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Original Article

miRNA-101 affecting proliferation and invasion of liver cancer by down-regulating COX-2 expression

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Abstract: Objective: to find out changes in the expression of miRNA-101 (miR-101) in the liver cancer tissues and cells and the effects on proliferation and invasion of the human liver cancer cells HepG2. Method: The expression of miR-101 and COX-2 in liver cancer and para-cancer tissues, human liver cancer cells HepG2 and normal human hepatocytes HHL-5 was detected in 10 patients with florescent quantitative real-time PCR. The HepG2 cells were transfected with miR-NC (Hep G2/NC) and miR-101 (HepG2/101) respectively, the expression of COX-2 in the transfected cells was detected with Western blotting, and proliferation and invasion of the transfected cells was detected using CCK-8 and clonogenic assays. A tumor model was created subcutaneously in BALB/C nude mice with HepG2, HepG2/NC and HepG2/101 cells, to compare the growth of tumor in mice. Results: The expression of miR-101 was obviously lower in liver cancer tissues than in the para-cancer tissues (t = 18.54, *P* = 0.005), and it was markedly lower in the HepG2 cells than in the HHL-5 cells (t = 26.95, *P* = 0.001). In contrast, the expression of COX-2 was obviously higher in liver cancer tissues and cells than in the para-cancer tissues (t = 20.47, *P* = 0.002) and normal human hepatocytes (t = 14.66, *P* = 0.010). After transfection with miR-101, the expression of COX-2 in the HepG2/101 cells was significantly down-regulated compared with the HepG2 cells (t = 26.47, *P* = 0.000). Results of CCK-8 and clonogenic assays showed that over-expression of miR-101 could remarkably inhibit proliferation (F = 5.756, P = 0.012) and invasion (Dunnett's test I-J = -0.28, *P* = 0.035) of the HepG2 cells. Results of tumor growth experiments *in vivo* revealed that after over-expression of miR-101, the growth of the HepG2/101 cells in subcutaneous tumor of mice was significantly slower than the HepG2 and HepG2/NC cells (F = 14.4, P = 0.003). Conclusion: There was down-expression of miR-101 and up-expression of COX-2 in liver cancer tissues and cells. Over-expression of miR-101 may inhibit proliferation and invasion of liver cancer cells by down-regulating the expression of COX-2 in HCC cells.

Keywords: miRNA-101, cyclooxygenase-2, liver cancer, cell proliferation

Introduction

Liver cancer is one of the commonly seen malignancies worldwide. Surgery is currently the most effective approach in treating liver cancer, but a quite few of patients are already at the middle-advanced stage at diagnosis and can not be cured with radical surgery [3]. Therefore, further clarification of the mechanism in development and progression of liver cancer is of important significance for improving treatment and prognosis of patients with liver cancer.

Cyclooxygenase-2 (COX-2) is an important rate-limiting enzyme in synthesis of prostaglandin E2 (PGE2) [4]. It has been shown that the expression of PGE2 increases in various tissues of malignant tumors; it inhibits immune function of the body and is closely related to the progression and prognosis of tumors [4-8]. MicroRNA (miRNA) is an endogenous small non-coding RNA molecule about 18~25 nt in length. miRNA participates in the regulation of 30% of the coding genes in human body. More and more studies suggest that miRNA plays an essential part in the development and progression of various malignant tumors.

In the present study, by comparing the expression of miR-101 in normal and liver cancer tissues and cells and by transfecting the HepG2 cells with miR-101, the effects of miR-101 over-expression on proliferation and invasion of the

Table 1. Reaction system of PCR experiment

Template RNA	3 μg
5 × MMLV RT Buffer	4 μl
10 mM dNTP	0.75 μl
1 μM miR-RT primers	1.2 μl
40 U/μl Rnasin	0.25 μl
200 Uμl MMLV Reverse Transcriptase	0.2 μl
RNase-free water	System complemented to 20 μl

HepG2 cells were observed to find out the action of mechanism in the development and progression of liver cancer.

Materials and methods

Cell culture

The human liver cancer cell strain HepG2 and the normal human embryonic liver fibroblast HHL-5 were purchased from American Type Culture Collection (ATCC) and stored in our department. Passage culture of the cells was performed in the DMEM medium containing 10% fetal bovine serum (GIBCO) at 37°C in a 5% CO_2 incubator.

Clinical data

Surgical specimens of 10 patients diagnosed pathologically with liver cancer and receiving surgical treatment in our hospital between September 2013 and November 2014 were selected for the study. The patients were 48-62 years old, 7 males and 3 females. The liver cancer and para-cancer tissues (liver tissues *in situ* 5 cm from the tumor border) were taken from the surgical specimens of patients and kept in liquid nitrogen within 10 min *ex vivo*, for use in experiment later. Patients were informed the objective and methods of the scientific experiment and signed the informed consent form before surgery.

Main reagents

The Total RNA Extraction Reagent (Trizol) was purchased from Dakewe Biotech Co., Ltd., the RNA Reverse Transcription Kit from Takara, the primary and secondary antibodies for Western blotting from Cell Signaling Technology, Inc. and the Cell Counting Kit (CCK-8) from Beyotime Institute of Biotechnology. Wright-Giemsa stain was purchased from Beijing Leagene Biotechnology Co., Ltd.

RNA extraction

The above tissue samples stored in liquid nitrogen was ground and added with 1 ml Trzol. The HepG2 and HHL-5 cells in passage culture were collected, washed with PBS and centrifuged (1200 r/min × 5 min), and then added with 1 ml Trzol. miRNA was extracted following the steps provided by the miRNA separation and extraction kit. Concentration and purity of RNA extracted was determined using the UV spectrophotometer.

Reverse transcription of RNA

RNA extracted in the above experiment was reversely transcribed into cDNA with a PCR instrument. The reaction system was presented in the **Table 1**, and the reaction conditions were: 25°C × 30 min, 42°C × 30 min, 85°C × 5 min.

Florescent quantitative real-time PCR

Using cDNA as the template, relevant reagents and primers were added following the steps provided by the RT-qPCR kit (Bi-Rad), and PCR amplification was performed on an ABI-7300 real-time fluorescence quantitative instrument with U6 as internal reference. Reaction conditions: 95°C × 3 min, (95°C × 12 s, 62°C × 40 s) × 40 cycles. Relative quantitative analysis on expression was performed with $2^{-\Delta\Delta Ct}$ for the experimental results.

Western blotting

With cells samples collected, protein samples after disruption with cell lysis buffer was transferred to the nitrocellulose membrane (Millipore) after 8% SDS-PAE electrophoretic separation, and then incubated with the corresponding primary antibodies and horse radish peroxidase (HRP)-labeled secondary antibodies, and coloration and luminescence of DAB occurred.

Transfection with miRNA

Using the method provided by the Lipofectamine 2000 Transfection Kit (Invitrogen), the HepG2 cells in logarithmic growth phase were transfected with miR-NC and miR-101, and 24 hours

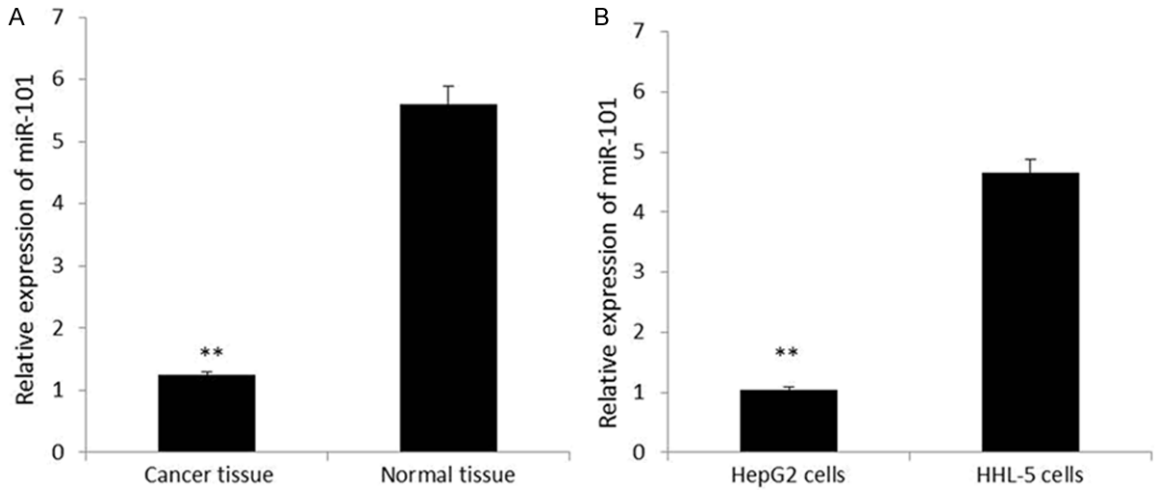

Figure 1. Expression of miR-101 in tissues and cells detected with RT-qPCR. A. Expression of miR-101 in the liver cancer and para-cancer tissues; B. Expression of miR-101 in the hepatoma cell line HepG2 cells and in the normal human embryonic liver fibroblasts, HHL-5 cells.

later, cryopreservation culture of the cells was carried out for subsequent use in experiment.

Determination of cell growth curve

The wild-type HepG2 cells and the HepG2 cells transfected with empty vector (HepG2/NC) and miR-101 (HepG2/101) were applied to a 48-well plate, with initial density of 3000 cells/well, cell vitality was determined following the steps provided by the CCK-8 kit on days 2, 3, 4 and 5, and statistical analysis was performed.

Clonogenic assay

The HepG2, HepG2/NC and HepG2/101 cells in logarithmic growth phase were collected and applied to a 6-well plate after cell count, with density of 100 cells/well. The cells were cultured as usual in an incubator, and 2 weeks later when clone was visible to naked eye in the cell culture plate, the culture was stopped, the cells were washed twice with PBS, staining with Wright-Giemsa stain was conducted for 30min after the cells were fixed with 4% neutral methanol for 15 min, and then the cells were dried with natural air after slow washing with running water. The number for clones more than 50 cells was counted under a microscope and cloning efficiency was calculated with the following formula:

$$\text{Cloning efficiency} = \frac{\text{Numbers of Clones}}{\text{Numbers of cell inoculated}} \times 100\%$$

[12]

Determination of tumor growth rate in vivo

The BALB/C nude mice of 6-8 weeks old were inoculated subcutaneously with 5×10^6 HepG2, HepG2/NC and HepG2/miR-101 cells. The long and short diameters of tumors in mice were measured weekly to calculate the tumorigenicity and growth of subcutaneous tumors. The volume of tumors in mice was calculated as follows:

Volume of tumor = 1/2 long diameter × short diameter^2 [13]

The mice were sacrificed 6 weeks later by cervical vertebra dislocation, and weight of the tumor tissues were measured, on which statistical analysis was performed.

Statistical analysis

Statistical analysis was performed with the software SPSS 11.0, and the unpaired t test was used for statistical analysis in comparison of means between two groups; one-way analysis of variance was used for statistical analysis in comparison of means among 3 or more groups, and Dunnett's test was employed for comparison between groups; repeated measures analysis of variance was used for statistical analysis on multiple measurement results (cell vitality detected with CCK-8 and growth curve of subcutaneous tumors in mice) at different time points for one observation

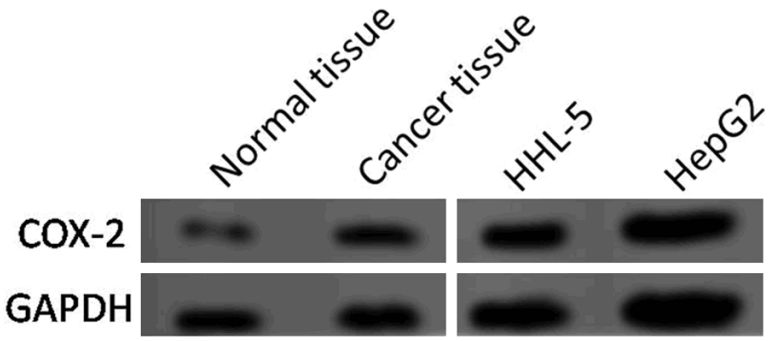

Figure 2. Expression of COX-2 compared with Western blotting in the liver cancer and para-cancer tissues, the HepG2 cells and the normal human embryonic liver fibroblasts, HHL-5 cells.

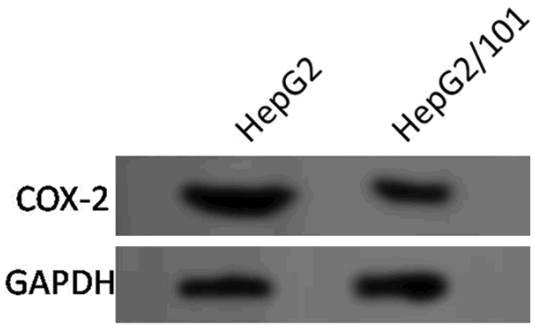

Figure 3. Changes in the relative expression of COX-2 in the HepG2 cells after miR-101 over-expression detected with Western blotting.

indicator of the same study subject, with test level $\alpha = 0.05$.

Results

Expression of miR-101 in the liver cancer and para-cancer tissues

Distribution relative to the housekeeping gene U6 of the expression of miR-101 in the liver cancer and para-cancer tissues in 10 patients selected for this study was shown in **Figure 1A**. It was indicated by statistical analysis results that the expression of miR-101 in the liver cancer tissues was (1.241 ± 0.11) relative to the housekeeping gene, lower than the relative expression of miR-101 in the para-cancer tissues (5.603 ± 0.709), with statistically significant difference ($t = 5.95$, $P = 0.001$).

Expression of miR-101 in liver cancer cells and normal human embryonic liver fibroblasts

As shown in **Figure 1B**, the expression of miR-101 in the hepatoma cell line HepG2 cells was 1.043 ± 0.181 relative to the housekeeping gene U6, lower than the relative expression of miR-101 in the normal embryonic liver fibroblasts HHL-5 cells (4.650 ± 0.62), with statistically significant difference ($t = 26.95$, $P = 0.001$).

Expression of COX-2 in liver cancer cells and normal human embryonic liver fibroblasts

After grayscale scanning for the Western blotting image (**Figure 2**) with Quality One, the ratio of COX-2 to GAPDH in gray scale was used to represent the relative expression and was used for statistical analysis. The results showed that the relative expression of COX-2 in the liver cancer tissues was 0.917 ± 0.103, significantly higher than that in the para-cancer tissues (0.204 ± 0.41) ($t = 20.47$, $P = 0.002$); the relative expression of COX-2 in the HepG2 cells was 0.616 ± 0.093, significantly higher than that in the normal human embryonic liver fibroblasts, HHL-5 cells (0.305 ± 0.032) ($t = 14.66$, $P = 0.010$).

Effects of miR-101 over-expression on the expression of COX-2 in HepG2 cells

The Western blotting results indicated that (**Figure 3**) after over-expression of miR-101, the relative expression of COX-2 in the HepG2 cells reduced significantly from 1.733 ± 0.214 to 0.919 ± 0.117, with statistically significant difference ($t = 26.47$, $P = 0.000$).

Effects of miR-101 over-expression on in vitro proliferation and invasion of the Hep G2 cells

As shown in **Figure 4A**, after culture for 5 days, vitality of the HepG2 cells and the HepG2/NC cells was 682.53 ± 92.7% and 713.95 ± 86.33% respectively of that at inoculation, with no statistical significant difference in between ($F = 1.41$, $P = 0.326$). However, vitality of the HepG2/101 cells over-expressing miR-101 was 327.01 ± 45.18% of that at inoculation, significantly lower compared to vitality of the HepG2 cells ($F = 5.783$, $P = 0.017$) and the HepG2/NC cells ($F = 5.756$, $p = 0.012$). Results of the clonogenic assays (**Figure 4B**) revealed that the clonogenic ability of the HepG2/101 cells decreased obviously compared with the HepG2

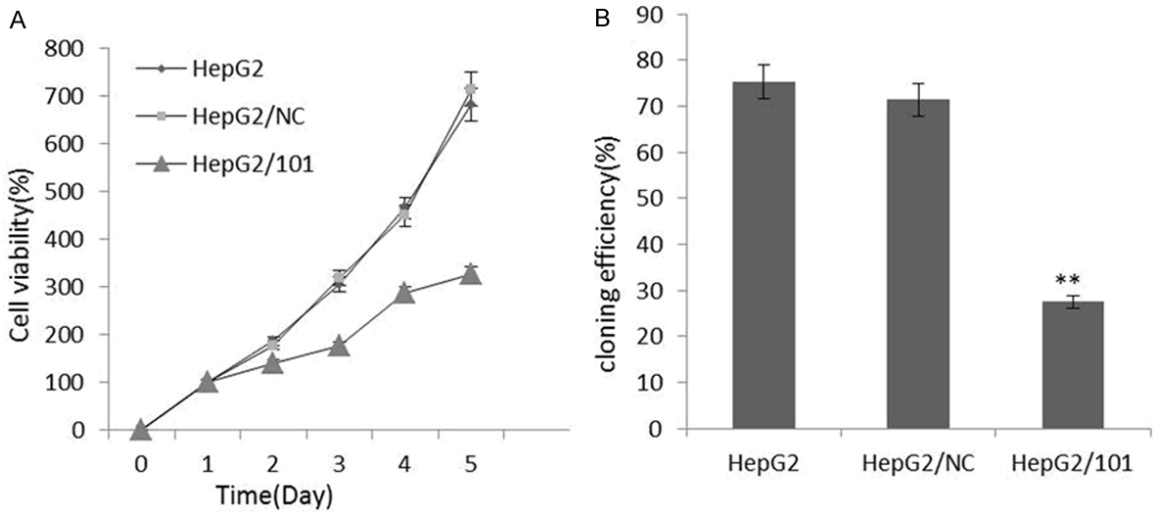

Figure 4. Effects of miR-101 over-expression on *in vitro* proliferation and invasion of the HepG2 cells. A. Effects of miR-101 over-expression on growth curve of the HepG2 cells. B. Effects of miR-101 over-expression on the *in vitro* clonogenic ability of the HepG2 cells.

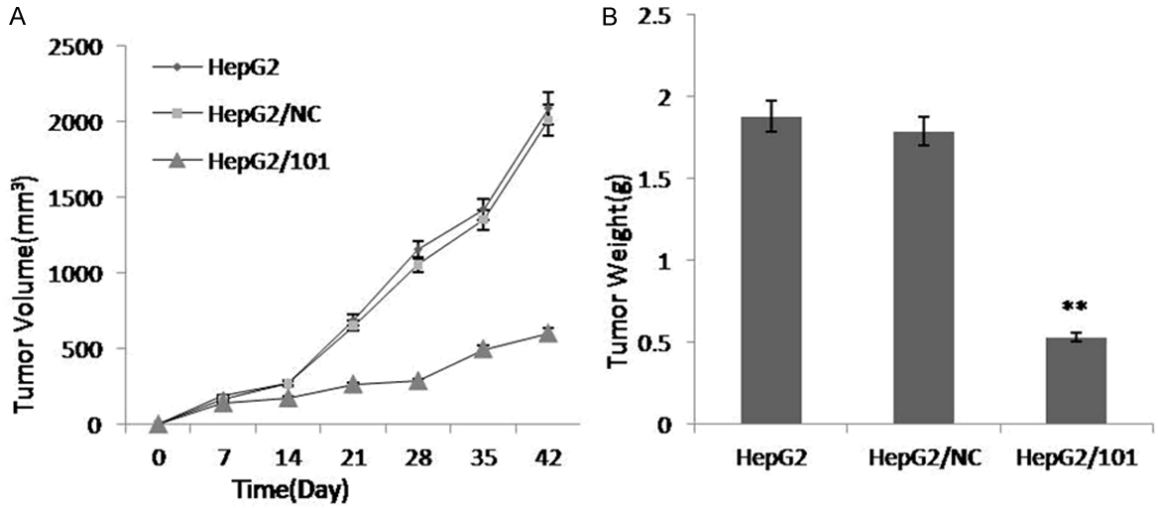

Figure 5. Effects of miR-101 over-expression on the *in vivo* tumor growth rate for the HepG2 cells. A. Growth of tumor for the HepG2, HepG2/NC and HepG2/101 cells after subcutaneous inoculation in BALB/Cl nude mice. B. At 42 days after inoculation, the mice were sacrificed and dissected, with the weight of tumor measured. Weight of tumor is expressed in mean ± SD (n = 4), $^{**}P < 0.01$.

cells and the HepG2/NC cells (cloning efficiency: 75.32 ± 5.09% for the HepG2 cells, 71.48 ± 8.23% for the HepG2/NC cells and 27.55 ± 4.01% for the HepG2/101 cells; Dunnett's test I-J = -0.28, $P = 0.035$).

Effects of miR-101 over-expression on the in vivo tumor growth rate for the HepG2 cells

To determine the effects of miR-101 over-expression on the *in vivo* tumor growth rate for the HepG2 cells, the present study established a subcutaneous tumor model of the HepG2 cells with BALB/C nude mice, and tumorigenicity and tumor growth was observed in the tumor model for the HepG2, HepG2/NC and HepG2/101 cells. Results suggested (**Figure 5A**) that at about 14 days after subcutaneous inoculation of the cells, obvious liver cancer mass could be detected subcutaneously in the nude mice. Then the tumor grew slowly, and at 42 days after inoculation, the volume of subcu-

taneous tumor in mice of the HepG2/101 group was 742.3 ± 195.4 mm^3, lower than that of the HepG2 group (2009.5 ± 41.5 mm^3) and the HepG2/NC group (1989.4 ± 457.9 mm^3). It was indicated by the statistical analysis results that there was no statistically significant difference in the tumor growth rate between the HepG2 group and the HepG2/NC group ($F = 1.57$, $P = 0.226$), while the tumor growth rate in the Hep G2/101 group was obviously lower than that in the Hep G2 group ($F = 14.8$, $P = 0.003$) and the Hep G2/NC group ($F = 15.31$, $P = 0.002$). After sacrifice of the mice, the weight of tumor was measured and results showed that the weight of tumor in mice of the HepG2/101 group was 0.61 ± 0.17 g, significantly lower than that in the HepG2 group (1.92 ± 0.35, Dunnett's test I-J = -0.47, $P = 0.005$) and the HepG2/NC group (1.84 ± 0.41, Dunnett's test I-J = -0.32, $P = 0.007$).

Discussions

miRNA plays an important part in the essential life processes such as embryonic development, cell cycle regulation, proliferation, differentiation and apoptosis [14]. miRNA controls the expression of target genes by influencing mRNA degradation and translation [15]. Bioinformatic study has suggested that miRNA participates in the regulation of approximately 30% of the coding genes in human body. One miRNA molecule can control the expression of several coding genes, and the expression of one coding gene can be regulated by several miRNA molecules, thereby forming a complex regulation network [9, 10]. More and more evidence have shown various abnormal expression of miRNA in human tumors, indicating that miRNA plays an important part in the development and progression of malignant tumors by regulating the expression of oncogenes and anti-oncogenes, which may be an effective approach for humans to overcome tumors [16, 17].

The present study demonstrates with qRT-PCR that the expression of miR-101 in the liver cancer tissues and cells of patients is significantly lower than that in the para-cancer tissue and the normal cells, whereas the expression of COX-2 increases significantly. The study by Chakrabarty A. et al. has suggested [16, 17] that miR-101 inhibits the expression of COX-2 at a post-transcription level. Bioinformatic analysis has confirmed that miR-101 has binding sites for COX-2 [11, 18]. To establish the relationship between down-expression of miR-101 and up-expression of COX-2 in the liver cancer tissues and cells, the present study has successfully transfected the HepG2 cells with miR-101 using Lipofectamine 2000, and it is shown that in the HepG2/101 cells over-expressing miR-101, the expression of COX-2 is up-regulated remarkably compared with the wild-type HepG2 cells.

Further experimental results have suggested that *in vitro* proliferation and invasion of the HepG2/101 cells over-expressing miR-101 decreases significantly compared with the wild-type HepG2 cells. *In vivo* experiments have further demonstrated that the HepG2/101 cells are tumorigenic in BALB/C mice, but the rate of tumor growth is obviously lower compared with the wild-type HepG2 tumor model. It is revealed in study that cyclooxygenase-2 (COX-2) is an important rate-limiting enzyme in synthesis of prostaglandin E2 (PGE2) [4], and the expression of PGE2 increases in various tissues of malignant tumors, it inhibits immune function of the body and is closely related to the development, invasion, metastasis and poor prognosis of tumors [4-8]. The study by Masferrer J.L. et al. [19] has suggested that COX-2 plays an important role in tumor microvessel formation and resistance to apoptosis of tumor cells. The present study has found out that there is a significant negative correlation between the expression of miR-101 and that of COX-2 in the liver cancer tissues and cells, and *in vitro* proliferation and invasion of liver cancer cells over-expressing miR-101 decreases significantly. These results indicate that miR-101 is most likely to play an important part in the development and progression of liver cancer by regulating the expression of COX-2 by target genes. Therefore, up-expression of miR-101 in the liver cancer tissues and cells may be an effective approach to the treatment of liver cancer.

Disclosure of conflict of interest

None.

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