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Perturbation of IGF2BP1 Transcriptome upon the Interplay between miR-486-5p and let-7a

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Abstract

Background: Activation of IGF-1/IGF-1R signaling cascade is a hallmark in Hepatocellular carcinoma (HCC). In our previous work, we showed that miR-486-5p acts as a tumor suppressor miRNA in HCC mainly by vertically blocking IGF-1/IGF-1R axis and its downstream signaling mediators STAT3, mTOR and c-Myc. Recently, it was reported that the proto-oncogene c-Myc directly down-regulates the tumor suppressor miRNA, let-7a, especially in HCC and that let-7 directly targets the oncogenic RNA binding protein IGF2BP1.

Aim: Therefore, the main aim of this study was to investigate the indirect interplay between microRNAs; miR-486-5p and miR-let-7a through c-MYC thereby its effect on a vital member of IGF-axis, IGF2BP1, in HCC.

Methods: Huh-7 cell lines were cultured and transfected using miR-486-5p mimics using lipofection technique. Forty-eight hours post transfection, total RNA was extracted, reverse transcribed into cDNA, and finally amplified and quantified using q-RT-PCR. Impact of miR-486-5p on cell cycle was assessed using cell cycle vectors carrying response elements for the cell cycle protein c-Myc.

Results: Efficient delivery of miR-486-5p in Huh-7 cells was obtained, where mimicked cells showed more than 8000 folds increase in miR-486-5p expression level. Ectopic expression of miR-486-5p in Huh-7 cells resulted in a significant decrease in c-Myc protein expression, an increase in the expression level of the tumor suppressor, let-7a and finally forcing the expression of miR-486-5p showed a significant repression of the oncogenic validated target of let-7a, IGF2BP1.

Conclusions: This study shows a novel mechanism of action of the tumor suppressor miR-486-5p. MiR-486-5p was found to indirectly repress an essential member of IGF-axis, the oncogenic RNA binding protein IGF2BP1, mainly through decreasing c-MYC expression and up regulating let-7a expression.

Keywords: Mir-486-5p; Let-7a; IGF2BP2; C-Myc; HCC

Introduction

Insulin-like growth factor-2 mRNA-binding proteins 1, 2, and 3 (IGF2BP1, IGF2BP2, IGF2BP3) are a family of cytoplasmic mRNA-binding proteins that are essential for development. Normally IGF2BP1 and IGF2BP3 were found to be highly expressed during embryogenesis, whereas they were reported in negligible levels during adulthood, with the exception of reproductive tissues [1]. On the other hand IGF2BP2 is suggested to be normally expressed in adult tissues and appears to have metabolic control and a role in type 2 diabetes and in liver metabolism via directing IGF2 mRNA fate [2-4].

IGF2BPs were found to be re-expressed (de novo synthesized) in several malignancies, including Hepatocellular carcinoma (HCC), where IGF2BP1 and IGF2BP3 were classified as oncofetal cytoplasmic mRNA-binding proteins that appear to have a pivotal role in cancer development and progression. Nonetheless, IGF2BPs expression level was directly correlated with tumor invasion, early recurrence, and poor prognosis of HCC patients [4-6]. Moreover, IGF2BPs were also reported to promote liver steatosis, non-alcoholic fatty liver diseases (NAFLD) which are considered as illustrious risk factors for HCC [7-9].

IGF2BP1 was also reported to inhibit IGF2 mRNA translation in-vitro [10] and in contrast it was shown to enhance IGF2 mRNA levels in mice [11]. On the other hand IGF2BP2 and IGF2BP3 were reported to enhance IGF2 mRNA translation [12]. Our research group has previously shown that the knockdown of the three oncogenic IGF2BPs (IGF2BP1, IGF2BP2, IGF2BP3) by miR-1275 and let-7i, resulted in marked reduction in Huh-7 cells proliferation, migration, cellular viability and clonogenicity properties [13, 14]. Moreover, specific knockdown of IGF2BP1 in particular in multiple liver cancer cell lines resulted in a noticeable reduction in their proliferation and induces apoptosis in these cells. This was attributed to its ability to stabilize the c-MYC and MKI67 mRNAs and increase their protein expression levels [15].On the other hand c-Myc was shown to be an upstream regulator to many miRNAs and validated as a repressor for let-7a [16], which highlights a potential feedback loop between c-Myc, Let-7a and IGF2BP1.

Recently we have shown that miR-486-5p acts as tumor

suppressor miRNA through directly targeting IGF-1, IGF-1R and its downstream signaling mediators c-Myc, mTOR, STAT3 in Huh-7 cells [17, 18].Thus hypothetically miR-486-5p may regulate IGF2BP1 through the manipulation of the expression of c-MYC–Let-7a axis, so the main aim of this study was to investigate the interplay between c-Myc and Let-7a via miR-486-5p and its impact on IGF2BP1.

Materials and Methods

Cell culture and oligonucleotide transfection

Huh-7 cells were cultured in DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% FBS (Fetal Bovine Serum), 1% penicillin/streptomycin/mycozap (Lonza, Switzerland). Cells were transfected with mimics of miR-486-5p (Qiagen, Germany) using HiPerFect Transfection Reagent (Qiagen, Germany), according to the manufacturer's protocol. Mock cells are cells exposed to transfection reagent only.

MicroRNA and mRNA extraction and quantification HuH-7 cell line

Total mRNA and microRNAs were extracted from Huh-7 cells using BIOZOL RNA Extraction Reagent (Bioer Technology, China). Complementary DNA was generated using reverse transcription (Applied Biosystems, USA). Relative expression of miR-486-5p was normalized to RNU6B in each sample, while IGF2BP1 relative expression was normalized to beta-2 microglobulin (B2M), and quantified using TaqMan Real-time quantitative polymerase chain reaction (RTqPCR) (Applied Biosystems). Relative expression was calculated using the 2-ΔΔCT method.

Reporter constructs and Luciferase assay

Huh-7 cells were transfected with a vector containing the enhancer element of cell cycle regulatory proteins, c-Myc, and containing an unspecific binding site as p-Luc vector (Clontech, Germany). Huh-7 cells were transfected with 2 µg of the constructs using SuperFect (Qiagen) then co-transfected 24 h later with miR-486-5p mimics using HiPerFect (Qiagen). Relative luciferase activity was measured after 48 h by the Luciferase Reporter Assay Kit (Biovision, USA). It was measured at 615 nm using Wallac 1420 Victor2 Multilabel Counter (Perkin Elmer, USA)

Statistical analysis

All experiments were performed in triplicates and repeated at least three times. The data were expressed as the mean ± standard error of the mean (SEM). All analyses, unless otherwise stated, were performed using GraphPad Prism version 5 and a two-tailed value of $P < 0.05$ was considered statistically significant with Student's t-test.

Result

Transfection Efficiency of miR-486-5p oligonucleotides

For the purpose of assessing the impact of miR-486-5p Huh-7 cell line, miR-486-5p mimics were transfected into cultured Huh-7 cells. In order to confirm efficient transfection, miR-486-5p was quantified 48 h post-transfection using qRT-PCR, where a dramatic up-regulation of miR-486-5p of about 8000 folds was observed in mimicked cells compared to mock untransfected cells (P =0.0040) as shown in Figure 1.

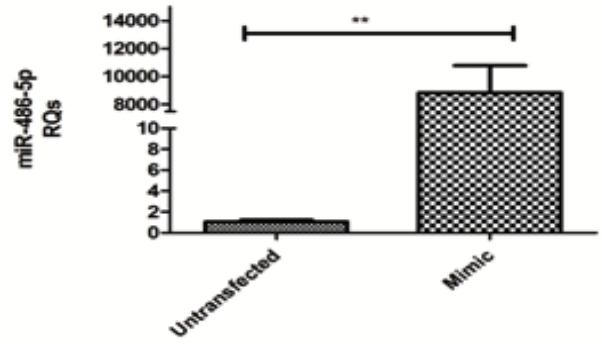

Figure 1: Transfection efficiency of miR-486-5p in Huh7 cells.
Mir-486-5p Expression Level Was Assessed Using Qrt-PCR And RNU6B Was Used As An Internal Control. Mir-486-5p Showed More Than 8000 Folds Increase In Mimicked Huh-7 Cells Compared To Mock Untransfected Cells

Impact of miR-486-5p on cell cycle progression through c-MYC expression

Mimicked miR-486-5p cells showed a significant (P=0.0456) down-regulation of c-MYC expression compared to mock untransfected cells using luciferase reported assay as shown in Figure 2

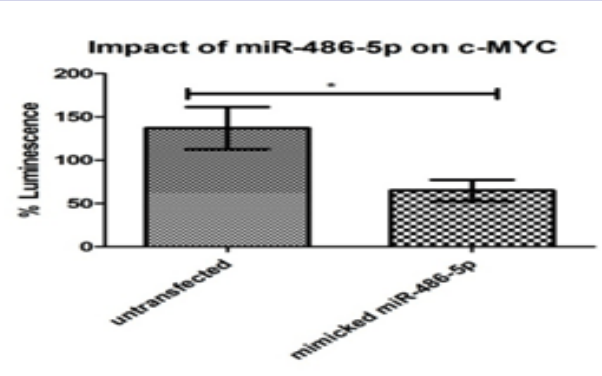

Figure 2: Impact of mi-486-5p on c-Myc protein level
Mir-486-5p Mimics Led To A Significant Decrease In C-Myc Luciferase Activity Compared To Mock Untransfected Cells In Huh-7 Cells Transfected With Pmyc-TA-Luc Vector.

Impact of miR-486-5p mimicked cells on Let-7a expression levels

Mimicking of miR-486-5p expression showed a significant up-regulation of let-7a expression in the Huh-7 cells (P=0.0471) compared to the mock untransfected cells as shown in Figure 3.

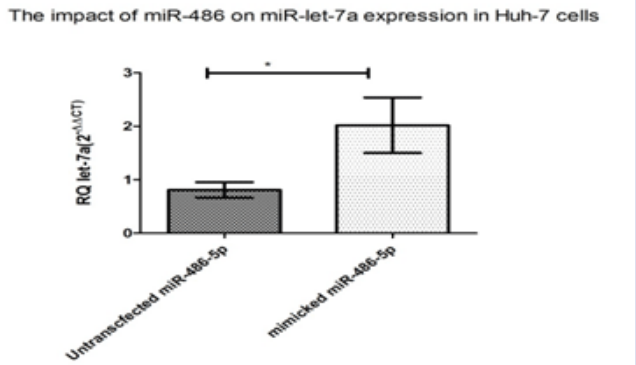

Figure 3: Impact of miR-486-5p on let-7a expression level in Huh7 cells Let-7a expression level was assessed using qRT-PCR and RNU6B was used as an internal control. Let-7a expression level was significantly augmented in mimicked Huh-7 cells compared to mock untransfected cells.

Impact of miR-486-5p on IGF2BP1 expression in Huh-7 cells

The impact of miR-486-5p manipulation was assessed using qRT-PCR, where mimicking of miR-486-5p in Huh-7 cells resulted in a significant down-regulation of IGF2BP1 mRNA levels (P=0.0044) compared to mock untransfected cells. However, Anti-miR-486-5p showed non-significant difference in IGF2BP1 expression levels compared to mock untransfected cells as shown in Figure 4.

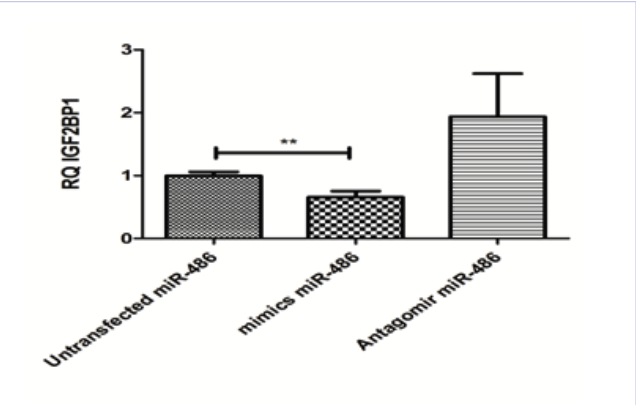

Figure 4: The mRNA level of IGF2BP1 was assessed using qPT-PCR and normalized to B2M as an internal control. Ectopic expression of miR-486-5p resulted in an increase in IGF2BP1 mRNA level in mimicked Huh7 cells compared to mock untransfected cells. While antagomirs showed no effect when compared to mock untransfected cells

Discussion

Recently we have shown that miR-486-5p could act as a tumor suppressor miRNA upon forcing its expression in HCC mainly through decreasing IGF-1/IGF-1R axis and its downstream mediators mTOR, STAT3, c-MYC [17]. On the other hand previous studies have shown that c-MYC regulates the expression of let-7a [16], which in turn regulates IGF2BP1[19, 20].

This study aimed at further characterizing the mechanistic role for miR-486-5p by examining its indirect impact on IGF2BP1 through c-MYC and let-7a loop. Loss and gain of function experiments for miR-486-5p in Huh-7 cells repressed the expression of c-MYC on the protein level, which supports previous findings where c-MYC expression was manipulated by miRNAs like miR-135b and miR-181a [21].Then the impact on let-7a via c-MYC was investigated through intended over expression of miR-486-5p in Huh-7 cells compared to the mock-untransfected cells, forced expression of miR-486-5p resulted in a significant up-regulation of let-7a in Huh-7 cells. This goes along with the idea that repressing c-MYC relives let-7a from its inhibitory effect.

The impact of miR-486-5p on IGF2BP1was then investigated expression through forcing its expression in Huh-7 cells, which resulted in a significant down regulation of IGF2BP1 mRNA. This might show indirect impact of miR-486-5p on IGF2BP1 through c-MYC and let-7a loop. This goes in line with another study where we have shown that c-MYC as an intermediate player between E2f and miR-17-5p creating a triad of E2F/c-MYC/miR-17-5p in Systemic lupus erythematosus (SLE) [22]. This also simulates another study where c-MYC was able to regulate CCDN2 through manipulating the expression of miRNAs let-7a, miR-16 and miR-29b in Ewing's sarcoma [16].

In conclusion This study showed that miR-486-5p interplays with let-7a through repressing c-MYC, which is an upstream regulator of let-7a hence relieving the inhibitory effect of c-MYC on let-7a enhancing its expression and increasing the

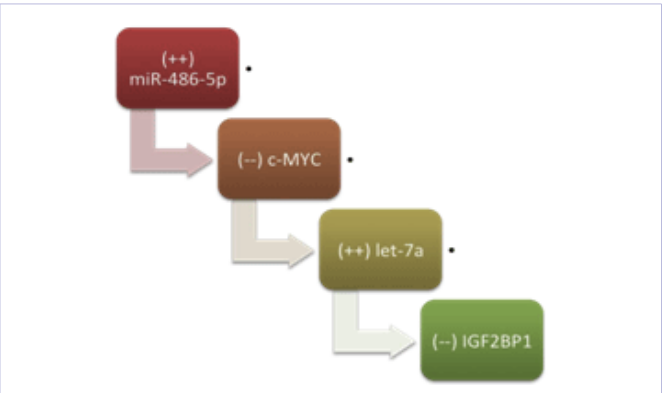

Figure 5: Schematic Representation of Interplay between miR-486-5p and let-7a in Huh-7 cells

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