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MiR-21 regulates the *ACAT1* gene in MCF-7 cells

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ABSTRACT

Aims: The purpose of the present study was to determine whether miR-21 regulates the human *ACAT1* gene. We also assessed whether transfection of MCF-7 cells with miR-21 mimic/inhibitor leads to changes in *ACAT1* mRNA/protein levels, cell proliferation rate, or apoptosis.

Main methods: Regulation of *ACAT1* 3′UTR by miR-21 was evaluated using a dual-luciferase reporter assay. The effect of miR-21 on mRNA/protein levels of *ACAT1* and *PTEN* (confirmed as an important target of miR-21 for comparison) was measured by qPCR/western blot analysis and immunostaining. Proliferation rate was determined by cell counting. Percentage of cells undergoing late apoptosis was determined by staining with Hoechst 33342/propidium iodide.

Key findings: Dual-luciferase reporter assay confirmed the regulation of *ACAT1* 3′UTR by miR-21. Furthermore, transfection of MCF-7 cells with miR-21 mimic decreased mRNA and protein levels of *ACAT1* and *PTEN* genes. In contrast, miR-21 inhibition increased the mRNA and protein levels of both genes studied. Finally, we observed an increase in cell proliferation and decrease in the percentage of cells in late apoptosis in MCF-7 cells transfected with miR-21 mimic, whereas transfection with miR-21 inhibitor led to the opposite effect.

Significance: Our data confirm the hypothesis that miR-21 regulates the human *ACAT1* gene. As the expression of this microRNA is altered in many types of cancers, the discovery of novel targets for miR-21 is of particular interest for diagnosis and treatment.

1. Introduction

MicroRNAs are small, noncoding, single-stranded RNAs 19–23 nucleotides in length that complementarily bind target mRNAs at their 3′-untranslated regions, and rarely at their 5′-regions, leading to translational repression or mRNA degradation [1]. These molecules regulate the expression of genes associated with many biological processes, such as cell proliferation, differentiation and apoptosis [2]. MiR-21, one of the most famous onco-microRNAs [3], is upregulated in a variety of cancers, including breast [4], prostate [5] and hepatocellular carcinoma [6]. However, miR-21 downregulation has also been demonstrated in cancer, in particular in endometrioid carcinoma [7]. Unsurprisingly, many studies have suggested that miR-21 could be used for cancer diagnosis [8,9]. Some important miR-21 target genes have been identified, including the tumor suppressor genes phosphatase and tensin homolog (PTEN) [6], programmed cell death 4 (PDCD4) [10] and maspin (SERPINB5) [11]. In addition, silencing of miR-21 expression by siRNA induces apoptosis of cervical cancer cells [12].

In our previous study we showed in DDT- and benzo[*a*]pyrene-treated rats that miR-21 levels in the liver were elevated [13]. Moreover, the observed increases in miR-21 corresponded with decreases in its target gene *Acat1*. Acetyl-CoA acetyltransferase 1 (ACAT1) is an enzyme that catalyzes the reversible formation of acetoacetyl-CoA from two molecules of acetyl-CoA [14]. In 2016, Jun Fan et al. hypothesized that ACAT1 plays a key role in the Warburg effect: it was shown that arecoline hydrobromide, an ACAT1 covalent inhibitor, leads to increased pyruvate dehydrogenase complex flux and oxidative phosphorylation with attenuated cancer cell proliferation and tumor growth. Additionally, increased expression of *ACAT1* was identified in different cancer cells [15]. Moreover, Yi-Wen Lo et al. have shown that *ACAT1* is involved in the induction of doxorubicin resistance in uterine cancer [16]. Until now, regulation of the *ACAT1* gene by miR-21 has not been examined. Meanwhile, our understanding of the epigenetic regulation of gene expression is crucial for the treatment and diagnosis of many diseases, including cancer. The objective of the present study was to determine whether miR-21 regulates the human *ACAT1* gene.

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2. Materials and methods

2.1. Cell lines, culture conditions and transfections

MCF-7 cells were obtained from the Laboratory of Xenobiotics Biochemistry, Institute of Molecular Biology and Biophysics, Russia. Cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM) (Invitrogen) supplemented with 10% FBS (HyClone), 1% Antibiotic-Antimycotic (100×) (Gibco) and maintained at 37 °C in 5% CO_2. For experiments, MCF-7 cells were seeded in 6-well plates at a density of 10^5 cells per well in complete medium. The following day, cells were transfected with mirVana miRNA mimic Negative Control (NC) or hsa-miR-21-5p mimic/inhibitor (Thermo Fisher Scientific) using Lipofectamine 3000 reagent (Thermo Fisher Scientific) according to the manufacturer's instructions otherwise indicated. Forty-eight hours after transfection, samples were used for further experiments.

2.2. Reporter plasmids

Targetscan and Pita were used for the *ACAT1* 3′-untranslated region (3′-UTR) analysis and querying miR-21 binding sites. Only one potential site was identified, position 89–95 in the 3′-UTR. We designed primers containing native and mutated miR-21 binding sites (sequences are shown below). The 3′UTR is underlined, the binding sites are highlighted in gray, and mutated nucleotides are highlighted in dark gray.

Native forward:
tcgagaagcttagtgtgactactgtgggtcagcttatattcagataagctgtttcag
Native reverse:
tcgactgaaacagcttatctgaatataagctgacccacagtagtcacactaagcttc
Mutated forward:
tcgagagatctagtgtgactactgtgggtcagcttatattcagattacatgtttcag
Mutated reverse:
tcgactgaaacatgtaatctgaatataagctgacccacagtagtcacactagatctc

Primers were obtained from Biosset, Novosibirsk. Annealed primers were cloned into *Sal*I and *Xho*I restriction sites of the pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega). Thus, we obtained reporter vectors containing native (ACAT1 3′UTR nat) and mutated (ACAT1 3′UTR mut) miR-21 binding sites from ACAT1 3′UTR.

2.3. Luciferase reporter assay

MCF-7 cells were transiently cotransfected with reporter vectors ACAT1 3′UTR nat/mut and mirVana miRNA mimic, negative control (NC) or hsa-miR-21-5p mimic/inhibitor (Thermo Fisher Scientific) using Lipofectamine 3000 reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. Briefly, one day before the transfection assay, cells were plated at a density of 104 cells per well in 96-well plates containing 0.1 ml DMEM with 10% FBS. At the time of transfection, the vectors, miR-21 mimic/inhibitor/NC and Lipofectamine 3000 reagent were diluted in Opti-MEM™ Reduced Serum Medium and GlutaMAX™ Supplement (Thermo Fisher Scientific) and incubated for 20 min at room temperature. Medium was replaced with Opti-MEM, and each well contained 0.3 ng of reporter vectors and 3 pmol of miR-21 mimic/inhibitor or NC. After 48 h, the Dual-Glo luciferase assay system (Promega) was used to detect luciferase activity according to the manufacturer's instructions. Raw luciferase activity was measured using the EnVision 2103 Multilabel Plate Reader (PerkinElmer). Raw values were normalized by dividing the FLuc values by the RLuc values.

2.4. RNA analysis

Total RNA was isolated using TRIzol reagent (Ambion, USA) following the supplier's recommendations. Quantity of total isolated RNA was determined spectrophotometrically at 260 nm using a P360 nanophotometer (Implen). Total RNA quality was evaluated by electrophoresis in a 1.5% agarose gel, and ratio $D_{260}/D_{280} > 1.8$. reverse transcription was performed to obtain cDNA from the RNA template using a M-MuLV–RH Reverse Transcription Kit (Biolabmix, Novosibirsk) following the supplier's recommendations. One microgram of total RNA was used per reaction. Relative gene expression levels of *ACAT1* and *PTEN* were determined by real time PCR using BioMaster HS-qPCR SYBR Blue (2×) and CFX96™ Real-Time PCR Detection System (Bio-Rad Laboratories). The housekeeping gene *GAPDH* was used as a reference gene. The following primers were used in this study:

ACAT1: Forward GAACAGAGGATCAACACCAT Reverse TGCTGCT TTACTTCTGGTAT
PTEN: Forward GTTTACCGGCAGCATCAAAT Reverse CCCCCACTT TAGTGCACAGT
GAPDH: Forward ACAACTTTGGTATCGTGGAAGGAC Reverse CAG GGATGATGTTCTGGAGAGC

For measurement of miR-21 levels, we performed reverse transcription using special stem-loop primers and the M-MuLV–RH Reverse Transcription Kit (Biolabmix, Novosibirsk) following the supplier's recommendations. Relative gene expression levels of miR-21 were determined by real time PCR using a BioMaster UDG HS-qPCR (2×) kit (Biolabmix, Novosibirsk) and the CFX96™ Real-Time PCR Detection System (Bio-Rad Laboratories) following the supplier's recommendations. The small nuclear RNA U_6 was used as a reference gene. We used the following primers:

U_6: Stem-loop GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTG GATACGACGGCCATGC
Forward GCCGCATACAGAGAAGATTA
Reverse AGTGCAGGGTCCGAGGTA
Probe (R6G)-TTCGCACTGGATACGACGGCCATGC-(BHQ1)
miR-21: Stem-loop GTCGTATCCAGTGCAGGGTCCGAGGTATTCGC ACTGGATACGACTCAACATC
Forward GCCGCTAGCTTATCAGACT
Reverse AGTGCAGGGTCCGAGGTA
Probe (R6G)-TTCGCACTGGATACGACTCAACATC-(BHQ1)

PCR specificity was controlled using melting curves. Relative gene expression levels were evaluated using cycle threshold (Ct) values and taking into consideration the reaction efficiencies (E).

2.5. Western blot analysis

Cells were incubated with RIPA lysis buffer on ice for 20 min and centrifuged at 17,500*g* for 20 min. The supernatant was collected, and protein concentration was measured using a Pierce BCA Protein Assay kit (Thermo Fisher Scientific). For western blot analysis, 50 μg of isolated protein was separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane. Membranes were stained with Ponceau S to verify loading and transfer efficiency. Immunodetection was performed with anti-PTEN antibody (Abcam ab79156), anti-ACAT1 antibody (Abcam ab168342), and goat anti-mouse/anti-rabbit IgG H&L (HRP) secondary antibodies (Abcam ab97040/6721). Bands were visualized colorimetrically using Opti-4CN Substrate Kit (Bio-Rad).

2.6. Immunostaining

Cells were seeded in 96-well plates at a density of 10^4 cells per well. Forty-eight hours after transfection with NC or miR-21 mimic/inhibitor, cells were fixed with paraformaldehyde. Immunodetection was performed with anti-PTEN (Abcam ab79156) and anti-ACAT1 (Abcam ab168342) primary antibodies and goat anti-mouse CF568 (Biotium)