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FOXN2 suppresses the proliferation and invasion of human hepatocellular carcinoma cells

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Abstract. – OBJECTIVE: The aim of this study was to explore the roles of FOXN2 (Fork head Box N2) in mediating the proliferation and invasion of hepatocellular carcinoma (HCC) cells.

PATIENTS AND METHODS: Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) was used to determine expression of FOXN2 in HCC tissues and cells. Transfection of plasmid containing FOXN2 was used to exogenously overexpress FOXN2 *in vitro*. Cell Counting Kit-8 (CCK-8) assay and transwell assay were applied to detect the proliferation and invasion of HCC cells, respectively.

RESULTS: FOXN2 expression decreased significantly in both HCC tissues and cells ($p<0.05$). Upregulation of FOXN2 significantly inhibited the proliferation and invasion of HCC cells ($p<0.05$).

CONCLUSIONS: FOXN2 acts as a regulator in the progression of HCC. Our findings suggest that FOXN2 may be a novel therapeutic monitoring and prognosis biomarker in HCC.

Key Words:
Hepatocellular carcinoma (HCC), Fork head Box N2 (FOXN2), Proliferation, Invasion.

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignant tumors worldwide, seriously threatening people's lives and health[1,2]. In recent years, the global incidence of HCC is slowly increasing. Meanwhile, multiple treatment options have been developed for HCC, including surgical resection, local chemoradiotherapy, and targeted drug therapy[3-6]. However, recurrence and metastasis still occur in patients due to its hidden disease process, high malignancy, and low sensitivity[7,8]. This may eventually affects the quality of life and survival time of HCC patients. Therefore, in-depth exploration of the molecular mechanism of HCC occurrence and development, along with the search for tumor markers with strong specificity and high sensitivity, will provide important guiding significance for the molecular diagnosis and targeted therapy of HCC.

The Fork head box (FOX) family is a family of transcription factors that were first discovered in fruit flies[9]. The FOX family protein is a monomer that binds to DNA binding sites with high affinity. Meanwhile, it functions as a transcription regulator to activate or inhibit transcription[10]. In recent years, several studies have indicated the relationship between FOX family proteins and tumors. For example, FOXQ1 can combine with the E-cadherin promoter, further blocking its expression and resulting in tumor invasion and metastasis[11]. Besides, FOXM1 upregulates the expression of matrix metalloproteinase (MMP), thereby improving cancer cell migration and invasion[12]. High expression of FOXN3 inhibits the growth, migration, and invasion of colon cancer cells. However, knocking down the expression of FOXN3 promotes the growth, migration, invasion and metastasis of colon cancer cells[13].

FOXN is a subclass of the FOX family composed of six members, including FOXN1-6. FOXN2 (Fork head Box N2) exists in the nucleus and is located at 2p16.3 with about 1296 bp in length. The FOXN2 protein is widely expressed in many organs and tissues of the human body[14]. Researchers have found that FOXN2 plays an important role in various malignancies, such as breast cancer[15], lung cancer[16], and cervical cancer[17]. However, few reports have illustrated the associations of FOXN2 with HCC, as well as its effect on the biological characteristics of HCC cells. Therefore, the aim of this study was to explore the role of FOXN2 in the development of HCC. All our findings might help to provide theoretical basis and technical support for the clinical treatment of HCC patients.

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Patients and Methods

Sample of Tissues

In this study, after the pathological diagnosis of HCC, paired tumor tissues and corresponding adjacent tissues (2 cm away from the tumor edge) were surgically resected and collected. All enrolled patients did not receive any of treatment, such as radiofrequency ablation, interventional therapy, radiotherapy and chemotherapy and drug targeted therapy before surgery. The selection of patients was based on the guideline proposed by the Union for International Cancer Control (UICC). This study was approved by the Ethics Committee of The First Affiliated Hospital of Gannan Medical University. Signed written informed consents were obtained from all participants before the study.

Culture of Cells

Human normal cell line L-02 and HCC cell lines, including Huh7, Hep-3B, HepG2, HCCLM3 were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). All cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) complete medium (HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA) and 1% penicillin-streptomycin mixture in a 5% CO_2 cell incubator at 37°C. When cell density reached 80-90%, cell passage was performed. In brief, the cells in the plate was cultured with approximately 1 mL of trypsin for digestion. Subsequently, 3 mL of Roswell Park Memorial Institute-1640 (RPMI-1640) complete medium was added to the plate to terminate the digestion process. After centrifuged at 800 rpm for 5 min, the cells were re-suspended with fresh RPMI-1640 complete medium and passaged at 1:3.

Cell Transfection

Cells under transfection were divided into three groups, including: Control group, plasma vector group, and Plasma FOXN2 group. 24 h before transfection, cells were seeded into 6-well plates. Subsequently, Lipofectamine™ 3000 (Invitrogen, Carlsbad, CA, USA) was diluted with DMEM basal medium, mixed and incubated at room temperature. At the same time, the plasma vector/plasmid FOXN2 was diluted with DMEM basal medium and mixed at room temperature. After 5 minutes, the above two mixtures were mixed at a ratio of 1: 1, followed by incubation at room temperature for 15 minutes. Next, 500 μL of the mixture was added to each well of a 6-well plate, shaken slightly, and placed in a cell incubator. 6 h later, DMEM basic medium was replaced with DMEM complete medium. Cell transfection process was terminated for subsequent experiments.

Cell Proliferation Assay

Cells of each group were collected, re-suspended and seeded into 96-well plates at a density of 2000 cells per well. Subsequently, the cells were cultivated at 37°C and 5% CO_2 for 24 hours. After that, 10 μL of Cell Counting Kit-8 (CCK-8) solution (Dojindo Molecular Technologies, Kumamoto, Japan) was added to each well, followed by incubation at 37°C for 1 hour in dark. Absorbance at 450 nm was detected by a micro-plate reader.

Transwell Assay

48 hours after transfection, the cells in each group was collected and re-suspended in serum-free RPMI-1640 medium. A total of 2×10^4 cells in each group was added into the upper side of the transwell chamber and placed in a 5% CO_2, 37°C incubator for 24 hours. Meanwhile, complete culture medium was added to the lower chamber. Afterwards, the cells in the upper chamber were fixed with formaldehyde for 15 min and stained with 0.1% crystal violet (Sigma-Aldrich, St. Louis, MO, USA) for 5 min. Cells not passing through the chamber membrane were wiped off with cotton swaps. Invasive cells were observed under a microscope, and 5 fields of view were randomly selected for each sample.

Quantitative Real Time-Polymerase Chain Reaction (RT-PCR) Analysis

Total RNA in tissues or cells was extracted according to the manufacturer's instructions of TRIzol reagent (Invitrogen, Carlsbad, CA, USA). RNA concentration was, then, determined. Subsequently, 1 μg of RNA was reversely transcribed into complementary deoxyribose nucleic acid (cDNA). To determine gene expression, RT-PCR assay was conducted with Applied Bio Step One Plus systems (Applied Biosystems, Foster City, CA, USA). Specific procedure was as follows: pre-denaturation at 95°C for 30 s; reaction at 95°C for 5 s, and at 60°C for 30 s (40 cycles), and dissociation. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal reference. The $2^{-\Delta\Delta CT}$ method were used to quantify gene expression based on CT values. Primers used in this study were as follows: GAPDH forward: 5′-GGCTCTCTGCTCCTCCCTGTT-3′ and re-

verse: 5′-GGCTCTCTGCTCCTCCCTGTT-3, FOXN2 forward: 5′-AGTCCATTGTATGACA-TAGAGGG-3′ and reverse: 5′-TTCCATTAAC-CTTGCCATGG-3′.

Western Blot Analysis

Total protein was first extracted from cells using radioimmunoprecipitation assay (RIPA) lysate [containing phenylmethylsulphonyl fluoride (PMSF) at the ratio of 100:1] (Beyotime, Shanghai, China). The concentration of protein samples was determined by the bicinchoninic acid (BCA) protein quantification kit (Pierce, Rockford, IL, USA). Protein samples (30 μg/well) were separated by 15% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blocking with 5% nonfat milk, the membranes were incubated with primary antibodies at 4°C overnight. Thereafter, the membranes were incubated with corresponding secondary antibody at room temperature for 1 h. Immuno-reactive bands were exposed by a chemiluminescence imager, and band density was analyzed using Image J software (NIH, Bethesda, MD, USA).

Statistical Analysis

Statistical data were analyzed by Graph Pad Prism 7.01 software (La Jolla, CA, USA). Experimental data were expressed as mean ± standard deviation ($\bar{x} \pm s$). 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). $p<0.05$ was considered statistically significant.

Results

FOXN2 was Abnormally Low-expressed in HCC Tissues

FOXN2 expression in HCC tissues and adjacent normal tissues was first determined by RT-PCR. As shown in Figure 1A, FOXN2 expression in HCC tissues decreased to 0.4-fold when compared with corresponding adjacent tissues ($p<0.05$). Human normal cell line L-02 and HCC cell lines, including Huh7, Hep-3B, HepG2, HCCLM3 were selected. The expression levels of FOXN2 in normal cells and HCC cells were determined as well. The results in Figure 1B showed that the expression of FOXN2 decreased significantly in the above four HCC cells compared with normal cells ($p<0.05$). These results indicated that FOXN2 was closely correlated with HCC. Meanwhile, low expression of FOXN2 in HCC tissues and cells might be implicated in the pathogenesis of HCC.

FOXN2 was up-regulated in HCC Cells After Transfection

To explore the association between the biological characteristics of HCC cells and different expression levels of FOXN2, we separately transfected plasmid vector and plasmid containing FOXN2 into HCC cells (Hep-3B cells and

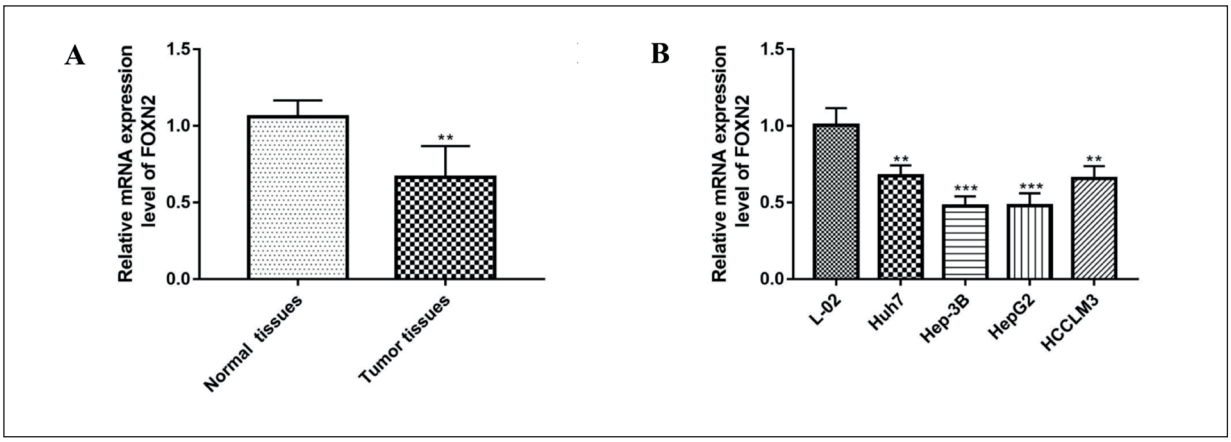

Figure 1. Expression level of FOXN2 in HCC tissues and cells. **A,** FOXN2 expression in HCC tissues decreased significantly compared wuth adjacent normal tissues detected by RT-PCR. **B,** FOXN2 was down regulated in HCC cells (Huh7, Hep-3B, HepG2 and HCCLM3) compared with L-02 cells detected by RT-PCR. (**$p<0.01$, ***$p<0.001$).

HepG2 cells). Meanwhile, HCC cells without transfection were regarded as blank control. RT-PCR results showed that compared with control group and plasmid-vector group, both Hep-3B cells and HepG2 cells in plasmid-FOXN2 group exhibited significantly upregulated FOXN2 expression (p<0.05, Figure 2A, 2B). Western blot analysis also showed the similar expression manuals of FOXN2 among the three groups (p<0.05, Figure 2C, 2D).

Enforced Expression of FOXN2 Inhibited the Proliferation Ability of HCC Cells

To examine whether exogenous high expression of FOXN2 could affect cell proliferation, CCK-8 assay was performed. Results in Figure 3A, 3B showed that, in contrast to Hep-3B cells and HepG2 cells in control group and plasmid-vector group, cells in plasmid-FOXN2 group with enforced expression of FOXN2 represented significantly inhibited cell proliferation ability (p<0.05). These results manifested that overexpression of FOXN2 inhibited the proliferation ability of HCC cells.

Effects of FOXN2 High Expression on the Invasion Ability of HCC Cells

To illustrate the role of FOXN2 in the invasion ability of HCC cells, transwell assay was employed. As shown in Figure 4A and 4B, the number of invasive Hep-3B cells in plasmid-FOXN2 group was significantly reduced in comparison with control group and plasmid-vector group, respectively (p<0.05). Reciprocally, HepG2 cells with high expression of FOXN2 exhibited remarkably decreased number of invasive cells (Figure 4C, 4D). These data implied that high expression of FOXN2 impaired the invasion ability of HCC cells.

Discussion

At present, significant progress has been made in the improvement of the prognosis of HCC patients[18,19]. However, due to its complex pathogenesis, the lack of typical symptoms in the early stage, low sensitivity to radiotherapy and chemotherapy, as well as high probability of recurrence after sur-

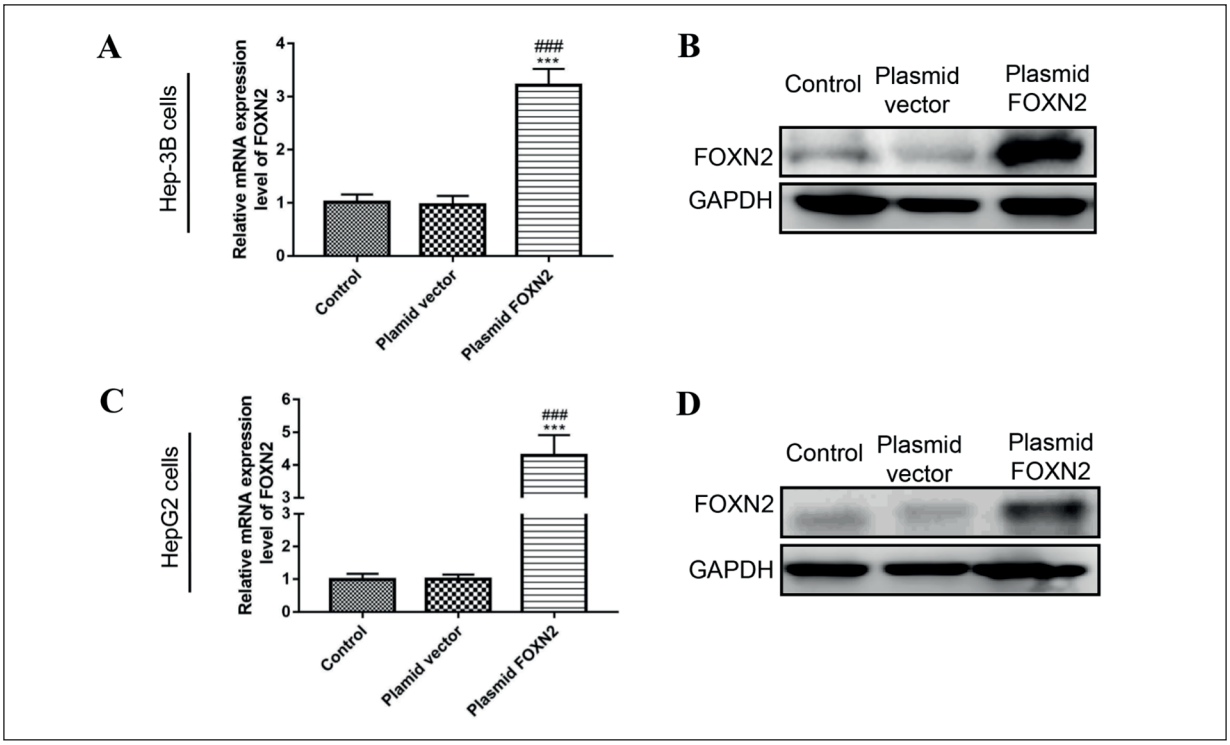

Figure 2. FOXN2 was significantly upregulated after plasmid transfection. The expression of FOXN2 in Hep-3B cells was remarkably upregulated in plasmid FOXN2 group than that in control group and plasmid vector group detected by RT-PCR (**A**) and Western blot (**B**), respectively. HepG2 cells under plasmid transfection exhibited significantly up-regulated expression of FOXN2 detected by RT-PCR (**C**) and Western blot (**D**), respectively. (***p<0.001 *vs.* control group, $^{###}p$< 0.001 *vs.* plasmid vector group).

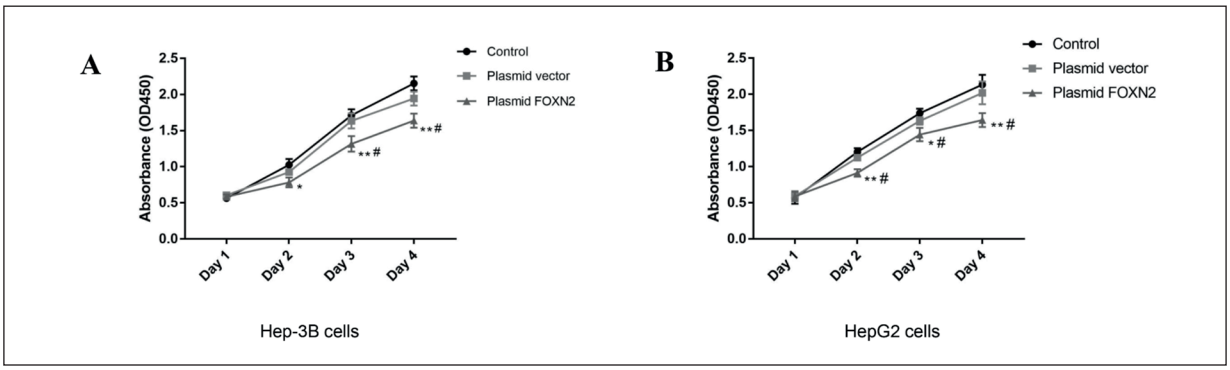

Figure 3. Effects of FOXN2 on the proliferation ability of HCC cells determined by CCK-8 assay. **A,** Hep-3B cells in plasmid FOXN2 group showed impeded proliferation ability compared to cells in control group and plasmid vector group. **B,** HepG2 cells with plasmid FOXN2 transfection presented reduced proliferation ability compared to normal HepG2 cells and HepG2 cells with plasmid vector transfection. (*p<0.05, **p< 0.01 *vs.* control group, $^{\#}p$< 0.05 *vs.* plasmid vector group).

gery, the overall therapeutic effect of HCC is still far from satisfactory. Meanwhile, the prognosis of patients is poor[20-22]. Over the past few decades, countless studies[23-25] have pointed out that many molecules are linked together like a network and can jointly participate in the occurrence and outcome of HCC. Therefore, it is extremely important to carry out further research on the molecular mechanism in the development of liver cancer, and to explore novel therapeutic targets and clinical diagnostic and therapeutic markers.

The FOX family plays different roles in many tumors. For example, when the activity of FOXO is reduced, the cells show increased cycle activity and reduced DNA damage repair capacity, leading to cancer development[26]. Meanwhile, decreased FOXN3 expression activates the β-catenin/TCF signaling pathway, thereby promoting the development of colon cancer[13]. Low expression of FOXN2 predicts poor prognosis of breast cancer. Knockdown of FOXN2 significantly promotes the proliferation, migration, and invasiveness of

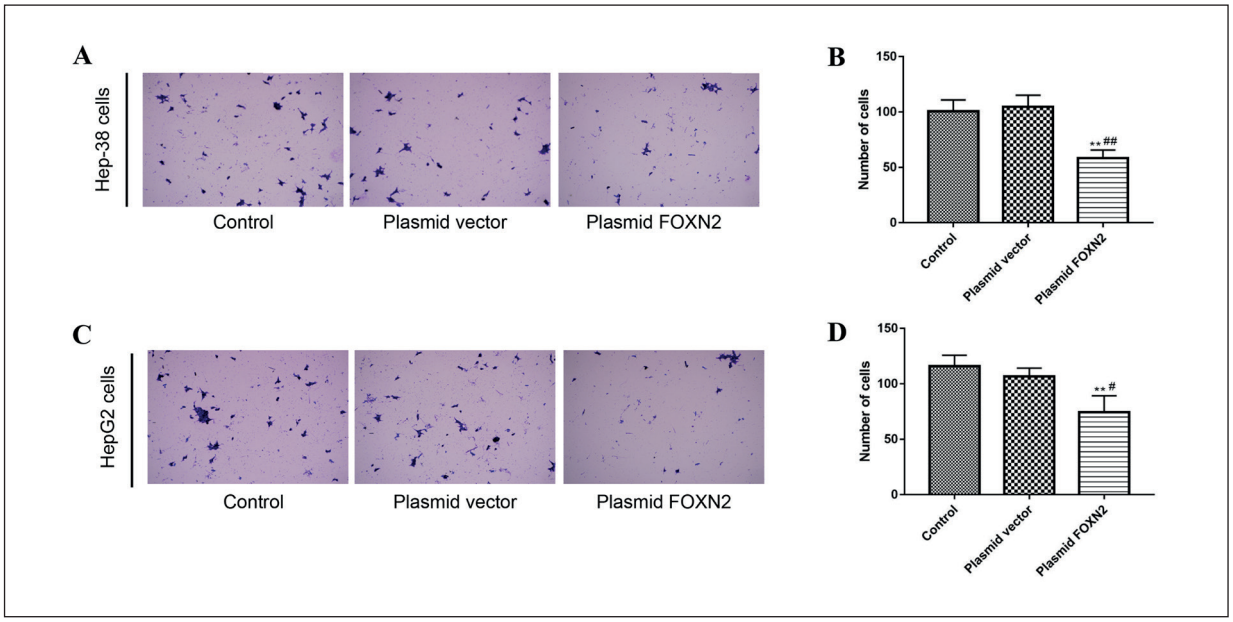

Figure 4. Effects of FOXN2 on the invasion ability of HCC cells determined by transwell assay. **A, B,** Hep-3B cells transfected with plasmid FOXN2 showed impaired invasion ability (magnification: 10×). **C, D,** Hep-3B cells with high expression of FOXN2 showed restricted invasion ability (magnification: 10×). (**p<0.01 *vs.* control group, $^{\#}p$<0.05, $^{\#\#}p$<0.01*vs.* plasmid vector group).

breast cancer cells, as well as represses epithelial-mesenchymal transition[15]. These researches fully demonstrate that FOX family proteins can affect and regulate tumorigenesis, which makes it a hot spot for many cancer researches.

Currently, few studies have reported the role of FOXN2 in HCC development. Therefore, in the present study, we initially collected tumor tissues and adjacent normal tissues from HCC patients. RT-PCR assay was performed to detect the expression of FOXN2. The results showed that FOXN2 expression in HCC tissues was dramatically lower than adjacent normal tissues. Similarly, FOXN2 was lowly expressed in human HCC cell lines (Huh7, Hep-3B, HepG2, HCCLM3) compared with normal cell line L-02. Thus, our data demonstrated that FOXN2 expression decreased remarkably in both HCC tissues and cells.

The status of gene expression in primary tumor tissues reflects the biology of the tumor and affects the biological behavior of tumor cells[27,28]. Therefore, we artificially overexpressed FOXN2 by transfection of the plasmid into HCC cell lines to explore the effect of FOXN2 on HCC cells. Notably, we observed that exogenously expression of FOXN2 resulted in remarkably decreased cell proliferation and invasion in contrast to cells transfected with plasmid vector or non-transfection. All these results proved that FOXN2 was implicated in the pathogenesis of HCC cells.

Conclusions

FOXN2 was downregulated in HCC tissues and cell lines. Overexpression of FOXN2 suppressed the proliferation and invasion of HCC cells. The novelty of this study was that FOXN2 acted as an important biomarker for the diagnosis and therapeutic response of HCC.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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