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ZNF471 inhibits nasopharyngeal carcinoma cell growth and stemness

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Abstract. We investigated how zinc finger protein 471 (ZNF471) affects nasopharyngeal carcinoma (NPC) cell growth, migration, invasion and stemness and offer possible treatment targets for NPC research. The GEO2R online dataset was used to query the expression of ZNF471 in NPC tissues. ZNF471 overexpression plasmid was transfected into NPC cell lines. Cell Counting Kit-8 (CCK8) was used to detect cell viability, 5-Ethynyl-2'-deoxyuridine (Edu) to detect cell proliferation, Wound Healing Assay to detect cell migration, Transwell Assay to detect cell invasion, and Spheroid Formation Assay to detect the stemness characteristics of NPC cells. Western blot assay was used to determine the downstream genes matrix metalloproteinase 7 (MMP-7) and Myelocytomatosis viral oncogene homolog (c-Myc), as well as the protein expression of β-catenin, a protein linked to the Wnt/β-catenin pathway. Overexpression of ZNF471 significantly inhibited NPC cell viability, reduced the number of Edu-positive cells, migration rate, cell invasion number, and tumor cell spheroid formation number. Besides, overexpression of ZNF471 reduced the protein expression of β-catenin and the downstream genes c-Myc and MMP-7. In conclusion, ZNF471 inhibits the growth, migration, invasion and stemness of NPC cells, which may be related to its inhibition of Wnt/β-catenin pathway activation.

Key words: Nasopharyngeal carcinoma — ZNF471 — Migration and invasion — Stemness — Wnt/β-catenin

Highlights

- ZNF471 is down-expressed in nasopharyngeal carcinoma
- ZNF471 inhibits the proliferation and cell migration of nasopharyngeal carcinoma cells
- ZNF471 inhibits the stemness characteristics of nasopharyngeal carcinoma cells
- ZNF471 inhibits the Wnt/β-catenin pathway

Introduction

Nasopharyngeal carcinoma (NPC) is a multidimensional spatiotemporal "eco-evolutionary unified" disease and evolutionarily adaptive pathological ecosystem composed of four interdependent parts: the primary ecosystem, the circulating ecosystem, the metastatic ecosystem, and the multidirectional ecosystem (Luo 2023). Southeast Asia and southern China are more frequent locations for NPC, a malignant tumor originating from the nasopharyngeal epithelium. Since early symptoms of NPC are not always evident, most instances are detected in the middle or late stages of the disease. Furthermore, NPC has a propensity to spread and invade. Nearly all advanced patients will develop

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invasively to the base of the skull, and the majority of NPC patients will have metastases from cervical lymph nodes. Chemotherapy and radiation are being used in combination as the current treatment. Despite improving the prognosis for NPC patients, the five-year survival rate of patients remains dismal. Thus, additional research into possible gene targets for NPC is necessary to create more potent treatment plans to fight this illness (Mo et al. 2021).

The unrestricted proliferation, invasion, and advanced stage of cancer in patients are caused by cancer stem cells (CSCs), a subset of cells within tumors with the capacity to self-renew and create new tumors. The existence of CSCs in NPC cells has been demonstrated by a wealth of evidence, indicating that NPC treatment strategies may focus on NPC CSCs (Hu et al. 2023).

In mammals, the biggest transcription factor family consists of zinc-finger proteins (ZFPs). By binding to gene promoters, their zinc-finger domains either activate or inhibit the expression of target genes. Zinc finger protein 471 (ZNF471) is a member of the ZFP family and is downregulated because of promoter hypermethylation in a number of tumor types. By suppressing genes involved in proliferation, growth, migration, and invasion, ZNF471 may function as a tumor suppressor in cervical cancer (Bhat et al. 2021). ZNF471 inhibits AKT and Wnt/β-catenin signaling, tumor cell stemness, and the epithelial-mesenchymal transition in order to carry out its tumor suppressor role (Tao et al. 2020).

The function and mechanism of ZNF471 in NPC are currently the subject of few studies. In NPC, ZNF471 suppresses tumor development and stemness by acting as a tumor suppressor gene, and this study established this for the first time. This information will be useful for future NPC anti-cancer research.

Materials and Methods

Bioinformatics analysis

Since NPC belongs to head and neck squamous cell carcinoma (HNSC), the expression of ZNF471 in HNSC was queried in the Ualcan database (https://ualcan.path.uab.edu/). In addition, using the Kaplan-Meier Plotter database (https://www.kmplot.com/analysis/), the impact of ZNF471 on the survival rate of HNSC patients was investigated. To determine the expression of ZNF471 in NPC, the GSE53819 expression profile was analyzed online by GEO2R.

Tissue collection

Tumor tissues and normal tissues were taken from 40 individuals who had nasopharyngeal tumor biopsy and were pathologically diagnosed with NPC. The clinical information is provided in Table 1. The Ethics Committee of the hospital

Table 1. The correlation between ZNF471 expression and clinicopathological characteristics of NPC patients

Characteristics	*n*	ZNF471 level		Chi-square (math.)	*p* value
		high	low		
Total case	40	20	20		
Gender					
male	26	12	14	0.4396	0.5073
female	14	8	6		
Age (year)					
≤50	19	11	8	0.9023	0.3422
>50	21	9	12		
Smoking history					
yes	22	12	10	0.4040	0.5250
no	18	8	10		
Degree of differentiation					
moderate and high	28	13	15	0.4762	0.4901
poor	12	7	5		
TNM stage					
I–II	22	17	8	8.6400	0.0033*
III–IV	18	3	12		
Lymph node metastasis					
yes	24	8	16	6.6667	0.0098*
no	16	12	4		

* $p < 0.05$

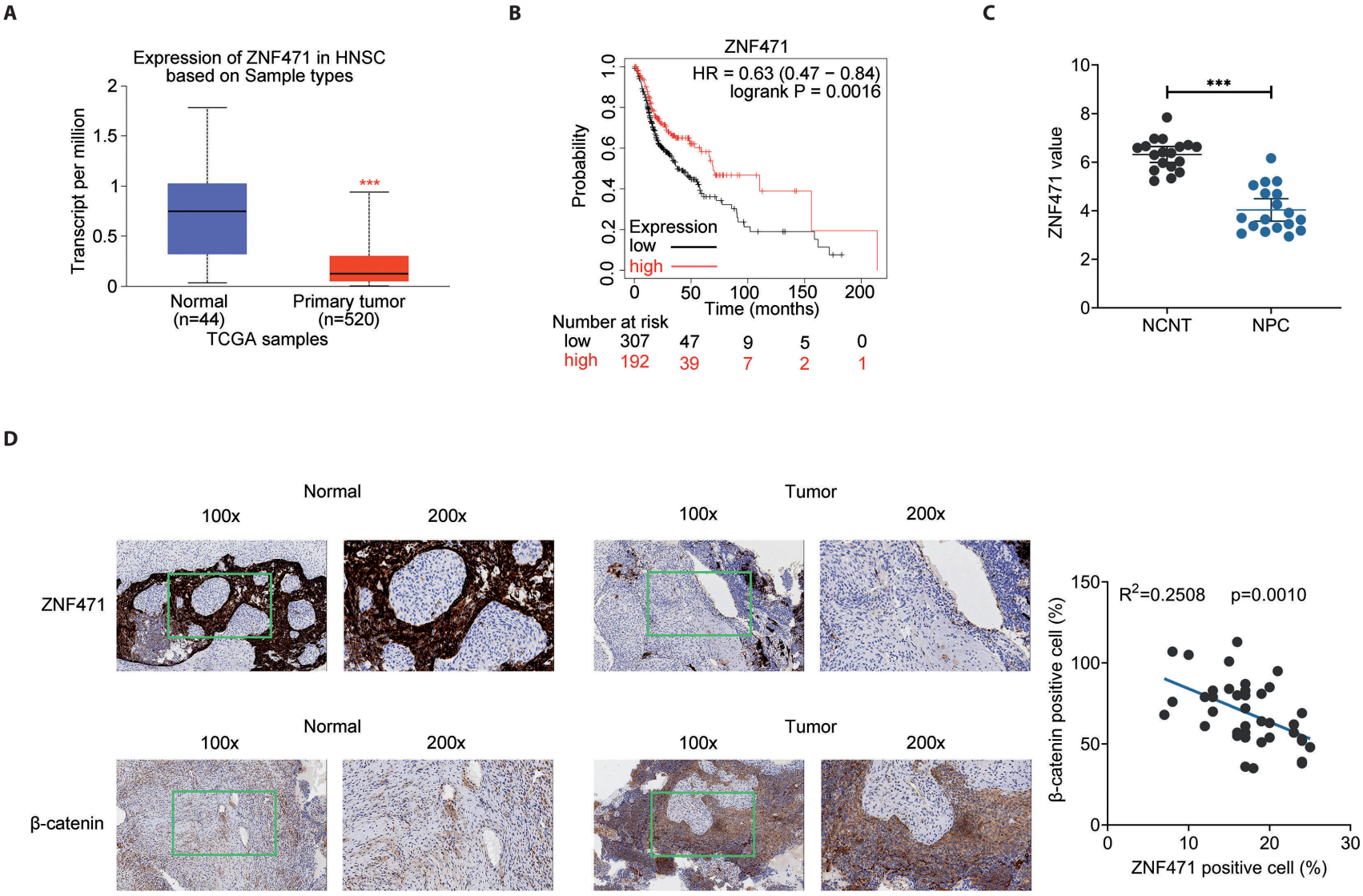

Figure 1. ZNF471 is down-expressed in NPC. **A.** Ualcan database shows that ZNF471 is lowly expressed in neck squamous cell carcinoma (HNSC) tissues. **B.** The effect of ZNF471 expression on the survival rate of HNSC patients by Kaplan-Meier plotter database query. **C.** GSE53819 expression profile shows that ZNF471 is lowly expressed in NPC tissues. **D.** Immunohistochemistry was used to detect the expressions of ZNF471 and β-catenin in non-tumor tissues and tumor tissues of NPC patients, and correlation analysis was performed. *** $p < 0.001$, *vs.* Normal; *** $p < 0.001$, *vs.* non-cancerous nasopharyngeal tissues (NCNT).

gave its approval to this study. Informed consent was given by each participant.

Cell culture and transfection

To develop the human NPC cell lines C666-1 and CNE1, RPMI-1640 media supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin was utilized. The cell lines were negative for mycoplasma. A Lipofectamine 3000 transfection was used to introduce ZNF471 overexpression plasmids into the cells.

CCK8 assay

Each well in a 96-well plate contained 4×10^3 cells. Following incubation for 24, 48, 72, or 96 h, 10 μl of CCK-8 reagent was added to each well, and the plate was incubated for an additional 2 hours. Next, the absorbance of each well at 450 nm was measured using a microplate reader.

Edu assay

A seeding density of 1×10^4 cells/well was used in 96-well plates. The medium was switched out for Edu medium and the cells were cultured for four hours after they had attached to the wall. Following incubation, the medium was disposed of, and the cells were fixed with 4% paraformaldehyde and the fixative was discarded. Glycine was added and incubated for 5 min, and the glycine solution was discarded. The cells were rinsed with PBS, and then the permeabilization agent (0.5% Triton X-100) was added and incubated for 10 min. After rinsing with PBS, Apollo staining solution was added and incubated at room temperature in the dark for 30 min, the staining solution was discarded, and the permeabilization agent was added and incubated for 10 min. Rinse with PBS, Hoechst was added to cells and incubated for ten minutes in the dark, and finally imaged under a fluorescence microscope.

Wound healing

After 24-h cell culture in 6-well plates, a straight line was scratched with a sterile pipette tip. Digital images from an Olympus camera were captured at 0 and 24 h following the scratching. Using ImageJ software, the scratch healing distance was calculated.

Transwell

The upper chamber of the Transwell was covered with matrigel, and 5×10^4 cells treated with serum-free media were sown there. After adding the entire medium, cells were incubated for 24 h at 37°C and 5% CO_2. After 30 min of methanol fixation, the cells were stained for further 30 min with 0.1% crystal violet. To count the cells at the bottom of the chamber, an inverted phase contrast microscope was used.

Spheroid formation assay

After trypsinization, the cells were suspended in serum-free DMEM/F-12, supplemented with 2% B27 supplement, 0.4% bovine serum albumin, 20 ng/ml epidermal growth factor, and 10 ng/ml basic fibroblast growth factor. After 14 days of culture, the number of spheres (≥ 40 μm in diameter) that formed from the cells that were implanted in ultra-low attachment plates was counted under a microscope.

Western blot

Following treatment of the cells with RIPA lysis buffer, a BCA kit was employed to measure the protein concentration. Proteins were separated by SDS-PAGE gel electrophoresis. After being placed on PVDF membranes, the protein bands were blocked with 5% skim milk. The membranes underwent an overnight incubation period at 4°C with the appropriate antibodies. The cells were supplemented with secondary antibodies the next day and allowed to incubate at room temperature. The target protein was seen using the ECL luminescence reagent and the imager ChemiDoc XRS system (Bio-Rad) (Xu et al. 2023). Antibody information: ZNF471 (Sigma-Aldrich, HPA066695, 1:2000); beta Catenin(Abcam, UK, Cambridge, ab32572, 1:5000); c-Myc (Abcam, ab32072, 1:1000); MMP-7 (Abcam, ab207299, 1:1000); β-actin (Abcam, ab8227, 1:1000); HRP IgG (Abcam, ab6721, 1:10000).

Immunohistochemistry

The samples were treated with the primary antibody ZNF471 (Sigma-Aldrich, 1:200) or beta Catenin (Abcam, ab32572, 1:500) at 4°C for a whole night after paraffin embedding, dewaxing, rehydrating, and antigen retrieval. They were then incubated with the HRP-conjugated secondary antibody for one hour at 37°C. Following DAB staining of each segment, pictures were captured using a light microscope.

Statistical analysis

With GraphPad Prism, the statistical analysis was performed. A one-way analysis of variance was used to assess significant differences for multiple comparisons, and the Student's *t*-test was used to assess any significant differences between any two sets of data. Mean ± SD was used to express all data. When $p < 0.05$, differences were deemed significant. The association between ZNF471 expression and the clinicopathological characteristics of NPC patients was evaluated using the Chi-Square test.

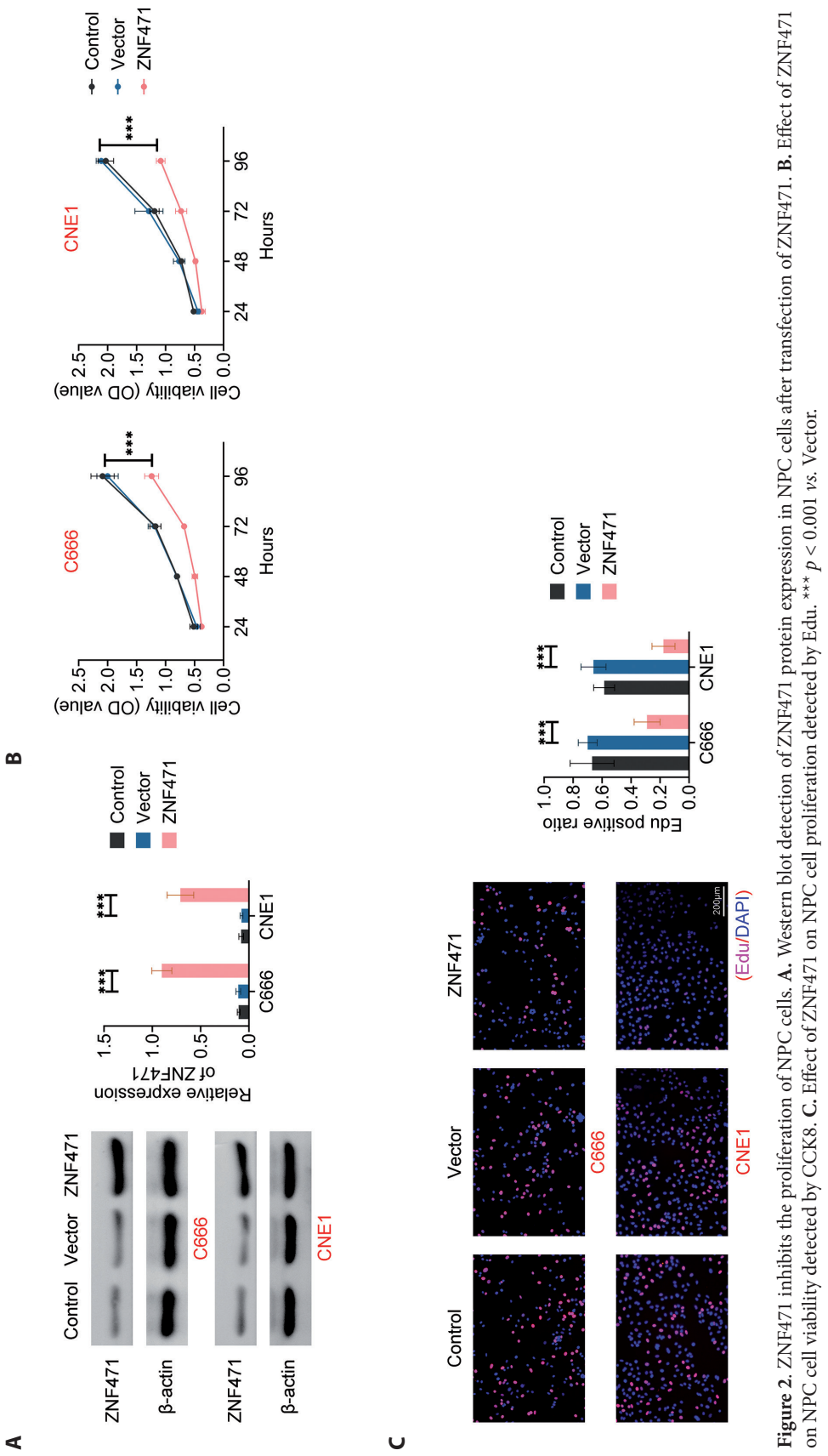

Figure 2. ZNF471 inhibits the proliferation of NPC cells. **A.** Western blot detection of ZNF471 protein expression in NPC cells after transfection of ZNF471. **B.** Effect of ZNF471 on NPC cell viability detected by CCK8. **C.** Effect of ZNF471 on NPC cell proliferation detected by Edu. *** $p < 0.001$ *vs.* Vector.

Results

ZNF471 is down-expressed in NPC

NPC is a type of squamous cell carcinoma of the head and neck (HNSC). ZNF471 was observed to express itself lowly in HNSC tissues when the Ualcan database was searched for ZNF471 expression in HNSC tissues (Fig. 1A). In addition, it was also found in the Kaplan-Meier Plotter database that there was a decreased survival rate among NPC patients with reduced expression of ZNF471, while patients with high expression of ZNF471 had a higher survival rate (Fig. 1B). Examined the GSE53819 expression profile to verify ZNF471 expression in NPC tissues, and the findings indicated that ZNF471 was expressed considerably less in NPC (Fig. 1C). In addition, the expression of ZNF471 and β-catenin in NPC tissues collected by the hospital was also detected. The results showed that the expression of ZNF471 and β-catenin was negatively correlated. ZNF471 was lowly expressed in tumor tissues of NPC patients, while β-catenin was highly expressed (Fig. 1D).

ZNF471 inhibits the proliferation of NPC cells

To validate the function of ZNF471 in NPC, ZNF471 overexpression plasmid was transfected into NPC cell line, and the effect of transfection was detected by Western blot. The

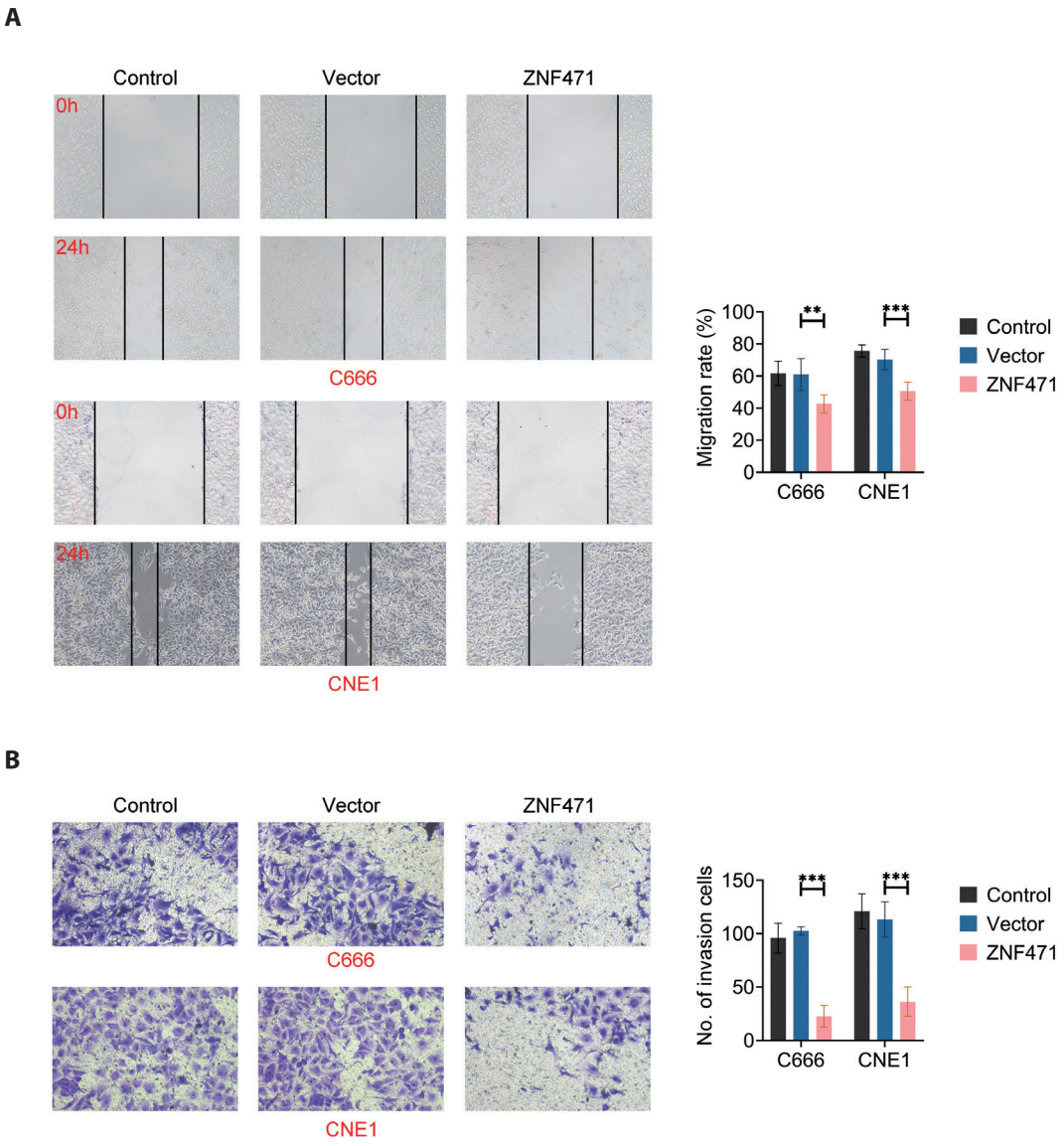

Figure 3. ZNF471 inhibits NPC cell migration. **A.** Wound healing assay to detect the effect of ZNF471 on NPC cell migration. **B.** Transwell assay to detect the effect of ZNF471 on NPC cell invasion. ** $p < 0.01$, *** $p < 0.001$ *vs.* Vector.

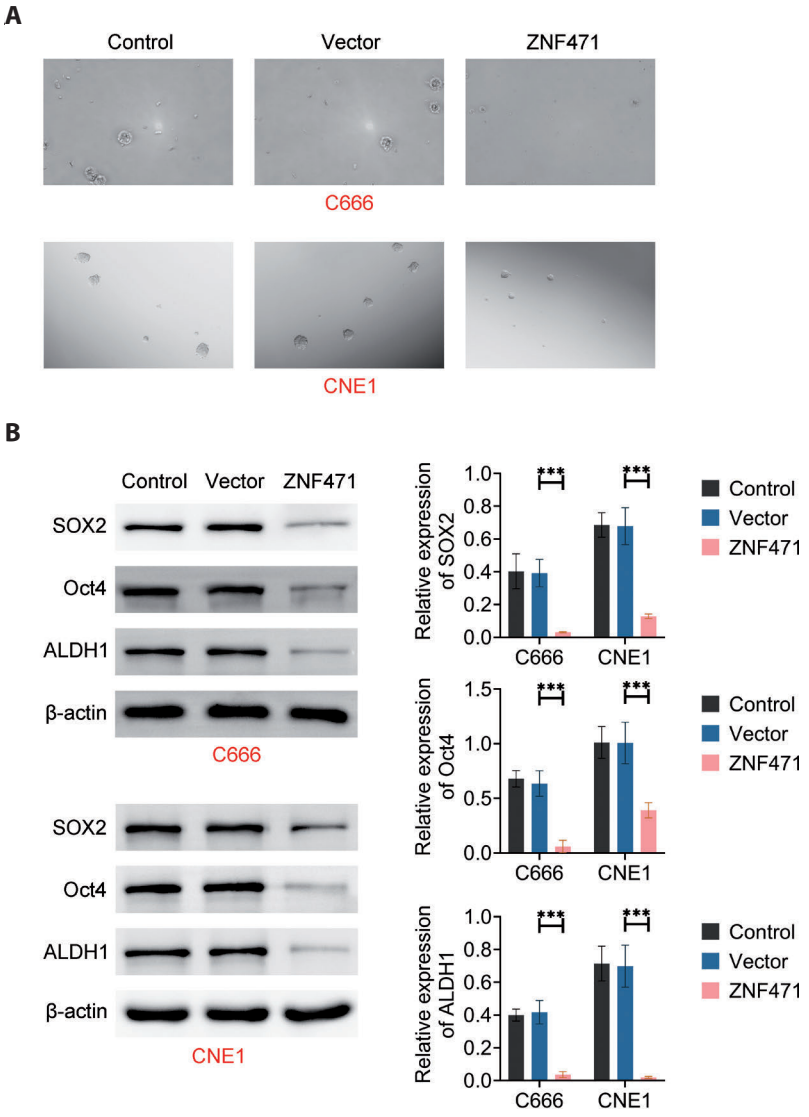

Figure 4. ZNF471 inhibits the stemness characteristics of NPC cells. **A.** Spheroid formation assay to detect the effect of ZNF471 on NPC cell stemness. **B.** Western blot detection of SOX2, Oct4 and ALDH1 protein expression in NPC cells.

outcomes demonstrated that the protein blotting of ZNF471 in the group transfected with ZNF471 overexpression plasmid increased significantly, indicating that the transfection effect was better (Fig. 2A). CCK8 measured cell viability, and the Edu experiment measured cell proliferation. The findings demonstrated that overexpressing ZNF471 dramatically decreased the vitality of NPC cells (Fig. 2B) and Edu-positive cells (Fig. 2C), suggesting that ZNF471 prevents NPC cells from proliferating.

ZNF471 inhibits NPC cell migration

The influence of ZNF471 on the migration and invasion of NPC cells was demonstrated using the wound healing test and the Transwell assay for cell invasion. Based on the results, the migration and invasion of NPC cells are inhibited by ZNF471 (Fig. 3A,B). Specifically, it dramatically reduced the number of invaded cells and the rate of cell migration.

ZNF471 inhibits the stemness characteristics of NPC cells

The number of spheres formed was measured using a sphere formation experiment to demonstrate the effect on tumor stemness. The outcomes showed that ZNF471 greatly decreased the quantity of NPC tumor spheres that developed (Fig. 4A), Western blot was used to detect the protein expression of stemness-related proteins SOX2, Oct4 and ALDH1. The results showed that ZNF471 reduced the expression of

stemness-related proteins (Fig. 4B), suggesting that ZNF471 inhibits the stemness characteristics of NPC cells.

ZNF471 inhibits the Wnt/β-catenin pathway

Western blotting was used to detect the protein expressions of β-catenin, c-Myc, and MMP-7 in order to demonstrate the connection between ZNF471 and the Wnt/β-catenin pathway. The findings demonstrated that ZNF471 strongly suppressed the β-catenin (Fig. 5A), c-Myc, and MMP-7 (Fig. 5B) protein expressions, suggesting that ZNF471 blocked the Wnt/β-catenin pathway.

Discussion

In many types of cancers, ZNF471 is methylated downregulated and functions as a tumor suppressor gene to prevent

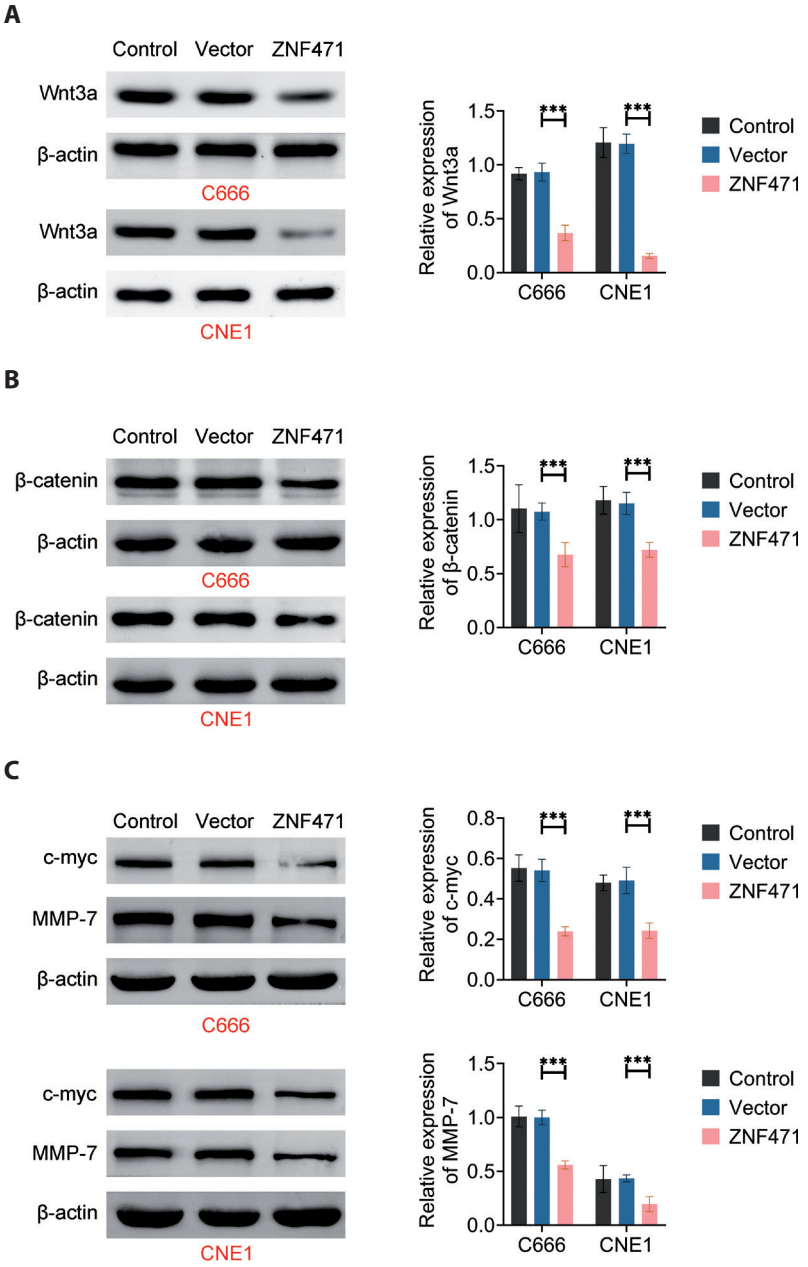

Figure 5. ZNF471 inhibits the Wnt/β-catenin pathway. Western blot assay to detect the effect of ZNF471 on Wnt3a (**A**), β-catenin (**B**) and c-Myc and MMP-7 (**C**) protein expression in NPC cells. *** $p < 0.001$ *vs.* Vector.

the growth of malignant tumors. ZNF471 can reverse epithelial–mesenchymal transition (EMT), induce G0/G1 arrest and death, and further inhibit esophageal squamous cell carcinoma (ESCC) cell invasion and migration to significantly slow down the growth of ESCC cells. According to studies that have revealed ZNF471 is downregulated as a methylation in ESCC (Sun et al. 2020). There has not been much research done on the function of ZNF471 in nasopharyngeal carcinoma up to this point. In this investigation, we verified that ZNF471 was little expressed in NPC tissues and that it prevented NPC cells from progressing malignantly. Moreover, ZNF471 blocked the Wnt/β-catenin pathway.

At the beginning of this study, data from the GEO database were used to analyze the bioinformatics data and found that ZNF471 was downregulated in NPC tissues. The function of ZNF471 in NPC cells was explored by transfecting ZNF471 overexpression plasmids in NPC cells. The findings indicated that ZNF471 strongly suppressed the proliferation, migration, and invasion of NPC cells. Within a tumor, there exists a tiny population of cells known as cancer stem-like cells that possess "stem cell properties" that encourage tumor growth, invasion, and resistance to treatment (Xu et al. 2024). Treating NPCs and understanding their recurrence and therapy resistance may be aided by research on tumor stemness (Cai et al. 2019). ZNF471 was found to decrease the tumor stemness properties of NPC cells in this investigation.

Several investigations have shown that Wnt/β-catenin activation can raise the level of β-catenin protein in the cell nucleus and combine with TCF/LEF to create a complex, which in turn controls the expression of target genes (c-Myc, MMP7, Axin2, SOX4, TCF7, and TCF7) (Tang et al. 2020). Cell migration, differentiation, and proliferation are just a few of the biological processes linked to the dysregulation of the Wnt/β-catenin signaling system (Zeng et al. 2021). One of the main mechanisms for cancer stem cell self-renewal is the activation of the important regulator β-catenin (Dai et al. 2020). The crucial pathway of Wnt/β-catenin signaling controls the growth and migration of NPC cells as well as the development of tumors. Important biomarkers for NPC cell prognosis include proteins associated with Wnt/β-catenin (Pang et al. 2019). Wnt/β-catenin signaling inactivation can reduce tumor growth and NPC cell invasion (Li et al. 2024). At the same time, the activation of Wnt/β-catenin signaling is also one of the important pathways affecting the stemness of NPC tumors (Wang et al. 2017). In this study, it was confirmed that ZNF471 inhibits the Wnt/β-catenin pathway.

Conclusion

ZNF471 is not highly expressed in NPC, as this work has demonstrated for the first time, and that ZNF471 has the function of inhibiting tumor growth, migration, invasion, and stemness characteristics in NPC cells. Mechanistically, the function of ZNF471 in NPC to suppress tumors may be related to its inhibition of Wnt/β-catenin pathway activation. This study still has some shortcomings. Due to some objective factors, NPC tissue samples were not collected to detect the expression of ZNF471, and no *in vivo* experiments were performed to verify the function of ZNF471. These will be further supplemented in future studies to provide a small contribution to the targeted treatment of NPC.

Availability of data and materials. All data generated or analyzed during this study are included in this published article. The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Conflict of interests. The authors state that there are no conflicts of interest to disclose.

Contribution of authors. XW and MC designed the study, completed the experiment and supervised the data collection; ZW analyzed the data, interpreted the data; QW and CL prepare the manuscript for publication and reviewed the draft of the manuscript. All authors have read and approved the manuscript.

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