Article

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The Expression of NXPH4 in Hepatocellular Carcinoma Tissues and Its Impact on the Biological Functions of Hepatic Cancer Cells

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Background: There is an urgent need for reliable biomarkers for early detection and effective therapeutic targeting of hepatocellular carcinoma (HCC). This investigation aimed to elucidate neurexophilin 4 (NXPH4) expression in HCC and explore its impact on the biological functions of HCC cells.

Methods: This research employed publicly available transcriptomic data from The Cancer Genome Atlas (TCGA) database to evaluate the expression profiles of the NXPH family members in HCC. We collected 70 HCC tissue samples and assessed NXPH4 expression levels using quantitative real-time polymerase chain reaction (qRT-PCR), Western blot analysis, and immunohistochemistry. Furthermore, HCC cell lines with overexpressed and knocked down NXPH4 were established. Methyl thiazolyl tetrazolium (MTT) assay and colony formation analysis were conducted to assess the proliferation and colony formation capabilities of the HCC cells. Additionally, scratch assays and transwell experiments were employed to elucidate the migration and invasion capabilities of these cells.

Results: NXPH4 showed significant differential expression in HCC compared to adjacent non-cancerous tissues ($p < 0.001$), which was associated with overall patient survival ($p < 0.001$). Moreover, the knockdown of NXPH4 (shNXPH4-1 and shNXPH4-2) significantly suppressed the proliferation and clonogenicity of HepG2 HCC cells ($p < 0.01$). Conversely, transfection with an overexpression vector (Ov-NXPH4) substantially elevated the proliferation and clonogenicity of Hub-7 cells ($p < 0.01$). Furthermore, knockdown of NXPH4 (shNXPH4-1 and shNXPH4-2) significantly suppressed the migration and invasion of HepG2 cells ($p < 0.01$), while its overexpression vector (Ov-NXPH4) significantly promoted these capabilities ($p < 0.01$).

Conclusion: Elevated expression of NXPH4 in HCC regulates crucial biological processes, such as proliferation, migration, and invasion, suggesting its potential role as a biomarker for the progression of hepatocellular carcinoma.

Keywords: hepatocellular carcinoma (HCC); NXPH4; proliferation; migration; invasion

Introduction

Hepatocellular carcinoma (HCC), characterized by its high malignancy and propensity for metastasis, poses a significant threat to human health, ranking among the top six in incidence and top three in mortality among malignant tumors [1]. Understanding the underlying mechanisms driving the development of HCC remains a pressing scientific challenge in current clinical practice. The progression of HCC often involves alterations in gene expression, dysregulation of metabolic pathways, and abnormalities in cell signaling [2]. Specifically, the enhanced capabilities of HCC cells in proliferation, invasion, migration, and resistance to anticancer treatments are closely associated with the molecular regulatory mechanisms of HCC [3]. Therefore, gaining a comprehensive understanding of the molecular-level regulatory mechanisms within HCC cells holds significant implications for developing targeted therapeutic drugs and improving patient prognosis.

Neurexophilin 4 (NXPH4) is a secretory protein in the synaptic cleft and belongs to the wider NXPH family, known for its regulatory roles within the central nervous system. Beyond its neural functions, recent findings have unveiled a pivotal role for NXPH4 in cancer biology, marking it as a contributor to tumor progression and metastasis across various cancer types. Notably, NXPH4 overexpression has been linked to poorer clinical outcomes in bladder cancer, serving as a robust prognostic marker correlated with both overall survival (OS) and progression-free survival (PFS) [4]. Further elucidation into molecular mechanisms has revealed that NXPH4 enhances glycolysis activation and reactive oxygen species by regulating NDUFA4L2 (reduced form of nicotinamide-adenine dinucleotid (NADH) dehydrogenase [ubiquinone] 1 alpha subcomplex, 4-like 2), thereby promoting gemcitabine resistance in bladder cancer patients [5]. In lung cancer, enhancer of zeste homolog 2 (EZH2) regulates the NXPH4/CDKN2A (cyclin-dependent kinase inhibitor 2A)

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axis, participating in the modulation of non-small cell lung cancer cell migration and proliferation [6]. Additionally, NXPH4 shows potential as a bio-index for early identification of HCC [7]. Given these multifaceted roles, exploring NXPH4's expression and function in HCC is a logical and essential extension of ongoing research efforts. HCC, characterized by metabolic reprogramming and the urgent need for reliable biomarkers for early detection and targeted therapy, presents an ideal context for investigating the potential of NXPH4 as a novel biomarker and therapeutic target. Therefore, this study aims to delve into the expression patterns and biological impacts of NXPH4 in HCC. By elucidating the role of NXPH4, we aspire to uncover novel insights into the molecular mechanisms underlying HCC and provide a reference for developing innovative diagnostic and therapeutic strategies.

Material and Methods

Acquisition and Processing of TCGA Data

RNA-seq data from The Cancer Genome Atlas-Liver Hepatocellular Carcinoma (TCGA-LIHC) project were downloaded from the TCGA database (https://portal.gdc.cancer.gov/). This project included RNA-seq data from 374 HCC tissue samples and 50 normal tissues. The data were accessed using the spliced transcripts alignment to a reference (STAR) processing pipeline, extracting in TPM (transcripts per million) format. Subsequently, the data were normalized using the TPM method. Moreover, the Wilcoxon rank-sum test was employed to assess the expression differences of NXPH family members between tumor samples and normal liver tissue samples. Finally, the ggplot2 package was used for data visualization (box plot visualization method) to intuitively display the expression of NXPH family members in liver cancer.

HCC Tissue Acquisition and HCC Cells

This study included seventy HCC patients treated at the First Affiliated Hospital of Zhengzhou University, China, from March 2020 to June 2022. All procedures involving human participants adhered to the Declaration of Helsinki (as revised in 2013), and the study design was approved by the ethics committee of the First Affiliated Hospital of Zhengzhou University, China (Approval number. 2023-KY-1008-002). After getting informed consent from the patients or their families, clinical specimens, including HCC and adjacent healthy tissues, were harvested and immediately stored in liquid nitrogen. The HCC cell lines (Hub7 (No. SCSP-526), HCC-LM3 (No. PWE-HU164), SMMC-7721 (No. C6865), and HepG2 (No. SCSP-510)) and normal liver cell line (THLE-2) (No. SCSP-5068) were purchased from the Institute of Cell Biology, Chinese Academy of Sciences, China. We tested the cell cultures for mycoplasma using the Applied Biosystems MycoSEQ detection kit (4460623, Thermo Fisher Scientific, Waltham, MA, USA) and found them negative. On the other side, STR identification was conducted by Wuhan Zhishan Biotech Co., Ltd., (Wuhan, China) on May 5th, 2023. The results of STR identification were consistent with those published in the German Collection of Microorganisms and Cell Cultures (DSMZ) STR database (http://www.dsmz.de/fp/cgi-bin/str.html).

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA from tissues and cells was isolated using Trizol reagent (15596026CN, Thermo Fisher Scientific, Waltham, MA, USA), and was quantified using a Nanodrop 2000 spectrophotometer (ND2000c, Thermo Fisher Scientific, Waltham, MA, USA), then stored at –80 °C. RNA was reverse transcribed into cDNA using a reverse transcription kit (K1266, Thermo Fisher Scientific, Waltham, MA, USA). The qRT-PCR was performed on a real-time PCR machine, using glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) as a reference gene [6]. The primer sequences used in qRT-PCR were as follows: *NXPH4*: Forward: 5′-GCAGCGAAAACTTGAGGGTAT-3′, Reverse: 5′-AAGGTCTTCGGACGGCCTA-3′; *GAPDH*: Forward: 5′-GAAGGTGAAGGTCGGAGTC-3′, Reverse: 5′-GAAGATGGTGATGGGATT-3′. The $2^{-\Delta\Delta Ct}$ method was employed to assess the relative expression levels of the target genes. Furthermore, to achieve statistical significance, each experiment was replicated three times.

Western Blot

For tissue samples, formalin-fixed, paraffin-embedded slices of HCC or adjacent tissues were prepared and subsequently underwent deparaffinization and hydration procedures. However, in the case of HCC cell samples, the cells were washed, and total cellular protein was extracted using lysis buffer. The total protein was quantified utilizing the BCA (bicinchoninic acid) protein quantification kit (701780, Cayman, Ann Arbor, MI, USA). After this, 80–60 µg of protein sample was loaded per lane and electrophoresed under constant pressure at 40 V (concentration gel)/100 V (separation gel). The protein was transferred onto the nitrocellulose membrane and subsequently blocked with 8% skim milk at ambient temperature for 2 hours. The membranes were incubated overnight at 4 °C with the primary antibody, diluted in 4% skim milk. The primary antibodies used in Western blot analysis were as follows: Rabbit anti-NXPH4 (1:1000, PA5-101711, Thermo Fisher Scientific, Waltham, MA, USA) and Rabbit anti-GAPDH (1:1000, #2118, mAb, Cell Signaling Technology, Danvers, MA, USA). The next day, the membranes were incubated with secondary antibody horseradish peroxidase-labeled secondary antibody immunoglobulin G (IgG) (1:2000, AS014, Abclonal, Wuhan, China) for 4 hours at ambient temperature followed by washing with TBST (YT8036, Beijing Ita Biotechnology

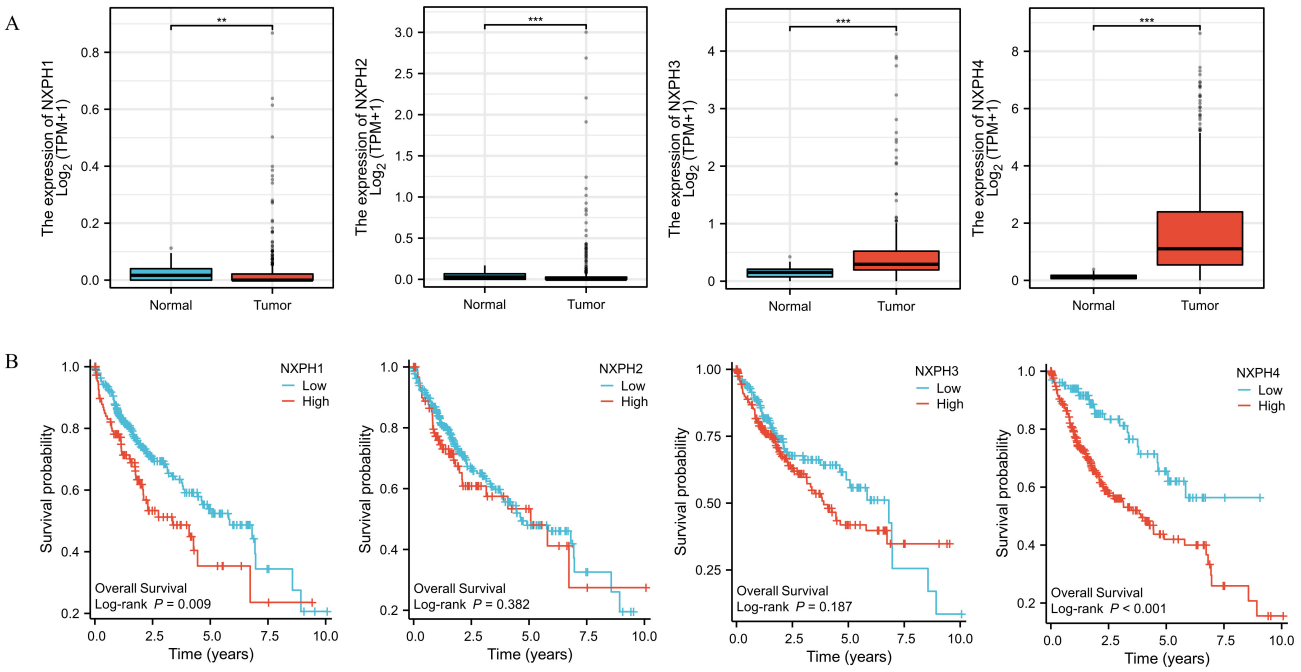

Fig. 1. Expression of NXPH family members in HCC tissues and their correlation with patient survival. (A) Volcano plot showing the mRNA expression levels of NXPH family members in 20 cases of HCC tissues and adjacent tissues; $^{**}p < 0.01$; $^{***}p < 0.001$. (B) TCGA data analysis of the correlation between the gene expression levels of NXPH family members and the overall survival of patients. HCC, hepatocellular carcinoma; NXPH, neurexophilin; TCGA, The Cancer Genome Atlas.

Co., Ltd., Beijing, China). After this, equal volumes of ECL solutions A and B were added to the membrane and protein bands were observed using a gel imaging system and recorded. The grayscale values of the protein bands were determined utilizing the Image J software (v1.8.0, NIH Image, Stapleton, MD, USA).

Immunohistochemistry

The formalin-fixed patient pathological tissues and adjacent normal tissues underwent various processes, including deparaffinization, gradient alcohol dehydration, antigen retrieval using citrate buffer, blocking, overnight incubation with the primary antibody at 4 °C, and subsequent treatment with the secondary antibody for two hours at ambient temperature. The tissue specimens were mounted with an anti-fluorescence quenching sealing agent and subsequently treated with Rabbit anti-NXPH4 (1:150, PA5-101711, Thermo Fisher Scientific, Waltham, MA, USA). The tissue specimens were stained employing Diaminobenzidine (DAB, goat anti-rabbit (IgG), T15132, Shanghai Shangbao Biotechnology Co., Ltd., Shanghai, China), followed by counterstaining with hematoxylin and subsequent differentiation. The pathological staining results were observed by three pathologists, and staining intensity was scored as follows: 0 (negative), 1 (1+), 1–2+ (2+), 2+ (3+). Positive staining rate was scored as follows: 0 (negative), 1 (1–50%), 2 (50%–90%), 3 (90–100%). The total score and grouping were assessed by multiplying the "staining intensity score" by the "staining positive rate score". The tissue specimen with a total score ≤6 was categorized as the low expression group, while that with a total score >6 was classified as the high expression group.

Construction of Overexpressing and Knockdown NXPH4 HCC Cell Lines

Following the instructions provided by Clontech Laboratories, Inc. (Mountain View, CA, USA), the cDNA of the NXPH4 was amplified, recovered, and purified using gel electrophoresis. After cloning cDNA into the T-vector, positive clones were screened and confirmed through sequencing. Furthermore, the target gene was incorporated into the pLVX-IRES-NEO plasmid through a double enzyme digestion. The plasmid was transformed into competent E. coli cells and subsequently extracted and validated. Moreover, 293T cells were co-transfected with the validated pLVX-IRES-NEO plasmid and the Lenti-X HTX packaging system to produce lentivirus carrying the *NXPH4* gene. The virus was harvested, and its titer was determined for subsequent use. Meanwhile, a control virus was prepared using the pLVX-IRES-NEO empty vector. Furthermore, HCC cell lines were infected with low expression or deletion of the *NXPH4* gene using the prepared NXPH4 gene lentivirus expression vector. After applying resistance selection, the cells were screened to obtain HCC cell lines stably expressing the *NXPH4* gene for future use.

Two different short hairpin RNA (shRNA) sequences (sh*NXPH4*-1-5′-AGAGTCACGCGCTTTCAATTG-3′,

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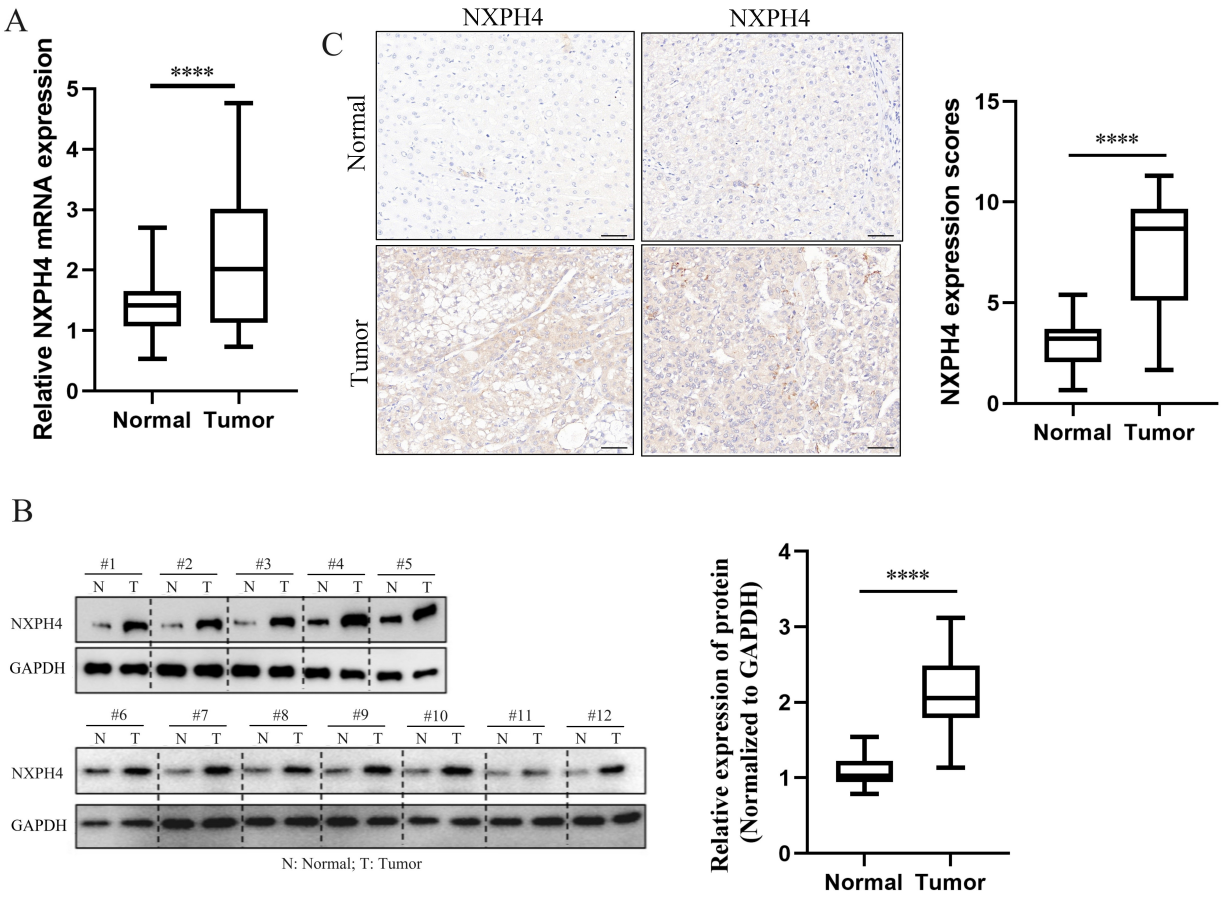

Fig. 2. NXPH4 expression in HCC and paracancerous tissues. (A) qRT-PCR and (B) Western blot analysis of the mRNA and protein expression levels of NXPH4 in HCC and paracancerous tissues, respectively. n = 12. (C) Immunohistochemistry of the NXPH4 levels in tissues. The scale bar is 50 μm. Immunohistochemistry results were scored. ****$p < 0.0001$; n = 3. HCC, hepatocellular carcinoma; NXPH4, neurexophilin 4; qRT-PCR, quantitative real-time polymerase chain reaction; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

sh*NXPH4*-2-5′-AGAAGGTGTGCCCAGACTATA-3′), and a negative control shRNA (5′-CAACAAGATGAAGAGCACCAA-3′) were obtained from GenePharma (GenePharma Co., Ltd., Shanghai, China). HCC cells were transfected with the shNXPH4-1 and shNXPH4-2 vectors using Lipofectamine 2000 reagent (YT1317, Beijing Ita Biotechnology Co., Ltd., Beijing, China). The NXPH4 overexpression and empty vector were purchased from WZ Biosciences (Shandong, China) [5].

Methyl Thiazolyl Tetrazolium (MTT) Assay

Initially, the cells were incubated in a 6-well plate at 37 °C and 5% CO_2 for 24 hours. After discarding the supernatant, 200 μL of different concentrations of drugs diluted in Dulbecco's modified eagle medium (DMEM) (YBM-D, Yizefeng Biotechnology (Shanghai) Co., Ltd., Shanghai, China) were added into each well (excluding the blank control group), and incubated for 12 hours or 24 hours. The blank control group only received 200 μL of DMEM for cultivation. Four hours before the end of incubation, 20 μL of MTT solution (5 g/L, YT1468, Beijing Ita Biotechnology Co., Ltd., Beijing, China) was added per well. In the next step, the supernatant was discarded and Dimethyl Sulfoxide (CAS#: 67-68-5, Sigma-Aldrich, St. Louis, MO, USA) was added to dissolve the formazan crystals. Finally, the absorbance values at 450 nm were assessed using a microplate reader, and the cell inhibition rate (%) was determined as follows: Cell Inhibition Rate (%) = (1–average OD value of the experimental group/average OD value of the control group) × 100%.

Clonogenic Assay

The cells were seeded in a 6-well plate at a density of 500 cells per well and cultured in a medium augmented with 10% fetal bovine serum (10099158, Thermo Fisher Scientific, Waltham, MA, USA). After 14 days, the cells were fixed using 1 mL of 4% paraformaldehyde (818715,

Table 1. Correlation analysis between NXPH4 expression and clinicopathological parameters of patients.

Clinical pathological feature	NXPH4 Expression		χ^2	p
	High (n = 42)	Low (n = 28)		
Sex			0.1020	0.7494
Male	30	19		
Female	12	9		
Age (Years)			1.373	0.2414
<60	18	16		
≥60	24	12		
Tumor Differentiation			6.793	0.0092
Moderate and well differentiated	11	16		
Poorly differentiated	31	12		
Tumor Diameter (cm)			4.613	0.0317
<5	16	18		
≥5	26	10		
BCLC stage			5.380	0.0679
A	10	14		
B	21	8		
C	11	6		
Lymph nodes metastasis			6.076	0.0137
Yes	29	11		
No	13	17		

BCLC, Barcelona Clinic Liver Cancer.

Sigma-Aldrich, St. Louis, MO, USA) at 4 °C for 60 minutes, followed by washing with phosphate buffer solution (PBS) (Abcam, Cambridge, UK). After this, the cells underwent staining for 2 minutes with 0.1% crystal violet dye and subsequently examined for the number of colonies formed (>50 cells).

Scratch Assay

Initially, HCC cells of each group were seeded in a 60 mm culture dish at a density of 1×10^6 cells/well. Once cells formed a monolayer, a pipette tip was employed to develop scratches in the vertical direction of the culture dish. Subsequently, the cells were washed with PBS (Abcam, Cambridge, UK), and serum-free DMEM (YBM-D, Yizefeng Biotechnology (Shanghai) Co., Ltd., China) was added followed by incubation. The cells were examined using an optical microscope to capture images at 0 hours and 48 hours. Moreover, the distance between the two scratches was observed utilizing ImageJ 1.50 (LOCI, University of Wisconsin, Madison, WI, USA). Furthermore, the scratch healing rate was assessed as follows: Scratch Healing Rate (%) = (Scratch width 0 hours – scratch width 24 hours) / scratch width 0 hours × 100%. Each experiment was independently replicated thrice for each group.

Transwell Invasion and Migration Assay

In the Transwell invasion assay, the upper chamber was pre-coated with Matrigel gel to simulate the 3D environment inside the tumor. Transfected HCC cells were resuspended in a serum-free medium. The upper chamber of the Transwell insert was filled with cells at a density of 1×10^4 cells per well. The lower chamber was filled with the medium supplemented with 10% fetal bovine serum (10099158, Thermo Fisher Scientific, Waltham, MA, USA) followed by incubation under normal conditions for 24 hours. In the next step, the old medium was gently aspirated using a cotton ball. The cells were fixed with 4% paraformaldehyde (P6148, Sigma-Aldrich, St. Louis, MO, USA) for 15 minutes followed by staining with crystal violet dye (Sigma-Aldrich, St. Louis, MO, USA) for 10 minutes. After this, the cells were washed with PBS (Abcam, Cambridge, UK), air-dried, and observed using an inverted microscope.

Furthermore, the Transwell migration assay followed similar procedures as the invasion assay, except for the pretreatment of the upper chamber with Matrigel.

Statistical Analysis

Statistical analyses were conducted using GraphPad Prism 8.0 software (GraphPad Software, Inc., La Jolla, CA, USA). The boxplots were analyzed using non-parametric tests. The association between NXPH4 expression and HCC patient's clinical parameters was assessed by the chi-square test. The quantitative data were expressed as the mean ± standard deviation. However, a comparison between the two groups was assessed using a *t*-test. A p-value < 0.05 was deemed statistically significant.

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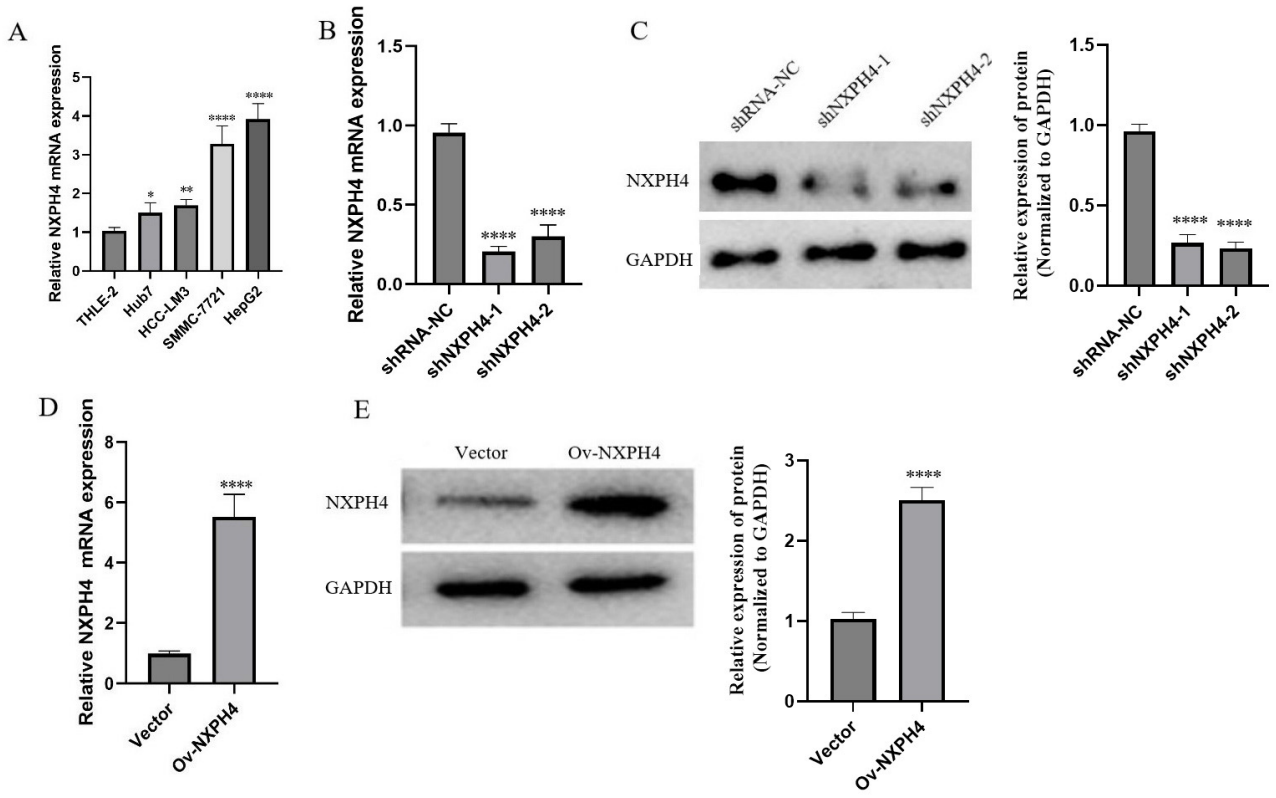

Fig. 3. Expression of NXPH4 in HCC cell lines. (A) qRT-PCR analysis of NXPH4 mRNA expression levels in HCC and normal liver epithelial cells. (B) qRT-PCR and (C) Western blot of the NXPH4 expression level in HCC cells following transfection with NXPH4 knockdown lentivirus. (D) qRT-PCR and (E) Western blot of the NXPH4 expression level in HCC cells following transfection with overexpressing NXPH4 lentivirus. *$p < 0.05$; **$p < 0.01$; ****$p < 0.0001$; n = 3. HCC, hepatocellular carcinoma; NXPH4, neurexophilin 4; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; qRT-PCR, quantitative real-time polymerase chain reaction; NC, negative control.

Results

Expression of NXPH Family Members in HCC Tissues and Their Correlation with Patient Survival

The expression profiles of NHPH family members, including NXPH1, NXPH2, NXPH3, and NXPH4, were analyzed utilizing published HCC transcriptome data accessed from the TCGA database. The results indicated that compared to adjacent normal tissues, these four genes (NXPH1, NXPH2, NXPH3, and NXPH4) exhibited elevated expression in HCC tissues ($p < 0.01$, Fig. 1A). Additionally, differential expression analysis (log2) revealed a significant difference in the expression levels of NXPH4 between HCC and adjacent tissues.

Furthermore, correlation analyses were performed to assess the relationship between the expression levels of NXPH1, NXPH2, NXPH3, and NXPH4 and the overall survival of HCC patients. The findings revealed that the expression levels of NXPH2 and NXPH3 did not correlate with the overall survival of HCC patients ($p > 0.05$). In contrast, the expression levels of NXPH1 and NXPH4 showed a significant correlation with shorter overall survival in HCC patients ($p < 0.01$), indicating that those with elevated NXPH1 and NXPH4 expression experienced a reduced overall survival period (Fig. 1B). Based on the expression levels of NXHP family members in HCC tissues and their correlation with patient survival, we hypothesized that NXPH4 might play a crucial regulatory role in the occurrence and development of HCC. Therefore, NXPH4 was processed in the subsequent analysis.

Expression of NXPH4 in HCC Tissues

The expression of NXPH4 mRNA in tissues was assessed using qRT-PCR. The results revealed a significant increase in the expression level of NXPH4 mRNA in HCC tissues (Tumor) compared to adjacent normal tissues (Normal) ($p < 0.0001$, Fig. 2A). Western blot analysis further validated the elevated expression of NXPH4 protein in HCC tissues (T) compared to adjacent tissues (N) (Fig. 2B).

Immunohistochemical analysis was performed to examine the expression of NXPH4 in HCC tissues and adjacent normal tissues. We observed lighter staining in ad-

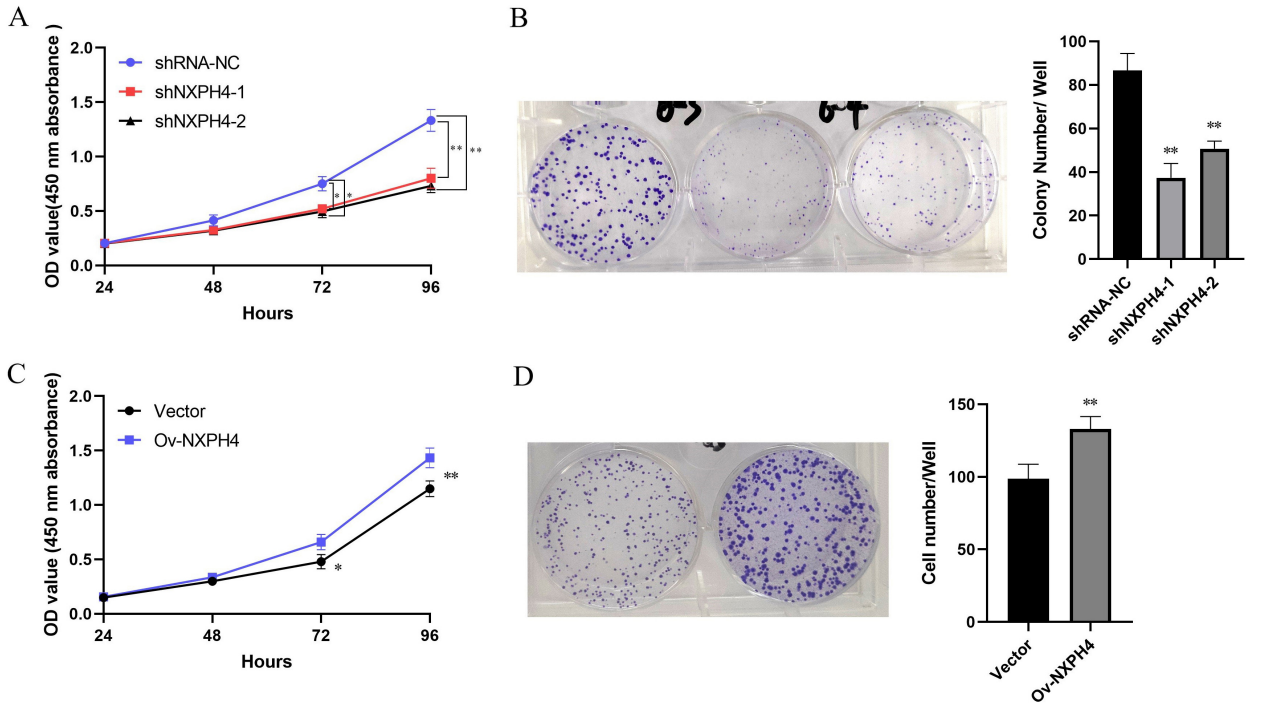

Fig. 4. Effects of knockdown or overexpression of NXPH4 on the proliferation of HCC cells. (A) MTT was used to detect the effect of NXPH4 knockdown on the proliferation of HCC cells; *$p < 0.05$; **$p < 0.01$ vs shRNA-NC. (B) Clone formation assay was used to determine the effect of NXPH4 knockdown on the clonogenic formation of HCC cells. **$p < 0.01$ vs shRNA-NC. (C) MTT assay was used to assess the effect of NXPH4 overexpression on the proliferation of HCC cells. *$p < 0.05$; **$p < 0.01$ vs Vector. (D) Clone formation assay was used to examine the effect of NXPH4 overexpression on the clonogenic formation of HCC cells. **$p < 0.01$ vs Vector; n = 3. HCC, hepatocellular carcinoma; NXPH4, neurexophilin 4; OD, Optical Density; MTT, methyl thiazolyl tetrazolium; NC, negative control.

jacent normal tissues (Normal), suggesting lower expression levels of NXPH4. In contrast, HCC tissues (Tumor) exhibited darker and predominantly cytoplasmic staining, indicating higher expression levels of NXPH4 (Fig. 2C). Scoring analysis of immunohistochemical staining results demonstrated a significantly elevated expression score in tumor tissues (Tumor) compared to adjacent normal tissues (Normal), highlighting substantially increased NXPH4 expression in HCC tissues ($p < 0.0001$, Fig. 2).

Correlation Analysis of NXPH4 Expression and Patient's Clinicopathological Parameters

According to the immunohistochemistry scoring results, 42 patients exhibited elevated NXPH4 expression, and 28 showed reduced NXPH4 expression. The association of NXPH4 levels with patient's clinicopathological parameters revealed that NXPH4 expression was correlated to the degree of tumor differentiation ($p = 0.0092$), tumor diameter ($p = 0.0317$), and lymph node metastasis ($p = 0.0137$). Moreover, patients with high NXPH4 expression showed poor differentiation, tumor diameter ≥5 cm, and lymph node metastasis (Table 1).

Construction of HCC Cell Lines with Knocked Down or Overexpressed NXPH4

The qRT-PCR analysis revealed that NXPH4 was highly expressed in HCC cell lines (Hub7, HCC-LM3, SMMC-7721, and HepG2) compared to normal liver epithelial cells (THLE-2) ($p < 0.05$, Fig. 3A).

In the knocked-down experiments, synthetic interference vectors (shNXPH4-1 and shNXPH4-2) were transfected into HepG2 cells, and the outcomes were examined using qRT-PCR (Fig. 3B) and Western blot analysis (Fig. 3C). Compared to the blank control group (shRNA-NC), both shNXPH4-1 and shNXPH4-2 groups significantly inhibited the expression of NXPH4 protein in HepG2 cells ($p < 0.0001$).

Furthermore, in the overexpression experiments, synthetic overexpression vectors (Ov-NXPH4) and blank control vectors (Vector) were transfected into Hub7 cells. qRT-PCR (Fig. 3D) and Western blot analysis (Fig. 3E) indicated that compared to the blank control vector (Vector), transfection with the overexpression vector (Ov-NXPH4) significantly promoted the expression of NXPH4 in the cells ($p < 0.0001$). This finding confirms the successful construction of HCC cell lines with either knocked down or overexpressed NXPH4.

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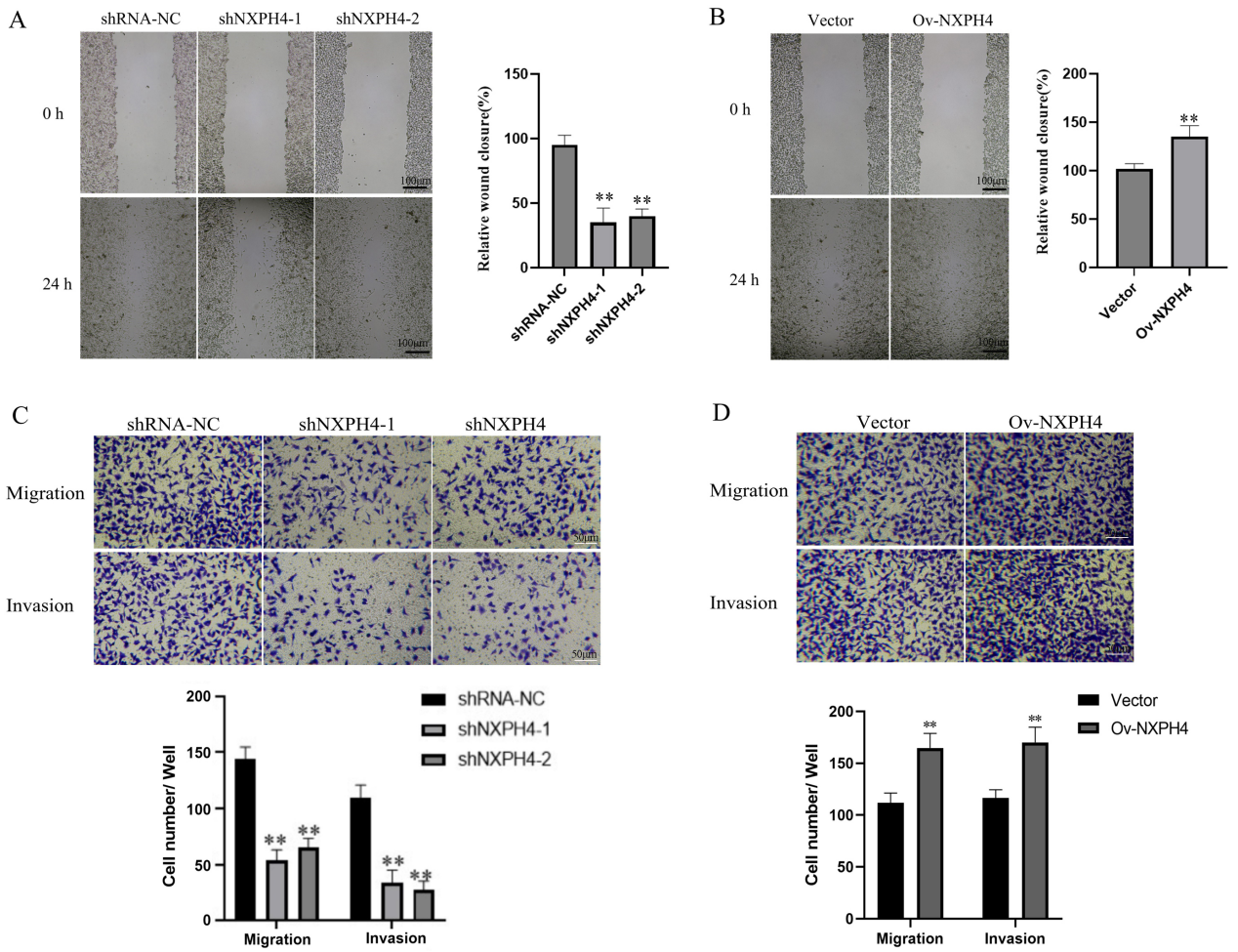

Fig. 5. Effects of knockdown or overexpression of NXPH4 on HCC cell's ability to migrate and invade. Scratch assay was used to elucidate the impact of knockdown (A) or overexpression (B) of NXPH4 on HCC cell's ability to migrate. Transwell assay was performed to elucidate the impact of knockdown (C) or overexpression (D) of NXPH4 on HCC cell's ability to migrate and invade. $^{**}p < 0.01$; n = 3. HCC, hepatocellular carcinoma; NXPH4, neurexophilin 4; NC, negative control.

Effects of Knockdown or Overexpression of NXPH4 on the Proliferation of HCC Cells

The MTT and clonogenic assays indicated that compared to the blank control group (shRNA-NC), knockdown of NXPH4 (shNXPH4-1 and shNXPH4-2) significantly inhibited both the proliferation (Fig. 4A) and clonogenic capabilities (Fig. 4B) of HepG2 HCC cells ($p < 0.05$). Conversely, compared to the blank control vector (Vector), transfection with the overexpression vector (Ov-NXPH4) significantly promoted the proliferation (Fig. 4C) and clonogenic capabilities (Fig. 4D) of Hub-7 HCC cells ($p < 0.05$).

Effects of Knockdown or Overexpression of NXPH4 on the Migration and Invasion of HCC Cells

The scratch assay revealed that, in comparison to the blank control group (shRNA-NC), the knockdown of NXPH4 (shNXPH4-1 and shNXPH4-2) led to a significant reduction in the migration ability of HepG2 HCC cells ($p < 0.01$, Fig. 5A). On the other hand, when compared to the blank control vector (Vector), transfection with the overexpression vector (Ov-NXPH4) markedly increased the migration ability of the cells ($p < 0.01$, Fig. 5B).

Similarly, the Transwell invasion and migration assays showed that the knockdown of NXPH4 (shNXPH4-1 and shNXPH4-2) substantially decreased the invasion and migration abilities of HepG2 HCC cells ($p < 0.01$, Fig. 5C). Conversely, relative to the blank control vector (Vector), transfection with the overexpression vector (Ov-NXPH4) significantly boosted the invasion and migration abilities of the cells ($p < 0.01$, Fig. 5D).

Discussion

In recent years, significant advancements have been made in molecular targeted therapies for HCC, offering multiple treatment avenues for patients in advanced stages. However, despite these developments, the overall clinical outcomes remain unsatisfactory [8,9]. Biomarkers of-

fer the potential to precisely identify patient populations most likely to benefit from specific treatments, thereby minimizing undesired side effects and improving the cost-effectiveness of therapies [10]. Currently, the identification of reliable biomarkers for the various molecular targeted therapies associated with HCC poses an ongoing challenge. The pursuit of novel therapeutic targets and the development of drugs targeting these specific sites promise to introduce groundbreaking options into the clinical management of HCC.

The NXPH family proteins, comprising NXPH1, NXPH2, NXPH3, and NXPH4, display considerable evolutionary conservation. Although these proteins share highly similar sequences at their C-termini, their N-terminal sequences show significant divergence [11,12]. Recent investigations highlight the significant overexpression of NXPH family proteins in tissues linked to various diseases and cancers, where they play distinct biological regulatory roles [13]. For instance, Wang *et al*. [14] demonstrated that miR-194-5 suppresses the invasion, growth, and inflammatory response in LPS-induced astrocytes by directly targeting NXPH1. This finding underscores the pivotal role of the miR-194-5p/NXPH1 axis in modulating astrocyte activity and neuroinflammation-related disorders. Furthermore, research by Fanlo *et al*. [15] identified a transcriptional module of the sympathetic-adrenal program that counteracts neuroblastoma malignancy by inhibiting metastasis, underscoring the NXPH1/α-NRXN signaling pathway as a valuable therapeutic target for high-risk neuroblastoma cases. These insights into the NXPH family's involvement in cancer development underscore their potential as biomarkers or therapeutic targets. Despite the growing body of research, the specific role of NXPH4 in cancer, particularly in HCC, remains underexplored. Our study seeks to bridge this gap by elucidating the function of NXPH4 in HCC. Through this study, we aim to identify novel targets for treatment and foster innovations in the diagnosis and therapeutic approaches for HCC.

This study thoroughly assessed the expression profiles of the NXPH family members (NXPH1, NXPH2, NXPH3, and NXPH4) in HCC tissues, utilizing publicly available transcriptomic datasets from the TCGA database. We observed upregulation of these four genes in HCC tissues compared to their normal counterparts, with NXPH4 demonstrating the most significant differential expression. Additionally, we established a substantial association between elevated expressions of NXPH1 and NXPH4 and reduced overall survival rates, suggesting their contributions not just to the onset of HCC but also to its prognosis. Based on these observations and the survival analysis outcomes, NXPH4 was selected for subsequent exploration. Our findings align with a previous bioinformatics analysis conducted by Tang *et al*. [16], proposing NXPH4 as a potential adverse prognostic indicator and a novel target for immunotherapeutic strategies in HCC.

The clinical findings of this study reveal the elevated level of NXPH4 in HCC tissues, consistent with our previous research. Furthermore, we found that NXPH4 expression correlates with tumor differentiation, tumor diameter, and lymph node metastasis. Additionally, *in vitro* cell experiments demonstrated that knockdown of NXPH4 substantially inhibited HepG2 HCC cell's growth and colony formation function. Conversely, overexpressing NXPH4 significantly promoted the Hub7 HCC cell's growth and colony formation function, indicating the impact of NXPH4 on the proliferative capacity of HCC cells. These findings suggest that NXPH4 may promote HCC cell proliferation by influencing cell cycle regulators or growth signaling pathways. Similarly, previous research has indicated that in colorectal cancer, Forkhead Box K1 (FoxK1)-regulated NXPH4 exhibits a promoting effect on the growth, migration, and glycolysis of colorectal cancer cells [17].

Furthermore, Scratch and Transwell invasion migration analysis demonstrated that knockdown of NXPH4 significantly inhibits HCC cell's capability to migrate and invade while overexpression of NXPH4 significantly enhances these properties. These findings emphasize the crucial role of NXPH4 in regulating the migration and invasion of HCC cells, suggesting that NXPH4 might modulate the HCC cell's invasion and metastasis through various mechanisms, including influencing cell cytoskeleton rearrangement, cell adhesion, or intercellular signaling pathways.

Conclusion

The elevated NXPH4 level in HCC and its association with several crucial clinical parameters suggest that it may be utilized as a biomarker for the progression of HCC. Our study explored the potential role of NXPH4 in modulating crucial biological functions, including growth, migration, and invasion of HCC cells. These findings not only provide a new perspective for understanding the molecular mechanisms of HCC but also offer valuable clues for developing novel therapeutic strategies targeting HCC. Future research efforts should uncover the molecular regulatory mechanisms of NXPH4 to comprehensively understand its role in developing HCC.

Availability of Data and Materials

All experimental data used to support the findings of this study are available from the corresponding author upon request.

Author Contributions

YP and HZ designed the research study. YP draft the manuscript. XL performed the research. RC provided help and advice on the experiments. JY analyzed the data. All authors contributed to editorial changes in the manuscript.

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All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

This study included seventy HCC patients treated at the First Affiliated Hospital of Zhengzhou University, China, from March 2020 to June 2022. All procedures involving human participants adhered to the Declaration of Helsinki (as revised in 2013), and the study design was approved by the ethics committee of the First Affiliated Hospital of Zhengzhou University, China (Approval number. 2023-KY-1008-002). We got informed consent from the patients or their families.

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Conflict of Interest

The authors declare no conflict of interest.

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