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ScienceDirect
Clinical Biochemistry 41 (2008) 712–716
CLINICAL BIOCHEMISTRY

The MTP −493TT genotype is associated with peripheral arterial disease: Results from the Linz Peripheral Arterial Disease (LIPAD) Study

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Received 23 November 2007; received in revised form 12 February 2008; accepted 13 February 2008
Available online 21 February 2008

Abstract

Objectives: Microsomal triglyceride transfer protein (MTP) transfers lipids into apoprotein B-containing lipoproteins for secretion from liver, intestine, and heart. We hypothesized the −493T single nucleotide polymorphism in the MTP promoter region to be associated with altered lipoprotein levels and with presence of peripheral arterial disease (PAD).

Design and methods: 433 patients with symptomatic PAD and 433 controls matched for sex and age from the Linz Peripheral Arterial Disease (LIPAD) study were genotyped cross-sectionally for the −493T single nucleotide polymorphism in the promoter region of the MTP gene.

Results: The frequency of the −493T allele in patients with PAD was 0.320, whereas it was 0.255 in controls ($p<0.001$). The MTP −493TT genotype was independently associated with PAD, even after adjustment for LDL cholesterol. The odds ratio of the −493TT MTP genotype for PAD was 3.18 (95% CI, 1.76–5.71) when adjusted for current smoking, arterial hypertension, LDL cholesterol, triglycerides, glycohemoglobin, C-reactive protein, and homocysteine. Furthermore, we found an association between the MTP promoter polymorphism and the apolipoprotein B-containing lipoproteins total-cholesterol ($p=0.011$), LDL cholesterol ($p=0.002$) and apolipoprotein B ($p=0.034$).

Conclusions: Our results provide preliminary evidence for a potential role of the MTP −493TT genotype in the pathogenesis of PAD.

Keywords: MTP; Peripheral vascular disease; Polymorphism; Risk factors

Introduction

The microsomal triglyceride transfer protein (MTP) lipidates the growing apolipoprotein B polypeptide chain and, thereby, allows for the assembly and secretion process of lipoproteins from liver, intestine, and heart [1–3]. MTP preferentially transfers triglycerides and cholesteryl esters into the lumen of the endoplasmatic reticulum to form very low density lipoproteins (VLDL) and chylomicrons. MTP is an intracellular heterodimer that contains a large 97-kDa subunit, which is responsible for the lipid transfer activity, and a protein disulfide isomerase subunit, which contains the endoplasmatic reticulum retention signal [4].

In humans, there are conflicting results about the association between the single nucleotide polymorphism −493T/G genotype in the promoter region of the MTP gene and the phenotype of plasma lipoprotein levels. The less common −493T allele was associated with lower LDL cholesterol levels in healthy young Caucasians [5], whereas the same genotype was associated with higher LDL cholesterol levels in young African Americans [6]. There was no association between MTP variants and the lipoprotein profile in the Framingham Offspring Study [7], and the Etude Cas-Temoin de l'Infarctus du Myocarde (ECTIM) Study [8]. Furthermore, visceral obesity and hyperinsulinemia seem to modulate the impact of the −493G/T polymorphism on plasma LDL cholesterol levels [9,10].

Independently of its interaction with plasma LDL cholesterol levels and visceral obesity, the −493T allele of the MTP gene was associated with an increased risk of coronary heart disease

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doi:10.1016/j.clinbiochem.2008.02.007

in the West of Scotland Coronary Prevention (WOSCOP) Study [11].

Inhibition of MTP by a synthetic MTP-inhibitor lowers atherogenic apolipoprotein B-containing lipoproteins in patients with homozygous familial hypercholesterolemia [12]. In mice, inhibition of MTP by drugs [13] or genetic engineering [14,15] also decreases plasma low density lipoprotein (LDL) cholesterol levels, whereas adenoviral overexpression of hepatic MTP increases secretion of triglyceride-rich apolipoprotein B-containing lipoproteins [16].

We tried to extend this issue to the vascular bed of the lower extremities and recruited participants of the Linz Peripheral Arterial Disease (LIPAD) population for this purpose [17]. We hypothesized the −493T single nucleotide polymorphisms in the MTP promoter region to be associated with an increased risk for peripheral arterial disease in the LIPAD population.

Methods

Study population

To test the hypothesis that MTP genotypes are associated with atherosclerotic PAD, we used data from the LIPAD study [17], which was designed to evaluate possible phenotypic and genotypic risk factors for atherosclerotic PAD. The study protocol was approved by the local ethics committee in accordance with the Declaration of Helsinki, and all study participants gave informed consent. The LIPAD study objectives, recruitment procedures, and characteristics have been described previously [17]. The study sample comprised 433 consecutive patients with symptomatic atherosclerotic PAD admitted to the St. John of God Hospital in Linz, Austria, for inpatient diagnostics and treatment of chronic limb ischemia, and 433 control subjects matched to the patients with PAD in a 1:1 design by sex, age (+/−2 years), and diabetes mellitus status (i.e., for each PAD patient enrolled in the study, an appropriate referent was recruited by the investigators). This series of study participants represents a Caucasian population [17] and underwent evaluation for the presence of risk factors for atherosclerosis and comorbid conditions, as recommended by Rutherford et al. [18].

PAD was defined as chronic atherosclerotic disease of the lower extremities associated with typical symptoms, such as claudication or leg pain on exertion, rest pain, or minor or major tissue loss, and was verified by interview; physical examination; Doppler segmental blood pressure of the lower limbs, including continuous-wave spectral analysis and resting ankle–brachial index measurements; and intra arterial aortofemoral angiography. Patients with PAD were included in the present study on the basis of the final clinical diagnosis established by the attending vascular surgeons. All cases with acute ischemia (i.e., peripheral arterial thrombosis of a native artery, popliteal artery aneurysm, or acutely thrombosed peripheral bypass grafts) were excluded. Additional exclusion criteria were PAD caused by nonatherosclerotic causes (i.e., cardioembolic disease, thromboangiitis obliterans, vasculitis, or congenital vascular disease) and a history or presence of any malignancy. Control subjects were free of manifest or previous atherosclerotic disease (i.e., coronary artery disease, cerebrovascular disease, or PAD). All controls had an ankle–brachial index >=1.0, no pathologic pattern of pulse waves in lower limbs by continuous-wave spectral analysis, no stenoses of the internal carotid artery >=50% by color duplex ultrasound scans, and no history or presence of any malignancy. None of the control subjects received a lipid-lowering medication, and took folate supplement or B vitamins by study design. The referents were patients of the St. John of God Hospital in Linz who had been admitted for treatment of minor health problems, such as cataract surgery, vertebragenic pain, or nonvascular surgery (e.g., herniotomy or varicose vein removal) and were recruited without knowledge of their laboratory data.

Coronary artery disease was defined as remote myocardial infarction by history, occult myocardial infarction by electrocardiography, previous coronary bypass surgery or percutaneous transluminal coronary angioplasty, and stable or unstable angina and acute coronary syndrome (cardiac troponin positive or negative). Cerebrovascular disease was defined as transient or temporary stroke, completed stroke with permanent neurological deficit, or acute stroke. Arterial hypertension, diabetes mellitus, and smoking were classified according to recommended standards [18].

Of the 433 patients with PAD, 359 had mild to severe leg pain upon exertion, and 74 had critical limb ischemia (i.e., 15 with ischemic rest pain and 59 with minor or major tissue loss). In this series of PAD patients the frequencies of concomitant coronary artery disease and cerebrovascular disease were 30% and 18%, respectively. Furthermore, 119 patients with PAD were classified as having carotid stenosis ≥50%. At enrolment, 116 patients with PAD had undergone remote percutaneous transluminal angioplasty with or without stenting, 86 patients had undergone vascular surgery, and 22 patients had undergone minor amputations. Per definition, none of the 433 control subjects matched to the patients with PAD for sex, age (±2 years), and diabetes had coronary artery disease, or cerebrovascular disease, or an internal carotid stenosis ≥50%, but many (n=365; 84%) had carotid plaques, indicating mild but not clinically relevant atherosclerosis. Of the 433 patients with PAD, 102 were on lipid-lowering medication (Simvastatin n=32, Atorvastatin n=31, Pravastatin n=22, Fluvastatin n=4, Cerivastatin n=1, Bezafibrates n=10, Atorvastatin and Bezafibrat n=1, Simvastatin and Gemfibrozil n=1).

Blood samples were taken from all study participants under standardized conditions after an overnight fasting period. We measured conventional cardiovascular risk factors, such as lipids, lipoproteins, fasting glucose, glycohemoglobin A_{1C} (HbA_{1C}), total homocysteine, and high-sensitivity C-reactive protein (hs-CRP) as previously described [17]. DNA was genotyped for the MTP polymorphism in all 866 participants of the LIPAD case-control study.

DNA analyses

The single nucleotide polymorphism −493T was detected by polymerase chain reaction (PCR) restriction fragment length polymorphism analysis, as previously described [5]. A 109 bp

fragment of the MTP gene was amplified. Forward and reverse primer sequences were 5′-GGA TTT AAA TTT AAA CTG TTA ATT CAT ATC AC-3′ and 5′-AGT TTC ACA CAT AAG GAC AAT CAT CTA-3′, respectively. DNA was denatured for 9 min at 94 °C, and then subjected to 35 amplification cycles. Each PCR cycle consisted of denaturation for 60 s at 94 °C, annealing for 95 s at 55 °C, and extension for 60 s at 72 °C; followed by a final extension at 72 °C for 10 min. The buffer contained 2 μmol/L of each primer, 200 μmol/L dNTP, 0.8 mmol/L $MgCl_2$, 1.5 U of Taq DNA polymerase, 60 mmol/L Tris–HCl, pH 8.3, and 100 ng of genomic DNA. PCR products were incubated overnight with 0.1 Ut/μL HphI (New England Biolabs, Beverly, MA, USA) at 37 °C, separated by electrophoresis in 4% agarose, and visualized by staining for 30 min in 5 μg/mL ethidium bromide. Only the MTP −493G allele is digested by HphI into two fragments of 89 and 20 bp, whereas the PCR product of the MTP −493T allele is not substrate of this restriction enzyme.

Statistical analyses

We tested if the genotype distribution was in Hardy–Weinberg equilibrium in the cases and controls of our study population by using chi^2 test. We estimated the sample size to be about 400 individuals in each group in order to detect an odds ratio of 2 in a multivariate logistic regression analysis with a 0.05 two-sided significance level and a power of 80%. Normally distributed quantitative values are expressed as means±SD, not-normally distributed variables as medians (25th–75th percentile). Differences in mean values between cases and controls were determined by two-tailed Student's *t*-test, those of medians by Mann–Whitney *U* test. χ^2-test was used to compare categorical variables. The *a priori* hypothesis of a relationship between the MTP genotypes and PAD was tested using χ^2-test and multivariate logistic regression analysis with PAD as the dependent variable. Current smoking, arterial hypertension, LDL cholesterol, triglycerides, HbA_{1C}, total homocysteine, and hs-CRP were included as covariates into the final model without variable selection technique (all variables were included simultaneously into the model). Inasmuch as patient and control groups were matched for age, sex, and diabetes status, these variables were not included into the logistic regression analysis. The associations of MTP genotypes with cholesterol and apolipoprotein values were evaluated using ANOVA and Kruskal–Wallis analyses. Two tailed tests were used for all statistical analysis, *p*-values <0.05 were considered to indicate statistical significance. Statistical analyses were performed using SPSS version 13.0 (SPSS, Inc., Illinois, USA).

Table 1
Characteristics of study subjects

Characteristic	Controls (*n*=433)	Patients with PAD (*n*=433)	*p*-value
Male sex‡, *n*	306 (71%)	306 (71%)	1.000†
Age‡, years	67.3±10.7	67.1±10.7	0.692
Body mass index, kg/m^2	26.8±4.0	26.5±4.1	0.211
Current smoking§, *n*	51 (12%)	193 (45%)	<0.001†
Arterial hypertension, *n*	178 (41%)	251 (58%)	<0.001†
Diabetes mellitus‡, *n*	115 (27%)	115 (27%)	1.000†
ABI, mmHg/mmHg	1.18 (1.09–1.29)	0.63 (0.47–0.79)	<0.001*
Biochemical markers			
Total-cholesterol, mg/dL	215 (182–243)	229 (195–258)	<0.001*
LDL cholesterol, mg/dL	136 (108–158)	150 (121–178)	<0.001*
HDL cholesterol, mg/dL	53±17	52±18	0.057
Apolipoprotein B#, g/L	0.84 (0.70–0.99)	0.97 (0.80–1.16)	<0.001*
Apolipoprotein A1#, g/L	1.44 (1.26–1.59)	1.47 (1.27–1.67)	0.14*
Triglycerides, mg/dL	117 (88–160)	132 (99–201)	<0.001*
Total homocysteine, μmol/L	14 (12–18)	16 (13–21)	<0.001*
Hs-CRP, mg/L	2.1 (0.9–6.0)	4.1 (1.8–9.2)	<0.001*
Creatinine, mg/dL	0.9 (0.8–1.0)	1.0 (0.9–1.1)	<0.001*

Statistical testing was performed with Student's t-test, *Mann–Whitney U test, and †χ2-test for categorical values. ‡Matched variables. §Current smoking was defined as any amount of tobacco use including abstinence less than one year (18). ¶46 of the 443 referents had been prescribed aspirin therapy by their general practitioners without any indication. #Apolipoprotein A1 and apolipoprotein B were measured in 300 patients with PAD and 300 matched control subjects. §§Related to persons with diabetes mellitus only (i.e., 115 controls and 115 PAD patients).
Abbreviations: ABI, resting ankle brachial index; hs-CRP, high-sensitivity C-reactive protein; HDL, high density lipoprotein; LDL, low density lipoprotein.

Table 2
MTP genotype distribution

−493T genotype	Controls (*n*=433)	Patients with PAD (*n*=433)	*p*-value*
GG	235 (54.3%)	217 (50.1%)	<0.001
GT	175 (40.4%)	155 (35.8%)	
TT	23 (5.3%)	61 (14.1%)	

*calculated by the χ2-test.

Results

Table 1 gives a summary of clinical and biochemical data of the patients with PAD and controls. The genotype distribution in the control group was found to be in Hardy–Weinberg equilibrium (chi^2=1.73; *p*=0.188), whereas the genotype distribution in the PAD group was not in the Hardy–Weinberg equilibrium (chi^2=13.6; *p*=0.000227). Genotyping was performed in all 433 cases and 433 controls. The frequency for the −493T allele of the MTP gene was 0.320 in patients with PAD, whereas it was 0.255 in controls (Table 2). This difference was statistically significant

Table 3
Multivariate logistic regression analysis for the presence of PAD

Risk factor	Odds ratios of PAD (95% CI)[a]	*p*-value
Current smoking[b] (vs. not)	8.64 (5.75–12.98)	<0.001
Arterial hypertension (vs. not)	3.08 (2.19–4.32)	<0.001
LDL cholesterol (+ 10 mg/dL)	1.12 (1.07–1.17)	<0.001
Glycohemoglobin A_{1C} (+ 1%)	1.13 (1.01–1.27)	0.029
Triglycerides (+ 10 mg/dL)	1.01 (0.99–1.03)	0.346
Total homocysteine (+1 μmol/L)	1.04 (1.02–1.07)	0.001
Hs-CRP (+ 1 mg/L)	1.01 (1.00–1.02)	0.002
−493 Genotype (TT vs. GT+GG)	3.18 (1.76–5.71)	<0.001

Abbreviations: hs-CRP, high-sensitivity C-reactive protein; LDL, low density lipoprotein.
[a] CI = confidence interval.
[b] Current smoking was defined as any amount of tobacco use including abstinence less than one year [18].

Table 4
Correlation of −493G/T MTP genotypes with lipoprotein profile

	GG	GT	TT	*p*-value
	(*n*=452)	(*n*=330)	(*n*=84)	
Total-cholesterol (mg/dL)	221±45	217±51	241±54	0.011*
LDL cholesterol (mg/dL)	141±38	141±42	156±47	0.002*
HDL cholesterol (mg/dL)	51 (41–62)	50 (40–61)	49 (38–63)	0.614†
Triglycerides (mg/dL)	127 (95–179)	123 (90–177)	128 (97–197)	0.341†
Apo A1 (mg/dL)	144±30	146±34	148±30	0.942*
Apo B (mg/dL)	87±24	91±27	101±35	0.034*

As compared by ANOVA* and by Kruskal–Wallis analysis†.
Abbreviations: ABI, resting ankle brachial index; hs-CRP, high-sensitivity C-reactive protein; HDL, high density lipoprotein; LDL, low density lipoprotein; Apo A1, Apolipoprotein A1; Apo B, Apolipoprotein B.

with a p-value of <0.001 as calculated by the χ^2-test and remained statistically significant after excluding patients with lipid-lowering medication ($p<0.001$).

Following univariate analysis we performed a multivariate logistic regression analysis. We compared homozygous carriers of the −493T allele to heterozygous patients and homozygous patients for the wild-type. Table 3 shows the multivariate logistic regression analysis for the presence of PAD in the LIPAD population. The MTP promoter polymorphism distribution remained significantly different after multivariate logistic regression analysis when adjusted for smoking, arterial hypertension, LDL cholesterol, triglycerides, HbA_{1C}, hs-CRP, and total homocysteine ($p<0.01$). The odds ratio of the −493TT genotype for peripheral arterial disease was 3.18 (95% confidence interval of 1.74–5.81). There were no gender-specific differences in MTP genotype distribution in our cohort.

Next, we performed a correlation analysis of the MTP polymorphism and the lipoprotein profile as outlined in Table 4. There was a positive correlation between the −493G/T variants of the MTP gene on the one hand, and total-cholesterol, LDL cholesterol and apolipoprotein B on the other hand. We did not find any correlation between the −493G/T MTP polymorphism and triglyceride or HDL cholesterol levels.

The percentage of patients homozygous for the putative MTP risk allele −493TT was 12.2% in the subgroup of patients with ischemic heart disease, 12.6% in the subgroup with carotid stenoses, and 18.8% in the subgroup with stroke and, thus, in a similar order of potency as in the whole study population with overt PAD (14.1%).

Discussion

The aim of the present study was to investigate the association between the −493G/T polymorphism in the MTP promoter region and the risk of developing PAD in the LIPAD study. The frequency of the MTP −493T allele in our control group was similar to that in other Caucasian populations, and the genotype distribution of the −493G/T polymorphism was in Hardy–Weinberg equilibrium [5,7,9,11]. Compared to controls, we found a significantly higher gene frequency of the −493T allele in patients with PAD ($p<0.001$). Furthermore, the MTP −493TT genotype was independently associated with symptomatic PAD. The odds ratio of this homozygous −493TT genotype for the presence of PAD in our population was 3.18, and of similar magnitude previously reported for patients with ischemic heart disease [11]. We, thus, provide evidence for the importance of the MTP −493TT genotype in the development of atherosclerosis in the lower limbs.

In the presented study, subjects homozygous for the MTP −493T allele had significantly higher total-cholesterol, LDL cholesterol and ApoB levels. Karpe et al. [5] have already shown that the MTP −493T allele has a 2-fold higher transcriptional activity when compared to the MTP −493G allele. Our data are also in line with data by Chuchel et al [12], who found a significant reduction of apolipoprotein B-containing lipoproteins in patients with homozygous familial hypercholesterolemia by inhibiting of MTP with a synthetic inhibitor.

Previous data on the association between the G −493T polymorphism and lipoprotein levels were contradictory. An association has been reported between the MTP −493 polymorphism and low levels of TC, LDL-C, and ApoB [5]. In contrast, the MTP −493 polymorphism was associated with increased levels of TC, LDL-C, TG, and ApoB in young African Americans [19], whereas the Framingham Offspring Study cohort failed to detect such an association [7].

In the presented study, subjects homozygous for the MTP −493T allele had significantly higher total-cholesterol, LDL cholesterol and ApoB levels. Differences between various studies may be due to different population characteristics. First of all, we had a relevant proportion of diabetic patients (27%) in our study population. Hyperinsulinemia is known to influence the effect of MTP variants on the lipoprotein profile [9,20,21]. Second, our patients were significantly older than previous study populations. Furthermore, there might also be other (racially-associated) genetic modifiers differing in the diverse populations. In a case-control study of U.S. Caucasians, a two-SNP-haplotype in the MTP gene was implicated as a modifier of human life span [22]. However, this association was not reproduced in a large European cohort [23,24]. Another possible explanation is that there is an interaction with the LDL-receptor gene or the hepatic cholesterol homeostasis [25]. Last but not least, strong environmental factors, such as diet and lifestyle may have contributed to the observed inconsistency between studies. In an interventional study, the effects of low-fat diet depended on MTP −493G/T polymorphism [26]. Homozygous carriers of the rare T-allele showed a threefold greater reduction in LDL cholesterol in comparison to carriers of the common G-allele.

Our study has some limitations. First, the study design was cross-sectional and the study population was a selected subgroup of the overall population of PAD as described in the method section (Caucasian patients admitted for inpatients diagnostics and treatment of atherosclerotic PAD). Thus, the findings cannot be generalized to non-Caucasian patients, asymptomatic patients with PAD, or patients who do not meet criteria for hospitalization. Moreover, the genotype distribution of the MTP −493G/T polymorphism was not in Hardy–Weinberg equilibrium in the

PAD group. This Hardy–Weinberg disequilibrium probably presents a selection bias. Prospective studies are needed to establish a direct relationship between MTP variants and the various atherosclerotic phenotypes. The LIPAD study was not designed for evaluation of cerebrovascular and cardiovascular events. Therefore, *post hoc* subgroup analyses of these phenotypes with MTP −493 genotypes might be underpowered.

Our data indicate that homozygosity for the less common MTP −493T allele confers a risk for PAD which is not purely reflected by the fasting lipoprotein profile. This may be due to the fact that MTP significantly modulates postprandial lipemia [27–29]. Taken together, the presented study provides evidence for an association between the MTP −493TT genotype and atherosclerosis in the vascular bed of the lower limbs.

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