Available online at www.sciencedirect.com
ScienceDirect
Biochemical and Biophysical Research Communications 354 (2007) 62–65
BBRC
www.elsevier.com/locate/ybbrc

Apolipoprotein C-II promoter T → A substitution at position −190 affects on the transcription of the gene and its relationship to hyperlipemia

Chun-Hua Chen [a], Ying-Lin Cao [a], Wei-Cheng Hu [b,*]

[a] Department of Immunology, School of Medicine, Shandong University, 44 Wenhuaxi Road, Jinan 250012, PR China
[b] Department of Pathophysiology, School of Medicine, Shandong University, 44 Wenhuaxi Road, Jinan 250012, PR China

Received 14 December 2006
Available online 29 December 2006

Abstract

A Chinese patient with severe hypertriglyceridemia was found to have similar clinical features to that of malignant hyperlipemia in infancy. DNA sequence analysis of the apoC-II gene from the patient's parents revealed a novel heterozygous mutation of T → A substitution at position −190 base in the apoC-II promoter. We speculated that the patient was a homozygote of the same mutation that resulted in the deficiency of apoC-II. In vitro expression studies showed T → A substitution in the apoC-II promoter leads to a decrease by approximately 20% in transcriptional activity compared with its counterpart that inserted the normal promoter. These results suggested that T → A substitution at position −190 in the apoC-II gene promoter only partly affected transcriptional activity of the apoC-II promoter, leading to decrease of apoC-II expression in quantity.

Keywords: Apolipoprotein C-II; Site-directed mutation; Promoter; Hyperlipemia; Luciferase expression

Human apolipoprotein C-II (apoC-II) is a 79-amino acid residue protein that has a crucial role in lipoprotein metabolism as an activator for the lipoprotein lipase (LPL) [1–4]. Human apoC-II gene has been mapped on the long arm of chromosome 19 in a gene cluster that contains the ApoE/C-I/C-I′/C-IV/C-II genes and spans 45 kb of chromosomal region [5–7]. The 0.55-kb intergenic region between the apoC-II and apoC-IV genes is a strong cell type-specific promoter [5,8]. The apoC-II promoter contains five footprints defined by hepatic nuclear extracts and designated C-II-A (−74/−44), C-II-B (−102/−81), C-II-C (−159/−116), C-II-D (−288/−265), and C-II-E (−497/−462) [9].

In previous study, we reported the first case of hyperlipemia syndrome in infancy [10–12]. The proband, a 30-day-old male infant, whose clinical features included severe anaemia, eruptive xanthomas, and fasting triglycerides of 50 mmol/L, died of infection in the lung at 50 days which conformed to the diagnosis of malignant hyperlipemia in infancy [13]. The apoC-II gene from the proband's parents without clinical feature has been sequenced after amplification by the polymerase chain reaction (PCR). DNA sequence analysis revealed a single base substitution A for T at position −190 in apoC-II promoter, which indicated that the proband's parents were heterozygous for the point mutation. Their plasma apoC-II concentration is below half the value of normal apoC-II, but this level of plasma apoC-II could effectively activate the lipoprotein lipase because their plasma triglycerides (TG) concentration is normal [10]. We speculated that the proband was a homozygote of the same mutation, resulting in preventing from transcript of the apoC-II gene. The deficiency of

Abbreviations: ApoC-II, apolipoprotein C-II; LPL, lipoprotein lipase; PLB, passive lysis buffer; LAR II, Luciferase Assay Reagent II; TG, triglycerides.

* Corresponding author. Fax: +86 531 88382502.
E-mail address: huweicheng@sdu.edu.cn (W.-C. Hu).

0006-291X/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2006.12.154

apoC-II protein in plasma could not activate LPL, which leads to hyperlipoproteinemia.

In this study, we have investigated the molecular mechanism for familial apolipoprotein C-II deficiency in the first case with the syndrome of hyperchylomicronemia by measuring the activity of the luciferase expression plasmid with the mutant apoC-II promoter in vitro. Our results showed that the T → A substitution at position −190 in the apoC-II promoter could not significantly reduce transcription activity of apoC-II promoter, and is only associated with molecular mechanism of malignant hyperlipemia. It is not crucial for transcription activity of apoC-II promoter to the mutation at position −190 in the apoC-II promoter.

Materials and methods

The human normal −545/+18 apoC-II promoter, which was blunted and cloned into the *Sma*I site of luciferase expression vector pGL3-Basic, was a gift from Dr. Dimitris Kardassis (University of Crete, Greece). The plasmid pGL3-control, pGL3-basic, and pRL-TK all were purchased from Promega. QIAprep Spin Miniprep Kit Protocol was purchased from Qiagen. QuikChange™ Site-Directed Mutagenesis Kit, which contains passive lysis buffer (PLB), Luciferase Assay Reagent II (LAR II), and Stop & Glo™Reagent, was purchased from Stratagene. LipofectAMINE™ 2000 Reagent was purchased from Invitrogen. Dual Luciferase Reporter Assay System was purchased from Promega.

Site-directed mutagenesis. The plasmid pGL3 with normal apoC-II promoter was used as the template, and a pair of completely complementary primers with the desired mutation was designed, which made the plasmid amplified and mutated on −190 base of the apoC-II promoter using Stratagene's site-directed mutagenesis kit. Primer 1: 5′ GAA TTC TCA GAG TGA GGG A*TC CCT GTC ACT TGA G 3′, Primer 2: 5′ CTC AAG TGA CA G GGA T*CC CTC ACT CTG AGA ATT C 3′ (The symbol * represent the desired mutant base). The reaction mixture contains: 5 μl of 10× reaction buffer, 10 ng of dsDNA template, 125 μg of oligonucleotide primer 1, 125 μg of oligonucleotide primer 2, 1 μl of dNTP mix (10 mmol/L), 1 μl of PfuTurbo DNA polymerase (2.5 U/μl), then add ddH_2O to a final volume of 50 μl. The reaction mixture was applied to 12 cycles of PCR (95 °C for 30 s; 55 °C for 1 min; 68 °C for 12 min), which was preceded by an additional denaturation. The 10 μl of amplified product was checked by electrophoresis of 2% agarose gels. The amplified plasmid was digested with 1 μl of *Dpn*I restriction enzymes at 37 °C for 1 h in order to eliminate parental non-mutated dSDNA. After being transformed into XL-Blue supercompetent cells, the nicked mutant plasmid was repaired in these cells. The desired mutation in apoC-II promoter was verified by DNA sequencing.

Cell cultures and transient transfection. HepG2 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Transfection steps were operated according to instruction of LipofectAMINE™ 2000 Reagent (Invitrogen). The plasmid pGL3 with mutant promoter or normal promoter was, respectively, cotransfected with pRL-TK, an internal control into HepG2 cells. As a positive control we used the plasmid pGL3-Control Vector, which contains an improved firefly luciferase gene (*luc*+) under the control of a SV40 promoter/enhancer region. As a negative control we used the pGL3-Basic Vector, containing the improved *luc*+ gene without a promoter or enhancer. To ensure independent genetic expression between experimental and control reporter genes, we optimized both the amount of vector pGL3 and the ratio of co-reporter vector pRL-TK added to the transfection mixture. Ratio of 25:1 for experimental vector pGL3:co-reporter vector pRL-TK combinations was choosed in the cotransfection system.

Assay of luciferase activity. After transient transfection, cells were cultured for 48 h and lysed using passive lysis buffer (PLB) at room temperature for 15 min. The firefly luciferase activity of pGL3 (M1) and the *Renilla* luciferase activity (M2) of pRL-TK were measured sequentially by the Luminoskan TL Plus Luminometer. The firefly luciferase activity was measured first by adding 100 μl Luciferase Assay Reagent II (LAR II) in 20 μl lysate of harvested cells. After quantifying the firefly luminescence, this reaction is quenched, and the *Renilla* luciferase reaction is initiated simultaneously by adding 100 μl Stop & Glo™Reagent to the same tube. Then, the *Renilla* luminescence was quantified. The luciferase activity from each group was normalized with control *Renilla* luciferase activity.

Statistical analysis. The statistical significance for the mean values between mutant and normal types was determined by Student's *t* test. The level of significance was set to $\alpha = 0.05$. All tests were two sided.

Results

Site-directed mutagenesis

The plasmid pGL3 with normal −545/+18 apoC-II promoter has 5381 base pairs in length (Fig. 1). The nicked circular plasmid with mutant site was amplified by site-directed mutagenesis, using a pair of completely complementary primers with the desired mutation. The 10 μl of the PCR product was run on 2% agarose gel, showed a corresponding band on 5381 bp position (Fig. 2). After digested the methylated and non-mutanted parental DNA template with *Dpn*I, the nicked plasmid was transformed into XL-Blue supercompetent cells, and repaired the nicks of the mutated plasmid in cells. The apolipoprotein C-II Gene T → A mutation at Position −190 in the apoC-II promoter was confirmed by DNA sequencing (Fig. 3).

Transient transfection and assays of luciferase activity

The experimental plasmid pGL3 and internal control pRL-TK were cotransfected into HepG2 cells. The luminescent activity in lysates of harvested cells was assayed 48 h later after transfection with the DLR™ Assay System. First, the firefly luminescence of pGL3 (M_1) was quantified; second, the *Renilla* luminescence of pRL-TK (M_2) was quantified in the same tube. Ratio of M_1:M_2 was the relative luciferase activity of the experimental plasmid pGL3. The normalized luciferase activity of mutant apoC-II promoter at the transcriptional level was reduced by approximately 20% relative to normal apoC-II promoter (Fig. 4).

Discussion

Plasma apolipoprotein C-II is a potent activator of LPL, which catalyzes the lipolysis of triglycerides in plasma chylomicrons and VLDL. ApoC-II deficiency is a rare genetic disorder that is inherited as an autosomal recessive trait. On hereditary deficiency of apoC-II, plasma levels of triglycerides are highly increased due to accumulation of chylomicrons and very low density lipoproteins in blood [14]. Clinical features also include lipemia retinalis, eruptive xanthomas, and an increased incidence of pancreatitis.

At present, the two major causes of severe hypertriglyceridemia are a deficiency of the enzyme, lipoprotein lipase,

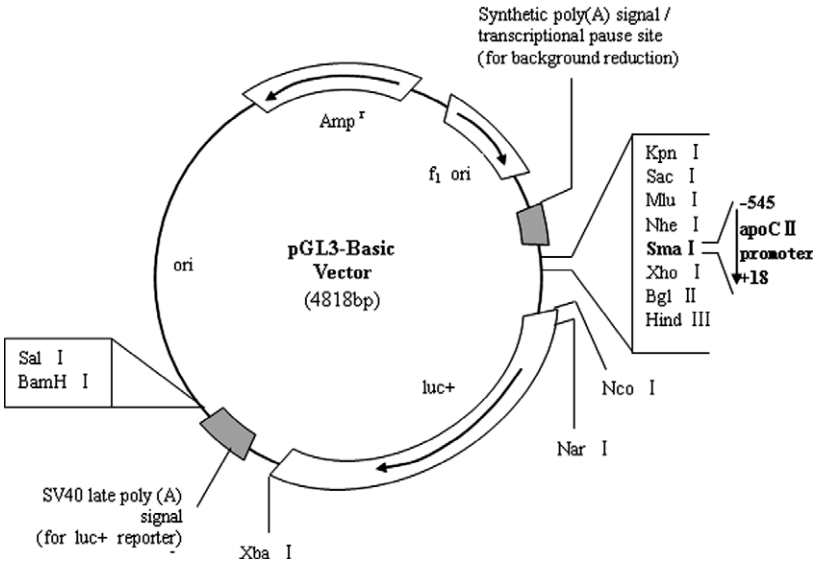

Fig. 1. Physical map of pGL3-Basic with the normal apoC-II promoter. The normal −545/+18 apoC-II promoter, which was blunted and cloned into the *Sma*I site of luciferase expression vector pGL3-Basic. The plasmid pGL3 with normal −545/+18 apoC-II promoter has 5381 base pairs in length.

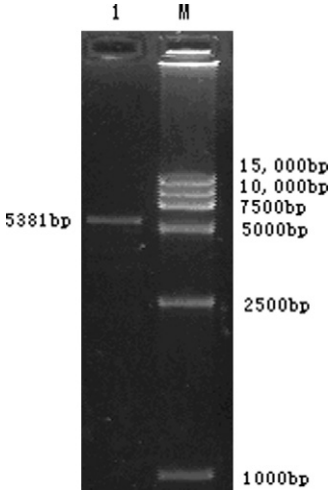

Fig. 2. Electrophoresis analysis of PCR products. PCR product amplified by site-directed mutagenesis was run on 2% agarose gel and showed a corresponding band on 5381 bp position according to the DNA marker DL15,000. Lane M, DNA marker (DL15,000); lane 1, plasmid pGL3 with the mutant apo C-II promoter (5381 bp).

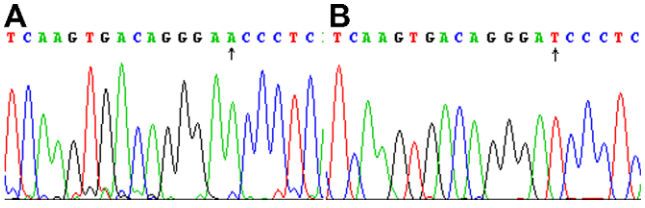

Fig. 3. DNA reverse sequencing of normal and mutant apoC-II promoter. (A) DNA reverse sequencing of the normal apoC-II promoter. (B) DNA reverse sequencing of the mutant apoC-II promoter. The T → A substitution is indicated by an arrow.

and its cofactor, apoC-II. Both were inherited as an autosomal recessive trait [15].

Hagberg et al. [13] first reported a very rare clinical syndrome including hypertriglyceridemia, intumesces of liver

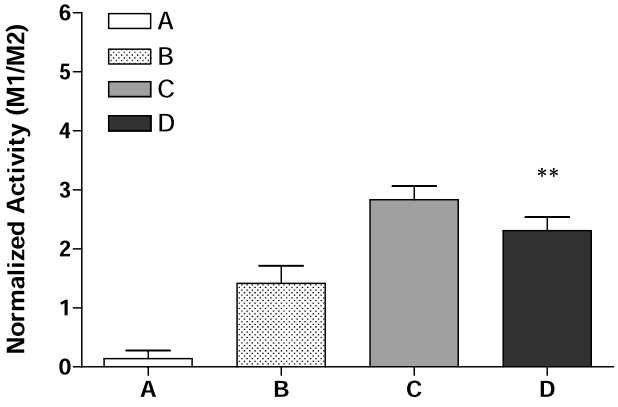

Fig. 4. Measurement of luciferase activities in HepG2 cells. The plasmid pGL3 with normal or mutant apoC-II promoter and internal control pRL-TK were co-transfected into HepG2 cells. After 48 h, cells were lysed with passive lysis buffer and assayed for luminescent activity with the DLR™ Assay System. Measurements were carried out in triplicate. Firefly luciferase activity (M1) was normalized to *Renilla reniformis* luciferase activity (M2). (A) Negative control pGL3-basic; (B) Positive control pGL3-control; (C) Plasmid pGL3 with normal apoC-II promoter; (D) Plasmid pGL3 with mutant apoC-II promoter. ($^{**}P < 0.01$).

and spleen, decrease of blood cells and died quickly in 1964, which was diagnosed as malignant hyperlipemia in infancy. In that time, the investigation of the case could not be done owing to the limited technology and knowledge. We reported the first case in China, whose clinical features were similar to malignant hyperlipemia in infancy. The plasma apoC-II concentration of the proband's parents was below half the value of normal apoC-II, but their plasma triglycerides concentration was normal. These results showed that half the value of plasma apoC-II could effectively activate the lipoprotein lipase which can hydrolyze triglycerides. Because the proband died quickly without DNA left, we analyzed the DNA sequence of the proband's parents. DNA analysis of the apoC-II gene

revealed that they were heterozygotes with T → A substitution at position −190 in the apoC-II promoter. The severe symptoms of this proband led us to suspect that the proband was a homozygote of the same mutation, which resulted in the deficiency of apoC-II.

A single base T → A transition at position −190 in the apoC-II promoter region was obtained by site-directed mutagenesis method. The role of the apoC-II promoter was examined by cloning the region into a luciferase reporter assay system and transfection into HepG2 cells. The experimental results indicated the constructed expression vector containing the mutant promoter had a decrease by approximately 20% in transcriptional activity compared with its counterpart that inserted the normal promoter. As a result of the complicated gene regulation system in eukaryote, we concluded a single base mutation T → A at position −190 in the apoC-II promoter did not diminish the binding of important transcription factors to relevant cis-acting elements in the promoter, only partly affected transcriptional activity of the apoC-II promoter leading to decrease of apoC-II expression in quantity.

Acknowledgments

We thank Dr. Dimitris Kardassis, University of Crete, Greece, for the plasmid pGL3 with the normal apoC-II promoter gene, Dr. J.Y. Zhang and Dr. P.J. Zhang, School of Medicine, Shangdong University, Jinan, China, for the plasmid pGL3 and pRL-TK. W.C. Hu is supported by the National Natural Science Foundation of China (No. 30070354).

References

[1] R.J. Havel, V.G. Shore, B. Shore, D.M. Bier, Role of specific glycopeptides of human serum lipoproteins in the activation of lipoprotein lipase, Circ. Res. 27 (1970) 595–600.
[2] J.C. LaRosa, R.I. Levy, P. Herbert, S.E. Lux, D.S. Fredrickson, A specific apoprotein activator for lipoprotein lipase, Biochem. Biophys. Res. Commun. 41 (1970) 57–62.
[3] A.V. Hospattankar, T. Fairwell, R. Ronan, H.B. Brewer Jr., Amino acid sequence of human plasma apolipoprotein C-II from normal and hyperlipoproteinemic subjects, J. Biol. Chem. 259 (1984) 318–322.
[4] W.C. Breckenridge, J.A. Little, G. Steiner, A. Chow, M. Poapst, Hypertriglyceridemia associated with deficiency of apolipoprotein C-I, N. Engl. J. Med. 298 (1978) 1265–1273.
[5] C.M. Allan, D. Walker, J.P. Segrest, J.M. Taylor, Identification and characterization of a new human gene (APOC4) in the apolipoprotein E, C-I, and C-II gene locus, Genomics 28 (1995) 291–300.
[6] A.J. Lusis, C. Heinzmann, R.S. Sparkes, J. Scott, T.J. Knott, R. Geller, M.C. Sparkes, T. Mohandas, Regional mapping of human chromosome 19: organization of genes for plasma lipid transport (APOC1, -C2, and -E and LDLR) and the genes C3, PEPD, and GPI, Proc. Natl. Acad. Sci. USA 83 (1986) 3929–3933.
[7] C.F. Wei, Y.K. Tsao, D.L. Robberson, A.M. Gotto Jr., K. Brown, L. Chan, The structure of the human apolipoprotein C-II gene. Electron microscopic analysis of RNA: DNA hybrids, complete nucleotide sequence, and identification of 5′ homologous sequences among apolipoprotein genes, J. Biol. Chem. 260 (1985) 15211–15221.
[8] P. Vorgia, V.I. Zannis, D.A. Kardassis, Short proximal promoter and the distal hepatic control region-1 (HCR-1) contribute to the liver specificity of the human apolipoprotein C-II Gene, J. Biol. Chem. 273 (1998) 4188–4199.
[9] D. Kardassis, E. Sacharidou, V.I. Zannis, Transactivation of the human apolipoprotein C-II promoter by orphan and ligand-dependent nuclear receptors. The regulatory element C-IIC is a thyroid hormone response element, J. Biol. Chem. 273 (1998) 17810–17816.
[10] C. Tian, W. Hu, R. Chen, B. Shen, The genetic analysis of apolipoprotein C-II in the parents of a malignant hyperlipidemia, Chin. J. Med. Genet. 20 (2003) 272.
[11] X. Feng, W. Hu, S. Zhang, F. Liu, L. Li, Y. Liu, Y. Zhu, J. Xu, The initial research of malignant hyperlipemia in infancy, Chin. J. Med. Genet. 12 (1995) 366–367.
[12] C. Chen, Y. Cao, W. Hu, A single base mutation (T to A) at position-190 in the apolipoprotein C-II promoter with apolipoprotein C-II deficiency, Atherosclerosis 11 (2003) 509–511.
[13] B. Haberg, G. Hultquist, L. Sevennerholm, Malignant hyperlipemia in infancy, Am. J. Dis. Chile 107 (1964) 267–276.
[14] S.S. Fojo, J.L. de Gennes, U. Beisiegel, G. Baggio, A.F. Stalenhoef, J.D. Brunzell, H.B. Brewer Jr., Molecular genetics of apoC-II and lipoprotein lipase deficiency, Adv. Exp. Med. Biol. 285 (1991) 329–333.
[15] S.S. Fojo, H.B. Brewer, Hypertriglyceridaemia due to genetic defects in lipoprotein lipase and apolipoprotein C-II, J. Intern. Med. 231 (1992) 669–677.