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RESEARCH ARTICLE

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Transcription factor FOXA3 promotes the development of Hepatoblastoma via regulating HNF1A, AFP, and ZFHX3 expression

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Abstract

Objective: In this research paper, we aimed to study the role of FOXA3 in hepatoblastoma (HB) and the molecular mechanism.

Methods: Immunohistochemistry was applied to determine the expression situation of FOXA3 and AFP in HB tissues and the adjacent normal tissues. FOXA3, HNF1A, and ZFHX3 expressions in HB tissues and the normal tissues were measured by Western blot. HB cell lines were randomly divided into 4 groups: Model, si-NC, si-FOXA3-1, and si-FOXA3-2 group. The HB cell viability and colony formation characteristics in the 4 groups were explored by CCK-8 and cell cloning formation assay, respectively. The expression of FOXA3, AFP, HNF1A, ZFHX3, and MYC in HB cells after knockdown of FOXA3 was measured.

Results: FOXA3, AFP, and HNF1A expressions were significantly up-regulated in HB tissues, while ZFHX3 expression was down-regulated. Knockdown of FOXA3 markedly inhibited HB cell viability and cloning formation ability. Knockdown of FOXA3 decreased FOXA3, AFP, and HNF1A/MYC expression, while increased ZFHX3 expression.

Conclusion: FOXA3 promotes the occurrence and development of HB by up-regulating AFP and HNF1A/MYC expression, and down-regulating ZFHX3 expression.

KEYWORDS
AFP, FOXA3, hepatoblastoma, HNF1A, transcription

1 | INTRODUCTION

Hepatoblastoma (HB) is the most common malignant tumor of liver in children. In recent years, with the progress of surgical techniques, the improvement of chemotherapy regimens, and the joint efforts of many childhood tumor organization around the world, the diagnosis and treatment of HB has made certain progress.[1] If neoadjuvant chemotherapy combined with pediatric liver transplantation is used, the 5-year overall survival rate of HB can reach to about 70%.[2] Even so, there about 20% of children with hepatoblastoma still have distant metastasis at initial diagnosis. The 5-year survival rate of HB children with recurrence, poor chemotherapy, and no liver transplantation is only 15%-20%.[3] Therefore, tumor recurrence and metastasis are the primary causes of death in children with HB and also the key factors affecting the long-term survival of children with HB.[4] Therefore, revelation of the mechanisms of proliferation and metastasis of HB cells by molecular biology and exploration of new treatments for HB to improve the overall survival rate of children with HB is an urgent

problem to be solved in the treatment of HB and also a hot spot in the research of HB in recent years.[5]

Malignant tumor cells often express some relatively small but specific protein molecules, which can be used as markers to identify malignant tumor cells.[6] The cell surface markers of different tumors are not exactly the same. These markers are closely related to the regulation of malignant tumor cell proliferation, chemotherapy resistance, cell migration, and tumor metastasis and play an extremely important role in the occurrence and development of tumors. The FOX gene family consists of 19 subfamilies, which are involved in cell signal transduction, cell cycle regulation, and metabolism regulation by binding with DNA, activating, or inhibiting the transcriptional activity of target genes.[7] Members of the FOXA family are necessary for endoderm formation and the normal development of related organs, such as the liver, pancreas, and prostate. They can also regulate energy metabolism by regulating multiple target genes in liver, pancreas, and adipose tissue and thus participate in the occurrence and development of many diseases.[8] Mammalian organogenesis is a highly dynamic process. During early organogenesis, Fox protein determines the formation of three layers, especially the endoderm and mesoderm. Liver development begins with the appearance of liver buds, the formation of hepatic progenitor cells, then the proliferation, differentiation, and migration of hepatic progenitor cells, and finally the formation of organs.[9] Liver development has undergone a highly complex process of spatiotemporal regulation of transcription factors and cellular signals. FOXA transcription factor family forms a highly complex regulatory network with HNF family, GATA family, and other intra-hepatic transcription factors and plays an important role in the development of the liver as well as its normal functions. Wang et al found FOXA2 expression was decreased in hepatocellular carcinoma (HCC) tissues, and further in vitro and in vivo experiments confirmed that FOXA2 inhibited the invasion and metastasis of HCC.[10] At present, there are many studies on FOXA1 and FOXA2, while there are few studies on FOXA3, which mainly focus on embryonic development, blood glucose, fat metabolism, and related diseases. FOXA3 is an important member of the hepatocyte nuclear factor (HNF) family and is expressed in large quantities in the liver.[11] It plays an important role in hepatocyte differentiation and function maintenance. It can regulate the expression of hepato-specific genes and affects the expression of a series of genes and proteins involved in the maintenance of glucose homeostasis.[12]

In this study, the expression of FOXA3, AFP, HNF1A and ZFHX3 in HB tissues and the adjacent normal tissues was explored. Si-FOXA3 plasmids were transfected to human HB cell lines. The cell proliferation and cloning formation situation in si-FOXA3 and si-NC group were determined.

2 | MATERIAL AND METHODS

2.1 | Tissue samples

Twenty children with HB who underwent pulmonary lobectomy in Shandong Provincial Hospital from January 2014 to December 2019 were selected for researching. There were 9 male cases and 7 female cases. The age of them ranged from 1-year old to 7-year old. All pathologic diagnoses were HB. Tumor tissue and normal tissue 5 cm from the edge of tumor tissue were collected. The expression characteristic of FOXA3 and AFP in HB tissues and control tissues was analyzed by immunohistochemistry. The expression of FOXA3, HNF1A,, and ZFHX3 was determined by Western blot.

2.2 | Western blot

Tissues were lysed with cell lysis buffer at ice for 30 min and then centrifuged at 12 000 r/min for 25 min. Supernatant was collected, and Bradford method was used for protein quantification. Protein of 25 μg was performed for SDS-polyacrylamide gel electrophoresis. The proteins were transferred to the PVDF membrane and sealed with 5% skim milk at room temperature for 1 h, and primary antibody was added and incubated overnight at 4°C. The next day, the secondary antibody was added and incubated at room temperature for 1 h, and then, ECL hypersensitive luminescence solution was added for developing.

2.3 | Immunohistochemistry

Tissues were dewaxed, and endogenous oxidase was inactivated by 3% hydrogen peroxide. After washing by PBS for 3 times, the antigen was repaired by microwave repair. The slices were placed in sodium citrate buffer with pH 6.0, heated to boiling by microwave, and then cooled. The slices were repeated for one time and then washed with PBS. After cleaning, sections were removed and sealed for 10min. Then, mouse anti-human FOXA3 and AFP monoclonal antibody (1:150) were added and incubated at 4°C for 12h. After washing, biotin-labeled secondary antibodies were added and incubated at 4°C for 20 minutes each. After incubation and washing, the samples were washed again and DAB color developing solution for 5 minutes. After that, the samples were washed, re-dyed, dehydrated, sealed, and observed under an optical microscope.

2.4 | Cell culture and transfection

Human HB cell lines HuH-6 were all purchased from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). They were cultured in DMEM medium containing 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 mg/ml streptomycin at 37°C in 5% CO_2. Cells were randomly divided into 4 groups: HuH-6, si-FOXA3-1, si-FOXA3-2, and si-control group. Si-FOXA3 was applied to knockdown FOXA3. Si-FOXA3 and its negative controls were obtained from Shanghai Jima Biotechnology Co. LTD (Shanghai, China). Cells at logarithmic growth stage were collected and inoculated to 6-well plates at a concentration of 1 × 106/ml, and transfection was performed using Lipofectamine 2000 (Thermo Fisher Scientific, CA, USA) when 75% of the cells were fused. After transfection for 48 h, cells were collected for subsequent experiments.

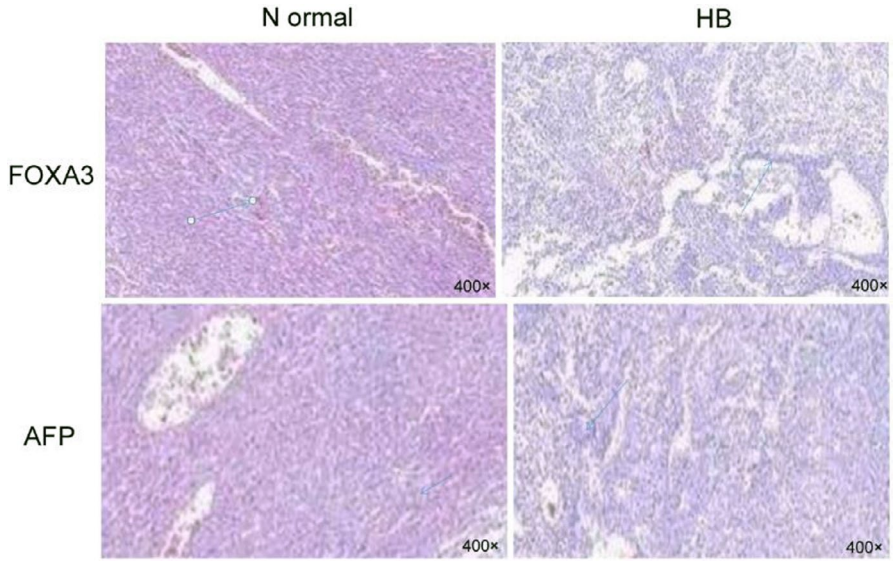

FIGURE 1 The expression situation of FOXA3 and AFP in HB tissues and the adjacent normal tissues by immunohistochemistry. FOXA3 and AFP expression was up-regulated in HB tissues

2.5 | CCK-8

Cells were collected after transfection for 24 h and inoculated into 96-well plates with 5×10^4 cells/well. CCK-8 detection was performed on the 1, 2, 3, and 4th day, respectively. Before detection, 10 μL CCK-8 solution was added to each well. After incubation for 4h, the absorbance value at 450 nm was detected by an ELIASA.

2.6 | Cell cloning formation assay

Cells were made to single cell suspension and inoculated in a 6-well plate with 1000 cells/well, and the total medium was 2.0 ml in per well. The plates were cultured at 37°C, 5% CO_2 for 15 days. When the visible clone appeared, the culture was stopped, the medium in the hole was removed, and the culture was carefully washed with PBS for 2 times. After air drying, the culture was fixed with 4% paraformaldehyde for 30 min, dried, and stained with 1% crystal violet dye for 30min. The dye was washed with PBS and photographed after drying.

2.7 | RT-PCR

Total RNA was extracted from cells by TRIzol method, and cDNA was synthesized according to the instructions of reverse transcription kit. PCR amplification was performed as follows: 95°C 5 min, 95°C 20s, 60°C 30s, and 72°C 20s for 40 cycles. U6 was taken as a control.

2.8 | Immunofluorescence

Tissue sections were put into the 12-well plate wells and washed with PBS phosphate buffer solution for 5 times with every 10min. Then, plates were sealed with 10% normal goat serum at 37°C for 90 min. After incubation with FOXA3 and AFP antibody at 4°C for 48 h, plates were washed with PBS phosphate buffer solution for 5 times with every 10min, dyed with DAPI staining solution at room temperature for 5min, and washed with PBS phosphate buffer solution for 3 times. The tissue sections were transferred to slides and sealed with anti-fluorescence quenching sealing solution (strong). The images were taken under an inverted fluorescence microscope.

2.9 | Statistical analysis

SPSS 19.0 software was used for statistical analysis. The t test was used for comparison between the two groups. $P < .05$ indicates the difference was statistically significant.

3 | RESULTS

3.1 | FOXA3 and AFP were up-regulated in HB

Immunohistochemistry was applied to determine the expression situation of FOXA3 and AFP in HB tissues and the adjacent normal tissues. As Figure 1 showed, FOXA3 and AFP were all located in the nucleus. The expression of FOXA3 and AFP in HB tissues was markedly up-regulated compared to that in the normal tissues, which indicated FOXA3 and AFP may be related to the development of HB.

3.2 | FOXA3, HNF1A and ZFHX3 expression

The protein expression of FOXA3, HNF1A,, and ZFHX3 in HB tissues and the normal tissues was measured by Western blot, and

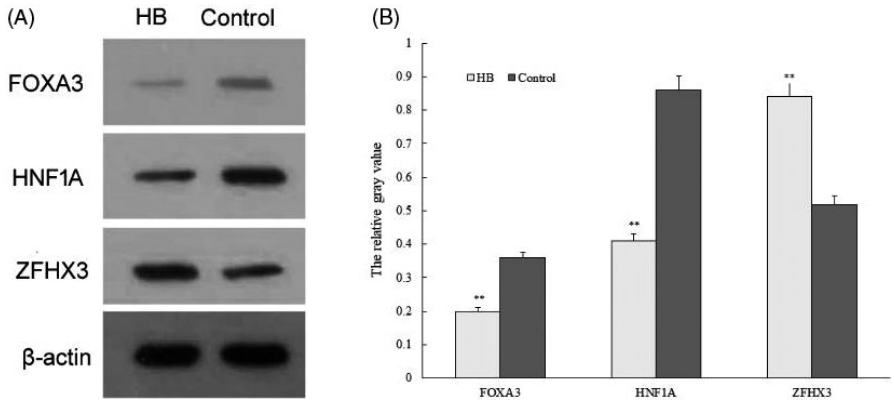

FIGURE 2 The protein expression of FOXA3, HNF1A, and ZFHX3 in HB tissues and the normal tissues determined by Western blot. A. The expression of FOXA3, HNF1A, and ZFHX3. B. The relative gray scale expression of FOXA3, HNF1A, and ZFHX3. $^{**}p < .01$ *VS* control group, indicating the difference is significant

results were showed in Figure 2. As showed in Figure 2A, FOXA3 and HNF1A were less expressed in HB tissues compared with normal tissues. However, the relative gray scale expression of ZFHX3 in HB tissues was significantly higher compared to that in normal tissues ($P < .01$). The statistical histogram data were shown in Figure 2B.

3.3 | Knockdown of FOXA3 inhibits HB cell viability

The HuH-6 cell viability at 0, 24, 48, and 72 h in the model, si-NC, si-FOXA3-1, and si-FOXA3-2 group was measured by CCK-8 assay. As Figure 3 showed, HuH-6 cell viability increased with the increase of culture time. HuH-6 cell viability in 24, 48, and 72 h in si-FOXA3-1 and si-FOXA3-2 group was all lower than that in model and si-NC group. Moreover, the difference in 48 and 72 h was significant ($p < .05$, $p < .01$). Those indicated knockdown of FOXA3 in human HB cells could decreased the cell viability.

3.4 | Knockdown of FOXA3 inhibits HB cell cloning formation

The colony formation characteristics of HuH-6 cells in the Model, si-NC, si-FOXA3-1, and si-FOXA3-2 group were measured by cell cloning formation assay. As Figure 4 showed, there was a lot of colony formation in model and si-NC group, while si-FOXA3-1 and si-FOXA3-2 group had low colony formation. Those indicated knockdown of FOXA3 in human HB cells could decreased the cell colony formation.

3.5 | Knockdown of FOXA3 inhibits FOXA3 and AFP expression

The expression situation of FOXA3 and AFP in HuH-6 cells in the model, si-NC, si-FOXA3-1, and si-FOXA3-2 group were measured by immunofluorescence, and results were showed in Figure 5. As shown, the expression of FOXA3 and AFP was markedly decreased in si-FOXA3-1 and si-FOXA3-2 group compared that in the model and si-NC group. Those indicated knockdown of FOXA3 inhibited FOXA3 and AFP expression in HB cells.

3.6 | Knockdown of FOXA3 influences FOXA3, HNF1A, ZFHX3 and MYC levels

The relative mRNA levels of FOXA3, HNF1A, ZFHX3, and MYC in the model, si-NC, si-FOXA3-1, and si-FOXA3-2 group were measured by RT-PCR. As Figure 6 showed, the relative mRNA level of FOXA3 in si-FOXA3-1 and si-FOXA3-2 group was markedly decreased compared to that in the model and si-NC group ($p < .05$, $p < .01$). The relative levels of HNF1A/MYC were also significantly reduced in si-FOXA3-1 and si-FOXA3-2 group compared to Model and si-NC group ($p < .05$, $p < .01$). ZFHX3 expression levels in si-FOXA3-1 and

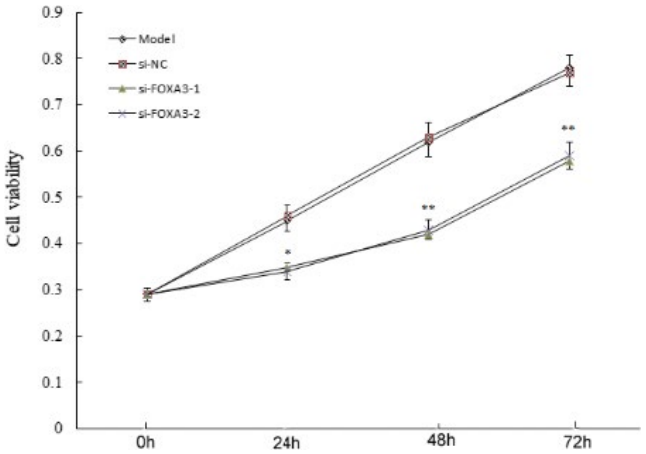

FIGURE 3 The HuH-6 cell viability in the Model, si-NC, si-FOXA3-1, and si-FOXA3-2 group measured by CCK-8 assay. Knockdown of FOXA3 in human HB cells could decrease the cell viability. $^{*}p < .05$ or $^{**}p < .01$ *VS* Model group, indicating the difference is significant

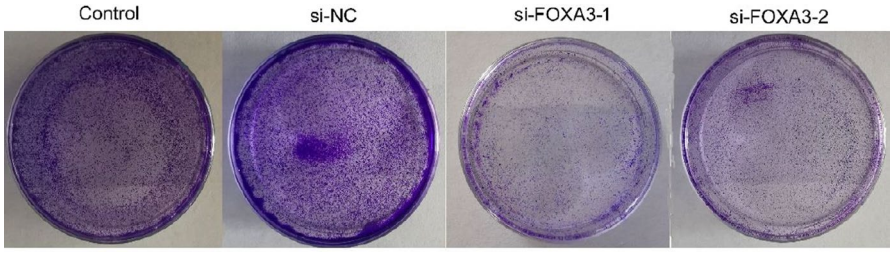

FIGURE 4 The colony formation characteristics of HuH-6 cells in the Model, si-NC, si-FOXA3-1, and si-FOXA3-2 group measured by cell cloning formation assay. Knockdown of FOXA3 in human HB cells could decreased the cell colony formation

si-FOXA3-2 group were dramatically increased compared to model and si-NC group ($p < .05$, $p < .01$).

4 | DISCUSSION

Human alpha-fetoprotein (AFP) is a kind of large molecular glycoprotein and is mainly produced by fetal liver cells and yolk sac. After birth, the synthesis of AFP gradually stops, but when liver cells become cancerous, the gene for this embryonic antigen is activated, regenerating large amounts of AFP.[13] AFP is a serological marker of HB, and its numerical changes often indicate whether there is an effective response to chemotherapy.[14] FOXA family, as transcription factors, is closely associated with the occurrence, proliferation, invasion, and metastasis of malignant tumors. The expression of FOXA family gene and its role are different in different tumors.[15] Previous study found overexpression expression of FOXA3 could induce fibrocyte translate into induced hepatoid parenchymal cells. The study revealed that FOXA3, as a key transcription factor, occupied a key site of hepatic fate determination in the transcriptional regulatory network of the whole gene.[16] Sayaka et al identified three specific combinations of the two transcription factors by screening 12 candidate factors, which could transform embryonic and adult fibroblasts in vitro into cells very similar to liver cells. This induced hepatocyte-like cell has a variety of hepatocyte-specific characteristics and can be transplanted to restore damaged liver tissue.[17] Our study found the expression of FOXA3 and AFP was up-regulated significantly in HB tissues, which indicated FOXA3 and AFP expression was related to the development of HB.

HNF1A is a member of the hepatocyte nuclear factor family. Hepatocyte nuclear factor is a group of transcription factors that are related to phylogeny and can regulate the transcription of different groups of genes and translates them into proteins.[18] Those proteins include coagulation factors and so on. In addition, enzymes and transporters are also involved in the transport and

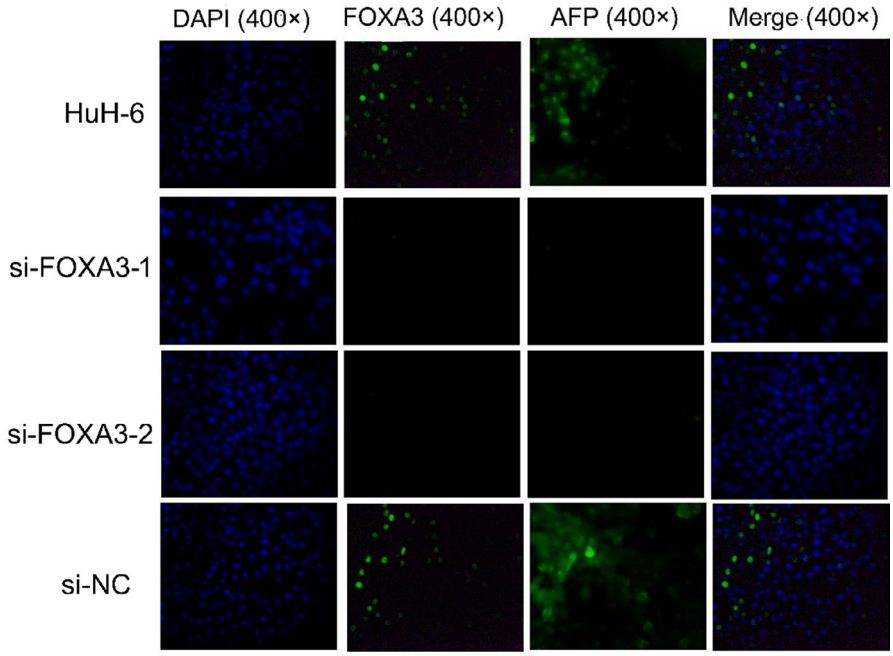

FIGURE 5 The expression situation of FOXA3 and AFP in HuH-6 cells in the Model, si-NC, si-FOXA3-1, and si-FOXA3-2 group measured by immunofluorescence. The expression of FOXA3 and AFP was markedly decreased in si-FOXA3-1 and si-FOXA3-2 group compared that in the Model and si-NC group

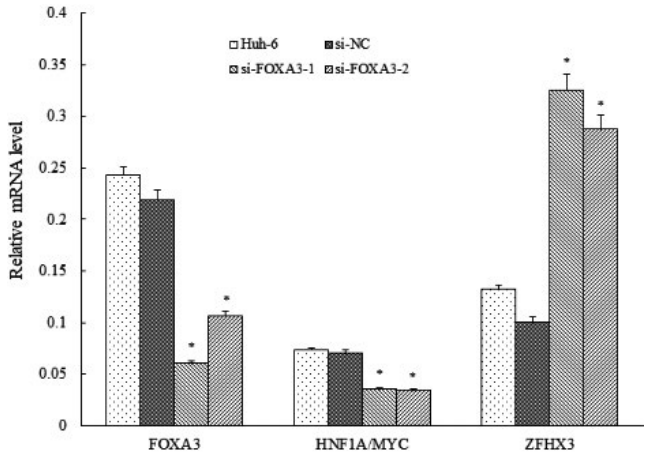

FIGURE 6 The relative mRNA levels of FOXA3, HNF1A, ZFHX3, and MYC in the Model, si-NC, si-FOXA3-1,, and si-FOXA3-2 group measured by RT-PCR. Knockdown of FOXA3 markedly decreased the relative mRNA levels of FOXA3 and HNF1A/MYC, while dramatically increased the relative mRNA levels of ZFHX3. $^{**}p < .01$ *VS* Huh-6 group, indicating the difference is significant

metabolism of glucose, cholesterol, and fatty acids.[19] HNF1A is expressed primarily in the liver, but it has also been found to be expressed in the pancreas, kidneys, and small intestine. Studies have also shown that HNF1A can regulate the expression of multiple target genes, including albumin secretion gene, anti-trypsin synthesis gene, and fibrinogen synthesis gene.[20] In addition, the promoter regions of more than 200 liver-specific genes are capable of binding to HNF1A.[21] HNF1A plays an important role in the development, maturation, and regeneration of the liver. Studies have also shown that many liver diseases are closely related to HNF1A expression.[22,23] The molecular mechanism of HNF1A regulating liver-specific gene expression is mainly controlled by its own indirect negative feedback regulation.[24] The ZFHX3 gene, located in Chromosome 16q22.3, codes for a transcription factor which is widely expressed in human tissues.[25] It is crucial for the angiogenic function of hypoxia-inducible factor 1α in liver cancer cells.[26] Our results showed the relative gray scale expression of FOXA3 and HNF1A in HB tissues wass significantly higher compared to that in normal tissues, while ZFHX3 was lower. To explore the role of FOXA3 on cell function, we determined the HB cell viability by CCK-8 assay and cell colony formation characteristics by cell cloning formation assay in after knockdown of FOXA3. Results showed knockdown of FOXA3 decreased the cloning formation ability and viability of HB cells. Moreover, results showed knockdown of FOXA3 markedly decreased FOXA3, AFP, HNF1A/MYC,, and expression and increased ZFHX3 expression.

In conclusion, FOXA3 expression was up-regulated in HB. It promotes the occurrence and development of HB by up-regulating AFP and HNF1A/MYC expression and down-regulating ZFHX3 expression.

DATA AVAILABILITY STATEMENT

Data were available on request from the corresponding author.

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