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Original Article

MiR-146a suppresses hepatocellular carcinoma by downregulating TRAF6

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Abstract: MicroRNAs have been proven to play important roles in many biological processes such as cellular growth and differentiation, apoptosis, and modulation of host response to viral infection. In the present study, we find that the expression of miR-146a was decreased in hepatocellular carcinoma (HCC) tissues compared with corresponding adjacent tissues, and the expression level in HCC cell lines was lower than in a normal liver cell. Over-expression suppressed the proliferation and invasion of HCC cells. In addition, luciferase reporter assays and western blotting confirmed that miR-146a directly target TRAF6 which attenuated the effect of miR-146a on cell proliferation and invasion in HepG2 and SMMC7721 cells. Meanwhile, lentivirus-mediated increased expression of miR146a repressed tumor formation in nude mice. Taken together, our findings demonstrate that miR-146a suppresses HCC by down-regulating TRAF6. We also discovered that miR-146a may represent a novel potential candidate of the HCC carcinoma diagnostic marker in the long term.

Keywords: Hepatocellular carcinoma, miR-146a, proliferation, invasion, TRAF6, xenografted tumor

Introduction

Liver cancer is the sixth most common cancer in the world, with 782,000 new cases diagnosed in 2012 alone. Hepatocellular carcinoma (HCC) is the dominant histological type of liver cancer, and accounts for about 80% of primary liver cancer occurences [1]. In China specifically, HCC is a common type of cancer, and is the second highest cause of mortality in the country. More than 110,000 people die from liver cancer each year, accounting for 45% of all HCC deaths worldwide [2-4]. Despite therapeutic advances, the five-year survival rate of HCC is still below 5% [5-7]. HCC is the result of a multistep process which involves the accumulation of several structural and genomic alterations, gene expression profile and intracellular signal pathway alterations [8, 9]. Hence, it is critical to clearly understand these alterations and develop novel strategies for the early diagnosis, prognosis prediction and therapeutic target of patients with HCC.

MicroRNAs (miRNAs) are a large family of short noncoding and evolutionarily conserved RNAs that can negatively regulate gene expression [10]. They can directly and imperfectly bind to the 3'untranslated region of target messenger RNAs (mRNAs), inducing the mRNA degradation, and eventually resulting in a decreased expression of the target gene at a posttranscriptional level. MicroRNAs have been shown to play an important role in malignancy by targeting various tumor suppressors and oncogenes, and by taking part in cancer stem cell biology, angiogenesis, and epithelial-mesenchymal transition [11-14]. MicroRNAs are regared as having applicable clinical values in the early diagnosis of carcinoma. Increased research has shown that microRNAs plays important roles in the initiation and progression

of many cancers such as breast cancer, gastric cancer, lung cancer and bladder cancer [15-19]. Therefore, it may provide new avenues for HCC diagnostic and treatment regiments, if we have a better knowledge of the miRNA gene expression changes in HCC and the function of microRNAs in the process of carcinogenesis and metastasis [20-24].

MiR-146a, which belongs to a member of microRNA, has been shown to play an important role in adaptive immunity and the differentiation of megakaryocytic and monocytic lineages [25]. Multiple groups have indicated that miR-146a may be an important negative regulator of immune responses, done by repressing two key target genes: TRAF6 and IRAK1 [26, 27]. Recent emerging evidence indicates that miR-146a acted as a tumor suppressor during tumor progression [25, 28]. over-expression of miR-146a could inhibit various cancer cell invasion and metastasis, such as breast [29], pancreatic [27] and prostate cancer [30]. However, the molecular mechanism is still unclear. Given the importance and specific functions of miR-146a in cancer cells, further investigation into the role and molecular mechanisms of miR-146a in cancer development and progression is needed.

In this study, we sought to determine the expression and function of miR146a in HCC, and investigate the molecular mechanism of miR146a in the initiation and progression of HCC.

Materials and methods

Samples

HCC tissues and adjacent tissues were kindly provided by Professor Jiangfeng Qiu of the Renji Hospital of Shanghai Jiao Tong University. The specimens were both handled and made anonymous according to ethical and legal standards. All the tissues were collected during surgery and immediately stored at -80°C until the RNA and protein were later extracted. The study was approved by the Medical Ethics Committee of Shanghai Jiao Tong University (Shanghai, China) and all participants provided a written consent for their information to be stored in the hospital database and used for research.

Immunohistochemistry

Immunohistochemical staining was performed for TRAF6 to evaluate the immunoreactivity in the above-mentioned tissue samples [21]. Paraffin-embedded tissue sections (5 μm thick) were prepared. Slides were deparaffinized, rehydrated and subjected to microwave heat antigen retrieval in 0.01 M citrate buffer (pH 6.0) for 20-25 min. After blocking the endogenous peroxidase activity with 3% H_2O_2 for 15 min, the sections were incubated overnight with primary antibodies against TRAF6 (1:300, Abcam, USA) at 4°C. After washing with phosphate-buffered saline (PBS), staining was performed by the Elivision™ plus two-step system (Maixin Bio, Fuzhou, China). We visualized the immunoreactivity using the chromogen, 3, 3'diaminobenzidine (DAB) (Maixin Bio, China).

The immunostaining frequency for each tumor was scored as follows: 0 (<10%), 1 (10%-30%), 2 (31%-60%) and 3 (>61%). The staining intensity was documented as 0 (no immunostaining), 1 (weak), 2 (moderate), and 3 (strong). A total immunostaining score results from the multiplication of both parameters. Total sample scores were based on the following scale: a scale of 0 (-, total immunostaining score = 0), 1+ (score range from 1 to 2), 2+ (++, total immunostaining score = 3~4), and 3+ (+++, total immunostaining score = 6~7). Immunostaining was assessed by an experienced pathologist who was blinded to the clinical data of the patients. The TRAF6 expression was determined by assessing the percentage and intensity of the stained tumor cells. For TRAF6 protein, immunostainings were scored as strong (2++ and 3+++), weak, or negative (1+ and 0), according to the rate of labeled tumor cells and membrane staining intensity.

Cell lines and cultures

Hepatocellular carcinoma cell lines (HCC-LM3, QGY-7701, SMMC7721, HepG2) and the human hepatocyte cell line (LO2) were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS, Gibco). 100 units/mL penicillin and 100 μg/mL streptomycin (Sigma, St-Louis, MO, USA) are supplemented. All these cell lines are incubated at 37°C in humidified atmosphere consisting of 5% CO_2 and 95% air.

RNA extraction and quantitative real-time PCR

Total RNA was extracted from cell lines or frozen tissues using trizol reagent and a miRNeasy mini kit (invitrogen, USA). The Qrt-PCR reactions were performed using an All-in-One™ miRNA

qRT-PCR Detection Kit (GeneCopoeia Inc, USA), and an iQ-5 (Bio-Rad) was used to monitor the PCR in real-time. The average Ct, from triplicate assays, was used for further calculations. Relative expression levels were normalized to control. The endogenous U6 snRNA was chosen as the internal control. The 2-ΔΔCt (ΔΔCt = (CtmiR-146a - CtU6RNA) - (control - CtU6RNA)) method was used to quantify the relative amount of miR-146a.

Oligonucleotides transfection and luciferase reporter assays

MiR-146a mimics and negative control (scramble control mimic) were purchased from GeneCopoeia Inc. (China, Guangzhou). Cells in 24-well plates (50% confluency) were co-transfected with miR-146a mimic and TRAF6 3'UTR Reporter (psiCHECK-TRAF6). A cell transfection was performed using Lipofectamine® RNAiMAX (invitrogen, USA) with a final concentration of 50 nM (miR-146a mimic) and 200 ng (psiCHECK-TRAF6). After being transfected for 6 hours, the medium was changed. The cells were collected for a luciferase reporter assay 48 hours later. Luciferase activity was detected using the Dual-Glo luciferase assay system (Promega, Fitchburg, WI, USA), according to the manufacturer's instructions.

Lentivirus production

The lentivirus vector of miR-146a and TRAF6 was obtained from the Shanghai R&S Biotechnology (Shanghai, China), and cotransfected with helper plasmids (pLP1-Gag-pol, pLP-VSVG, and pLP2-Rev) into human embryonic kidney 293T cells. The supernatant was collected at 48 hours (48 h) after transfection, and fresh medium was added to the culture flask. After the cells were cultured for another 24 h, the supernatant was collected again. The supernatants collected from 48 and 72 h were mixed, and the mixture was then centrifuged at 3000 rpm for 15 min at 4°C. The liquid was filtered by a 0.45-mm filter membrane, and the acquired virus was stored at -80°C until use. The titers of the lentivirus were 3*10^8 transfer units per ml (TU/ml).

Lentivirus infection

The cells were planted into 10 cm dishes (10^6 cells/dish) one day before the lentivirus infection. The next day, when the confluence reached 70%, the lentivirus was added into the dishes, with a MOI (multiplicity of infection) of 15, to infect HepG2 and SMMC-7721. The infection efficiency was detected by a fluorescence microscopy analysis of GFP 24 h after infection, and the efficiency was ensured higher than 90%.

Cell proliferation assay

A Cell Counting Kit (CCK-8) assay was performed to analyze the cell proliferation. All cells were cultured in 10% CCK-8 (DOJINDO) and diluted in normal culture media at 37°C. When visual color conversion appeared, a quantification was carried out on a microtiter plate reader (Thermo, MuLTiSKAN MK3), and the data were used to draw a growth curve.

Cell invasion assay

Transwell invasion chambers coated with Matrigel (50 μl/filter) (BD Biosciences, Franklin Lakes, NJ, USA) were used to analyze the invasion capabilities of HepG2 and SMMC-7721 according to the manufacturer's instructions. After being infected with the lentivirus for 48 h, the cells were transferred to the top of chambers in a 1% fetal calf serum DMEM (4*10^4 cells/well). 500 μl DMEM with 10% fetal calf serum was added into the lower chambers. Cells were incubated for 24 h at 37°C in an atmosphere containing 5% CO_2. Invaded cells on the lower surface were then stained with a crystal violet stain.

Cell cycle assay

Following a 24 h transfection, the cells were seeded into 6-well plates having a density of 1 × 10^5 cells/well, and then maintained in DMEM containing 10% FBS. After being cultured for 72 h, the cells were harvested and fixed in 70% (v/v) ice-cold ethanol. They were then stored overnight at -20°C. The fixed cells were washed twice with PBS (1mL) and stained in propidium iodide solution (PI) (Invitrogen) (10 μg/mL) with 0.5 mg/mL RNase for 30 min at 4°C in the dark. A flow cytometry was then performed using a FACSCalibur cytometer (BD Biosciences, USA) to examine the cell cycle distribution. We performed these experiments in triplicate.

Western blotting

Total proteins of cells and tissues were extracted according to the protein extraction kit

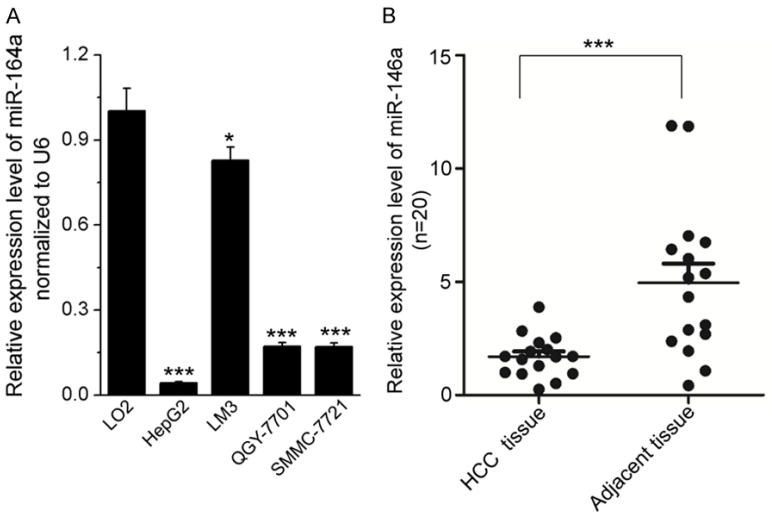

Figure 1. MiR-146a expression in HCC cell lines and tissues. A. Real-time PCR was used to determine relative miR-146a levels in four HCC cell lines and one normal liver cell. B. Relative expression of miR-146a levels in HCC tissues and respective adjacent non-cancer tissues. Each dot represents a single sample.

(KEYGEN, China) guidelines, and the protein concentration of each sample was determined using the bicinchoninic acid protein assay kit (Pierce Biotechnology, USA). Equivalent quantities of protein were separated by 12% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were blocked with 10% defatted milk and then incubated overnight with the appropriate primary antibody. Next, they were washed and incubated for 1 h with the corresponding horseradish peroxidase (HRP)-conjugated secondary antibody. The Bound secondary antibody was visualized using an enhanced chemiluminescence (ECL) system (Pierce Biotechnology, Rockford, IL). The primary antibodies used were: anti-GAPDH and anti-TRAF6 (CST, USA).

In vivo studies

We investigated the carcinogenesis of miR-146a and TRAF6 in nude mice that harbored a hepatocellular tumor. HepG2 cells (2*10^6) were injected subcutaneously into the posterior flanks of female nude mice aged 6 weeks, to establish a tumor-bearing animal model. Following this injection, the tumor diameters were measured every 3 days. Tumor volume was calculated as length × width2 × ½ mm^3. When the tumor size reached 50 mm^3, 50 μl lentivirus were injected into the tumors every 3 days, for five times. The mice were killed one week after the last lentivirus injection, and the tumors collected and weighted.

Statistical analysis

The data were presented as the mean ± standard deviation (SD) of three separate experiments. When two groups were compared, the differences were analyzed using Student's t-test. When more than two groups were compared, a one-way analysis of variance (ANOVA) was used instead. All statistical analyses were performed with SPSS 16.0 (SPSS Inc., USA). The difference was considered statistically significant at $P < 0.05$.

Result

MiR-146a is downregulated in HCC cell lines and tissues

To explore the difference of the miR-146a expression levels between HCC cells and normal liver cells, we detected miR-146a expression in HCC-LM3, QGY-7701, SMMC7721, HepG2 and LO2 cell. The expression of miR-146a was significantly down-regulated in HCC cell lines compared with the LO2 cell (**Figure 1A**). We then examined the expression in 20 pairs of HCC tissues and matched the tumor adjacent tissues. As shown in **Figure 1B**, the expression of miR-146a in HCC tissues was lower than in adjacent tissues (**Figure 1B**). In our study, miR-146a expression levels in the QGY-7701, SMMC7721 and HepG2 cell lines were much lower than in LO2 cells. SMMC7721 and HepG2 cells were then selected for further studies.

Over-expression of miR-146a inhibits HCC cells proliferation

To investigate the effect of miR-146a on HCC cell proliferation, we used the miR-146a over-expression lentivirus to infect HepG2 and SMMC-7721 (**Figure 2A**). The result showed that the expression of miR-146a was remarkably up-regulated in HepG2 and SMMC-7721

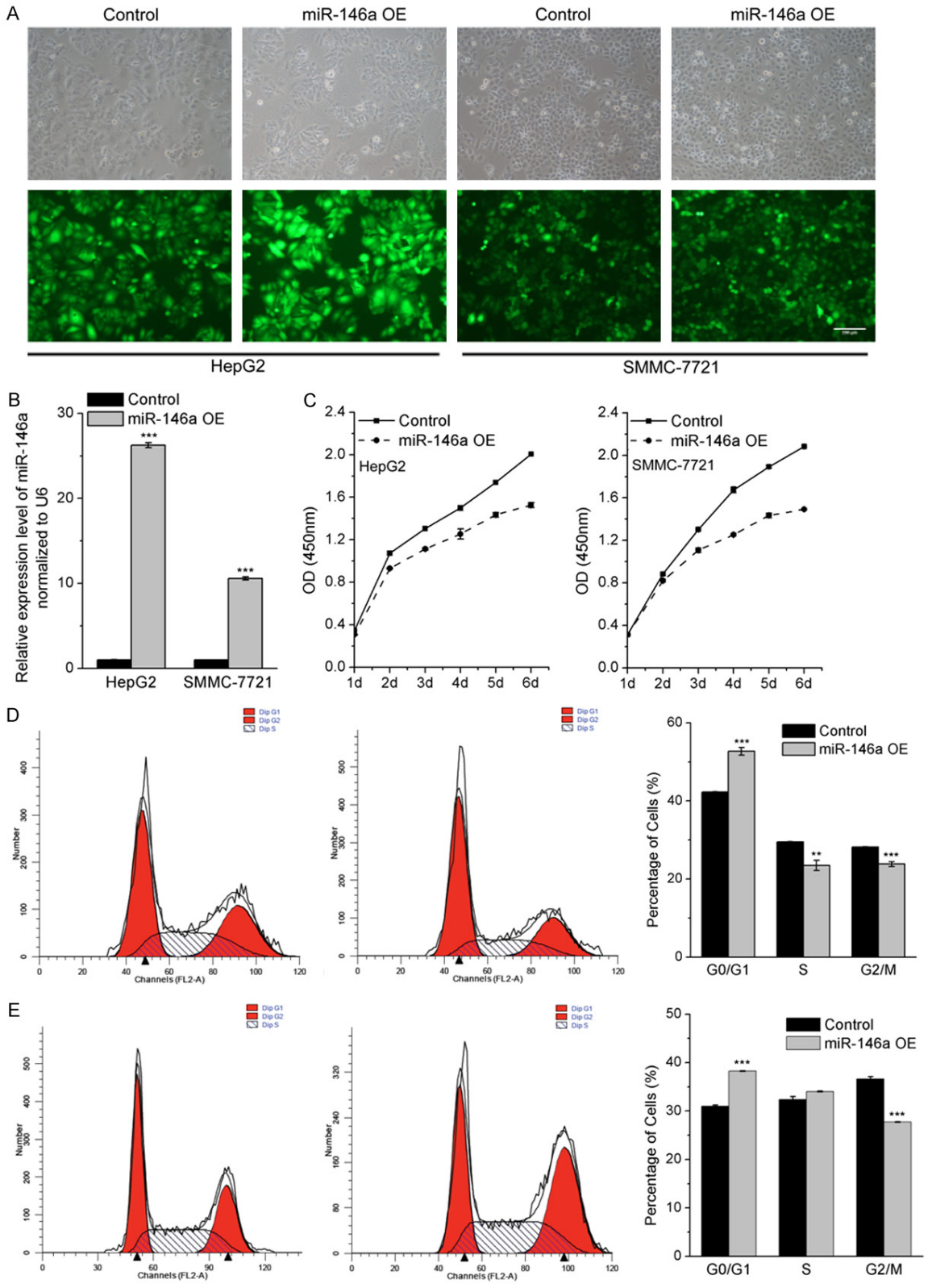

Figure 2. Over expression of miR-146a inhibits growth of HCC cells. (A) HepG2 and SMMC-7721 were infected with a scramble lentivirus (Control) over-expression lentivirus of miR-146a (miR-146a OE), and then imaged by fluorescence microscopy after 24 h. Magnifcation × 200; scale bar, 200 mm. (B) Real-time PCR was used to measure the relative expression level of miR-146a in HepG2 and a SMMC-7721 infected with indicated lentivirus. (C) The prolif-

eration rate of HepG2 and SMMC-7721 infected with indicated lentivirus was measured by a CCK-8) assay. (D and E) The cell cycle distribution of HepG2 (D) and SMMC-7721 (E) infected with indicated lentivirus was analyzed using flow cytometry. The number of cells in G0/G1, S and G2/M phases are shown (n = 3).

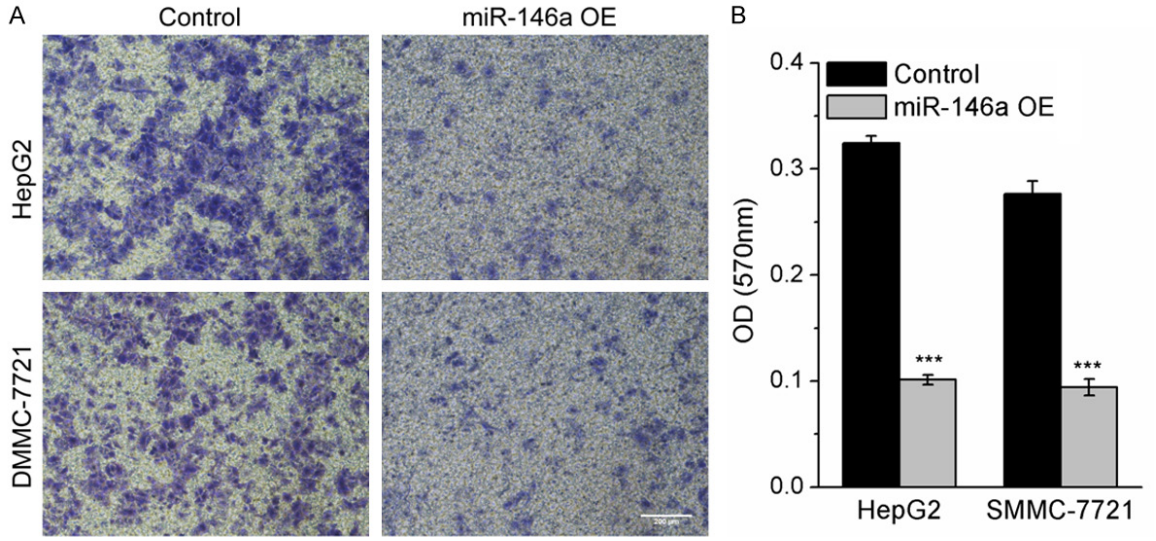

Figure 3. Over-expression of miR-146a inhibits invasion of HCC cells. A. Matrigel invasion chambers were used to determine the invasiveness of HepG2 and SMMC-7721 cells, stained with crystal violet and then photographed. B. The invading cells were lysed and the OD570 is measured.

cells after the lentivirus infection (**Figure 2B**). Further, the up-regulation of miR-146a significantly inhibited cell growth in HepG2 and SMMC-7721 (**Figure 2C**).

A cell cycle analysis was performed with HCC cells infected by miR-146a over-expression lentivirus. We observed a significant increase in the G1-phase cell population at 72 h post-infection when compared with the negative control. Meanwhile, a concomitant decrease of the S/G2-phase population in HepG2 and SMMC-7721 also occurred (**Figure 2D** and **2E**) and led to cell cycle arrests in the HCC cells. All the results demonstrated that an over-expression of miR-146a inhibits HCC cell proliferation.

Over-expression of miR-146a inhibits HCC cell invasion

To investigate the effect of miR-146a on the invasion capability of HCC cells, we performed a Transwell assay in HCC cells. **Figure 3** shows the changes in the invasion capacity of HCC cells. HepG2 and SMMC-7721 cells infected with miR-146a over-expression lentivirus showed a significantly decreased invasion capability when compared with the negative control lentivirus infected cells.

TRAF6 is a direct target of miR-146a

To explore the mechanisms of miR-146a in HCC cells, we used bioinformatics analysis to find the direct target of miR-146a. We found that the 3'-UTR of TRAF6 contained a conserved putative target site for miR-146a (**Figure 4A**). Meanwhile, we discovered that TRAF6 was inversely expressed with miR-146a in HCC tissues and cell lines (**Figure 4B** and **4C**). Thus, we constructed luciferase vectors carrying 3'-UTR of TRAF6 behind the firefly luciferase gene coding region. As shown in **Figure 4D**, a miR-146a mimic repressed the luciferase activity, and the repress phenomenon was meanwhile largely abolished when the miR-146a binding site was deletion mutated in the TRAF6 3'-UTR. In addition, real-time PCR and western blot analysis also showed that the transfected miR-146a mimic remarkably suppressed TRAF6 expression in HepG2 and SMMC-7721 cells (**Figure 4E**).

The expression of TRAF6 is increased in HCC tissue

To further assess the expression of TRAF6, we immunohistochemically stained TRAF6 in the tissue sections of HCC and in their correspond-

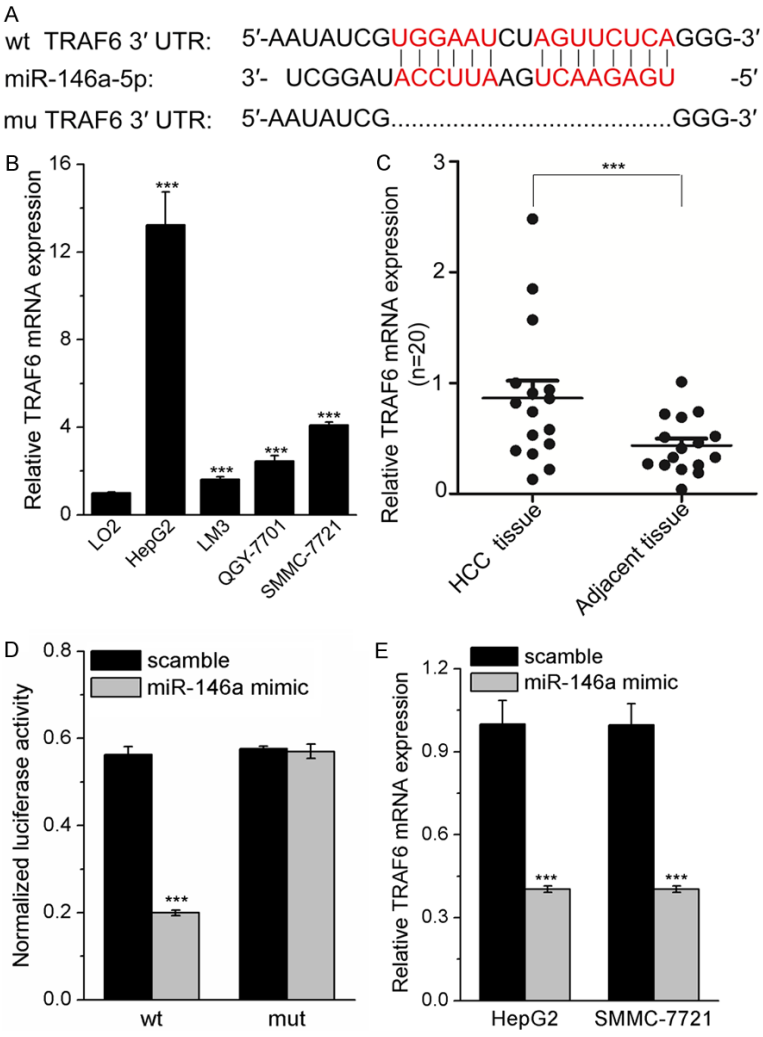

Figure 4. The direct inhibition of miR-146a on TRAF6. (A) the target site of TRAF6. 3'-UTR for miR-146a. (B and C) Real-time PCR was used to determine relative TRAF6 mRNA levels in HCC cell lines (B) and tissues (C). (D) The different effects of miR-146a on TRAF6 3'-UTR and its mutant was measured by the relative luciferase activity in HepG2 cells. (E) Real-time PCR was used to assess relative TRAF6 mRNA in HepG2 and SMMC-7721 cells treated as indicated.

Table 1. TRAF6 expression detected by immunohistochemistry in HCC tissues

Histological type	Number of patient	TRAF6 expression n(%)			
		-	+	++	+++
Normal tissue	5	2 (40%)	0 (0.0)	0 (0.0)	3 (60%)
HCC	5	0 (0.0)	0 (0.0)	0 (0.0)	5 (100%)

ing adjacent non-cancerous tissues. **Table 1** and **Figure 5** show that the TRAF6 protein is abundantly expressed in the HCC tissue, which is similar to the PCR result. It is worth noting that more expressions of TRAF6 was found in the normal adjacent tissue.

MiR-146a suppressed cell proliferation and invasion through inhibiting TRAF6 expression in HepG2

To investigate the role of miR-146a through inhibiting TRAF6, a TRAF6 over-expression lentivirus was used to restore TRAF6 expression in HepG2 cells (**Figure 6A**). An over-expression of TRAF6 promoted HepG2 cell proliferation and invasion (**Figure 6B** and **6C**), and the over-expression of TRAF6 also rescued the miR-146a-mediated inhibitory effect on the proliferation and migration of HepG2 cells.

MiR-146a inhibited the growth of HepG2-engrafted tumors

To further explore the effect of miR-146a on tumor growth in vivo, we injected HepG2 cells into nude mice. Lentivirus were injected into tumors every 3 days, with a dose of 10^7 TU administered per nude mice when the tumor volume reached 50 mm^3. Compared with the negative control lentivirus injected group, the tumors in the miR-146a-5p over-expression lentivirus injected group grew in significance only slightly. While the TRAF6 over-expression lentivirus infection accelerated the growth of tumors and reversed the inhibitory effect of the miR-146a-5p over-expression lentivirus (**Figure 7A** and **7B**).

Discussion

The roles of miRNAs in cancer has been reported by many researchers in recent decades. Our study showed that the expression of miR-146a was downregulated in HCC cells and HCC tissues. By contrast, a lentivirus-mediated up-expression of miR-146a inhibits the prolifera-

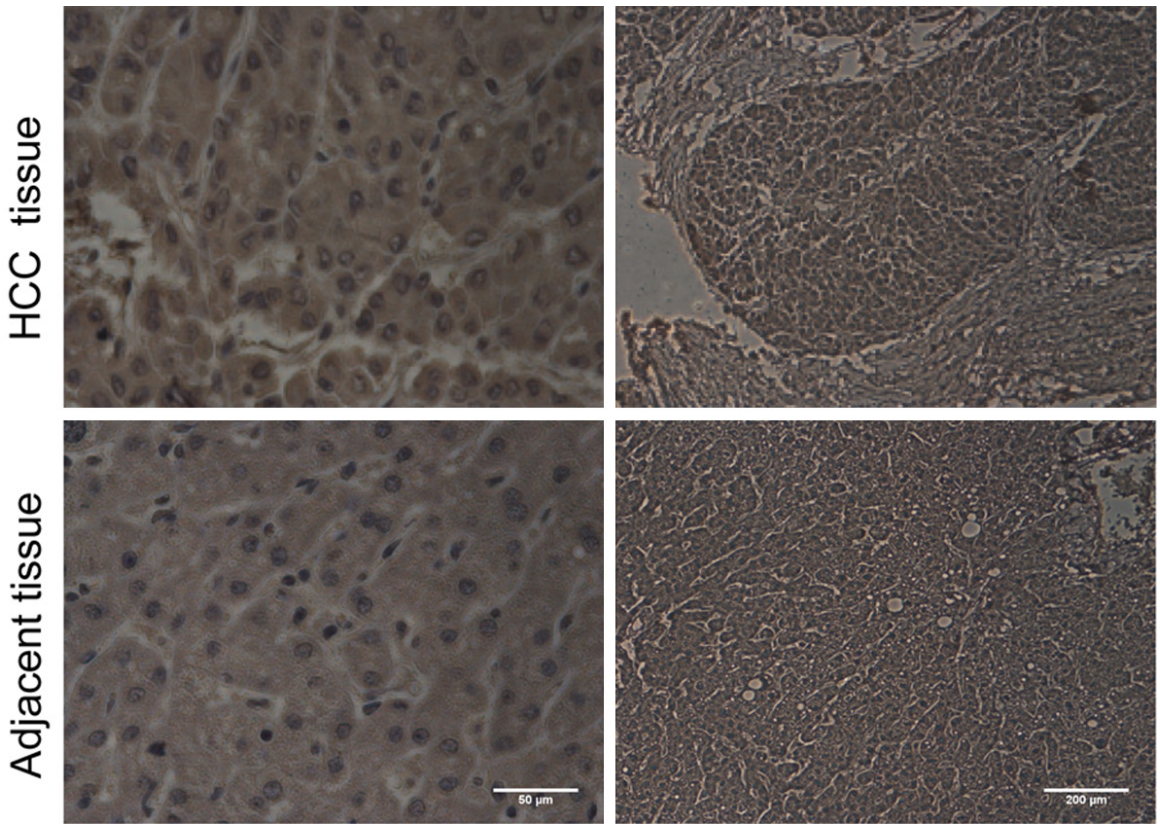

Figure 5. TRAF6 expression in HCC tissues. Immunohistochemistry images showing the expression level of TRAF6 in HCC tissue and the corresponding adjacent non-cancerous tissue.

tion and invasion of HCC cells. With regard to the mechanism, our results indicated that miR-146a directly targeted TRAF6 to inhibit cell proliferation and invasion in HCC cells. More importantly, the TRAF6 rescued the miR-146a-mediated inhibition of cell invasion and proliferation. Moreover, we detected the expression of TRAF6 and miR-146a in HCC tissues and HCC cells, and the result indicated that there was an inverse correlation between the miR-146a and TRAF6 expression. We also performed a subcutaneous tumor forming assay on nude mice and our results prove that miR-146a significantly repressed the growth of HCC cells. Further western blot analysis demonstrated the negative regulation of miR-146a to TRAF6. These results indicate that miR-146a may play an important role in promoting the carcinogenesis of HCC, and more importantly, the findings suggest that miR-146a is a potential therapeutic choice in HCC.

MicroRNAs (miRNAs) are a type of small conserved RNA molecules, approximately 17-22 nucleotides in length. Due to its important roles in physiology and pathology, miRNA has attracted more and more attention in the past ten years [31-33]. miRNAs are involved in the regulation of cell proliferation, migration and apoptosis, which are associated with the development and progression of various types of cancer. Some of the miRNAs function as tumor suppressor genes and oncogenes [34-36]. MiR-146 has been reported as a potential tumor suppressor, which was identified as one of the miRNAs downregulated in HCC [31], breast cancer [37], pancreatic cancer [38], and hormone refractory prostate cancer [27]. It has also been reported that miR-146a inhibited cancer cell metastasis by targeting IRAK-1 [39-41], and regulated migration and invasion of HCC cells through inhibiting HAb18G. The results of our study indicate that the expression of miR-146a was downregulated in HCC cells and HCC tissues. Our study also shows that the up-expression of miR-146a inhibits cell proliferation and invasion in HCC cells. Over-expression of miR-146 also inhibits tumor growth in vivo,

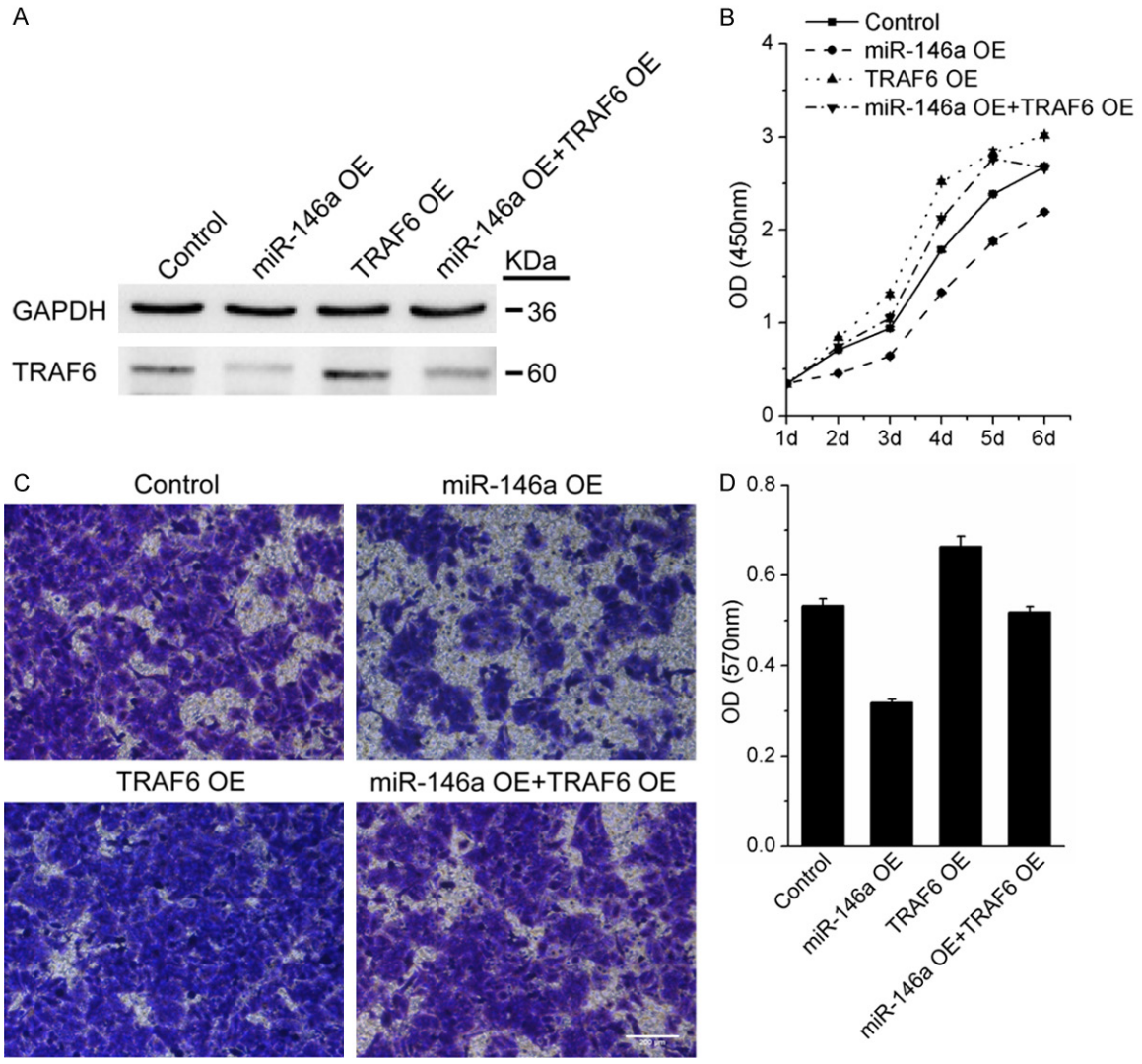

Figure 6. Over expression TRAF6 rescues growth and invasion inhibitory effect of over-expression miR-146a. (A) A Western blot analysis was used to determine relative levels of TRAF6 protein in HepG2 cells infected with the indicated lentivirus. GAPDH was used as the loading control. (B) The proliferation rate of HepG2 from each treatment group was measured by a CCK-8 assay. (C and D) The invading HepG2 cells were photographed (C) and the OD570 was measured (D).

which confirmed miR-146a was a potential tumor suppressor.

TRAF6 is a member of the TNF receptor-associated factor (TRAF) family of proteins. Other research has shown that TRAF6 was a commonly amplified oncogene in many cancers, such as lung cancer [42], papillary thyroid carcinoma [43], pancreatic cancer [44] and esophageal squamous cell carcinoma [45]. TRAF6 is reported as a target of miR-146a [43]. Moreover, using a bioinformatics analysis, we discovered that the 3'-UTR of TRAF6 contained a conserved putative target site for miR-146a. A TRAF6 over-expression-Lentivirus was used to investigate the molecular mechanism of miR-146a in HCC in vitro and in vivo. The data of luciferase reporter assay indicated that TRAF6 is a direct target of miR-146a. Our study showed the up-regulated expression of TRAF6 in HCC tissue and cells. An over-expression of the TRAF6 lentivirus can promote HCC cell proliferation, invasion and tumor growth, and reverse the effect of miR-146a on HCC in vitro and in vivo. All the above results imply that miR-146a may function as a negative regulator and tumor suppressor for cell proliferation, invasion and

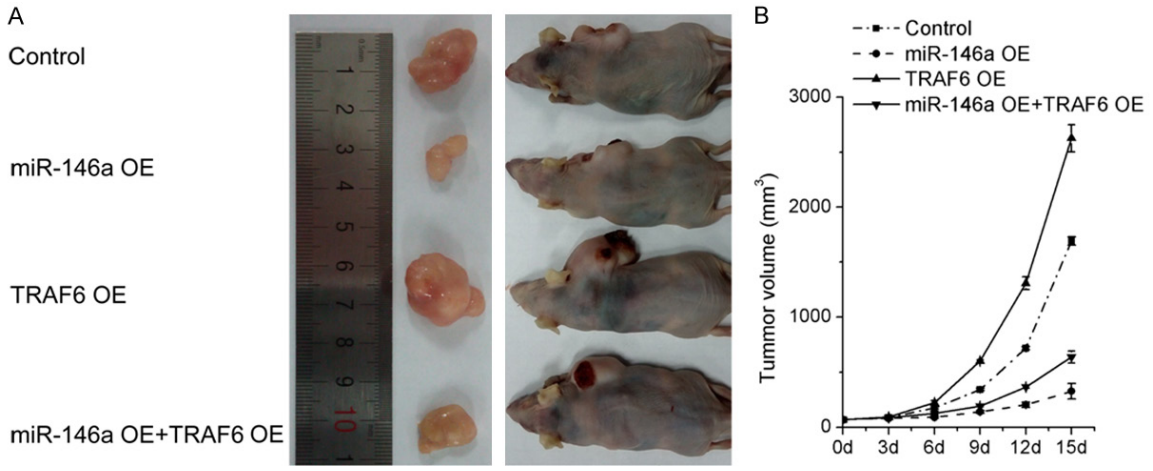

Figure 7. MiR-146a regulates growth of HepG2 cells in a subcutaneous mouse model. A. Photograph shows tumor size in each treatment group. B. Tumor growth was measured by tumor volume.

tumor growth in HCC. And TRAF6 is an oncogene in HCC as a target of miR-146a.

As with previous reports, we confirm that miR-146a act as a tumor suppressor miRNA in HCC tumorigenesis and progression. MiR-146a inhibits HCC cell proliferation, invasion and tumor growth partly through the downregulation of TRAF6. Given that the reintroduction of miR-146a inhibited tumor formation in the nude mice HCC model, this mature miRNA could serve as a potential therapeutic strategy for HCC. Our findings are encouraging and suggestting that miR-146a could be targeted for the development of novel HCC treatment in the future.

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Disclosure of conflict of interest

None.

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References

[1] Perz JF, Armstrong GL, Farrington LA, Hutin YJ, Bell BP. The contributions of hepatitis B virus and hepatitis C virus infections to cirrhosis and primary liver cancer worldwide. J Hepatol 2006; 45: 529-538.

[2] Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. CA Cancer J Clin 2005; 55: 74-108.

[3] El-Serag HB, Rudolph KL. Hepatocellular carcinoma: epidemiology and molecular carcinogenesis. Gastroenterology 2007; 132: 2557-2576.

[4] Huang J. Current progress in epigenetic research for hepatocarcinomagenesis. Sci China C Life Sci 2009; 52: 31-42.

[5] Jung CJ, Iyengar S, Blahnik KR, Ajuha TP, Jiang JX, Farnham PJ, Zern M. Epigenetic modulation of miR-122 facilitates human embryonic stem cell self-renewal and hepatocellular carcinoma proliferation. PLoS One 2011; 6: e27740.

[6] Furuta M, Kozaki K, Tanimoto K, Tanaka S, Arii S, Shimamura T, Niida A, Miyano S, Inazawa J. The tumor-suppressive miR-497-195 cluster targets multiple cell-cycle regulators in hepatocellular carcinoma. PLoS One 2013; 8: e60155.

[7] Ladeiro Y, Couchy G, Balabaud C, Bioulac-Sage P, Pelletier L, Rebouissou S, Zucman-Rossi J. MicroRNA profiling in hepatocellular tumors is associated with clinical features and oncogene/tumor suppressor gene mutations. Hepatology 2008; 47: 1955-1963.
[8] Furuta M, Kozaki KI, Tanaka S, Arii S, Imoto I, Inazawa J. miR-124 and miR-203 are epigenetically silenced tumor-suppressive microRNAs in hepatocellular carcinoma. Carcinogenesis 2010; 31: 766-76.
[9] Lang Q, Ling C. MiR-124 suppresses cell proliferation in hepatocellular carcinoma by targeting PIK3CA. Biochem Biophys Res Commun 2012; 426: 247-252.
[10] Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 2004; 116: 281-297.
[11] Zhang B, Pan X, Cobb GP, Anderson TA. microRNAs as oncogenes and tumor suppressors. Dev Biol 2007; 302: 1-12.
[12] Takebe N, Harris PJ, Warren RQ, Ivy SP. Targeting cancer stem cells by inhibiting Wnt, Notch, and Hedgehog pathways. Nat Rev Clin Oncol 2011; 8: 97-106.
[13] Wang X, Hu G, Zhou J. Repression of versican expression by microRNA-143. J Biol Chem 2010; 285: 23241-23250.
[14] Kong YW, Ferland-McCollough D, Jackson TJ, Bushell M. microRNAs in cancer management. Lancet Oncol 2012; 13: e249-e258.
[15] Yang W B, Chen PH, Hsu T 1st, Fu TF, Su WC, Liaw H, Chang WC, Hung JJ. Sp1-mediated microRNA-182 expression regulates lung cancer progression. Oncotarget 2014; 5: 740.
[16] Wang Z, Wang N, Liu P, Chen Q, Situ H, Xie T, Zhang J, Peng C, Lin Y, Chen J. MicroRNA-25 regulates chemoresistance-associated autophagy in breast cancer cells, a process modulated by the natural autophagy inducer isoliquiritigenin. Oncotarget 2014; 5: 7013-7026.
[17] Itesako T, Seki N, Yoshino H, Chiyomaru T, Yamasaki T, Hidaka H, Yonezawa T, Nohata N, Kinoshita T, Nakagawa M, Enokida H. The microRNA expression signature of bladder cancer by deep sequencing: the functional significance of the miR-195/497 cluster. PLoS One 2014; 9: e84311.
[18] Xu Y, Jin J, Liu Y, Huang Z, Deng Y, You T, Zhou T, Si J, Zhuo W. Snail-regulated MiR-375 inhibits migration and invasion of gastric cancer cells by targeting JAK2. PLoS One 2014; 9: e99516.
[19] Zhao G, Cai C, Yang T, Qiu X, Liao B, Li W, Ji Z, Zhao J, Zhao H, Guo M, Ma Q, Xiao C, Fan Q, Ma B. MicroRNA-221 induces cell survival and cisplatin resistance through PI3K/Akt pathway in human osteosarcoma. PLoS One 2013; 8: e53906.
[20] Giovannini C, Minguzzi M, Baglioni M, Fornari F, Giannone F, Ravaioli M, Cescon M, Chieco P, Bolondi L, Gramantieri L. Suppression of p53 by Notch3 is mediated by Cyclin G1 and sustained by MDM2 and miR-221 axis in hepatocellular carcinoma. Oncotarget 2014; 5: 10607.
[21] Zhang K, Chen J, Chen D, Huang J, Feng B, Han S, Chen Y, Song H, De W, Zhu Z, Wang R, Chen L. Aurora-A promotes chemoresistance in hepatocelluar carcinoma by targeting NF-kappaB/microRNA-21/PTEN signaling pathway. Oncotarget 2014; 5: 12916.
[22] Zhou HC, Fang JH, Luo X, Zhang L, Yang J, Zhang C, Zhuang SM. Downregulation of microRNA-100 enhances the ICMT-Rac1 signaling and promotes metastasis of hepatocellular carcinoma cells. Oncotarget 2014; 5: 12177.
[23] Liu LL, Lu SX, Li M, Li LZ, Fu J, Hu W, Yang YZ, Luo RZ, Zhang CZ, Yun JP. FoxD3-regulated microRNA-137 suppresses tumour growth and metastasis in human hepatocellular carcinoma by targeting AKT2. Oncotarget 2014; 5: 5113-24.
[24] Heo MJ, Kim YM, Koo JH, Yang YM, An J, Lee SK, Lee SJ, Kim KM, Park JW, Kim SG. microRNA-148a dysregulation discriminates poor prognosis of hepatocellular carcinoma in association with USP4 over-expression. Oncotarget 2014; 5: 2792.
[25] Rusca N, Monticelli S. MiR-146a in immunity and disease. Mol Biol Int 2011; 2011: 437301.
[26] Zhao JL, Starczynowski DT. Role of microRNA-146a in normal and malignant hematopoietic stem cell function. Front Genet 2014; 5: 219.
[27] Saba R, Sorensen DL, Booth SA. MicroRNA-146a: a dominant, negative regulator of the innate immune response. Front Immunol 2014; 5: 578.
[28] Lin SL, Chiang A, Chang D, Ying SY. Loss of mir-146a function in hormone-refractory prostate cancer. RNA 2008; 14: 417-24.
[29] Bhaumik D, Scott GK, Schokrpur S, Patil CK, Campisi J, Benz CC. Expression of microRNA-146 suppresses NF-κB activity with reduction of metastatic potential in breast cancer cells. Oncogene 2008; 27: 5643-5647.
[30] Li Y, Vandenboom TG 2nd, Wang Z, Kong D, Ali S, Philip PA, Sarkar FH. miR-146a suppresses invasion of pancreatic cancer cells. Cancer Res 2010; 70: 1486-1495.
[31] Zhang Z, Zhang Y, Sun XX, Ma X, Chen ZN. microRNA-146a inhibits cancer metastasis by downregulating VEGF through dual pathways in hepatocellular carcinoma. Mol Cancer 2015; 14: 5.
[32] Calin GA, Croce CM. MicroRNA signatures in human cancers. Nat Rev Cancer 2006; 6: 857-866.

[33] Esquela-Kerscher A, Slack FJ. Oncomirs-microRNAs with a role in cancer. Nat Rev Cancer 2006; 6: 259-269.
[34] Varnholt H. The role of microRNAs in primary liver cancer. Ann Hepatol 2008; 7: 104-113.
[35] Shenouda SK, Alahari SK. MicroRNA function in cancer: oncogene or a tumor suppressor? Cancer Metastasis Rev 2009; 28: 369-378.
[36] Li S, Fu H, Wang Y, Tie Y, Xing R, Zhu J, Sun Z, Wei L, Zheng X. MicroRNA-101 regulates expression of the v-fos FBJ murine osteosarcoma viral oncogene homolog (FOS) oncogene in human hepatocellular carcinoma. Hepatology 2009; 49: 1194-1202.
[37] Bhaumik D, Scott G K, Schokrpur S, Patil CK, Campisi J, Benz CC. Expression of microRNA-146 suppresses NF-κB activity with reduction of metastatic potential in breast cancer cells. Oncogene 2008; 27: 5643-5647.
[38] Li Y, Vandenboom TG 2nd, Wang Z, Kong D, Ali S, Philip PA, Sarkar FH. miR-146a suppresses invasion of pancreatic cancer cells. Cancer Res 2010; 70: 1486-1495.
[39] Cui JG, Li YY, Zhao Y, Bhattacharjee S, Lukiw WJ. Differential regulation of interleukin-1 receptor- associated kinase-1 (IRAK-1) and IRAK-2 by microRNA-146a and NF-κB in stressed human astroglial cells and in Alzheimer disease. J Biol Chem 2010; 285: 38951-38960.
[40] Chatzikyriakidou A, Voulgari PV, Georgiou I, Drosos AA. A polymorphism in the 3'-UTR of interleukin-1 receptor-associated kinase (IRAK1), a target gene of miR-146a, is associated with rheumatoid arthritis susceptibility. Joint Bone Spine 2010; 77: 411-413.
[41] Chatzikyriakidou A, Voulgari PV, Georgiou I, Drosos AA. The role of microRNA-146a (miR-146a) and its target IL-1R-associated kinase (IRAK1) in psoriatic arthritis susceptibility. Scand J Immunol 2010; 71: 382-385.
[42] Lin G, Huang C, Su G, Hu H, Xu H, Huang C. Effect of TRAF6 Downregulation on Malignant Biological Behavior of Lung Cancer Cell Lines. Zhongguo Fei Ai Za Zhi 2015; 18: 661.
[43] Cong D, He M, Chen S, Liu X, Liu X, Sun H. Expression profiles of pivotal microRNAs and targets in thyroid papillary carcinoma: an analysis of The Cancer Genome Atlas. Onco Targets Ther 2015; 8: 2271.
[44] Rong Y, Wang D, Wu W, Jin D, Kuang T, Ni X, Zhang L, Lou W. TRAF6 is over-expressed in pancreatic cancer and promotes the tumorigenicity of pancreatic cancer cells. Med Oncol 2014; 31: 1-10.
[45] Han Q, Yao F, Zhong C, Zhao H. TRAF6 promoted the metastasis of esophageal squamous cell carcinoma. Tumor Biology 2014; 35: 715-721.