Allele-specific regulation of MTTP expression influences the risk of ischemic heart disease

Anna Aminoff,* Helena Ledmyr,† Petra Thulin,* Kerstin Lundell,* Leyla Nunez,† Elisabeth Strandhagen,† Charlotte Murphy,* Ulf Lidberg,** Jukka Westerbacka,** Anders Franco-Cereceda,†† Jan Liska,** Lars Bo Nielsen,** Mats Gåfvels,§ Maria Nastase Mannila,* Anders Hamsten,* Hannele Yki-Järvinen,** Dag Thelle,**†† Per Eriksson,* Jan Borén,** and Ewa Ehrenborg†††.

Atherosclerosis Research Unit, Department of Medicine,§ Center for Molecular Medicine, Karolinska Institutet, Stockholm, Sweden; Sahlgrenska School of Public Health and Community Medicine,† Institute of Medicine, University of Gothenburg, Gothenburg, Sweden; Division of Clinical Chemistry, Department of Laboratory Medicine,§ Karolinska Institutet, Stockholm, Sweden; Sahlgrenska Center for Cardiovascular and Metabolic Research, Wallenberg Laboratory,** University of Gothenburg, Gothenburg, Sweden; Department of Medicine,†† Division of Diabetes, University of Helsinki, Helsinki, Finland; Department of Cardiothoracic Surgery and Anaesthesia,§§ Karolinska University Hospital, Stockholm, Sweden; Department of Clinical Biochemistry, Rigshospitalet, and Department of Biomedical Science,*** University of Copenhagen, Copenhagen, Denmark; and Department of Biostatistics, Institute of Basic Medical Sciences, Faculty of Medicine,††† University of Oslo, Oslo, Norway

Abstract Promoter polymorphisms in microsomal triglyceride transfer protein (MTTP) have been associated with decreased plasma lipids but an increased risk for ischemic heart disease (IHD), indicating that MTTP influences the susceptibility for IHD independent of plasma lipids. The objective of this study was to characterize the functional promoter polymorphism in MTTP predisposing to IHD and its underlying mechanism. Use of pyrosequencing technology revealed that presence of the minor alleles of the promoter polymorphisms -493G>T and -164T>C result in lower transcription of MTTP in vivo in the heart, liver, and macrophages. In vitro experiments indicated that the minor -164C allele mediates the lower gene expression and that C/EBP binds to the polymorphic region in an allele-specific manner. Furthermore, homozygous carriers of the -164C were found to have increased risk for IHD as shown in a case-control study including a total of 544 IHD patients and 544 healthy control subjects. We concluded that carriers of the minor -164C allele have lower expression of MTTP in the heart, mediated at least partly by the transcription factor CCAAT/enhancer binding protein, and that reduced concentration of MTTP in the myocardium may contribute to IHD upon ischemic damage.—Aminoff, A., H. Ledmyr, P. Thulin, K. Lundell, L. Nunez, E. Strandhagen, C. Murphy, U. Lidberg, J. Westerbacka, A. Franco-Cereceda, J. Liska, L. B. Nielsen, M. Gåfvels, M. N. Mannila, A. Hamsten, H. Yki-Järvinen, D. Thelle, P. Eriksson, J. Borén, and E. Ehrenborg.


Supplementary key words promoter activity • lipotoxicity • lipid accumulation • myocardium

The microsomal triglyceride transfer protein (MTTP) is essential for formation of apolipoprotein B (apoB)-containing lipoproteins. The liver and the intestine have the highest tissue expression of MTTP and secrete triacylglycerol (TAG)-rich VLDLs and chylomicrons, respectively (1). MTTP is also expressed in other cells such as cardiomyocytes (2) and macrophages (3). The heart secretes apoB100-containing lipoproteins and it has been proposed that cardiac lipoprotein secretion protects the heart against accumulation of lipids that are toxic to the myocardium (4). This theory is supported by the finding that MTTP expression is...
pression is inversely correlated with the concentration of TAGs in the myocardium. Thus, ischemia-induced TAG accumulation in the myocardium might be mitigated by increased MTTP expression in the myocardium (5).

The -493G>T single nucleotide polymorphism (SNP) in MTTP (rs1800591) has been associated with metabolic traits and ischemic heart disease (IHD) (6). Homozygosity for the minor -493T allele appeared to confer an increased risk for IHD in two studies, the West of Scotland Coronary Prevention Study (WOSCOPS) and the Uppsala Longitudinal Study of Adult Men (ULSAM), together comprising 680 patients with IHD (6).

The -493G>T polymorphism is in linkage disequilibrium (LD) with two other SNPs in MTTP, the -164T>C (rs1800804) and Ile128Thr (rs1816873) (7, 8). It is not known which of these three SNPs are functional in vivo, and few studies have addressed tissues other than the liver and intestine. The MTTP promoter is well characterized, and it has been shown that the first 200bp in the proximal promoter determines the basal expression and regulation of MTTP (9).

We hypothesized that MTTP promoter polymorphisms alter the expression of the gene in the heart, thereby affecting lipid accumulation in the myocardium and thus, susceptibility to ischemia. Against this background, we investigated the in vivo allele-specific mRNA expression of MTTP in human heart, liver, and macrophages. We also characterized the underlying mechanism to the allele-specific gene expression and investigated the association between the MTTP polymorphisms and IHD in a Swedish case-control study.

MATERIALS AND METHODS

Biopsies from the human heart

Nine left ventricular biopsies were obtained from subjects who were heterozygous for the -493G>T, -164T>C, and Ile128Thr SNPs. The biopsies were from a biobank of left ventricular biopsies taken from patients undergoing heart valve surgery. The nine biopsies obtained were all from patients with tricuspid valves. The nine patients were of Caucasian origin and none of them had type 2 diabetes (T2D). Basic characteristics are shown in Supplementary Table I. Subjects had given their written informed consent to participate. The study was approved by the ethics committee at the Karolinska Institutet, Stockholm, Sweden.

Liver biopsies from individuals with hepatic steatosis

Liver samples were obtained from 25 Caucasian subjects with various degrees of liver steatosis. Total RNA was isolated and cDNA generated according to standard protocols. Twelve of these individuals were found to be heterozygous for the MTTP polymorphisms and were subsequently examined. None of the 12 individuals suffered from T2D. Basic characteristics are shown in Supplementary Table I. Subjects had given their written informed consent to participate. The study was approved by the ethics committee of the Helsinki University Hospital (10).

Primary monocytes from healthy individuals

A total of 16 healthy individuals with known genotypes for the -493G>T, -164T>C and Ile128Thr polymorphisms were recruited among Caucasian participants from a previous population screen-
Table 1. Primers and oligonucleotides used in current study

<table>
<thead>
<tr>
<th>Used in Experiment</th>
<th>Oligonucleotide Sequence (5' → 3')</th>
<th>Position</th>
<th>MTTP Region</th>
<th>Direction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Allele-Specific Gene Expression</td>
<td>GAGAAGAGCATTCTCAAAGG&lt;sup&gt;a&lt;/sup&gt;</td>
<td>+378 – +397</td>
<td>Exon 3</td>
<td>Forward</td>
</tr>
<tr>
<td>Amplification of genomic DNA and cDNA</td>
<td>GAGAAAAAGTTGGGAAATCTAA</td>
<td>+479(+13) – +479(+35)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Intron 3</td>
<td>Reverse</td>
</tr>
<tr>
<td>Amplification of genomic DNA</td>
<td>TTCTATGGCCACTGCCTCATT</td>
<td>+504 – +524</td>
<td>Exon 4</td>
<td>Reverse</td>
</tr>
<tr>
<td>Amplification of cDNA</td>
<td>GTTCAATTTTTTTCACCCA</td>
<td>+470 – +479(+7)</td>
<td>Exon 3 – Intron 3</td>
<td>Reverse</td>
</tr>
<tr>
<td>Sequencing of genomic DNA</td>
<td>CTTTACCTTTCAGG</td>
<td>+470 – +486</td>
<td>Exon 3 – Exon 4</td>
<td>Reverse</td>
</tr>
<tr>
<td>Sequencing of cDNA</td>
<td>CCTTTACCTTTCACCCA</td>
<td>+504 – +524</td>
<td>Exon 4</td>
<td>Reverse</td>
</tr>
<tr>
<td>Luciferase Assay</td>
<td>CATTTAAAGTTTCCTCA</td>
<td>+504 – +524</td>
<td>Exon 4</td>
<td>Reverse</td>
</tr>
<tr>
<td>Minimal promoter construct</td>
<td>GATCTTAATTTTTTTCACCCA</td>
<td>-178 – -148</td>
<td>Promoter</td>
<td>Forward</td>
</tr>
<tr>
<td>Minimal promoter construct</td>
<td>GATCTTAATTTTTTTCACCCA</td>
<td>-178 – -148</td>
<td>Promoter</td>
<td>Forward</td>
</tr>
<tr>
<td>Minimal promoter construct</td>
<td>GATCTTAATTTTTTTCACCCA</td>
<td>-178 – -148</td>
<td>Promoter</td>
<td>Forward</td>
</tr>
<tr>
<td>Amplification of long promoter construct</td>
<td>GCTAGCGCTGATTTGCTCCAAC</td>
<td>-592 – -577</td>
<td>Promoter</td>
<td>Forward</td>
</tr>
<tr>
<td>Amplification of long promoter construct</td>
<td>GATACCGCTGATTTGCTCCAAC</td>
<td>+9 – +27</td>
<td>Exon 1</td>
<td>Reverse</td>
</tr>
</tbody>
</table>

<sup>a</sup> Positions in the MTTP gene are given relative to transcription start site (+1) according to NM_000253.1 (mRNA). <sup>b</sup> primer is 5'-biotinylated, primer is located in intronic region, primer covers the exon/intron boundary. Rs1800804:T>C is indicated by bold characters. Enzymatic restriction sites are underlined (BgII, Nhe, BamHI).

In order to investigate if CCAAT/enhancer binding proteins (C/EBPs) had any direct effect in the allele-specific expression of MTTP, HeLa cells, which do not express MTTP, and only express C/EBPs at low levels, were cotransfected with plasmids comprising the MTTP promoter (-93 to +27) and PCMV5 expression vectors containing C/EBPs C/EBP<sub>a</sub>, C/EBP<sub>b</sub>, or C/EBP<sub>c</sub>. The C/EBP expression vectors were a kind gift from Dr. Magnus Nord, Karolinska Institute. Transient transfections and luciferase assay were performed as described above.

EMSA

For electrophoretic mobility shift assays (EMSA), complementary oligonucleotides containing the -164 polymorphic site, C/EBP consensus, and a sterol response element (SRE), which binds sterol response element binding protein 1 (SREBP-1), were annealed. Nuclear extracts were prepared from HepG2 or HuH7 liver cell lines as described (14), and competitive EMSAs were performed with labeled oligonucleotide corresponding to 50,000 cpm and 100-fold excess for competition with cold oligonucleotide. To investigate the potential binding of SREBP-1, HepG2 cells were transfected with an SREBP-1 expression vector before harvesting. Successful cloning was verified by sequencing (ABI). For the supershift assay, 1 μg/lane of human anti-SREBP-1 antibody was added to the mixture and incubated for 10 min at room temperature before loading onto the gel. The antibodies were a kind gift from Dr. Jay Horton, Southwestern Medical Center, Dallas, TX. The EMSA experiment was performed as described (15). Primer sequences are shown in Table 1.

Case-control study (INTERGENE)

The case-control study is part of the INTERGENE study and includes 544 validated IHD patients and 544 age- and sex-matched control subjects living in the Västra Götaland region of Sweden. INTERGENE is a cohort designed to study the interplay between genetic susceptibility, environmental factors, lifestyle, gender, and established risk factors in cardiovascular disease. The patients were survivors of acute coronary syndrome (ACS) and were identified from the same source population as the sampled cohort. There was no lower age limit for the first event and the upper age limit was 75. ACS comprises both acute myocardial infarction [International Classification of Diseases (ICD) 10: I21.0, I21.9] and unstable angina pectoris (ICD 10: I20.0). Previously-known coronary cases with new attacks as well as first time patients were included. The patients and controls were 61.1 ± 8.4 and 61.2 ± 8.4 years old, respectively, and 28% were women. The participants had given their informed written consent to participate. The study was approved by the local ethics committee. Full description of the study is available at www.sahlgrenska.gu.se/intergene (16).

Genotyping

Genomic DNA from subjects included in the case-control cohort and in the allele-specific gene expression assay was isolated from venous blood according to standard protocols. The patients were genotyped for the -493G>T (rs1800591G>T) and the -164T>C (rs1800804T>C) polymorphisms by TaqMan Technology (ABI). The call rates were above 96% for both the -493G>T and -164T>C polymorphisms.

Hardy-Weinberg equilibrium and differences in genotype distributions were assessed using χ<sup>2</sup> tests. Pairwise linkage disequilibrium was estimated using a maximum-likelihood Expectation Maximization algorithm implemented in the Haploview 4.0 software (17). Continuous variables that did not adhere to a normal distribution were normalized by logarithmic transformation prior to statistical analy-
in vivo, the allele-specific gene expression of MTTP was analyzed by pyrosequencing technology in human tissue biopsies from the heart, liver, and macrophages.

The pyrosequencing technology was employed because the method can detect small differences in relative gene expression from each chromosome with high accuracy. Analyses need to be performed in material from individuals heterozygous for the polymorphism(s) of interest. Any imbalance in expression levels between two alleles is, thus, measured within the same individual, hence, minimizing the cis- and trans-acting effects. The allele-specific gene expression of MTTP was measured in individuals heterozygous for the -493G>T, -164T>C, and Ile128Thr polymorphisms that are in perfect LD. The results show that the expression of the minor alleles of the MTTP polymorphisms was significantly lower compared with the major alleles in the heart (P = 0.004), liver (P = 0.001), and macrophages (P = 0.031) (Fig. 1). Genomic DNA from the same individuals was used as control and the allele ratio for the DNA was 50% as expected (Fig. 1). Thus, the minor -493T/-164C alleles are expressed at lower levels than the major -493G/-164T alleles in vivo.

Allele-specific MTTP promoter activity

In contrast to the above in vivo results, our previous in vitro results showed that the minor -493T allele has increased promoter activity compared with the -493G allele when using minimal promoter constructs (13). Inasmuch as the -493G>T and -164T>C polymorphisms are on the same haplotype, we set out to determine whether the -164T>C SNP influences transcriptional activity per se or in combination with the -493G>T SNP. For this purpose, transient transfection assays were performed in a hepatoma cell line (HepG2) that endogenously expresses MTTP. Transfections using minimal promoter constructs spanning only the -164 site showed that the minor -164C allele conferred a 30% decrease in transcriptional activity compared with the major -164T allele (P = 0.004) (Fig. 2). Constructs containing both the minor -493T and the -164C alleles (-593 to +27) also resulted in a 30% decrease in transcriptional activity compared with constructs containing both major -493G/-164T alleles (P = 0.02) (Fig. 2). In conclusion, differences between groups were estimated by unpaired t-test and one-way ANOVA using Fisher’s protected least significant difference test for post hoc analysis. Odds ratios (ORs) with corresponding 95% confidence intervals (CI) were calculated using logistic regression analysis and adjusted for age and gender. Linear regression was applied on the standard curve used in the allele-specific gene expression assay. The Wilcoxon sign rank test was employed to detect any differences in allelic ratio as measured by pyrosequencing technology. P values < 0.05 were considered significant.

RESULTS

Allele-specific gene expression of MTTP in the human heart, liver, and macrophages

To investigate whether the -493G>T and -164T>C SNPs influence transcriptional activity in vivo, the allele-specific gene expression of MTTP was measured by pyrosequencing technology in human tissue biopsies from the heart, liver, and macrophages. The pyrosequencing technology was employed because the method can detect small differences in relative gene expression from each chromosome with high accuracy. Analyses need to be performed in material from individuals heterozygous for the polymorphism(s) of interest. Any imbalance in expression levels between two alleles is, thus, measured within the same individual, hence, minimizing the cis- and trans-acting effects. The allele-specific gene expression of MTTP was measured in individuals heterozygous for the -493G>T, -164T>C, and Ile128Thr polymorphisms that are in perfect LD. The results show that the expression of the minor alleles of the MTTP polymorphisms was significantly lower compared with the major alleles in the heart (P = 0.004), liver (P = 0.001), and macrophages (P = 0.031) (Fig. 1). Genomic DNA from the same individuals was used as control and the allele ratio for the DNA was 50% as expected (Fig. 1). Thus, the minor -493T/-164C alleles are expressed at lower levels than the major -493G/-164T alleles in vivo.

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In contrast to the above in vivo result, our previous in vitro results showed that the minor -493T allele has increased promoter activity compared with the -493G allele when using minimal promoter constructs (13). Inasmuch as the -493G>T and -164T>C polymorphisms are on the same haplotype, we set out to determine whether the -164T>C SNP influences transcriptional activity per se or in combination with the -493G>T SNP. For this purpose, transient transfection assays were performed in a hepatoma cell line (HepG2) that endogenously expresses MTTP. Transfections using minimal promoter constructs spanning only the -164 site showed that the minor -164C allele conferred a 30% decrease in transcriptional activity compared with the major -164T allele (P = 0.004) (Fig. 2). Constructs containing both the minor -493T and the -164C alleles (-593 to +27) also resulted in a 30% decrease in transcriptional activity compared with constructs containing both major -493G/-164T alleles (P = 0.02) (Fig. 2). In conclusion, differences between groups were estimated by unpaired t-test and one-way ANOVA using Fisher’s protected least significant difference test for post hoc analysis. Odds ratios (ORs) with corresponding 95% confidence intervals (CI) were calculated using logistic regression analysis and adjusted for age and gender. Linear regression was applied on the standard curve used in the allele-specific gene expression assay. The Wilcoxon sign rank test was employed to detect any differences in allelic ratio as measured by pyrosequencing technology. P values < 0.05 were considered significant.

Allele-specific binding of C/EBP to the -164T>C polymorphic site

The -164 polymorphic site comprises both a putative C/EBP response element and a putative SRE (Fig. 3A). The major -164T allele is homologous to the C/EBP response element. MatInspector predicted a C/EBP binding site across the -164 polymorphic region when the promoter sequence containing the major T-allele was used (Core Similarity = 1.000). When the T allele was changed to the C allele, MatInspector could not predict the C/EBP binding site. MatInspector did not predict any SRE across the -164 polymorphic site with either the T or C allele, respectively. However, earlier studies showed that a putative SRE similar to the one found in the LDL-receptor (LDLR) gene is present across the -164 region (9, 19). The minor -164C allele shows higher homology with the putative SRE (Fig. 3A). EMSAs were performed to investigate potential binding of these nuclear factors.

Fig. 1. In vivo allele-specific gene expression of MTTP in heart, liver, and macrophages. The pyrosequencing assay measured the relative amount (%) of the major 128Ile and the minor 128Thr alleles in heterozygous individuals, thus revealing the allele-specific effect on gene expression of the -493G>T and the -164T>C polymorphisms. Measurement on DNA from the same individuals is included as controls and showed a perfect 50:50 allele ratio as expected, reflecting the two chromosomes. Allele ratios measured on the cDNA (generated from RNA isolated from subjects heterozygous for the three SNPs) show that the minor -493T/-164C alleles have a lower transcriptional activity compared with the major alleles (-493G/-164T). Measurements were performed in triplicate on at least two different occasions for each subject. Bars indicate mean ± SD.
The major -164T allele oligonucleotide bound more strongly to nuclear factors compared with the minor -164C allele oligonucleotide. A 100-fold excess of an unlabeled -164T oligonucleotide competed against this binding whereas a nonspecific DNA sequence had no effect on the binding (Fig. 3B). Competitive EMSA with labeled C/EBP consensus oligonucleotide and -164T and -164C oligonucleotides showed that an excess of unlabeled C/EBP oligonucleotide competes the binding of nuclear factors to the -164T oligonucleotide. In contrast, we could not detect any binding of C/EBP to the -164C allele (Fig. 3C).

The SRE across the -164 polymorphic site, similar to the one found in the LDLR promoter, is known to bind SREBP-1 (19). The binding of SREBP-1 to the polymorphic site at position -164 was investigated using nuclear extracts from HepG2 cells overexpressing SREBP-1. The LDLR-SRE consensus oligonucleotide was used as a positive control. Supplementation with SREBP-1 antibody generated a clear supershift of the LDLR-SRE complex but no supershift was detected for the nuclear complexes containing the -164T or the -164C oligonucleotides (Fig. 4, A and B). These results indicate that C/EBP bound more strongly to the major -164T allele than the minor -164C allele and that SREBP-1 did not bind to the -164C polymorphic site in an allele-specific manner.

C/EBPs mediate allele-specific gene expression of the MTTP gene

In order to investigate if C/EBPs affect the promoter activity of MTTP per se in an allele-specific manner, transient transfection assays were performed in HeLa cells. HeLa cells do not have endogenous expression of MTTP, which minimizes the background signals and allows detection of any direct influences of C/EBPs on the transcription of the two promoter variants (i.e., -493G/-164T and -493T/-164C), respectively. For this purpose, HeLa cells were cotransfected with pGL3-basic containing the promoter constructs (-593 to +27) and expression vectors containing C/EBPa, C/EBPβ, or C/EBPδ, respectively. In accordance with the results based on EMSA experiments, the presence of C/EBPa and C/EBPδ increased the transcriptional activity for the major -493G/-164T alleles compared with the minor -493T/-164C alleles (Fig. 5). We conclude that C/EBPa and C/EBPδ are critical for the effect on transcriptional activity mediated by the -164T>C polymorphism.

Association of the MTTP -164T>C promoter polymorphism with IHD

The relationship between the -164T>C SNP and IHD was investigated in a Swedish case-control cohort (INTERGENE) consisting of 544 patients and 544 sex- and age-matched control subjects. The subjects were genotyped for the -493G>T and -164T>C SNPs, which were found to be in perfect LD (D' = 1.0, r^2 = 1.0).

The minor allele frequency of the -164T>C polymorphism in the MTTP promoter was 0.25 in patients and 0.22 in controls and the genotype distributions adhered to the Hardy-Weinberg equilibrium in both groups. Homozygous carriers of the minor -164C allele (or the -493T allele) were found to have a higher risk for IHD after adjustment for age and gender: 1.76 (1.03–2.99) [OR (95% CI)], P = 0.038. The OR for heterozygous individuals was 1.13 (0.88–1.45), P = 0.36.

### DISCUSSION

In this study, we demonstrated an allele-specific expression of MTTP predisposing to IHD. In summary, the minor alleles of the -493G>T and -164T>C SNPs conferred lower expression of MTTP in the human heart, liver, and macrophages. In vitro experiments revealed that the minor -164C allele is critical in mediating the lower transcriptional activity. C/EBP did not bind to the minor -164C allele but had strong affinity for the major -164T allele. Finally, homozygosity for the -164C allele was associated with increased risk of IHD. On the basis of these results, we propose that the -164T>C SNP alters the MTTP expression and, thus, influences the susceptibility to IHD.

**Fig. 2.** Transcriptional activity of MTTP promoter constructs. Relative luciferase activity from constructs spanning the -164T>C polymorphic site from position -179 to -148 in the MTTP promoter and from constructs spanning both the -164T>C and -493G>T polymorphic site (-593 to +27). All constructs were transiently transfected into the human HepG2 liver cell line. Cells transfected with longer promoter constructs were also cultured in the presence of 100 mM insulin for 24 h. Constructs containing the major allele(s) were set to 100% and used as reference. Bars indicate mean ± SEM. All assays were performed in triplicate. Empty pGL3-vector was used as control.
It is postulated that MTTP is important in the heart for secretion of intracellular lipids, which may be toxic to the myocardium at high concentration (5, 20). The finding that the minor -164C allele conferred decreased MTTP mRNA expression compared with the major -164T allele is consistent with our previous study showing that individuals carrying the -493T allele, and hence also the minor -164C allele, have lower MTTP mRNA concentrations in the atrium of the heart compared with carriers of the common allele (6). Of note, homozygote individuals also had electrocardiogram abnormalities independent of preexisting heart disease (6). Furthermore, it has been shown that decreased transcription of MTTP mRNA is inversely correlated with intracellular TAG concentrations in human hearts (5). Cardiac lesions and excessive deposition of lipochrome pigment have been reported in abetalipoproteinema, a rare recessive monogenic disease caused by mutation in MTTP (21, 22). Furthermore, tissue-specific knockout mice that lack MTTP expression in the heart show cardiac lipid accumulation (4). Taken together, these results suggest that a functional MTTP protein is important for cardiac lipid metabolism and that the minor

![Fig. 3. Allele-specific binding of nuclear factor to the -164 polymorphic site. A: DNA sequence from position -173 to -156 in the MTTP promoter. The -164T>C site is shadowed in gray. Sequence containing the major T-allele shows homology to the C/EBP binding site as predicted by MatInspector. The C-allele sequence shows homology to the LDLR-SRE (9). The core sequence for C/EBP binding site is 5′-ATTGCGCAAT and the binding site for the LDLR-SRE is 5′-ATCACCCCAC. Stars indicate homology nucleotides. B: EMSA with nuclear extract from the liver cell line HepG2 and radioactive probes with either the major -164T allele (lanes 1–5) or the minor -164C allele (lanes 6–10), as indicated at the top row. The nuclear extract was added in all lanes except 1 and 6 which contain only free labeled oligonucleotide. In the competition row, the minus sign (−) indicates no competition (lanes 2 and 7), T indicates competition with 100-fold unlabeled -164T oligonucleotide (lanes 3 and 8), C indicates competition with 100-fold unlabeled -164C oligonucleotide (lanes 4 and 9), and X shows competition with 100-fold unlabeled random oligonucleotide (3 and 10). Arrows show the allele-specific binding of nuclear factors. C: EMSA with nuclear extract from the liver cell line Huh7, and the radioactive probe comprising the C/EBP consensus sequence (lanes 1–3), the major -164T allele (lanes 4 and 5), and the minor -164C allele (lanes 6 and 7), as indicated in the top row. First lane, no nuclear extract added, only free labeled oligonucleotide. Arrow indicates the supershift with SREBP-1 antibody and the LDL-SRE consensus sequence. B: Magnification of the supershift. Arrow indicates the band representing the supershift.

![Fig. 4. Binding of SREBP-1 to LDLR-SRE but not to the -164 site. A: EMSA with nuclear extract from the liver cell line HepG2, overexpressing SREBP-1 and the radioactive probe comprising the LDLR-SRE consensus sequence (lanes 1–3), the major -164T allele (lanes 4 and 5) or the minor -164C allele (lanes 6 and 7), as indicated in the top row. Supplementation of antibody against SREBP-1 is indicated with plus signs (+) (lanes 3, 5, and 7). No nuclear extract was added in lane 1, only free labeled oligonucleotide. Arrow indicates the supershift with SREBP-1 antibody and the LDL-SRE consensus sequence. B: Magnification of the supershift. Arrow indicates the band representing the supershift.

Supplemental Material can be found at: https://www.jlr.org/content/suppl/2009/07/01/M900195-JLR209.DC1.html
Allele-specific gene expression of MTTP and IHD

The formation of VLDL (1). The expression of the minor allele of the -164T>C SNP resulted in lower transcription of the gene. This is in contrast with our earlier in vitro results from HepG2 cells, showing that the minor allele of the -493G>T polymorphism was associated with higher promoter activity compared with the major allele, using minimal promoter constructs comprising only the -493G>T SNP (13). However, an additional effect of the -493G>T polymorphism cannot be excluded from these experiments, which would require constructs of the same length with only one polymorphic site included in each promoter construct. Nonetheless, genetic linkage studies have shown that these two polymorphisms are always inherited together. Rubin et al. (27) recently reported that the minor alleles of the -493G>T and -164T>C SNPs result in higher promoter activity compared with the major alleles when Caco-2 cells originating from human intestine were transfected with MTTP promoter constructs spanning from -966 to -1 not including the transcriptional start site. However, they could not detect any significant differences in allele-specific promoter activity when HuH7 liver cells were used (27). Of note, these two promoter studies did not include in vivo analyses and it has recently been shown that in vitro analyses often fail to predict in vivo effects of regulatory polymorphisms (28), indicating the need for assessing the effect of polymorphisms in native tissues.

We previously reported that the Ile128Thr polymorphism influences the interaction between MTTP and apoB. The threonine allele had decreased thermal stability and weakened binding to apoB compared with the isoleucine allele (29). Taken together, our results suggest that homozygosity for the minor -164C and 128Thr alleles has both reduced MTTP expression and a less functional protein.

Here, we showed that the -164T>C SNP predisposed to IHD in a study of 544 patients and 544 control subjects. We also showed that the -164T>C and -493G>T SNPs were in perfect allelic association. This is in agreement with previous results from the WOSCOOPS and ULSAM studies where the -493TT genotype was associated with higher risk of IHD (6). Of note, analyses based on combined data from the INTERGENE, WOSCOOPS, and ULSAM studies yielded a common Mantel-Haenszel OR of 1.64 (1.20–2.25) with a Cochran-Mantel-Haenszel Statistic of 9.49 (P = 0.002), the odds ratios being homogeneous across all studies (P = 0.14, Breslow-Day test).

In contrast to the above result, there was no significant association between the -493G>T or -164T>C SNPs and cardiovascular end points in the Framingham Offspring Study (consisting of 212 IHD patients and 2,510 control subjects) (30) and the ECTIM study (consisting of 622 male patients with myocardial infarction and 728 age-matched controls from Ireland and France) (31). Possible explanations for the lack of association in these studies...
might be different study designs and ethnicities. Validation in prospective studies is needed as well as more in-depth knowledge about the function and regulation of MTTP in the heart and macrophages. In addition, the influence of other polymorphisms that are in allelic association with the studied SNPs cannot be excluded. However, the -493G>T, -164T>C, and Ile128Thr are in perfect LD and lie in a haplotype clearly distinguished from the 3’ region of the gene, including the 3’-UTR in MTTP (Supplementary Fig. II). Any influences of potential functional polymorphisms located 3’ in MTTP are, thus, unlikely. The -164T>C polymorphism lies in the proximal promoter of MTTP that has been shown to regulate the basal transcription of the gene (9). Therefore, it is likely that the -164T>C polymorphism has a profound effect on the observed allele-specific gene expression. However, other polymorphic sites may also affect the expression and regulation of MTTP.

In summary, we have shown that the minor MTTP-164C variant resulted in lower expression of MTTP in the heart, liver, and macrophages, most likely due to a reduced binding of C/EBPs. The -164C allele was associated with increased risk for IHD and we postulate that lower concentration of MTTP results in increased lipid accumulation in the myocardium and, thus, increased susceptibility to IHD. We hypothesize that MTTP inhibitors could have severe long-term side effects related to myocardial function and it is important not only to study MTTP in the liver, but also in the heart and other tissues, to fully understand its potential side effects when used as a drug target.

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