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Original Article

Overexpression of ERBB3 promotes proliferation, migration, and angiogenesis in nasopharyngeal carcinoma

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Abstract: The purpose of this study is to investigate the role of ERBB3 in nasopharyngeal carcinoma (NPC). We investigated the expression level of ERBB3 in NPC by immunohistochemistry and western blot. CCK-8, cell cycle analysis, transwell assay and wound healing assay were used to detect the effect of ERBB3 on the proliferation and migration of NPC cells. HUVECs were used to study the effects of ERBB3 on angiogenesis in NPC. Our results showed that the expression level of ERBB3 in NPC was much higher than that in normal controls. ERBB3 promoted the proliferation and migration of NPC cells, while suppression of ERBB3 reduced angiogenesis. These results indicated that overexpression of ERBB3 promotes tumorigenesis and angiogenesis in NPC, which may provide an effective target in gene therapy for NPC.

Keywords: Nasopharyngeal carcinoma, ERBB3, proliferation, migration, angiogenesis

Introduction

Nasopharyngeal carcinoma (NPC), a solid tumor arising from epithelial cells of the nasopharynx, is the most common malignant tumor of the head and neck. It is also one of the most common cancers in East and Southeast Asia [1, 2]. The primary pathogenic factors of NPC include Epstein-Barr virus (EBV) infection, genetic predisposition, environmental factors and lifestyle [3, 4]. With the progress in radiotherapy and chemotherapy, the 5-year survival rate of NPC patients is about 50% [5]. However, since most patients with NPC are diagnosed at advanced stage, the treatment of NPC is not effective enough. Therefore, in order to improve the quality of life and survival rate of NPC patients, it is important to investigate the molecular mechanisms underlying NPC progression for early diagnosis and the development of effective therapeutic agents.

The ERBB protein family, whose members are type 1 tyrosine kinase receptors, contains four receptor tyrosine kinases, structurally related to the epidermal growth factor receptor (EGFR) [6]. In humans, this family includes ERBB1 (also known as EGFR/HER1), ERBB2 (HER2/Neu), ERBB3 (HER3) and ERBB4 (HER4), and represents a key control of fundamental cellular processes, such as cell proliferation, migration, and metabolism [7, 8]. The combination of ligands and ERBB triggers homodimerization or heterodimerization of ERBB, resulting in autophosphorylation of the receptors and induceing downstream signaling events. The activated ERBB proteins recruit various signalling complexes such as those in MAPK, PI3K/Akt, STATs, Src kinase, and mTOR pathways [9]. Also, these complexes have been closely associated with pathogenesis and progression of several cancers, including breast, gastric, lung, and head and neck cancer [10-13]. Alterations of ERBB in tumors can result in aberrant receptor expression and different kinds of mutations that regulate the activation of receptors and inducce specific intracellular signaling [14, 15]. Several

studies have evaluated the prognostic values of ERBB genes and protein expression in NPC [10]. Cao et al. found that the overexpression of ERBB1 was related to poor prognosis in NPC [16]. Gene amplification and protein overexpression of ERBB2, likewise, was reported in NPC and was linked to poor prognosis [17, 18]. Thus, we suspected that ERBB3 and ERBB4 may have similar effects in NPC, but their expression and functional roles in NPC remained unclear.

Angiogenesis is an extremely complex and multistep process including growth, migration, adhesion, differentiation, and vascularization of vascular endothelial cells [19, 20]. Full execution of angiogenesis requires complex signaling through vascular endothelial growth factor (VEGF) and its receptors [21]. Angiogenesis not only is a normal process in development, but also is necessary for tumor progression [22, 23]. In colon cancer, overexpression of ERBB2 and ERBB3 was associated with advanced stage and poor prognosis [24, 25]. Expression levels of VEGF in tumor and serum have also been related to stage and prognosis [26, 27]. Recent studies have shown that overexpression of ERBB2 in human breast cancer is closely associated with angiogenesis and the expression of vascular endothelial growth factor (VEGF), a potent stimulator of angiogenesis [28-30], suggesting that the activation of the ERBB2/ERBB3 signaling pathway may upregulate VEGF expression and lead to angiogenesis. We theorize that ERBB protein family member ERBB3 may also interact with VEGF and its receptors in NPC.

In this study, we investigated the role of ERBB3 in NPC. Our results indicated that overexpression of ERBB3 could promote NPC cell proliferation, migration, and angiogenesis. Our findings provide unique insight into the pathogenesis of NPC and suggest ERBB3 might be of value for clinical treatment in NPC.

Materials and methods

Cell lines and cell culture

CNE2 cell line used in this research was purchased from the Wuhan Institute of Cell Biology, China Center for Type Culture Collection, and was maintained in RPMI 1640 (GibCo BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS, GibCo). The immortalized normal nasopharyngeal epithelial cell line NP-69 was cultured in Keratinocyte-SFM (Invitrogen, Carlsbad, CA). Human umbilical vein endothelial cells (HUVECs) were purchased from Gene Company Ltd. (Shanghai Distributor of Lonza) and cultured in EGM-2 Endothelial Cell Growth Medium according to the protocol provided by the manufacturer (Lonza). All these cell lines were incubated at 37°C in a humidified chamber containing 5% CO_2.

Transfection and siRNA

ERBB3 small-interfering RNA (siRNA) and silencer negative control siRNA (snc-RNA) were obtained from Biomics Biotech (Nantong, Jiangsu Province, China). The sequences of ERBB3 siRNAs were: si-h-ERBB3_001 antisense: 5'-GGTCTACGATGGGAAGTTT-3'; si-h-ERBB3_002 antisense: 5'-TCGTCATGTTGAACTATAA-3'; si-h-ERBB3_003 antisense: 5'-GGCCATGAATGAATTCTCT-3'. According to the manufacturer's instructions, siRNAs were transfected into cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) and incubated for 6 h at 37°C in medium with no serum or antibiotics. Transfected cells were used for subsequent experiments 48 h after transfection. The efficiency of siRNAs was determined by the protein levels of ERBB3 in the 60 h post-transfected cells.

Cell proliferation assay and cell cycle analysis

Cells were seeded onto 96-well cell culture cluster plates (Corning, NY, USA) at a density of 1×10^4 cells per well in volumes of 100 µl and incubated for 24 h. Cell viability rate was assessed using Cell Counting Kit-8 (CCK-8 Kit, BBI Life Sciences), and the absorbance was read at 450 nm using a microplate reader. For cell cycle analysis, cells were fixed in 70% ethanol overnight at -20°C and then incubated with 1 mg/ml RNase A for 20 min. Propidium iodide (PI, 50 µg/ml) (Bectone-Dickinson, San Jose, CA, USA) in PBS-Triton® X-100 was used to stain the cells and which were analyzed by BD FACScan (BD Biosciences, USA) and Cell Quest acquisition and analysis programs. All the experiments were carried out three times.

Transwell assay

Cell migration assays were performed with a Millipore chamber with a polycarbonate filter of

8 μm pore size (Millipore). 1 × 10^5 cells were seeded in medium without serum in the upper chambers, and medium containing 10% FBS in the bottom chambers. After 18 h to 22 h of incubation, the reverse sides of the upper chambers were washed with PBS, fixed with methanol, and stained using crystal violet. Digital images were obtained from the membranes, and four random fields were counted. The results were averaged from three independent experiments.

Wound healing assay

Cells were transfected with either ERBB3-siRNA or negative control. After cell confluence reached 90% at 24 h post transfection, the monolayer cells were wounded by scraping off the cells using a 100 μl pipette tip. The 6-well plates were then incubated at 37°C and the migrated distance was observed by microscopy at different time points. The relative distance of cells was measured by the wound width/the distance measured at 0 h. Duplicate wells for each condition were examined, and the experiments were carried out three times.

Western blot

Cells were lysed by RIPA Lysis Buffer containing protease inhibitor PMSF on ice. The concentration of total protein was measured by BCA Protein Assay Kit. 20 μg of total cellular protein was separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto poly vinyllidine difluoride filter (PVDF) membranes (Millipore). The membrane was incubated with the anti-ERBB3 primary antibody (1:1000, Abclonal) or anti-VEGFA polyclonal antibody (1:300, BBI Life Sciences) overnight at 4°C and then incubated with HRP-tagged secondary antibodies (1:2000, BBI Life Sciences) at room temperature for 1 h. Immunoreactivity was detected by ECL reagent (Millipore) and quantitative data were obtained using Image J software. GADPH (1:6000, Flarebio Biotech LLC) was used as a loading control. All experiments were carried out three times.

Enzyme-linked immunosorbent assay (ELISA)

Serum VEGF-A was measured using Human VEGF-A Precoated ELISA Kit (Dakewe Bioengineering Co., LTD) according to the manufacturer's instructions. The samples and biotinylated detection antibody were added to the wells which were precoated with human VEGF-A specific monoclonal antibody. Then Streptavidin-HRP, diluted with Dilution buffer (1 ×), was added to the wells and incubated for 2 hours. TMB was used to visualize the HRP enzymatic reaction. The VEGF-A levels were measured in 5 patients with NPC and 5 healthy controls. All experiments were independently repeated three times.

Immunohistochemical staining

Human tissues were collected from Department of Pathology, Affiliated Hospital of Nantong University (Jiangsu, China). This study was approved by the Ethics Committee of theHospital. All participants gave their consent. Non-cancerous nasopharyngeal tissues were collected from patients with clinical symptoms suggestive of NPC but in whom NPC was ruled out by biopsy. The patients had not received any therapy before the biopsy. NPC and non-cancerous nasopharyngeal specimens were sectioned at 4 μm thickness. The primary antibody used for IHC was anti-ERBB3 (diluted 1:200, Abclonal). Color reaction was used with 3, 39-diaminobenzidine tetrachloride (DAB) chromogen solution. Slides were counterstained with hematoxylin, dehydrated, and cover-slipped. For assessment of ERBB-3, the staining intensity and relative percentage of immunostained cells were analyzed and evaluated by two pathologists blind to disease status.

Zebrafish

The study was conducted conforming to the local institutional laws, and the Chinese law for the Protection of Animals. Zebrafish embryos of Tg (fli1a: EGFP) line were raised and staged as described [31]. Embryos were staged according to somite number or hours postfertilization (hpf). For angiogenesis assays in vivo, 1 ng ERBB3 siRNAs or DMSO supplied in the fish water were injected into Tg (fli1a: EGFP) transgenic zebrafish 1-2-cell stage fertilized eggs. 3 days after injection, embryos were fixed and green fluorescent signals were analyzed using confocal microscopy (Leica, TCS-SP5 LSM). Analysis was performed using Imaris software.

Statistical analysis

Statistical analysis was performed using SPSS17.0 software (SPSS Inc., Chicago, IL, USA).

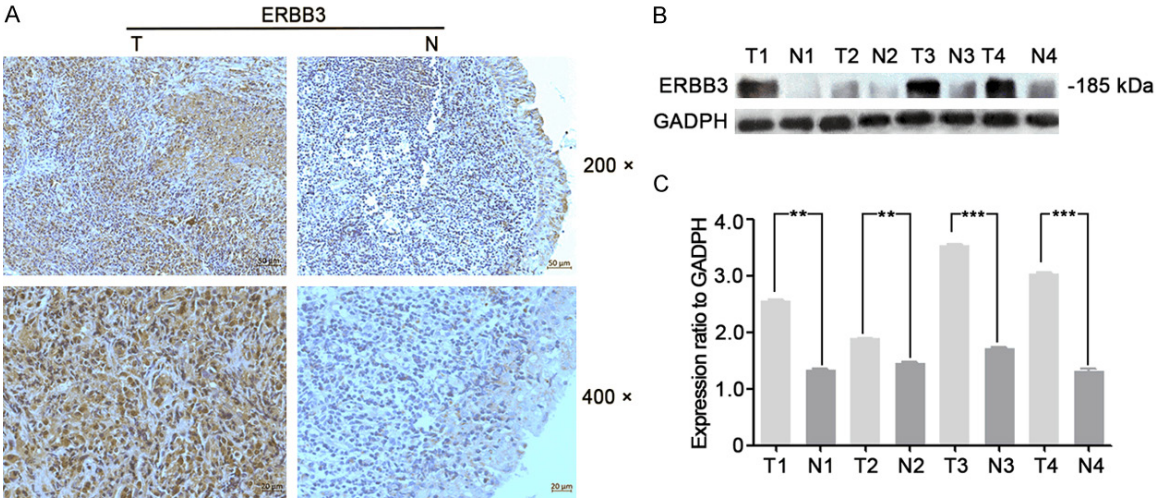


Figure 1. Expression levels of ERBB3 in NPC tissues and cell lines. A. Expression levels of ERBB3 in NPC tissues and normal nasopharyngeal epithelial tissues. Some representative results are shown. B, C. Protein levels of ERBB3 in 4 NPC tissues and 4 normal nasopharyngeal tissues by western blot analysis. *P < 0.05. **P < 0.01.

Each experiment was carried out at least three times. Data are presented as mean ± SEM, where differences were evaluated using Student's t-test. χ^2 test was used to analyze the relationship between ERBB-3 expression and clinicopathologic features of NPC. The results are expressed as the mean ± standard error (SE). A value of $P < 0.05$ was considered significant.

Results

High expression of ERBB3 in NPC tissues

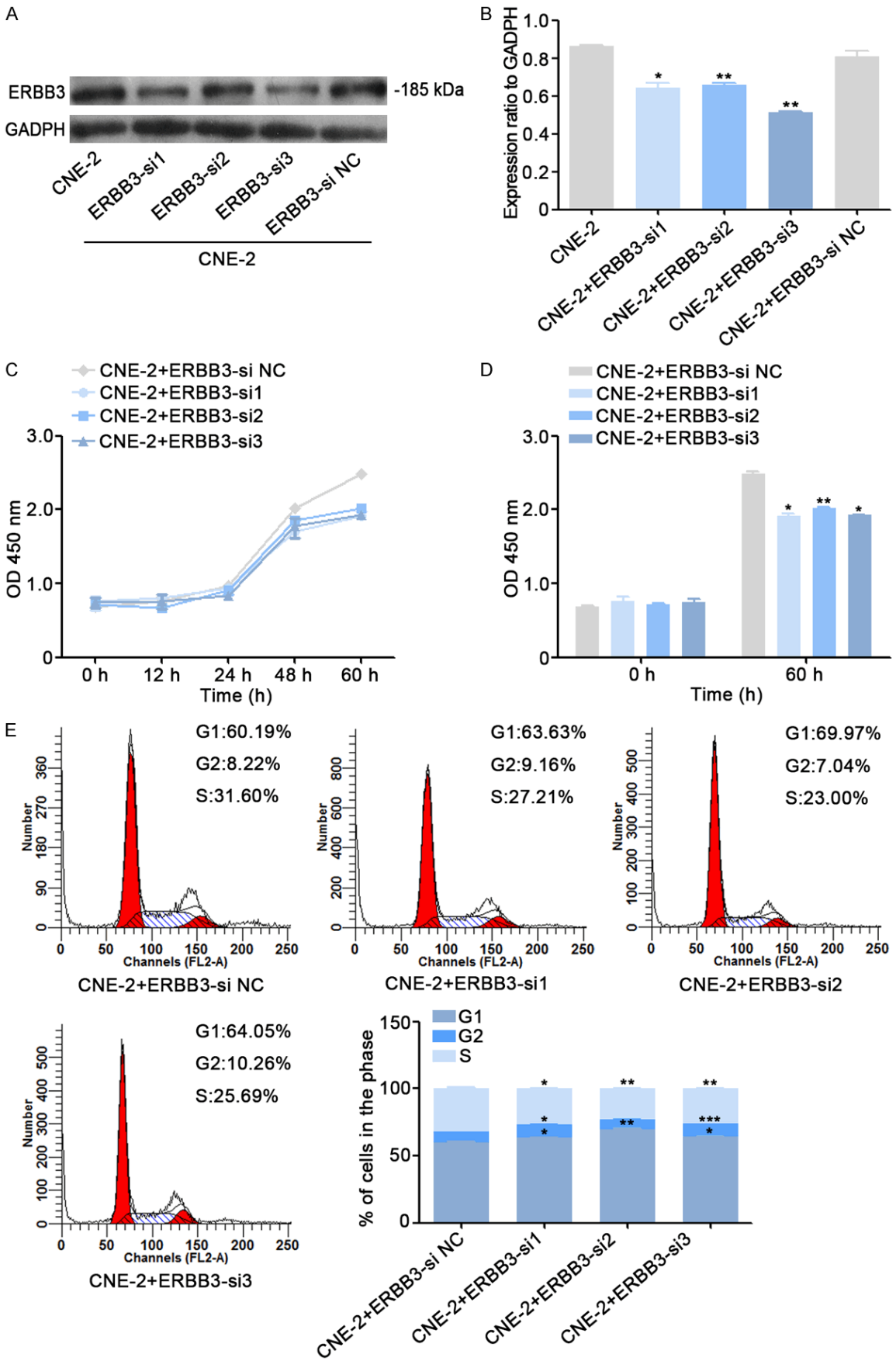
We used immunohistochemical analysis to investigate the expression of ERBB3 in nasopharyngeal carcinoma (NPC) and non-cancerous nasopharyngeal tissues. The results showed that compared with non-cancerous nasopharyngeal tissues, ERBB3 was highly expressed in NPC tissues (**Figure 1A**), which was consistent with previous studies of Tulalamba et al. [32]. Furthermore, we confirmed the high expression of ERBB3 by western blot using 4 paired fresh NPC and non-cancerous nasopharyngeal tissues (**Figure 1B**, **1C**). These findings suggested that ERBB3 may contribute to malignant progression of NPC.

Knockdown of ERBB3 suppressed proliferation and migration of NPC cells

To explore the role of ERBB3 in NPC, CNE-2 cells were transfected with ERBB3-specific siRNAs (ERBB3-si1, ERBB3-si2, ERBB3-si3) and a negative control siRNA. As expected, ERBB3 was reduced in cells transduced by the three different ERBB3-siRNAs (**Figure 2A**, **2B**). CCK8 assay demonstrated that down-regulating the expression of ERBB3 caused a significant decrease of the proliferation rate in CNE-2 cells (**Figure 2C**, **2D**). Cell cycle analysis showed that the percentage of cells in G1 phase increased after transfecting with ERBB3-siRNAs while the S phase was decreased (**Figure 2E**). These data suggested that ERBB3 was able to regulate proliferation of CNE-2 cells by influencing the G1-S transition. The results of transwell assay and wound healing assay indicated that the downregulation of ERBB3 could decrease the migration of NPC cells (**Figure 3A-D**).

ERBB3 regulates human umbilical vein endothelial cell (HUVECs) viability

Previous studies showed a relationship between ERBB3 and angiogenesis [33, 34]. To verify this finding, Human Umbilical Vein Endothelial Cells (HUVECs) were transfected with ERBB3 siRNAs. As expected, ERBB3 were significantly decreased after transfection (**Figure 4A**, **4B**). We thereby investigated the role of ERBB3 in HUVECs proliferation by CCK-8 assay. The results showed that ERBB3-siRNAs specifically decreased the proliferation of HUVECs (**Figure 4C**). Given this significant change, we further investigated the role of ERBB3 in vivo. After injection of ERBB3 siRNA and negative


A
ERBB3
GADPH
-185 kDa
CNE-2
ERBB3-si1
ERBB3-si2
ERBB3-si3
ERBB3-si NC
CNE-2
B
Expression ratio to GADPH
CNE-2
CNE-2+ERBB3-si1
CNE-2+ERBB3-si2
CNE-2+ERBB3-si3
CNE-2+ERBB3-si NC
C
CNE-2+ERBB3-si NC
CNE-2+ERBB3-si1
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CNE-2+ERBB3-si3
OD 450 nm
0 h
12 h
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CNE-2+ERBB3-si NC
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CNE-2+ERBB3-si2
CNE-2+ERBB3-si3
OD 450 nm
0 h
60 h
Time (h)
E
G1:60.19%
G2:8.22%
S:31.60%
CNE-2+ERBB3-si NC
G1:63.63%
G2:9.16%
S:27.21%
CNE-2+ERBB3-si1
G1:69.97%
G2:7.04%
S:23.00%
CNE-2+ERBB3-si2
G1:64.05%
G2:10.26%
S:25.69%
CNE-2+ERBB3-si3
Number
Channels (FL2-A)
G1
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CNE-2+ERBB3-si1
CNE-2+ERBB3-si2
CNE-2+ERBB3-si3

Figure 2. Knockdown of ERBB3 inhibited the proliferation of CNE-2 cells. A, B. Western blot showed the expression of ERBB3 after CNE-2 cells transfected with ERBB3-specific siRNAs and a negative control siRNA. C. Proliferation was detected by CCK-8 assay after CNE-2 cells were treated with ERBB3-siRNAs or negative control siRNA for the indicated time. D. Histogram shows CNE-2 cell survival at 0 h and 60 h. E. Cell cycle analysis by flow cytometry in CNE-2 with ERBB3 downregulation. The data shown represent at least three independent experiments. *P < 0.05. **P < 0.01.

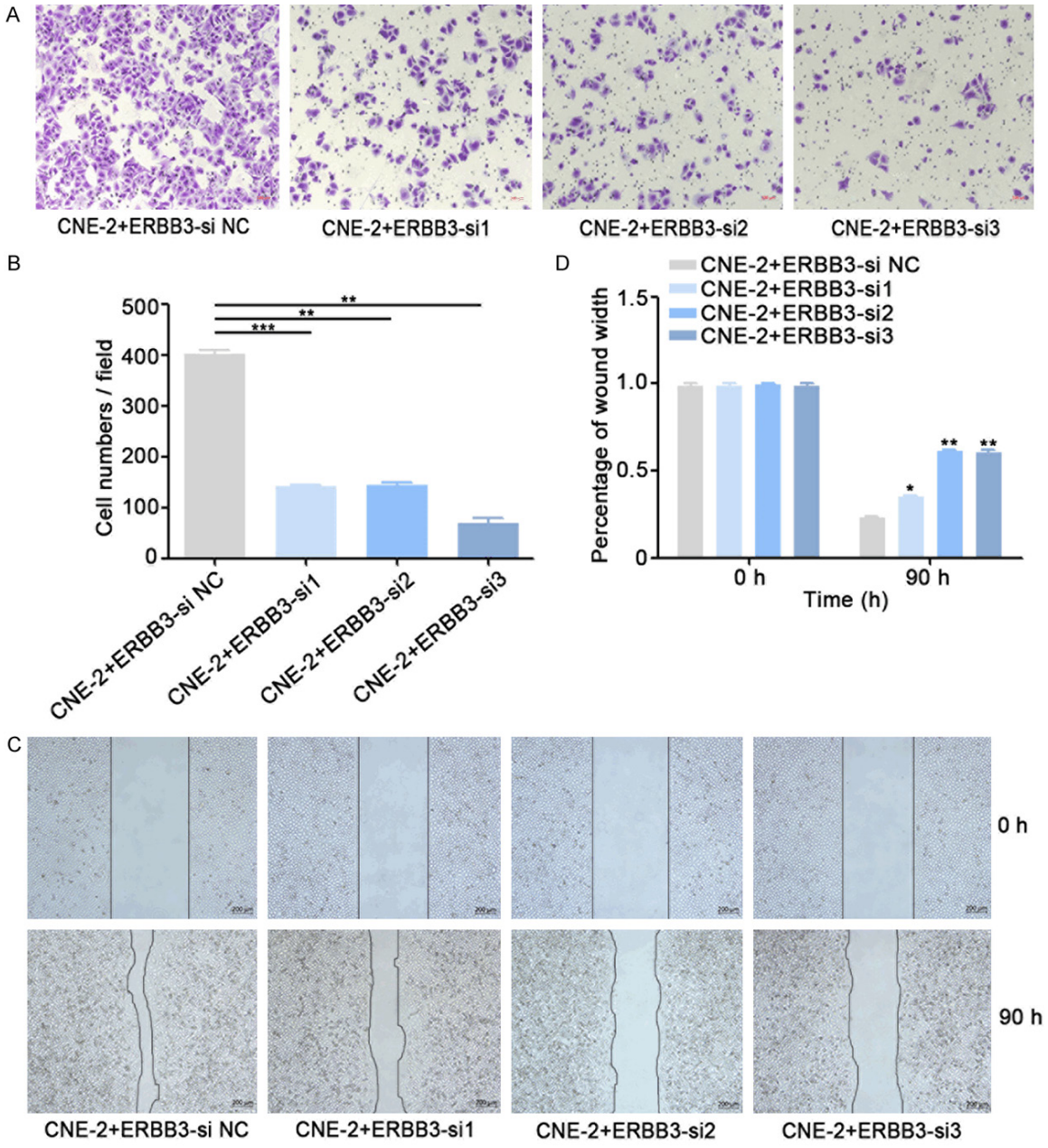

Figure 3. Suppression of ERBB3 inhibited the migration of CNE-2 cells. A. Transwell assay used to detect the penetration of ERBB3-silencing cells through the membrane compared with controls. B. Absolute number of cells migrated through the membrane. C. Silenced and control cells were grown until confluence and their migratory capabilities were analyzed by wound-healing assay. Representative images of wound-healing assay are shown at 0 h and 90 h. D. Histogram shows the relative migration distance of cells. Migration speed of the cells was analyzed by the wound width/distance measured at 0 h. The data shown represent at least three independent experiments. *P < 0.05. **P < 0.01.

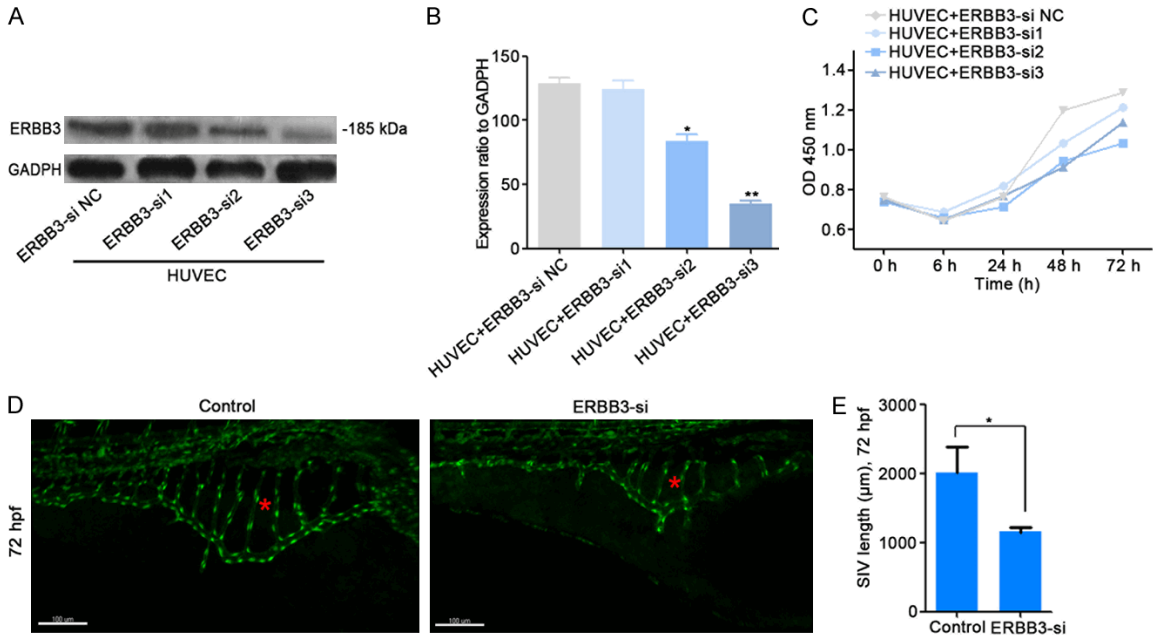

Figure 4. Knockdown of ERBB3 impacts the proliferation of HUVECs and the length of blood vessels. A, B. Western blot shows the expression of protein levels after knocking down ERBB3 in HUVECs. C. Cell proliferation was measured by CCK-8 assay after HUVECs were treated with ERBB3-siRNAs or negative control siRNA for the indicated time. D. The statistics of SIV length in 72 hpf Tg (fli1a: EGFP) embryos injected with negative control siRNA or ERBB3-siRNAs. E. Morphology of SIVs in 72 hpf Tg (fli1a: EGFP) embryos injected with negative control siRNA or ERBB3-siRNAs. The data shown represent at least three independent experiments. *P < 0.05. **P < 0.01.

control siRNA into Tg (fli1a: EGFP) transgenic zebrafish embryos, embryos injected with ERBB3 siRNAs showed abnormal length of blood vessels among subintestinal vessels (SIVs) at 72 hours post-fertilization (hpf) (**Figure 4D**, **4E**). These results indicate that knockdown of ERBB3 inhibited the activity of endothelial cells.

Suppression of ERBB3 reduced NPC angiogenesis by downregulating vascular endothelial growth factor A (VEGF-A)

Several studies indicated the need for angiogenesis for the progression of tumors [22, 23]. Full execution of angiogenesis requires complex signaling by vascular endothelial growth factor (VEGF) and its receptors [21]. To investigate the importance of angiogenesis in the progression of NPC, we measured serum VEGF-A levels in 5 patients with NPC and 5 healthy controls. The results showed higher level of serum VEGF-A in NPC as compared to controls (**Figure 5A**). We further tested the level of VEGF-A in HUVECs transfected with ERBB3-si. Western blot showed that after knocking down ERBB3 expression, VEGF-A protein levels were significantly decreased (**Figure 5B**, **5C**). These results suggest that ERBB3 can promote angiogenesis by regulating VEGF-A.

Discussion

As one of the most common cancers of the head and neck, NPC is a highly metastatic cancer with high incidence and mortality rates in China and Southeast Asia [35]. Despite encouraging advances in the molecular mechanism of NPC, the prognosis for patients with advanced NPC remains unsatisfactory. It is important to explore the potential mechanisms associated with NPC progression.

ERBB3 is structurally related to epidermal growth factor receptor (EGFR) [6] and represents a key control of fundamental cellular processes [7, 8]. Aberrant ERBB expression leading to dysregulation of downstream signalling events evidently results in enhanced uncontrolled proliferation of tumor cells [9, 36, 37]. These members have been closely associated with pathogenesis and progression of several cancers, including breast, gastric, lung, and head and neck cancer [10-13]. Moreover, studies have found that ERBB3 contributes to malignant

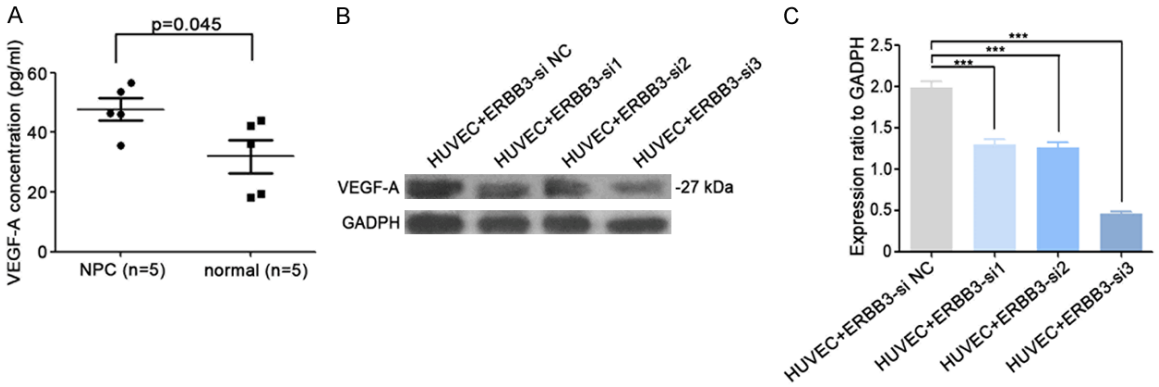

Figure 5. Knockdown of ERBB3 reduced angiogenesis by downregulating vascular endothelial growth factor A (VEGF-A). A. Human VEGF-A Precoated ELISA Kit was used to measure serum VEGF-A levels in 5 NPC patients and 5 healthy controls. B, C. Western blot shows the expression of VEGF-A after knocking down ERBB3 in HUVECs. The data shown represent at least three independent experiments. *P < 0.05. **P < 0.01.

tumor characteristics, including proliferation and migration. In this study, we demonstrated that ERBB3 is an important regulator in NPC. Using IHC and western blot, we discovered the upregulation of ERBB3 in NPC tissues (**Figure 1**). Previous studies of Tulalamba et al., considered ERBB3 as an independent prognostic marker for NPC and it was correlated with patients' overall survival time [32]. To further explore the function of ERBB3, we undertook studies in NPC cell lines. We found that the downregulation of ERBB3 suppressed the proliferation and migration of NPC cells at the same time (**Figures 2**, **3**). These indicated that ERBB3 overexpression might be associated with the progression of NPC and act as a clinical biomarker for evaluating prognosis.

Angiogenesis is a crucial step for the development of tumors, in which the neovascularization is indispensable to formation of solid cancer [38]. Previous research suggested that pathologic angiogenesis is closely related to NPC [39]. Also, Peng et al. have found that anti-angiogenesis could normalize NPC vasculature and enhance the radiation response [40]. The above suggested the importance of angiogenesis in the development of NPC. Recent research has shown that overexpression of ERBB in human breast cancer is closely related to angiogenesis and the expression of VEGF [29, 30]. Therefore, does ERBB3 exert any vascular functions in NPC in addition to its role in carcinogenesis? These studies promoted us to further explore the function of ERBB3 in angiogenesis. We knocked down ERBB3 in HUVECs and this caused a significant decrease in the activity of endothelial cells (**Figure 4**). We then measured the expression level of VEGF-A in serum of patients and healthy controls. The results showed that serum VEGF-A was higher in NPC as compared to controls (**Figure 5**), as we predicted. Also, downregulation of ERBB3 represented a significant decrease of VEGF-A protein levels in HUVECs (**Figure 5**). All our results were similar to previous studies that ERBB3 was closely related to angiogenesis [33, 34]. Thus ERBB3 appears to mediate progression and migration of NPC and actas an angiogenesis agent in NPC.

In summary, our data indicate that ERBB3 is overexpressed in NPC tissues and is associated with NPC progression and migration. These results also indicate that ERBB3 may play a role in promoting angiogenesis and have potent oncogenic activity in NPC. Therefore, the results suggest that ERBB3 may serve as a novel therapeutic target for the treatment of NPC.

Acknowledgements

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Disclosure of conflict of interest

None.

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