European Review for Medical and Pharmacological Sciences 2020; 24: 12144-12152

MicroRNA-30a-3p inhibits malignant progression of hepatocellular carcinoma through regulating IGF1

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Abstract. – OBJECTIVE: The purpose of this study was to investigate the expression level of microRNA-30a-3p in hepatocellular carcinoma (HCC), and to further study its relationship with HCC clinical parameters and prognosis and the underlying mechanisms.

PATIENTS AND METHODS: Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) was performed to examine microRNA-30a-3p level in 44 tumor tissue specimens and paracancerous normal ones collected from HCC patients, and the interplay between microRNA-30a-3p expression and clinical indicators, as well as prognosis of HCC patients was analyzed. Meanwhile, qPCR was also used to further verify microRNA-30a-3p expression in HCC cell lines. In addition, microRNA-30a-3p overexpression and knockdown models were constructed in HCC cell lines, and the impacts of microRNA-30a-3p on HCC cell functions was evaluated by cell counting kit-8 (CCK-8), transwell and cell wound healing assays. Finally, the Luciferase reporting assay was conducted to uncover the underlying mechanism.

RESULTS: In this study, qRT-PCR results showed that the expression level of microRNA-30a-3p in tumor tissues of HCC patients was markedly lower than that in adjacent ones. Compared with patients with high expression of microRNA-30a-3p, the patients with low expression of microRNA-30a-3p had a higher incidence of lymphatic or distant metastasis and a lower overall survival rate. In the Bel-7402 cell line, the proliferation, invasion, and metastasis ability of HCC cells were decreased markedly after microRNA-30a-3p overexpression, while in Hep3B cell line, knockdown of microRNA-30a-3p enhanced the cell proliferation and invasion capacity. In addition, Luciferase reporting assay demonstrated that microRNA-30a-3p could specifically bind to IGF1. Furthermore, Western Blot results also verified a reduced expression of IGF1 after overexpression of microRNA-30a-3p, and an elevated one after knockdown of microRNA-30a-3p. Finally, cell recovery experiment verified that microRNA-30a-3p and IGF1 may regulate each other and thereby together inhibit the malignant progression of HCC.

CONCLUSIONS: MicroRNA-30a-3p expression is significantly decreased in HCC tumor tissue samples, which is associated with lymph node or distant metastasis rate, as well as the poor prognosis of HCC. In addition, this research suggests that microRNA-30a-3p may inhibit the malignant progression of HCC by regulating IGF1.

Key Words:
MicroRNA-30a-3p, IGF1, Hepatocellular carcinoma, Malignant progression.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies of the digestive system in humans[1-3]. At present, the incidence of liver cancer is increasing worldwide, ranking 5th in the global incidence of malignant tumors. There are about 560,000 new liver cancer patients every year, among which more than 50% occur in China[4,5]. HCC is characterized by insidious onset, high degree of malignancy, rapid progress, poor prognosis and strong aggressiveness, and its mortality is extremely high[6,7]. Globally, it ranks third in the cause of malignant tumor death, second only to lung cancer and gastric cancer, and its five-year survival rate is less than 5%[7]. Therefore, seeking effective treatment methods for liver cancer and measures to prevent metastasis and recurrence is of great significance to improve the treatment efficiency of liver cancer[8,9].

MicroRNA (miRNA) is a non-coding single-stranded small-molecule RNA found in eukaryotes in recent years, which plays a post-transcriptional regulatory role[10,11]. Currently, miRNAs are believed to directly regulate the expression of more than one-third of the genes in the genome and play a pivotal role in the life activities such as stem

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cell maintenance, cell differentiation, proliferation, apoptosis, metabolism, embryonic development, and immune response[11,12]. Meanwhile, the abnormal expression of miRNA is closely related to the occurrence and development of many diseases including cancer, cardiovascular disease and viral infection[13,14]. Fu et al[15] and Tutar et al[16] have shown the changes in the expression levels of miRNAs in various tumors, which can play an essential role in tumorigenesis and development by regulating the expression of corresponding oncogenes and tumor suppressor genes. In recent years, with the continuous discovery and in-depth research of new miRNA molecules, the great potential of some miRNA in the occurrence of HCC has been gradually recognized[17,18]. Recently, microRNA-30a-3p has been indicated as one of the miRNAs with low expression in HCC cancer tissues. Furthermore, bioinformatics analysis revealed that IGF1 is a possible target gene of microRNA-30a-3p.

Insulin like growth factor (IGF) is a crucial regulatory factor related to growth and development in the body, which can participate in embryonic development, glucose metabolism, lipid metabolism, bone development, atherosclerosis, myocardial infarction, and cardiovascular diseases[19]. IGF1 is a small molecule of 7,500 kDa single chain polypeptide composed of 70 amino acids and is one of the main members of the IGF family[20]. It is engaged in the development of bone, new blood vessels and nervous system, and plays a vital regulatory role in cancers[21,22]. In this study, quantitative Real Time- Polymerase Chain Reaction (qRT-PCR) was performed to examine microRNA-30a-3p and IGF1 expressions in tumor tissue specimens and paracancerous ones of HCC patients, and *in vitro* as well as *in vivo* experiments were designed to explore the influences of microRNA-30a-3p on the growth and invasion ability of HCC cells, uncovering that microRNA-30a-3p may affect the molecular biological behavior of HCC cells by targeting IGF1 gene and its downstream signaling pathway.

Patients and Methods

Patients and HCC Samples

The surgically resected tumor tissue samples and corresponding adjacent ones were collected from 44 HCC patients, who had not accepted any anti-tumor therapy such as radiotherapy or chemotherapy before surgery. According to the 8th edition of UICC/AJCC liver cancer tumor node metastasis (TNM) staging criteria, all patients were diagnosed with HCC by postoperative pathological analysis. This investigation had been approved by the Ethics Oversight Committee, and patients and their families had been fully informed that their specimens would be used for scientific research. This study was conducted in accordance with the Declaration of Helsinki.

Cell Lines and Reagents

Six human HCC cells (Bel-7402, HepG2, MHCC44H, SMMC-7221, Huh7, Hep3B) and one human normal liver cell line (LO2) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA), and Dulbecco's Modified Eagle's Medium (DMEM) medium and fetal bovine serum (FBS) were purchased from American Life Technologies. The cell was cultured in a DMEM high glucose medium containing 10% FBS, penicillin (100 U/mL), and streptomycin (100 μg/mL) in a 37°C, 5% CO_2 incubator. When the cells grew to 80%-90% confluence, they were digested with 1×trypsin+EDTA (ethylenediaminetetraacetic acid).

Transfection

The control group (NC or Anti-NC) and microRNA-30a-3p (microRNA-30a-3p or Anti-microRNA-30a-3p) containing the microRNA-30a-3p lentiviral sequence were purchased from Shanghai Jima Company (Shanghai, China). Cells were plated in 6-well plates and grew to a cell density of 30%-40%, and then lentiviral transfection was performed according to the manufacturer's instructions. After 48 h, cells were collected for qRT-PCR analysis and cell function experiments.

Cell Counting Kit-8 (CCK-8) Assay

The cells after 48 h of transfection were collected and plated into 96-well plates at 2000 cells per well. After cultured for 24 h, 48 h, 72 h, and 96 h respectively, 10 μL of CCK-8 solution (Dojindo Molecular Technologies, Kumamoto, Japan) was added per well for incubation for 2 h, and then, the optical density (OD) value of each well was measured in the microplate reader at 490 nm absorption wavelength.

Transwell Assay

After transfection for 48 h, the cells were trypsinized and resuspended in serum-free medium. After cell counting, the diluted cell density was adjusted to 5.0×10^5/ml, and 200 μL (1 x 10^5 cells) of the cell suspension was added in the upper chamber, and 700 μL of a medium containing 20%

FBS was added to the lower chamber. After incubated in a 37°C incubator for 48 h, the chamber was removed, fixed with 4% paraformaldehyde for 30 min, and stained with 0.2% crystal violet for 15 min. Subsequently, cells were washed with phosphate-buffered saline (PBS), and the inner surface of the basement membrane of the chamber was carefully cleaned to remove the inner layer cells. Finally, the perforated cells stained in the outer layer of the basement membrane of the chamber were observed under the microscope, and 5 fields of view were randomly selected.

Cell Wound Healing

After transfection for 48 h, cells were digested, centrifuged and resuspended in medium without FBS to adjust the density to 5×10^5 cells/mL. The density of the plated cells was determined according to the size of the cells (the majority of the number of cells plated was set to 50000 cells/well), and the confluency of the cells reached 90% or more the next day. After the stroke, cells were rinsed gently with PBS for 2-3 times and observed again after incubation in low-concentration serum medium for 24 h.

QRT-PCR

The total RNA was extracted from HCC cell lines and tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and RNA was reversely transcribed into cDNA using Primescript RT Reagent (TaKaRa, Otsu, Shiga, Japan). QRT-PCR was performed using SYBR® Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan) and StepOne Plus Real-time PCR System (Applied Biosystems, Foster City, CA, USA). The following primers were used for qPCR reaction: microRNA-30a-3p: forward: 5'-CGCTTTCAGTCGGATGTTTG-3', reverse: 5'-GTGCAGGGTCCGAGGT-3'; U6: forward: 5'-CTCGCTTCGGCAGCACA-3', reverse: 5'-AACGCTTCACGAATTTGCG-3'; IGF1: forward: 5'-ACTGAGCTCTGATGAGTTAATGTGCAACC-3', reverse: 5'-ACTCTCGAGCCTCTGATCCTTGAGGTGA-3'; β-actin: forward: 5'-CCTGGCACCCAGCACAAT-3', reverse: 5'-TGCCGTAGGTGTCCCTTTG-3'. Data analysis was performed using ABI Step One software (Applied Biosystems, Foster City, CA, USA) and the relative expression levels of mRNA were calculated using the 2-ΔΔCt method.

Western Blot

The transfected cells were lysed using cell lysis buffer, shaken on ice for 30 min, and centrifuged at 14,000 × g for 15 min at 4°C. Total protein concentration was calculated by bicinchoninic acid (BCA) Protein Assay Kit (Pierce, Rockford, Il, USA). Then, the extracted proteins were separated using a 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and subsequently transferred to a polyvinylidene difluoride membrane (PVDF; Millipore, Billerica, MA, USA). After that, Western blot analysis was performed according to standard procedures. The primary antibodies against IGF1 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and the secondary antibodies anti-mouse and anti-rabbit, were all purchased from Cell Signaling Technology (Danvers, MA, USA).

Dual-Luciferase Reporter Assay

HCC Bel-7402 and Hep3B cells were seeded in 24-well plates and co-transfected with microRNA-30a-3p mimic/NC and pMIR Luciferase reporter plasmids. A reporter plasmid was constructed in which a specific fragment of the target promoter was inserted in front of the Luciferase expression sequence. The plasmid was then introduced into the cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. After 48 h of transfection, the reporter Luciferase activity was normalized to control.

Statistically Analysis

Statistical analysis was performed using GraphPad Prism 5 V5.01 software (La Jolla, CA, USA). Differences between two groups were analyzed by using the Student's *t*-test. Comparison between multiple groups was done using One-way ANOVA test followed by post-hoc test (Least Significant Difference). Kaplan-Meier method followed by the log-rank test were used to compare the survival curves. Independent experiments were repeated at least three times for each experiment and data were expressed as mean ± standard deviation. $p<0.05$ was considered statistically significant.

Results

MicroRNA-30a-3p was Lowly Expressed in HCC Tissues and Cell Lines

To determine the role of microRNA-30a-3p in HCC, a total of 44 pairs of tumor tissue samples and paracancerous ones of HCC patients were collected and microRNA-30a-3p expression was analyzed by qPCR. The results showed that microR-

NA-30a-3p level was lower in tumor tissues than that in adjacent tissues (Figure 1A), suggesting that microRNA-30a-3p may act as a tumor suppressor in HCC. At the same time, immunohistochemical analysis revealed the low expression of microRNA-30a-3p in tumor tissues of HCC patients (Figure 1B). In addition, in the commonly used HCC cell lines, microRNA-30a-3p level was also examined. Among them, Bel-7402 cell line had the lowest while Hep3B had the highest microRNA-30a-3p expression (Figure 1C), so they were selected for subsequent experiments.

MicroRNA-30a-3p Expression was Correlated with Lymph Node and Distance Metastasis and Overall Survival in HCC Patients

According to the qPCR results, the above-mentioned tissue specimens were divided into two groups, namely, the high-microRNA-30a-3p expression group and the low-microRNA-30a-3p expression group, and the interplay between microRNA-30a-3p level and the age, gender, pathological stage, lymph node or distant metastasis HCC patients was analyzed by Chi-square test. As shown in Table I, low expression of microRNA-30a-3p was positively correlated with HCC lymph node or distant metastasis, but not with other indicators. In addition, to explore the interplay between microRNA-30a-3p expression and prognosis of HCC, Kaplan-Meier survival curve was plotted, which uncovered that low expression of microRNA-30a-3p was markedly relevant to poor prognosis of HCC patients. In other words, the lower the microRNA-30a-3p, the worse the prognosis ($p<0.05$; Figure 1D).

MicroRNA-30a-3p Inhibited HCC Cell Migration and Invasion

To explore the impact of microRNA-30a-3p on the invasiveness and migration ability of HCC cells, microRNA-30a-3p overexpression and knockdown models were first successfully constructed, and performed CCK-8, transwell, and cell wound healing

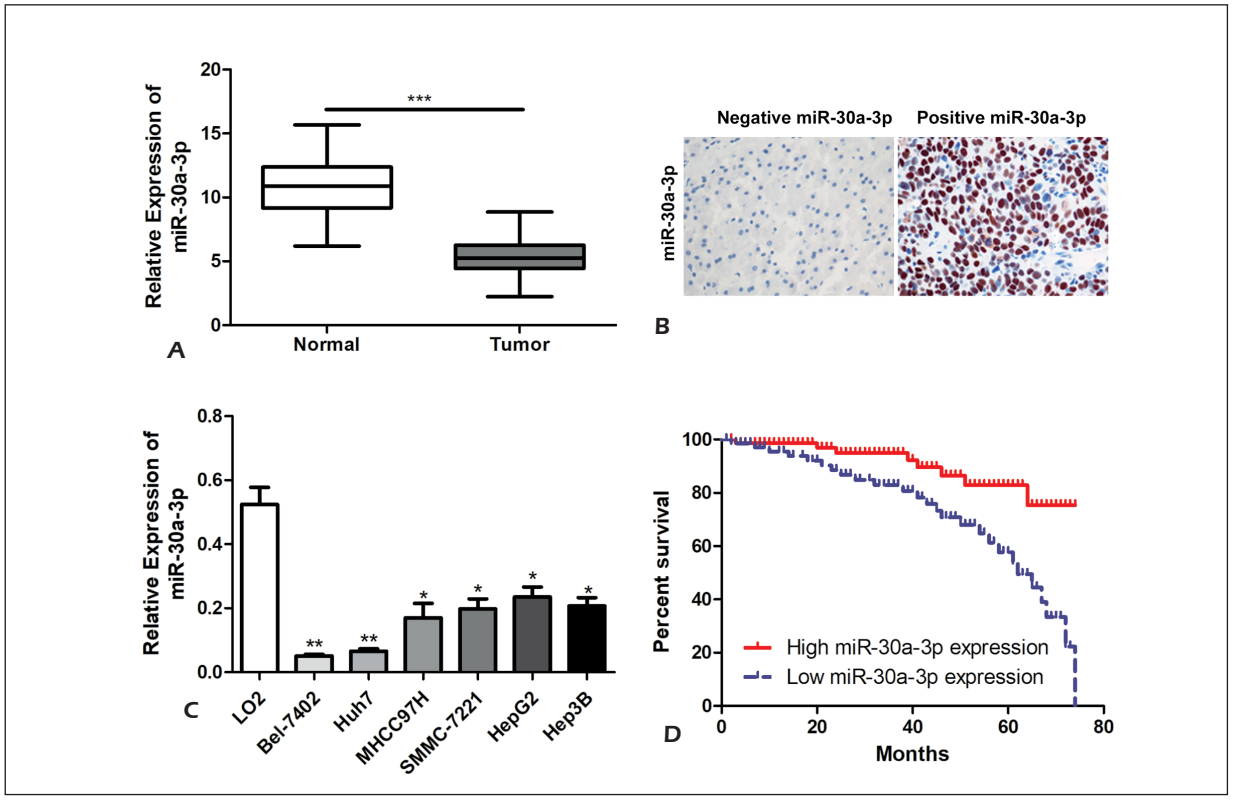

Figure 1. MiR-30a-3p is under expressed in HCC tissues and cell lines. **A**, qRT-PCR is used to detect the differential expression of miR-30a-3p in HCC and adjacent non-tumor tissues. **B**, Immunohistochemistry shows differences in the expression of miR-30a-3p in HCC (magnification: 200×). **C**, qRT-PCR is used to detect the expression level of miR-30a-3p in hepatoma cell lines. D, The Kaplan Meier survival curve of liver cancer patients based on miR-30a-3p expression is shown. The prognosis of patients with high expression of miR-30a-3p is significantly worse than those with low expression. Data are mean ± SD, *$p<0.05$, ** $p<0.01$, *** $p<0.001$.

Table I. Association of miR-30a-3p expression with clinicopathologic characteristics of hepatocellular carcinoma.

Parameters	Number of cases	MiR-30a-3p expression		*p*-value
		High (%)	Low (%)	
Age (years)				0.888
<60	19	11	8	
≥60	25	15	10	
Gender				0.220
Male	22	15	7	
Female	22	11	11	
T stage				0.263
T1-T2	24	16	8	
T3-T4	20	10	10	
Lymph node metastasis				0.009
No	25	19	6	
Yes	19	7	12	
Distance metastasis				0.013
No	33	23	10	
Yes	11	3	8	

assays were subsequently performed (Figure 2A). As shown in Figure 2B-2D, in Bel-7402 cells, overexpression of microRNA-30a-3p markedly attenuated the cell proliferation, invasiveness and metastasis ability, while in Hep3B cell line, compared with the anti-NC group, the ability of HCC cells to proliferate and invade or metastasize was conversely enhanced after knocking down microRNA-30a-3p. These results suggest that microRNA-30a-3p can inhibit HCC cell migration and invasion.

MicroRNA-30a-3p was Bound to IGF1

To further explore the way in which microRNA-30a-3p inhibited the malignant progression of HCC, bioinformatics analysis predicted that there existed a certain interaction between IGF1 and microRNA-30a-3p, which was subsequently verified through Luciferase reporting gene assay (Figure 3A and 3B). Western Blot and qPCR detected that overexpression of microRNA-30a-3p significantly decreased IGF1 expression while knockdown of it enhanced IGF1 expression (Figure 3C). In addition, it was found that HCC tumor tissues contained higher IGF1 expression compared with adjacent tissues (Figure 3D). Meanwhile, qPCR indicated that the expression levels of microRNA-30a-3p and IGF1were negatively correlated in HCC (Figure 3E).

MicroRNA-30a-3p Modulated IGF1 in HCC

To further figure out whether microRNA-30a-3p functioned in HCC through IGF1, IGF1 was upregulated in Bel-7402 cells with microRNA-30a-3p overexpression and downregulated in Hep3B cells with microRNA-30a-3p knockdown, and the co-transfection efficiency was verified by Western blot and qPCR (Figure 4A, 4B). Subsequently, transwell experiment revealed that upregulation or downregulation of IGF1 could offset the enhanced or weakened cell invasive ability induced by overexpression or knockdown of microRNA-30a-3p (Figure 4C and 4D), indicating that microRNA-30a-3p may act through IGF1 in the malignant progression of HCC.

Discussion

HCC is one of the most common malignancies in humans, and its incidence ranks sixth in terms of morbidity and is the third leading cause of cancer-related deaths[1-4]. China is a region with a high incidence of liver cancer, and the number of patients with primary HCC accounts for about 55% of the world, posing a serious threat to the life and health of Chinese people[4,5]. In recent years, great progress has been made in the diagnosis and treatment of liver cancer. However, due to the difficulty in the early diagnosis and the easy recurrence after surgery, patients' prognosis still remains poor[6-8]. Therefore, it is necessary to strengthen the research on the molecular mechanism of liver cancer and find more effective targets for the HCC diagnosis and treatment, so as to improve the early diagnosis rate and efficacy of liver cancer patients[8,9].

MicroRNA is evolutionally conservative and widely exists in animals, plants, fungi, viruses and other organisms[10-13]. The genes encoding miRNAs

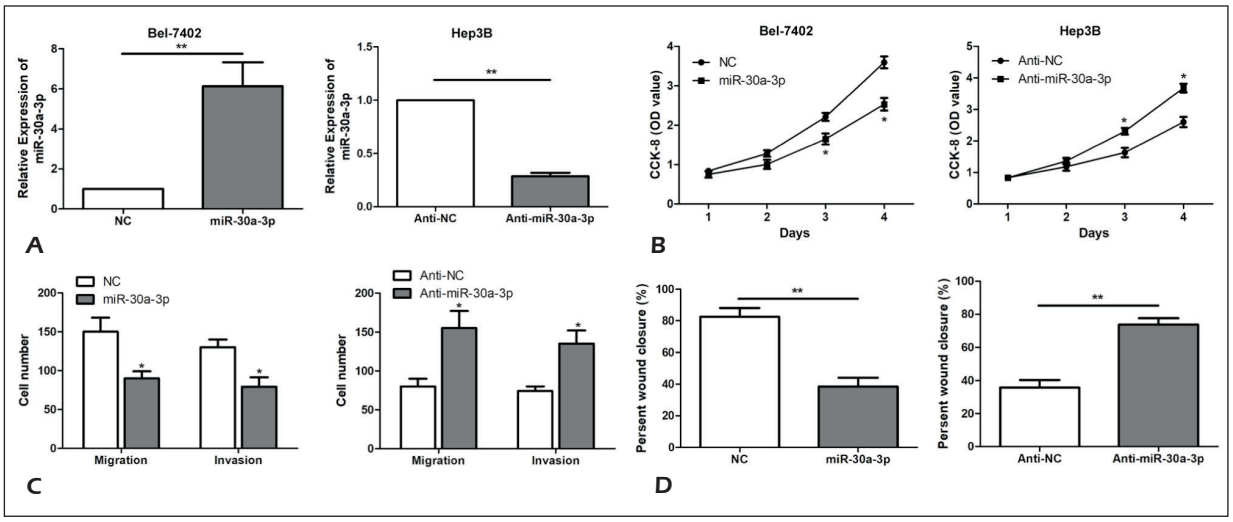

Figure 2. MiR-30a-3p inhibits proliferation, invasion, and migration of HCC cells. **A**, qRT-PCR verifies transfection efficiency after transfection of miR-30a-3p overexpression or knockdown vector in Bel-7402 and Hep3B cell lines. **B**, CCK-8 assay detects the effect of transfection of miR-30a-3p overexpression or knockdown of the vector on the proliferation of hepatoma cells. **C**, Transwell invasion and migration assays are performed to detect the invasion and migration of hepatoma cells after transfection of miR-30a-3p overexpression or knockdown of vectors. **D**, Cell wound healing assay detects the effect of miR-30a-3p overexpression or knockdown of the vector on the cell crawling of Bel-7402 and Hep3B cell lines. Data are mean ± SD, *p<0.05, **p<0.01.

are mostly located in the intron regions of genes or coding genes, and exist in the form of single copy, multiple copies or gene clusters[13,14]. Mature miRNAs mainly pair with incomplete bases of the miRNA regulatory element (MRE) located at the target gene mRNA 3'UTR through seed sequence at its 5 'terminal (2-8 nt) and recognize each other, and achieve post-transcriptional regulation of target gene expression by inhibiting protein translation and triggering mRNA degradation[14,15]. MiRNA with different expression patterns is also closely correlated with tumor classification and prognosis, and its expression patterns can be used as molecular markers for tumor diagnosis and prognosis[15,16]. Therefore, it is of great significance to study the changes of miRNA expression related to the occurrence and progression of HCC and to further uncover the underlying mechanism[17,18]. At present, the abnormal expression of microRNA-30a-3p has been found in a variety of tumors with different organs throughout the body, which may be related to the malignant progression of tumors. In the present study, a large number of clinical specimens of HCC were used for the first time to detect microRNA-30a-3p level at the transcriptional level in fresh HCC surgical specimens and cell lines, and to investigate the role of microRNA-30a-3p in the occurrence and development of HCC. It was found that microRNA-30a-3p level in most HCC tissues and cell lines was reduced to different degrees. Besides, immunohistochemistry was applied to detect microRNA-30a-3p level in paraffin specimens of 44 cases of liver cancer, so as to distinguish the high and low expression of microRNA-30a-3p. It was found that patients with low expression of microRNA-30a-3p have higher incidence of lymphatic metastasis and distant metastasis, and lower overall survival rate. To verify the effect of microRNA-30a-3p on the function of HCC cells, CCK8, transwell and cell wound healing assays were performed and the results revealed that HCC cell proliferation, invasion and metastasis ability were significantly reduced after overexpression of microRNA-30a-3p, while the opposite result was observed after its knockdown. The above experimental results indicate that microRNA-30a-3p can inhibit the development of HCC and perform important functions in HCC. However, the specific mechanism remains elusive.

IGF1, also known as somatomedin for growth, is a single-chain peptide composed of 70 amino acids, which has 50% homologous sequence with insulin and plays a pivotal role in bone, nervous system and tumor, inhibiting cell apoptosis, inducing cell cycle, and promoting angiogenesis and tumor metastasis through its corresponding receptors[19,20]. In this study, it was found that tu-

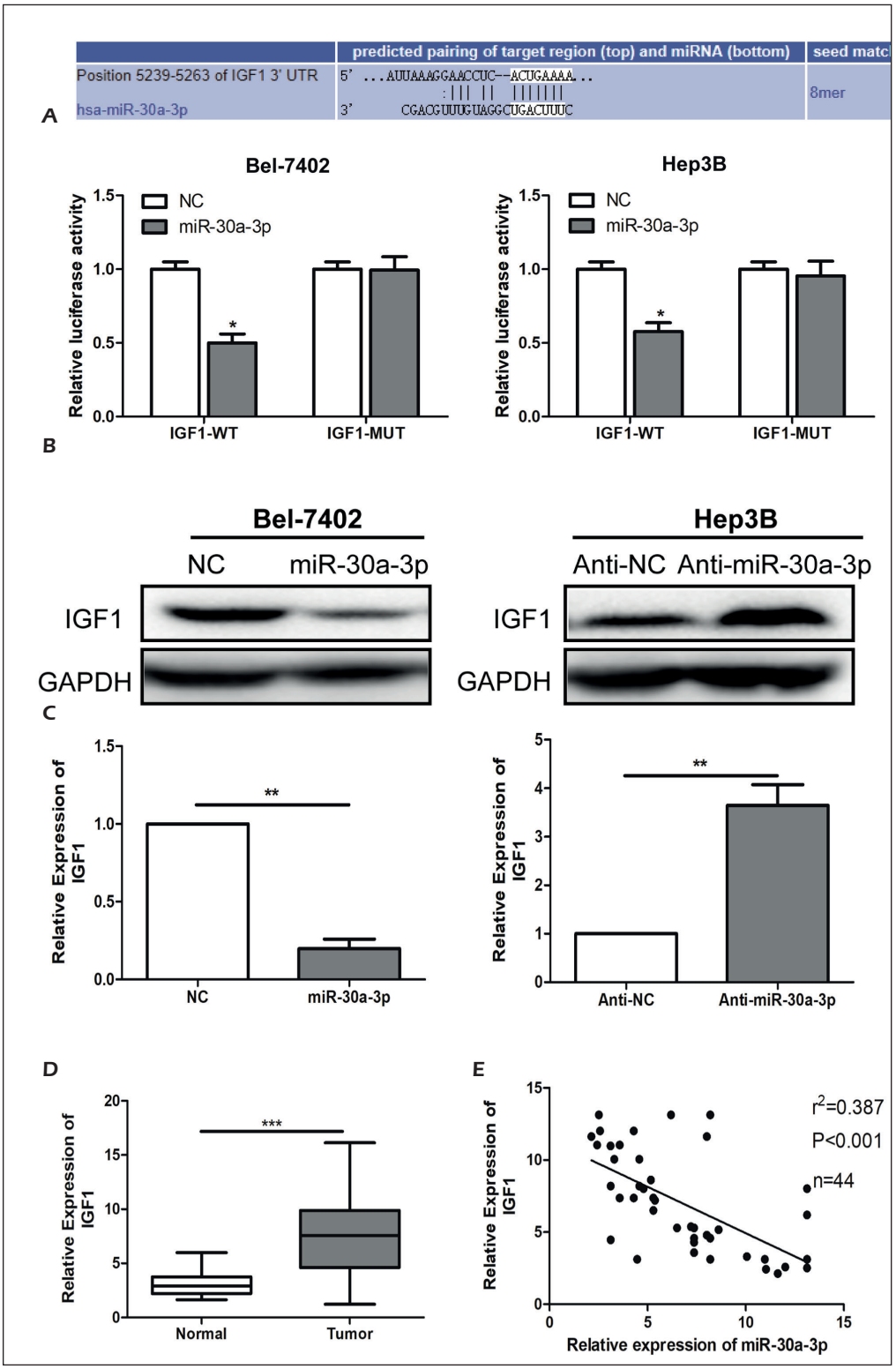

Figure 3. MiR-30a-3p regulates the expression of IGF1. **A**, Schematic diagram of the targeted binding site of miR-30a-3p to IGF1. **B**, Dual-Luciferase reporter gene assay verifies the direct targeting of miR-30a-3p to IGF1. **C**, Western blot and qRT-PCR verifies the expression level of IGF1 after transfection of miR-30a-3p overexpression or knockdown of vectors in Bel-7402 and Hep3B cell lines. **D**, qRT-PCR is used to detect the differential expression of IGF1 in liver cancer tumor tissues and adjacent non-tumor tissues. E, There is a significant negative correlation between miR-30a-3p and IGF1 expression in HCC tissues. Data are mean ± SD, *p<0.05, **p<0.01, ***p<0.001.

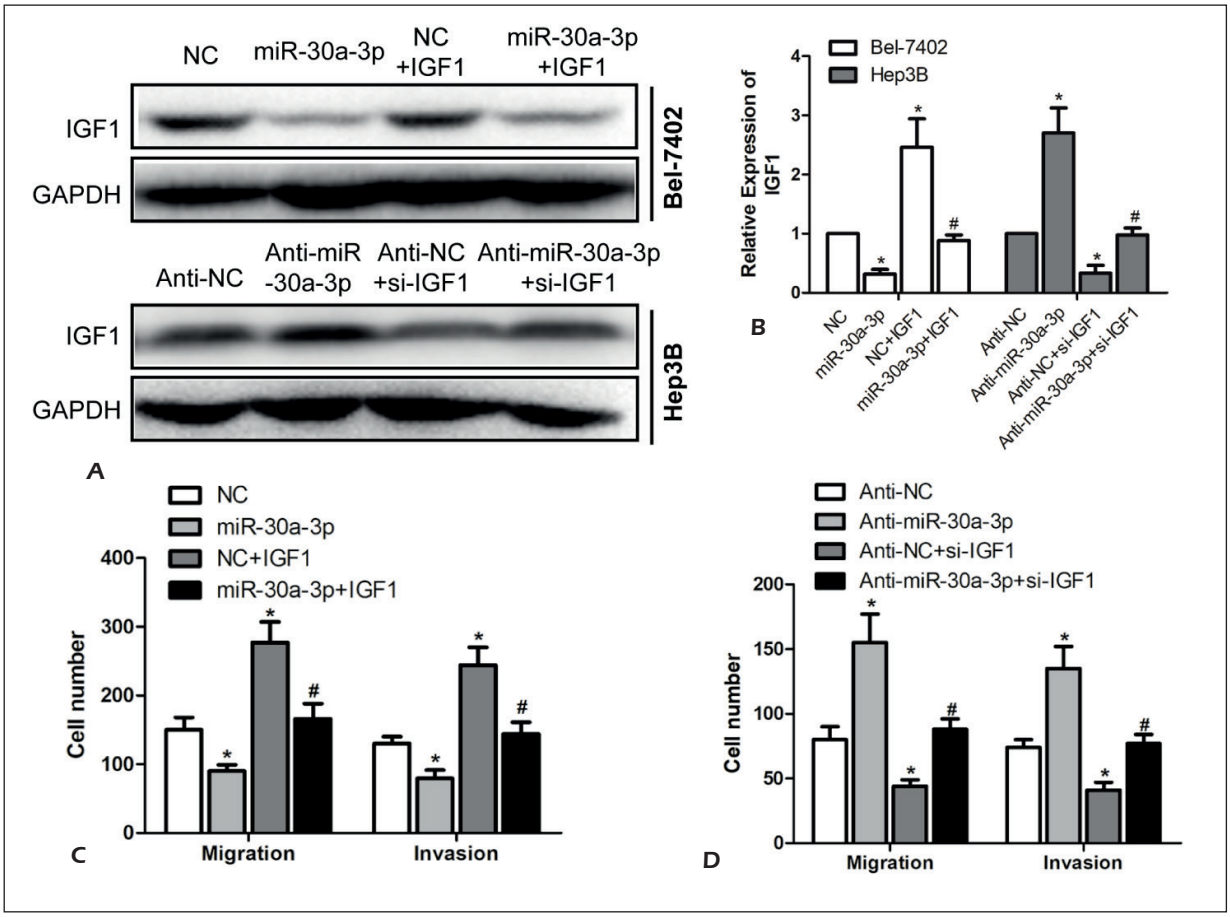

Figure 4. MiR-30a-3p regulates the mechanism of action of IGF1 in hepatoma cells. **A**, Protein expression level of IGF1 in HCC lines co-transfected with miR-30a-3p and IGF1 is detected by Western blot. **B**, mRNA expression level of IGF1 in hepatoma cell lines co-transfected with miR-30a-3p and IGF1 is detected by qRT-PCR. **C**, Transwell invasion and migration assay are used to detect the invasion and migration of HCC Bel-7402 cell line after co-transfection of miR-30a-3p and IGF1. **D**, Transwell invasion and migration assay are used to detect the invasion and migration of HCC Bel-7402 cell line after co-transfection of miR-30a-3p and IGF1. Data are mean ± SD, $*^{\#}p<0.05$.

mors with low positive expression rate of IGF1 protein were characterized by poor differentiation, high incidence of lymph node metastasis and distant metastasis, which was consistent with previous researches[21,22]. To prove whether microRNA-30a-3p inhibited the development of HCC by regulating IGF1, IGF1 expression was detected by Western Blot and qPCR and was found to be markedly reduced after the over-expression of microRNA-30a-3p. After knockdown of microRNA-30a-3p, IGF1 expression was dramatically increased. In addition, according to Luciferase reporter gene and recovery experiment, microRNA-30a-3p and IGF1 had a mutual regulatory effect. Finally, IGF1 can counteract the effect of microRNA-30a-3p on invasion and migration of HCC cells. With the continuous deepening of research, further understanding of the biological functions of microRNA-30a-3p and IGF1 genes and their roles in the occurrence and development of HCC will be more conducive to the diagnosis, treatment and prognosis evaluation of tumor, and this research has brought new hope and dawn for mankind to conquer cancer.

Conclusions

In summary, microRNA-30a-3p level was markedly decreased in the tumor tissues of HCC patients, which was relevant to the incidence of lymph node or distant metastasis and poor prognosis of HCC

patients. Additionally, this study demonstrated that microRNA-30a-3p may inhibit the malignant progression of HCC *via* regulating IGF1.

Conflict of Interests

The authors declare that they have no conflict of interests.

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