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miR-152-5p inhibits proliferation and induces apoptosis of liver cancer cells by up-regulating FOXO expression

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Currently, a lot of microRNAs (miRNAs) have been confirmed to be closely related with liver cancer occurrence and development. This study was aimed to explore the role of miR-152-5p in liver cancer. HepG2 and MHCC97 cells were transfected with miR-152-5p mimic, inhibitor or corresponding scramble controls, respectively. The expression level of miR-152-5p in transfected cells was detected by qPCR. Cell viability, apoptosis, migration and invasion of miR-transfected cells were measured to determine the effect of miR-152-5p on the activity of hepatoma cells. The protein expressions of fork head transcription factor O (FOXO) and apoptosis related factors in miR-transfected cells were detected by western blot assay. In addition, western blot was used to detect the relationship of FOXO expression and mainly factors of the JNK signaling pathway after concurrent treatment with miR-152-5p mimic and JNK inhibitor. The results showed that the miR-152-5p was effectively overexpressed or repressed in both HepG2 and MHCC97 cells. Overexpression of miR-152-5p inhibited cell viability, promoted apoptosis, and reduced migration and invasion. In these cells, miR-152-5p overexpression activated the expression of apoptosis-related factors and upregulated the expression of FOXO by activating the phosphorylation of mainly factors in the JNK pathway. miR-152-5p might be a potential anti-tumor factor for liver cancer treatment.

1. Introduction

Hepatic carcinoma is one of the most common malignancies in the world with the third mortality rate after stomach cancer and lung cancer (Valencia and Rodriguezwulff 2013). Due to the lack of specific clinical manifestations, patients with liver cancer are usually diagnosed at late stages. Until now the treatment of liver cancer is still insufficient (Adamov 2012). The development of cancer is usually a multistage process with activation of oncogenes and inactivation of tumor suppressor genes (Laudański et al. 2011). After receiving foreign carcinogens, liver cells will firstly activate oncogenes. For instance, activation of Ras gene is necessary to maintain the malignant phenotype, C-myc overexpression is related to cancer cell differentiation (Nagl et al. 2006; Sun et al. 2013). In later stages of liver cancer, deletion and mutation of p53 genes are seen in up to 50% of cases (Winkler et al. 2014). PTEN gene as tumor suppressor gene was deleted or weakly expressed in primary hepatocellular carcinoma, which was probably related to its tumorigenesis (Lu et al. 2003).

The microRNAs (miRNAs) are in the forms of cancer researches since they have shown to be functional as potent post-transcriptional regulators of gene expression. The miRNAs were likely to influence over one third of all protein-encoding human genes and participate in many cellular processes including carcinogenesis (Carthew 2006). Many miRNAs have been identified as oncogenic miRNAs or tumor suppressors over the past decade (Grammatikakis et al. 2013). The differentially expressed miRNAs contribute to enable the cancer traits including miR-21, the cluster miR-17-92, miR-155, miR-193a, miR-221, miR-222 and *etc.* (Gonzales et al. 2009; Jaiswal et al. 2012; Wahdan-Alaswad 2014). Recently, many studies have focused on miRNAs expression profiling in liver cancer. Some specific miRNAs were associated with clinic pathological features of cancer (Huang and He 2011). For example, the expressions of miR-151, miR-221/222, and miR-143 were upregulated in liver cancer cells, while miR-122, miR-203, and miR-124 were downregulated (Ding et al. 2010; Furuta et al. 2010; Gramantieri et al. 2007; Gramantieri et al. 2009; Zhang et al. 2009). In numerous studies, miR-152 has been proved to influence cell behaviors of ovarian cancer, prostate cancer, neuroblastoma and *etc*. (Lefever et al. 2009; Zhou et al. 2012; Zhu et al. 2013). However, the role of miR-152-5p in liver cancer has not been reported.

In this study, we focused on the effect of miR-152-5p on liver cancer cells, and detected the relationship between miR-152-5p expression and cell proliferation and apoptosis via differential expression of miR-152-5p *in vitro*. The effect of miR-152-5p on apoptosis related factors was also investigated. We also explored the potential relevant mechanism by which miR-152-5p regulated the expression of fork head transcription factor O (FOXO) as a tumor suppressor factor which is also involved in cancer cell proliferation and apoptosis.

2. Investigations and results

2.1. Efficient overexpression and inhibition efficiency of miR-152-5p in HepG2 and MHCC97 cells

In this study, the miR-152-5p mimic, inhibitor or scramble controls were transfected into HepG2 and MHCC97 cells, respectively (Fig. 1) expression of miR-152-5p was effectively inhibited by miR-152-5p inhibitor ($P < 0.05$), and also been significantly increased after transfected with miR-152-5p mimic ($P < 0.01$). It suggested that miR-152-5p expression in these two cells could be effectively regulated by intracellular transfection.

2.2. Overexpression of miR-152-5p inhibited HepG2 and MHCC97 cells viability and promoted apoptosis

The effect of miR-152-5p on cell viability was detected by MTT assay. The results shown in Figs. 2 A and 2B suggest that both

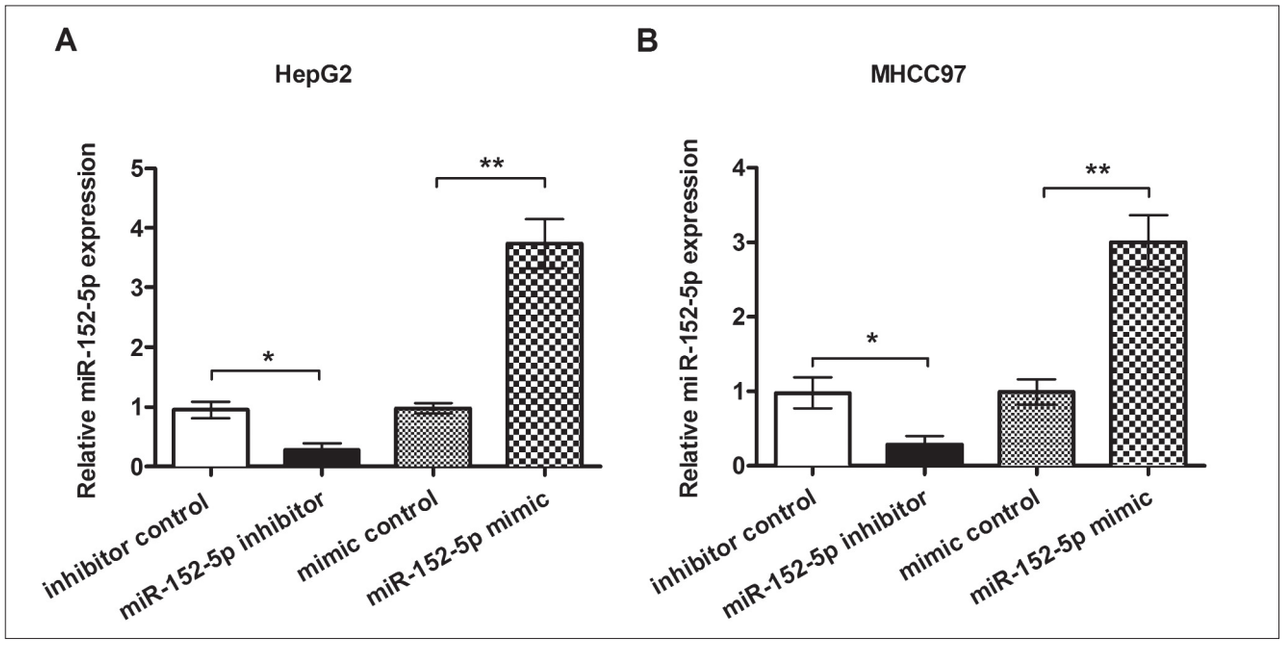

Fig. 1: Effects of transfection on miR-152-5p expression in HepG2 (A) and MHCC97 (B) cells. The mRNA levels of miR-152-5p expression in miR152-5p mimic or inhibitor transfected cells were measured by qPCR. U6 acted as an internal control.
qPCR, quantitative polymerase chain reaction; *, P < 0.05; **, P < 0.01.

HepG2 and MHCC97 cells viability were increased after been transfected with miR-152-5p inhibitor ($P < 0.05$). While after been treated with miR-152-5p mimic, cell viability was reduced in both two kinds of cells ($P < 0.05$). This indicated that the expression of miR-152-5p was negatively correlated with HepG2 and MHCC97 cells viability.

Flow cytometry analysis results (Figs. 2 C and D) showed that the apoptosis of both HepG2 and MHCC97 cells were significantly increased by overexpression of miR-125-5p ($P < 0.01$, or $P < 0.001$). Transfection of miR-152-5p inhibitor showed no significant effect on apoptosis of both two kinds of cells. Therefore, overexpression of miR-152-5p could improve apoptosis of both HepG2 and MHCC97 cells.

2.3. Overexpression of miR-152-5p inhibited cell migration and invasion of HepG2 and MHCC97 cells

We detected the migration and invasion of HepG2 and MHCC97 cells after miR-transfection by Transwell assay. In Figs. 3 A and 3B, the migration of these two kind of cells were increased after miR-152-5p inhibitor transfection ($P < 0.05$), while in miR-152-5p mimic treated groups, the migration of cells was reduced both in HepG2 and MHCC97 cells ($P < 0.05$). The same situation accorded in invasion analysis (Figs. 3 C and D). After been treated with miR-152-5p inhibitor, the invasion of both two kinds of cells was significantly improved ($P < 0.05$), and miR-152-5p mimic transfection suppressed cell invasion ($P < 0.05$). Therefore, miR-152-5p expression was negatively related with cell migration and invasion of HepG2 and MHCC97 cells.

2.4. Expression of miR-152-5p affected apoptosis-related factors expression

To detect the protein expressions of FOXO and apoptosis-related factors in miR-transfected cells, Western blot assay results showed that in miR-152-5p inhibitor treated groups, the expressions of FOXO and Bax were decreased in both HepG2 and MHCC97 cells, while they were increased in miR-152-5p mimic transfection groups (Figs. 4 A and B). Meanwhile, the expression of Bcl-2 showed the opposite trend, miR-152-5p inhibitor transfection increased the Bcl-2 expression and miR-152-5p overexpression suppressed Bcl-2 expression. For caspase 3, the cleaved caspase 3 was only expressed in HepG2 and MHCC97 cells after been transfected with miR-152-5p mimic, and no expression was found in other groups. The results suggested that overexpression of miR-152-5p was positively related with FOXO expression, and associated with apoptosis-related factors to promote cell apoptosis.

2.5. Overexpression of miR-152-5p upregulated the FOXO expression via the JNK signal pathway

We used SP600125 as the inhibitor of JNK, which could inhibit the phosphorylation of JNK, to explore the possible mechanism of miR-152-5p affect the expression of FOXO. As shown in Fig. 5, in both HepG2 and MHCC97 cells after miR-152-5p mimic transfection, the expression of p-JNK1, p-JNK2, p-c-Jun and FOXO were increased. However, after the cells treated with miR-152-5p mimic combined with SP600125, the expression of these proteins were obviously decreased. It signified that SP600125 inhibited the effect of miR-152-5p mimic on the up-regulation of FOXO expression by inhibiting the phosphorylation of JNK and c-Jun. It suggested that miR-152-5p affected FOXO expression by regulating the expression of related proteins in JNK signaling pathway.

3. Discussion

The prognosis and survival rates of liver cancer might be significantly improved in cases of been early diagnosed. Currently, the finding of early biomarkers and drug targets as well as novels therapeutic intervention strategies aroused considerable attention (Temirak et al. 2012). Many studies indicated that some miRNAs, such as miR-199, miR-151, miR-23b and *etc*. were abnormal expressed in liver disease and could be potential biomarkers (Hou J 2011; Murakami et al. 2006; Ura et al. 2009). In this study, we assessed the regulation of miR-152-5p on hepatoma cell growth *in vitro* and found that overexpression of miR-152-5p inhibited cell viability, migration and invasion, also promoted apoptosis in HepG2 and MHCC97 cells. In addition, we confirmed that miR-152-5p positively regulated the expression of FOXO via regulation of the JNK signaling pathway.

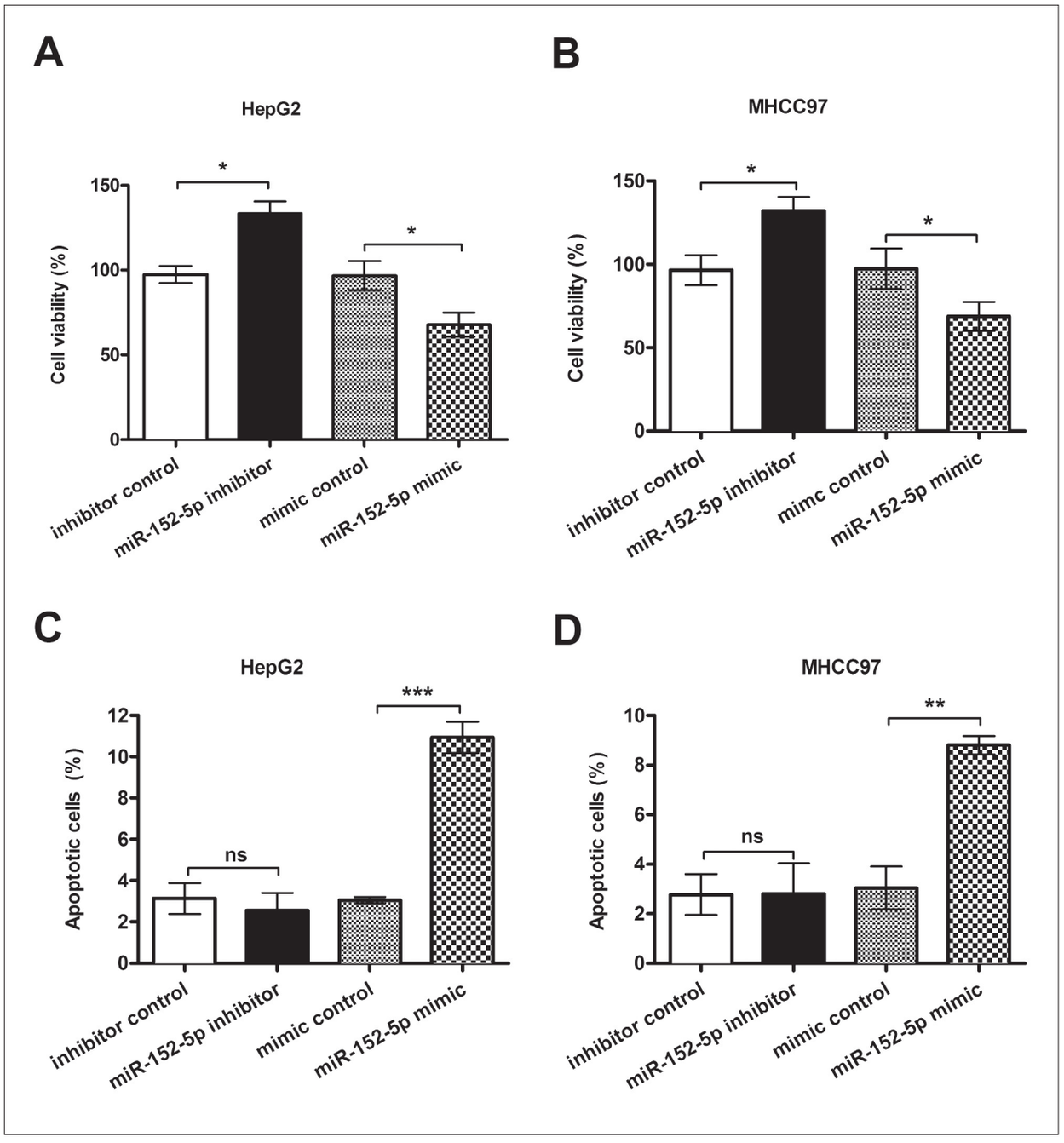

Fig. 2: Overexpression of miR-152-5p suppressed cell viability while promoted apoptosis of HepG2 and MHCC97 cells. miR-152-5p mimic, inhibitor and their controls were transfected into cells, respectively. The transfected cells viability were determined by CCK-8 assay in HepG2 cells (A) and MHCC97 cells (B). The apoptotic cells rat was detected by fly cytometry in HepG2 cells (C) and MHCC97 cells (D).
CCK-8, Cell Counting Kit-8; ns, no statistical differences; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$

MicroRNAs (miRNAs), short non-coding RNAs, play a major role in many fundamentally important biological processes (Hummel et al. 2010). miRNA expression is highly specific for the tissue and the developmental stage via suppressing the expression of protein-encoding genes at posttranscriptional level (Brennecke et al. 2005). The possible relationship between miRNA expression and tumor development attracted growing attentions. miR-152, a member of the miR-148/152 family, was aberrantly expressed in various types of cancer (Zhou Zhao, 2012) and might be a tumor suppressor which associated with cell proliferation, migration and invasion in human cancer by regulating its target genes, in ovarian, endometrial and breast cancer (Azizi et al. 2014; Xiang et al. 2014; Xu et al. 2013). A recent study has investigated the inhibitory effects of miR-152 in cell proliferation and colony formation of liver cancer stem cells CD133 and Hep3B (Huang et al. 2015). However, only few studies have reported the association between miR-152-5p and liver cancer. The potential functions of miR-152-5p and its correlation with liver cancer needed further studies. In our studies, we found that overexpression of miR-152-5p in both HepG2 and MHCC97 cells could efficiently inhibit cell viability, migration and invasion, and promote apoptosis of these cells. It suggested that miR-152-5p might be a tumor suppressor that could regulate the cell proliferation and promote apoptosis of liver cancer cells.
Our results also showed that FOXO expression was upregulated by overexpression of miR-152-5p in the HepG2 and MHCC97 cells, and the expression of apoptosis-related factors were also influenced. FOXO has been confirmed to activate caspase and induced apoptosis through various pathways such as Fas, Bcl-2 and *etc*.

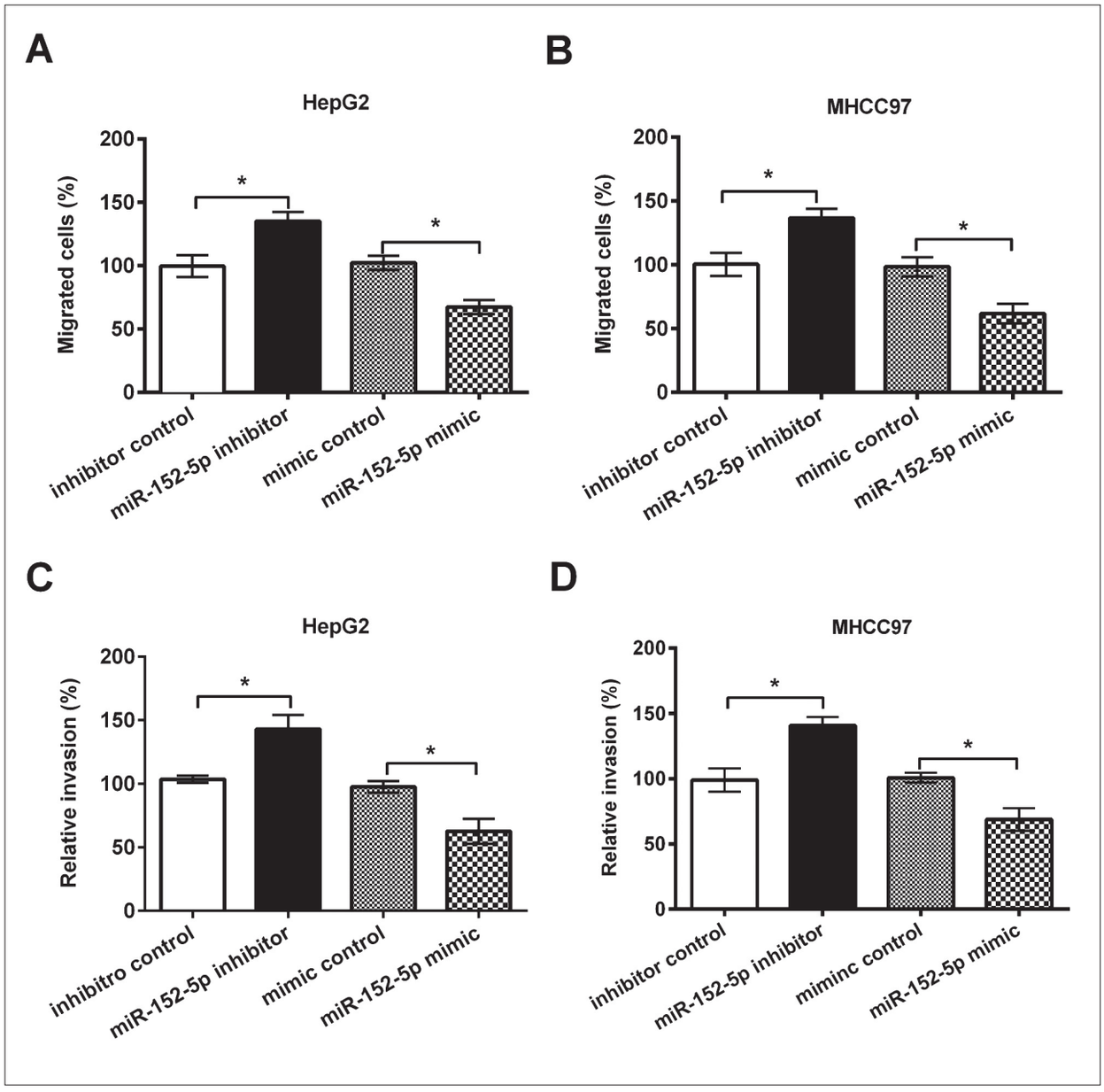

Fig. 3: Overexpression of miR-152-5p suppressed cell migration and invasion in both HepG2 and MHCC97 cells. The cells were transfected with miR-152-5p mimic, inhibitor or control. (A and B) The migration of transfected cells were measured by Transwell assay. (C and D) The invasion of transfected cells were measured by Transwell assay using matrigel invasion chamber. *, $P < 0.05$.

(Tanaka-Nakanishi 2014). In our study, miR-152-5p mimic transfection reduced the expression of Bcl-2 which was an important anti-apoptotic protein encoded by pro-oncogene and regulated cell death, while increased the expression of Bax that could antagonized the protective effect of Bcl-2 and made cells tend to die. In addition, cleaved caspase 3 was only significantly expressed in the miR-152-5p mimic treated group. All these results suggested that upregulation of FOXO by miR-152-5p mimic transfection activated caspase 3 and induced liver cancer cell apoptosis through the Bcl-2 apoptotic pathway.

FOXO, a tumor suppressor factor, plays a key role in cell apoptosis via regulation of multiple downstream target genes (Weidinger et al. 2008). It has been reported that FOXO was an important factor between PI3K/AKT and MAPK/ERK signaling pathways in tumor cells and was involved in cell proliferation and apoptosis (Roy et al. 2010). In our studies, although miR-152-5p overexpression could significantly increase FOXO protein expression, FOXO expression was significantly lower than that in the normal group once the JNK pathway was inhibited by JNK inhibitor SP600125, even in the presence of miR-152-5p mimic. Therefore, we inferenced that miR-152-5p regulated FOXO expression through the JNK signaling pathway in both HepG2 and MHCC97 cells.

In conclusion, the results of this study suggested that miR152-5p inhibited liver cancer cell proliferation and inducted apoptosis which was related with FOXO expression. So we speculated that miR-152-5p might be a tumor suppressor in liver cancer and hope our preliminary explorations could provide basic data support and a novel direction for further researches. Nevertheless, further investigations are needed to explore the deep and exhaustive mechanisms of miR-152-5p effect on liver cancer which might provide a novel therapeutic strategy for the treatment of liver disease.

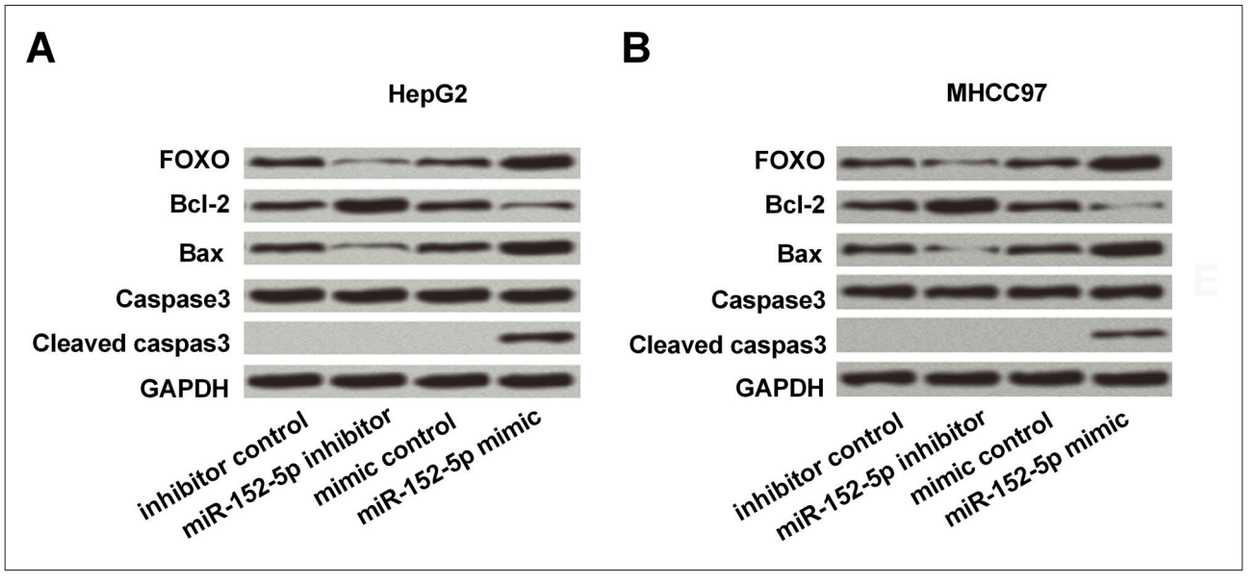

Fig. 4: miR-152-5p overexpression upregulated the expression of FOXO and activated apoptotic pathway. HepG2 and MHCC97 cells were transfected with miR-152-5p mimic, inhibitor or control. The protein expression levels of FOXO, Bcl-2, Bax, Caspase 3 and cleaved Caspase 3 were detected by western blot assay. (A)Western blot result of HepG2 cells. (B) Western blot result of MHCC97 cells. GAPDH acted as an internal control.
FOXO, fork head transcription factor O; Bcl-2, B cell lymphoma-2; Bax, BCL2-Associated X.

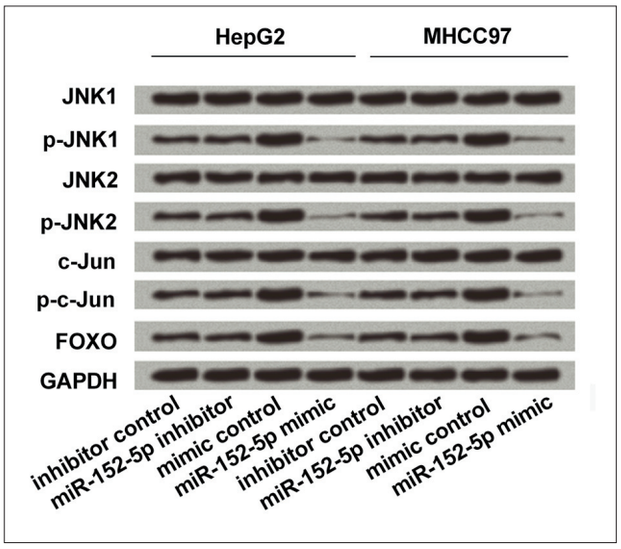

Fig. 5: miR-152-5p upregulated FOXO expression through the JNK signaling pathway. HepG2 and MHCC97 cells were transfected with miR-152-5p mimic alone or combined with SP600125. The protein expression level of p/t JNK1 or 2, p/t c-Jun and FOXO were detected by western blot. GAPDH acted as an internal control.
FOXO, fork head transcription factor O; JNK, c-Jun N-terminal kinase; p-c-Jun, phosphorylated c-Jun; SP600125; the JNK inhibitor.

4. Experimental

4.1. Cell culture and transfection

The human hepatoma cell lines HepG2 and MHCC97 were obtained from Cell Resource Center of Shanghai Institutes for Biological Sciences (Shanghai, China). The cells were cultured in high glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% feral bovine serum (FBS) (Gibco, Carlsbad, CA, USA) at 37 °C in an humidified incubator with 5% CO_2.
The cells were seeded in 6-well plat at 5×10^4 per well and incubated for 24 h at 37 °C. After the cells grown to about 70% confluence, miR152-5p mimic, miR-152-5p inhibitor or control were transfected into cells, respectively. The transfection was performed by using Lipofectamine 3000 reagent (Invitrogen, USA), according to the manufacturer's instructions (Jo et al. 2014). The miR152-5p mimic, miR-152-5p inhibitor and control were synthesized by GenePharma (Shanghai, China).

4.2. Cell viability assay

After miR-transfection, cells were seeded into 96-well plate with 5×10^3 per well and pre-cultured for the following test. Cell viability was determined using Cell Counting Kit-8 (CCK-8, Dojindo, Kumamoto Prefecture, Kyushu, Japan), according to the manufacturer's instruction. In brief, 48 h after transfection, 10 µl of CCK was added in each well and cells in 96-well plat were incubated for another 4 h at 37 °C. The absorbance at 450 nm was measured by a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA).

4.3. Apoptosis assay

Cell apoptosis analysis was performed using Annexin V-FITC- PI apoptosis detection kit (Beijing Biosea Biotechnology, Beijing, China) according to the manufacturer's instruction. The miR-transfected cells were collected and suspended in 200 µl of binging buffer containing 10 µl Annexin V-FITC and 5 µl PI, then incubated for 1 h at room temperature in the dark. Apoptotic cells were measured with Flow cytometry analysis by using a FACS can (Beckman Coulter, Fullerton, CA, USA) (Zhang et al. 2013).

4.4. Cell migration and invasion assay

Cell migration was determined by using a modified two-chamber Transwell culture chamber with pore size of 8.0 µm (Greiner 662638). The transfected cells were suspended in 200 µl of serum-free culture medium and added into the upper compartment of 24-well culture chamber. Then 600 µl complete medium was added into the lower compartment, the chamber was incubated at 37 °C for 12 h. After that, cells were fixed with methanol (4%, NIST, USA) for 30 min, non-traversed cells were removed from the upper surface of the filter with a cotton swab carefully. Traversed cells in the lower were stained with 0.1% crystal violet for 20 min and counted under microscope (Leica Microsystems, Wetzlar, Germany).
The invasion behavior of transfected cells was determined in a 24-well BD BioCoat™ Matrigel™ Invasion Chamber with 8.0 µm pore size polycarbonate filters membranes (BD, Biosciences,). In brief, after cells were treated with miR-transfection, 5×10^4 cells in 200 µl serum-free DMEM medium were added into the upper of chambers, while completed culture medium with was added into the lower chamber. After been incubated for 48 h, the non-invaded cells were removed with a cotton swab; the invaded cells were fixed in methanol and stained with crystal violet solution. Cells were counted under a microscope (Leica Microsystems, Wetzlar, Germany) (Walter et al. 2009).

4.5. Quantitative polymerase chain reaction (qPCR)

Total RNA was extracted from cells using Trizol reagent (Life Technologies Corporation, Carlsbad, CA, USA) and RNA quality was evaluated according to the manufacturer's instructions. The reversed transcription and qPCR analysis were performed by Taqman MicroRNA Reverse Transcription Kit and Taqman Universal Master Mix II (Biosystems, Foster City, CA, USA) to test the expression level of miR-152-5p in the transfected cells. The qPCR was performed in triplicate, including no template control. The miR-152-5p expression was normalized to U6 using $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). All primers were synthesized by Gene Pharma (Shanghai, China). The Date were analyzed by Real-Time Stat Miner (Integromics).

4.6. Western blot

The proteins expressed in miR-treated cells were extracted using PIPA lysis buffer (Beyotime Biotechnology, Shanghai, China) supplemented with protease inhib-

itors (Roche, Guangzhou, China). All the protein simples were quantified by the BCA™ Protein Assay Kit (Pierce, Appleton, WI, USA). Equal amounts of simples were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes. The membranes were incubated at 4 °C overnight with primary antibodies (all 1:1000): FOXO1A (ab12161), B cell lymphoma-2 (Bcl-2, ab32124), BCL2-Associated X (Bax, ab70407), caspase 3 (ab13847), cleaved-caspase 3 (ab49822) c-Jun N-terminal kinase (JNK, ab179461), phosphorylated JNK (ab76572), c-Jun (ab32137), phosphorylated c-Jun (p-c-Jun, ab32385), GAPDH (ab8245) (Abcam, USA). Then the membranes were washed and incubated with horseradish peroxidase-conjugated (HRP) secondary antibodies (1:5000, Abcam, USA) for 1 h at room temperature. The signals of blots were captured and the intensity of the bands was quantified by Image Lab™ Software (Bio-Rad, Shanghai, China).

4.7. Statistical analysis

All experiments were repeated at least three times in triplicate. The results of multiple experiments were presented as the mean±standard deviation (SD), and date were analyzed by using GraphPad Prism 6 software (GraphPad Software, La Jolla, CA, USA). Student's t-test was used for pairwise comparisons, one-way analysis of variance (ANOVA) was used for multiple group comparisons. P value of < 0.05 was considered to indicate a statistically significant result.

Conflicts of interest: All authors declare that they have no conflict of interests and financial. There is no funding to support the work.

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