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*Original Article*

# NCYM facilitates cell proliferation and invasion in Wilms tumor by regulating SIX1/β-catenin axis

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**Abstract:** Recently, accumulating studies demonstrate that some long non-coding RNAs (lncRNAs) contain open read frames and have protein/peptide-coding potential. NCYM is a 109-amino acid product encoded by lncRNA MYCNOS variant 2 that is an antisense transcript of MYCN oncogene. NCYM is amplified in human neuroblastomas and associated with poor prognosis. However, its functional role in Wilms tumor (WT) remains unclear. In this study, we identified lncRNA MYCNOS as a promising prognostic factor in Wilms tumor through bioinformatics analysis. The expression of NCYM and downstream genes was determined by western blotting. Cell proliferation, migration, and invasion were measured by CCK-8, wound healing and Transwell assays, respectively. Cell apoptosis was evaluated by flow cytometry assay. The subcutaneous xenograft and lung metastasis mouse model were established by the armpit injection or tail intravenous injection of WT cells, respectively. Our results showed that NCYM was validated to be abundantly expressed in Wilms tumor cell lines and tissues. The exogenous overexpression of NCYM promoted WT cell proliferation, migration, and invasion. The silencing of SIX1 expression abolished the pro-growth effect of NCYM and downregulated β-catenin in WT. Additionally, NCYM facilitated tumor growth and formation of lung metastasis in vivo. In summary, the exogenous overexpression of NCYM could play a critical role in WT progression by mediating SIX1 and β-catenin as an oncopromoting factor.

**Keywords:** Wilms tumor, lncRNA, MYCNOS, peptide

## Introduction

Wilms tumor (WT), also known as nephroblastoma, is indeed the most frequently occurring primary malignant renal tumor in childhood, accounting for about 90% of renal tumors in children. It is believed to arise from nephrogenic rests, which are remnants of embryonal development that can lead to malignant transformation [1]. The primary age of WT onset is 2-5 years old, 95% of WT patients are under 10 years old [2]. In the United States, approximately 500 new cases of WT are reported annually [3]. Multidisciplinary comprehensive therapy including surgery, chemotherapy and radiotherapy has made it possible to achieve overall survival rate of over 90% [4]. However, post-operative recurrence and high-risk subtypes still pose challenges to the survival and treatment of children with WT. It is important to note that early diagnosis and treatment are crucial for the effective management of this type of tumor. Recurrence rate of WT is approximately 15% in children, and the long-term survival rate was less than 50% in relapsed cases [5]. Thus, investigations on the molecular pathogenesis are critical to deepen the understanding of the therapy and prognosis of patient with WT.

In mammalian cells, most transcribed RNAs are considered non-coding without protein-coding sequences. Non-coding RNAs (ncRNAs) are classified into long ncRNA (lncRNA), small nucleolar RNAs (snoRNAs), microRNAs (miRNAs) and circular RNAs (circRNAs) [6]. Long non-coding RNAs (lncRNAs) is a group of long RNA transcripts with the length longer than 200 nucleotides that lack protein-coding open reading frames (ORFs). Emerging studies have documented that lncRNAs participate a number of cellular biological processes, including gene regulation, telomeres maintenance, chromosome inactivation, cellular survival et al, playing an important role in cellular develop-

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ment and human diseases [7]. lncRNAs naturally integrate genetic networks to mediate a range of protein regulators that induce specific cellular biological process and determine cell fate. Some lncRNAs exhibit tissue-specific expression characteristics and can be used as biomarkers. Thus, they are potential to be monitor as indicator in assessment of disease therapy and prognosis [8, 9].

For decades, lncRNAs are widely considered as "noise transcripts" transcribed from genomic DNA without protein-coding capability. With development of bioinformatics and proteomics technologies, accumulative evidences suggest some lncRNA transcripts contain short open read frames (sORFs) and have small peptide/micropeptide coding potential which have been reported to be involved in the pathogenesis of multiple disease [10, 11]. For instance, A 17-amino-acid micropeptide, encoded by the long non-coding RNA (lncRNA) Dleu2 induces the generation of regulatory T cells through its interaction with SMAD3 and enhances its binding to the conserved non-coding region of Foxp3 [12]. LncRNA MIR7-3 host gene encodes a 125-amino-acid-long short peptide, which is able to reverse dexamethasone-induced β-cell dysfunction through activation of PI3K/AKT signaling pathway [13]. LncRNA DLX6-AS1-encoded peptide serves as a tumor promoter to facilitate proliferation, migration, and invasion by activating the wnt/β-catenin pathway in non-small-cell lung cancer [14]. LncRNA AFAP1-AS1 encoded 90-amino acid peptide inhibits transportation of NIPSNAP1 in mitochondria to regulate autolysosome formation by peptide-protein interaction [15].

LncRNA MYCNOS, an antisense transcript originating from the opposite strand of MYCN, has been found to be related to progression of multiple cancers, including glioblastoma [16], ovarian adenocarcinoma [17], hepatocellular carcinoma [18]. MYCNOS also mediates MYCN promoter usage and recruits various proteins to the upstream MYCN promoter to affect MYCN expression [19]. NCYM, a 109-amino acid product encoded by MYCNOS variant 2, was confirmed to be translated from the ORF in exon 3 region of MYCNOS transcripts and co-amplified with MYCN in neuroblastoma cells [20]. NCYM could induce OCT4 and stem cell-related genes via stabilizing MYCN in neuroblastoma [21]. These evidences suggest that the small peptide NCYM may play a pivot role in pathogenesis of cancers, especially in tumors with low-differentiated features.

Given the potential roles of NCYM in cancer progression, lncRNAs associated with WT progression were identified by bioinformatics in this study. We further explored whether NCYM could affect cell viability and migration. Additionally, the molecular basis of NCYM was preliminarily investigated.

## Materials and methods

### *Data acquisition and bioinformatics analysis*

The RNA-Seq gene expression profiles of patients with WT and corresponding clinical information were downloaded from TARGET-WT cohort in The Cancer Genome Atlas (TCGA, https://portal.gdc.cancer.gov/) data portal using GDC-client download tool. 130 WT samples and 6 adjacent non-cancerous samples were included in this study. 11 WT cases were excluded in prognostic analysis due to incomplete clinical information. R software (R Foundation for Statistical Computing, Vienna, Austria) was used for data extraction and analysis. The R package "edgeR" was used to normalize the expression level of each lncRNA and screened differentially expressed lncRNA. *P* value <0.05 and |logFC|>1 were used as the cutoffs for filtering the differentially expressed genes. Survival analysis and univariable/multivariable Cox regression analysis were conducted using the "survival" R package. Survival-related genes determined in multivariable Cox regression were used to further calculate the risk score by following formula: *Riskscore (RS)* = $\sum_{i=1}^{n}$ (*Expi*Coefi*), where Expi is the expression value of lncRNA, and Coei is the coefficient.

### *Cell culture*

Human Wilms tumor cell line WiT49 (BLUEFBIO Biotechnology, Shanghai, China) and HFWT (RIKEN BioResource Center, Tsukuba, Japan) were included in this study. Embryonic kidney cell line HEK293T was obtained from Procell (Wuhan, China). Cells were cultured in DMEM medium (Procell, Wuhan, China) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. All cells were maintained at 37°C in a 5% $CO_2$ humidified air incubator.

*Clinical samples acquisition*

WT tissue and adjacent normal kidney tissues were obtained from patients undergoing radical nephrectomy at Hunan Children's Hospital. The use of these WT specimens was approved by the Ethics Committee of Hunan Children's Hospital (No. HCHLL-2022-83), and informed consent was obtained from all patients.

*Plasmid construct*

The coding sequence of NCYM was constructed into backbone vector pcDNA3.1 (Invitrogen). The sequence of the ORF starting with start codon ATG of NCYM was shown as follow: 5-'ATGCAGCACCCACCCTGCGAGCCTGGCAATTGCTTGTCATTAAAAGAAAAAAAAATTACGGAGGGCTCCGGGGGGTGTGTGTTGGGGAGGGGAGACCGATGCTTCTAACCCAGCCCCCGCTTTGACTGCGTGTTGTGCAGCTGAGCGCGAGGCCAACGTTGAGCAAGGCCTTGCAGGGAGGTTGCTCCTGTGTAATTACGAAAGAAGGCTAGTCCGAAGGTGCAAAATAGCAGGGAGAGGACGCGCCCCCTTAGGAACAAGACCTCTGGATGTTTCCAGTTTCAAATTGAAAGAAGAGGGGCGCCCCCCTTGTTTGAAAATAAATAAATAA-3'. For knockdown of SIX1 in WT cell lines, the specific shRNA targeting SIX1 was transfected into cells. The sequence of SIX1 shRNA is listed as follows: 5'-CCAGCTCAGAAGAGGAATT-3'. Transfection was performed via using Lipofectamine 3000(Invitrogen, USA). Stable cells were kept in medium containing puromycin (1.5 μg/ml) for 4 weeks.

*Western blot*

Total protein was extracted through RIA lysis buffer (Biosharp, China) and quantified. Approximately 20 μg of protein was loaded in per lane for SDS-polyacrylamide electrophoresis and then transferred onto a PVDF membrane. The following antibodies were used: anti-NCYM (DF9068, Affinity, China), anti-Bcl2 (26593-1-AP, Proteintech, China), anti-Bax (50599-2-Ig), anti-SIX1 (10709-1-AP, Proteintech, China), anti-Beta catenin (51067-2-AP, Proteintech, China), anti-GAPDH (390035, Zenbio, China).

*Cell counting kit-8 (CCK-8) assay*

The CCK-8 assay was employed to evaluate the cell viability (Dojindo, Japan). Briefly, cells were counted and seeded in a 96-well plate with 4000 cells per well at 37°C. OD values were measured at 450 nm every 24 h. Subsequently, 10 ul CCK-8 reagent was added to each well and cultured for 3 h. The absorbance at 450 nm was measured using a microplate reader (Thermo Fisher Scientific, Massachusetts, United States).

*Colony formation assay*

Cells were seeded at low density in 6-well plates. Cells were cultured to recover for 14 days to allow colony formation. 4% paraformaldehyde was used to fix and cells were stained with 0.5% crystal violet. The plates were photographed, and the colonies were then counted.

*Apoptosis detection*

Cell apoptosis was detected by Annexin V-FITC/PI apoptosis detection kit (Keygenbio, Nanjing, China). Treated cells were cleaned twice with cold PBS and re-suspended in 1× binding buffe at the concentration of $1\times10^6$ cells/ml. Then, cells were double-labelled with Annexin V-FITC and PI staining according to the instruction. Finally, cells were analyzed by flow cytometry on BD FACSCanto II analyser (Becton Dickinson, USA).

*Wound healing and invasion assay*

Wound healing assay was performed to evaluate cell migration. $5\times10^5$ cells were seeded in a six-well plate and grown to nearly 100% confluence; Then, a linear scratch was made with a 200-μl pipette tip. The migration was determined by the percentage of cells filling the scratched area after 24 h.

Matrigel Transwell membrane filters (Corning, USA) were inserted into 24-well tissue culture plates for the invasion assay. Cells suspended in serum-free medium were added to the upper chamber of filters. Medium with 10% FBS was added to the lower chamber as a chemoattractant. The cells across the membrane were fixed with 4% formaldehyde, stained with Crystal Violet after 24 h.

*Dual-luciferase reporter assay*

In dual reporter luciferase assays, SIX1 promoter sequences were amplified and cloned into the pGL3 vector. pTK-Renilla was used as an

internal control. HEK293T cells were co-transfected with SIX1 luciferase vector combined with NC or NCYM-overexpressed vectors. The cells were lysed and the luciferase activities were measured with Dual-Luciferase Reporter Assay System (Promega, USA) 48 h later.

*Animal experiment*

Six-week-old BALB/c nude mice were purchased from The SJA Laboratory Co., Ltd. (Changsha, China) and housed in a specific pathogen-free facility of our institute. Our animal experiment was implemented in strict accordance with the Guideline for the Care and Use of Laboratory Animals of China. Twenty-four mice were acclimated for 5 days with free access to food and water. All mice were randomly divided into four groups of six mice. For subcutaneous xenograft group, each mouse was given a unilateral armpit subcutaneous injection of $6\times10^6$ cells. Tumor volumes were measured every four days and estimated according to the formula: tumor volumes ($mm^3$) = length × width$^2$ × 0.52. Tumors were removed and weighed after 21 days. For the metastasis models, $2\times10^6$ cells were suspended in 200 μL PBS and injected into the tail veins of each mouse. Lungs were harvested after 21 days and examined as secondary sites to analyze the events of metastasis.

*Statistical analysis*

All statistical analyses were performed using GraphPad Prism 8.0 (GraphPad Inc., USA). Data are presented as mean ± SD. The statistical significance between two groups was analyzed by Student's t test or one-way ANOVA. Mann-Whitney U test was applied for nonparametric variables. Statistical difference was defined as significance level of $P<0.05$.

## Results

*Identification of candidate lncRNAs based on TCGA database*

The TARGET-WT cohort consisted of 130 WT tissues and 6 adjacent tissues. To screen the aberrantly expressed lncRNAs in WT, the expression levels of lncRNAs in WT samples from TARGET-WT cohort were analyzed by using the R package "edgeR". Finally, according to the criteria of FDR<0.05 and [$\log_2$ FC]>1, a total of 2470 lncRNAs were found to be aberrantly expressed in WT, including 1143 down-regulated lncRNAs and 1327 upregulated lncRNAs (**Figure 1A**, **1B**). Gene ontology analysis was performed for differentially expressed lncRNAs to explore potential biological processes associated with WT. The result showed differentially expressed lncRNAs were mainly associated with structure constituent of ribosome, tubulin/microtube binding, DNA-dependent ATPase activity and ATP-dependent DNA helicase activity (**Figure 1C**).

*Identification of prognosis-related lncRNAs*

To explore the prognostic value of lncRNAs in WT, we performed univariate Cox regression analysis on the 2470 differentially expressed lncRNAs. MYCNOS (Hazard ratio (HR) =1.474, $P$=0.000), Z84485.1 (Hazard ratio (HR) =2.196, $P$=0.000) and AC007879.3 (Hazard ratio (HR) =1.283, $P$=0.000) were shown to have significant prognostic value under the strict screening criteria ($P<0.001$). Furthermore, multivariate Cox regression analysis also demonstrated that these 3 lncRNAs exhibited unfavorable prognostic value for WT ($P<0.05$) (**Table 1**).

The K-M analysis of these lncRNAs indicated that the upregulation of MYCNOS, Z84485.1 and AC007879.3 was correlated with worse overall survival (OS) ($P<0.05$) (**Figure 2A**). In addition, the Area Under Curve (AUC) suggested that MYCNOS, Z84485.1 and AC007879.3 indicated good diagnostic efficiency for WT, of which MYCNOS had highest AUC with nearly 1.00 (**Figure 2B**).

Moreover, the expression levels of the 3 lncRNAs between WT and adjacent tissues were analyzed. The expression levels of all the 3 lncRNAs were remarkably higher in WT tissues, compared with normal tissues (**Figure 2C**).

*Developing a three-lncRNAs gene signature for predicting OS*

According to the multivariate Cox regression analysis, we established a prognostic risk scoring system based on these three lncRNAs expression levels and coefficients. We divided the cohort into high-risk and low-risk groups based on their risk score value. The distribution of the prognostic risk score and survival outcome in the WT cohort were displayed in **Figure**

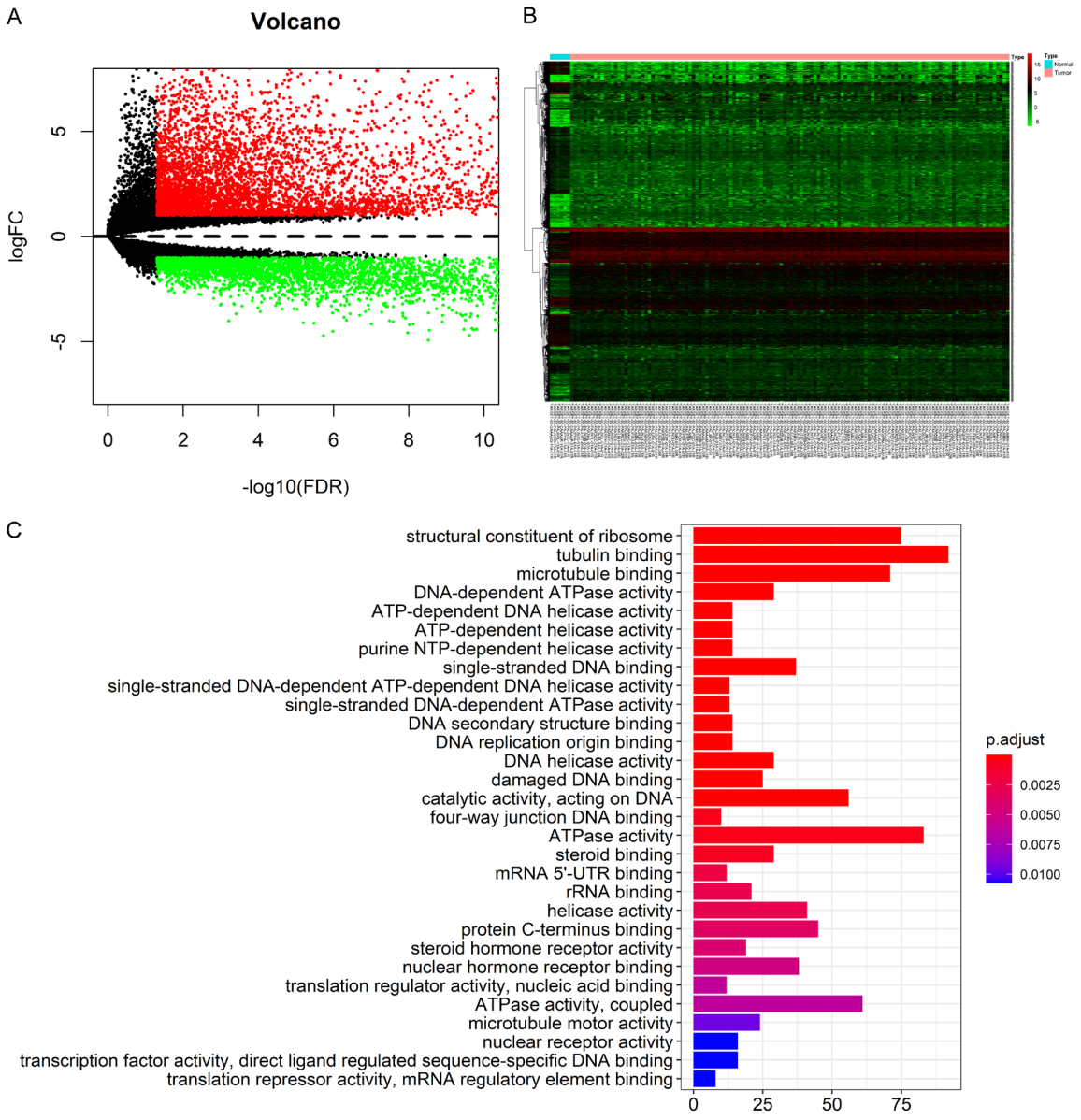


**Figure 1.** Identification of potential lncRNAs with protein-coding ability in human Wilms tumor (WT). A. The volcano plot visualizing the differentially expressed lncRNAs in TCGA database. Red dots represent upregulated genes, green dots represent downregulated genes. B. Heatmap of the expression profiles of differentially expressed lncRNA in tumors and adjacent non-cancerous tissues. C. The Gene Ontology analysis of differentially expressed lncRNAs in WT.

**Table 1.** Univariate and multivariate COX regression analysis of lncRNAs for WT patients' overall survival (OS)

| Gene | Univariate | | | Multivariate | | |
|---|---|---|---|---|---|---|
| | Hazard ratio | Z value | *P* value | Hazard ratio | Z value | *P* value |
| MYCNOS | 1.474 | 3.293 | 0.000 | 1.302 | 2.077 | 0.037 |
| Z84485.1 | 2.196 | 3.352 | 0.000 | 1.871 | 2.759 | 0.006 |
| AC007879.3 | 1.283 | 3.317 | 0.000 | 1.194 | 2.274 | 0.023 |

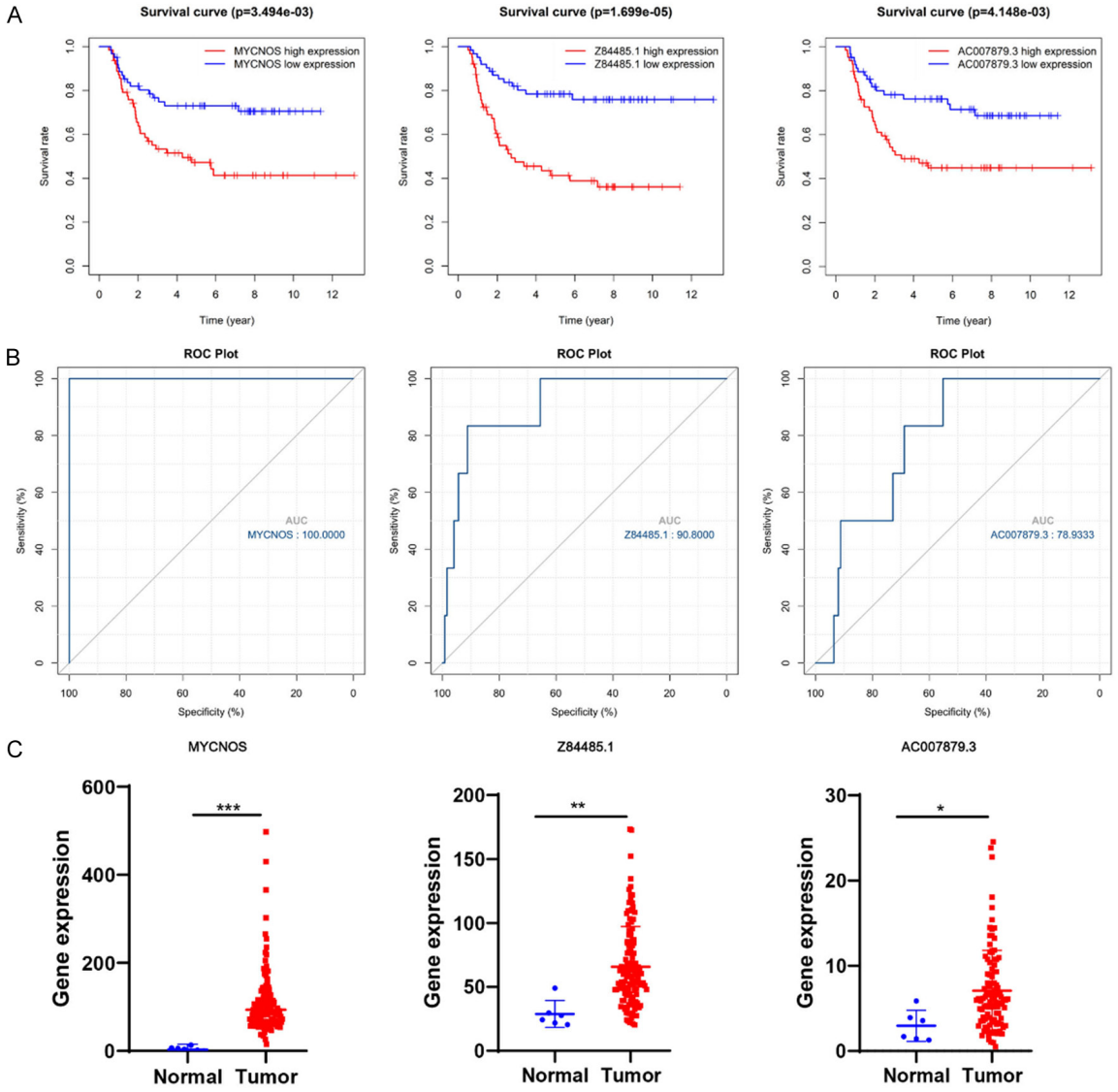


**Figure 2.** Selection of lncRNAs related with the survival of WT patients. A. Kaplan-Meier survival curves revealed expression levels of MYCNOS, Z84485.1 and AC007879.3 were significantly associated with the overall survival of patients with WT. B. Receiver operating characteristic (ROC) curves indicated good performance of MYCNOS, Z84485.1 and AC007879.3 expression levels for predicting overall survival of WT patients. C. MYCNOS, Z84485.1 and AC007879.3 expression levels between WT tumors and adjacent non-cancerous tissues in TCGA database. **P*<0.05, ***P*<0.01, ****P*<0.001.

**3A**, **3B**. The AUC value of the three-lncRNAs risk scoring system was 0.752 for 5-years survival suggested by **Figure 3C**. The result of K-M analysis indicated that high-risk group had a poor OS of WT patients (*P*=0.003) (**Figure 3D**).

*Expression of NCYM encoded by lncRNA MYCNOS in WT*

Expression analysis of online RNA-seq data of WT revealed that lncRNA MYCNOS expression was markedly increased in WT samples and exhibited high diagnostic accuracy compared with other lncRNA. Recently, accumulating evidences demonstrates that some lncRNAs have potential protein coding potential. Thus, bioinformatic screening for possible MYCNOS lncRNA-encoding ability was performed using ORFfinder online tool. We discovered that lncRNA MYCNOS harbored 3 short open reading frames (ORF) (**Table 2**). Interestingly, we found that a 109-amino acid peptide named

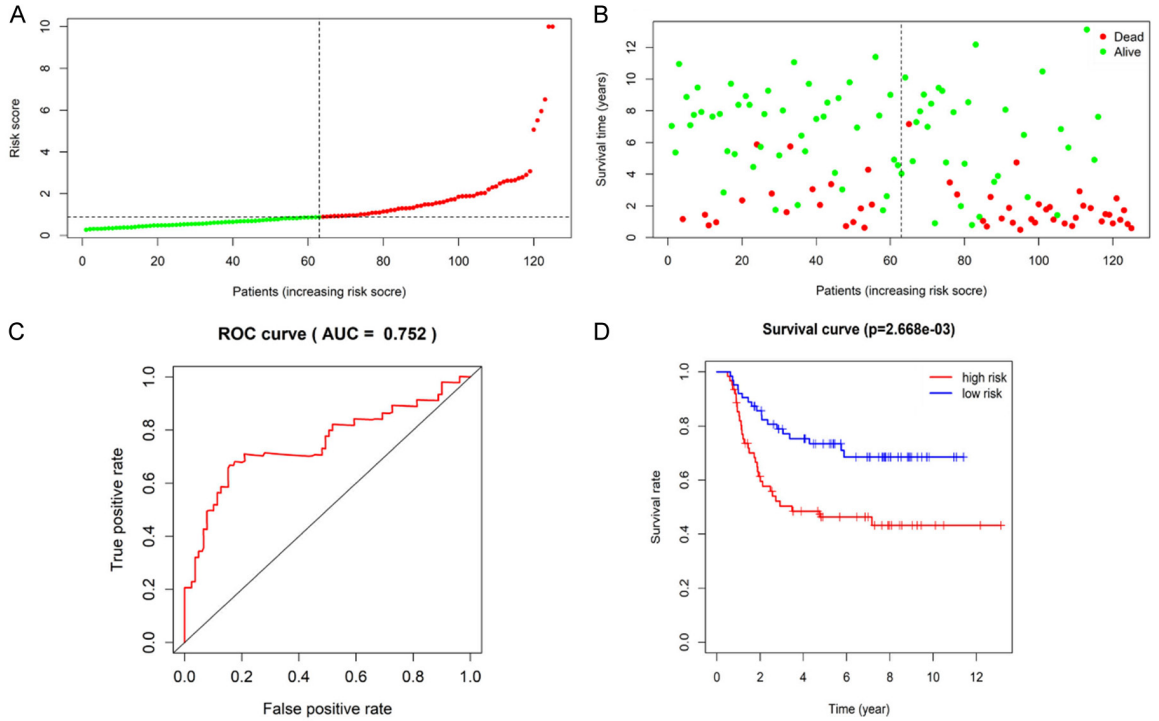


**Figure 3.** Characteristics of the prognostic lncRNA signature. A. lncRNA signature risk score distribution. B. Survival status of patients based on risk scores. C. ROC curves to evaluate the prognostic significance of the lncRNAs signature for 5-year survival. D. Kaplan-Meier analysis revealed the relationship between the risk score and the overall survival of WT patients.

**Table 2.** Characteristics of potential peptides encoded by MYNCOS ORFs

| ORF | Sequence | Strand | Start | Stop | Length (nt/aa) |
|---|---|---|---|---|---|
| 1 | MQHPPCEPGNCLSLKEKKITEGSGGVCWGGETDASNPAPALTAC-CAAEREANVEQGLAGRLLLCNYERRLVRRCKIAGRGRAPLGTRPLDVSS-FKLKEEGRPPCLKINK | + | 426 | 755 | 330/109 |
| 2 | MGHPLEKNPSLGWGRTGSPTAQTQTDLLEPREFLFAEAITPPER-RLQSWEAQGVLLPPFWTPGLAPAAL | + | 190 | 399 | 210/69 |
| 3 | MTSNCQARRVGAALHRSARSWFSECSRCKPGGPKGREEHTLGF-PALQPSLGGSNGFCEKKFPRL | - | 465 | 271 | 195/64 |

NCYM encoded by MYCNOS variant 2, was reported to be translated from the ORF1 in exon 3 region of MYCNOS transcripts and upregulated in neuroblastoma [20].

To confirm the endogenous expression of NCYM in WT clinical samples, we detected the peptide expression by Western-blotting using polyclone antibody. The results revealed that this peptide was highly expressed in WT tumor samples than in normal adjacent tissue (**Figure 4A**). Immunoblotting of cell lines also demonstrated the upregulated expression of the NCYM in HFWT and WiT49 cells compared with HEK293T cell line (**Figure 4B**).

To further examine the exogenous expression of NCYM, NCYM coding ORF fragment was cloned into pcDNA3.1 vector containing Flag epitope tag. Then we transfected this plasmid into WT cell lines WiT49 and HFWT. Western blot analysis revealed a notable overexpression (OE) of NCYM in cells transfected with NCYM-OE plasmid (**Figure 4C**), demonstrating that NCYM overexpression plasmid could translate peptide NCYM in WT cell lines efficiently.

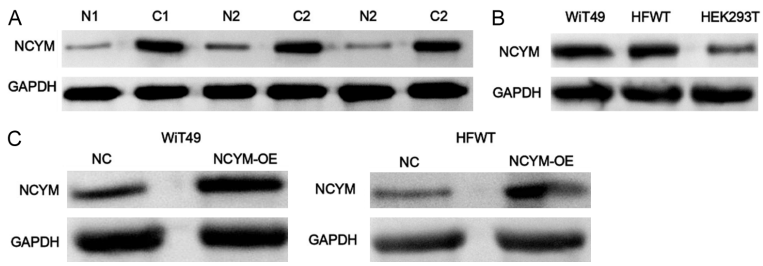


**Figure 4.** Identification of NCYM expression level in clinical tissues and cell lines. High expression level of NCYM was observed in tumor samples (A) and WT cell lines (B) via Western-blotting assay. (C) The expression of NCYM was measured after the expression construct was transfected into cell lines. N, normal tissue; C, cancer tissue.

*The effect of NCYM on WT cell proliferation, apoptosis, migration, and invasion*

Next, CCK-8 assay showed that the overexpression of NCYM led to approximately 23.9% or 25.9% increase in the viability of in HFWT and WiT49 cells, respectively (**Figure 5A**). In addition, we used colony formation assay to evaluate the effect of NCYM on long-term viability in these two cell lines. **Figure 5B** showed overexpression NCYM significantly enhanced colony formation of WT cells. These results implied that the potential tumor-promoting role of NCYM in WT progression. Flow cytometry was performed to detect apoptotic in WT cell lines after NCYM overexpression. The overexpression of NCYM could decrease cell apoptosis rate in WiT49 and HFWT cells (**Figure 5C**). The wound healing assay disclosed that the upregulated expression of NCYM led to marked increase of cell migration area (**Figure 5D**). Similarly, transwell assay revealed that the number of invasive cells in NCYM-OE group was higher than in the control group (**Figure 5E**). These data suggested that NCYM could enhance WT cell migration and invasion ability.

*Overexpression of NCYM mediates WT progression through activating SIX1*

SIX1 as WT-specific oncogenes, is related to the unfavorable WT subtype with high-risk histology. 89% blastemal components in WT showed higher expression level of SIX1 [22]. SIX1 mutations result in worse prognostic outcome with a significant higher relapse rate and a higher mortality [23]. Additionally, a critical progression mechanism of Wilms tumors is that activation of WNT/β-catenin (CTNNB1) pathway leads to abnormal induction or persistent progenitor proliferation [24]. Hence, we further investigated whether NCYM could regulate WT cell biological process through SIX1 and β-catenin.

Spearman correlation revealed high positive correlation of lncRNA MYCNOS with key WT oncogenes, including MYCN (ρ=0.858), IGF2 (ρ=0.446), SIX1 (ρ=0.600), β-catenin (CTNNB1) (ρ=0.400) (**Figure 6A**). Herein, we evaluated whether NCYM could regulate SIX1 and β-catenin expression in WT cells. Western-blotting showed SIX1, β-catenin and anti-apoptotic gene Bcl-2 expression level was notably increased and pro-apoptotic gene Bax expression level was markedly reduced in cells transfected with NCYM-OE plasmid than in control group (**Figure 6B**), suggesting that NCYM likely modulate WT through activation of SIX1 and β-catenin pathway. To validate our presumption, SIX1-specific shRNA was introduced to knockdown SIX1 expression. Western-blotting showed knockdown of SIX1 inhibited the upregulation of β-catenin and Bcl-2 expression induced by NCYM and alleviated NCYM inhibitory effect on Bax2 expression (**Figure 7A**). Moreover, dual luciferase activity assay showed that, compared to NC group, NCYM-OE group showed significantly higher luciferase activity, suggesting NCYM may participate SIX1 transcriptional activation (**Figure 7B**). Remarkably, knockdown of SIX1 reversed NCYM-driven cell viability promotion (**Figure 7C**), cell apoptosis repression (**Figure 7D**) in WiT49 and HFWT cells. Due to low HFWT cell viability in serum-free medium, WiT49 cells were used to evaluate cell migration and invasion. Furthermore, SIX1 downregulation also abrogated NCYM-mediated migration ability enhancement (**Figure 7E**) and invasion cell number increase (**Figure 7F**).

*NCYM promotes in-vivo tumor formation and lung metastasis*

To validate the role of NCYM under in vivo conditions, stably transfected NCYM-OE plasmid WiT49 cells and control cells were subcutaneously injected into the nude mice to develop subcutaneous xenograft tumors. Six mice were randomly assigned to each group. Mice were

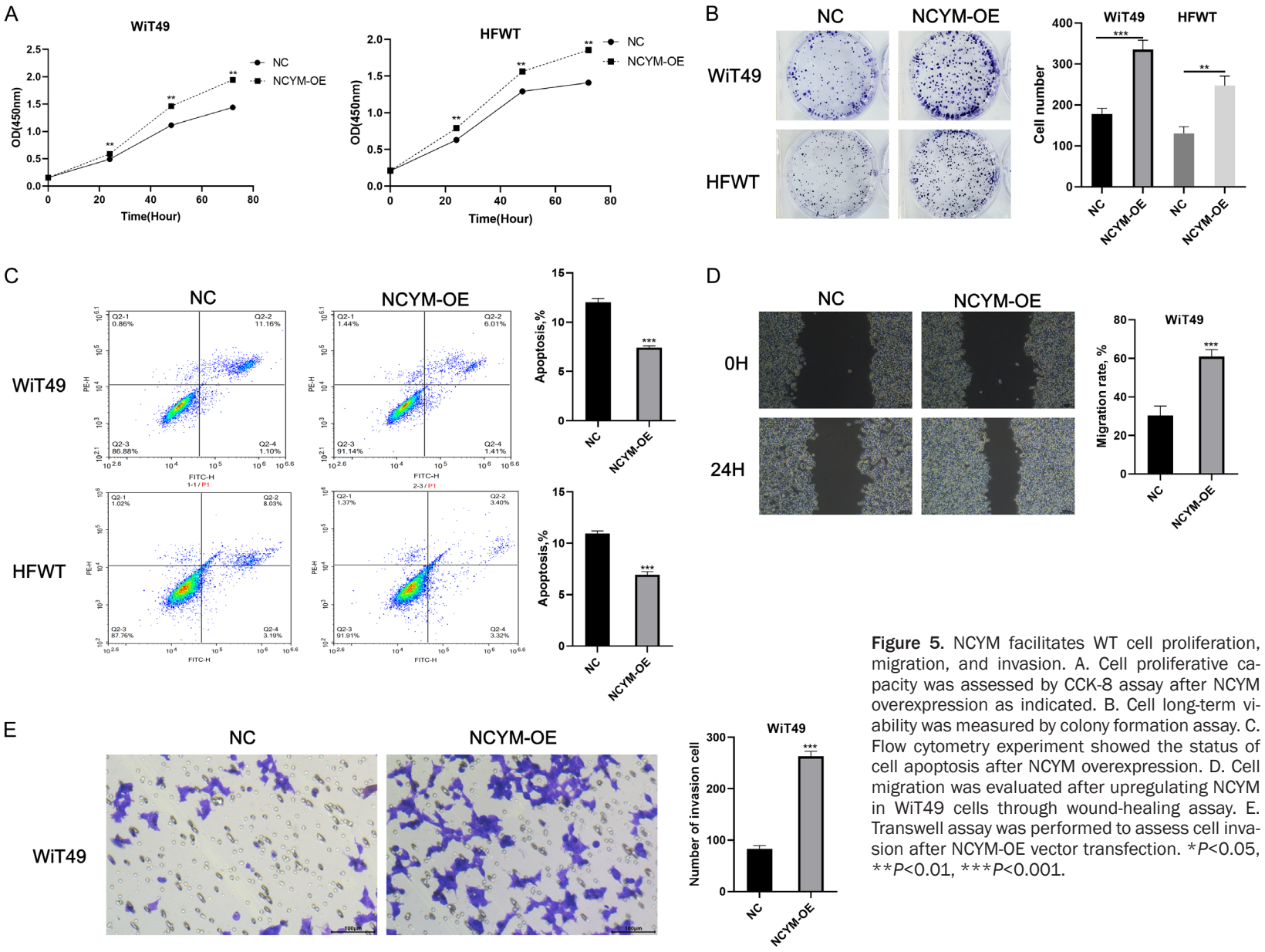


**Figure 5.** NCYM facilitates WT cell proliferation, migration, and invasion. A. Cell proliferative capacity was assessed by CCK-8 assay after NCYM overexpression as indicated. B. Cell long-term viability was measured by colony formation assay. C. Flow cytometry experiment showed the status of cell apoptosis after NCYM overexpression. D. Cell migration was evaluated after upregulating NCYM in WiT49 cells through wound-healing assay. E. Transwell assay was performed to assess cell invasion after NCYM-OE vector transfection. **P*<0.05, ***P*<0.01, ****P*<0.001.

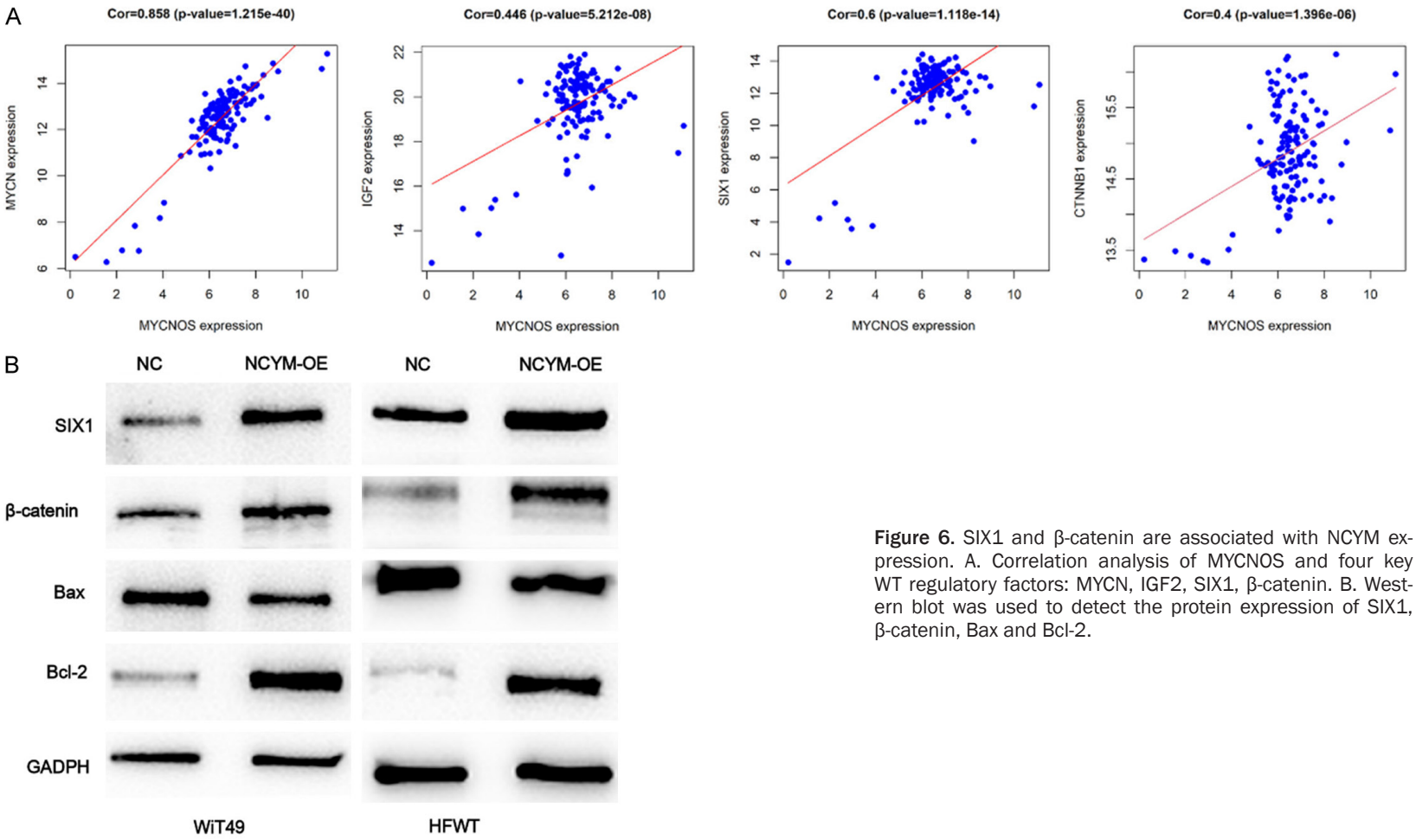


**Figure 6.** SIX1 and β-catenin are associated with NCYM expression. A. Correlation analysis of MYCNOS and four key WT regulatory factors: MYCN, IGF2, SIX1, β-catenin. B. Western blot was used to detect the protein expression of SIX1, β-catenin, Bax and Bcl-2.

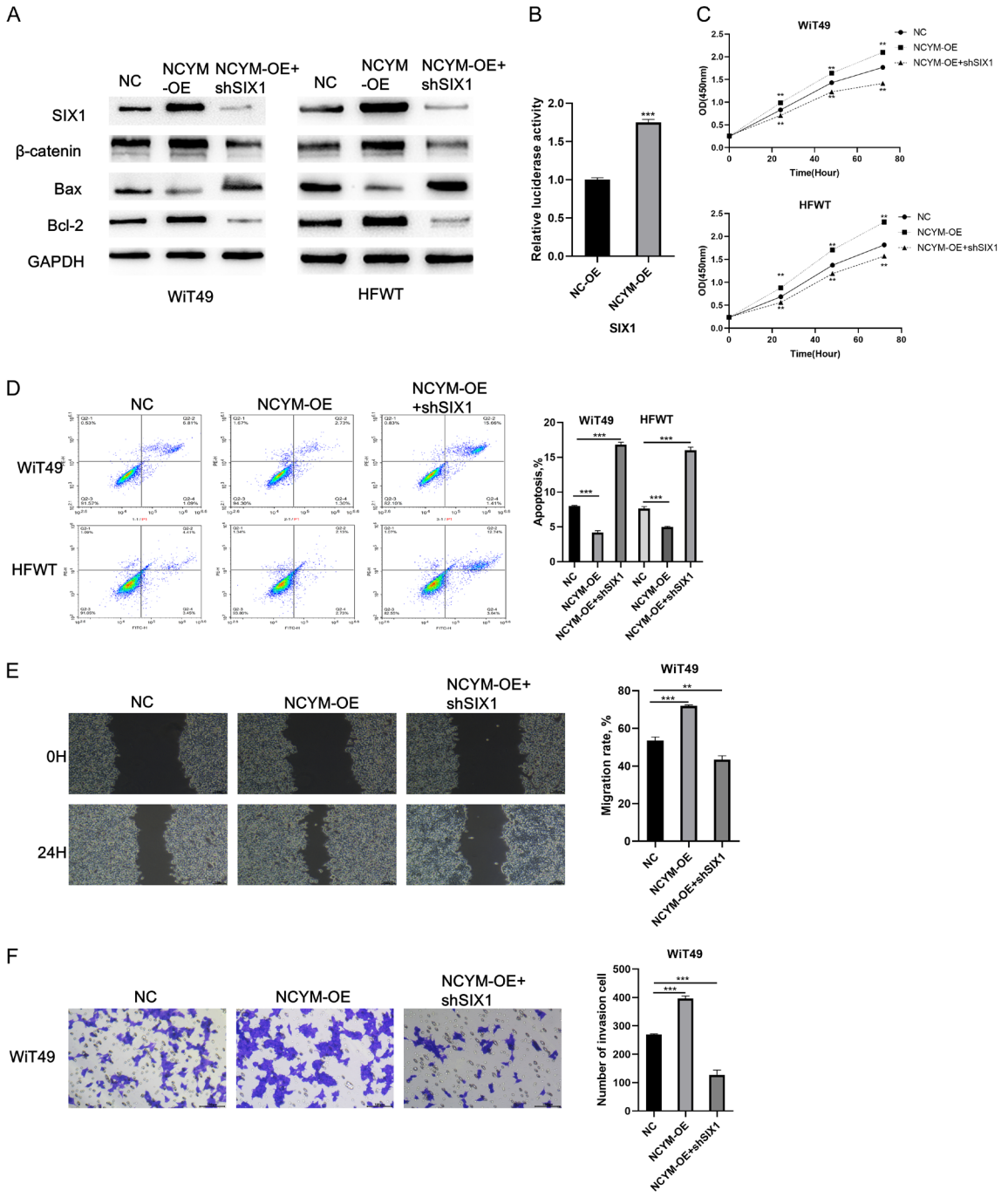


**Figure 7.** NCYM promotes viability, migration and invasion of Wilms tumor cells by mediating SIX1 expression. A. Western blot was used to determine the protein expression of SIX1, β-catenin, Bax and Bcl-2 after NCYM overexpression and silencing of SIX1. B. Luciferase reporter assays confirmed an interaction between NCYM and the SIX1 promoter. C. Cell viability was detected by CCK-8 assay after NCYM overexpression and silencing of SIX1. D. Cell apoptosis was assayed by flow cytometry. E. Wound healing assay was applied to assess cell migration after NCYM overexpression and silencing of SIX1. F. Transwell assay was applied to assess cell invasion after NCYM overexpression and silencing of SIX1. *$P$<0.05, **$P$<0.01, ***$P$<0.001.

sacrificed and tumor tissues were collected three weeks later (**Figure 8A**). Our result showed that xenografted tumors formed by NCYM-OE cells had much larger volume and

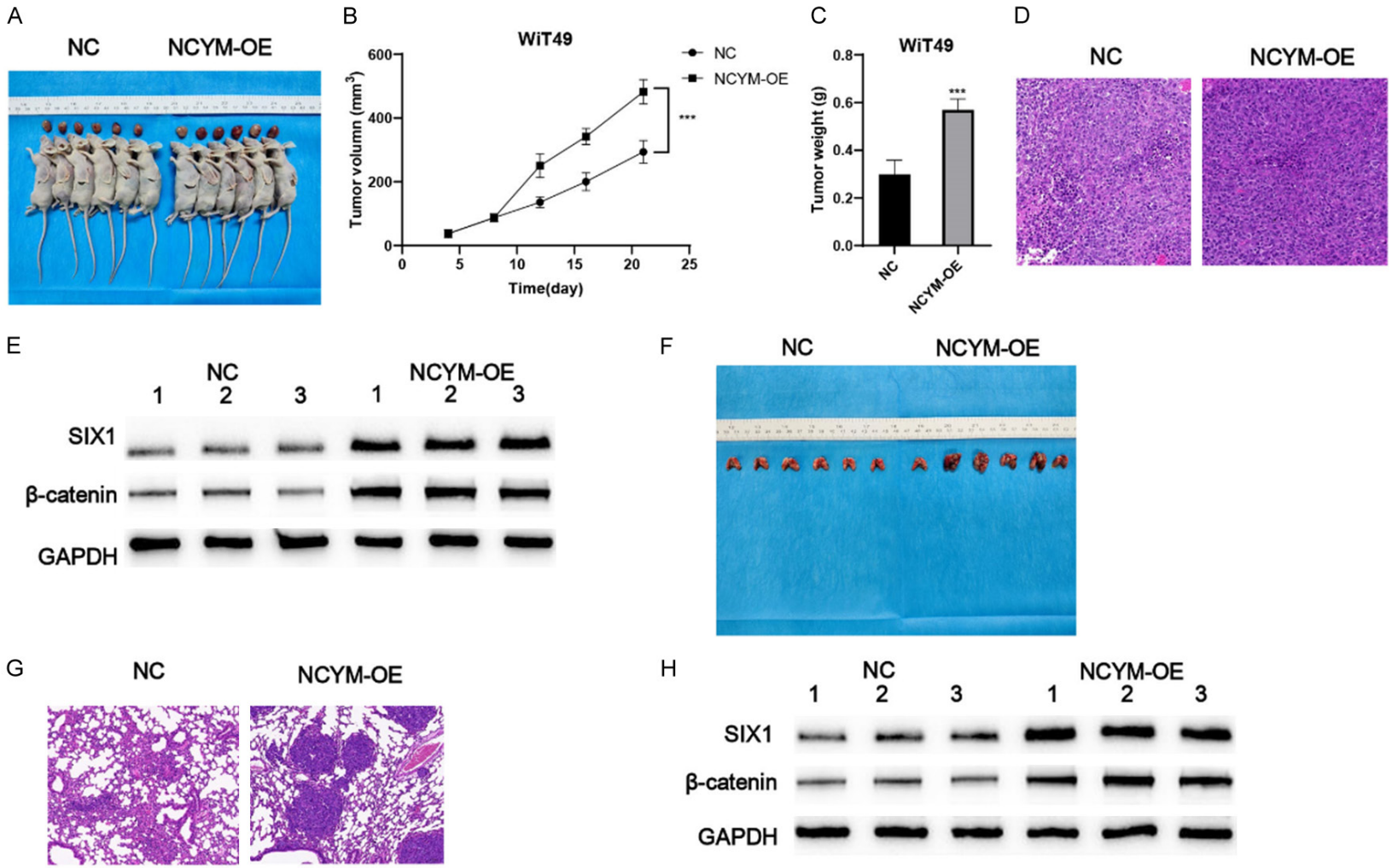


**Figure 8.** Overexpression of NCYM promotes the tumor growth and lung metastasis of Wilms tumor in vivo. A. Representative image of mice sacrificed at day 21 after xenografted and tumor. B. The growth curve of tumor volumes. C. The growth curve of tumor weight. D. HE staining of subcutaneous tumors. E. SIX1 and β-catenin expression were detected by Western-blotting in subcutaneous tumors tissue. F. Representative image of mice lungs of each group at day 21 after tail intravenous injection of cells. G. HE staining of lung metastasis colonies. H. SIX1 and β-catenin expression were detected by Western-blotting in lung metastasis nodules. ***$P$<0.001.

substantially higher tumor weight, compared to the control group (**Figure 8B**, **8C**). Tumor formation in mice was confirmed by hematoxylin/eosin (HE) staining (**Figure 8D**). Western-blotting of xenograft tumor showed that the level of SIX1 and β-catenin in NCYM-OE xenograft were higher than in control xenograft (**Figure 8E**). For the lung metastasis model (**Figure 8F**), it was found that more lung tumor formed in NCYM-OE group (6 of 6, 100%) compared with that of the control group (3 of 6, 50%). Metastatic tumors were also observed via HE staining (**Figure 8G**). We obtained similar SIX1 and β-catenin expression results to subcutaneous xenograft model (**Figure 8H**).

## Discussion

In this study, we reported that a 109-amino acid protein named NCYM encoded by lncRNA MYCNOS variant 2 exhibited tumor-promoting effect in Wilms tumor. We use a range of in-vitro experiments to verify the pro-proliferation features of NCYM in WT cell lines. Mechanically, we preliminary demonstrate that NCYM may mediate WT malignant biological process via upregulating SIX1 and β-catenin. In-vivo experiments validated the pro-growth and metastasis-promoting role of NCYM in WT. Therefore, NCYM may become a critical molecule for exploration of Wilms tumor tumorigenesis.

lncRNAs are RNA transcripts with length over 200 nucleotides that lacking canonical protein-coding open reading frames (ORFs). Originally considered as “noise” from genomic transcription, lncRNAs have gradually been proven to play important roles in the regulation of multiple biological processes, including transcriptional, and post-transcriptional level and epigenetic modulation [25, 26]. Previously, it was believed that lncRNAs did not have the ability to translate proteins or peptides due to the absence of an open reading frame. With the advances in translation omics technology and bioinformatics, lncRNAs have been reported to contain short ORFs encoding small functional peptides with biological functions in numerous diseases [27, 28]. However, whether there are small peptides encoded by lncRNAs are involved in the pathogenesis of Wilms tumor has not been reported. In this study, initially bioinformatics analysis was employed to identify differentially expressed lncRNAs in WT to screen out lncRNAs with potential translation ability. Our findings identify lncRNA MYCNOS, Z84485.1 and AC007879.3 are associated with overall survival of WT patients as risk factors. Using ROC analysis, we obtained AUC of 0.79-1.00 for differentiating tumor and normal tissue based on expression level of these three lncRNA, demonstrating they could serve as early predictor for the survival of WT. After integrating these lncRNAs expression with COX regression coefficients, we construct a gene signature with a strong prognostic value. The signature may be used as a potential prognostic parameter and therapeutic target in WT.

lncRNA MYCNOS exhibits highest diagnostic value and expression level among these three survival-related lncRNAs. Previous reports have documented that MYCNOS promotes cell proliferation, migration and invasion in several cancers, including glioblastoma, retinoblastoma and ovarian adenocarcinoma [16, 17, 29]. Thus, translation ability of MYCNOS was examined in this study currently. Interestingly, previous publications revealed the existence of open reading frame (ORF) in exon 3 region of MYCNOS transcripts and the peptide translated from ORF, named NCYM due to the ‘reverse reading’ of MYCN gene locus [20, 30]. In neuroblastoma NCYM stabilizes MYCN through inhibition of GSK3b-mediated MYCN phosphorylation, and MYCN facilitates NCYM transcription [31]. NCYM is reported to regulate chemotherapy sensitivity through the NOTCH pathway in small cell lung cancer [32]. Therefore, we examined the expression of NCYM in WT tissue and cell lines. Elevated NCYM expression was found in WT tumor tissue compared to adjacent normal tissue. Moreover, WT cell lines WiT49 and HFWT exhibited higher NCYM level than HEK293T cells. To evaluate the function of NCYM in WT, the plasmid containing NCYM ORF sequence was generated and transfected to construct stably NCYM-expressed cells. Our results showed exogenous overexpression of NCYM could noticeably enhance WT cells proliferative, migratory, and invasive abilities.

Next, it is essential to identify the underlying mechanisms by which NCYM is involved in cancer biology. SIX1, an embryologic transcription factor, is critical for progenitor renewal by regulating the expression of various genes involved

in embryogenesis. In tumor biology, SIX1 promotes cell growth via mediating cancer stem cell and epithelial-to-mesenchymal transition-phenotypes in Pancreatic ductal adenocarcinoma [33]. SIX1 also facilitates the proliferation of non-small lung cancer via activating the Notch signaling pathway [34]. Cell cycle genes was found to be upregulated in SIX1-mutant WT mice, and loss of SIX1 led to mesenchymal apoptosis [23]. Specificity of the overexpression of SIX1 in WT tissue was confirmed by microarray compared to other multiple tissues including fetal heart, kidney and peripheral blood [35]. Significant amplification of SIX1 was also observed in Wilms tumor tissue via array comparative genomic hybridization, implying its important role in the tumorigenesis of Wilms tumor [36]. In addition, β-catenin is a core component of the canonical WNT signal transduction pathway. In bladder cancer, NCYM could bind GSK3b to prevent β-catenin from phosphorylation-dependent degradation [37]. Activation of β-catenin in both the nephron progenitor and stromal lineages leads to profoundly disrupted kidney development characterized by the formation of bone-like tissue, a phenotype previously reported in human Wilms tumor [38]. MUC6 exerts its tumor-suppressive function via autophagy-dependent β-catenin degradation in Wilms tumor [39]. Here, our work demonstrated that exogenous overexpression of NCYM could trigger the notable increase of SIX1 and β-catenin expression level, while knockdown of SIX1 could reverse the induction overwhelmingly. The inhibition of SIX1 abrogated NCYM-mediated promotive effects on cell proliferation, migration and invasion in WT cells. Moreover, NCYM-mediated pro-proliferative and pro-metastasizing abilities were also confirmed in xenograft model.

In summary, we demonstrate that the NCYM is a oncoprotein that promotes cell proliferation, migration, and invasion partly through induction of SIX1 and β-catenin in WT. This is the first report of the small peptides NCYM exerts certain function in WT. NCYM may have a potential value and offer a novel strategy for the treatment of WT.

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## Disclosure of conflict of interest

None.

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