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Clinical and genetic analysis of a family diagnosed with familial hypobetalipoproteinemia in which the proband was diagnosed with diabetes mellitus

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ABSTRACT

Objective: To perform clinical and genetic analysis of a family with familial hypobetalipoproteinemia in which the proband had been diagnosed with diabetes mellitus.
Methods: Direct sequencing was performed on candidate genes such as *APOB*, *PCSK9*, and *ANGPTL3*. The effect of the mutant gene on lipid profile was investigated using biochemical methods.
Results: A novel mutation Y344S in *ANGPTL3* was identified but no variants were found in *PCSK9* or *APOB*. Lipid profiles showed the levels of TG, TC, and LDL-C to be significantly lower in Y344S carriers than in non-carriers in this family. The levels of HDL-C and plasma concentrations of ANGPTL3 showed no significant differences. Western blot analysis revealed that the mutant ANGPTL3 proteins could not be secreted into the medium.
Conclusion: A novel mutation Y344S was found in *ANGPTL3* gene in two diabetic patients with familial hypobetalipoproteinemia. The family study and genetic analysis suggest that this set of gene mutation may be a genetic basis for the lipid phenotypes, and may become a vascular protective factor in the probands with high risk of atherosclerosis.

1. Introduction

Familial hypobetalipoproteinemia (FHBL) refers to group of inherited heterogeneous diseases characterized by low-density lipoprotein cholesterol (LDL-C) and apolipoprotein B (ApoB) levels below 5% of the population [1]. The genetic etiology in FHBL is mainly associated with the mutations in *APOB* gene and in the proprotein convertase subtilisin kexin type 9 (*PCSK9*) gene [2,3]. There are a variety of reductions in LDL-C and ApoB levels in FHBL patients [4], however, most are difficult to detect due to the lack of clinical manifestations. A previously undiscovered familial combined hypolipidemia (FHBL2) was recently reported, it was characterized by decreased levels of very-low-density lipoprotein (VLDL), low-density lipoprotein (LDL), high-density lipoprotein (HDL) and triglyceride (TG) [5]. The genetic etiology of FHBL2 has been shown to be associated with the angiopoietin-like 3 (*ANGPTL3*) gene [6–9].

The *ANGPTL3* gene is located on human chromosome 1 (1p31.1-p22.3). It encodes a 460-amino-acid protein, which is secreted by the liver and has an inhibitory effect in lipoprotein lipase (LPL) and endothelial lipase (EL) [10]. For this reason, *ANGPTL3* defects may cause excessive activity of these enzymes accelerating the metabolism of VLDL and HDL [11,12]. However, the mechanism by which *ANGPTL3* defects decreases LDL-C levels has not yet been elucidated [13].

There are several cases of FHBL2 reported in the literature. However, the relationship between *ANGPTL3* gene mutation and lipid phenotype has not yet been fully explored [5,9]. One study has shown that patients with homozygous mutations in the *ANGPTL3* gene have relatively low fasting blood glucose levels and low risk of diabetes, but the number of cases was small, which limits its applicability [9].

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Table 1
Primer name and sequence.

Primer	Primer sequence 5′→3′
PCR- ANGPTL3 cDNA -S-	CACCATGTTCACAATTAAGCTCCTTC
PCR- ANGPTL3 cDNA -AS-	TTCAAAGCTTTCTGAATCTGTTGGAT
Muta- ANGPTL3- Y344S -S-	GACAACAAACATTCTATTGAATATTC
Muta- ANGPTL3- Y344S -AS-	GAATATTCAATAGAATGTTTGTTGTC
Muta- ANGPTL3- E375K -S	CAATGCAATCCCGAAAAACAAAGATTC
Muta- ANGPTL3- E375K -AS	GAATCTTTGTTTTTCGGGATTGCATTG
Muta- ANGPTL3- N173S -S	GAAAAACAAGATAGTAGCATCAAAGACC
Muta- ANGPTL3- N173S -AS	GGTCTTTGATGCTACTATCTTGTTTTTC

The present study reports a pair of twins diagnosed with diabetes. Their TC, LDL-C, and TG levels were lower than normal, as determined by lipid assays. This was consistent with FHBL lipid phenotype. Re-sequencing of the genes related to the hypobetalipoproteinemia, such as *APOB*, *PCSK9*, and *ANGPTL3*, revealed a missense mutation, Y344S, in exon 6 of *ANGPTL3*. No mutations were detected in *APOB* or *PCSK9*. *In vitro* studies and analyses of genetic variation showed that the FHBL clinical phenotype may be associated with *ANGPTL3* gene mutations in these twins.

2. Materials and methods

2.1. Clinical data

The 38-year-old female proband had a six-year history of type 2 diabetes. The patient used insulin to control blood glucose at the time of the study. Her glycated hemoglobin (Hb) was 10.2%, as determined in the hospital's outpatient department. Her twin sister, who had a four-year history of type 2 diabetes, also used insulin to control blood glucose, and her glycated hemoglobin was 8.4%. There were a total of six sisters in the family, all born to the same mother but to two different fathers (Fig. 2). None were vegetarian. They had no history of using other medications, no malnutrition, and no family history of diabetes, hypertension, or coronary heart diseases. The Hospital Ethics Committee of the First Hospital of China Medical University approved the study, and all the patients provided written informed consent.

2.2. Laboratory tests

Fasting blood glucose and glycated Hb were measured using the standard methods. Lipids (TC, TG, LDL-C, HDL-C) and apolipoprotein B were measured using an automatic biochemical analyzer. The ANGPTL3 ELISA test kit (AdipoGen) was used to assess ANGPTL3 content in serum.

Carotid intima-media thickness (IMT) was measured by using a Color Doppler ultrasound (iE33, Philips).

2.3. Genetic analysis

A blood genomic DNA extraction kit (Tian Jing Biochemical Technology Beijing, Ltd.) was used to extract genomic DNA. The PCR primers for amplification of *APOB*, *PCSK9*, and *ANGPTL3* genes are as shown in previous reports [9,14,15] and in Table 1. The PCR products were purified using a Tiangen Biotech, Ltd. agarose gel extraction kit, followed by DNA sequencing by the Beijing Liuhe Genomics Technology Co. The restriction enzyme HhaI was used to identify the *ApoE* genotype [16].

2.4. Construction of ANGPTL3 cDNA wild-type and mutant expression vectors

Total RNA was extracted with TRIzol reagent (Invitrogen) from HepG2 cells, followed by reverse transcription using a cDNA reverse transcription kit (Applied Biosystems). A cDNA containing no termination codon was amplified by PCR using a pair of primers (Table 1). The PCR products were further subcloned into T vector (pCR2.1-TOPO, Invitrogen) and verified by sequencing. The resulting T vector and expression vector pcDNA3.1/mys-His A (Invitrogen) containing *ANGPTL3* cDNA were digested with HindIII and XhoI restriction enzymes, and the digested products were purified with a gel purification kit (QIAquick Gel Extraction Kit, Qiagen). This was followed by ligation using T4 ligase (DNA Ligation Kit, Ver.2.1, TaKaRa). The *ANGPTL3* expression construct carrying the wild-type cDNA and C-terminal poly-His and myc was verified with DNA sequencing. A point mutagenesis kit (QuickChange® Site-Directed Mutagenesis Kit, Stratagene) was used to introduce mutations to the wild-type constructs to construct *ANGPTL3* mutant (Y344S) expression vector. The *ANGPTL3* E375K (reduced secretion) and N173S (normal secretion but reduced function) mutant recombinant plasmids were used as a control for subsequent experiments [17]. The accuracy of the sequences of the mutant plasmids was verified by DNA sequencing.

2.5. ANGPTL3 cDNA in transiently transfected cell lines

HEK293 cells at 1×10^5 cells/well was placed in DMEM medium containing 10% fetal calf serum (Dulbecco's modified Eagle's medium, Sigma), and cultured at 37 °C, 5% CO_2 and 95% air in a 6-well culture dish in an air-conditioned chamber until confluence reached 80%. The expression plasmids were transiently transfected into HEK cells, 4 μg/well, using a transfection kit (SuperFect®

Table 2
Clinical and biochemical data of the family members.

	Age(y)	Sex	BMI	FBG	HbA1c (%)	TG	TC	LDL-C	HDL-C	ANGPTL3 level	IMT	ANGPTL3 genotype
I1	69	M	–	–	–	–	–	–	–	–	–	–
I2	65	F	24.8	6.42	5.7	1.53	4.63	2.59	1.51	112.51	0.88	344Y
I3	71	M	25.2	4.87	5.4	0.93	3.18	1.92	1.15	94.52	0.79	344Y/S
II1	56	F	23.6	5.21	5.3	1.66	4.21	2.23	1.39	123.45	0.68	344Y
II2	53	F	23.2	4.82	5.4	1.58	4.51	2.51	1.59	124.42	0.72	344Y
II3	53	F	24.5	4.91	5.3	2.19	4.33	2.62	1.21	97.65	0.69	344Y
II4	42	F	23.8	5.22	5.2	1.91	3.38	1.68	1.21	87.68	0.64	344Y
II5	38	F	22.1	9.38	10.2	0.45	2.88	1.16	1.47	79.65	0.59	344Y/S
II6	39	M	–	–	–	–	–	–	–	–	–	–
II7	38	F	23.3	8.74	8.4	0.55	2.37	1.13	1.49	84.65	0.62	344Y/S
II8	43	M	–	–	–	–	–	–	–	–	–	–
III1	16	M	19.5	4.11	5.1	2.13	4.32	2.41	0.93	108.44	0.44	344Y
III2	14	F	18.6	4.53	5.2	1.65	4.31	2.1	0.95	91.88	0.42	344Y

BMI, body mass index (kg/m^2); FBG, fasting blood glucose (mmol/L); TG, triglyceride (mmol/L); TC, total cholesterol (mmol/L); LDL-C, low-density lipoprotein cholesterol (mmol/L); HDL-C, high-density lipoprotein cholesterol (mmol/L); ANGPTL3, angiopoietin-like 3 (ng/ml); IMT, intima-media thickness (mm).