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Title: A novel microdeletion affecting the CETP gene raises HDL-associated cholesterol levels

Emma Hitchcock, BSc, University of British Columbia, Child and Family Research Institute, 950 W 28th Ave, A4-151, Bay 17, Vancouver, BC, V5Z 4H4
Phone: (604) 875-2000 ext. 6783, Fax: (604) 875-2373, email: ehitchcock@cfri.ca

Jay V. Patankar, PhD, Postdoctoral Fellow, Centre for Molecular Medicine and Therapeutics

Christine Tyson, PhD, FCCMG, Clinical Cytogeneticist. Royal Columbian Hospital

Monica Hrynchak, MD, FRCPC, FCCMG, Medical Director Cytogenetic Laboratory, Royal Columbian Hospital

Michael R. Hayden, MB, ChB, PhD, FRCP(C), FRSC, CM, OBC, University Killam Professor, Department of Medical Genetics, UBC, Senior Scientist, Centre for Molecular Medicine and Therapeutics

William T. Gibson, MD, PhD, FRCPC, FCCMG, Associate Professor, Department of Medical Genetics, University of British Columbia, Senior Clinician Scientist, Child and Family Research Institute

All authors declare no conflict of interest.

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Abstract

We describe a novel, inherited 16q13 microdeletion that removes CETP and several nearby genes. The proband was originally referred for severe childhood-onset obesity and moderate developmental delay, but his fasting lipid profile revealed relatively high HDL-cholesterol and low LDL-cholesterol for age, despite his obesity. Testing first-degree relatives identified two other microdeletion carriers. Functional assays in affected individuals showed decreased CETP mRNA expression and enzymatic activity. This microdeletion may or may not be pathogenic for obesity and developmental delay, but based on the lipid profile, the functional studies, and the phenotype of other patients with loss-of-function mutations of CETP, we believe this microdeletion to be antipathogenic for cardiovascular disease.

Key Words

Cardiovascular disease, cholesteryl ester transfer protein, common disease, copy-number variant, microdeletion, obesity, structural variant

Introduction

Cholesteryl ester transfer protein (CETP) exchanges cholesteryl ester (CE) for triglycerides (TG). CE is transferred from apolipoprotein A-I (ApoA-I)-containing high-density lipoproteins (HDL) to the apolipoprotein B (ApoB)-containing molecules, namely very low-density lipoproteins (VLDL) and low-density lipoproteins (LDL), while TG are transferred into HDL. Through this exchange CETP can mediate the amount, size, and composition of plasma HDL particles. In humans, CETP is an integral component of a primary reverse cholesterol transport (RCT) pathway, in which CE is transported back from peripheral tissue to the liver via the CETP-mediated movement of CE from HDL to LDL.

CETP (OMIM #118470) is a highly polymorphic gene, with both rare and common polymorphisms. Heterozygous and homozygous deficiency of CETP in humans results in hyperalphalipoproteinemia (HALP – OMIM #143470), with high levels of circulating HDL-associated cholesterol and low levels of circulating LDL-associated cholesterol. HDL particles in CETP-deficient patients are larger and richer in apolipoprotein E (ApoE) and CE than are HDL particles from non-CETP deficient patients; such a profile is believed to be anti-atherogenic and cardioprotective. The observed HDL levels depend on which variant(s) are present, because different polymorphisms have different effect sizes on protein expression and/or activity. As expected, heterozygous CETP deficiency produces an intermediate phenotype.

We have identified a novel microdeletion at 16q13 affecting the CETP gene in a 9-year-old boy, and in his brother and mother (Figure S1). Although all three had a Body-Mass Index (BMI) in the obese range, each was found to have a lipid phenotype of relatively high HDL cholesterol (HDL-C) and relatively low LDL cholesterol (LDL-C).
**Materials and Methods**

All participants gave written informed consent. Clinical-grade microarray analysis was done in the proband using an Affymetrix CytoScan HD array and Chromosome Analysis Suite (ChAS) v1.2.2 with annotation file na32.1.v1 (Affymetrix, Santa Clara, CA). Call thresholds were 200 kb for microdeletions and 400 kb for microduplications. Fluorescence in situ hybridization (FISH) validation used BAC probe RP11-892K8 (Centre for Applied Genomics, Toronto).

Lipid levels were tested clinically at BC Children’s Hospital and St. Paul's Hospital (Vancouver, Canada). Enzymatic activity was assayed using the fluorometric CETP activity assay kit (Abcam, Toronto, ON, Canada).

RNA was isolated from non-fasting peripheral blood mononuclear cells (PBMCs) using the RNeasy RNA isolation kit (Qiagen, Toronto). Real time quantitative PCR (q-PCR) used the Power SYBR Green qPCR master mix (Applied Biosystems, Warrington, UK) on cDNAs reverse transcribed using the SuperScript III, first strand cDNA synthesis kit (Invitrogen, Carlsbad, CA, USA). Expression of endogenous housekeeping mRNAs for Ubiquitin C (UBC) and β-2 microglobulin (B2M) were used as input controls (primer sequences in Table S1).

Total cholesterol (TC) and free cholesterol (FC) levels were estimated using the Amplex red cholesterol assay kit (Invitrogen Canada). Cholesteryl ester (CE) levels were calculated by subtracting the free cholesterol levels from those for total cholesterol.

**Patient Case and Results**

The proband was referred to the Medical Genetics clinic at age 9 years, 2 months for developmental delay, moderate intellectual disability, anxiety, mixed receptive and expressive language disorder, and obesity with macrosomia. Height was 155.0 cm (+3.55 standard deviations (SD)) and weight was 130.4 kg (+6.28 SD), giving a BMI of 54.3 kg/m² (+5.17 SD)

Microarray revealed a previously-undescribed 291 Kb single copy loss at 16q13 from nucleotide 57,000,937-57,292,408 (GRCh37/hg19), containing most of the OMIM gene CETP (chr16:56,995,834-57,017,756), as well as four other OMIM genes, NLRC5, CPNE2, ARL2BP and PLLP, and two RefSeq genes, FAM192A and SPRY1 (Figure S2). The first ~5103 nucleotides of the CETP genomic sequence, including coding exons 1 and 2, remain. Familial FISH cascade testing in the proband, his brother, mother and half-sister confirmed the deletion in all family members except the maternal half-sister.

The proband’s father is of African-American descent, and was unavailable for genetic or biochemical studies. The proband’s mother is of European descent and is
currently 43 years old. She had undergone gastric bypass surgery at 27 years to manage obesity (lifetime maximum weight was 172 kg). Due to side effects she had had the bypass reversed and replaced with a gastric sleeve. Her BMI is now 32.7 kg/m². Although a clinical IQ test was not done, she did not report ever having learning difficulties. The brother had been diagnosed with obesity, Osgood-Schlatter disease (OSD), and mild learning disabilities. OSD is characterized by pain and inflammation at the tibial tubercle caused by repetitive strain on the patellar tendon. At age 13 the brother’s height, weight, and BMI were 182.4 cm (+2.82 SD), 165.0 kg, and 49.6 kg/m² (+3.99 SD), respectively. The proband’s 19-year-old maternal half-sister had a height of 170.5 cm (+1.12 SD), a weight of 90.7 kg, and a BMI of 31.2 kg/m² (+2.27 SD).

Non-fasting and fasting lipid profiles, were measured in all participants (Table 1). Previous non-fasting blood panels on the proband revealed high HDL-C of 2.1 mmol/L at age 7 years, 3 months, and of 2.3 mmol/L at age 8 years, 6 months, demonstrating persistently elevated HDL-C in childhood. Cholesteryl ester (CE) levels, measured as percent of total cholesterol (%CE), were lower in affected family members, but not significantly different from controls (Figure S3).

To establish that hemizygosity for CETP decreased CETP mRNA and protein activity, we performed mRNA and enzymatic assays. CETP mRNA among microdeletion carriers was significantly lower than CETP mRNA in the half-sister (Figure S5A and S5B). The proband, his brother, and their mother also showed significantly decreased plasma CETP activity versus controls under non-fasting conditions (Figure 1A), and decreased activity when compared to the half-sister in both fasting and non-fasting states (Figure S4). Our controls consist of both healthy and obese individuals that have an age range of 2-62 years, and a BMI range of 18.5-58.0 kg/m² (Figure 1B).

Lastly, highly-sensitive C-Reactive Protein (CRP) levels suggested the proband, his brother, and half-sister to be at average cardiovascular risk, whereas the mother was assessed to be low risk (Table 1).

**Discussion**

Despite controversy regarding the role of CETP in atherosclerosis, previous investigations show that high levels of HDL, including those caused by CETP deficiency, correlate with a decreased risk of coronary heart disease (CHD). Moriyama et al. determined that among individuals with high HDL, the decrease in prevalence of CHD correlated with HDL levels irrespective of CETP genotype. This suggests that the cardioprotection conferred by CETP deficiency is directly due to the elevated HDL levels and not by some other mechanism.

Plasma CETP activity correlates positively with BMI, and is typically elevated in obese children and adults. Increased plasma CETP has been proposed as a
mechanism for generation of atherogenic lipid profiles seen in obese individuals, due to its enzymatic activity exchanging CE and TG between HDL and Apo-B containing particles.\textsuperscript{9,11} TG-rich particles accept the CE molecules being transferred by CETP. Because adipose tissue secretes CETP, a direct effect of adipose mass on CETP levels and activity may partly explain the concurrent increase in plasma CETP with obesity.\textsuperscript{12} In addition, an excess of TG-rich substrate particles (e.g. in obese individuals) is thought to drive CETP activity, thereby enhancing exchange of CE for TG.\textsuperscript{12} Gomaraschi \textit{et al.}\textsuperscript{13} demonstrated that plasma HDL increases in a gene-dose dependent manner when heterozygous carriers of CETP deficiency are compared to homozygotes for loss-of-function alleles. Our participants appear to have decreased CETP activity in the non-fasting state (Figure S4). CETP levels have been observed to increase in postprandial plasma, but only under lipemic conditions.\textsuperscript{14} Samples were collected several hours after our participants' last meal, and we would not expect individuals lacking one \textit{CETP} gene to produce as robust a postprandial response compared to those with two functioning \textit{CETP} genes. Compared to controls, the proband, his brother and mother showed low CETP activity for both their BMI (Figure 1C) and age (Figure 1D). CETP enzymatic activity correlated directly with CETP mRNA levels in all participants (Figure S5C). We conclude that the copy-number variant in this family affected \textit{CETP} mRNA levels, with downstream effects on enzyme activity and lipid profile.

HDL-C is typically low among obese children and adults, whereas LDL-C is high.\textsuperscript{15,16} Our CETP-hemizygous subjects showed a phenotype that contrasted with what would be expected on the basis of their body weight. For normative values, we used age-dependent HDL-C and LDL-C reference ranges from obese children (BMI >95\textsuperscript{th} percentile).\textsuperscript{16} In that study, the average HDL-C levels for boys between ages 5-11 years and ages 12-17 years were 1.17 ± 0.27 mmol/L and 1.05 ± 0.24 mmol/L, respectively. These are significantly lower than the HDL-C levels seen in our proband and his brother, who also had LDL-C levels significantly lower than expected for their weight, at 3.87 ± 0.86 mmol/L and 3.16 ± 0.93 mmol/L, respectively. Furthermore, Norris \textit{et al.}\textsuperscript{17} found that CRP (an early marker of cardiovascular disease that is considered representative of inflammation), increased with BMI among severely obese children. None of our CETP-deficient participants displayed a CRP value indicative of high cardiovascular disease risk adjusted for their current age. The proband and his brother appear to have lower than expected CRP compared to the mean CRP of children and adolescents grouped by their BMI (Figure 2). The mother also appears to show low CRP when compared to the mean CRP of adults grouped by BMI, gender, and ethnicity.

Elevated levels of ApoA-I have previously been reported among CETP deficient patients.\textsuperscript{3} Given that ApoA-I is largely associated with HDL particles and that ApoB is largely associated with LDL particles, the proband’s ApoB/A-I ratio would be considered highly favourable from a cardiovascular perspective based on age and sex, and is particularly remarkable in light of his BMI.
Relatively little is known about the other deleted genes. To date, \textit{FAM192A} and \textit{RSPRY1} have not been associated with any phenotypes. \textit{NLRC5} (OMIM \#613537) is thought to be a transcriptional regulator of major-histocompatibility complex class I genes, with no obvious role in lipid metabolism or neural development. \textit{CPNE2} (OMIM \#604206) belongs to the copine family of calcium-dependent lipid-binding proteins. Recessive loss-of-function variants in the ciliary protein \textit{ARL2BP} (OMIM \#615407) are implicated in retinitis pigmentosa (RP), though heterozygotes are unaffected by RP.

Plasmolipin, \textit{PLLP} (OMIM \#600340), is a membrane-bound protein expressed in brain and kidney. Hamacher \textit{et al.}\textsuperscript{18} suggested that \textit{PLLP} could be a candidate gene for Bardet-Biedl syndrome (BBS – OMIM \#209900), an autosomal recessive condition that features truncal obesity and learning disabilities.\textsuperscript{18} Our participants do not meet the diagnostic criteria for BBS. However, we cannot exclude the possibility that \textit{PLLP} may have an effect on our participants’ obesity and cognitive profiles. The locus for Gitelman syndrome (GS - OMIM \#600968) is nearby, but is unaffected by the microdeletion.

Our participants show relatively high HDL-C levels, despite also presenting with significant obesity. Based on their profiles of lipids and other inflammatory biomarkers, we would expect their partial CETP deficiency to offer some protection against atherosclerosis and coronary heart disease in the long term. However, the protection may be incomplete and may also attenuate with age.\textsuperscript{8} We thus conclude that the absence of one copy of \textit{CETP} confers an antipathogenic phenotype for cardiovascular disease, though without additional cases it remains a variant of uncertain significance for obesity and developmental delay.

References


**Figures and Tables**

**Figure 1.** (A) Non-fasting enzymatic activity of CETP in the affected proband, his brother, and his mother compared to his unaffected half-sister and a control cohort. **(B)** The control cohort is composed of both male and female individuals with a range of BMI and age in order to obtain representative controls for this family. **(C)** The proband and his brother show low CETP activity for their BMI, while their half-sister shows CETP activity similar to controls. **(D)** Affected participants all show low CETP activity for their age compared to controls. Squares denote male controls. Crosses denote female controls. (PR: proband; B: brother; M: mother; S: half-sister)
Figure 2. Fasting C-reactive protein (CRP) levels of the proband, his brother and his mother appear to be low for their BMI compared to pediatric and adult reference values. Pediatric reference points were taken from Weiss et al., who measured fasting CRP in healthy and obese individuals ages 4 to 20 years, and reference values for white, female, adults were adapted from Wee et al., who measured CRP in adults over age 20.


(PR: proband; B: brother; M: mother; S: half-sister)

Table 1. Fasting and non-fasting lipid profiles of the proband, his brother, his mother and his half-sister. Reference ranges for each individual are shown in brackets to the right of their results.

<table>
<thead>
<tr>
<th></th>
<th>Proband</th>
<th>Brother</th>
<th>Mother</th>
<th>Half-Sister</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>non-fasting</td>
<td>fasting</td>
<td>non-fasting</td>
<td>fasting</td>
</tr>
<tr>
<td>TC (mmol/L)</td>
<td>4.35</td>
<td>4.18</td>
<td>[2.60-5.20]</td>
<td>3.98</td>
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<tr>
<td>HDL-C (mmol/L)</td>
<td>2.02*</td>
<td>1.95</td>
<td>[0.96-1.97]</td>
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<td>LDL-C (mmol/L)</td>
<td>1.34</td>
<td>1.64</td>
<td>[1.30-3.40]</td>
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<tr>
<td>TG (mmol/L)</td>
<td>2.17</td>
<td>1.29*</td>
<td>[0.45-1.10]</td>
<td>2.13</td>
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<tr>
<td>non-HDL-C (mmol/L)</td>
<td>2.33</td>
<td>2.23</td>
<td>[1.50-3.70]</td>
<td>2.77</td>
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<tr>
<td>ApoA-I (g/L)</td>
<td>1.71</td>
<td>1.40</td>
<td>[1.02-1.91]</td>
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<tr>
<td>ApoB (g/L)</td>
<td>0.59</td>
<td>0.78</td>
<td>[0.45-1.19]</td>
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<td>ApoB/ApoA-I</td>
<td>0.35</td>
<td>0.56</td>
<td>[0.30-0.95]</td>
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<tr>
<td>Lp(a) (mg/L)</td>
<td>106</td>
<td>122</td>
<td>[&lt;300]</td>
<td>122</td>
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<tr>
<td>CRP (mg/L)</td>
<td>2.0</td>
<td>[&lt;3.1]</td>
<td>2.3</td>
<td>[&lt;3.1]</td>
</tr>
</tbody>
</table>

* Above the 95th percentile
** Below the 10th percentile

Reference ranges for pediatric HDL-C levels are between the 5th and 95th percentiles and were derived from Hickman et al. Reference ranges were defined as being...
within three standard deviations of the mean for age and gender and were derived from Ritchie et al.\textsuperscript{22} (HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; TC: total cholesterol; TG: triglyceride; ApoA-I: apolipoprotein A I; ApoB: apolipoprotein B; Lp(a): lipoprotein (a); CRP: C-Reactive Protein)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
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<tr>
<td>CETP</td>
<td>5’GGCCAAGTCAAGTATG GGTG 3’</td>
<td>5’ACAGACACGTCTGAATGGAGA 3’</td>
</tr>
<tr>
<td>UBC</td>
<td>5’CTGGAAGATGGCTACCTCG 3’</td>
<td>5’GGTCTTGCCAGTGAGTGTCT 3’</td>
</tr>
<tr>
<td>B2M</td>
<td>5’GAGGCTATCCAGGCTACTCCA 3’</td>
<td>5’CGGCAGGCTACTCATCTTTT 3’</td>
</tr>
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</table>

**Table S1.** Primer sequences used for the amplification of CETP, UBC, and B2M. All primers were purchased from integrated DNA technologies (San Diego, CA, USA).

**Figure S1.** Pedigree of family affected with hemizygous 16q13 microdeletion resulting in deficiency for Cholesteryl Ester Transfer Protein (CETP) gene. The proband is indicated by an arrow.
Figure S2. (A) Microarray image showing single nucleotide polymorphism (SNP) probe binding indicating a 291 Kb microdeletion at 16q13. (B) Enhanced view of microdeletion at 16q13, showing affected genes, including partial deletion of CETP. (C) FISH imaging of microdeletion on chromosome 16, indicated by arrow.
Figure S3. Cholesteryl ester levels, displayed as percent of total cholesterol (%CE), in the affected participants are not seen to be significantly different from those seen in the unaffected half-sister and non-fasting control samples. The %CE levels varied between affected individuals with the mother having the highest with 65.98%. The proband, his brother, and half-sister had %CE levels of 53.29%, 59.86%, and 66.85% respectively.
Figure S4. (A) Non-fasting, and (B) fasting enzymatic activity of CETP in the affected proband, his brother and their mother compared to the unaffected half-sister.
Figure S5. CETP mRNA levels of the affected participants compared to the unaffected half-sister, normalized to (A) Beta-2 Microglobulin, and (B) Ubiquitin C. (C) Positive correlation of CETP mRNA and CETP enzymatic activity.