufacturer's protocol.

2.4. Immunohistochemistry staining

After dewaxing and blocking, the paraffin-embeded atherosclerotic vascular sections underwent microwave-based antigen retrieval. After that, the sections were stained with mouse monoclonal anti-bodies against SMAD7 (Abcam, Cambridge, UK) at 4 °C overnight, followed by incubating with goat anti-mouse antibodies (ZSGB-BIO, China). The percentage of positive staining for SMAD7 was measured by using a quantitative image-analysis system (Image-Pro Plus 6.5, Media Cybernetics, Silver Spring, MD).

2.5. DNA extraction and bisulfite treatment

Genomic DNA was respectively extracted from paraffin-embeded arterial tissues and peripheral blood using DNA Extraction Kit (BioTeKe, China), following the instructions recommended by manufacturer. The quality and concentration of DNA were assessed by gel electrophoresis and a NanoDrop 2000 spectrophotometer (Thermo Scientific, America). Bisulfite conversion of genomic DNA was performed with EpiTect Bisulfite Kit (Qiagen, Switzerland), according to the manufacturer's guidelines.

2.6. Methylation-specific polymerase chain reaction (MSP)

Methylation degrees of Smad 7 gene promoters from paraffin-embeded arterial tissues were determined by MSP. The designed primer sets contained the eighth and the fifteenth CpG sites (Fig. 1), which were the methylated ones (*SMAD7* -M, forward: 5′-TTGCGAGAGTTTTTTTATTTTGTTAGAC-3’; reverse: 5′- CTCTATATCCTTAGTAACCGAATCTCCT -3′) and the unmethylated ones (*SMAD7* -U, forward: 5′- TTGTGAGAGTTTTTTTATTTTGTTAGAT -3’; reverse: 5′- CTCTATATCCTTAGTAACCAAATCTCCT -3′). The polymerase chain reactions with bisulfite-modified DNA were carried out under such conditions:95 °C for 5 min, followed by 35 cycles of 95 °C for 30 s, 64 °C for 30 s, and 72 °C for 30 s, with a final extension at 60 °C for 30 min. The methylated (M) and unmethylated (U) PCR products were detected by gel electrophoresis. Relative intensity of bands was quantified with Image J (National Institutes of Health, Bethesda, MD, USA) and ratios (M/U) were calculated.

2.7. Primer design and Sequenom MassArray analysis

Methylation levels of Smad 7 gene promoters of peripheral blood samples were analyzed with Sequenom MassArray. We used EpiDesigner to design the primers for *SMAD7* gene [17]. The designed primers covered most *SMAD7* promoter region with lots of CpG sites. The amplified region was located nearby the transcription start site, from −619 to −196 (Fig. 1). For PCR reaction, each reverse primer had a T7 promoter tag (5′-cagtaatacgactcactatagggagaaggct- 3′) and the forward primer contained a 10-mer tag (5′-aggaagagag-3′) (Table 1). The genomic DNA treated with bisulfite was amplified in such conditions: denaturation for 4 min at 94 °C, followed by 45 cycles of 94 °C for 20 s, 56 °C for 30 s, and 72 °C for 60 s, with a final extension for 3 min at 72 °C. The products then underwent SAP treatment and T-cleavage assay followed by being mixed with Clean Resin and transferred to SpectroCHIP by Nanodispenser. Then, DNA methylation was quantified by Sequenom MassArray [18] and spectra of methylation ratios were analyzed with EpiTYPER system (Sequenom).

2.8. Statistical analyses

Data obtained from this study were expressed as mean ± SEM. Statistical analyses were performed by using SPSS version 16.0 software (SPSS Inc., Chicago, USA). Student's *t*-test was applied for two-group comparison. The associations between *SMAD7* methylation percentages and levels of tHcy, carotid plaque scores were performed with correlation analysis, respectively.