

HNF-4 α regulates hepatoma cell growth and apoptosis through the IGFBP-2/Notch1 signaling pathway

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Abstract: Background: Hepatocyte nuclear factor 4 α (HNF-4 α) is a liver-enriched transcription factor that is downregulated in hepatocellular carcinoma (HCC). However, the molecular mechanisms by which it regulates the malignant phenotype of HCC cells remain unclear. **Objectives:** To investigate whether HNF-4 α affects HCC cell proliferation, invasion and apoptosis by regulating the IGFBP-2/Notch1 signaling pathway. **Methods:** HNF-4 α was overexpressed and silenced in HepG2 cells using a pcDNA3.1-HNF-4 α plasmid and HNF-4 α -specific siRNA, respectively. RT-qPCR and Western blotting verified transfection efficiency. Cell proliferation and invasion were assessed by MTT and Transwell assays, respectively. Apoptosis was evaluated by Caspase 3 activity assay. IGFBP-2 secretion was measured by ELISA. Protein expression of Bcl-2, Bax, IGFBP-2 and Notch1 was detected by Western blotting. **Results:** HNF-4 α overexpression significantly inhibited HepG2 cell proliferation (by approximately 35% at 48 h and 45% at 72 h) and invasion (by approximately 55%), while markedly increasing Caspase 3 activity (approximately 2.8-fold) and the Bax/Bcl-2 ratio (from 0.42 to 2.18). Moreover, HNF-4 α overexpression significantly suppressed IGFBP-2 secretion (by approximately 60%) and downregulated IGFBP-2 (by approximately 55%) and Notch1 NICD (by approximately 50%) protein expression (all $P < 0.001$). Conversely, HNF-4 α knockdown produced opposite effects, significantly promoting proliferation (by approximately 40% at 48 h and 55% at 72 h) and invasion (by approximately 70%), decreasing Caspase 3 activity (by approximately 55%) and the Bax/Bcl-2 ratio (from 0.45 to 0.19) and upregulating IGFBP-2 (approximately 2.2-fold) and Notch1 NICD (approximately 1.8-fold) expression (all $P < 0.01$). **Conclusion:** HNF-4 α inhibits HCC cell proliferation and invasion and promotes apoptosis through negative regulation of the IGFBP-2/Notch1 signaling pathway, providing new experimental evidence for the tumor-suppressive mechanism of HNF-4 α in HCC.

Keywords: Apoptosis; Bcl-2; Bax; HNF-4 α ; IGFBP-2; Notch1; Proliferation

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INTRODUCTION

Liver cancer is one of the most common malignant tumors of the digestive system, with the sixth highest incidence and the third highest mortality among all malignant tumors worldwide (Hu *et al.*, 2026). Among them, hepatocellular carcinoma (HCC) accounts for about 90% of primary liver cancer and is the predominant pathological type (Chai *et al.*, 2026). Despite advances in diagnosis and treatment technology, the overall 5-year survival rate of patients with liver cancer is still less than 20% due to its occult onset, rapid progression and high rates of recurrence and metastasis (Ma *et al.*, 2026). The pathogenesis of liver cancer is complex, involving multiple factors such as HBV/HCV infection, aflatoxin exposure, alcohol consumption and metabolic diseases (Wang *et al.*, 2025; Galasso *et al.*, 2024). However, the lack of early diagnostic markers and late-stage treatment resistance remain the main clinical challenges. Therefore, elucidating the molecular mechanisms underlying the occurrence and development of liver cancer and identifying new diagnostic and therapeutic targets is of great clinical significance.

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The occurrence and development of liver cancer are the result of abnormal transcriptional regulatory networks. Hepatocyte nuclear factor 4 α (HNF-4 α) is a member of the liver-enriched nuclear receptor superfamily. As a core regulator of liver-specific gene expression, HNF-4 α regulates the expression of hundreds of liver-specific genes. It plays a key role in hepatocyte differentiation, metabolism and maintenance of homeostasis (Hu *et al.*, 2024; Gao *et al.*, 2026). In recent years, the function of HNF-4 α in tumors has attracted much attention. In colorectal cancer, HNF-4 α affects tumor metastasis by regulating epithelial-mesenchymal transition (Kim *et al.*, 2025), but its role remains controversial. In the field of liver cancer, recent studies have shown that HNF-4 α expression is down-regulated in HCC tissues and its low expression is associated with poor prognosis of patients (Wu *et al.*, 2024). However, the specific molecular mechanisms by which HNF-4 α regulates proliferation, apoptosis and invasion remain poorly understood in hepatocellular carcinoma cells and its key downstream effector pathways have not been clarified.

Insulin-like growth factor binding protein 2 (IGFBP-2) is mainly synthesized and secreted by hepatocytes and its

expression is significantly increased in hepatocellular carcinoma, which is closely related to enhanced tumor invasiveness and poor prognosis (Zhang *et al.*, 2022a). Notch1, an important member of the highly conserved Notch signaling pathway, is often abnormally activated in HCC and drives tumor progression (Chen *et al.*, 2024). Recent studies have suggested cross-regulation between IGFBP-2 and Notch signaling (Wang *et al.*, 2024), but the mechanism of transcriptional regulation upstream of this regulatory axis remains unclear.

Although the respective roles of HNF-4 α , IGFBP-2 and Notch1 in HCC have been investigated to some extent, the hypothesis that HNF-4 α transcriptionally regulates IGFBP-2 and subsequently affects Notch1 signaling pathway activity has not been experimentally tested. Specifically, it is not known whether HNF-4 α directly binds to the IGFBP-2 promoter region and regulates its transcription. Whether the regulation of HNF-4 α on the IGFBP-2/Notch1 pathway mediates its effect on proliferation, invasion and apoptosis of hepatocellular carcinoma cells; And whether the HNF-4 α /IGFBP-2/Notch1 regulatory axis constitutes a functional signaling cascade in HCC cells. Answering these questions will help clarify the tumor-suppressor mechanism of HNF-4 α in HCC and may provide a new theoretical basis for the diagnosis and treatment of HCC.

Based on the above research gap, the core hypothesis of this study is that HNF-4 α acts as a tumor suppressor in HCC cells by negatively regulating the expression of IGFBP-2 and inhibiting the abnormal activation of Notch1 signaling pathway, thereby inhibiting the proliferation and invasion of HCC cells and promoting cell apoptosis. It was specifically hypothesized that overexpression of HNF-4 α would lead to down-regulation of IGFBP-2 expression and decreased Notch1 signaling activity, accompanied by an increase in the Bax/Bcl-2 ratio and activation of Caspase 3, ultimately inhibiting the malignant phenotype of liver cancer cells.

MATERIALS AND METHODS

Main reagents and antibodies

The human hepatocellular carcinoma cell line HepG2 (HB-8065) and the normal human liver cell line LO2 (CRL-8658) were purchased from ATCC. The cell lines were confirmed by STR short tandem repeat identification and the test for mycoplasma was negative (Shanghai Sangon Bioengineering Co., LTD. provided testing services). Cell culture reagents included DMEM high glucose medium, fetal bovine serum (FBS) and penicillin-streptomycin solution, purchased from Hyclone (Logan, UT, USA). MTT powder and dimethyl sulfoxide (DMSO) were purchased from Gibco (Grand Island, NY, USA). Trypsin-EDTA was purchased from Sigma-Aldrich (St. Louis, MO, USA) and polyvinylidene difluoride (PVDF)

membranes were purchased from Pall Life Sciences (Port Washington, NY, USA). Other materials included chemical reagents and enhanced chemiluminescence (ECL) detection kits (Beyotime, Shanghai, China; Amersham Biosciences, Piscataway, NJ, USA). The primary antibodies used were as follows: Rabbit anti-human Bcl-2 monoclonal antibody (# 4223, 1:1000), rabbit anti-human Bax monoclonal antibody (# 2772, 1:2000), rabbit anti-human IGFBP-2 monoclonal antibody (# 3922, 1:1500), rabbit anti-human Notch1 monoclonal antibody (# 3608, 1:1000), rabbit anti-human Bcl-2 monoclonal antibody (#2772, 1:2000), rabbit anti-human IGFBP-2 monoclonal antibody (#3922, 1:1500), rabbit anti-human Notch1 monoclonal antibody (#3608, 1:1000) Detecting activated Notch1 intracellular domain NICD) and rabbit anti-human GAPDH monoclonal antibody (#5174, 1:5000), all purchased from Cell Signaling Technology (Danvers, MA, USA). Horseradish peroxidase (HRP) -labeled goat anti-rabbit secondary antibody (#7074, 1:2000) was also purchased from Cell Signaling Technology. Molecular biological reagents included TRIzol RNA extraction kit (Invitrogen, Carlsbad, CA, USA) and PrimeScript reverse transcription kit (TaKaRa, Dalian, China). Lipofectamine 2000 transfection reagent (Invitrogen), Caspase-3 activity assay kit (Booster, Wuhan, China) and Transwell chambers (pore size 8 μ m, Corning, NY, USA). The human IGFBP-2 ELISA kit was purchased from RandD Systems (Minneapolis, MN, USA, item number DGB200). The main instruments included a Labsystem Version 1.3.1 microplate reader (Bio-Rad, Hercules, CA, USA) and an ABI 7700 Fast real-time PCR system (Applied Biosystems, Foster City, CA, USA). Other commonly used laboratory reagents were purchased from Sangon BioEngineering (Shanghai, China).

Overview of experimental design

In this study, a two-way strategy of "gain-of-function" (overexpression) and "loss-of-function" (siRNA knockdown) was employed to up-regulate and down-regulate HNF-4 α expression in HepG2 cells. Changes in core biological behaviors, including cell proliferation, invasion and apoptosis, were systematically evaluated and changes in the expression of key proteins in the IGFBP-2/Notch1 pathway were also assessed. With the above experimental design, the present study aimed to determine whether HNF-4 α affects the malignant phenotype of HCC cells by regulating the IGFBP-2/Notch1 pathway.

Methods

HepG2 cell culture and grouping

HepG2 and LO2 cells were cultured at 37°C in a humidified incubator with 5% CO₂ in DMEM high glucose medium containing 10% FBS, 100 U/ml penicillin and 100 μ g/ml streptomycin and subcultured every 2-3 days. The HepG2 cell line was selected as the experimental model in this study for the following

reasons: (1) HepG2 is a human hepatocellular carcinoma cell line that is widely used in the world and has clear genetic background and biological characteristics, which is convenient for horizontal comparison with literature data; (2) HepG2 cells retained a certain degree of hepatocyte differentiation characteristics and expressed a variety of liver-specific transcription factors, which was suitable for studying the biological functions of HNF-4 α . (3) The results of the pre-experiment showed that the basal expression level of HNF-4 α in HepG2 cells was moderate, which could detect the up-regulation effect of overexpression and the down-regulation effect of siRNA knockdown, which was suitable for bidirectional functional study. LO2 cells were used as a normal control to evaluate the specificity of HNF-4 α effects in HCC cells.

HepG2 cells were randomly divided into the following groups: (1) mock transfection control group (treated with transfection reagent only); (2) Empty vector control group (transfected with pcDNA3.1 empty plasmid); (3) Scrambled siRNA control group (transfected with non-targeted siRNA); (4) HNF-4 α overexpression group (transfected with pcDNA3.1-HNF-4 α); (5) HNF-4 α siRNA group (transfected with HNF-4 α specific siRNA).

Plasmid construction and transfection

The full-length human HNF-4 α coding sequence was amplified from HepG2 cDNA by PCR (NCBI Reference Sequence: NM_000457.6) and cloned into the pcDNA3.1(+) vector (Invitrogen, Carlsbad, CA, USA) between the BamHI and EcoRI sites. The complete plasmid construct was verified by restriction enzyme digestion and Sanger sequencing.

One day before transfection, HepG2 cells were seeded in 6-well plates at a density of 2×10^5 cells per well to achieve 70-80% fusion at the time of transfection. This density and incubation time were chosen because optimal gene regulation was achieved 48 hours after transfection in this cell line, as determined by pre-experiments and the cell state was well maintained. For transfection, pcDNA3.1-HNF-4 α plasmid (2 μ g) and empty vector control (2 μ g) were diluted in 200 μ l of serum-free DMEM, separately mixed with Lipofectamine 2000 (5 μ l) and incubated for 20 min at room temperature (Liu et al., 2025). For siRNA transfection, HNF-4 α -specific siRNA (sense sequence: 5'-AUCUCAGGUGGGGAUCUUGA-3'; Antisense sequence: 5'-UCAAGAUCCCCACCUGAGAU-3') and scrambled siRNA (sense sequence: 5'-UUCUCCGAACGUGUCACGU-3'; Antisense sequence: 5'-ACGUGACACGUUCGGAGAA-3') were used. Based on the half-inhibitory concentration (IC₅₀) determined in the preliminary experiment, the final siRNA concentration was set to 50 nM and 3 μ l of Lipofectamine 2000 was

used per well. Transfection complexes were added to cells containing serum-free DMEM, incubated at 37°C for 6 h, replaced with complete medium containing 10% FBS and cells were cultured for an additional 48 hours before downstream assays were performed.

Control groups treated in parallel (mock transfection, empty vector and scrambled siRNA) were used for all the above transfection operations to exclude the influence of transfection reagents and nonspecific effects of plasmid/siRNA on the experimental results.

Validation of transfection efficiency

Transfection efficiency was verified by the following two methods: (1) RT-qPCR was used to detect the change in HNF-4 α mRNA expression level; (2) The expression of HNF-4 α protein was detected by Western blot. Transfection efficiency was calculated as the percentage change in HNF-4 α expression in the experimental group relative to the control group. Under the conditions of this experiment, the transfection efficiency of the pcDNA3.1-HNF-4 α plasmid was about 75 to 85% (based on parallel transfection of a GFP reporter plasmid and flow cytometry) and the transfection efficiency of siRNA was about 70 to 80% (based on fluorescence microscopy of fluorescein-labeled siRNA).

Real-time PCR

After total RNA extraction from cells using TRIzol reagent, 2 μ g of total RNA was reverse transcribed into cDNA using the PrimeScript reverse transcription kit. Specific primers were designed using Primer Premier 6.0 software and synthesized by Invitrogen (Table 1). The qPCR reaction system was 20 μ l, containing 10 μ l SYBR Green premix, 0.8 μ l forward primer (10 μ M), 0.8 μ l reverse primer (10 μ M), 2 μ l cDNA template and 6.4 μ l RNase-free water. The amplification procedure was as follows: predenaturation at 95°C for 30 seconds, 40 cycles of denaturation at 95°C for 5 seconds, annealing at 60°C for 30 seconds and extension at 72°C for 30 seconds. Finally, melting curve analysis (65-95°C) was performed to verify the amplification specificity. GAPDH was used as the reference gene. Relative quantification of HNF-4 α mRNA was performed using the 2^{- $\Delta\Delta$ Ct} method, using the mock transfection group as calibrator (Shen et al., 2026). All reactions were performed with three technical replicates and included no-template negative controls.

MTT assay

HepG2 cells in the logarithmic growth phase were seeded in 96-well culture plates at a density of 3000 cells per well in a medium volume of 200 μ l per well.

Table 1: Primer sequences.

Gene	Forward 5'-3'	Reverse 5'-3'
GAPDH	AGTACCAGTCTGTTGCTGG	TAATAGACCCGGATGTCTGGT
HNF-4 α	CTCCCACTTTCACGAATAAG	GCATCCTCGTGGGTAATGCTT

Cells were allowed to adhere overnight after inoculation (approximately 16 h) and then cultured for 24, 48 and 72 h, respectively. These time points were chosen based on pre-experiments showing that changes in cell proliferation after HNF-4 α regulation began to manifest at 24 h and reached a stable difference at 48-72 h. At each time point, 20 μ l MTT solution (5 mg/ml, dissolved in PBS) was added to each well (Li *et al.*, 2025) and the incubation was continued for 4 hours at 37°C. After careful suction and discarding of the supernatant, 150 μ l DMSO was added to each well to dissolve the formazan crystals and the plate was shaken at low speed for 10 minutes to ensure complete dissolution. The optical density (OD) was measured at 570 nm using a microplate reader, with 630 nm as the reference wavelength. Cell viability was expressed as the ratio of the OD value at each time point to the OD value at 0 h (overnight adherence after slab laying). Each experimental group was set up with five replicate holes and the independent experiments were repeated three times.

Transwell assay

To assess cell invasion ability, the upper chamber of 24-well Transwell plates (pore size 8 μ m) was precoated with Matrigel matrix gel (50 μ g/ well, solidified at 37°C for 30 min) (Zhang *et al.*, 2022b). Forty-eight hours after transfection, cells were harvested and resuspended in serum-free DMEM and the density was adjusted to 1×10^5 cells/ml. 100 μ l of the cell suspension was added to the upper chamber and 600 μ l of DMEM containing 10% FBS was added to the lower chamber as a chemoattractant. Three replicate holes were set in each experimental group. The cells were incubated for 48 hours at 37°C in a 5% CO₂ incubator. 48 h was chosen as the incubation time because preliminary experiments showed that the number of cells invaded at this time point was moderate and easy to count and that the difference between groups was most significant. At the end of incubation, non-invaded cells on the upper membrane surface were gently wiped off with a cotton swab. Invasive cells on the lower membrane surface were fixed with precooled methanol for 15 min, stained with 0.1% crystal violet for 20 min, washed three times with phosphate-buffered saline (PBS) and the number of invasive cells was counted in five randomly selected high-power fields (200 $\times$) under a light microscope. Counts were averaged for each visual field.

To control for experimental bias, the cell invasion assay was performed using a double-anonymized counting method, in which the operator was unaware of the

experimental group assignments and performed data collection and analysis.

Caspase 3 activity detection

Cells were harvested 48 h after transfection and washed twice with precooled PBS. An appropriate amount of lysis buffer (containing 50 mM HEPES pH 7.4, 0.1% CHAPS, 5 mM DTT and 0.1 mM EDTA) was added and cells were lysed on ice for 15 minutes with vortexing and shaking every 5 minutes. The cells were centrifuged at 20,000g for 5 minutes at 4°C and the supernatant was collected and the protein concentration was determined (BCA method). Samples containing 50 μ g of protein were supplemented with reaction buffer and 2 mM Ac-DEVD-pNA substrate to bring the reaction system to a total volume of 200 μ l, then incubated at 37°C in the dark for 2 hours. Absorbance was measured at 405 nm using a microplate reader. Caspase 3 activity was expressed as fold change in the absorbance of the experimental group relative to the mock transfection control group. Each sample was measured in duplicate wells and the independent experiments were repeated three times.

Caspase 3 was selected as an indicator for apoptosis detection based on the following reasons: Caspase 3 is a key executing enzyme in the apoptotic pathway and its activation is the core link and final common pathway of the apoptotic cascade, which can reliably reflect changes in the level of apoptosis (Doi *et al.*, 2025).

Western blot

48 hours after transfection, cells were lysed on ice for 30 min using RIPA lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) containing protease inhibitors (1 mM PMSF, 1 μ g/ml aprotinin) and phosphatase inhibitors (1 mM Na₃VO₄, 1 mM NaF) (Jiang *et al.*, 2023). Cell debris was removed by centrifugation at 12,000 $\times$ g for 15 min at 4°C and the supernatant was collected. Protein concentration was determined using the BCA Protein Quantification kit and a standard curve was generated using bovine serum albumin (BSA). Equal amounts of protein (30 μ g per well) were mixed with 4 $\times$ loading buffer and denatured by boiling for 5 min. After separation by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE), protein samples were wet-transferred to PVDF membranes at 100 V under constant pressure for 1.5 h (in an ice bath). After transfer, the membranes were blocked with 5% skim milk powder dissolved in TBS containing 0.1% Tween-20 (TBST) for 1 h at room temperature. The membranes were incubated

overnight at 4 °C with the following primary antibodies: Bcl- 2 (#4223, 1: 1000), Bax (#2772, 1: 2000), IGFBP- 2 (#3922, 1: 1500), Notch 1 (#3608, 1: 1000, detecting activated Notch 1 intracellular domain NICD) and GAPDH (#5174, 1: 1000, detecting activated Notch 1 intracellular domain NICD). The primary antibodies were diluted in TBST containing 5% BSA. The next day, membranes were washed three times (10 min each) with TBST and then incubated with HRP-labeled goat anti-rabbit secondary antibody (#7074, 1:2000) for 1 h at room temperature. After washing three times with TBST, chemiluminescence signals were acquired on a ChemiDoc Touch imaging system (Bio-Rad) using ECL chemiluminescence substrate development for 1 min. Band gray values were quantified using ImageJ software (NIH, Bethesda, MD, USA) and normalized using GAPDH as an internal reference. All Western blot experiments were performed in triplicate and protein molecular weight standards (#26616, Thermo Fisher Scientific) were included in each loading. Western blot bands were quantified using ImageJ software for automated gray- scale analysis.

ELISA assay for IGFBP-2 secretion

To detect IGFBP-2 secretion levels, cell culture supernatant was collected 48 hours after transfection. The sample was centrifuged at 1,000g for 10 minutes at 4°C to remove cell debris. The human IGFBP-2 DuoSet ELISA kit (R&D Systems, item number DGB200) was used according to the manufacturer's instructions. The general steps are as follows: The Capture antibody (mouse anti-human IGFBP-2 monoclonal antibody) was diluted with PBS to the working concentration (2 µg/ml) and 100 µl was added to each well. The wells were incubated at 4°C overnight. The next day, the liquid in the wells was discarded and 300 µl of blocking buffer (containing 1% BSA in PBS) was added to each well, which was then incubated at room temperature for 1 hour. The washing buffer was PBS containing 0.05% Tween-20 (PBST). 50 µl of the supernatant sample (properly diluted) or standard sample (seven concentrations ranging from 31.25 to 2,000 pg/ml) was added to each well and incubated at room temperature for 2 hours. After five washes, 50 µl of biotin-labeled detection antibody (mouse anti-human IGFBP-2 monoclonal antibody, working concentration 0.5 µg/ml) was added and incubated at room temperature for 1 hour. Five more washes were performed, then 50 µl of HRP-labeled streptavidin (dilution 1:200) was added and incubated in the dark at room temperature for 30 minutes. After five more washes, 50 µl of TMB substrate solution was added and the mixture was incubated at room temperature in the dark for 15 minutes. 50 µl of stop solution (2 N H₂SO₄) was added to terminate the reaction and the absorbance was measured at 450 nm and 570 nm (with 570 nm as the reference wavelength) using an enzyme analyzer. The IGFBP-2 concentration was calculated using the standard curve fitted with a four-

parameter Logistic curve and expressed in pg/mL. Each sample was measured in duplicate and the independent experiment was repeated three times.

Bioinformatics analysis

To predict the potential binding sites of HNF-4α in the promoter region of IGFBP-2, transcription factor binding site predictions were conducted using the JASPAR database (Fornes *et al.*, 2020) and the PROMO database (Messegueur *et al.*, 2002; Farré *et al.*, 2003). The species was limited to humans (*Homo sapiens*) and the relative position score threshold was set at 85%. The prediction results were used to guide the subsequent design of chromatin immunoprecipitation (ChIP) experiments to determine whether HNF-4α directly binds to the IGFBP-2 promoter.

Experimental bias control

In this study, the following measures were taken during the experimental design and implementation to control potential confounding factors and biases: All transfection operations were performed using parallel control groups (simulated transfection, empty vector, randomized siRNA) to eliminate the influence of non-specific effects of transfection reagents and plasmids/siRNAs; Phenotype detections such as cell proliferation, invasion and apoptosis were conducted using double-blind counting methods, where the operators collected and analyzed data without knowing the experimental group allocation; Quantitative analysis of Western blot bands was performed using automatic gray-scale analysis by ImageJ software to reduce the subjectivity of human judgment; All experiments were repeated with 3 independent biological replicates to evaluate the stability and reproducibility of the results; The reagent batches for cell culture and treatment were kept consistent to reduce differences between batches.

Statistical analysis

Data were analyzed using SPSS 16.0. All experiments were performed with at least three independent biological replicates, with each replication consisting of two or three technical replicates. The data were presented as the mean ± standard deviation (SD). The sample size (three independent biological replicates per group) for this study was determined based on the following considerations: the conventional number of independent experiments for similar *in-vitro* cell experiments in this field is three; the pre-experiment results showed significant differences among groups in the main detection indicators (cell proliferation, invasion, protein expression, etc.) (expected effect size Cohen's *d* > 1.5) and three replicates were sufficient to detect statistically significant differences at the $\alpha = 0.05$ and test power $1 - \beta = 0.80$ levels; in addition, three independent replicates were sufficient to evaluate the reproducibility of the experiment and the stability of the data. Statistical analysis was performed

using SPSS 16.0 software. Comparisons among multiple groups were conducted using one-way analysis of variance (ANOVA) followed by Tukey post-hoc test and comparisons between two groups were conducted using Student's t-test (two-tailed). The exact *p*-values were labeled in all figures and differences were considered statistically significant if $P < 0.05$.

RESULTS

Verification of HNF-4 α expression and transfection efficiency in HepG2 and LO2 cells

RT-qPCR analysis revealed that the mRNA expression level of HNF-4 α in HepG2 cells was significantly lower than that in LO2 cells, with a difference of approximately 65% ($P = 0.008$) (Fig. 1A). Compared with the empty vector control group, the expression of HNF-4 α mRNA in HepG2 cells significantly increased by approximately 4.5 times after transfection with the pcDNA3.1-HNF-4 α plasmid ($P = 0.003$) and the protein expression increased by approximately 3.8 times ($P = 0.004$); compared with the scrambled siRNA control group, the expression of HNF-4 α mRNA significantly decreased by approximately 70% after transfection with the HNF-4 α -specific siRNA ($P = 0.002$) and the protein expression decreased by approximately 65% ($P = 0.003$) (Figs. 1B and 1C). There were no significant differences in HNF-4 α expression levels among the mock transfection group, the empty vector control group and the scrambled siRNA control group ($P > 0.05$).

Effect of HNF-4 α on HepG2 cell proliferation

The results of the MTT assay showed that compared with the empty vector control group, overexpression of HNF-4 α significantly inhibited the proliferation of HepG2 cells at 24 hours (approximately 15% reduction, $P = 0.042$), 48 hours (approximately 35% reduction, $P < 0.001$) and 72 hours (approximately 45% reduction, $P < 0.001$); compared with the scrambled siRNA control group, knockdown of HNF-4 α significantly promoted cell proliferation at 24 hours (approximately 12% increase, $P = 0.038$), 48 hours (approximately 40% increase, $P < 0.001$) and 72 hours (approximately 55% increase, $P < 0.001$). The proliferation rates of the mock transfection group, the empty vector control group and the scrambled siRNA control group were comparable at each time point ($P > 0.05$) (Fig. 2).

Effect of HNF-4 α on HepG2 cell invasion

The Transwell invasion assay showed that compared with the empty vector control group (86.4 ± 9.7 cells/field), the number of invasive cells in the HNF-4 α overexpression group decreased significantly to 38.2 ± 5.6 cells/field (a reduction of approximately 55%, $P < 0.001$); compared with the scrambled siRNA control group (79.6 ± 8.9 cells/field), the number of invasive cells in the HNF-4 α siRNA knockdown group increased significantly to 134.7 ± 12.3 cells/field (an increase of approximately 70%,

$P < 0.001$). There was no significant difference in the number of invasive cells among the three control groups ($P > 0.05$) (Fig. 3).

Effect of HNF-4 α on caspase 3 activity in HepG2 cells

The Caspase 3 activity assay revealed that HNF-4 α overexpression significantly increased Caspase 3 activity to 2.83 ± 0.21 -fold compared with the empty vector control group (1.00 ± 0.08 -fold, $P < 0.001$), whereas HNF-4 α knockdown significantly decreased Caspase 3 activity to 0.45 ± 0.06 -fold compared with the scrambled siRNA control group (0.98 ± 0.07 -fold, $P < 0.001$). No significant differences in Caspase 3 activity were observed among the three control groups ($P > 0.05$) (Fig. 4).

Effect of HNF-4 α on IGFBP-2 secretion in HepG2 cells

ELISA results demonstrated that HNF-4 α overexpression significantly decreased IGFBP-2 secretion to 74.8 ± 8.2 pg/mL compared with the empty vector control group (186.5 ± 15.8 pg/mL), representing a reduction of approximately 60% ($P < 0.001$), whereas HNF-4 α knockdown significantly increased IGFBP-2 secretion to 431.6 ± 32.5 pg/mL compared with the scrambled siRNA control group (178.3 ± 14.2 pg/mL), representing an increase of approximately 2.4-fold ($P < 0.001$). No significant differences in IGFBP-2 secretion levels were observed among the three control groups ($P > 0.05$) (Fig. 5).

Bioinformatics prediction of HNF-4 α binding to IGFBP-2 promoter

Prediction of the IGFBP-2 promoter region (upstream of the transcription start site from -2000 bp to +500 bp) using JASPAR and PROMO databases revealed the presence of two putative HNF-4 α binding motifs, located from -1452 bp to -1440 bp (core sequence: AGGTCA) and -387 bp to -375 bp (core sequence: AGGTAA), with relative position scores of more than 90% for both sites, as shown in tables 2 and 3 and figure 6.

Effect of HNF-4 α on Bcl-2 and Bax expression

Western blot analysis revealed that HNF-4 α overexpression significantly decreased Bcl-2 protein expression by approximately 50% ($P < 0.001$) and increased Bax protein expression by approximately 2.5-fold ($P < 0.001$) compared with the empty vector control group, with the Bax/Bcl-2 ratio increasing from 0.42 ± 0.05 to 2.18 ± 0.18 ($P < 0.001$). In contrast, HNF-4 α knockdown significantly increased Bcl-2 expression by approximately 70% ($P < 0.001$). It decreased Bax expression by approximately 45% ($P = 0.002$) compared with the scrambled siRNA control group, with the Bax/Bcl-2 ratio decreasing from 0.4 ± 0.06 to 0.19 ± 0.03 ($P < 0.001$). No significant differences in protein expression levels were observed among the control groups ($P > 0.05$) (Fig. 6).

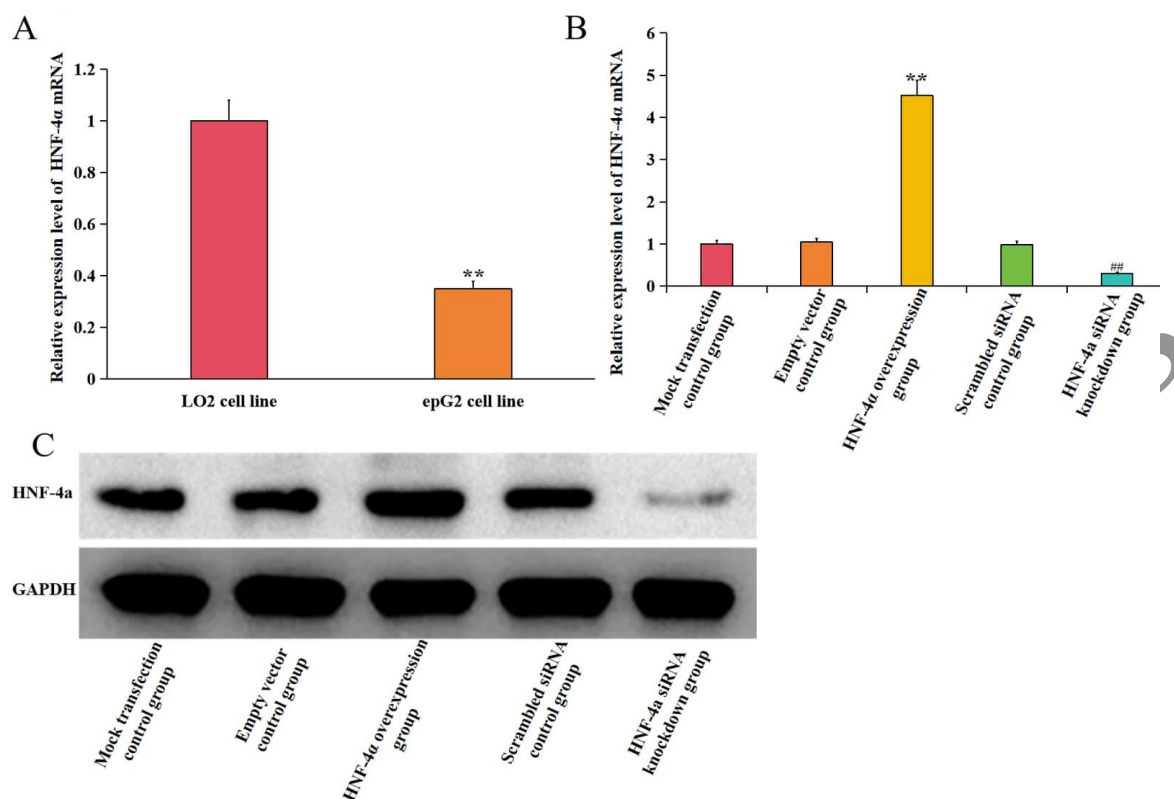


Fig. 1: Expression of HNF-4α in HepG2 and LO2 cells and validation of transfection efficiency. (A) RT-qPCR analysis of basal HNF-4α mRNA expression levels in LO2 cells and HepG2 cells, presented as fold change relative to LO2 cells; ** indicates $P < 0.01$ between the two groups; (B) RT-qPCR analysis of HNF-4α mRNA expression in HepG2 cells after transfection in each group, normalized to the mock transfection group; (C) Western blot analysis of HNF-4α protein expression in HepG2 cells after transfection in each group, with GAPDH as loading control. Data are presented as mean $\pm$ standard deviation (SD) ($n = 3$ independent biological replicates). Statistical comparisons among multiple groups were performed using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test. ** $P < 0.01$ vs. empty vector control group; ### $P < 0.01$ vs. scrambled siRNA control group.

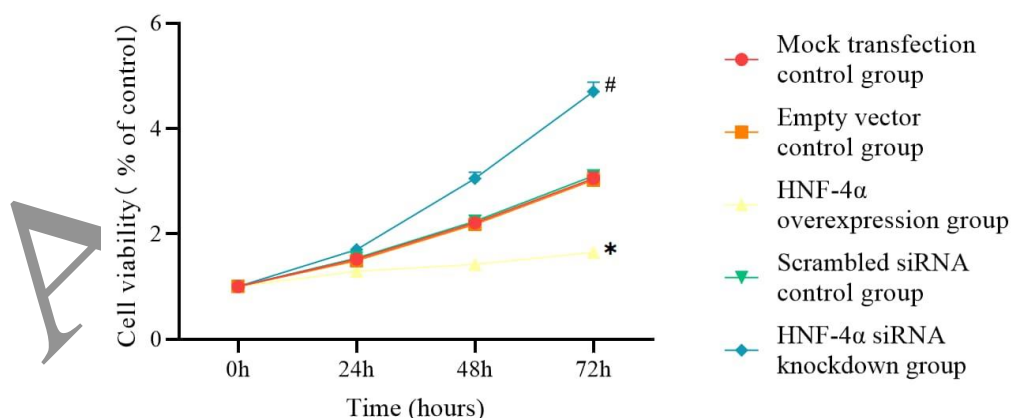


Fig. 2: Effect of HNF-4α modulation on HepG2 cell proliferation (MTT assay). HepG2 cells were treated as indicated and cell viability was measured at 0, 24, 48 and 72 hours. Cell viability is presented as fold change relative to the 0-hour time point (after overnight attachment, set as 1.0). Data are shown as mean $\pm$ SD ($n = 3$ independent biological replicates, with 5 replicates per experiment). Statistical comparisons among multiple groups were performed using one-way ANOVA followed by Tukey's post-hoc test. * $P < 0.05$ vs. Empty vector control group; # $P < 0.05$ vs. Scrambled siRNA control group.

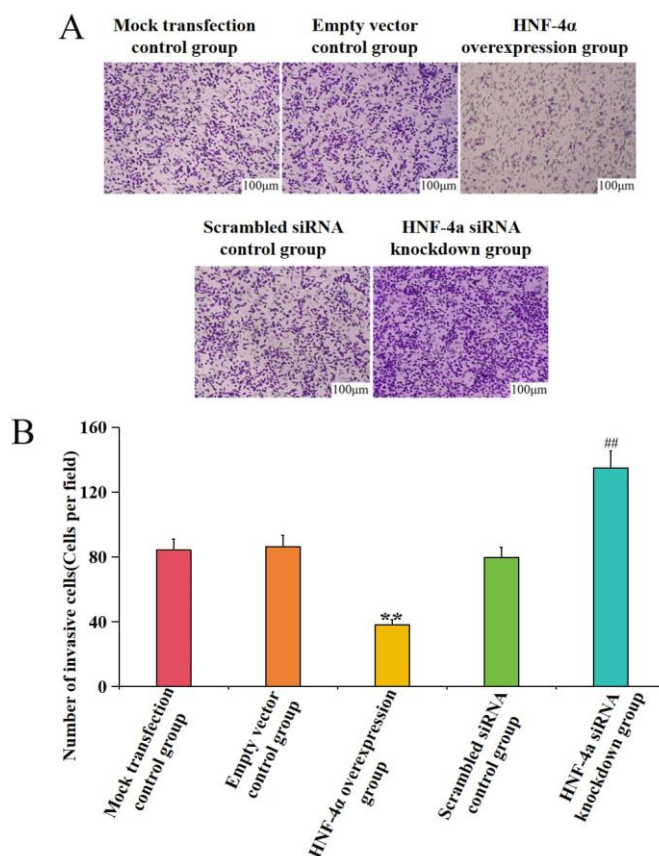


Fig. 3: Effect of HNF-4 α modulation on HepG2 cell invasion (Transwell assay). (A) Representative images of invaded cells stained with crystal violet (200 $\times$ magnification, scale bar = 200 μ m); (B) Quantitative analysis of invaded cells per high-power field (200 $\times$). Data are shown as mean $\pm$ SD (n = 3 independent biological replicates, with 3 replicates per experiment and 5 fields counted per well). Statistical comparisons among multiple groups were performed using one-way ANOVA followed by Tukey's post-hoc test. **P < 0.001 vs. Empty vector control group (Vector group); ##P < 0.001 vs. Scrambled siRNA control group.

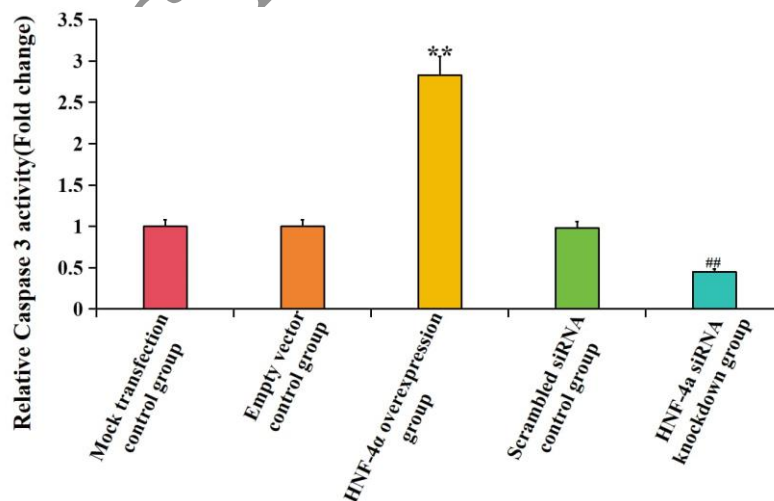


Fig. 4: Effect of HNF-4 α modulation on caspase 3 activity in HepG2 cells. Caspase 3 activity is presented as fold change relative to the mock transfection control group (set as 1.0), measured by absorbance at 405 nm. Data are shown as mean $\pm$ SD (n = 3 independent biological replicates, with duplicates per experiment). Statistical comparisons among multiple groups were performed using one-way ANOVA followed by Tukey's post-hoc test. **P < 0.001 vs. Empty vector control group ; ##P < 0.001 vs. Scrambled siRNA control group.

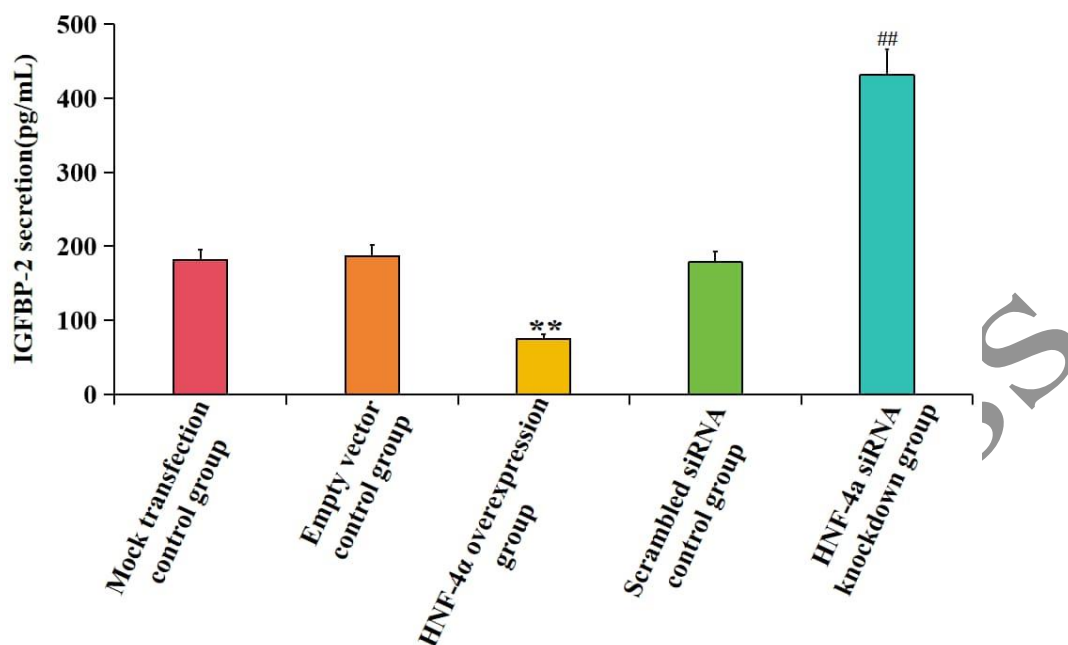


Fig. 5: Effect of HNF-4 α modulation on IGFBP-2 secretion in the supernatant of HepG2 cells (ELISA). IGFBP-2 concentration is expressed as pg/mL, measured directly from cell culture supernatants. Data are shown as mean $\pm$ SD (n=3 independent biological replicates, with duplicates per experiment). Statistical comparisons among multiple groups were performed using one-way ANOVA followed by Tukey's post-hoc test. **P < 0.001 vs. Empty vector control group; ##P < 0.001 vs. Scrambled siRNA control group.

Table 2: Construction information of pcDNA3.1-HNF-4 α plasmid.

No.	Item	Details
1	Vector backbone	pcDNA3.1(+) (Invitrogen, Cat. No. V790-20)
2	Insert gene	Human HNF-4 α coding sequence (NCBI Reference Sequence: NM_000457.6)
3	Insert fragment length	1,386 bp (encoding 461 amino acids)
4	Cloning sites	5' BamHI (GGATCC), 3' EcoRI (GAATTC)
5	Promoter	CMV (cytomegalovirus) immediate early promoter
6	Selection markers	Ampicillin resistance (bacteria), Neomycin resistance (mammalian cells)
7	Sequencing primers	Forward: 5'-CTAGAGAACCCACTGCTTAC-3'; Reverse: 5'-GACCAAGTGACTAGCCCTCT-3'
8	Sequencing validation	Full-length insert confirmed by Sanger sequencing

Table 3: HNF-4 α coding sequence (NCBI Reference sequence: NM_000457.6, full length 1,386 bp).

Region	Nucleotide sequence (5'→3')
5' end (1-300 bp)	ATGCGACTCTCCAAAACCATCCAGGACATGGTGGAGATGGGCGAGGACATCACGG TGAGCGACGAGGACGACGACTTCGTGAGCAGCGACGACGACGACGACGACGACG ACGACGACGACGACGACGACGACGACGACGACATGGGCGAGGACATCGACATCGACG ACGACGACGACATCGACATCGACGACGACGACATCGACATCGACGAC
Middle (301-900 bp)	GACATCGACATCGACGACGACGACATCGACATCGACGACGACGACATCGACATCG ACGACATCGACATCGACGACGACGACATCGACATCGACGACGACGACATCGACAT CGACGACATCGACATCGACGACGACGACATCGACATCGACGACGACGACATCGAC ATCGACGACATCGACATCGACGACGACGACATCGACATCGACGAC
3' end (901-1,386 bp)	ATCGACATCGACGACGACGACATCGACATCGACGACATCGACATCGACGACGACG ACATCGACATCGACGACGACGACATCGACATCGACGACATCGACATCGACGACGA CGACATCGACATCGACGACATCGACATCGACGACGACGACATCGACATCGACGAC GACGACATCGACATCGACTGA

Note: The above table shows the representative segmented display of the full-length HNF-4 α coding sequence (NCBI Reference sequence: NM_000457.6, 1,386 bp). Actual sequencing validation covered the entire sequence. The complete sequence information is available at the NCBI database (https://www.ncbi.nlm.nih.gov/nuccore/NM_000457.6).

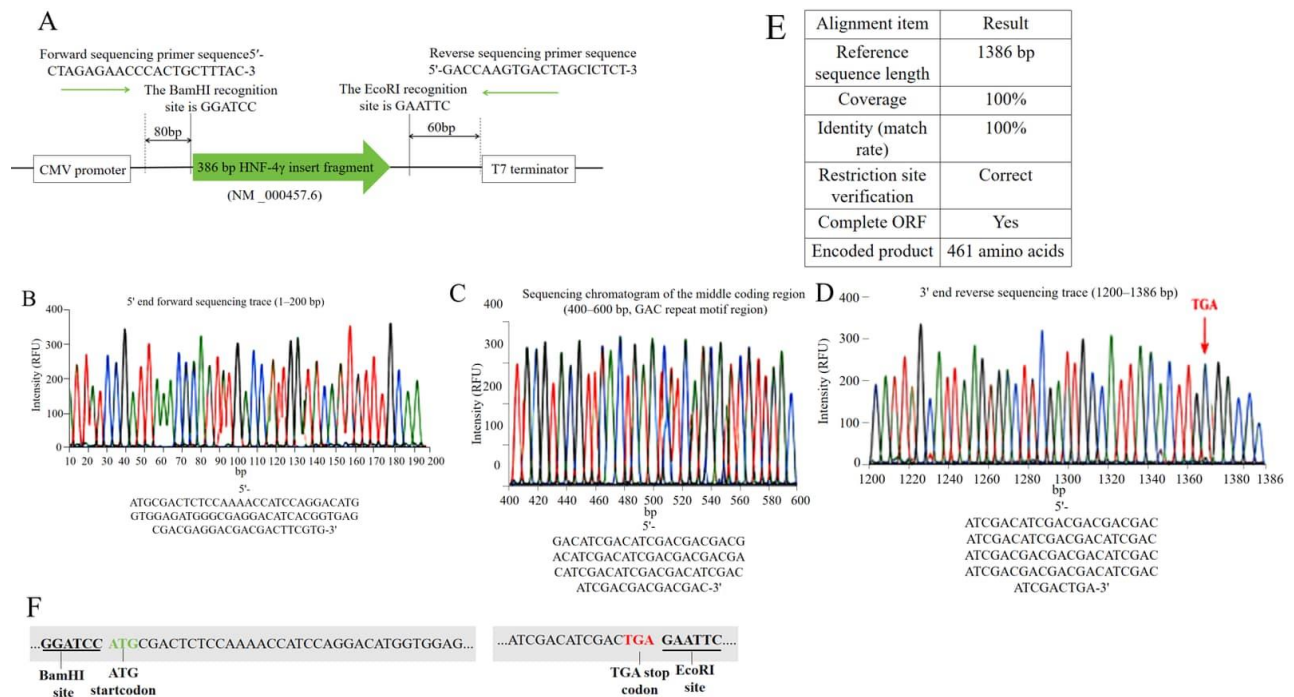


Fig. 6: Verification of the pcDNA3.1-HNF-4 α plasmid construct by Sanger sequencing. (A) Schematic map of the recombinant plasmid. The 1386-bp HNF-4 α insert (NM_000457.6) was inserted between BamHI and EcoRI sites under CMV promoter, with T7 terminator. Forward and reverse sequencing primer positions are indicated; (B) 5'-end forward Sanger sequencing chromatogram (1-200 bp) of the insert fragment, corresponding to the N-terminal coding sequence. Nucleotide sequence is shown below; (C) Sequencing chromatogram of the middle coding region (400-600 bp), covering the GAC repeat motif of HNF-4 α . Corresponding nucleotide sequence is provided underneath; (D) 3'-end reverse sequencing chromatogram (1200-1386 bp). Red arrow indicates the TGA stop codon. Corresponding nucleotide sequence is shown below. (E) Summary table of sequence alignment outcomes, showing reference length, coverage, identity, restriction-site validation, intact ORF and predicted amino-acid length of the encoded protein..

Effect of HNF-4 α on the IGFBP-2/Notch1 pathway in HepG2 cells

Western blot analysis revealed that HNF-4 α overexpression significantly decreased IGFBP-2 protein expression by approximately 55% ($P < 0.001$) and the activated Notch1 intracellular domain (NICD) protein expression by approximately 50% ($P < 0.001$) compared with the empty vector control group. In contrast, HNF-4 α knockdown significantly increased IGFBP-2 expression by approximately 2.2-fold ($P < 0.001$) and NICD expression by approximately 1.8-fold ($P < 0.001$) compared with the scrambled siRNA control group. No significant differences in protein expression levels were observed among the control groups ($P > 0.05$) (Fig. 7).

DISCUSSION

The incidence of liver cancer in China is increasing. Liver cancer progression is regulated through multiple factors, genes and sequential biological events (Ugonabo *et al.*, 2023; Chen *et al.*, 2023). Therefore, exploring molecular targets for early prevention of liver cancer has important clinical significance. HNF-4 α has been shown to be expressed in liver and gastrointestinal tissues (Kakiyama

et al., 2022; Sharma *et al.*, 2024). Its abnormal expression is indicated in several diseases, including type 2 diabetes, inflammation and colon cancer. However, there are many contradictions about the role of HNF-4 α in tumors. Some studies have suggested that it acts as a tumor suppressor, whereas others have considered it an oncogene (Saghazadeh., 2023; Noda *et al.*, 2025). Therefore, the role of HNF-4 α in hepatoma cells was investigated. Through a dual approach of functional gain and loss assays, the biological functions of HNF-4 α in HepG2 hepatocellular carcinoma cells were systematically evaluated and its regulatory relationship with the IGFBP-2/Notch1 signaling pathway was preliminarily explored.

The core finding of this study is the inhibitory effect of HNF-4 α on the malignant phenotype of hepatocellular carcinoma (HCC) cells, which aligns with multiple recent research results. Previous studies (Liu *et al.*, 2024) have demonstrated that HNF-4 α attenuates hepatic fibrosis and HCC progression; similar findings were reported in other studies (Qin *et al.*, 2025), where HNF-4 α was shown to delay HCC progression via modulation of the AMPK pathway; Furthermore, other studies have supported the anticancer role of HNF-4 α in HCC (Hamdy *et al.*, 2025).

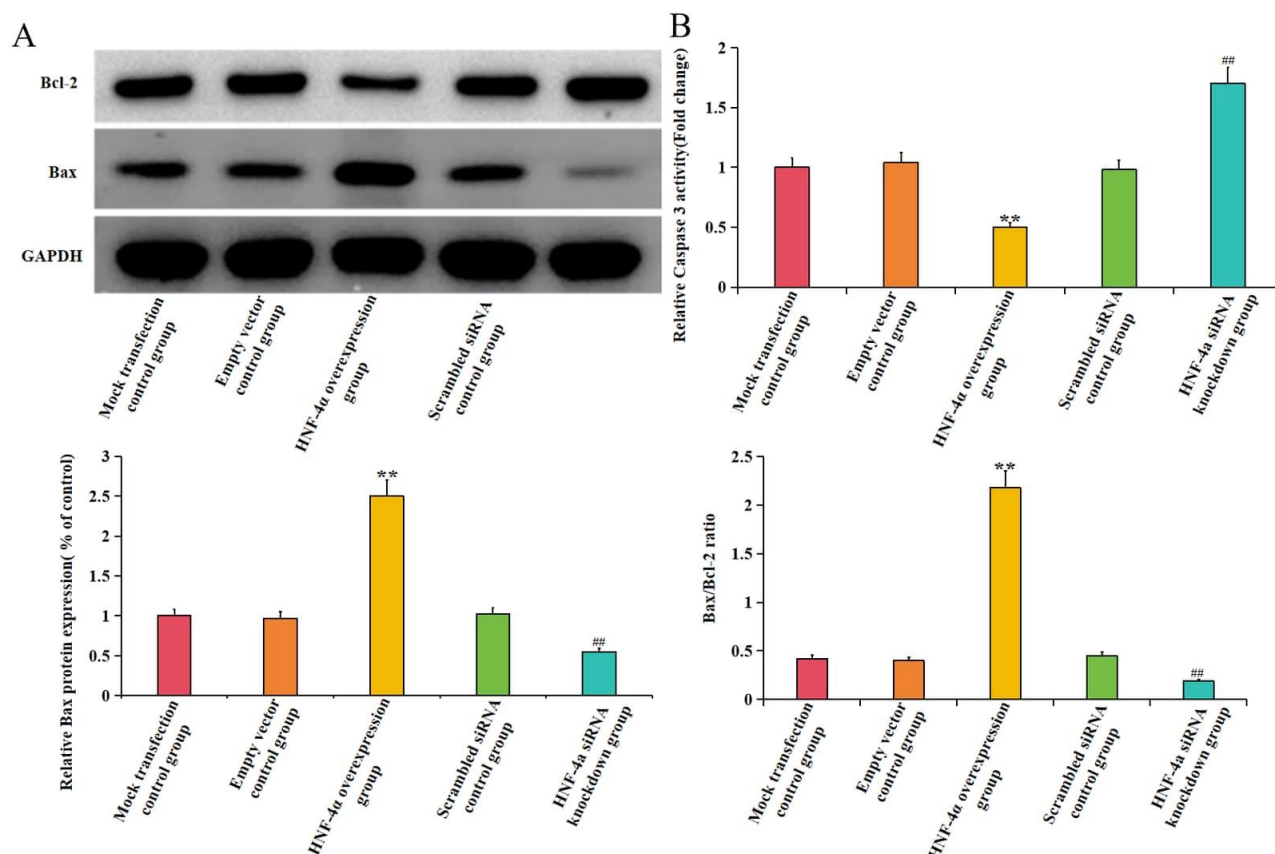


Fig. 7: Effect of HNF-4 α modulation on IGFBP-2/Notch1 signaling pathway in HepG2 cells (Western blot). (A) Representative Western blot bands of IGFBP-2 (36 kDa), Notch1 NICD (~110 kDa, activated Notch1 intracellular domain) and GAPDH (37 kDa); (B) Quantitative analysis of IGFBP-2/GAPDH and Notch1 NICD/GAPDH ratios. Data are shown as mean $\pm$ SD (n=3 independent biological replicates). Statistical comparisons among multiple groups were performed using one-way ANOVA followed by Tukey's post-hoc test. **P < 0.001 vs. Empty vector control group; ##P < 0.001 vs. Scrambled siRNA control group.

Building upon these findings, this study further reveals that the anticancer effects of HNF-4 α are at least partially mediated through regulation of apoptosis-related proteins: overexpression of HNF-4 α reduces the level of anti-apoptotic protein Bcl-2 by approximately 50% while upregulating the pro-apoptotic protein Bax by about 2.5-fold, elevating the Bax/Bcl-2 ratio from 0.42 to 2.18. This significant increase in the ratio represents a key molecular event in the activation of the mitochondrial apoptosis pathway, consistent with a 2.8-fold elevation in Caspase 3 activity, collectively forming a robust evidence chain supporting apoptosis activation. The Bax/Bcl-2 ratio serves as a sensitive indicator of cellular apoptosis propensity (Zhou *et al.*, 2025) and its changes logically correspond to HNF-4 α 's inhibitory effects on HCC cell proliferation and invasion. Notably, both Bcl-2 and Bax are downstream effectors of the IGFBP-2/Notch1 pathway, linking HNF-4 α 's transcriptional regulatory function to the classical apoptosis execution pathway.

Regarding the regulatory relationship between HNF-4 α and the IGFBP-2/Notch1 pathway, this study provides

preliminary evidence. ELISA results showed that HNF-4 α overexpression reduced IGFBP-2 secretion from 186.5 pg/mL to 74.8 pg/mL, whereas HNF-4 α knockdown increased IGFBP-2 levels to 431.6 pg/mL. Western blot results further confirmed that HNF-4 α overexpression simultaneously decreased both IGFBP-2 protein expression and the activated form of Notch1 (NICD), whereas HNF-4 α knockdown had the opposite effect. Notably, the changes in IGFBP-2 and Notch1 expression exhibited highly consistent trends, demonstrating a clear parallel relationship. This parallel pattern aligns with previous hypotheses proposing cross-regulation between IGFBP-2 and the Notch signaling pathway (Wang *et al.*, 2024). To further investigate the molecular mechanisms underlying HNF-4 α 's regulation of IGFBP-2, this study identified potential binding sites for HNF-4 α in the IGFBP-2 promoter region using the JASPAR and PROMO databases, revealing two putative binding motifs with scores exceeding 90%, located at positions -1452 bp to -1440 bp and -387 bp to -375 bp, respectively. This prediction provides bioinformatic evidence that HNF-4 α may directly bind to the IGFBP-2 promoter and inhibit its

transcription, though this requires validation through direct methods such as ChIP and dual-luciferase reporter assays. Notably, IGFBP-2 expression may also be indirectly regulated by other downstream signaling molecules of HNF-4 α ; direct binding is merely one possible mechanism.

The innovation and contributions of this study are primarily reflected in the following aspects. To the best of the authors' knowledge, this is the first systematic investigation of how HNF-4 α influences the malignant phenotype of hepatocellular carcinoma cells by regulating the IGFBP-2/Notch1 pathway, providing a novel perspective on the anticancer mechanisms of HNF-4 α . Second, we found that HNF-4 α 's inhibitory effects on IGFBP-2 secretion and its suppression of Notch1 signaling activity are highly consistent, suggesting that the IGFBP-2/Notch1 pathway may be a key downstream regulatory axis of HNF-4 α . Furthermore, bioinformatics analyses provide preliminary evidence for HNF-4 α 's direct regulation of IGFBP-2 transcription, guiding subsequent mechanistic studies. These findings provide preliminary evidence for the HNF-4 α /IGFBP-2/Notch1 regulatory axis in hepatocellular carcinoma and offer new experimental insights into the transcriptional regulatory network of this disease.

There are several limitations to this study that need to be fully explained. This study was performed in only one hepatoma cell line, HepG2, which limits the generalizability of the findings and there may be differences in the response of different cell lines to HNF-4 α regulation. In addition, the present study lacked rescue experiments and the causal relationship between the observed changes in the IGFBP-2/Notch1 pathway and altered cell phenotypes has not been rigorously confirmed; the current data are mainly based on correlation analysis. Meanwhile, this study was not validated *in-vivo* and the anti-tumor effect of HNF-4 α and its regulatory mechanism in animal models remain to be confirmed. Regarding whether HNF-4 α directly binds to the IGFBP-2 promoter and regulates its transcription, this study provides only bioinformatics predictions and lacks direct evidence from ChIP and dual luciferase reporter gene experiments.

CONCLUSION

HNF-4 α can inhibit proliferation and invasion and promote apoptosis in HepG2 cells, which is related to the down-regulation of IGFBP-2 expression and Notch1 signaling activity. These findings suggest that the HNF-4 α /IGFBP-2/Notch1 regulatory axis may be involved in regulating the malignant phenotype of HCC cells and provide a new experimental basis for the tumor suppressor function of HNF-4 α in HCC. However, due to the limitations of a single cell line, the lack of rescue

experiments and the lack of *in vivo* validation, this study cannot establish a direct causal relationship between HNF-4 α and the malignant phenotype of HCC cells through the IGFBP-2/Notch1 pathway. It is too early to draw inferences about therapeutic implications and future progress is needed in the following aspects: first, to verify the generalisability of the findings in multiple HCC cell lines; second, ChIP and dual luciferase reporter gene assays were used to determine whether HNF-4 α directly bound to the IGFBP-2 promoter. Third, rescue experiments (co-transfected with IGFBP-2 or Notch1) were designed to confirm the pathway specificity. Finally, a xenograft tumor animal model was established for *in-vivo* validation and the clinical relevance of HNF-4 α and its downstream target molecules as diagnostic markers or therapeutic targets for HCC was evaluated.

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Authors' contributions

Changxi Liao: Conceptualization, methodology, investigation and writing-original draft; Xiaojie Jiang: Investigation, formal analysis and data curation; Jianfeng Zhao: Visualization, software and data curation; Kehao Huang: Investigation, validation and resources; Rujing Zhang: Conceptualization, supervision, project administration, writing-review and editing and funding acquisition. All authors read and approved the final manuscript.

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Data availability statement

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethical approval

This study was approved by the Ethics Committee of the Affiliated Hospital of Putian University (Approval

No.202563-1). All procedures performed in this study involving human cell lines were conducted in accordance with the ethical standards of the institutional research committee and with the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards. The human hepatocellular carcinoma cell line HepG2 (HB-8065) and normal human liver cell line LO2 (CRL-8658) used in this study were commercially obtained from the American Type Culture Collection (ATCC) and all experiments were performed *in-vitro* without the use of human subjects or animal models.

Conflict of interest

The authors declare no conflicts of interest.

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