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Lack of evidence for a liver or intestinal miRNA regulation involved in the hypertriglyceridemic effect of *APOC3* 3′UTR variant SstI

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ABSTRACT

Background and aims: *APOC3* is a major regulator of triglycerides metabolism. Several *APOC3* variants are associated with hypertriglyceridemia (HTG). Our aim was to establish the potential regulation of *APOC3* 3′UTR variants associated with HTG by liver or intestinal miRNAs.
Methods: We sequenced *APOC3* 3′UTR in 100 type 2 diabetic (TD2) patients with severe HTG (TG > 15 mmol/L) (HTG group) compared to 100 normotriglyceridemic patients (NTG group). We performed *in silico* studies to identify potential loss of miRNA binding induced by *APOC3* 3′UTR variants. We also performed *in vitro* studies to test the functionality of miRNA/*APOC3* variants interactions: *APOC3* 3′UTR plasmids coupled with a firefly luciferase reporter were transfected in HepG2, HuH-7 and Caco-2 cells.
Results: We identified only two variants: SstI (rs5128) and BbvI (rs5225) in *APOC3* 3′UTR in the 2 groups of patients. Only the SstI-S2 rare allele was significantly associated with HTG (allele frequency 19,5% in HTG group *vs.* 9,5% in NTG group, $p = 0.0045$). *In silico* studies predicted a potential loss in the binding of 5 miRNAs induced by the S2 variant. These 5 miRNAs are all endogenously expressed in human liver and intestine, as well as in the cell models studied. However, *in vitro*, the S2 variant did not modulate *APOC3* 3′UTR reporter gene expression in HepG2, HuH-7 and Caco-2 cells.
Conclusions: Our results do not confirm the hypothesis of a direct regulation of the *APOC3* SstI variant by hepatic or intestinal miRNAs.

1. Introduction

Apolipoprotein CIII (apo CIII), expressed mainly in the liver and intestine, is carried on very low density lipoprotein (VLDL), chylomicrons (CM) and high density lipoprotein (HDL) [1]. Apo CIII is a major regulator of plasma triglycerides (TG) metabolism by promoting liver VLDL assembly and secretion, inhibiting hydrolysis of TG-rich lipoproteins by lipoprotein lipase and decreasing the uptake of TG-rich remnant lipoproteins by the liver [2–4].

There is a strong positive correlation between plasma TG and apo CIII levels. Rare *APOC3* loss-of-function mutations are associated with lower TG [5] whereas overexpression of *APOC3* induces HTG in transgenic mice [6]. Elevated concentration of plasma apo CIII is also closely associated with hypertriglyceridemia in humans [7]. Antisens oligonucleotides anti-*APOC3* have been shown to consistently decrease plasma TG in humans in phase 1 and 2 clinical studies [8]. Several *APOC3* gene non-coding variants, such as promoter variants (rs2854117 and rs2854116) or 3′UTR SstI (rs5128), are tightly associated with both moderate and severe hypertriglyceridemia, but their functionality is not clearly established [9–12].

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The discovery of miRNAs, small non-coding RNAs that post-transcriptionally down-regulate gene expression by binding target 3′UTR mRNA sequences, provides new opportunities to explain the functionality of the 3′UTR variant [13]. We previously showed that 3′UTR variants of key genes involved in the regulation of TG metabolism could either create or suppress miRNA binding sites and modulate plasma TG concentration [14,15].

Therefore, we aimed to assess whether TG associated *APOC*3 3′UTR variants could be involved in miRNA dysregulation of *APOC*3: the loss of a miRNA binding site could increase *APOC*3 mRNA expression and raise plasma TG concentration.

2. Materials and methods

2.1. Population studied

100 type 2 diabetic (T2D) patients with documented hyperchylomicronemia (TG > 15 mmol/L) and 100 T2D normotriglyceridemic patients from the DIACOR cohort were studied, as previously described [16]. Detailed inclusion and exclusion criteria are available in reference [17]. Baseline characteristics and lipid parameters are presented in Supplemental Table 1.

2.2. APOC3 3′UTR polymorphisms analysis

Genomic DNA was extracted as previously described [12]. The *APOC*3 3′UTR DNA sequence (0.2 μg) was amplified by PCR using the following primers (Life Technologies, Invitrogen Inc., Carlsbad, CA, United States): forward 5′-CTGGGATTTGGACCCTGAG-3′; reverse 5′-CATGCCCAGACACAGATGG-3′. PCR products were analyzed by using denaturing high-performance liquid chromatography (dHPLC, Transgenomic, Glasgow, United Kingdom) in a 1:1 mixture with a control (wild type) amplicons. Variant sequences were confirmed by direct sequencing (CEQ™ 8000 Genetic analysis system Beckman Coulter ™, Villepinte, France). Pairwise linkage disequilibrium was calculated using Lewontin's parameter of LD, D′ [18].

2.3. Bio-informatic studies

We compared the miRNA binding-site predictions of two software programs for miRNA target prediction PITA [19] and RegRNA [20], whose characteristics are summarized in a previous study [14].

2.4. Human APOC3 3′UTR luciferase constructs

In order to study the miRNA regulation of the two *APOC*3 3′UTR polymorphisms identified SstI (c.*40G > C, rs5128) and BbvI (c.*71 G > T, rs4225), the *APOC*3 3′UTR full length wild sequence (c*40C (S1) and c*71T (B1)) was synthesized and inserted in pEZX-MT01 vector (GeneCopoeia™, Labomics S.A, Nivelles, Belgium) downstream a firefly luciferase reporter gene, creating a pEZX-S1B1 vector. Site-directed mutagenesis was performed to introduce c*40G (S2) and/or c*71G (B2) alleles in pEZX-S1B1, creating variants vectors: pEZX-S1B2, pEZX-S2B1, pEZX-S2B2 (GenScript, Piscataway, USA). All constructs were confirmed by sequencing.

2.5. MicroRNA expression

Total RNA was extracted from cell lines: HepG2, HuH-7, and Caco-2 and from human intestine and liver tissues (generous gift from anathomopathology laboratory South Hospital, Lyon), using the TriPure technical (Roche Applied Science, Penzber, Germany), in accordance with the manufacturer's instructions. miRNA-specific reverse transcription was performed with 500 ng of purified total RNA, using the TaqMan MicroRNA Reverse Transcription kit and miRNA qPCR using TaqMan 2x Universal PCR master Mix (Life Technologies – Invitrogen Inc., Carlsbad, CA, United States) on Rotor-Gene Q thermocycler (QIAGEN, Courtaboeuf, France). miRNA expression was expressed in 1/cycle threshold, proportional to the miRNA concentration. All experiments were performed in triplicate.

2.6. APOC3 3′UTR luciferase reporter assays

Human hepatic (HepG2) and intestinal (Caco-2) cell lines were cultured in DMEM high glucose (25 mM) medium; HuH-7 human hepatic cell line was cultured in DMEM low glucose (5,5 mM); all media were supplemented with 10% fetal bovine serum. Cells were plated onto 12-well plates ($4{,}5 \times 10^5$ cells/well for HepG2, $2{,}5 \times 10^5$ cells/well for Caco-2, and $1{,}5 \times 10^5$ cells/well for HuH-7) and were transfected with *APOC*3 3′UTR luciferase constructs. All transient transfections were performed with jetPRIME® transfection reagent (Polyplus transfection™, Illkirch, France) using 2 μl for 1 μg of DNA according to the manufacturer's protocol.

Transfection efficacy was controlled using *APOA5* 3′UTR luciferase constructs pEZX-MT01-*APOA5*-SNP1-T vector (pEZX-T) and pEZX-MT01-*APOA5*-SNP1-C vector (pEZX-C), which was previously demonstrated to be regulated by miR485-5p in HuH-7 [14].

Luciferase activity was measured 24 h after transfection as previously described [14]. Results were expressed as a percentage of the control condition. All transfection experiments were performed in triplicate and repeated at least three times.

2.7. Statistical analysis

Statistical analyses were performed using SPSS 13.0 software. Continuous variables were expressed in mean ± standard error of the mean (SEM) and categorical variables as frequencies and percentages. Means of quantitative variables were compared using one-way ANOVA test or Student's t-test, while categorical variables were analyzed with the Chi-squared test or Fisher's exact test when effectives were under 10. A p value < 0.05 (two sided) was considered as significant.

3. Results

3.1. Screening of APOC3 3′UTR variants associated with hypertriglyceridemia

A genetic analysis in 2 groups of type 2 diabetic patients either normotriglyceridemic (NTG, n = 100) or severely hypertriglyceridemic (HTG, n = 100) was performed. In both groups, we identified only 2 genetic variations: SstI (c.*40G > C, rs5128) and BbvI (c.*71 G > T, rs4225) (Refseq accession number NM_000237.2). Allele and genotype distribution of the rare variant of BbvI polymorphism (B2) was not significantly different between the 2 groups, whereas the rare variant S2 of the SstI polymorphism was twice as frequent in group HTG as in group NTG (Table 1). The S2 variant was found to be in strong linkage disequilibrium (D′ = 1) with the B2 variant as no carrier of the S2B1 haplotype was identified among the 400 chromosomes of this study (Table 2) Thus, only the SstI *APOC*3 3′UTR variant was significantly associated with severe HTG in this cohort.

3.2. Bioinformatic prediction of APOC3 3′UTR SstI variant effect on miRNA binding sites

We used 2 prediction software tools to investigate the putative