The Review of DIABETIC STUDIES

SHORT REPORT

Evaluation of Apolipoprotein M Serum Concentration as a Biomarker of HNF-1alpha MODY

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Manuscript submitted January 22, 2008; resubmitted February 19, 2008; accepted February 22, 2008.

■ Abstract

Apolipoprotein M (apoM) is a 26-kDa protein expressed mainly in the liver and kidneys. It is present predominantly in high-density lipoproteins (HDL). ApoM expression is influenced by the hepatocyte nuclear factor-1α (HNF-1α), which is a transcription factor associated with the pathogenesis of MODY. Some earlier data suggested that apoM levels were lower in the serum of HNF-1α MODY subjects, than in that of other diabetics and healthy controls. The aim of this study was to evaluate apoM as a biomarker for HNF-1α MODY. We included in this study 48 HNF-1α mutation carriers (40 diabetic patients and 8 subjects with normal glucose levels in the fasted state) from the Polish Nationwide Registry of MODY. In addition, we examined 55 T2DM patients and 55 apparently healthy volunteers who had normal fasting glucose levels. ApoM was measured by the sandwich dot-blot technique with recombinant apoM (Abnova) as a protein standard, mouse anti-human apoM monoclonal primary antibody and rat anti-mouse HRP-conjugated secondary antibody (BD Biosciences). Mean apoM level in the MODY group was 13.6 μg/ml, SD 1.9 (13.5 μg/ml, SD 1.7 in diabetic subjects and 13.9 μg/ml, SD 2.0 in non-diabetic mutation carriers respectively). In the T2DM group, mean apoM level was 13.7 μg/ml, SD 2.1, while it reached 13.8 μg/ml, SD 2.0 in healthy controls. There was no difference between apoM serum concentrations in all the study groups. In summary, our study showed no association between HNF-1α mutations resulting in MODY phenotype and apoM levels. Thus, we cannot confirm the clinical usefulness of apoM as a biomarker of HNF-1α MODY.

Keywords: maturity onset diabetes of the young · MODY · apolipoprotein M · HNF-1alpha

Introduction

Apolipoprotein M (apoM) is a ~26 kDa glycoprotein of 188 amino acids, which was subsumed to the lipocalin family [1]. This protein is expressed in the liver and kidneys, and it is present in the serum, mainly in high density lipoproteins (HDL) [2], though it was first discovered in triglyceride-rich lipoproteins [3]. Its physiologic role remains incompletely understood. It seems to play a role in the anti-atherogenic function of HDL by influencing the accumulation of cholesterol in these particles and by promoting the formation of preβ-HDL [4]. ApoM expression is regulated by hepatocyte nuclear factor-1α (HNF-1α), a transcription factor [5]. Interestingly, mutations in the HNF-1α gene cause the phenotype of maturity onset diabetes of the young in humans and the specific form associated with HNF-1α mutations

 DOI 10.1900/RDS.2007.4.231

was formerly designated MODY3 [6]. As shown in an animal model, apoM expression is decreased by approximately 50% in mice with one functional allele of HNF-1α. Moreover, the expression of apoM was missing in homozygous HNF-1α knock-out mice. In the same study, it was found that HNF-1α MODY patients have about a 36% lower apoM serum concentration than HNF-4α MODY subjects and non-diabetic controls [5]. This finding was not confirmed by the initial results of another study [7]. Therefore, the relationship between apoM and HNF-1α gene mutations in humans requires further investigation.

Clarifying this issue could influence clinical practice. Scientific evidence has shown that specific treatment of diabetes could be successfully tailored on the basis of a proper differential diagnosis, which, in many instances, also includes genetic testing. However, as the latter requires expensive and laborious laboratory procedures, the identification of a biochemical biomarker for initial screening would be very beneficial. Thus, additional approaches are needed to help identify the different forms of diabetes. For example, because of the pleiotropic effect of the gene, HNF-1α MODY is characterized by the phenotype of glycosuria [8]. There is potential for utilizing certain markers of low glucose renal threshold in screening for HNF-1α MODY, with the most promising results for the 1,5-anhydroglucitol particle (Skupien and Malecki, unpublished data).

Interestingly, the tubulopathy seen in HNF-1α MODY also includes aminoaciduria [8]. Since it has been shown that apoM is reabsorbed in the kidneys [10], it is possible to hypothesize that the renal phenotype of HNF-1α MODY could additionally influence the metabolism of this lipoprotein in humans. This may provide further support for the hypothesis that serum or urine apoM measurement could be useful in HNF-1α MODY diagnosis.

While testing apoM level, it is important to be aware that HNF-1α MODY can be influenced by various clinical factors, such as hyperglycemia or hypoinsulinemia. For example, it was found in an animal model that hyperglycemia impaired apoM expression [11], while insulin administration reversed this effect [12]. With the aim of evaluating serum apoM as a biomarker for HNF-1α MODY, we assessed its levels in the mutation carriers of this gene, in subjects with type 2 diabetes mellitus (T2DM) and in non-diabetic individuals. We also examined renal excretion of apoM in the patients with diabetes who were included into our study.

Methods

Study groups

We examined 48 HNF-1α mutation carriers (40 with diabetes and 8 subjects with normal glucose levels in the fasted state) from the Polish Nationwide Registry of MODY [13]. Among them, there were 8 minors, aged 11-17 years, 4 of whom were diabetic patients and the other 4 had normal glucose levels in the fasted state. There were 5 patients with Arg131Gln, 3 with Ser249Pro, 6 with Asn257Thr, 4 with Arg263His, 2 with Arg271Trp and 2 with Pro447Leu substitution. In addition, 5 subjects carried Ser225fsdelC, 2 Pro291fsinsC, 1 Pro379fsdelT, 3 Arg171X, 13 IVS2nt^{+}1G>A and 2 IVS7nt^{-}6G>A mutations. 55 T2DM patients and 55 apparently healthy subjects with normal glucose levels in the fasted state (including 4 minors, 16-17 years old) were included in this study as comparative groups.

Blood specimens for the apoM assay were taken in the fasted state. Anthropometric measurements, as well as biochemical analyses, including plasma fasting glucose, serum creatinine level and lipid profile, glycated hemoglobin (HbA1c) and urinary albumin excretion were recorded. Glucose concentration was measured with an enzymatic GOD-PAP (glucose oxidase-phenol 4-aminophenazone peroxidase) method, creatinine with the Jaffe method, total cholesterol and HDL-cholesterol with CHOD-PAP (cholesterol oxidase phenol 4-aminoantipyrine peroxidase) assays, triglycerides with a GPO-PAP (glycerol phosphate oxidase phenol 4-aminoantipyrine peroxidase) assay and LDL-cholesterol level was calculated using the Friedewald formula. Albumin urinary excretion was assessed nephelometrically. An albumin concentration between 30 and 300 mg/l was regarded as microalbuminuria and one above 300 mg/l was considered to be overt proteinuria. HbA1c was measured using a high performance liquid chromatography method (BioRad).

The study protocol and informed consent procedures were consistent with the Helsinki Declaration and were approved by Jagiellonian University Ethical Committee.

Apolipoprotein M assay

Serum apoM concentration was determined with a dot-blot sandwich technique. Each serum sample was diluted in Tris buffered saline (TBS) (dilution ratio 1:20). The protein standard, recombinant human apoM with a GST-tag (glutathione S-transferase) of molecular weight 44.4 kDa (Abnova) at a concentration of

0.41 μg/μl, was diluted in TBS, in ratios of 1:100, 1:200, 1:400, 1:600, 1:800, 1:1000 and 1:2000. 1 μl of the serum specimens was placed on the nitrocellulose membrane (Amersham) in three repetitions and 1 μl of the recombinant apoM dilutions was placed in two repetitions. Distilled water and a 3% solution of bovine serum albumin (BSA) were used as a negative control. The membrane was blocked with a 5% solution of dry milk. The membranes were then incubated at room temperature in a 1:10000 dilution of mouse anti-human apoM monoclonal antibody (BD Biosciences) in Tween-TBS for one hour. Subsequently they were incubated in a 1:3000 dilution of horseradish peroxidase (HRP) conjugated rat anti-mouse antibody (BD Biosciences). The chemoluminescent reaction was performed and visualized by autoradiography (Amersham). The films were scanned and mean signal densities within the repetitions of each specimen and protein standards were measured with ImageJ 1.38x software. ApoM concentration was calculated from the standard curves computed on the basis of recombinant apoM dilutions.

The apoM concentration in undiluted urine samples was measured using the same protocol. The inter- and intra-assay imprecision of the measurements was evaluated with 10 and 20 repeated measurements respectively for three samples. The precision was presented as coefficient of variation (CV). To test for the specificity of the detection, a Western-blot assay was performed with 10 μl of 1:200 dilutions of serum samples, 1:4000 recombinant apoM dilution, 0.3% BSA and distilled water. A precast 12% denaturing polyacrylamide gel (BioRad) was utilized and the electrophoresis products were transferred to a nitrocellulose membrane using the tank transfer technique. The membranes were then processed as described above.

Statistical analysis

Comparisons of sex proportions were performed with the chi² test. The frequencies of proteinuria and microalbuminuria were tested with the Fisher exact test. For quantitative traits that fitted into normal distribution, i.e. apoM concentration, age, BMI, fasting glucose and HbA1c, the Student *t*-test or one-way ANOVA was applied. Otherwise, for age of diagnosis with diabetes, lipid parameters and serum creatinine concentration, the Mann-Whitney U-test or Kruskal-Wallis tests were utilized. Normality was tested with the Kolmogorow-Smirnow test. Spearman rank correlation coefficient (ϱ) was computed to analyze relationships between apoM concentration and the other quantitative traits, i.e. age, age of diagnosis, BMI, fasting glucose, HbA1c, serum creatinine and lipid parameters. The values of p estimated to be <0.05 were assumed to be significant. The computations were performed using MiniTab 14.20 statistical software.

Table 1. Clinical characteristics of the study groups

Characteristic	T2DM (n = 55)	p	MODY (n = 48)	p	Controls (n = 55)
Females (%)	58.2	n.s.	62.5	n.s.	54.5
Age (yr)	58.4; 9.7	<0.0005	36.1; 17.4;	n.s.	38.4; 16.6;
Age of diagnosis (yr)	48.8; 47.0; 6.0	<0.00005	24.1; 19.0; 8.8	n.a.	n.a.
BMI (kg/m²)	34.0; 6.7	<0.00005	22.7; 3.2	n.s.	24.5; 4.3
Total cholesterol (mmol/l)	5.6; 5.3; 1.08	0.013	4.7; 4.6; 0.93	n.s.	4.8; 4.9; 0.82
LDL-cholesterol (mmol/l)	3.3; 3.1; 0.92	<0.026	2.7; 2.4; 0.75	n.s.	2.8; 2.8; 0.75
HDL-cholesterol (mmol/l)	1.3; 1.1; 0.21	<0.00005	1.6; 1.5; 0.25	n.s.	1.4; 1.4; 0.27
Triglycerides (mmol/l)	2.4; 2.0; 0.59	<0.00005	1.1; 0.9; 0.41	n.s.	1.3; 1.0; 0.39
Fasting glucose (mmol/l)	7.2; 2.1	n.s.	7.2; 3.1	<0.00005	4.6; 0.5
HbA1c (%)	8.1; 1.9	0.02	7.2; 1.7	n.a.	n.a.
Serum creatinine (μmol/l)	80.1; 75.1; 11.8	n.s.	84.2; 71.3; 12.5	n.s.	69.6; 66.6; 10.0
Microalbuminuria (n)	9	n.s.	6	n.a.	n.a.
Proteinuria (n)	3	n.s.	4	n.a.	n.a.
Serum apoM (μg/ml)	13.7; 2.1	n.s.	13.6; 1.9	n.s.	13.8; 2.0

Legend: Age, BMI, fasting glucose, HbA1c and serum apoM are presented as mean and standard deviation. The other quantitative variables, which do not fit into normal distribution, are presented as mean, median and interquartile deviation. p-values refer to comparisons of HNF-1α MODY with T2DM and HNF-1α MODY with controls, respectively. n.a.: not applicable. n.s.: not significant. n: number of individuals.

Results

As shown in Table 1, there were no differences between the MODY group and control group in terms of sex ratio, age, body mass index (BMI), serum creatinine concentration and lipid profile. The subjects in the T2DM group were older, more obese, with later diabetes onset than MODY subjects, which represents typical differences between these types of diabetes. T2DM patients had also higher serum total cholesterol, LDL-cholesterol and triglyceride levels, as well as lower HDL-cholesterol level. The differences in HbA1c level and fasting glucose between diabetic MODY and T2DM groups were not significant (7.5 % vs. 8.1 %, $p = 0.18$ and 7.9 mmol/l vs. 7.2 mmol/l, $p = 0.25$, respectively). The prevalence of subjects with microalbuminuria and with overt proteinuria was similar in HNF-1α MODY and T2DM groups.

The Western-blot assay for apoM produced one band corresponding with a 26 kDa protein for serum samples and a 44 kDa for recombinant apoM conjugated with GST. Neither distilled water, nor albumin, produced a detectable signal in Western-blot and dot-blot. Mean inter-assay imprecision of dot-blot, evaluated as CV, was 7.9% and intra-assay imprecision (CV) was also 7.9 %.

The mean serum apolipoprotein M level in the HNF-1α mutation carrier group was 13.6 μg/ml, standard deviation (SD) 1.9, range 10.0-17.0 μg/ml. It was no different from apoM serum concentration in the T2DM group (mean 13.7 μg/ml, SD 2.1, range 10.0-20.0 μg/ml, $p = 0.70$), and in the non-diabetic control group (mean 13.8 μg/ml, SD 2.0, range 10.0-19.0 μg/ml, $p = 0.47$). Excluding non-diabetic patients (mean apoM concentration 13.9 μg/ml, SD 2.0) from the MODY group did not significantly influence the study outcome, nor did excluding minors from the MODY and control groups (mean apoM level 13.6 and 13.5 μg/ml respectively for two additional analyses). There was no difference between all subjects with diabetes, MODY and T2DM, and individuals from the control group (13.6 vs. 13.8 μg/ml respectively, $p = 0.62$). Sex did not influence apoM level, either in diabetic patients, or in non-diabetic individuals (13.8 vs. 13.4 μg/ml, $p = 0.53$ for males and females in the MODY group, 14.1 vs. 13.4 μg/ml, $p = 0.29$, in the T2DM group and 13.6 vs. 14.0 μg/ml, $p = 0.42$ in the controls respectively). Serum apoM level correlated significantly with age of diabetes diagnosis ($\varrho = 0.33$, $p = 0.038$) and fasting glucose ($\varrho = 0.30$, $p = 0.048$) in the MODY group, and with HbA1c ($\varrho = 0.22$, $p = 0.031$) in pooled diabetic groups. No other significant correlations were found.

The apoM protein in the undiluted urine of most subjects was undetectable by the method used in this study. The lowest concentration of apoM detectable with the applied dot-blot technique was about 0.1 μg/ml. Apolipoprotein M was detected in the urine of diabetic patients characterized by overt proteinuria at concentrations of about 0.2 to 1 μg/ml. Among them were 4 subjects from the MODY group and 3 patients from the T2DM group, whose mean serum apoM concentration was 12.5 μg/ml and 14.0 μg/ml respectively. There was no significant difference in apolipoprotein M concentration between these patients and the remaining diabetic subjects from both groups.

Discussion

In this study, we found no difference in serum apoM concentration between HNF-1α mutation carriers, T2DM patients and non-diabetic control individuals. Thus, the putative influence of the HNF-1α deficiency reported earlier from an animal study and from a small group of HNF-1α MODY patients could not be confirmed [5]. The initial results of the only other study that has examined this issue were discordant with respect to both HNF-1α and HNF-4α human mutation carriers, with the latter tending to show lower apoM levels [7]. We were also unable to confirm the influence of diabetes on apoM level, which was postulated by animal studies [11, 12]. The positive correlation of apoM with fasting glucose and HbA1c suggest the opposite effect in humans, although no difference was recorded between the diabetic study groups and control subjects. We were also unable to find a significant correlation between apoM serum level and lipid parameters or BMI. Earlier studies showed very discordant results: positive correlation [14] and lack of correlation [15] with BMI, or positive [15] and negative [14] correlation with cholesterol level respectively. Our findings did not support the hypothesis that HNF-1α mutation carriers might be characterized by renal loss of apolipoprotein M. It was found that, in the course of diabetic nephropathy, proteinuria caused an increased urinary excretion of apoM. However, this did not significantly affect their level of examined protein.

The discrepancy between the results of this study and the previous report [5] warrants discussion. It should be pointed out that the number of individuals examined in the earlier study was much smaller and the borderline difference could have constituted a type 1 error. The other possible cause is that our dot-blotting

method was less sensitive compared to the Western-blotting used in Richter's study. In addition, no details of clinical characteristics of diabetic HNF-1α subjects were provided, including the presence of diabetic nephropathy. Another reason could be the presence of overt proteinuria with urinary loss of apoM. However, this is implausible in the light of our results, which showed no evidence of proteinuria influencing serum level of apoM.

One potential criticism may arise from our choice of T2DM patients for the comparison. Another group that should be considered comprises subjects with other forms of MODY, particularly the carriers of HNF-4α (MODY1) mutations. These individuals seem to be the most appropriate for the comparison because of the latter condition's clinical similarity with HNF-1α MODY and its relatively high frequency [16]. However, the Polish Registry of MODY has not yet been screened for HNF-4α MODY. Additionally, the lack of standardization of the apoM assay is an important issue in the apolipoprotein M studies.

In summary, we were unable to confirm the clinical usefulness of apoM as a biomarker of HNF-1α MODY.

Acknowledgments: This study was supported by the Polish Ministry of Education and Science (Grant 2 P05B 070 28) and the Polish Diabetes Association.

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