Nur77 Inhibit β-catenin Expression to Mediate Hepatoblastoma Progression and Therapeutic Effect of Cisplatin

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Abstract

Hepatoblastoma (HB) is the most common malignant tumor in children under 5 years old, but its pathogenesis remains unclear. Nur77 has been reported to be an important regulator for cancer progression in various cancer types. This study found that Nur77 was downregulated in HB tumors, compared with paracancer tissue. Knockout or overexpression of Nur77 in HB tumor cell line HepG2 and HuH6 could significantly enhance or inhibit the proliferation, migration and invasion of tumor cells both in vitro and in vivo. Further studies illustrated that Nur77 regulated the proliferation of tumor cells by affecting the expression of β-catenin. Nur77 agonist Cns-A effectively enhanced the therapeutic effect of cisplatin on HB tumors both in vitro and in vivo. This study proved that Nur77 could act as a tumor suppressor gene in HB tumors, providing a new direction for improving the clinical responses of HB.

Simple Summary

Nur77 belongs to the nuclear hormone receptor superfamily and was classified as an early immediate-response gene. Many studies have found that the low expression of Nur77 is related to tumor development, tumor metastasis and drug resistance in cancer cells. Our study revealed that Nur77, as a suppressor gene and β-catenin directly interacting with each other, plays an important role in the proliferation, migration, invasion and apoptosis of hepatoblastoma (HB). Meanwhile, our study demonstrated that the Nur77 agonist could significantly enhance the therapeutic effect of cisplatin against HB both in vitro and in vivo, bringing new hope for the treatment of HB.

Introduction

HB is a rare malignancy but accounts for the majority of liver malignancies in children (1). According to statistics, the number of HB cases increased by 2.2% per year between 2000 and 2015 (1, 2). Current therapeutic strategies for HB mainly include chemotherapy, surgical resection and liver transplantation (3). Although the prognosis of HB patients has been greatly improved over the years, the overall survival rate of children with advanced disease remains poor, with a 5-year survival rate of 39%-57% (4–7). Therefore, to improve the therapeutic effect and prognosis of HB patients, there is an urgent need to study the mechanism of HB occurrence and development.

The orphan nuclear receptor Nur77, also known as TR3 or NGFI-B, is encoded by the immediate early gene N\textit{r}4\textit{a}1. It is an important regulator in cancer progression in various cancer types (8–12). Studies have found that Nur77 induces the expression of lncRNA WFDC21P in hepatocellular carcinoma (HCC) cells, thereby inhibiting HCC cell proliferation and metastasis in vitro and in vivo (13). In addition, Nur77 directly interacts with RAR β, and simultaneous targeting of Nur77 and RAR β can effectively induce apoptosis in HCC cells (14). Given the important regulatory role of Nur77 in HCC, we hypothesized that Nur77 may also play a crucial role in HB. Therefore, this study aimed to investigate the function of Nur77 in HB tumor cells.
In this study, we analyzed the expression of Nur77 in cancer and paracancer tissues of HB patients. Then we studied the effects of knockout and overexpression of Nur77 on proliferation, migration and apoptosis of HB cell lines HepG2 and HuH6 both in vivo and in vitro. We found that Nur77 could act as a tumor suppressor gene by regulating the proliferation, migration and apoptosis of HB cells by affecting the expression of β-catenin. Besides, the Nur77 agonist could significantly enhance the therapeutic effect of cisplatin against HB both in vitro and in vivo, which provided a new idea for improving the clinical responses of HB.

Materials and Methods

Patient Cohort and Sample Collection

Tumor and paracancer tissues of HB patients were collected from patients (n = 30) treated at the Children's Hospital Affiliated to Nanjing Medical University (Nanjing, China) from September 2021 to March 2023. Each pathological diagnosis of HB patients was independently confirmed by ≥ 3 experienced pathologists. Before starting the experiment, each patient has signed an informed consent to participate in the study. The Ethics Committee of Children's Hospital Affiliated to Nanjing Medical University approved this study (Approval number: NJCH2020137).

Cell culture and drug administration

HepG2 and HuH6 cell lines were purchased from American Type Culture Collection (ATCC). Cells were cultured in DMEM medium (Gibco, USA) containing 10% fetal bovine serum (HyClone, USA) in a 37°C incubator with 5% carbon dioxide. In this research, cisplatin (DDP 15ug/ml) was used alone (Soleibao, China) or combined with Nur77 agonist Cns-A (Sigma-Aldrich, USA) (DDP 15ug/ml + Cns-A 10uM) according to the grouping for 48 hours.

Plasmid construction

We constructed the CRISPR/Cas9 system plasmids to knock out Nur77. We designed 5 single guide (sg) RNAs targeting NR4A1 exons and one scrambled sgRNA as a wild-type control. The sequences of the 5 NR4A1-targeting sgRNAs and the scrambled sgRNA were listed as follows: sgRNA1: 5'-gggacaccagcaccgagtccggg-3'; sgRNA2: 5'-gcttgatgaactcaggggtcagg − 3'; sgRNA3: 5'-accttcatggacggctacacagg-3'; sgRNA4: 5'-agcgggggctgcctcggggctgg-3'; sgRNA5: 5'-catggacctggccagccccgagg-3'; scrambled sgRNA: 5'-gcactcacatcgctacatca-3'. The above sgRNAs were cloned into the lentiCRISPRv2-puro plasmid (98290, Addgene. Inc.) in pairs. For Nur77 overexpression experiments, Nur77 was cloned in frame into the pCDH-EF1 plasmid (72266, Addgene. Inc.), Nur77 overexpression was carried out by using lentiviral packaging and transfection system in HB cell lines. All constructed plasmids were confirmed by DNA sequencing in this study.

Animal and Xenograft Models
This study strictly followed the guidelines of the Institutional Animal Care and Use Committee of the Children's Hospital Affiliated to Nanjing Medical University. Nude mice (aged, 6–8 weeks old) were obtained from the Model Animal Research Center of Nanjing University. In the subcutaneous xenograft tumor model, $1 \times 10^6$ HepG2 cells were injected into nude mice subcutaneously. Tumor diameters were measured twice a week. In drug treatment experiments, cisplatin (2.5 mg/kg body weight) was injected intraperitoneally every other day (4–6 doses in total). Mice were sacrificed 4 weeks after drug treatment by carbon dioxide asphyxiation. The death of mice was confirmed by lack of heartbeat, pupil fixation, and pupil dilation. Tumors are isolated for further evaluation afterward. If mice experienced distress (weight loss or wrinkled hair) or moving difficulty, they will be sacrificed immediately out of humanitarianism.

**Western Blotting**

Cells and tissues were fully lysed with RIPA lysis buffer supplemented with protease inhibitors. An equal amount of protein was loaded for SDS-PAGE protein electrophoresis, and transferred to a PVDF membrane. After blocking with TBST-Tween20 containing 5% skim milk for 2 hours, the PVDF membrane was incubated with the corresponding primary antibody overnight at 4°C and incubated with HRP-conjugated secondary antibody for 2 hours at room temperature. Signals were detected using a chemiluminescence imaging system (BioRad, USA). The primary antibodies involved in this study are listed as follows: Nur77 (Proteintech, 12235-1-AP, 1:1000), GAPDH (Proteintech, 60004-1-Ig, 1:2000), GSK3β (Proteintech, 22104-1-AP, 1:1000), P53 (Proteintech, 60283 -2-Ig, 1:1000); β-catenin (Cell Signaling Technology, D10A8, 1:1000). The secondary antibodies involved in this study are listed as follows: HRP-conjugated AffiniPure Goat Anti-Mouse IgG (H + L) (Proteintech, SA00001-1, 1:5000); HRP-conjugated AffiniPure Goat Anti-Rabbit IgG (H + L) (Proteintech, SA00001-2, 1 : 5000).

**Real-time Quantitative PCR**

Trizol (Invitrogen, USA) was used to extract total RNA from tissues or cells according to the instructions. The cDNA was prepared using a reverse transcription kit (Vazyme, China). Real-time quantitative PCR was performed in ABI Rapid Quantitative PCR 7900HT System (Applied Biosystems, USA) using ChamQ Universal SYBR qPCR Master Mix (Vazyme, China). The primer sequences used in the research are listed as follows: Nur77-F: 5'-GGCTCGGGGATACTGGATACA-3'; Nur77-R: 5'-CTGGCATGAAGCGTTGTCC-3'; β-actin-F: 5'-CATGTACGTTGCTATCCAGGC-3'; β-actin-R: 5'-CTCCTTAATGTCACGCAGC-3'.

**Immunofluorescence staining**

We used the Tyramide SuperBoost kit (Thermo Fisher Scientific, USA) to detect the expression of Nur77 in tumor and paracancerous tissues in HB patients according to manufacturer's instructions. Paraffin sections of HB tissues were deparaffinized and rehydrated by progressively passing the slides through xylene, different concentrations of alcohol and water. Slides were immersed in Tris-EDTA buffer (pH 9.0) and microwaved for 15min to perform antigen retrieval. Then, the slides were incubated in 3% H$_2$O$_2$ to block endogenous peroxidase. Next, sections were incubated in 2% BSA for 1 hour at room temperature and primary antibody overnight at 4°C. After incubation with HRP-conjugated secondary antibodies, slides were incubated with Alexa fluorescence-conjugated tyramide reagent, and counterstained nuclei
with DAPI. Images were captured with an inverted confocal fluorescence microscope. The antibodies used in this study were listed as follows: Nur77 (Proteintech, 12235-1-AP, 1:100); β-catenin (Cell Signaling Technology, D10A8, 1:200).

**Co-immunoprecipitation assay (Co-IP)**

In this study, Dynabeads Antibody Coupling Kit (Invitrogen, USA) was used to detect the binding of Nur77 and β-catenin by Co-IP. Primary antibodies were first coupled to the Dynabeads according to the manufacturer’s instructions. Cell lysates were pre-cleaned with protein-G for 1 hour and incubated with antibody-coupled beads overnight at 4°C. After that, antibody-coupled beads were washed and analyzed by WB.

**TOPFLASH Reporter Plasmid System**

In this study, the TOPFLASH reporter gene plasmid system was used to detect the expression of β-catenin in HB cells. The construction of the reporter system was performed as previously described [15], whose working mechanism is based on the stability and activity changes of TCF/LEF1 (T-cell factor/lymphoid enhancer-binding factor 1) and β-catenin complex. We transfected the TOPFLASH reporter gene plasmid carrying TCF/LEF-1 into Nur77 knockdown and overexpressed HB cell lines, and measured β-catenin by detecting the fluorescence intensity.

**Data Analysis**

All experiments were performed at least 3 times in this study. Data were presented as mean ± SEM. The statistical differences were analyzed using two-tailed Student’s t test or a one-way ANOVA and Tukey’s post hoc T test with GraphPad Prism (GraphPad Software, Inc.). Overall survival (OS) and recurrence-free survival (RFS) were assessed using the Mantel-Cox test, and significance was determined using the log-rank test. Statistical significance was set at p-value < 0.05.

**Results**

**Nur77 was downregulated in HB**

To identify tumor suppressor genes in HB, we first analyzed differential expressed genes between HB tumor and paracancer tissues. We chose 2 HB cohorts with available transcriptomic data: 67 samples from Hiroshima University (GSE131329) and 55 samples from Baylor College of Medicine (GSE75271). We identified 739 genes that were significantly downregulated in tumors, compared with paracancer tissues (Fig. 1A, B). NR4A1 was downregulated in HB tumor and negatively correlated with AFP expression in both cohorts (Fig. 1C, D). To confirm the downregulation of NR4A1 in HB tumors, we detected the expression of Nur77 in the cancer and paracancer tissues of HB patients. Both WB and RT-qPCR results demonstrated that the expression of Nur77 in HB tumor tissues was significantly lower than that in paracancer tissues (Fig. 1E, F). Immunohistochemical staining of Nur77 also demonstrated the reduced expression of Nur77 in tumors (Fig. 1G). Next, we divided the HB patients into a middle-low-risk
group and a high-risk group according to clinical diagnosis and treatment norms, and analyzed the expression of Nur77 in those two groups by RT-qPCR. Results showed that the expression of Nur77 in the tumor tissues of HB patients from the middle and low-risk group was higher than that from the high-risk group (Fig. 1H). Those results proved that Nur77 was downregulated in HB tumors, indicating that Nur77 may act as a tumor suppressor gene in HB.

**Nur77 knockout significantly enhanced the proliferation, migration, and invasion of HB cell lines, and decreased cell apoptosis**

To study the functional role of Nur77 in HB, we knocked out Nur77 in HepG2 and HuH6 cell lines by utilizing the CRISPR/Cas9 system. We picked clones with high knock-out efficiency by WB in 2 cell lines separately for further studies (Nur77 KO) (Fig. 2A). Nur77-KO cells formed more clones *in vitro*, suggesting enhanced cell survival and growth after Nur77 knockout (Fig. 2B). Next, we used EdU incorporate assay and CCK-8 growth assay to further confirm the growth advantage of Nur77-KO cells. Nur77 knockout resulted in a higher EdU corporation rate and more rapid growth in both cell lines (Fig. 2C, D). Those results demonstrated that Nur77 deficiency could promote HB cell growth. Wound healing assay as well as transwell migration and invasion assay proved that Nur77 knockout significantly enhanced the migration and invasion of both HepG2 and HuH6 cell lines (Fig. 2E, Supplementary Fig. 1A). Finally, apoptosis experiments demonstrated that Nur77 deletion significantly reduced the proportion of apoptosis in HepG2 and HuH6 cells (Supplementary Fig. 1B). These results showed that Nur77 knockout in HB cells significantly enhanced the proliferation, migration, and invasion of HB tumor cells, and inhibited HB cell apoptosis.

**Overexpression of Nur77 significantly inhibited the proliferation, migration, and invasion of HB cell lines, and increased cell apoptosis**

Next, we further explored the function of Nur77 in Nur77 overexpressed HepG2 and HuH6 cells, which were validated by WB and RT-qPCR (Nur77 OE) (Fig. 3A, Supplementary Fig. 2A). Nur77 overexpression decreased the colony formation of HB cells *in vitro* (Fig. 3B). What’s more, Nur77 overexpression reduced the EdU incorporation and resulted in suppressed cell growth in HB cell lines (Fig. 3C, D). Next, wound healing assay along with transwell migration and invasion assay was performed to illustrate the effect of Nur77 overexpression on the ability of migration and invasion in HB cells, which were significantly suppressed (Fig. 3E, Supplementary Fig. 2B). Finally, the apoptosis assay showed that Nur77 overexpression increased the apoptotic ratio of HepG2 and HuH6 cells (Supplementary Fig. 2C). Constant with the results of Nur77-KO cells, these data demonstrated that Nur77 negatively regulated the proliferation, migration, and invasion ability and was positively correlated with the apoptosis ratio of HB tumor cells *in vitro*.

**Nur77 inhibited the proliferation of HB tumor cells in vivo**

The above experiments proved that Nur77 can regulate the proliferation, migration and apoptosis of HB tumor cells *in vitro*. Next, we verified the regulatory role of Nur77 on HB tumor cells *in vivo*. Results of the
xenograft model revealed that Nur77 knockout could significantly promote the growth of HB tumor cells \textit{in vivo} (Fig. 4A). Higher tumor weight and larger tumor size were observed in the Nur77-KO group, as well as worse survival of mice (Fig. 4B, C). In addition, we detected the proportion of Ki67-positive cells in xenograft, which was significantly higher in the Nur77-KO group (Fig. 4D). In contrast, compared with control HepG2 cells, the growth of Nur77 OE HepG2 xenograft was inhibited \textit{in vivo}, along with lower tumor weight, smaller tumor size and better survival of mice (Fig. 4E-G). Meanwhile, a lower proportion of Ki67-positive cells was detected in Nur77-OE xenograft, suggesting the restriction of cell proliferation (Fig. 4H). Taken together, those results proved that Nur77 inhibited the proliferation of HB tumor cells \textit{in vivo}.

**Nur77 inhibited the expression of β-catenin through direct interaction in HB cells**

It has been reported that Nur77 efficiently induces β-catenin degradation through a GSK3β-independent mechanism (15). Whereas in HCC, high levels of NDRG1 can competitively bind with Nur77, enabling β-catenin to escape degradation and ultimately lead to increased levels of downstream oncogenes (16). Given the important role of Wnt/β-catenin in HB (17, 18), we investigated whether Nur77 inhibited the growth of HB tumors by affecting β-catenin. First, we detected the expression of β-catenin in tumor and paracancerous tissues by WB and immunofluorescence staining, which showed a significantly higher level of β-catenin in HB tumor tissues compared with that in paracancerous tissues (Fig. 5A, B). Next, we explored whether Nur77 can regulate β-catenin through direct protein binding. Nur77 antibody was used to conduct co-immunoprecipitation (Co-IP) experiments in control and Nur77 knockout HepG2 and HuH6 cells, which proved that Nur77 could directly interact with β-catenin in HepG2 and HuH6 cells. As a negative control, no β-catenin band could be detected by Nur77-IP in Nur77-KO cells (Fig. 5C). Next, the TOPFLASH reporter gene plasmid carrying TCF/LEF-1 was transfected into the Nur77 knockout and Nur77 overexpressed HepG2 and HuH6 cells. We found that Nur77 knockout could significantly enhance the transcriptional activity of the TOPFLASH reporter gene plasmid, while overexpression of Nur77 significantly inhibited its activity (Fig. 5D). In addition, Nur77 knockout directly upregulated β-catenin, while Nur77 overexpression directly downregulated β-catenin in HepG2 and HuH6 cells (Fig. 5E). These results demonstrated that Nur77 could inhibit the expression and activity of β-catenin through direct interaction. We also investigated the expression level of GSK3β and P53 in HepG2 and HuH6 cells, which showed no significant changes (Fig. 5E). These results indicated that Nur77 inhibited β-catenin expression through a GSK3β independent mechanism in HB.

**β-catenin was the key to Nur77 suppressing the proliferation of HB tumor cells**

To further verify the mechanism of Nur77 inhibiting HB progression, we next explored if β-catenin overexpression could rescue the growth restriction induced by Nur77 overexpression in HepG2 cell. We first overexpressed β-catenin in both control and Nur77-OE HepG2 cells, and examined the overexpression...
efficiency of Nur77 and β-catenin by WB (Fig. 6A). Consistent with previous results, Nur77 overexpression resulted in decreased colony formation and inhibited growth in HepG2 cells, which was significantly restored by β-catenin overexpression (Fig. 6B-D). Previous studies have proved that the N-terminal region of Nur77 protein is necessary for its induction of β-catenin degradation (15). So we overexpressed mutated Nur77 with N-terminal region deletion (Nur77 Mutant) in HepG2 cells. Consistent with previous results, Nur77 overexpression decreased β-catenin expression, while no obvious change of β-catenin was observed in mutated Nur77 overexpressed HepG2 cells (Fig. 6E). Colony formation assay showed that mutated Nur77 couldn't suppress the clonogenicity of HepG2 cells like wild-type Nur77 (Supplementary Fig. 3A). Similarly, no obvious changes in EdU incorporation rate and growth rate cells were detected in mutated Nur77 overexpressed HepG2 cells (Supplementary Fig. 3B-C). Taken together, these results demonstrated that β-catenin was the key of Nur77 suppressing the proliferation to HB tumor cells.

Nur77 agonist enhanced the therapeutic effect of cisplatin on HB tumors both in vitro and in vivo

Cisplatin (DDP) is currently a commonly used chemotherapeutic drug in the clinical treatment of HB (4). But about 20% of HB patients are cisplatin resistant, who show poor prognosis and high recurrence rates. Studies have reported that Nur77 can promote cisplatin-induced tumor cell apoptosis by inhibiting the expression of anti-apoptotic genes BRE and RNF-7 (19). Considering the inhibitory effect of Nur77 overexpression on the proliferation of HB tumor cells, we next explored the feasibility of improving the effect of cisplatin on HB by using Nur77 agonist Cns-A (Cytosporone A n-amyl ester). Administration of DDP and Cns-A (DDP + Cns-A) significantly decreased β-catenin expression in HepG2 cells in vitro (Fig. 7A). Results showed that DDP treatment could inhibit the proliferation of HepG2 cells, while the combination of DDP and Cns-A significantly enhanced the inhibitory effect (Fig. 7B, C). Cns-A also enhanced the apoptosis induction of DDP on HB cells (Fig. 7D). These results proved that overexpression of Nur77 induced by Cns-A significantly enhanced the anti-tumor function of DPP in vitro. Next, we further validated whether Cns-A could improve the anti-tumor function of DDP in vivo. DDP administration significantly suppressed tumor growth as expected, as well as reduced tumor size and prolonged lifespan of tumor-bearing mice. Compared with DDP alone, the combination of DDP and Cns-A obviously improved the anti-tumor effect, which led to smaller tumor size, reduced tumor weight and longer lifespan of tumor-bearing mice (Fig. 7E-H). Taken together, these results proved that overexpression of Nur77 by using Cns-A significantly enhanced the therapeutic effect of cisplatin on HB tumor cells both in vivo and in vitro.

Discussion

This study demonstrated the tumor-suppressing function of Nur77 in HB. Through detecting the expression of Nur77 in tumor and paracancer tissues of HB patients, we found that Nur77 was downregulated in HB tumor tissues. Knockout and overexpressing of Nur77 in the HB cell lines could significantly enhance or inhibit the proliferation, migration, and invasion abilities of the HB cells, both in vitro and in vivo. Further experiments revealed that Nur77 regulated the proliferation of HB cells by
affecting the expression of β-catenin. In addition, Nur77 agonist enhanced the therapeutic effect of cisplatin on HB in vitro and in vivo.

Nur77 belongs to the nuclear hormone receptor (NR) superfamily and was classified as an early immediate-response gene. It can be induced by various signals, including cytokines, growth factors, hormones, fatty acids and physical stimuli. Nur77 plays an important regulatory role in cancer cell growth, survival, differentiation, and apoptosis (20–22). Numerous studies have shown that Nur77 is overexpressed in precancerous or cancerous cells to maintain their growth and survival (23–26). However, many studies have found that the low expression of Nur77 is related to tumor development, tumor metastasis and drug resistance in cancer cells (24, 27, 28). Overexpression of Nur77 in transgenic mice resulted in massive thymocyte apoptosis (29). Transgenic mice lacking both Nur77 and the associated Nor-1 gene developed fatal acute myeloid leukemia (30). Thus, Nur77 can exert tumorigenic and suppressive effects, depending on cell type and cellular environment. In this study, Nur77 was proved to function as a tumor suppressor gene in HB tumor cells, regulating the proliferation ability of HB tumor cells by mediating the degradation of β-catenin. However, whether Nur77 plays a regulatory role through other signaling pathways in HB tumor cells remains to be further studied.

In recent years, immunotherapy has become one of the hot spots in oncology research. Studies have shown that Nur77 is related to the regulation of anti-tumor immunity (31, 32). It has been reported that Nur77 can function as a key molecule mediating T cell dysfunction (33), and a high level of Nur77 expression effectively blocks the development and function of constant NKT cells (34). Simultaneous knockdown of the nuclear hormone receptor superfamily member NR4A1, NR4A2, and NR4A3 promoted the antitumor effect of CAR-T cells in several solid tumors (35). In addition, it is reported that NR4A1 can mediate the anti-tumor dysfunction of NK cells in HCC (12). The present research focused on the role of Nur77 in HB tumor cells, but did not explore the role of Nur77 in HB-related immune cells. Considering the important role of Nur77 in anti-tumor immunity, whether it also plays an important regulatory role in the process of HB tumor-related immunity, and whether regulating the expression of Nur77 can enhance the effect of HB-related immunotherapy will be the direction of our further studies.

Conclusions

In summary, this study proved that Nur77 can function as a tumor suppressor gene in HB. Knocking out and overexpressing Nur77 can significantly enhance or inhibit the proliferation ability of HB tumor cells in vitro and in vivo. Further research clarifies that Nur77 regulates the function of HB tumor cells by affecting the expression of β-catenin. Nur77 agonist Cns-A can effectively enhance the therapeutic effect of cisplatin on HB both in vitro and in vivo. This study preliminarily explained the mechanism of Nur77 in regulating the progression of HB, and provided a new research idea for improving the clinical efficacy of HB and improving the prognosis of patients.

Declarations
Ethics approval and consent to participate

This study was reviewed by the ethics committee of the Children's Hospital of Nanjing Medical University, batch number: NJCH2020137. Animal ethics were reviewed by Nanjing Medical University Animal Experiment Base, batch number: IACUC-1705026-2. The patients involved in the study signed an informed consent form to voluntarily participate in this study.

Consent for publication

Written informed consent was obtained from the patient for publication of this case report and any accompanying images. A copy of the written consent is available for review by the Editor-in-Chief of this journal.

Availability of data and materials

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

Competing interests

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in, or the review of, the manuscript entitled.

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Authors' contributions

Jianfeng Zhou, Xingyu Liu and Hanjun Yin performed the conceptualization, methodology, data creation, writing - original draft and writing - review & editing. Yong Zhou, Zhongya Xu and Kai Zhou performed the resources, investigation, formal analysis and visualization. Tao Li, Yongjun Fang and Qiyang Shen performed the validation, supervision and project administration.

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Figures
Figure 1

Nur77 was downregulated in HB. (A) Venn diagram showing the number of downregulated genes in HB tumor in 2 cohorts. (B) Dot plot showing the downregulated genes in HB tumor in 2 cohorts. (C) Violin plot showing the expression of NR4A1 in tumor and paracancer tissues in 2 cohorts. (D) Pearson's correlation test of NR4A1 and AFP expression in 2 cohorts. (E) Nur77 expression was analyzed in tumor and paracancer tissues of HB patients by western blotting. GAPDH was used as an internal reference gene.
Nur77 mRNA expression was analyzed in HB tumor and paracancer tissues by RT-qPCR (n=30). Nur77 protein expression in HB tumor and paracancer tissues by immunofluorescence staining. Scale bar represents 50 μm. HB patients were divided into middle-low risk group (n=18) and high-risk group (n=12) according to the clinical diagnostic criteria. The expression of Nur77 was detected by RT-qPCR in two groups of patients. Values are mean ± standard error of three independent experiments. P values were generated using unpaired t-test, ****p < 0.0001.
Figure 2

Nur77 knockout significantly enhanced the proliferation, migration, and invasion of HB cell lines, and decreased cell apoptosis. (A) The efficiency of Nur77 knockout in HepG2 and HuH6 cells were detected by western blotting. GAPDH was used as an internal reference gene. (B-D) Colony formation (B), EdU incorporation assay (C) and CCK-8 assay (D) were performed to validate the effect of Nur77 knockout on the proliferation of HepG2 and HuH6 cells. Scale bar represents 100 µm. (E) The effect of Nur77 knockout on the migration ability of HepG2 and HuH6 cells was detected by wound healing assay. Scale bar represents 200 µm. Values are mean ± standard error and of three independent experiments. P values were generated using unpaired t-test, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.
**Figure 3**

**A**

Nur77 overexpression efficiency was detected by western blotting in HepG2 and HuH6 cells. GAPDH was used as an internal reference gene.

**B-D**

The effects of Nur77 overexpression on the proliferation of HepG2 and HuH6 cells were detected by colony formation (B), EdU incorporation assay (C) and CCK-8 assay (D). Scale bar represents 100 μm.

**E**

The effect of Nur77 overexpression on cell apoptosis was detected by Annexin V-FITC/PI double staining.

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**Overexpression of Nur77 significantly inhibited the proliferation, migration, and invasion of HB cell lines, and increased cell apoptosis.** (A) Nur77 overexpression efficiency was detected by western blotting in HepG2 and HuH6 cells. GAPDH was used as an internal reference gene. (B-D) The effects of Nur77 overexpression on the proliferation of HepG2 and HuH6 cells were detected by colony formation (B), EdU incorporation assay (C) and CCK-8 assay (D). Scale bar represents 100 μm. (E) The effect of Nur77 overexpression on cell apoptosis was detected by Annexin V-FITC/PI double staining.
knockout on the migration ability of HepG2 and HuH6 cells was detected by wound healing assay. Scale bar represents 200 μm. Values are mean ± standard error of three independent experiments. P values were generated using unpaired t-test, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

**Figure 4**

A

B

C

D

E

F

G

H
Nur77 inhibited the proliferation of HB tumor cells in vivo. (A) Xenograft tumors were collected 1 month after subcutaneous transplantation of control or Nur77 KO HepG2 cells in nude mice (n = 5). (B) Statistics of the tumor weight (left) and tumor volume (right) of the subcutaneous xenografts in control or Nur77 knockout group. (C) Statistical analysis of the survival of tumor-bearing mice in control or Nur77 knockout group (n=10). (D) Representative immunohistochemical staining of Ki67 in control and Nur77-KO xenograft tumors. (E) Xenograft tumors were collected 1 month after subcutaneous transplantation of control or Nur77 OE HepG2 cells in nude mice. (n=5). (F) Statistics of the tumor weight (left) and tumor volume (right) of the subcutaneous xenografts in control or Nur77-OE group. (G) Statistical analysis of the survival of tumor-bearing mice in control or Nur77-OE group (n=10). (H) Representative immunohistochemical staining of Ki67 in control and Nur77-OE xenograft tumors. Values are mean ± standard error and represent three independent experiments. P values were generated using unpaired t-test (panel B, D, F, H) or mantel-cox test (panel C, G), *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001.
**Figure 5**

**Nur77 inhibited the expression of β-catenin through direct interaction in HB cells.** (A) β-catenin expression in tumor and paracancer tissues of HB patients by western blotting. GAPDH was used as an internal reference gene. (B) Immunofluorescence staining of β-catenin in tumor and paracancer tissues of HB patients was detected by immunofluorescence staining. Scale bar represents 50 μm. (C) The direct binding of Nur77 and β-catenin was detected by Co-IP experiments in control and Nur77 KO HepG2 and
HuH6 cells. (D) The effect of Nur77-KO and Nur77 OE on β-catenin activation was detected using TOPFLASH reporter plasmid system in HepG2 and HuH6 cells. (E) β-catenin expression was detected in control or Nur77-KO HepG2 and HuH6 cells using western blotting. GAPDH was used as an internal reference gene. Values are mean ± standard error and represent three independent experiments. P values were generated using unpaired t-test, ***p < 0.001, ****p < 0.0001.
β-catenin was the key to Nur77 suppressing the proliferation of HB tumor cells. (A) Overexpression efficiency of β-catenin and Nur77 was detected using western blotting. GAPDH was used as an internal reference gene. (B-D) Colony formation assay (B), EdU incorporation assay (C) and CCK-8 assay (D) were used to detect the effects of NUR77 OE and NUR77 OE + β-catenin OE on the proliferation of HepG2 cells. Scale bar represents 100 μm. (E) Expression of Nur77 and β-catenin was detected in Nur77-OE and Nur77 mutant OE cells using western blot. GAPDH was used as an internal reference gene. Values are mean ± standard error and represent three independent experiments. P values were generated using one-way ANOVA followed by Tukey's post hoc test analysis, ns, no significant difference, * p < 0.05, *** p < 0.001, **** p < 0.0001.
Figure 7

Nur77 agonist enhanced the therapeutic effect of cisplatin on HB tumor both in vitro and in vivo. (A) The expression of Nur77 and β-catenin was analyzed in DDP and DDP + Nur77 agonist Cns-A treated HepG2 cells by western blotting. GAPDH was used as an internal reference gene. (B-D) Colony formation assay (B), CCK-8 assay (C) and apoptosis detection assay (D) were used to detect the proliferation and apoptosis of HepG2 cells in DDP and DDP + Cns-A treated cells. (E) Image of control, DDP treated and
DDP + Cns-A treated subcutaneous tumors in nude mice (n=5). (F-H) Statistics of tumor weight (F), tumor volume (G) and survival of tumor-bearing mice (n=10) after administration of DDP or DDP + Cns-A (H). Values are mean ± standard error and represent three independent experiments. P values were generated using one-way ANOVA followed by Tukey’s post hoc (panel B-D, F, G) test or mantel-cox test (panel H), *p < 0.05, ***p < 0.001, ****p < 0.0001.

Supplementary Files

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