Correlation analysis between ApoM gene-promoter polymorphisms and coronary heart disease

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Abstract

Objectives: Apolipoprotein M (ApoM), a 25-kDa plasma protein belonging to the lipocalin protein family, is predominantly associated with high-density lipoprotein cholesterol (HDL-C). Studies have suggested ApoM to be important for the formation of pre-β-HDL and to increase cholesterol efflux from macrophage foam cells. The aim of this study was to explore the association of single-nucleotide polymorphisms (SNPs) in the ApoM promoter with coronary atherosclerotic disease (CAD), and the contribution of latent factors.

Methods: ApoM was measured in samples from two separate case-control studies, of whom 88 patients developed CAD and 88 were controls. Whole-blood samples from subjects were genotyped by PCR-restriction fragment length polymorphism (PCR-RFLP). Luciferase activities were measured for HepG2 cells with two SNPs, rs805296 (T-778C) and rs940494 (T-855C), and after interfering with or overexpressing the predicted transcription factors. The ability of the SNPs to combine with nucleoproteins was analysed by electrophoretic mobility shift assay (EMSA).

Results: Mean plasma ApoM concentrations in the CAD and non-CAD groups were 9.58 ± 4.30 and 12.22 ± 6.59 μg/ml, respectively. Correlation studies of ApoM concentrations with several analytes showed a marked positive correlation with HDL-C, fasting plasma glucose and triglyceride levels. The CC genotype showed lower luciferase activities compared with HDL-C, fasting plasma glucose and triglyceride levels. The ApoM-855 mutant-type could bind to the AP-2α. Interference and overexpression of AP-2 increased and decreased luciferase activities of the wild and mutant types to different degrees.

Conclusion: ApoM may be a biomarker of CAD. ApoM-855 T→C substitution provides binding sites for AP-2α and reduces ApoM transcription activity.

Keywords: coronary heart disease, ApoM, SNP, luciferase activity

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Apolipoprotein M (ApoM) is a novel lipocalin superfamily protein. Although also found in low-density lipoprotein (LDL), very low-density lipoprotein and chylomicrons, ApoM is primarily found in high-density lipoprotein (HDL) where it binds to sphingosine-1-phosphate (S1P) anchors.

Recent studies have suggested that ApoM may affect HDL metabolism, increasing the formation of pre-β-HDL particles. ApoM has been shown to protect LDL against Cu²⁺-induced oxidation, and to contribute to the anti-inflammatory function of HDL. Small circulating HDLs are involved in reverse cholesterol transport, and ApoM may affect this process by regulating pre-β-HDL. The binding of ApoM to S1P in HDL particles may also have an antioxidant role.

By affecting the immune and anti-inflammatory functions of HDL, ApoM may reduce atherosclerosis-related inflammation, preventing the onset and development of atherosclerosis. We used the online prediction software, TRANSFAC, to predict transcription factor (TF) binding sites for the normal and mutated ApoM-855 and ApoM-778 sites, and found ApoM T-855C provided binding sites for activating protein 2 (AP-2).

Activating protein-2α (AP-2α) was one of the first identified and studied TFs. The AP-2 gene, encoding a 437-amino acid protein of ~52 kDa, regulates the transcription of various genes regulating embryonic development, cell growth and differentiation. Vertebrates possess five subtypes of AP-2, α, β, γ, δ and ε.

The AP-2 protein has been shown to regulate atherosclerosis-associated genes, including matrix metalloproteinase-2, vascular endothelial growth factor, ApoE, tryptase and adiponectin ATP-binding cassette transporter A1 (ABCAI). In addition, AP-2α plays a role in atherosclerosis. It may mediate foam cell formation in mouse and human atherosclerotic lesions. ApoE mouse lesions within the artery wall, but was not detected in mouse arteries without atherosclerotic lesions. Similarly, AP-2α was observed in the human atherosclerotic aortic wall, mainly within the atherosclerotic plaque.

Recent studies involving the genetics of ApoM have led to major breakthroughs in metabolic and disease characteristics. In particular, associations have been found between diabetes and polymorphisms in the promoter region of the ApoM gene.

To explore the association of ApoM gene polymorphisms with coronary heart disease (CHD) in a Chinese Han population, we performed a population-based case-control study. We examined whether ApoM promoter polymorphisms could lead to changes in TF binding, and therefore, changes in promoter activity.
Methods

All relevant ethical and clinical approvals were obtained for this study. A total of 88 patients with coronary atherosclerotic disease (CAD) (63 males; mean age 60.80 ± 9.27 years) and 88 unrelated control individuals (53 males; mean age 58.18 ± 10.43 years) were retrospectively enrolled from among in-patients at the Anhui Cancer Hospital of Bengbu Medical College, Bengbu City, Anhui Province, China. All participants were of Han Chinese descent.

The criterion for inclusion in the CAD group was ≥ 50% stenosis in at least one major segment of the coronary arteries, determined by coronary artery angiography. Individuals in the control group had negative coronary artery angiography results (used to rule out CAD). A history of conventional risk factors for CAD or hypercholesterolaemia (total cholesterol ≥ 5.7 mmol/l) was obtained from the medical records. Exclusion criteria for both groups were familial hypercholesterolaemia, diabetes mellitus, cancer, renal disease and any other chronic illness.

For lipid analysis, whole blood samples were drawn from all participants in the morning after a 12-hour fast. Fasting plasma glucose (FPG), triglyceride (TG), HDL and LDL cholesterol (HDL-C and LDL-C), and total cholesterol (TC) levels were determined for each subject using an automated chemistry analyser (AU2000; Olympus Promarketing, Tokyo, Japan).

For ApoM analysis, 5-ml blood samples were collected in EDTA (as anticoagulant) after an overnight fast. Samples were centrifuged at 3 000 rpm for 10 minutes at room temperature. Separated sera were stored at −40°C. The serum ApoM level was quantified with ELISA, using horseradish peroxidase and 3,3′,5,5′-tetramethylbenzidine as substrates. The optical density was measured at 450 nm with an automatic microtitre plate reader (AU2000; Olympus Promarketing, Tokyo, Japan).

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Genotyping of ApoM T-778C and T-855C polymorphisms

Genomic DNA was extracted from peripheral blood using a Genomic DNA extraction kit (GeneChem). All constructs used in this study were confirmed by DNA sequencing (Shengong Bio Company, Shanghai, China).

Cell culture, transfection and reporter gene assays

The HepG2 hepatoma cell line was propagated in Dulbecco’s modified Eagle’s medium (DMEM; Hyclone) with 10% foetal calf serum (FCS) and maintained in 5% CO₂ at 37°C. HepG2 cells were seeded in 24-well plates at 5 × 10⁵ cells/well, respectively. After 24 hours, the cells were transfected with pGL3-basic (a promoterless control), pGL3-promoter (a promoter control), and two pGL3-basic constructed plasmids. The pRL-SV40 plasmid (Promega) was co-transfected as a normalising control.

To prevent transcription of ApoM by endogenous liver cell extracts, recombinant vector transfection groups were set up with and without liver cell extracts. After six hours, the AP-2α siRNA and AP-2α siRNA negative control fragments (Gima) were co-transfected into the two pGL3-basic constructed plasmid groups. The AP-2α expression vector (GeneChem)
groups were transfected into cells in the same way. Transfections were performed in triplicate and repeated three times. After 48 hours of incubation, the cells were collected and analysed for luciferase activity with the Dual-Luciferase Reporter assay system (Promega).

**Nuclear extract preparation**

HepG2 cells were propagated in DMEM with 10% FCS under 5% CO₂ at 37°C. Cells were cultured to a density of 1 × 10⁷ cells/ml, according to the manufacturer’s instructions.

HepG2 cells were collected in a 15-ml centrifuge tube and centrifuged at 500 g for five minutes at 4°C. The supernatant was discarded, and the cells were washed three times with 2 ml of pre-cooled phosphate-buffered saline (PBS). For every 20 μl of cell sedimentation, 200 μl of reagent A were added. The mixture was vortexed for five seconds, which caused the cell sedimentation to disperse completely, and placed in an ice bath for 15 minutes. Then 10 μl of cell plasma protein extraction reagent B was added to the solution. The mixture was vortexed for five seconds, placed in an ice bath for one minute, vortexed again for five seconds, and centrifuged at 16,000 g for five minutes at 4°C.

The supernatant was removed and 50 μl of phenylmethylsulfonyl fluoride (PMSF) nuclear protein extraction reagent was added. The mixture was vortexed for 30 seconds, placed on ice for two minutes, and vortexed again for 30 seconds. This cycle was repeated for a total of 30 minutes. After a final centrifugation at 16,000 g for 10 minutes at 4°C, the nuclear extract was drawn into a pre-cooled 1.5-ml centrifuge tube.

**Electrophoretic mobility shift analysis (EMSA)**

Sequences used for T1 and T1 wild-type and C1 and C2 mutant-type probe synthesis (Table 1) were labelled and unlabelled with biotin, respectively. Nuclear cell extracts were incubated with biotin-labelled double-stranded (ds) oligonucleotide probes containing the wild-type or mutant AP-2α binding sites in the ApoM promoter (wild-type T1 and mutant-type C1 probes in Table 1). Competition analysis was performed using the mutant probe with the AP-2α site. Supershifts were performed with antibodies against AP-2α and Sp1 (Abcam).
Fig. 2. pGL3 recombination vector sequencing. PGL3-TT means the regions –855T to –778T; PGL3-TC means the regions –855T to –778C; PGL3-CT means the regions –855C to –778T; PGL3-CC means the regions –855C to –778C.
Small interference (siRNA) transfection and efficiency

Three pairs of ds siRNA oligonucleotides were obtained from Gima Biotechnology Company, China (Table 1). Five groups were designated: a blank control group, a negative control group and three siRNA groups. Cells were seeded in six-well plates at 1 × 10^5 cells/well, and then 1 μl of siRNA and 2 μl of siRNA-Mate were added to each well, corresponding to a density of 70 to 80% at the time of transfection. All of the steps were strictly performed according to the manufacturer’s specifications. Cells were harvested at 48 hours. The Sp1 siRNA and AP-2α expression vectors were transfected into cells in the same way.

Six hours after fluorescein amidite (FAM)-labelled siRNA transfection, fluorescence was observed under an ultraviolet fluorescence microscope. The transfection efficiency was determined as: efficiency = number of fluorescent cells/total number of cells × 100%.

Semi-quantitative RT-PCR

Total RNA was extracted from the cell clones using TRIzol reagent (Invitrogen). The cDNAs were reverse-transcribed from total RNA. The primers used are shown in Table 1. The sizes of the PCR products for AP-2α and GAPDH were 385 bp and 496 bp, respectively. PCR products were checked by agarose gel electrophoresis. The abundance of each mRNA was detected and normalised to that of GAPDH mRNA.

Western blotting analysis

Cells in all groups were collected after 72 hours, and the total protein was extracted with RIPA lysis buffer (Beyotime Institute of Biotechnology). The protein was measured with the BCA protein assay and diluted with cell lysate to an equal concentration in each group (40 μg protein/group). A 10% SDS-PAGE analysis was performed. The proteins were transferred to a PVDF membrane (250 mA, 2 h), blocked with 5% BSA in PBS containing Tween-20 (PBST), and incubated overnight at 4°C.

The membrane was washed with TBST and incubated with a peroxidase-conjugated secondary antibody (1:1 000) for two hours. Specific antibody binding was detected using a chemiluminescence detection system, according to the manufacturer’s recommendations. Net intensities of the bands on the Western blots were quantified using Tanon GIS software.

After development, the membrane was stripped and re-probed with antibody against β-actin (1:1 000) to confirm equal sample loading.

Statistical analysis

Unless otherwise noted, results are reported as mean ± standard deviations (SD). General characteristics in the two groups and serum ApoM levels for different genotypes were statistically evaluated using the unpaired Student’s t-test (Prism software, version 4; GraphPad Inc, La Jolla, CA, USA). Significance was established at a p-value < 0.05.

Results

Association between risk factors for CAD and ApoM levels in CAD patients

The general characteristics of age- and gender-matched non-CAD and CAD patients are shown in Table 2. The mean ages of the non-CAD and CAD patients were 60.80 ± 9.27 and 58.18 ± 10.43, respectively (p > 0.05). The CAD patients had higher TG (1.97 ± 1.28 mmol/l) and FPG levels (6.40 ± 2.40 mmol/l), and lower HDL-C levels (1.05 ± 0.25 mmol/l) than the non-CAD patients (all p < 0.000).

According to their clinical symptoms and the American College of Cardiology (ACC)/American Heart Association (AHA) diagnostic guidelines, patients in the CAD group were divided into two subgroups: acute coronary syndrome (ACS group, n = 31) and stable angina pectoris (SAP group, n = 57). The Gensini score was calculated for the CAD group and both CAD subgroups, according to CHD severity (Table 2). The ACS group had a higher average Gensini score (80.48 ± 72.46, p = 0.017), higher TC level, and lower ApoM level than the SAP group (all p > 0.05).

Analysis of the serum ApoM lipid levels of the patients showed that the serum level of ApoM was positively correlated with HDL-C and negatively correlated with LDL-C and TG levels (p < 0.05). The correlation with TC was not significant (Table 3).

We used the multiple linear stepwise regression method to describe the relationship between serum ApoM and HDL-C and LDL-C levels. Linear dependencies and other related indicators
were used as dependent variables (\(y\)). ApoM was used as the independent variable (\(x\)). When HDL-C, FPG and TG were used as three indicators in the regression equation (\(x_1, x_2\) and \(x_3\), respectively), the resulting general multiple regression equation was \(y = 3.592 + 9.767x_1 - 0.539x_2 - 0.464x_3\), with the regression coefficient test, with \(p = 0.000\) and a coefficient of determination of 0.651. The standardised regression equation was \(y = 0.573x_1 - 0.301x_2 - 0.138x_3\). The closest relationship was between serum HDL-C and ApoM levels (Table 3).

**Association of polymorphisms in the ApoM proximal promoter region with CAD**

DNA sequencing of the polymorphic regions in the proximal promoter of the ApoM gene revealed that SNPs T-778C and T-855C of the ApoM gene were valid in the Han Chinese. The SNPs were accurately detected by PCR-RFLP (Fig. 1). Table 4 shows the plasma lipid and ApoM levels in each group according to the SNP status.

In the non-CAD group, we found no significant difference in lipid plasma levels between the TT and TC+CC groups. In the CAD group, the TC, TG and ApoM levels were significantly different between the TT and TC/CC groups. The ApoM plasma levels in the TT and TC+CC groups were 10.35 \(\pm\) 4.41 and 6.46 \(\pm\) 4.06 \(\mu\)g/ml, respectively in T-778C, and 10.17 \(\pm\) 4.41 and 6.45 \(\pm\) 3.57 \(\mu\)g/ml, respectively in T-855C (both \(p < 0.05\); Table 4).

**PCR amplification of the human ApoM gene promoter region**

From the above results, that patients with the TC/CC genotype showed lower plasma ApoM levels compared to those with the TT genotype, and that ApoM levels were lower in CAD compared to non-CAD patients, we inferred that the ApoM promoter variation may alter the promoter activity. To verify whether the −778T→C and −855T→C variation affected the ApoM promoter activity, we applied a reporter gene assay to detect the luciferase expression with transfection of the specific ApoM promoter activity, we applied a reporter gene assay to determine the Sp1 antibody activity of Sp1 was verified by immunohistochemistry (Fig. 5C). The supershift test showed that incubating the C probe with the supershift antibody produced a more lagging band (Fig. 5B), whereas the Sp1 antibody did not have this effect (Fig. 5C). The antibody activity of Sp1 was verified by immunohistochemistry analyses (Fig. 6A).

**Table 4. Lipid profiles according to genotype**

| Lipid parameter | CAD       | Control    | p-value | CAD       | Control    | p-value |
|-----------------|-----------|------------|---------|-----------|------------|---------|
| TC (mmol/L)     | 4.61 ± 1.37 | 4.77 ± 1.86 | 0.030   | 4.33 ± 0.49 | 4.31 ± 0.52 | 0.900   |
| TG (mmol/L)     | 1.93 ± 2.01 | 2.03 ± 2.02 | 0.002   | 1.03 ± 0.82 | 1.02 ± 0.32 | 0.157   |
| LDL-C (mmol/L)  | 2.78 ± 1.18 | 2.60 ± 1.46 | 0.610   | 2.53 ± 0.41 | 2.60 ± 0.44 | 0.655   |
| HDL-C (mmol/L)  | 1.05 ± 0.23 | 1.01 ± 0.34 | 0.557   | 1.32 ± 0.22 | 1.35 ± 0.19 | 0.706   |
| ApoM (μg/ml)    | 10.35 ± 4.41 | 6.46 ± 4.06 | 0.009   | 13.22 ± 9.18 | 3.57 ± 3.86 | 0.007   |

**Influence of SNPs T-778C and T-855C on ApoM promoter activity with liver cell extracts**

**Transcription factor prediction**

The TRANSFAC online prediction software was used to predict the combined TF binding sites upstream of the ApoM gene (Fig. 4A, B). The binding sites were identified on the ApoM promoter for some TFs, including Sp1 and AP-1. The AP-2 binding sites were located at -479 to -488 bp, -349 to -358 bp and -104 to -113 bp on the ApoM promoter. TRANSFAC was used to predict TF binding sites for the normal and mutated ApoM-855 and ApoM-778 sites (Fig. 4C–F).

ApoM T-855C provided binding sites for AP-2. ApoM-778T but not ApoM-778C had binding sites for hepatocyte nuclear factor-3 (HNF-3), CCAAT enhancer binding protein (C/EBP) and TATA box-binding protein (TBP). We also used the TESS online software to predict TF binding sites of the normal and mutated ApoM-855 and ApoM-778 sites (Fig. 4G, H). The results showed that ApoM T-855C provided binding sites for AP-2.

**EMSA results**

Small molecular weight chains have faster mobility in EMSA, whereas the electrophoretic velocity will vary with probe and protein binding. The velocity of the electrophoretic band indicates the presence or absence of binding.

We observed a hysterisis band when the C probe but not the T probe was incubated with nuclear protein (Fig. 5A). The competitive inhibition test showed that the hysterisis band of the C probe incubated with nuclear protein could be suppressed by unlabelled C probe, but not by unlabelled T probe (Fig. 5A).

The supershift test showed that incubating the C probe with the AP-2 antibody produced a more lagging band (Fig. 5B), whereas the Sp1 antibody did not have this effect (Fig. 5C). The antibody activity of Sp1 was verified by immunohistochemistry analyses (Fig. 6A).
Luciferase activity detection with AP-2α overexpression or interference

To examine the role of the TF AP-2α in mediating ApoM promoter activity, we treated HepG2 cells expressing the mutant-type or wild-type allele of the ApoM-855 site with siRNAs against AP-2α (Fig. 7A, B) or with an AP-2α expression vector (Fig. 7C–F), and examined changes in the luciferase activity of the -855 site (Fig. 8).

With AP-2α interference, the luciferase activity in cells expressing the wild-type allele (1.63) was less elevated compared to cells expressing the mutant-type allele (2.99). The luciferase activities of treated cells were increased compared to the
untreated control cells ($p < 0.05$).

With AP-2α overexpression, the activity levels of cells expressing the wild-type allele were more reduced than those of cells expressing the mutant-type (0.25 vs 0.38). The luciferase activity levels of AP-2α overexpressing cells were lower than those of the untreated cells ($p < 0.05$). Taken together, these results suggest that AP-2α was a negative regulatory factor for ApoM expression.
Discussion

The results of this study confirm the previously identified association between CHD and SNPs T-855C and T-778C in the promoter region of the ApoM gene. Luciferase activity associated with the -855 T→C substitution was significantly less than that of the promoter with -855 TT. With software predictive analysis we found the possible reason for this finding was that the -855 T→C substitution permitted the TFs AP-2α and Sp1 to bind to the promoter.

When HepG2 cells were transfected with the ApoM promoter containing the -855 T→C substitution, AP-2α combined with the ApoM-855 area, thereby decreasing promoter activity. These findings confirm that changes in the activation of the ApoM promoter region may induce variations in the ApoM plasma concentration.

Our results suggest that C alleles at the ApoM promoter -855 and -778 were associated with increased CHD risk. In our population-based case–control study, we enrolled 88 CHD patients (63 males, mean age: 60.80 ± 9.27 years) and 88 unrelated individuals (53 males, mean age: 58.18 ± 10.43 years) as a control group. The CHD group was divided into ACS and SAP patients (63 males, mean age: 60.80 ± 10.43 years) and 88 unrelated individuals (53 males, mean age: 58.18 ± 10.43 years) as a control group. The CHD group was divided into ACS and SAP groups, and the plasma levels of TG, TC, HDL-C, FPG and LDL-C were evaluated. Genomic DNA from whole blood of these subjects was subjected to PCR amplification and restriction enzyme digestion to determine genotype with regard to the ApoM T-855C and T-778C polymorphisms.

CHD patients had higher TG (1.97 ± 1.28 mmol/l; \(p = 0.000\)) and FPG levels (6.40 ± 2.40 mmol/l; \(p = 0.000\)) than non-CHD patients. The allele frequencies were in Hardy–Weinberg equilibrium.

After adjustment for age, gender and serum glucose level, multiple logistic regression analysis showed that, compared to the wild-type TT genotype of the two SNPs, carriers of the C allele had an increased risk of CHD, with an odds ratio (OR) of 1.819, 95% confidence interval (CI) of 1.142–2.898, and \(p = 0.012\) (T-855C: OR = 3.206, 95% CI = 1.139–2.204, \(p = 0.037\); T-778C: OR = 3.290, 95% CI = 1.487–7.280, \(p = 0.004\)). Luciferase activities of the promoter constructs with CC were significantly lower than those of the constructs with TC and TT.

To detect whether different alleles of the ApoM proximal promoter region may affect the expression of target genes, thereby affecting the metabolism of ApoM, we constructed different genotypes of the promoter reporter gene to examine how mutations in the ApoM proximal promoter would affect promoter activity. The presence of a C allele at -855 or -778 bp of the ApoM promoter region may lead to lower ApoM levels thereby affecting the metabolism of ApoM, we constructed different genotypes of the promoter reporter gene to examine how mutations in the ApoM proximal promoter would affect promoter activity. The presence of a C allele at -855 or -778 bp of the ApoM promoter region may lead to lower ApoM levels, and allow prediction of disease severity in the patient. However, due to the small number of cases analysed, more clinical data are needed for this conclusion to be confirmed.

We investigated whether the DNA sequence of ApoM from -844 to -869 bp was involved in transcriptional regulation of the ApoM gene. Using EMSA experiments, we showed that the mutant allele (-855C) could bind with nuclear proteins, whereas the wild-type allele (-855T) could not. Competitive inhibition experiments showed that the combination was due to specific binding by the TF AP-2α.

To explore the role of AP-2α in ApoM promoter activity, we examined the luciferase activities of the wild-type and mutant-type alleles after interference of AP-2α. Whereas AP-2α interference increased the luciferase activities of the treated cells, the wild-type was elevated to a lesser extent than the mutant-type. There were other AP-2α binding sites in addition to the -855 site. These results suggest that AP-2α may be a negative regulatory factor of ApoM. The increased luciferase activity of the mutant type with Apo-2α interference compared to the wild-type may indicate that the mutant had more binding sites for AP-2α, or that the mutated -855 site can bind with AP-2α.

Multiple epidemiological studies have shown that serum HDL levels are negatively correlated with the risk of early CHD. Generally, clinical CAD is divided into two major types, ACS and SAP. Patients with ACS had significantly lower ApoM levels, probably due to the fact that ApoM is a major apolipoprotein of HDL.

It has been confirmed that ApoM is required for pre-β-HDL formation and cholesterol efflux to HDL, and that it protects against atherosclerosis. ApoM increased formation of pre-β-HDL particles and had a profoundly protective effect on atherosclerotic lesion formation in hypercholesterolaemic Ldlr-/- mice. Atherosclerotic lesion areas in aortic roots and the thoracic aorta were reduced in Ldlr-/- mice infected with Ad-ApoM.

The unstable lesion (also vulnerable plaque, the formation being mainly due to dyslipidaemia) is the basic pathological aetiological factor of ACS. Therefore the presence of an enlarged unstable lesion may be because the decreased serum ApoM level prohibited the formation of sufficient amounts of mature, functional HDL to promote the mobilisation of cellular cholesterol in vivo.

The serum glucose level was different between CHD patients and normal control, and serum ApoM and serum glucose levels were negatively correlated (both \(p < 0.05\)). Very low ApoM levels increase the risk of atherosclerosis. Therefore the serum ApoM level may be a valuable marker for identifying high-risk groups.

TFs are the most important regulators of protein expression by genes and the most important factors to influence ApoM expression. Gene promoter regulation may underlie the low ApoM levels in CHD patients. Recent studies have shown that several TFs participate in the regulation of ApoM expression, such as HNF-1α, liver receptor homolog-1 (LRH-1), forkhead box A2 (Foxa2), liver X-activated receptor (LXR), lepion, interleukin-1 (IL-1), transforming growth factor (TGF) and epidermal growth factor (EGF).

Our study has some limitations. Although our results confirm findings on the effect of the T-778C polymorphism on CHD, our analysis of the ApoM promoter region SNPs with CHD was limited to a single locus. Such single-locus associations may be different in different populations. We found that the ApoM plasma concentration was decreased in CHD patients, the rs805296 and rs9404941 SNPs were associated with CHD occurrence and severity, and the rs9404941 SNP was associated with plasma TC and TG changes. However, the small sample size of this study limits its statistical power, and the results should be replicated in studies with larger sample sizes to avoid false positives. Expression of the ApoM protein and its relationship with diseases need to be further studied and discussed.

Conclusion

ApoM may be a biomarker of CAD. ApoM-855 T→C substitution provides binding sites for AP-2α and reduces ApoM transcription activity.
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References
1. Xu N, Dahlback B. A novel human apolipoprotein (apoM). J Biol Chem 1999; 274: 31286–31290.
2. Dahlback B, Nielsen LB. Apolipoprotein M – a novel player in high-density lipoprotein metabolism and atherosclerosis. J Curr Opin Lipidol 2006; 17: 291–295.
3. Christoffersen C, Obinata H, Kumaraswamy SB, Galvani S, Ahnström J, Sevmana M, et al. Endothelium-protective sphingosine-1-phosphate provided by HDL-associated apolipoprotein M. Proc Natl Acad Sci U S A 2011; 108: 9613–9618.
4. Wolfrum C, Poy MN, Stoffel M. Apolipoprotein M is required for pre beta-HDL formation and cholesterol efflux to HDL and protects against atherosclerosis. Nature Med 2005; 11: 418–422.
5. Christoffersen C, Nielsen LB, Axler O, Andersson A, Johnsen AH, Dahlbäck B. Isolation and characterization of human apolipoprotein M-containing lipoproteins. J Lipid Res 2006; 47: 1833–1843.
6. Christoffersen C, Nielsen LB. Apolipoprotein M – a new biomarker in sepsis. Crit Care 2012; 16: 126.
7. Bjørn D, Nielsen LB. Apolipoprotein M: a novel player in high density lipoprotein metabolism and atherosclerosis. Curr Opin Lipidol 2006; 17: 291–295.
8. Fenggold KR, Shigenaga JK, Chui LG, Moser A, Khovdinhunkit W, Grunfeld C. Infection and inflammation decrease apolipoprotein M expression. Atherosclerosis 2008; 199: 19–26.
9. Xu N, Hurtig M, Zhang XY, Ye Q, Nilsson-Ehle P. Transforming growth factor-beta downregulates apolipoprotein M in HepG2 cells. Biochem Biophys Acta 2004; 1683: 33–37.
10. Han M, Zhong YL. Transcription factor AP-2 and its Roles in Cancer. Acta Laser Biol Sinica 2004; 13: 462–464.
11. Huang QC, Cao JH. The role of activating protein-2 transcription factors in breast cancer. Basic Clinic Med 2012; 32: 841–843.
12. Hilger-Eversheim K, Moser M, Schorle H, Bueetner R. Regulatory roles of AP-2 transcription factors in vertebrate development, apoptosis and cell cycle control. Gene 2000; 260: 1–12.
13. Zhao F, Satoda M, Lichten CD, Hayashizaki Y, Gelb BD. Cloning and characterization of a novel mouse AP-2 transcription factor, AP-2delta, with unique DNA binding and transactivation properties. J Biol Chem 2001; 276: 40755–40760.
14. Tummala R, Romano RA, Fuchs E, Sinha S. Molecular cloning and characterization of AP-2 epsilon, a fifth member of the AP-2 family. Gene 2003; 321: 93–102.
15. Lu G. The role of AP-2alpha in atherosclerosis and HeLa apoptosis. Hunan: Biochemistry and molecular biology of Hunan Normal University Teaching and Research Section, 2008: 70–74.
16. Zhou JW, Tsai SK, Ng MC, Geng H, Li SK, So WY, et al. Apolipoprotein M gene (APOM) polymorphism modifies metabolic and disease traits in type 2 diabetes. PLoS One 2011; 6: e17324.
17. Wu X, Niu N, Bismar K, Zhu X, Wang X, Efendic S, et al. Apolipoprotein M promoter polymorphisms alter promoter activity and confer the susceptibility to the development of type 1 diabetes. Clin Cim Biol 2009; 42: 17–21.
18. Xu WW, Zhang Y, Tang YB, Xu YL, Zhu HZ, Ferro A, et al. A genetic variant of apolipoprotein M increases susceptibility to coronary artery disease in a Chinese population. Clin Exp Pharmacol Physiol 2008; 35: 546–551.
19. Jiao GQ, Yuan ZX, Xue YS, Yang CJ, Lu CB, Lu ZQ, et al. A prospective evaluation of apolipoprotein M gene T-778C polymorphism in relation to coronary artery disease in Han Chinese. Clin Biol Chem 2007; 40: 1108–1112.
20. Gordon T, Castelli WP, Hjortland MC, Kannel WB, Dawber TR. High density lipoprotein as a protective factor against coronary heart disease. The Framingham Study. Am J Med 1977; 62: 707–714.
21. Elseo S, Christoffersen C, Luchooon J, Turner S, Nielsen LB. Apolipoprotein M promotes mobilization of cellular cholesterol in vivo. Biochim Biophys Acta 2013; 1831: 1287–1292.
22. Faber K, Hvidberg V, Moestrup SK, Dahlbäck B, Nielsen LB. Megalin is a receptor for apolipoprotein M, and kidney-specific megalin-deficiency confers urinary excretion of apolipoprotein M. Mol Endocrinol 2006; 20: 212–218.
23. Lee CS, Friedman JR, Fulmer JT, Kaestner KH. The initiation of liver development is dependent on Foxa transcription factors. Nature 2005; 435: 944–947.
24. Wolfrum C, Howell JJ, Ndungo E, Stoffel M. Foxa2 activity increases plasma high density lipoprotein levels by regulating apolipoprotein M. J Biol Chem 2008; 283: 16940–16949.
25. Zhang X, Zhu Z, Luo G, Zheng L, Nilsson-Ehle P, Xu N. Liver X receptor agonist downregulates hepatic apom expression in vivo and in vitro. Biochem Biophys Res Commun 2008; 371: 114–117.
26. Calayir E, Becker TM, Kratzer A, Ebner B, Panzenböck U, Stefujl J, et al. LXR-agonists regulate ApoM expression differentially in liver and intestine. Curr Pharm Biotech 2008; 9: 516–521.
27. Xu N, Nilsson-Ehle P, Hurtig M, Ahrén B. Both leptin and leptin-receptor are essential for apolipoprotein M expression in vivo. Biochem Biophys Res Commun 2004; 321: 916–921.
28. Wolfrum C, Asilazma E, Luca E, Friedman JM, Stoffel M. Foxa2 regulates lipid metabolism and ketogenesis in the liver during fasting and in diabetes. Nature 2004; 432: 102710–102732.