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Effect of co-transfection of miR-520c-3p and miR-132 on proliferation and apoptosis of hepatocellular carcinoma Huh7

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ABSTRACT

Objective: To investigate the effects of co-transfection of miR-520c-3p and miR-132 on proliferation and apoptosis of hepatocellular carcinoma Huh7.

Methods: Hepatocellular carcinoma Huh7 was cultured *in vitro* and lipidosome was used to transfect miR-520c-3p and miR-132, respectively or together. The effects of transfection of miR-520c-3p and miR-132 on proliferation and apoptosis of Huh7 were detected by CCK8 and Annexin V staining and flow cytometry, and the expression level of the targeted gene of over-expressed miR-520c-3p and miR-132 was determined by Western blot and realtime PCR.

Results: Compared with the control group, the proliferation ability of Huh7 of the single transfected and co-transfected miR-520c-3p and miR-132 decreased significantly, and the apoptosis ratio increased distinctly ($P < 0.05$). Besides, the effect of the co-transfection group was better than that of the single transfection group. The protein levels of GPC3 (Glypican-3) and YAP (Yes-associated protein), the target genes transfected only by miR-520c-3p and miR-132, respectively, reduced obviously ($P < 0.05$), which was similar with the co-infected cells, but cells transfected by miR-132 only showed a decrease of YAP.

Conclusions: The co-transfection of miR-520c-3p and miR-132 can target-regulate the expression of GPC3 and YAP, enhance the exhibition effect on proliferation of hepatocellular carcinoma Huh7 and induce cell apoptosis synergistically.

1. Introduction

Liver cancer is a common malignancy worldwide with high morbidity and mortality rates which brings heavy burdens to societies and families. In our country, there is a severe trend of carcinogenic risks such as hepatitis B infection. Its morbidity rate reaches about 5–10 times as much as that in developed countries. The death toll from liver cancer accounts for about 50% of the total [1,2]. Traditional surgery, chemotherapy and radiotherapy do not work ideally. A large amount of researches have proved that many microRNA are closely related to the growth, metastasis and microenvironment of tumors [3–5]. Therefore, microRNA has been the effective target tool for inhibiting the growth and metastasis of tumors. There are researches demonstrating that miR-520c-3p and miR-132 express abnormally in hepatocellular carcinoma and are related to the proliferation, apoptosis and metastasis of hepatocellular carcinoma [6,7]. This study aimed to investigate the correlation of miR-520c-3p and miR-132 in the proliferation and

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apoptosis of hepatocellular carcinoma by using them to co-transfect hepatocellular carcinoma Huh7 and explore its mode of action.

2. Materials and methods

2.1. Materials

Human hepatocellular carcinoma cell line Huh7 used in this study was provided by Shanghai Cell Bank of Chinese Academy of Sciences; fetal bovine serum, antibiotics, trypsin, DMEM medium and Opti-MEM were all purchased from Gibco-BRL and transfection reagent Lipofectamine™ 2000, Trizol reagent and Annexin V-FITC apoptosis detection kits were bought from Invitrogen; the primer was designed and compounded by Sangon Biotech; reverse transcription kits and fluorescence quantitation kits were from Takara; miR-520c-3p, miR-132 mimics and mimics negative controls were all bought from Ribobio (Guangzhou, China); rabbit-anti-human GPC3, YAP, GAPDH primary antibody and HRP secondary antibody were from Abcam (USA); ECL luminescent solution was purchased from Pierce.

2.2. Methods

2.2.1. Cell culture

A DMEM medium containing 10% fetal bovine serum was prepared. The frozen Huh7 cells were taken out from the liquid nitrogen tank and thawn repeatedly and rapidly in a water bath at 37 °C. Then, 10 mL preheated complete culture medium absorbed from the super clean bench was taken to a centrifuge tube, washed to remove DMSO of the cell suspension and centrifuged at 1 000 r/min for 5 min. After that, the supernatant was discarded. Moderate culture solution was added to re-suspend cells and then it was moved to the culture flask and cultured conventionally at 37 °C in 5% CO_2 incubator. The solution was changed on the next morning. When Huh-7 grew and fused to a single layer, it was washed by PBS to have digestive transfer culture by 0.25% trypsin.

2.2.2. MiR transfection

miR-520c-3p, miR-132 mimics and mimics negative control freeze-dried powder was made into 20 μM stock solution by RNase-free H_2O and stored respectively to avoid repetitive freeze-thawing. The day before transfection, Huh-7 cells were incubated to 24-well plate or 96-well plate holes containing complete medium and transfected when the cell concentration reached 70% with the mimic concentration of 100 nM and three complex holes. A total of 50 μL Opti-MEM was applied to dilute miR and Lipofectamine™ 2000 blowing and absorbing slightly for 3–5 times till it was mixed evenly. The transfection reagent and miR diluent were mixed, also blew and absorbed slightly for 3–5 times till it was mixed evenly, and placed still at room temperature for 5 min. Then, the mixed solution was added into the primary culture medium. The transfection rate was detected after 24 h and the cell biological characteristics were tested after 48 h.

2.2.3. Detection of CCK8 cell viability

A total of 10 μL CCK8 solution was added into every well on the 96-well plate after transfecting for 48 h with the greatest extent to avoid bringing air bubbles. The culture plate was incubated in an incubator for 2 h. A microplate reader was employed to determine the absorbance at 450 nm. Cell viability (%) = [A (mimic) − A (blank)]/[A (negative control) − A (blank)] × 100.

2.2.4. Examination of cell cycle

The cells were washed by 0.25% trypsin-digested pre-cooling PBS 3 times after transfected for 48 h to make monoplast suspension with a adjusted cell number of (1–5) × 10^6 cells/mL. Pre-cooling absolute ethanol solution was added in it slowly to a final concentration of 75%. The sample was blended uniformly and placed in the freezer at −20 °C overnight. At the next day, the fixed sample was taken out and centrifuged at 1 000 r/min for 5 min. The supernatant was discarded. Then, 3 mL pre-cooling PBS resuspended cells was added and centrifuged at 800 rpm for 5 min. Also, the supernatant was discarded. In every tube, 200 μL RNase A (300 g/mL) resuspended cells were added respectively and incubated for 30 min. RNA in the sample was removed. In addition, 150 L PI dye liquor was added in every tube and stained in dark place at 4 °C for 20 min. After washed, flow cytometry (BD FACSCalibur) was used for the detection.

2.2.5. Apoptosis detected by Annexin V and PI stain

Cells were collected after trypsin digestion and centrifuged at 1 000 r/min for 5 min and the supernatant was discarded. Cells were suspended gently by PBS and counted. 1 × Annexin V bind buffer was prepared. Then, 150 μL of the buffer was added into every tube to suspend cells. The mixed solution of 45 μL buffer and 5 μL Annexin V-FITC was added into every tube, blended equally and dyed in dark place at 4 °C for 20 min. After that, 10 μL PI solution was added into every tube and mixed evenly. After incubated in dark place at room temperature for 10–20 min, 200 μL buffer was added into every tube and finally flow cytometry was applied to test the apoptosis condition.

2.2.6. Realtime PCR

After transfection of Huh7, Trizol method was used to extract the total RNA. Takara reverse transcription kits were used to conduct reverse transcription in accordance with the instruction strictly. Primer sequences used for reverse transcription and realtime PCR were designed and compounded by Ribobio (Guangzhou, China). SYBR realtime PCR was conducted by ABI7500. U6 was used as a internal reference for the expression levels of miR-520c-3p and miR-132, while GAPDH served as the internal reference for the expression levels of GPC3 and YAP. The experiment results were analyzed by the $2^{-\Delta\Delta Ct}$ method.

2.2.7. Western blot

The culture medium was removed after transfection for 48 h. The cells were washed by PBS. A total of 200 μL 1 × RIPA lysis buffer was added into every well respectively to incubate at 4 °C for 30 min. Afterwards, a pipettor was used to buffet the cells repeatedly and the lysis buffer was moved to the EP tube and stored at −80 °C. After testing the protein concentration by Bradford method, equal protein was used to conduct SDS denatured polyacrylamide gelelectrophoresis with a constant voltage electrophoresis of 60 V for 30 min and 120 V for 1 h. After that, the protein was transferred nitrocellulose membrane (Millipore) with transmembrane of 350 mA for 2 h. Five percentage of skim milk was enclosed to incubate specific GPC3 or YAP primary antibodies at 4 °C overnight and HRR secondary antibody was incubated after washing. ECL developer was used.

2.2.8. Data analysis

Streamed data were analyzed by FlowJo software. All data in this study were statistically analyzed with Graphpad Prism 4.0. Means between groups were tested by Student's *t*-test. Means of three or more than three groups were analyzed by ANOVA ($P < 0.05$). Differences were statistically significant. Measurement data were expressed by mean ± SD.

3. Results

3.1. The transfection effect of miR-520c-3p and miR-132 mimic

The results of realtime PCR showed that as compared to the negative control group the level of miR-520c-3p of Huh7 transfected only by miR-520c-3p mimic or co-transfected increased by about 150 times, while the expression level of miR-132 of Huh7 transfected only by miR-132 mimic or co-transfected increased by about 240 times (Table 1), which indicated the success of mimic transfection.

3.2. Cell viabilities of hepatoma carcinoma Huh7 after co-transfected by miR-520c-3p and miR-132

CCK8 was used to detect the cell viabilities of Huh7 after transfected 48 h. The results showed that the cell viabilities of Huh7 of the miR-520c-3p group and miR-132 group decreased significantly as compared with the control group. The cell viability of the co-transfection group declined most distinctly (Table 1), which proved that the over expression of miR-520c-3p and miR-132 could inhibit the proliferation of Huh7.

3.3. Effect of the co-transfection of miR-520c-3p and miR-132 on the cell cycle of hepatoma carcinoma Huh7

In order to confirm the correlation of the cell cycle and decrease of cell viability of Huh7 caused by miR-520c-3p and miR-132, Ethidium bromide was used to dye the transfected cells to analyze the cell ratios of all cell cycles. It was found that single transfection of miR-520c-3p or miR-132 could lead to the increase in the cell ratio during G1 phase and decreases in the phases starting from S ($P < 0.05$), while the cell cycle of the co-transfection group showed a apparent block phenomenon (Table 2), which indicated that the co-transfection of miR-520c-3p and miR-132 could inhibit cell cycle effectively.

Table 1
The expression of miR-520c-3p and miR-132 and cell viabilities of Huh7 after transfected with miR-520c-3p and miR-132.

Group	Relative expression		Cell viability (%)
	miR-520c-3p	miR-132	
Negative control	0.98 ± 0.14	1.05 ± 0.11	98.83 ± 4.21
miR-520c-3p	145.21 ± 12.31*	0.96 ± 0.13	77.63 ± 5.10*
miR-132	1.56 ± 0.29	237.00 ± 28.10*	74.21 ± 7.92*
Co-transfection	157.23 ± 23.95*	252.88 ± 19.76*	55.34 ± 7.43*

*$P < 0.05$ compared with the negative control group.

Table 2
Cell cycle distribution of Huh7 after transfected with miR-520c-3p and miR-132 (Mean ± SD, %).

Group	G1	S	G2
Negative control	44.07 ± 5.32	39.23 ± 5.08	15.62 ± 1.94
miR-520c-3p	58.77 ± 6.34*	30.14 ± 2.27*	10.83 ± 4.06
miR-132	61.30 ± 4.51*	28.3 ± 3.94*	13.13 ± 3.20
Co-transfection	74.32 ± 8.19*	17.23 ± 4.25*	11.42 ± 3.14

*$P < 0.05$ compared with the negative control group.

Table 3
Apoptosis of Huh7 cells after transfected by miR-520c-3p and miR-132 (Mean ± SD, %).

Group	Early	Late	Total
Negative control	4.06 ± 0.32	1.22 ± 0.04	5.71 ± 0.47
miR-520c-3p	9.33 ± 0.60*	6.29 ± 0.48*	17.32 ± 0.91*
miR-132	8.02 ± 0.72*	5.92 ± 0.40*	14.23 ± 2.44*
Co-transfection	13.09 ± 2.15*	12.71 ± 1.31*	25.79 ± 5.02*

Table 4
Expression of GPC3 and YAP in Huh7 cells after transfected by miR-520c-3p and miR-132.

Group	Protein expression		mRNA relative expression	
	GPC3	YAP	GPC3	YAP
Negative control	0.93 ± 0.14	1.02 ± 0.11	0.95 ± 0.08	0.97 ± 0.08
miR-520c-3p	0.47 ± 0.03*	0.59 ± 0.15*	0.92 ± 0.21	0.55 ± 0.14*
miR-132	0.94 ± 0.09	0.55 ± 0.04*	1.01 ± 0.16	0.23 ± 0.06*
Co-transfection	0.42 ± 0.03*	0.24 ± 0.07*	1.14 ± 0.22	0.17 ± 0.01*

Compared with the negative control group, *$P < 0.05$.

3.4. Effect of the co-transfection of miR-520c-3p and miR-132 on apoptosis of hepatoma carcinoma Huh7

In order to further explore the effect of co-transfection of miR-520c-3p and miR-132 on apoptosis of Huh7, we applied Annexin V and PI staining to test its apoptosis level. It was showed in Table 3 that transfections of miR-520c-3p and miR-132 could all cause apoptosis of hepatoma carcinoma Huh7, and the cell proportion between early apoptosis and late apoptosis increased obviously ($P < 0.05$), and the apoptosis of the co-transfection group presented most significant.

3.5. The expressions of GPC3 and YAP of Huh7 after co-transfected by miR-520c-3p and miR-132

Meanwhile, western blot and realtime PCR were used respectively to detect the protein levels of target molecule GPC3 and YAP and the level of mRNA of Huh7 after transfected by miR-520c-3p and miR-132. As a result, we found that the protein level of GPC3 in Huh7 transfected by miR-520c-3p mimic decreased distinctly, while its mRNA level did not changed, and its protein level of YAP and its mRNA level all declined significantly. The levels of GPC3 and mRNA of the co-transfection group remained unchanged, while its protein level and YAP expression level reduced in the same time (Table 4).

4. Discussion

MicroRNA is a non-coding single-stranded small RNA with a total length of 18–25 bp which regulates the expression of target genes by inducing the degradation of target gene RNA or

interdicting the translation process [8]. With the maturity of high throughput sequencing technology and the analysis of the expression profile of miRNA in human tumors, the differential expression of miRNA and its relationship with the early diagnosis and prognosis of tumors have attached more and more attentions. Researchers have manifested that multiple miRNA expresses abnormally in liver cancer. They participate in the occurrence and development of cancers by adjusting apoptosis and cell cycle or regulating the expression of oncogenes and tumor suppressor genes. The expression level of miRNA can be used a mark for tumor diagnosis and prognosis evaluation. For instance, the expressions of miR-21, miR-122 and miRNA-101 are correlated to tumor invasion and the increase of recurrence risks [9–11]. Moreover, miRNA in blood or serum cannot be dissociated by RNA enzyme, which made it more convenient to get and easier to operate. MiRNA tested by peripheral blood is of great importance to the early diagnosis of liver cancer. For example, miR-223, miR-122 and miR-192 possess high accuracy in detecting early hepatocellular carcinoma [12,13]. Results of animal experiments also prompted that miR-26a, miR-122 and miR-124 could serve as potential therapeutic targets to extend the survival time for mice. However, the interaction and mode of action of different miRNA in hepatoma carcinoma cells still remain unclear. It was found in this study the co-infection of miR-520c-3p and miR-132 on hepatoma carcinoma Huh7 could inhibit proliferation and stimulate apoptosis of hepatoma carcinoma cells.

miR-520c-3p is considered as a negative regulatory factor in the growth of tumors, which presents a low expression in diffuse B cell lymphoma, transcribes initiation factor eIF4GII by targeting regulation and inhibits the formation of cell colonies and the growth of tumors [14]. GPC3 is a transmembrane glycoprotein of cell surface inducing physiological functions such as lymphocytes homing and extracellular matrix adhesion, and its high expression is closely related to tumorigenesis and neoplasm metastasis [15]. There were researches confirming that miR-520c-3p could inhibit the expression of GPC3 by targeting in hepatoma carcinoma to inhibit the proliferation and metastasis of hepatoma carcinoma [7]. It was also found in this study that the cell cycle of miR-520c-3p-over-expressed hepatoma carcinoma Huh7 was inhibited significantly, its apoptosis ratio increased obviously, and the protein level of its target gene GPC3 declined distinctly. At the same time, we found that after the over expression of miR-520c-3p, the expression of the core molecule YAP of the Hippo signal pathway reduced dramatically, which indicated that the expression of YAP was inhibited at the time the expression of GPC3 reduced. The results were in agreement with those of the previous studies.

MiR-132 usually expresses high in nerve-related tissues participating in the growth of axon, proliferation and differentiation of synapse and angiogenesis and possessing certain functions in immune regulation [16,17]. Studies have revealed that miR-132 can also participate in the occurrence and development of tumors and expresses low in tumor tissues of prostate cancer, liver cancer and so on [18]. In prostatic cancer cells, miR-132 can decrease the expression of pro-survival genes HB-EGF and TALIN2 to facilitate tumor apoptosis [19]. MiR-132 can significantly inhibit the *in vitro* migration and invasion of lung carcinoma cell through the silence of the expression of ZEB2 [20]. In hepatoma carcinoma cells, dual-luciferase report experiments proved that miR-132 could combine with 3′UTR of YAP gene directly to inhibit the expression of YAP so as to inhibit the proliferation of tumor cells [6]. We have obtained similar results in this study that Huh7 with over-expressed miR-132 presented cell cycle arrest and a increase of apoptosis and the levels of mRNA and protein of YAP reduced significantly.

Hippo signal pathway is a cell signal pathway discovered in fruit flies. The pathway participates in the growth, differentiation and regeneration of tissues and organs and the maintenance of the stability of proliferation and apoptosis of body cells. Researches have pointed out that the abnormal regulation of Hippo signal pathway is closely related to the occurrence, development, apoptosis and metastasis of tumors [21,22]. YAP is the downstream protein playing a key role in the Hippo pathway. The increase of its expression can lead to the increase of the downstream transcription factor cyclin E [23], which means that YAP can facilitate cell proliferation, inhibit cell apoptosis and promote malignant transformation. More and more evidences have implied that YAP, as a oncogene, presents high expression in many human tumors such as colon cancer, lung cancer, ovarian cancer, liver cancer, etc., and the high expression of YAP in liver cancer is closely related to the differentiated degree of liver cancer and high-serum AFP [24–26]. After YAP over expresses in normal mammary epithelium, cells can be over-productive, which facilitates the epithelial–mesenchymal transition and inhibits cell apoptosis [27]. Disturbing the expression of YAP in tumor cells can inhibit the growth and metastasis of tumors [28]. Hence, to explore the intervention of the YAP expression lever to reduce tumor cells may provide theoretical basis for treating liver cancer. The results of this study revealed that single transfection of miR-520c-3p and miR-132 can decrease the levels of YAP protein and mRNA significantly, inhibit the proliferation of hepatoma carcinoma cells and induce apoptosis. The effect of the co-transfection of miR-520c-3p and miR-132 was more remarkable, which indicated the regulation effect of the target protein GPC3 of miR-520c-3p on YAP and the silence effect of miR-132 on YAP had a synergistic anti-tumor efficacy in hepatoma carcinoma Huh7.

In conclusion, it is found in this study that the co-transfection of miR-520c-3p and miR-132 can inhibit the expression level of YAP together, suppress the proliferation of hepatoma carcinoma synergistically and promote apoptosis of hepatoma carcinoma. The study results of this paper also suggest that the effects of different miRNA are networked in tumor cells. Based on their different action modes, their biological functions may overlap with each other, which provides the theoretical basis for the molecular intervention method for oncotherapy.

Conflict of interest statement

We declare that we have no conflict of interest.

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