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Genetic variants affecting alternative splicing of human cholesteryl ester transfer protein

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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.

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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.

electrophoretically transferred to nitrocellulose membranes for Western blots. Western blots of human liver samples and plasma were conducted with rabbit anti-CETP (Sigma–Aldrich, USA) detected with goat ARGG-HRP (anti-rabbit gamma-globulin-horseradish peroxidase) (Sigma–Aldrich, USA) using the ECL + detection reagent (Amersham, USA) [17].

3. Results

3.1. RT-PCR analysis of full-length CETP mRNA and Δ9-CETP mRNA

CETP mRNA levels were quantitated by RT-PCR. Accuracy of the assay was tested using a standard curve with known ratios of cDNA fragments spanning exons 8–10 and mini-gene fragment with exons 8 and 10 (Δ9-*CETP*). The standard curve showed a slope equal to 1 with $r^2 = 0.99$.

3.2. Native CETP mRNA expression and splicing in HEK293 and HepG2 cells

Expression of *CETP* mRNA in human embryonic kidney (HEK293) and hepatocyte (HepG2) cells was measured using RT-PCR. The long and short forms of *CETP* were amplified to determine the extent of splicing in non-transfected cells. *CETP* mRNA was present at very low levels in HepG2 and HEK293, 3.6×10^{-4} and 3.8×10^{-4} times that of β-actin mRNA used for normalization, respectively. Alternative splicing in HEK293 and HepG2 occurred at similar levels, with the full length mRNA accounting for 53 ± 1% and 54 ± 4% of the total, respectively, indicating that these cells are competent in alternative splicing of *CETP*.

3.3. Mini-gene constructs expressing the genomic region of CETP surrounding exons 8–10

To study the effect of each SNP on *CETP* mRNA splicing, mini-gene constructs were developed with all four combinations of the 2 SNPs (rs9930761 *T/C* alleles, and rs5883 *C/T* alleles). Mini-gene constructs spanning exon 8 through intron 10 of *CETP* were created using the pCMV-Tag2B expression vector.

Initial results revealed a high level of the Δ9-*CETP* splice isoform of the mini-gene in HEK293 cells (83% after 24 h transfection for the wild-type construct) when cells were transfected with 1.6 μg cDNA vector. To determine whether the splicing machinery was affected by the high expression level, 100-fold less (0.016 μg) plasmid DNA was transfected resulting in a proportional decrease of expressed mRNA, indicating that mRNA expression was not saturated. In addition, the level of splicing remained similar after the 100-fold dilution of plasmid DNA used for transfection (82% Δ9-*CETP* after a 24 h transfection for the wild-type construct). Expression of the *CETP* mini-gene constructs (1.6 and 0.016 μg) yielded mRNA levels 0.1–50 times that of β-actin in HepG2 and HEK293, respectively. This represents a 300 to 1×10^5 higher expression compared to native *CETP* mRNA levels in HepG2 and HEK293. Therefore, native expression of *CETP* mRNA was minimal compared to the mini-gene mRNA levels and was ignored in the analysis of mini-gene mRNA splicing. An amount of 0.016 μg of plasmid DNA was used in each subsequent transfection.

HEK293 cells transfected with 0.016 μg of plasmid DNA were harvested at different time points (2, 4, 7, and 24 h). Vector expressed mRNA increased between 4 and 7 h and peaked at 24 h (wild-type construct was normalized to β-actin expression 2 h = 0.2%, 4 h = 0.6%, 7 h = 2%, 24 h = 23%). The level of splicing was also relatively high in HepG2 cells, remaining similar between 7 and 24 h (7 h = 79% Δ9-*CETP*, 24 h = 84% Δ9-*CETP*). Therefore a 24 h incubation period was selected for subsequent experiments.

3.4. Exon9 splicing of CETP mini-gene constructs in HEK293 and HepG2 cells

All four possible combinations generated by the rs5883 and rs9930761 alleles were transfected into HepG2 and HEK293 cells, and relative expression of the mRNAs containing exons 8, 9, and 10 (representing wild-type *CETP*) and exon 8 and 10 (representing Δ9-*CETP*) was measured. Shown in Fig. 1, constructs that contain the minor allele of rs5883 (*T*) express significantly more Δ9-*CETP* mRNA than those with the major *C* allele in both HEK293 and HepG2 cells. In contrast, splicing efficiency was indistinguishable between the minor *C* allele and the major *T* allele of rs9930761. Moreover, the combination of the minor alleles of rs5883 and rs9930761 did not differ from the splicing levels observed with the minor *T* allele of rs5883 alone in HepG2 cells, indicating that rs9930761 was not required. In HEK293 cells, the combined effect of the two minor alleles was even reduced compared to splicing levels observed with the minor *T* allele of rs5883 alone. Experiments were repeated with different plasmid preparations, yielding the same results. These results indicate that rs5883 alone results in enhanced splicing to yield the Δ9-*CETP* isoform of the mini-gene *in vitro*.

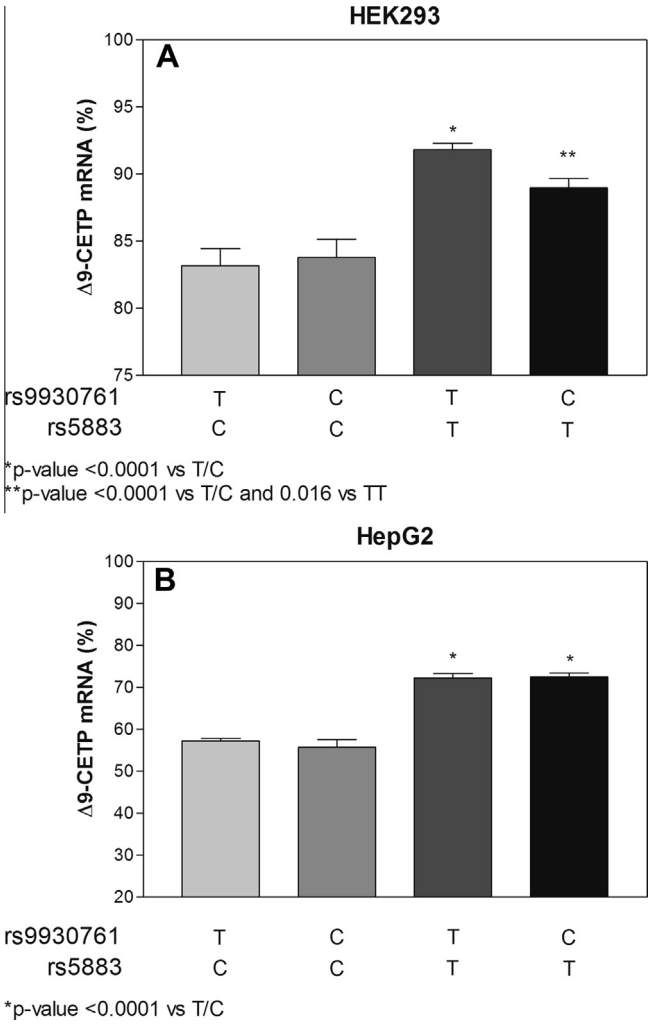

Fig. 1. CETP mRNA Splicing in cell lines. Mini-gene constructs were transfected into (A) HEK293 and (B) HepG2 cells. RT-PCR was used to determine amounts of full length and Δ9-*CETP* mRNA present in each sample. The percentage of Δ9-*CETP* was calculated by dividing the expression of Δ9-*CETP* by the sum of the expression of Δ9-*CETP* and full length.

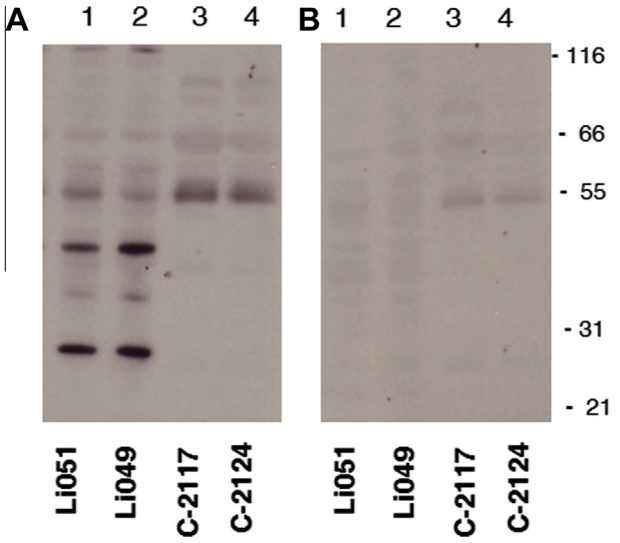

Fig. 2. Western blots with a CETP antibody in liver protein extract and plasma. Anti-CETP antibody and secondary antibody (ARGG-HRP) were used in (A), and secondary antibody only in (B). Liver tissues (heterozygous for rs5883) are denoted as Li051 and Li049. Plasma samples are denoted as C-2117 and C-2124. Lanes in panel (A and B) representing liver samples were loaded with 5 μg protein, and plasma with 2.5 μg protein based on protein measurements of each sample.

3.5. CETP protein in liver and plasma

CETP protein from human liver tissue homogenates was assessed by Western blot using anti-CETP antibody. Bands corresponding to the full length CETP protein of 55-kDa were observed in liver and plasma. Liver samples known to contain the Δ9-*CETP* mRNA splice isoform of *CETP* contained a 47-kDa band (Fig. 2A, lanes 1 and 2) corresponding in molecular weight (MW) to the Δ9-*CETP* splice variant. The minor 100-kDa band may represent a dimer of CETP protein. Lower MW bands may represent truncated or degraded forms of CETP. It is noted that plasma contains only the 55-kDa CETP protein and not the 47-kDa Δ9-*CETP* splice variant (Fig. 2A lanes 3 and 4), indicating that the Δ9-CETP protein is not secreted efficiently. Control experiments without the CETP antibody were devoid of these bands (Fig. 2B).

We also made an attempt to quantitate the relative amounts of CETP and Δ9-CETP isoform in livers with different *CETP* genotypes. However, the intensity of the staining of full length CETP and its Δ9-CETP isoform was highly sensitive to experimental conditions, impeding attempts to attain reproducible quantitation. In multiple experiments, the Δ9-CETP isoform tended to be robustly expressed in liver tissues, often yielding more intense bands on the gel than the full length CETP protein. This result is consistent with the notion that CETP is excreted from the liver while the Δ9-CETP isoform is not. An example of Western blots of two liver tissues heterozygous for rs5883 and rs9930761 is provided in Fig. 2A. In this case, the intensities of the 55 kDa and 47 kDa bands vary corresponding to the measured splicing levels of *CETP* mRNA in the same livers. Yet, multiple experiments showed that Western blots could not provide reliable quantitation of CETP protein isoforms. Genotypes of liver and plasma samples as well as Δ9-*CETP* mRNA percentage for livers used in Western blots is provided in Table 2.

4. Discussion

We report the results of mini-gene experiments testing the ability of rs5883 and rs9930761 to affect splicing of *CETP* to its Δ9-*CETP* isoform. The results indicate that an effect on splicing is largely attributable to rs5883 rather than rs9930761. The latter also does not appear to be necessary for the effect of rs5883, and in HEK293 cells, even diminishes the effect of rs5883.

These results also demonstrate that the effect of rs5883 on alternative splicing is not unique to liver cells, where *CETP* is primarily expressed, but also occurs in kidney cell lines, suggesting the regulation of splicing is not dependent on liver-specific factors. Determined with Western blots, the presence of both full length CETP and Δ9-CETP protein in the liver supports earlier findings that the Δ9-CETP protein is sufficiently stable but is not secreted from the liver as it is absent in the circulation [15]. The relative amounts of CETP and Δ9-CETP protein in the liver are subject to multiple factors, including the rate of transfer of full-length CETP into the circulations, expected to be slowed by higher levels of Δ9-CETP via formation of a heterodimer [15]. In addition, CETP and Δ9-CETP could be subject to differential degradation rates, confounding the interpretation of relative amounts of CETP *versus* Δ9-CETP. Whether Δ9-CETP has independent intracellular physiological functions remains to be determined. Further evaluation of the mechanism by which rs5883 influences alternative splicing may help to inform our understanding of *CETP* regulation and its potential role in CAD risk and treatment response.

CETP variants rs5883 and rs9930761 are in high linkage disequilibrium (LD) with each other ($D' = 1.0$) while rs9930761 is slightly more abundant (MAF 6% *versus* 5%) [9]. Therefore, the haplotype allele carrying only the minor *C* allele of rs9930761 is present at a MAF of ~1%, whereas the haplotype carrying only the minor *T* allele of rs5883 was not detected *in vivo* (but was tested here *in vitro*) and the haplotype with both minor alleles occurs at ~5%. Therefore, *in vivo* associations observed with rs5883 are not independent of any effects of rs9930761. We cannot rule out such combined effect, but the results presented here indicate that rs5883 has the main effect and support the use of only rs5883 in clinical association studies.

These *in vitro* results are consistent with previous association studies, showing that rs5883 is more strongly associated with HDL levels and risk of CAD in hypertensive patients than rs9930761. Despite a slightly lower MAF, rs5883 had shown greater significance in association and effect size with HDL-C levels and adverse CAD outcomes than rs9930761 [9], supporting the conclusion that rs5883 is the main active variant [9,10,18]. As CETP levels may be optimized for maintaining sufficient HDL levels while also enabling reverse cholesterol transport, robust genetic effects on CETP activity are critical for assessing its clinical relevance, in view of ongoing developments of CETP inhibitors. Also, an interaction of *CETP* splicing with statin therapy requires further study.

The clinical relevance of alterative splicing to Δ9-*CETP* mRNA on *CETP* activity remains to be studied further. If the alternatively spliced isoform truly acts in a dominant-negative manner, as suggested previously [15], CETP activity could decrease considerably with increased rates of alterative splicing in the presence of rs5883, in particular in homozygous carriers of the *T* allele. As clinical trials of CETP inhibitors progress, it may be important to analyze results with this genetic variant in mind as the more than 10% of the population heterozygous for rs5883, and ~0.25% homozygous, already show reduced CETP function. In some ethnic groups, including those of African descent, rs5883 MAF is higher (7–11%), enhancing its potential clinical significance. In addition, the interaction between rs5883 and *CETP* enhancer variants needs to be considered.

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