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Functional Study of -724I/D Polymorphism in Apolipoprotein M (apoM) Gene Promoter Region and its Association with Myocardial Infarction

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
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Background: The aim of this study was to detect the function of -724I/D polymorphism in the apolipoprotein M (apoM) gene promoter region and to determine its relationship with myocardial infarction (MI).

Material/Methods: We selected 309 patients with MI and 309 healthy controls for this case-control study. The PCR products of the apoM gene promoter region were directly sequenced to analyze the -724I/D polymorphism. Differences in frequency distributions of genotype and allele were compared between the MI group and the control group. We used gene recombination and site-directed mutagenesis technique to observe the impact of -724 I/D on transcription activity of apoM gene promoter *in vitro*.

Results: The allele frequency of the -724Del in the MI group was higher than that in the control group (9.5% *vs.* 3.2%, OR=3.156, 95% CI (1.876~5.309), P<0.001). Compared to the I/I genotype carriers, the apoM levels decreased but the total cholesterol (TC) levels increased significantly in the -724Del allele carriers in plasma. The activity of apoM I/I genotype promoter decreased significantly after the deletion mutation at -724 position in apoM gene.

Conclusions: -724 I/D polymorphism decreases the apoM promoter activity, down-regulates the apoM protein expression level, and increases the risk of MI.

MeSH Keywords: Amplified Fragment Length Polymorphism Analysis • Apoprotein(a) • Coronary Artery Disease

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Background

Apolipoprotein M (apoM) is one of the components of high-density lipoproteins (HDL) [1,2]. Previous studies have confirmed that apoM is a prerequisite for the formation and maturation of pre-β-HDL, whose main function is to promote intracellular cholesterol efflux. Therefore, lack of apoM significantly affects the reverse transport of cholesterol *in vivo* and causes atherosclerosis [2,3].

The promoter region is the "switch" for gene expression. The single-nucleotide polymorphism (SNP) locating on this region may directly influence the expression level of the protein *in vivo*. Some scholars have studied part of the SNPs in the apoM promoter region [3–7]. In the Chinese Han population, mutation of T-778C in the apoM promoter region is related to plasma cholesterol and fasting serum glucose and may increase the risk of type 2 diabetes mellitus [8]. Further studies have found that apoM T-778C may be related to the susceptibility to rheumatoid arthritis [6] and MI [5]; Xu et al. [9] have demonstrated the T-855C in apoM promoter region increases the risk of MI in the Han population. MI is not a conclusive genetic disease but does have an obvious familial tendency. Mutation in the apoM gene promoter region may influence the expression of apoM, further affecting the metabolism of lipoprotein (especially cholesterol and HDL) and thereby increasing the susceptibility to MI [9]. Zheng et al. [10] have recently found a new polymorphism, -724Ins/Del, in the apoM gene promoter region. The present study aimed to investigate the function of this polymorphic locus and its association with MI in a Chinese Han population.

Material and Methods

Subjects

The present study was reviewed and approved by the Ethics Study Board of Jinling Affiliated Hospital of the Second Military Medical University. Informed written consent was obtained from all subjects before enrollment and 309 patients with MI and 309 healthy controls were enrolled in this study. Diagnosis for patients with MI [214 males and 95 females, mean age 60.3±9.5 years old] was consistent with the MI diagnostic criteria of the World Health Organization (WHO) [11]. Each patient was confirmed to have more than 50% stenosis in at least one blood vessel according to the results of coronary angiography. We selected 309 healthy controls [210 males and 99 females, mean age 60.1±9.2 years old] who were either healthy people who passed a medical examination or those who were confirmed to be without pathological change in the coronary artery according to the results of coronary angiography. All the above subjects were unrelated Han people without cancer and systemic liver and kidney diseases from Jinling Affiliated Hospital of the Second Military Medical University. The characteristics of the two groups are shown in Table 1.

Methods

We obtained informed consent from all subjects before they were enrolled in the study. Their individual general conditions, previous medical illnesses, family history, and smoking and drinking history were recorded in detail. Their body weight, height, and blood pressure were measured and the body mass index (BMI) was calculated according to: body mass (kg)/height^2 (m^2). After fasting for 12 hours, 2–5 ml venous blood was collected to detect triglyceride (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), fasting blood glucose (FBG), and other biochemical indicators utilizing the biochemical analyzer.

Detection of the apoM -724 ins/del polymorphism

We collected 2 ml of peripheral blood [treated with ethylene diamine tetra-acetic acid (EDTA)] to extract genomic DNA using a DNA purification kit from Shanghai Biological Technology Company (Shanghai, China). The genomic DNA were dissolved in the TE solution and then stored at –20°C. Primers for the apoM promoter region were designed according to the literature [10] and the PCR products were directly sequenced to identify their genotypes using a DNA sequencer from Shanghai Ying Chun Company (ABI 3730, Shanghai, China).

Table 1. Characteristics of participants.

Groups	N	Age (years)	BMI Kg/m^2	Hypertension (n, %)	Diabetes (n, %)	Smoking (n, %)	TG (mmol/L)	TC (mmol/L)	HDL-C (mmol/L)	LDL-C (mmol/L)
MI group	309	60.3±9.5	25.1±3.3	118 (38.2)	123 (39.8)	115 (37.2)	3.6±1.4	5.6±2.1	1.0±0.9	3.6±1.3
Control group	309	60.1±9.2	24.5±3.2	34 (11.0)	46 (14.9)	45 (14.6)	1.6±1.1	4.3±0.9	1.5±0.8	2.4±0.9
P		0.542	0.041	<0.001	0.001	<0.001	<0.001	<0.001	<0.001	<0.001

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Detection of plasma apoM levels in patients with different genotypes

The plasma apoM levels in patients with different genotypes were detected by Western blot analysis: We diluted the plasma at a ratio of 1:25, mixed them with 4× sample loading buffer at a volume ratio of 3:1 and took a 16-ul denatured protein sample for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) detection; electrotransferred the protein bands to the PVDF membrane and blocked them for 1 h at room temperature; added homemade rabbit anti-human apoM polyclonal antibodies and incubated them overnight at 4°C; added horseradish peroxidase-labeled antibody as the secondary antibody and incubated them for 1 h at room temperature; finally completed sensitization, developing and fixing using the Pierce ECL Western Blotting Kit. The results were analyzed using Quantity One software (Version 4.6.2), taking gradation × area (INT × mm^2) as a unit.

Detection *in vitro* of the apoM promoter activity

Construction of recombinant plasmid (pGL3-apoM) carrying apoM promoter gene and luciferase reporter gene

According to the method provided by Zheng et al. [10], recombinant plasmid (pGL3-apoM) carrying apoM promoter gene and luciferase reporter gene was constructed to further clarify the impact of the SNP sites on apoM promoter activity, summarized as follows: One pair of primers were designed according to the apoM promoter gene sequences (-2165~-29): Sense primer: 5'ggggtaccGTGGCGCAATCACAACTC-3 '; antisense primer: 5'ccaagcct GACCCTITCACCTGCTAATG-3'. Kpn I and HindIII restriction sites (underlined bases) with appropriate protective bases were introduced to the sense and anti-sense primers, respectively. The 25 ul PCR reaction system contained 40~80 ng genomic DNA template, 2.5 ul 10× PCR reaction buffer, $MgCl_2$, 0.5 ul 4× dNTPs (1.5 mmol/L), 1.25 U Taq DNA polymerase, and 10 pmol primers. The above reaction system was first pre-denatured for 1 min at 95°C, and then followed by 40 cycles of 95°C for 10 s, 60°C for 90 s, and 72°C for 2 min. To obtain the recombinant plasmid, the PCR products were double- digested by the Kpn I/Hind III and then directly cloned into pGL3-basic vector (Promega) carrying a luciferase reporter gene with the help of T4 DNA ligase. The recombinant plasmid was confirmed as -724 position wild-type homozygotes (Ins/Ins genotype) using the digesting and sequencing method. Recombinant plasmid carrying apoM promoter gene with -724 position deletion mutant and luciferase reporter gene was constructed from the wild-type homozygotes (Ins/Ins genotype) using site-directed mutagenesis and finally identified as -724 position deletion mutant homozygotes (del/del genotype) by PCR.

Cell culture, transfection, and detection of luciferase activity

Human embryonic kidney cell line 293T cells (HEK293T) were cultured in RPMll640 (Gibco) medium containing 10% fetal bovine serum. When the cells were growing in good condition, we adjusted the cell density to 1×10^6/well in the 6-well cell culture cluster, rinsed the cells with serum-free and antibiotic-free RPMI 1640 culture solution, transfected 2 ug of plasmid DNA to the cells with the help of Lipofectamine 2000 in each well, and incubated them for 12 h. Then we added 1.2 m1 RPMI 1640 culture solution containing 10% fetal bovine serum to each well, gently mixed them, and incubated them for another 48 h. Finally, we collected the cells to detect the luciferase activity changes utilizing the Lumat LB9507 fluorescence analyzer (EG & G Berthhold, Germany Gilberto Company). The results were represented as Firefly (Firefly luciferase) activity *vs.* Renilla (Renilla reniformis luciferase) activity (F/R).

Statistical analysis

Measurement data of the two groups were compared utilizing the t-test. All subjects were tested utilizing the Hardy-Weinberg equilibrium analysis. Allele and genotype frequencies, odds ratio (OR), and 95% confidence intervals (CI) were analyzed utilizing the chi-square test. Differences in luciferase activity were tested utilizing the t-test. Western blot results were analyzed utilizing one-factor analysis of variance. $P < 0.05$ was considered as statistical significance.

Results

Genotype frequency distribution of -724 ins/del polymorphism in apoM gene promoter region in MI group and control group

The -724ins/del polymorphism in apoM gene promoter region was in Hardy-Weinberg balance. Distribution of the three genotypes in the two groups was statistically significant (Table 2). D allele carriers have a 3.156-fold increased risk for MI compared with I allele carriers (OR=3.156, 95% CI: 1.876~5.309, P<0.001).

Comparison of blood lipids levels in patients with different genotypes

In the MI group and healthy control group, the serum total cholesterol (TC) levels in patients with -724Ins/Ins genotype were significantly lower than that in the -724Del allele carriers (5.3±2.0 mmol/L *vs.* 6.4±2.3 mmol/L, P=0.017). In the healthy control group, serum HDL-C levels of -724Del allele carriers were lower than that in patients with -724Ins/Ins genotype (1.6±0.7 mmol/L *vs.* 0.9±0.4 mmol/L, P=0.011) (Table 3).

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Table 2. Distributions of -724 I/D polymorphism (N=309).

Groups	Genotypes (n, %)			*P* value	Allele (n, %)		OR (95% CI)	*P* value
	D/D	D/I	I/I		D	I		
MI group	5 (1.6)	49 (15.9)	255 (82.5)	0.012	59 (9.5)	559 (90.5)	3.156 (1.876~5.309)	<0.001
Control group	1(0.3)	18 (5.8)	290 (93.9)		20 (3.2)	598 (96.8)		

Table 3. Serum lipid levels between II and DI+II genotype in MI group and control group.

Group	N	TG (mmol/L)	TC (mmol/L)	HDL-C (mmol/L)	LDL-C (mmol/L)
MI group	309	3.6±1.4	5.6±2.1	1.0±0.9	3.6±1.3
I/I	255	3.5±1.1	5.3±2.0	1.2±0.8	3.5±1.4
DI+DD	54	3.8±1.4	6.4±2.3	0.8±0.5	3.8±1.6
P value	–	0.097	0.017	0.119	0.105
Control group	309	1.6±1.1	4.3±0.9	1.5±0.8	2.4±0.9
I/I	290	1.6±1.0	4.2±2.1	1.6±0.7	2.3±1.3
DI+DD	19	1.7±0.9	4.4±2.3	0.9±0.4	2.7±1.4
P value	–	0.768	0.217	0.011	0.145

Detection of plasma ApoM levels in patients with different genotypes

Western blot results of the plasma samples from patients with the three genotypes show that the plasma apoM levels in patients with deletion mutation homozygous (D/D genotype) were lower than that in patients without deletion mutation homozygous (I/I genotype) (P=0.015) (Figure 1).

Impact of -724 position polymorphism on apoM promoter activity

Vector with wild-type apoM promoter was constructed by linking the wild-type apoM promoter gene sequences (-2165~-29) to the pGL3-basic vector-carrying luciferase gene. Vector with -724 position deletion mutant was reformed from the vector with wild-type apoM promoter by utilizing the site-directed mutagenesis technique. The above vectors were transfected into HEK293 cells to analyze the activity of luciferase on them. Activity of the apoM promoter with -724Del allele was obviously decreased compared with that of wild-type apoM promoter (1.18±0.16 *vs.* 2.23±0.14, P=0.004) (Figure 2).

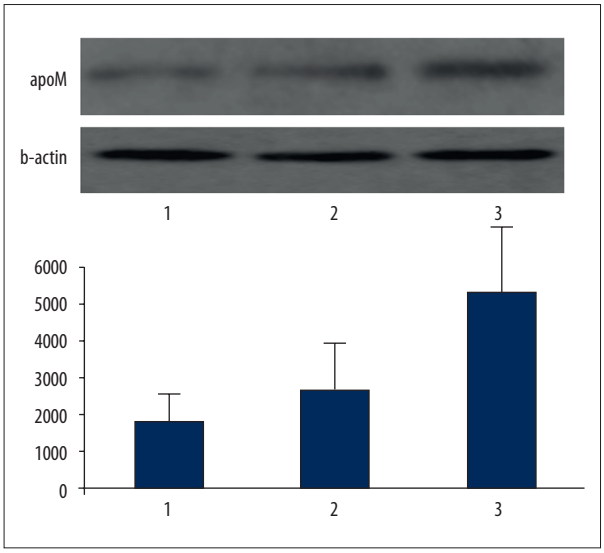

Figure 1. Western blot showed the difference in apoM expression between each genotype. 1 represents D/D genotype; 2 represents D/I genotype; 3 represents I/I genotype. Ordinate of histogram represents strip grayscale × area (INT × mm^2).

Discussion

In the present study, we found -724 I/D polymorphism influences the apoM promoter activity, down-regulates the apoM protein expression level, and increases the risk of MI.

ApoM is found in TG-rich lipoproteins initially, but it is an apolipoprotein composition second only to the apoAI (approximately the 10% of apoAI) in the HDL and mainly transit with the HDL [12]. The expression of the apoM, which is closely related to the synthesis of the HDL, plays a special role in cholesterol transport and lipid metabolism *in vivo* [13]. Wolfrum et al. [14]

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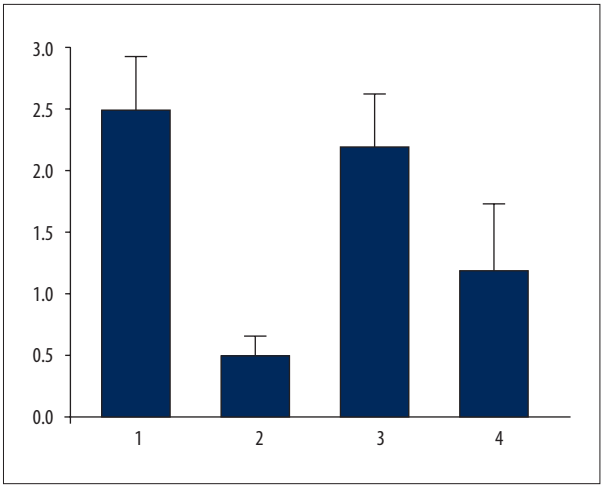

Figure 2. Influence of -724I/D polymorphism on promoter activity of apoM. 1: Positive control: carrying the luciferase gene, and the SV40 promoter and enhancer; 2: Negative control: carrying a luciferase gene but no promoter and enhancer; 3: I/I genotype: Carrying the luciferase gene and a recombinant plasmid of the -724 wild-type promoter; 4: Carrying luciferase gene and a recombinant plasmid of the -724 mutant-type promoter.

demonstrated that shortage of apoM expression in mice causes the accumulation of HDLl macrobeads in plasma and the decrease of the pre-β-HDL synthesis; on the other hand, the formation of pre-β-HDL particles increased in mice overexpressing apoM, which indicates that apoM plays an important role in the formation of HDL [15,16]. Christoffersen et al. [16] demonstrated that apoM+ HDL particles contain more cholesterol than the apoM– HDL particles in human plasma and that the apoM+ HDL particles are more effective in promoting cholesterol efflux, suggesting that the anti-atherosclerosis mechanism of apoM is accomplished through reverse cholesterol transport. Recently, Zheng et al. [10] have found a novel deletion mutant (-724ins/del) in the promoter region of the apoM gene. The distribution of this polymorphic locus is significantly higher in patients with coronary heart disease than in the healthy control group, showing the association between the SNP and coronary heart disease.

In our study, we also found that risk of MI significantly increased in people carrying -724Del allele. Functional studies found that the plasma apoM levels in patients with -724Del/del genotype apoM promoter are significantly lower than that with wild-type apoM promoter. We also found that the plasma TC levels in patients with the -724Del/del genotype are significantly higher than that with wild-type. Detecting the apoM promoter activity *in vitro*, we confirmed that -724Del mutation significantly decreases apoM promoter activity.

Conclusions

Our results suggest that -724Del mutation may down-regulate the expression level of apoM gene by decreasing the ApoM promoter activity, thus affecting the HDL synthesis and the reverse cholesterol transport, and finally increasing susceptibility to MI.

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