A Novel Mutation in the Intron 1 Splice Donor Site of the Cholesterol Ester Transfer Protein (*CETP*) Gene as a Cause of Hyperalphalipoproteinemia

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The exchange of cholesterol ester (CE) between lipoproteins occurs through the action of cholesterol ester transfer protein (CETP). The human *CETP* gene is composed of 16 exons encompassing 25 kbp on chromosome 16q13. The objective of this study was to determine whether a mutation in the *CETP* gene accounted for severe hyperalphalipoproteinemia in an 80-year-old subject. As a secondary objective, we also investigated the allelic frequency of D442G and Int14A mutation in 224 random Han Chinese subjects. DNA sequence analysis of the *CETP* gene in the patient revealed a peculiar nucleotide pattern in intron 1. To determine whether this peculiarity results in abnormally spliced mRNA, we used reverse-transcriptase polymerase chain reaction (RT-PCR) to amplify and sequence the patient's cDNA using *CETP*-specific primers that spanned this splice junction. Both the wild-type and mutant cDNA were detected, and the mutant cDNA showed that its 5′-splice site shifted 4 nucleotides upstream. This change results in a frame-shift and premature termination at amino acid residue 22, and thus predicts a markedly truncated protein product. Although this patient did not have either the D442G or Int14A allele, we found that the allelic frequency of D442G in 224 subjects was 4.46%. No subjects had the Int14A allele. In conclusion, a novel intron 1 splice site mutation in the *CETP* gene in 1 patient with hyperalphalipoproteinemia and D442G allelic frequency of 4.46% was found among a normal population in Taiwan.

PLASMA LIPOPROTEINS are continuously remodeled via transfer and exchange of lipids among lipoprotein subfractions. In humans, the main role of cholesterol ester transfer protein (CETP) is to transfer cholesterol ester (CE) from high-density lipoprotein (HDL) to lower density lipoproteins in exchange for triglyceride.[1] This triglyceride is hydrolyzed on HDL by hepatic lipase. Although CETP is also effective in transferring CE between HDL molecules, the CETP reaction may increase circulating very-low-density lipoprotein cholesterol (VLDL-C) and low-density lipoprotein-cholesterol (LDL-C) levels. Transgenic mice expressing CETP had much worse atherosclerosis than did nonexpressing controls.[2] As a result, CETP has been often considered a "proatherogenic" factor.

Plasma CETP is an extremely hydrophobic glycoprotein with a relative molecular mass of 74,000, and consists of 476 amino acids with 4 N-linked glycosylation sites.[3] The human *CETP* gene spans approximately 25 kb, encompasses 16 exons, and is found on chromosome 16q13.[4,5]

In some families, deficiency of CETP or increased production apolipoprotein (Apo) A1 have been identified as the genetic factor that causes elevated concentrations of HDL-C.[6,7] In addition, environmental factors such as alcohol consumption, female sex, African American race, exercise, and estrogen are positive factors for increasing HDL-C concentration.[8] By contrast, other factors such as smoking, male sex, obesity, diabetes, and diet high in polyunsaturated fat may lower it.[8,9] These environmental factors often influence HDL-C levels by increasing or reducing lipases or lipid transfer protein activities.[9,10] HDL has several beneficial biological effects, including an important role as an antiatherogenic agent via its ability to transfer cholesterol from tissues to the liver.[11] HDL also promotes prostacyclin secretion and stabilization[11] and acts as a major carrier of lipid hydroperoxides, leading wash-out oxidized lipids in LDL, and reducing its atherogenic potential.[12]

Variant as well as defective *CETP* alleles have been associated with hyperalphalipoproteinemia. The first reported human *CETP* gene mutation was a splicing defect of intron 14 (Int14A mutation) in a Japanese subject with hyperalphalipoproteinemia and low levels of CE transfer activity.[6,13] In addition, in the subjects with the D442G (Asp^{442} to Gly) mutation in exon 5, HDL concentration is increased 3-fold and plasma CETP mass and activity are markedly decreased, suggesting that the mutation has a dominant effect on CETP and HDL in vivo.[14] Subsequently, the Int14A mutation as well as a D442G variant allele have been found to be the most common mutations in the Japanese population to cause yperalphalipoproteinemia.[15,16] The prevalence of *CETP* mutations among patients with hyperalphalipoproteinemia is highly variable among different populations.[17-19] The objectives of this study are to describe a Han Chinese man with hyperalphalipoproteinemia carrying a novel mutation in the *CETP* gene and to find the allelic frequency of D442G and Int14A mutations in the normal population.

MATERIALS AND METHODS

Subjects

A patient with hyperalphalipoproteinemia and 224 random subjects with various concentrations of HDL-C were studied. The protocol was approved by institutional review board of the hospital, and informed consent was obtained from the patient and normal subjects.

Samples

Blood samples were collected from patients and normal subjects who had fasted overnight for 10 hours. The samples were centrifuged at 2,000 × *g* for 20 minutes. The sera obtained were measured immediately for triglyceride and total cholesterol using a Hitachi-911 automated analyzer (Hitachi Ltd, Tokyo, Japan). HDL-C was measured by

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Table 1. Sequences of Primers Used to Amplify and Directly Sequence Exons of the Human *CETP* Gene

Exon	Forward Primer (5′-3′)\Reverse Primer (5′-3′)	Product Size (bp)
1	TACGGGCTCCAGGCTGAACG\CTCCTCTCTCTGGGCTAGTG	295
2	AAGACCTGGGAGCCTCATCT\TCCAAATCAAGGGGAGAGAC	350
3-4	CTGCCTGGTGTTCCACTTTTCG\GAACCCTCTGCCTGCACAACC	681
5	TGGGCAGCATGTGGATACCATCTGATAGCG\TGGGCACGACAGGAATGGGGGCACAG	194
6-7	GGTCTGAGGCTCCGTGTTCT\TTCTGGCTCCCACTGCTGTC	565
8	ACGGTGACTCAGGGCAATTCC\GACTCTTGGCTGCCATAAGGT	264
9	TGAATGAGTGAAAGCCGCTGG\CCACCACCAAGTTTCCGAGTT	436
10	CCCTGCGAATTCTTCTTCTGAGGAGTGGAC\ATAATTGGATCCATTGGTGGTGTTATTGGC	227
11	TCCGAGGGCATGGACTGCTGC\TGGTGAGAAGGATCTGGAGGCTTCAGAGAC	337
12	GGTTGCTCTCTGCTTCGGGAA\ATGGACCACCTGGTCACATTC	614
13	CTGGCTGCTATTCTTAGAGTTTCTTTCCC\GACTTATTTCCCCCAGTCTATCC	158
14	CACGGATGGGCATGAGGATG\CCGCCAGCCCACACATA	225
15-16	CTTCCTCATCCTCAGAAGCAC\CCACGCTGGAGGAGACACCA	599

direct method, using commercial kits from Kyowa (Tokyo, Japan). The total Apo A1 and Apo B levels were measured using the nephelometry method (Behring Nephelometry Analyzer II, Marburg, Germany).

Polymerase Chain Reaction Amplification

The 16 exons and partial sequences of the flanking introns of the *CETP* gene were amplified by polymerase chain reaction (PCR). PCR was performed in a 25-μL volume containing 200 ng of genomic DNA prepared from whole blood in a K3 EDTA Vacutainer (Amersham Pharmacia Biotech, Inc, Piscataway, NJ), 0.2 mmol/L of each dNTP, 1.5 mmol/L $MgCl_2$, 50 mmol/L KCl, 0.15 μm of each primer, 10 mmol/L Tris-HCl (pH 7.4), 0.1% Triton X-100, 2 units of Taq DNA polymerase (Protech Technology Co, Taipei, Taiwan). The primers used in the present study are listed in Table 1.

Direct Sequencing

PCR products were purified using a GFX™ PCR DNA and Gel Band Purification Kit (Amersham Pharmacia Biotech) and sequenced using an ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (ABI Biosystems, Inc, Foster City, CA) on the ABI prism 377 DNA sequencer (Perkin Elmer, Foster City, CA).

Reverse-Transcriptase PCR

Total RNA was extracted from peripheral whole blood using GIBCOBRL TRIzoL reagent (Life Technologies, Inc, Rockville, MD). First-strand cDNA was generated with the GIBCOBRL SUPERSCRIPT reverse transcriptase (RT) system (Life technologies, Inc., Rockville, MD) using pd(T)12-18 primer and used as a template for PCR. The forward primer (E1F: CCACAGTCCTGAC CCTGGCCCTG) is located in exon 1 and the reverse primer (E2R: GCATCATG GCCTTCTCG CCCGTG) is located in exon 2. PCR products were cloned into pGEM-T vector (Promega, Inc, Madison, WI) and 10 clones were selected for sequence analysis (ABI Biosystems).

Allelic Frequency of D442G and Int14A Mutation in Normal Subjects

Regions of the *CETP* gene containing the D442G and Int14A mutations were amplified by PCR using previously described primers and conditions.[14] The PCR products were analyzed by restriction digest (*Nde*I for Int14A or *Msp*1 for D442G) as previously described.[16]

Case Report

An 80-year-old, man, born in China and emigrated to Taiwan in 1949, underwent the routine biochemical screening during treatment for prostate cancer in 1996. Laboratory analysis showed albumin of 47 g/L(normal, 37 to 53), globulin of 38 g/L (normal, 27 to 31), fasting glucose of 5.27 mmol/L (normal $<$ 7.0), cholesterol of 4.24 mmol/L (normal 3.23 to 6.20), triglyceride of 0.62 mmol/L (0.23 to 2.26), HDL-C of 2.48 mmol/L (0.78-1.81), LDL of 1.47 mmol/L (normal $<$ 4.14), and prostate-specific antigen of 355 ng/mL (normal $<$ 3.32 ng/mL). He was an heavy smoker in the past, but had stopped 10 years previously. His blood pressure was normal. Chest x-ray showed tortuosity of thoracic aorta, and calcification of aortic knob. The patient was taking Madopar (United Biomedical, Inc Asia for Roche Product Ltd, Hsinchu, Taiwan) 125 mg twice per day for parkinsonism. In 1996, he underwent total prostatectomy for adenocarcinoma.

The biochemical and clinical evaluation suggested a possible defect in CETP, and thus we analyzed his *CETP* gene.

Calculation

All data are presented as the mean ± SEM. Comparisons between groups were calculated using Student's nonpaired *t* test.

RESULTS

All 16 exons and exon-intron junctions of *CETP* gene were sequenced in the patient. We found a peculiar nucleotide pattern in intron 1. To study the effect of this peculiarity, we used RT-PCR to analyze *CETP* mRNA using total RNA isolated from control and patient's lymphocytes. Sequence analysis of cloned RT-PCR products showed normal intron 1 splicing in the control sample, whereas the patient's cDNA showed 6

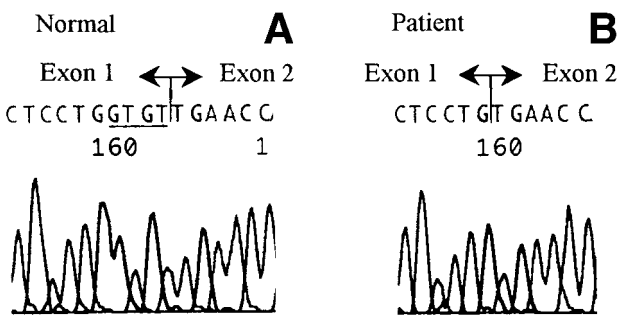

Fig 1. cDNA sequences of exon 1–exon 2 junction. The cDNA PCR product of patient D91 was cloned into pGEM-T and sequenced. The wild-type allele (A) and the patient's mutant allele (B) are shown. The mutant allele showed an abnormally spliced mRNA in which the 5′-splice site of intron 1 has shifted 4 nucleotides upstream.

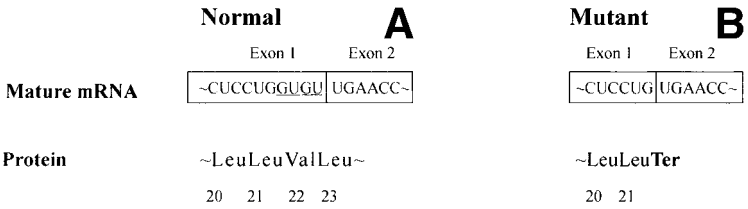

Fig 2. Schematic representation of wild-type (A) and mutant (B) alleles, including mature mRNA sequences and predicted proteins. The mutant allele generates an abnormal 5′splice site with a consequent truncated protein.

clones with normally spliced cDNA (Fig 1A) and 4 clones with an abnormal spliced cDNA, in which the 5'-splice site of intron 1 had shifted 4 nucleotides upstream (Fig 1B). This frame-shift results in a premature termination codon of *CETP* at amino acid residue 22 (Fig 2). No other mutations were found within the coding sequence, including the D446G and Int14A mutations. This abnormal nucleotides pattern in intron 1 was not present in DNA from any of the 224 normal controls.

None of the 224 normal subjects was found to have the Int14A variant allele. By contrast, the D442G allele was present with a total allelic frequency of 4.46%, including heterozygous (3.57%) and homozygous (0.89%) (Table 2). There was no relationship between the frequency the D442G allele and the concentration of serum HDL-C. By contrast, serum levels of HDL-C were significantly greater in subjects who were heterozygous for the D442G allele (1.99 ± 0.05 mmol/L) than in subjects with the wild-type allele (1.72 ± 0.03 mmol/L, $P < .0002$) (Table 3). Only 2 subjects were homozygous for the D442G allele, and thus the sample size was too small to perform statistical analysis.

DISCUSSION

The total cholesterol and HDL-C levels of the patient we describe were 4.24 mmol/L and 2.48 mmol/L, respectively, when first identified. Despite the high HDL-C level, tortuosity of thoracic aorta and calcification of aortic knob werre also present on x-ray examination. As is common to patients with CETP deficiency, this patient also showed significant hyperalphalipoproteinemia.

Several lines of evidence indicate that the mutation is causally related to hyperalphalipoproteinemia and the elevated level of HDL-C. The observed mutation was not present in the *CETP* gene of 224 unrelated subjects, indicating that this mutation is not a common polymorphism. The mutation is predicted to disrupt the normal 5' splicing donor site, and RT-PCR analysis demonstrated abnormally processed *CETP* mRNA in which a frame-shift causes premature termination of the predicted translational product after amino acid 22. Other splicing defects[20] as well as missense and point mutations that cause premature stop codon (eg, the truncated CETP protein and with Tyr57Stop, G181Stop, and Arg268Stop) have been demonstrated to cause hyperalphalipoproteinemia and elevated levels of serum HDL-C.[18,21,22] Thus, the truncated protein that is predicted to result from this mutation lacks 455 amino acid residues and would be expected to be inactive.

The patient described here has no family history of hyperalphalipoproteinemia and no surviving relatives. Thus, we could not determine whether the mutation was inherited or arose de novo. Of all mutations identified to date, both the D442G and Int14A mutational "hot spots" have been found in about 5% of the Japanese populations.[15,16] The D442G mutation was not present in this patient, but was found in 4.46% of normal Chinese controls. In addition, the D442G allele was not found in any subjects whose HDL concentration was higher than 2.35 mmol/L. These results suggest that environmental influences may overcome the relatively modest functional consequences of the D442G mutation.[16] By contrast, no Int14A mutation was found in the normal population. There was no significant relationship between frequency and serum lipid concentration. Furthermore, analysis of serum lipid in heterozygous subjects for the D442G allele showed that HDL-C levels were significantly greater than those in normal subjects.

In conclusion, a novel intron 1 splice site mutation in the *CETP* gene was found in 1 patient with hyperalphalipoproteinemia and a D442G allelic frequency of 4.46% was found in a normal population in Taiwan.

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Table 2. Allelic Frequency of D442G Among HDL-C Levels

HDL-C Level (mmol/L)	No. of Subjects	D442G			Allelic Frequency of D442G (%)
		Ho	He	N	
2.61-3.12	7			7	0.0
2.34-2.60	13			13	0.0
2.09-2.33	36		6	30	8.33
1.83-2.08	66	1	8	57	7.58
1.57-1.82	17		1	16	2.94
1.32-1.56	30	1	1	28	5.00
1.06-1.31	36			36	0.0
0.80-1.05	16			16	0.0
<0.79	3			3	0.0
All	224	2	16	206	4.46

Abbreviations: Ho, homozygotes; He, heterozygotes; N, no mutation.

Table 3. HDL-C Concentration in *CETP* D442G Genotypes

	D442G (mean ± SEM)		Wild-Type (mean ± SEM) (n = 206)
	Ho (n = 2)	He (n = 16)	
Total C (mmol/L)	5.14 ± 0.69	5.83 ± 0.32	5.54 ± 0.09
HDL-C (mmol/L)	1.77 ± 0.22	1.99 ± 0.05*	1.72 ± 0.03
LDL-C (mmol/L)	2.83 ± 0.19	3.44 ± 0.29	3.33 ± 0.07
Triglyceride (mmol/L)	1.15 ± 0.58	0.86 ± 0.09	1.07 ± 0.05

Abbreviation: Total C, total cholesterol.
*$P < .0002$ *v* wild-type.

REFERENCES

1. Tall AR: Plasma cholesteryl ester transfer protein. J Lipid Res 34:1255-1274, 1993

2. Marrotti KR, Castle CK, Boyle TP, et al: Severe atherosclerosis in transgenic mice expressing simian cholesteryl ester transfer protein. Nature (Lond) 364:73-75, 1993

3. Drayna D, Jarnagin AS, McLean J, et al: Cloning and sequencing of human cholesteryl ester transfer protein cDNA. Nature (Lond) 327:632-634, 1987

4. Agellon LB, Quinet EM, Gillette TG, et al: Organization of human cholesteryl ester transfer protein gene. Biochemistry 29:1372-1376, 1990

5. Callen DF, Hildebrand CE, Reeders S: Report of the Second International Workshop on Human Chromosome 16 Mapping. Cytogenet Cell Genet 60:158-167, 1992

6. Brown ML, Inazu CB, Hesler CB, et al: Molecular basis of lipid transfer protein deficiency in a family with increased high-density lipoproteins. Nature (Lond) 342:448-451, 1989

7. Rader DJ, Schaefer JR, Lohse P, et al: Increased production of apolipoprotein A-1 associated with elevated plasma levels of high density lipoproteins and apolipoprotein A-I in a patient with familial hyperalphalipoproteinemia. Metabolism 42:1429-1434, 1993

8. Gordon DJ, Rifkind BM: High-density lipoprotein—The clinical implications of recent studies. N Engl J Med 321:1311-1316, 1989

9. Tall AR: Plasma high density lipoproteins. Metabolism and relationship to atherogenesis. J Clin Invest 86:379-384, 1990

10. Patsch JR, Prasad S, Gotto AM, et al: High density lipoprotein2, relationship of the plasma levels of this lipoprotein species to its composition to the magnitude of postprandial lipemia, and to the activities of lipoprotein lipase and hepatic lipase. J Clin Invest 80:341-347, 1987

11. Tall AR, Small DM: Plasma high-density lipoproteins. N Engl J Med 299:1232-1236, 1978

12. Fleisher LN, Tall AR, Witte LD, et al: Stimulation of arterial endothelial cell prostacyclin synthesis by high density lipoprotein. J Biol Chem 257:6653-6655, 1982

13. Koizumi J, Mabuchi A, Yoshimura A, et al: Deficiency of serum cholesteryl-ester transfer activity in patients with familial hyperalphalipoproteinemia. Atherosclerosis 58:175-186, 1985

14. Takahashi K, Jiang XC, Sakai N, et al: A missense mutation in the cholesteryl-ester transfer protein gene with possible dominant effects on plasma high density lipoproteins. J Clin Invest 92:2060-2064, 1993

15. Inazu A, Jiang XC, Haraki T, et al: Genetic cholesterol ester transfer protein deficiency caused by two prevalent mutations as a major determinant of increased levels of high density lipoprotein cholesterol. J Clin Invest 94:1872-1882, 1994

16. Akita H, Chiba H, Tsuchihashi K, et al: Cholesterol ester transfer protein gene: Two common mutations and their effect on plasma high-density lipoprotein cholesterol content. J Clin Endocrinol Metab 79:1615-1618, 1994

17. Tall AR: Plasma cholesterol ester transfer protein and high-density lipoproteins: New insight from molecular genetic studies. J Intern Med 237:5-12, 1995

18. Teh EM, Dolphin PJ, Breckenridge WC, et al: Human plasma CETP deficiency: Identification of a novel mutation in exon 9 of the *CETP* gene in a caucasian subject from North America. J Lipid Res 39:442-456, 1998

19. Akita H, Chiba H, Hui SP, et al: Cholesterol ester transfer protein deficiency. Am J Med Genet 59:399-400, 1995

20. Sakai N, Santamarina-Fojo S, Yamashita S, et al: Exon 10 skipping caused by intron 10 splice donor site mutation in cholesterol ester transfer protein gene results in abnormal downstream splice site selection. J Lipid Res 37:2065-2073, 1996

21. Risch A, Drexel H, Amann WF, et al: Deficiency of cholesterol ester transfer protein: Description of the molecular defect and the dissociation of cholesterol ester and triglyceride transport in plasma. Arterioscler Thromb Vas Biol 17:3433-3441, 1997

22. Arai T, Yamashita S, Sakai N, et al: A novel nonsense mutation(G181X) in the human cholesteryl ester transfer protein gene in Japanese hyperalphalipoproteinemic subjects. J Lipid Res 37:2145-2154, 1996