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Genetic variants affecting alternative splicing of human cholesteryl ester transfer protein

Adam Suhy [a,*], Katherine Hartmann [a], Leslie Newman [a], Audrey Papp [a], Thomas Toneff [b], Vivian Hook [b], Wolfgang Sadee [a]

[a] Center for Pharmacogenomics, Department of Pharmacology, College of Medicine, The Ohio State University Wexner Medical Center, 5184A Graves Hall, 333 West 10th Avenue, Columbus, OH 43210, USA
[b] Skaggs School of Pharmacy and Pharmaceutical Sciences, School of Medicine, Departments of Neuroscience and Pharmacology, University of California, San Diego, 9500 Gilman Dr., La Jolla, CA 92093, USA

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ABSTRACT

Cholesteryl ester transfer protein (CETP) plays an important role in reverse cholesterol transport, with decreased CETP activity increasing HDL levels. Formation of an alternative splice form lacking exon 9 (Δ9-*CETP*) has been associated with two single nucleotide polymorphisms (SNPs) in high linkage disequilibrium with each other, namely rs9930761 *T* > *C* located in intron 8 in a putative splicing branch site and rs5883 *C* > *T* in a possible exonic splicing enhancer (ESE) site in exon 9. To assess the relative effect of rs9930761 and rs5883 on splicing, mini-gene constructs spanning *CETP* exons 8 to 10, carrying all four possible allele combinations, were transfected into HEK293 and HepG2 cells. The minor *T* allele of rs5883 enhanced splicing significantly in both cell lines whereas the minor *C* allele of rs9930761 did not. In combination, the two alleles did not yield greater splicing than the rs5883 *T* allele alone in HepG2 cells. These results indicate that the genetic effect on *CETP* splicing is largely attributable to rs5883. We also confirm that Δ9-*CETP* protein is expressed in the liver but fails to circulate in the blood.

1. Introduction

Cholesteryl ester transfer protein (CETP) is a key protein in reverse cholesterol transport, a process that moves cholesterol from the periphery to the liver for excretion. Expressed most highly in the liver and released into the circulation [1], CETP mediates the exchange of cholesteryl esters from high-density lipoproteins (HDL) with triglycerides to low-density lipoproteins (LDL). Increased CETP activity reduces the HDL/total cholesterol ratio, associated with increased risk for coronary artery disease (CAD) [2–4]. Accordingly, CETP inhibitors are currently in clinical trials to determine their ability to increase HDL levels and reduce the risk of CAD. However, initial results have been disappointing, in some cases even demonstrating enhanced CAD risk [5,6]. While off-target effects of CETP inhibitors are suspected to play a role, it is also possible that genetic *CETP* variants affect disease risk and treatment outcomes. Subjects lacking functional *CETP* expression display multiple cardiovascular abnormalities [6], showing that the reverse cholesterol pathway serves important physiological functions. Therefore, optimal CETP activity should balance negative and positive downstream events. A detailed understanding of the genetic architecture of the *CETP* locus is critical in guiding therapeutic intervention.

Numerous genetic studies of *CETP* have focused on frequent non-synonymous SNPs including I405 V (rs5882) and promoter region SNPs within ~1 kb of the transcription start site [7,8]; however, the mechanism underlying any effect on CETP activity remained uncertain. GWAS studies have also implicated SNPs in the promoter enhancer region as being associated with circulating CETP and HDL levels, existing in high LD with each other on a long haplotype. The most significant SNPs were found to reside in regions 5–10 kb upstream of *CETP* [9,10], with Taq1B in intron 1 serving as a marker SNP. Despite highly significant association with HDL, any effect of *CETP* variants on CAD risk remained weak at best [11]. Carriers of the minor *Taq1B* allele, associated with reduced CETP activity, may benefit less from statin therapy, suggesting a possible gene–drug interaction [5,12]. We have studied the molecular genetics of *CETP,* showing that a frequent SNP 6.2 kb upstream (rs247616) is the most likely variant responsible for reduced *CETP* mRNA expression associated with the long upstream haplotype [9]. This same SNP also has shown a strong association with HDL levels [9,13], but additional regulatory mechanisms are likely to be operative. Another enhancer SNP in high LD with rs247616 has also shown an association of CAD outcome with statin therapy [14], supporting the notion that CETP activity has clinical relevance.

* Corresponding author. Address: 5168 Graves Hall, 333 West 10th Avenue, Columbus, OH 43210, USA.
E-mail address: suhy.2@osu.edu (A. Suhy).

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Alternative splicing of *CETP* mRNA has been shown to result in a protein isoform lacking exon 9 (Δ9-*CETP*), which appears to be sequestered in the ER and may act in a dominant-negative fashion by binding to full length CETP preventing its secretion [15]. We have identified two SNPs of intermediate minor allele frequency (MAF 4–8%) to be associated with increased formation of the Δ9-*CETP* mRNA isoform in livers, one in intron 8 (rs9930761) interrupting a putative splicing branch point, and the other in exon 9 (rs5883) creating a putative exonic splicing enhancer (ESE) sequence [9]. In high LD with each other, these two SNPs reside on opposite alleles to the upstream promoter/enhancer alleles and were found to be associated with increased HDL levels, with an effect size similar to that of the upstream enhancer SNPs [9]. This strong effect had previously remained hidden because the splicing SNPs reside on opposite haplotypes as the enhancer SNPs, resulting in underestimation of the splicing effect on expressed CETP activity unless the enhancer SNP effect is accounted for [9]. Moreover, rs5883, has been associated with increased risk of CAD in hypertensive patients, a finding that still requires replication [9].

The goal of the present study was to test further the influence of rs9930761 and rs5883 on *CETP* exon 9 splicing. The former has slightly higher allele frequency (MAF ~ 6%) than the latter (~5%), but associations with mRNA expression favor rs5883 [9]. Therefore, rs9930761 could either have a relatively small effect, it could contribute to or be necessary for the rs5883 effects, or rs5883 alone could be the main variant affecting splicing. Our experiments with mini-gene constructs favor this third hypothesis.

2. Materials and methods

2.1. Mini-gene construction

A genomic DNA region was amplified with PCR using Advantage HD (Clonetech, USA) according to manufacturer's protocol using primers Exon-8F infusion and Exon-10R infusion (Table 1). This region extending from exon 8 to just downstream of exon 10 was inserted by In-Fusion Dry-Down PCR cloning kit (Clonetech, USA) into a pCMV-Tag2B expression vector in frame. The procedure was completed by transforming into Stellar competent *E. coli* (Clonetech, USA).

Site-directed mutagenesis was carried out with the Quikchange lightning II (Agilent, USA) system. Each SNP (rs5883 and rs9930761) was mutated using primers rs9930761-SDM C-T and rs5883-SDM C-T (sequences shown in Table 1), transformed, and isolated sequentially to create all four haplotype combinations. All constructs were sequenced to confirm proper insertion, showing that the insert sequence was otherwise that of the wild-type *CETP*. Multiple plasmid constructs were generated, yielding similar results in test transfection experiments. The haplotype plasmids were transformed into XL-10 gold competent cells (Agilent, USA), and three clones of each haplotype were collected and again sequenced. Each of the identical three clones was combined for the transfection assays.

Table 1
Primers used in PCR reactions, site directed mutation procedures, and splicing assay.

Primer	Sequence (5'-3')
Exon8-F infusion	CTGCAGGAATTCGATATCGCCAGCATCCTTTCAGATGG
Exon10-R infusion	ATCGATAAGCTTGATATCAGGGGGCAGTTACCTCTTGGAA
rs9930761-SDM C-T	CTGAAGCTGGACCTGAGCCCAGTAGGG
rs5883-SDM C-T	TGGTTCTCTGAGCGAGTCTTTCACTCGCTGGC
β-actin-R	GCCGATCCACACGGAGTACT
Exon10-R	AAGATTTCCTGGTTGGTGTTGA
Exon8_10-F	GGAGTCCCATCACATGGCAG
Exon9_10-F	GGGAGACGAGTTCATGGCAG

2.2. Cell lines

Human Embryonic Kidney (HEK 293) and HepG2 were grown to 70–80% confluence in low glucose DMEM + 10% FBS and 1% Penicillin/Streptomycin. For passaging, HEK293 and HepG2 cells were treated with 0.05% and 0.25% Trypsin in EDTA, respectively.

2.3. Transfection

Cells were grown to 70–80% confluence in T75 flasks, trypsinized and plated at ~2.5×10^5 cells on 6-well plates, and allowed to grow overnight in 2 mL of growth medium. Cells were transfected using Lipofectamine 2000 (Invitrogen, USA). Transfection was optimized, and 7.2 μL of Lipofectamine 2000 reagent was brought to 150 μL/well total volume in Optimem (Gibco, USA). 16 ng of haplotype construct DNA, 1.6 μg of empty vector, and 200 ng pcDNA vector expressing EmGFP (Life Technologies, USA) were mixed in a final volume of 150 μL/well with Optimem. This was added to the Lipofectamine dilution and incubated for 5 min at room temperature. 275 μL was added to each well, followed by incubation at 37 °C cells for 7–24 h.

2.4. RNA isolation and cDNA synthesis

Media were aspirated from cells and cells lysed and stored in 500 μL Trizol (Invitrogen, USA). RNA was isolated and washed using chloroform and isopropyl alcohol. 50 μL nuclease free water was added to dissolve pellet, RNA integrity assayed with a Bioanalyzer 2100 (Agilent, USA), and concentration measured with Qubit (Invitrogen, USA) spectroscopy.

1 μg RNA was treated with Amplification grade DNase I (Invitrogen, USA). cDNA was generated with poly-dT and gene-specific primers (β-actin-R, Ex10-R – Table 1) using SuperScript Reverse Transcriptase III (Invitrogen, USA).

2.5. CETP and Δ9-CETP mRNA assay

The splicing assay relies on measuring the relative expression of the Δ9-*CETP* and full-length mRNA from the same cDNA sample. The measurements were made using RT-PCR (Life technologies 7500) with SYBR Green. Primer Exon8_10F, which is specific for the Exon 8 to Exon 10 junction of the short form, and Exon9_10-F specific to the exon 9 to exon 10 junction of the long form were used with the Exon10-R (Table 1). PCR cycles included an initial incubation at 95 °C for 20 s, followed by a maximum of 40 cycles of 95 °C for 3 s and 60 °C for 30 s. Cycle thresholds were compared between the two reactions and used to determine the relative quantities of the short and long form of the mini-gene (Table 2).

2.6. CETP Western blots

Liver samples were prepared as homogenates in buffer with a cocktail of protease inhibitors to prevent protein degradation [16]. Liver and plasma samples were subjected to denaturing gel electrophoresis (4–12% NuPage Bis–Tris gels, Novex) and

Table 2
Liver and plasma sample genotypes and mRNA splicing percentages in the liver.

Sample	rs9330761	rs5883	Percent Δ9-*CETP* mRNA
Li049	CT	CT	32
Li051	TT	CC	17
C-2117	TT	CC	ND
C-377	CC	TT	ND

ND – not determined.