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MiR-205 modulates abnormal lipid metabolism of hepatoma cells *via* targeting acyl-CoA synthetase long-chain family member 1 (ACSL1) mRNA

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ABSTRACT

The abnormal lipid metabolism is considered as a hallmarker of tumorigenesis. Liver is the central organ for metabolic homeostasis. Hence, the development of hepatocellular carcinoma (HCC) always exhibits alterations of metabolism. MicroRNAs emerge as key post-transcriptional modulators of gene expression in physiologic and pathologic states. Here, we aim to explore the mechanism of abnormal lipid metabolism of hepatoma cells. Previously, our group reported that miR-205 as a tumor suppressor was down-regulated in HCC. Therefore, we supposed that miR-205 might be involved in the event. Interestingly, in this study we uncover that miR-205 deregulates lipid metabolism in HCC through targeting acyl-CoA synthetase long-chain family member 1 (ACSL1) mRNA, which is an important and abundant lipid metabolism enzyme in liver. We identified that miR-205 was able to down-regulate ACSL1 *via* targeting its 3′UTR in the cells. Oil red O staining showed that miR-205 disordered the lipogenesis in hepatoma cells and anti-miR-205 resulted in the accumulation of triglyceride in the cells depending on ACSL1. Moreover, we validated that the low levels of miR-205 were negatively related to high levels of ACSL1 in clinical HCC tissues. The expression levels of ACSL1 and its metabolite triglyceride levels were remarkably increased in hepatitis B virus X protein (HBx)-induced liver cancer tissues from the HBx transgenic mice model. Thus, we conclude that miR-205-targeted ACSL1 may contribute to the abnormal lipid metabolism of liver cancer. Our finding provides new insights into the dysregulation of lipid metabolism in HCC mediated by miR-205 targeting ACSL1 mRNA.

1. Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer and the third leading cause of cancer death worldwide [1]. Up to date, there is mounting evidence that cancer cells exhibit dramatically altered metabolic circuitry. These alterations can affect the availability of structural lipids for the synthesis of membranes, the synthesis and degradation of lipids that contribute to the energy homeostasis and the abundance of lipids with signaling functions [2]. As a central organ for metabolic homeostasis, liver is a major site for synthesis, metabolism, storage and redistribution of carbohydrates, proteins and lipids [3]. Hence, the advent of HCC is always accompanied by metabolism reprogramming [4]. However, the mechanism of metabolism reprogramming, such as abnormal lipid metabolism in liver cancer remains ill-identified. Acyl-CoA synthetase long-chain family member (ACSL) catalyzes the ATP-dependent acylation of fatty acids into long-chain acyl CoAs (LCA-CoAs), which is the first step in lipid metabolism after fatty acid entry into the cell [5]. ACSL1 is one of five isoforms, which is abundant in adipose tissue, liver, heart and important in activating fatty acid destined for triacylglycerol synthesis [6].

MicroRNAs (miRNAs) are small, non-coding RNAs which emerge as key post-transcriptional modulators of gene expression. Hundreds of miRNAs have been identified in vertebrates, with varying patterns of expression that range from ubiquitous to highly tissue- or developmental-stage-restricted. In addition, numerous miRNAs are involved in human cancer and undertake opposite roles of oncogenes or tumor suppressor genes depending on the different target genes [7,8]. As a miRNA, miR-205 is regarded as a

Abbreviations: HCC, hepatocellular carcinoma; miRNA, microRNA; ACSL1, acyl-CoA synthetase long-chain family member 1; 3′UTR, 3′untranslated region; qRT-PCR, quantitative real-time polymerase chain reaction; RT-PCR, reverse transcription-polymerase chain reaction; HBx, hepatitis B virus X protein.

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tumor suppressor gene since its disease-specific expression pattern. It has been reported that down-regulation of miR-205 contributes to the tumorigenesis of melanoma and breast cancer [9,10]. Previously, our group found that miR-205 as a tumor suppressor was down-regulated in the tissues of liver cancer mediated by Hepatitis B virus X protein (HBx) [11]. However, whether miR-205 participates in abnormal lipid metabolism is poorly understood.

In this study, we aimed to illuminate the mechanism of abnormal lipid metabolism in HCC. Strikingly, our data demonstrate that low levels of miR-205 contribute to the event. MiR-205 targeted ACSL1 is involved in the abnormal lipid metabolism in HCC. Our finding provides fascinating insights into the mechanisms of abnormal lipid metabolism mediated by miR-205 in HCC.

2. Materials and methods

2.1. Patient samples

Twenty-five HCC tissues utilized in this study were obtained from Tianjin First Center Hospital (Tianjin, China) after surgical resection. Written consents approving the use of their tissues for research purposes were obtained from patients. The study protocol was approved by the Institute Research Ethics Committee at the Nankai University.

2.2. Cell lines and cell culture

A human hepatoma cell line, HepG2 was maintained in Dulbecco's modified Eagle's medium (Gibco, CA, USA). The cells were supplemented with heat inactivated 10% fetal bovine serum (FBS, Gibco, CA, USA), 100 U/ml penicillin and 100 mg/ml streptomycin in 5% CO_2 at 37 °C.

2.3. Plasmid construction

One about 400 bp fragment of ACSL1 3′UTR was subcloned into pGL3-control vector (Promega Madison, WI, USA) immediately downstream of the stop codon of the luciferase gene to generate pGL3-ACSL1. Mutant construct of ACSL1 3′UTR (named as pGL3-PPARA-mut), carrying a substitution of 8 nucleotides within the core seed sequence of miR-205, was conducted by using overlapping extension PCR. The primers used in this study for construction were as follows: pGL3-ACSL1 forward, 5′-CTAGTCTAGATGGGA TTCACTTCTCCAGGGAT-3, reverse, 5′-GGGGGCCGGCCTCACCTGAAA TGCAGAAC-3′; pGL3 -ACSL1-mut forward, 5′-GGATTCACTTCTC-CAGGGATTTTTTAAAGTTAATTTGGGAA ATT-3, reverse, 5′-TCACCT-GAAATGCAGAAATATTTATTTTGGTTTGGAAGTAAGTT TT-3′.

2.4. Cell transfection

The cells were cultured in a 6-well or 24-well plate for 24 h and then were transfected with plasmids, miRNAs or siRNAs. All transfections were performed using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. ACSL1 siRNA oligonucleotides and a non-specific scrambled control (si-Ctrl), miR-205 (or anti-miR-205), miRNA control (miRNA Ctrl) and anit-miRNA control (anit-miRNA Ctrl) were synthesized by RiboBio (Guangzhou, China). The siRNA duplexes sequences used were as follows: ACSL1 siRNA, 5′-GGGCAGAUCCAACUCAGA AdTdT-3′.

2.5. Quantitative real-time polymerase chain reaction (qRT-PCR), reverse transcription-PCR (RT-PCR)

Total RNA was extracted from the cells (or tissues) using Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. For mature miR-205 detection, total RNA was polyadenylated by poly (A) polymerase (Ambion, Austin, TX, USA) as described previously [12]. Reverse transcription was performed using poly (A)-tailed total RNA and reverse transcription primer with ImPro-II Reverse Transcriptase (Promega, Madison, WI, USA), according to the manufacturer's instructions. The qRT-PCR was performed as described in the method of Fast Start Universal SYBR Green Master (Rox) (Roche Diagnostics GmbH Mannheim, Germany). The primers used were as follows: ACSL1 forward, 5′-ATCTGCAAGCCAGGAAGAGTC-3′, reverse, 5′-CTTGCTTGATGCTT TGGTCTGT-3′; GAPDH forward, 5′-CATCACCATCTTCCAGGAGCG-3′, reverse, 5′-TGACCTTGCCCACAGCCTTG-3′; miR-205 forward, 5′-TCCTTCATTCCACCGGAGTCTG-3′, reverse, 5′-GCGAGCACAGAAT-TAATACGAC-3′; U6 forward, 5′-AGAGCCTGTGGTGTCCG-3′, reverse, 5′-CATCTTCAAAGCACTTCCCT-3′.

2.6. Luciferase reporter gene assays

Luciferase reporter gene assays were performed using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) according to the manufacturer's instructions. Cells were transferred into 24-well plates at 3×10^4 cells per well. After 24 h, the cells were transiently co-transfected with the pRL-TK plasmid (Promega, Madison, WI, USA) containing the Renilla luciferase gene, which was used for internal normalization, and with various constructs containing the seed sequence or mutant seed sequence of ACSL1 3′UTR, or pGL3-control. All experiments were performed at least three times.

2.7. Western blotting analysis

The Western blotting analysis protocol was described previously [1]. The primary antibodies used were mouse anti-β-actin (Sigma, St. Louis, MO, USA), rabbit anti-ACSL1 (Proteintech Group, USA). All experiments were repeated three times.

2.8. Oil red O staining

Cells were seeded in 6-well plates and incubated overnight. After cells were transfected with plasmid (siRNA or miRNA) for 48 h, cells were washed twice with phosphate saline and fixed with 10% formalin. The oil red O staining was performed according to the manufacturer's instructions.

2.9. Total triglyceride assay

The levels of triglyceride in cellular and tumorous (obtained from the transgenic mouse) were assayed using Tissue triglyceride assay kit (Applygen Technologies Inc., Beijing, China). All of the experiments were performed according to the manufacturer's recommended protocol.

2.10. Statistical analysis

Each experiment was repeated at least three times. Statistical significance was assessed by comparing mean values (6 standard deviation; SD), using a Student's t test for independent groups, and was assumed for $^{*}P < 0.05$, $^{**}P < 0.01$, No significant (NS). Pearson's correlation coefficient was used to determine the correlation between the expressions of each gene in HCC clinical tissues.

3. Result

3.1. MiR-205 is able to down-regulate ACSL1 in hepatoma cells

It has been reported that HCC impairs homeostasis of lipid and lipoprotein metabolism, leading to alterations in plasma lipid and lipoprotein patterns [13]. Our group has pointed out that miR-205 is down-regulated in HCC tissues comparing with their peritumor tissues [11]. Here, to explore the roles of miR-205 in the development of liver cancer, especially in abnormal lipid metabolism, we screened the target genes of miR-205 using Targetscan and microRNA.org (http://www.targetscan.org/, http://www.microrna.org). ACSL1 grabbed our attentions, as it is an important enzyme for lipid metabolism in liver tissues. Then, HepG2 cells were transiently transfected with miR-205. Our observation revealed that miR-205 was capable of down-regulating ACSL1 at the levels of mRNA and protein in a dose-dependent manner in the cells (Fig. 1A). Moreover, the opposite results were obtained when the cells were treated with anti-miR-205 (Fig. 1B), suggesting that miR-205 modulates the expression of ACSL1 *in vitro*. Meanwhile, the transfection efficiency of miR-205 and anti-miR-205 was validated by qRT-PCR in the cells (Fig. 1A and B). Thus, we conclude that miR-205 is able to down-regulate ACSL1 in hepatoma cells.

3.2. MiR-205 restrains the expression of ACSL1 via targeting ACSL1 3′UTR

To gain insights into the mechanism of miR-205 decreasing ACSL1, we identified the miR-205 binding site in the ACSL1 3′UTR (Fig. 2A) and constructed plasmids of pGL3-ACSL1 and pGL3-ACSL1-mut, respectively (Fig. 2B). Cotransfection of miR-205 remarkably suppressed the firefly luciferase activity of pGL3-ACSL1 but failed to influence that of pGL3-ACSL1-mut in HepG2 cells (Fig. 2C). Furthermore, inhibition of endogenous miR-205 by anti-miR-205 resulted in increasing firefly luciferase activity of the wild-type reporter in the cells, but the mutant not (Fig. 2D). Taken together, our data suggest that miR-205 can attenuate the expression of ACSL1 by directly targeting its 3′UTR in hepatoma cells.

3.3. MiR-205 suppresses the lipogenesis in hepatoma cells relying on ACSL1

Lipid droplets regulate the storage and hydrolysis of neutral lipids, such as triglyceride [14]. Therefore, lipid droplets can be used as a marker for lipogenesis [15]. In this study, we examined the activity of lipogenesis in HepG2 cells by oil red O staining. The results manifested that cellular lipogenesis was decreased when the cells were treated with miR-205, but anti-miR-205 could increase lipid droplets. Meanwhile, cotransfection of ACSL1 siRNA was able to block the lipogenesis induced by anti-miR-205 (Fig. 3A), suggesting that miR-205 resulting in deregulation of lipid metabolism in hepatoma cells depends on the lipid enzyme ACSL1 at least in part. To move forward a step to reinforce our conclusion, we detected the levels of cellular triglyceride which was one of metabolites of ACSL1 as well. Notably, the outcome revealed that miR-205 or ACSL1 siRNA was able to decrease the levels of cellular triglyceride (Fig. 3B), furthermore, the increasing triglyceride induced by anti-miR-205 could be blocked by ACSL1 siRNA (Fig. 3C), suggesting that miR-205 is able to increase the levels of cellular triglyceride dependent on ACSL1. In addition, the efficiency of ACSL1 siRNA was validated by Western blotting analysis in the cells (Fig. 3D). Taken together, we conclude that miR-205 is able to disorder the lipid metabolism in hepatoma cells through targeting ACSL1.

3.4. The levels of miR-205 are negatively associated with those of ACSL1 in clinical HCC tissues and the animal model

Next, we used 25 cases of clinical HCC samples to assess the relationship between miR-205 and ACSL1. As expected, the anti-correlation between miR-205 and ACSL1 was exhibited in these clinical HCC samples, supporting that miR-205 down-regulates ACSL1 in HCC tissues (Fig. 4A). Previously, we reported that HBx was capable of inhibiting the expression of miR-205 in the liver cancer from HBx transgenic mice (HBx-tg mice) [11]. There-

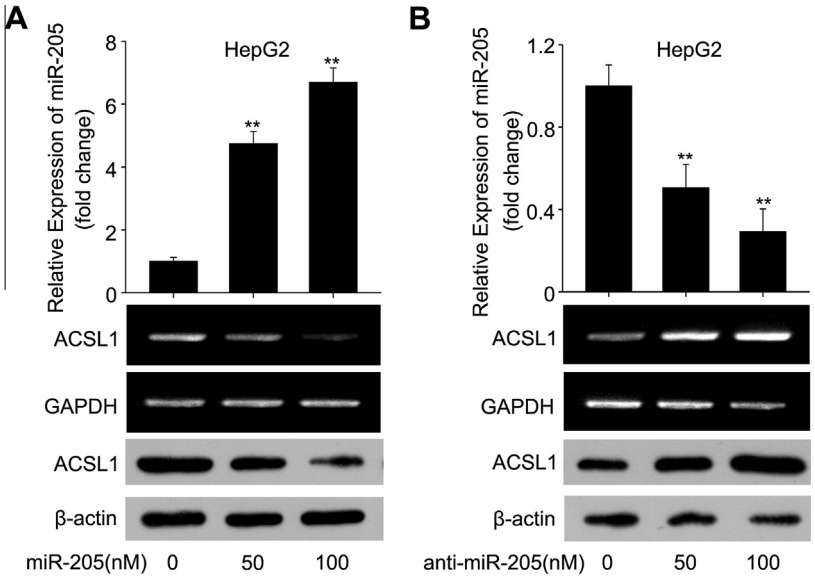

Fig. 1. MiR-205 is able to down-regulate ACSL1 in hepatoma cells. (A) The mRNA and protein levels of ACSL1 were examined in HepG2 cells transfected with miR-205 by RT-PCR and Western blotting, respectively. The transfection efficiency of miR-205 was detected by qRT-PCR. (B) The mRNA and protein levels of ACSL1 were examined in HepG2 cells transfected with anti-miR-205 by RT-PCR and Western blotting, respectively. The transfection efficiency of anti-miR-205 was detected by qRT-PCR. Statistically significant differences are indicated: $^{**}P < 0.01$; Student's *t* test.

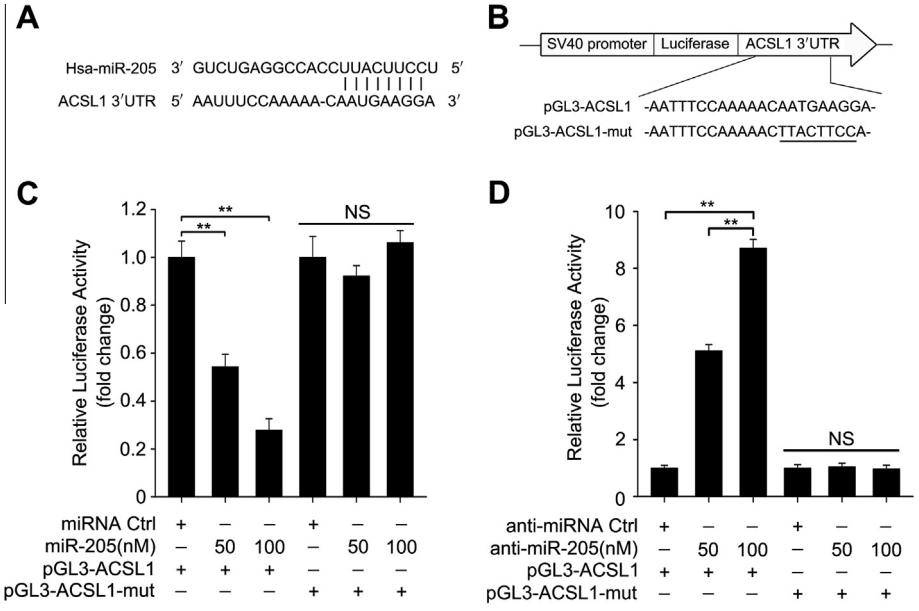

Fig. 2. MiR-205 restrains the expression of ACSL1 *via* targeting ACSL1 3′UTR. (A and B) A model shows the binding site of miR-205 in ACSL1 mRNA 3′UTR by prediction. Schematic diagram shows the generated mutant site at the ACSL1 3′UTR seed region binding to miR-205 and the inserted sites of wild type ACSL1 3′UTR (or mutant) into the downstream of the luciferase reporter gene in pGL3-control vector. (C and D) The effect of miR-205 (or anti-miR-205) on reporters of pGL3-ACSL1 and pGL3-ACSL1-mut in HepG2 cells was measured by luciferase reporter gene assays. Statistically significant differences are indicated: $^{**}P < 0.01$; NS; Student's *t* test. The experiment was repeated at least three times.

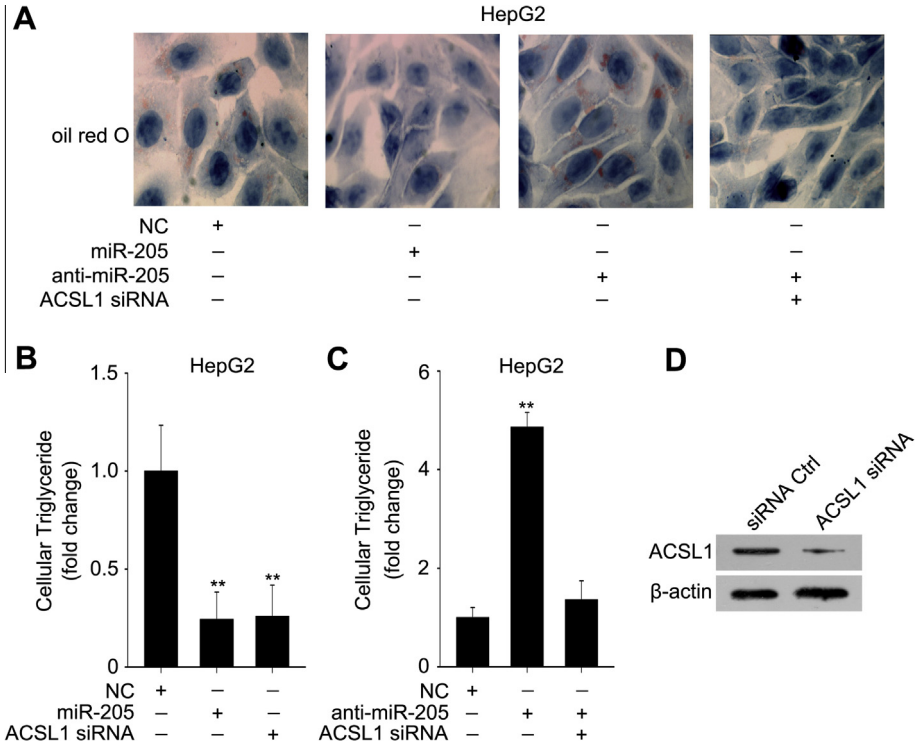

Fig. 3. MiR-205 suppresses the lipogenesis in hepatoma cells relying on ACSL1. (A) Effect of miR-205 (or anti-miR-205, ACSL1 siRNA) on lipogenesis was determined by oil red O staining in HepG2 cells. (B) Effect of miR-205 (or ACSL1 siRNA) on cellular triglyceride was measured in HepG2 cells by Tissue triglyceride assay kit. (C) Effect of anti-miR-205 (or anti-miR-205 and ACSL1 siRNA) on cellular triglyceride was measured in HepG2 cells by Tissue triglyceride assay kit. (D) Interference efficiency of ACSL1 siRNA was detected by Western blotting analysis in HepG2 cells. Statistically significant differences are indicated: $^{**}P < 0.01$; Student's *t* test.

fore, we used HBx-tg mice as an animal model to validate the findings in the HBx-induced liver cancer tissues. On the basis of the consequence, we found that the expression levels of ACSL1 were elevated in different month-aged HBx-tg mice comparing with wild-type littermate mice (WT mice) (Fig. 4B), suggesting that miR-205 is able to modulate the expression of ACSL1 in the development of HCC *in vivo*. In addition, we examined the levels of triglyceride in liver tissues from the HBx-tg mice and WT mice. Our data revealed that the levels of triglyceride were consistent with those of ACSL1 in the system (Fig. 4C), implying that miR-205 is capable of deregulating lipid metabolism depending on ACSL1 *in vivo*. Overall, our observations indicate

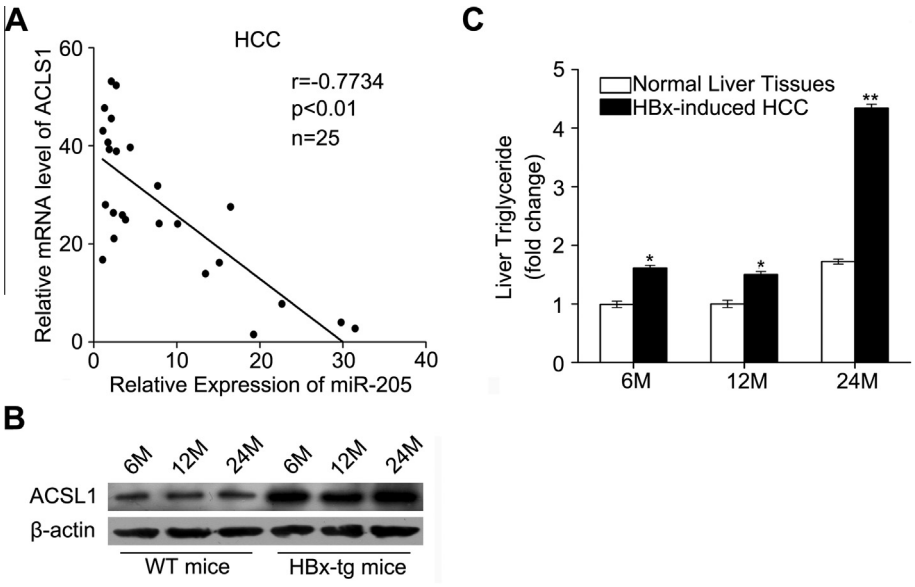

Fig. 4. The levels of miR-205 are negatively associated with those of ACSL1in clinical HCC tissues and the animal model. (A) Correlation of miR-205 levels with ACSL1 mRNA levels was examined by qRT-PCR in 25 cases of clinical HCC tissues (**$P < 0.01$; Pearson's correlation coefficient, $r = -0.7734$). (B) The expression levels of ACSL1 in the liver tumors from HBx transgenic mice aged 6 months (6 M), 12 months (6 M) and 24 months (6 M) were assessed by Western blotting analysis. (C) Levels of triglyceride were measured by Tissue triglyceride assay kit in the liver tissues from HBx transgenic mice aged 6, 12 and 24 months. Statistically significant differences are indicated: *$P < 0.05$; **$P < 0.01$; Student's t test.

that the levels of miR-205 are negatively associated with those of ACSL1in clinical HCC tissues and the animal model.

4. Discussion

It has been well recognized that cancer cells exhibit a higher rate of metabolism to support their accelerated proliferation rate [16]. Meanwhile, excessive caloric intake is associated with an increased risk for cancers [17]. Thus, metabolism reprogramming is a common phenomenon in numerous cancers. However, the leading cause of abnormal lipid metabolism in HCC remains poorly elucidated. In this study, we try to explore the mechanism of the event in liver cancer.

Strikingly, shifting lipid acquisition from lipid uptake toward *de novo* lipogenesis dramatically changes membrane properties and protects cells from both endogenous and exogenous insults [18], which is able to maintain the tumorigenesis. Therefore, cancer cells always display increasing lipogenesis. Given that the advent of HCC is accompanied by metabolic changes in the liver that are reflected in alters in gene expression, microRNA profiles, together with modified circulating protein and small metabolite concentrations [4]. Previous studies indicated that the oncogenic signaling was able to drive metabolic reprogramming, such as PI3K/Akt/mTORC1, HIF-1 and MYC [19,20]. In addition, our group reported that HBx accelerated lipogengesis *via* up-regulating oncogene Rab18 [15]. Interestingly, here we identified that low levels of miR-205 were involved in the abnormal lipid metabolism in HCC.

MiR-205 is widely explored since its expression pattern in pathologic states. It has been reported that low levels of miR-205 are not able to inhibit its target genes in breast cancer, such as HER3 [10], ErbB3, VEGF-A [21] and HMGB3 [22], which can promote cell proliferation and invasion. In human prostate, miR-205 exerts as a tumor suppressor to target protein kinase Cε, which plays a crucial role in regulating epithelial-to-mesenchymal transition [23]. Recently, our group has reported that miR-205 is involved in the HBV-mediated HCC through restraining HBx [11]. Therefore, we focused on the other roles of miR-205 in hepatocarcinogenesis, such as modulating abnormal lipid metabolism. Then, we screened the target genes of miR-205 by bioinformatic tools. Strikingly, ACSL1 stood out since its particular functions and expression pattern. As expected, our data demonstrated that miR-205 was able to directly bind to the 3′UTR of ACSL1 to regulate the expression of ACSL1 (Figs. 1 and 2). Moreover, we uncovered that miR-205 targeted ACSL1 could contribute to abnormal lipid metabolism. Oil red O staining indicated that miR-205 participated in lipogenesis in hepatoma cells depending on ACSL1. Meanwhile, the total triglyceride levels, a metabolite of ACSL1, could be influenced by miR-205 in the hepatoma cells (Fig. 3), supporting that low levels of miR-205 lead to deregulation of lipid metabolism in HCC relying on its target gene ACSL1. Next, we used HCC clinical samples and a mouse model to validate the above conclusions as well. Interestingly, low levels of miR-205 were negatively correlated with high levels of ACSL1 in clinical HCC samples. We reported that the levels of miR-205 were decreased in HBx-induced liver cancer from HBx-tg mice [11]. In this study, we showed that the levels of ACSL1 and its metabolite triglyceride were increased in the system. Therefore, the above data are consistent with that low levels of miR-205 contribute to abnormal lipid metabolism through increasing ACSL1 in hepatoma cells.

It has been reported that the overexpression of ACSL1 is able to increase the uptake of fatty acid in hepatoma cells [24]. However, in this study we showed that the overexpression of ACSL1 mediated by miR-205 was capable of increasing the levels of triglyceride in hepatoma cells. It suggests that ACSL1 plays crucial roles in lipogenesis of hepatoma cells. Taken together, we conclude that low levels of miR-205 contribute to the abnormal lipid metabolism through up-regulating ACSL1 in hepatoma cells, which result in the acceleration of lipogenesis and accumulation of triglyceride in the cells. Thus, our finding provides new insights into the mechanism of abnormal lipid metabolism in the development of liver cancer.

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