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Original Article

WWOX suppresses proliferation and induces apoptosis via G2 arrest and caspase 3 pathway in nasopharyngeal carcinoma cells

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Abstract: Background: WWOX plays crucial roles in various tumors. However, so far, minimal research into the role of WWOX in the development of nasopharyngeal carcinoma (NPC) has been reported. The present study investigates the effects of WWOX overexpression on cell proliferation, migration, and invasion in human NPC cell line CNE1. Materials and methods: A lentiviral vector carrying WWOX was transfected into CNE1 cells. The mRNA abundances of WWOX, MMP9, E-cadherin and WWOX protein were detected using quantitative RT-PCR and Western blotting in the transfected cells compared with the control cells (cells transfected using the empty vector and untransfected cells), respectively. Cell proliferation rates were assessed by plate colony formation assays and methyl thiazolyl tetrazolium (MTT). Cell migration and invasion were tested through wound healing assays and/or transwell migration and invasion assays. Cell cycle progression and apoptosis assays were performed by flow cytometry. The protein abundances of activated fragments of caspase-3, cleaved caspase-3 and AKT, phosphorylated p-AKT (Ser473) were measured using Western blotting. Results: Overexpression of WWOX significantly inhibited cell proliferation, migration and invasion and induced apoptosis. Moreover, WWOX overexpression led to cell proliferation inhibition via induction of cell cycle arrest in G2/M phase. WWOX suppressed migration and invasion via downregulation of MMP9 and upregulation of E-cadherin. Meanwhile, WWOX could downregulate the phosphorylation of Akt protein kinase and upregulate cleavage of Caspase-3, contributing to inhibition of proliferation and promotion of apoptosis. Conclusion: WWOX gene may be a novel target for gene therapy in NPC.

Keywords: WWOX, nasopharyngeal carcinoma, proliferation, cell cycle, cell apoptosis

Introduction

Nasopharyngeal carcinoma (NPC) is a highly invasive malignant tumor that mostly occurs in South-Eastern China and Southeast Asia compared with global distribution [1]. Because of the secluded anatomical sites and atypical early symptoms, 80-90% NPC patients are not diagnosed until the late advanced stage [2]. NPC patient five-year survival rate is 80-90% at the early stage. In the advanced stage, the prognosis is much poorer with a 61-66% five-year survival rate [3]. Strong migration and invasion capability is a major cause of the poor prognosis of patients with advanced NPC [4]. Therefore, to find novel key molecules involved in the progression of NPC and to understand their underlying roles is essential for the development of novel and effective therapeutic strategies to fight NPC.

WW domain containing oxidoreductase (WWOX) is a tumor suppressor gene, located on chromosome 16q23.3-24.1, spanning common fragile site FRA16D [5-7]. WWOX gene includes nine exons and encodes a 46 kDa protein, containing 2 N-terminal WW domains, a nuclear localization sequence (NLS) and a C-terminal short-chain alcohol dehydrogenase/reductase (ADH/SDR) domain [5, 8]. Inactivation of tumor suppressors is a well-established hallmark of cancer [9], and several studies have demonstrated that WWOX expression is lost or reduced in a wide variety of cancer types, such as breast, prostate, ovarian, hepatic cancer, gastric, osteosarcoma, and lung cancer [10-16]. Iliopoulos et al. [17] reported that overexpression of exogenous WWOX largely inhibits proliferation of breast cancer cells. Furthermore, Mare et al. [18] showed that ectopic expression of WWOX clearly attenuates migration and inva-

sion in vitro and metastasis in vivo of osteosarcoma. Nevertheless, the mechanism of WWOX in NPC remains poorly understood, and few studies have reported the effects of WWOX on NPC.

The enhancement of cell proliferation ability and cell cycle dysregulation are important to biological characteristics of tumor cells. The cell cycle is a complex and highly controlled process related to cell growth and division, DNA damage response and cancer [19]. Furthermore, it has been shown that overexpression of WWOX could inhibit cell proliferation via induction of cell cycle arrest G1 phase in many cancer cells [20]. Furthermore, the activation of cell cycle arrest at a particular checkpoint, and consequently induced apoptosis, is a normal mechanism for cytotoxic actions in cancer cells [21]. Cells progress through the phases of the cell cycle by interacting with various cyclins and their particular kinases and CDKs which can be negatively controlled by p27Kip1 and p21Cip1 [22]. In addition, the mammalian cell cycle is accurately managed by cell cycle checkpoints that permit progress through the cell cycle or arrest the cells in the G2/M phase in reaction to DNA damage for DNA repair [23].

Our preliminary studies have demonstrated that the decreased abundance of WWOX in NPC tissues and the lower abundance of WWOX are associated with NPC progression and lymph node metastasis [24]. Hu et al. [20] reported that WWOX could significantly inhibit cell proliferation of the hepatic carcinoma cell. In addition to this, Zheng et al. [25] found that WWOX markedly inhibited lung cancer cell migration and invasion. On the basis of these previous findings, and with the knowledge that WWOX might be involved in NPC tumorigenesis, the effects of WWOX on these characteristics of CNE1 NPC cells were further investigated. This study investigates the impact of WWOX overexpression on proliferation, migration, invasion, and apoptosis of NPC cells.

Materials and methods

Cell lines and cell cultures

The NPC cell line CNE1 was obtained from the Department of Otolaryngology, Guangxi Medical University (Nanning, China). The cells were cultured in RPMI-1640 medium (Gibco, USA) and supplemented with 10% fetal bovine serum (FBS) (Gibco, USA) and 1% penicillin-streptomycin antibiotic mixture (Beyotime, China). Cells were incubated at 37°C in 5% CO_2 and a humidified atmosphere. In all subsequent experiments, cells were used in an exponential growth state. Each experiment was performed in triplicate.

In vitro lentiviral transfection and establishment of stable cell lines

The WWOX-overexpressing lentiviral LV-WWOX and empty LV-CON145 vectors were purchased from GeneChem (Shanghai, China). CNE1 cells were transfected with GFP fluorescent protein of LV-WWOX and LV-CON145 at appropriate multiplicities of infection according to the manufacturer's instructions. Polybrene from Genechem was added to the infection medium to improve efficiency of the process. At 10 h after transfection, the medium containing infection reagents was replaced with fresh medium. At 72 h post-transfection, a lentiviral expression vector containing the WWOX gene (CNE1/LV-WWOX) was used to produce WWOX expression. Cells transfected with the empty vector (CNE1/LV-CON145) and untransfected cells (CNE1) were used as controls. The transfection efficiency was observed under an Olympus inverted fluorescence microscope. Transfection efficiency (%) = the number of cells under fluorescent/under incandescent lamps. The expression of WWOX mRNA and protein levels was evaluated by real-time PCR and western blot analysis, respectively.

RNA isolation and quantitative real time PCR (qRT-PCR) analysis of mRNA

The total RNA from cultured cells was extracted using the TRIzol reagent (TaKaRa, Dalian, China) according to the manufacturer's protocol. The concentration of RNA was measured by a NanoDrop spectrophotometer (NanoDrop, USA). The cDNA was synthesized using the Prime-Script RT reagent kit (TaKaRa, Dalian, China). The mRNA expression of WWOX, MMP9 and E-cadherin were determined using SYBR® Premix Ex Taq™ II (TaKaRa, Dalian, China) under an ABI-StepOnePlus™ Real-Time PCR System (Applied Biosystems, USA) following the manufacturer's instructions. The sequences of the primers used for the RT-PCR included: WW-

OX forward, (5'-TCGCAGCTGGTGGGTGTAC-3') and reverse, (5'-AGCTCCCTGTTGCATGGACTT-3'); MMP9 forward (5'-GACGCAGACATCGTCATCCA-3'), reverse, (5'-CACAACTCGTCATCGTGAAA-3'); E-cadherin forward, (5'-GTCTCTCTCACCACCTCCACAG-3') and reverse, (5'-CTCGGACACTTCCACTCTCTTT-3'); β-actin forward (5'-TTGCCGACAGGATGCAGAAGGA-3') and reverse, (5'-AGGTGGACAGCGAGGCCAGGAT-3'). The RT-PCR cycle parameters were as follows: 95°C for 10 min, 40 cycles of denaturation at 95°C for 30 s, 60°C for 1 min, and 95°C for 15 s, with a final extension step at 60°C for 1 min. The primers were synthesized by TaKaRa (Dalian, China). The relative quantity expression of mRNA was determined based on the threshold cycle (Ct) and was calculated using the $2^{-\Delta\Delta CT}$ method. β-actin served as the internal control.

Western blot analysis

Western blotting was performed as described previously with some modifications [20]. In brief, total protein was extracted from cultured cells using RIPA lysis buffer containing protease inhibitors (both from Solarbio, China). An equal amount of protein (25 μg) was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 10% polyacrylamide gels and transferred electrophoretically onto polyvinylidene fluoride (PVDF) membranes (Solarbio, China). The membranes were incubated with the primary antibodies at 4°C overnight, separately. The primary antibodies utilized were from Cell Signaling Technology (CST, USA), as follows: Rabbit anti-WWOX (1:800), Rabbit anti-caspase3 and cleaved-caspase3 (1:300), Rabbit anti-AKT (1:1500) and anti-pAKT (1:1600). Rabbit Anti-GAPDH (1:800, Beyotime, Shanghai, China) was acted as a control. The second-fluorescence anti-rabbit secondary antibody (1:10000, Abcam, USA) was applied to the membranes at room temperature for 1 h. The specific protein bands were detected by Western blot detection system (Bio-image, System, Israel) and analyzed using ImageJ software ((National Institutes of Health, Bethesda, MD, USA). The optical density of the specified protein was presented as the ratio between target protein and loading control (GAPDH).

Plate colony formation assay

Approximately 500 cells were cultured in each well of a six-well plate with complete culture medium. After incubation in a 5% CO_2 atmosphere at 37°C for 12 d, cells were washed twice with PBS and fixed with 4% paraformaldehyde (30 min at room temperature). Colonies were stained with crystal violet (Solarbio, Beijing, China) for 20 min at room temperature. The number of colonies including ≥ 50 cells was calculated under an optical microscope [plate clone formation efficiency (%) = (number of colonies/500) × 100%].

Cell proliferation assay

Cell proliferation was assessed using the methyl thiazolyl tetrazolium (MTT) assay (Sigma, USA). For this experiment, 3×10^3 cells were seeded onto 96-well plates with five replicates for each group. After culturing cells respectively 0, 1, 2, 3, and 4 days, MTT reagent was added into each well at a 25 μL volume, and the cells were allowed to continue incubating for an additional 4 h. Thereafter, the supernatant was carefully discarded from each well. The formazan crystals were dissolved completely in 100 μL of DMSO via shaking for 10 min. Subsequently, the optical density (OD) values of 490 nm wavelength were measured using a microplate reader (Bio-Tek, USA).

Wound healing assay

The migration activity of NPC cells was assessed by wound-healing assays. Cells were planted at 5×10^4 cells/well in 6-well plates. When cells were grown to a confluent monolayer in a 6-well plate, a 100-μL plastic pipette tip was used to scratch an artificial wound. Subsequently, the cells were cultured in serum-free medium. Images of the gap length at the time of scratching 0 h and 24 h later were observed under an inverted microscope. Quantification of the healing rate was performed using ImageJ software. The wound-healing rate was calculated as the percentage of reduction of gap distance after 24 h.

In vitro transwell migration assay and invasion assay

Cell migration and invasion assays were performed with 8-μm-pore transwell chambers (Corning, Acton, USA). For the invasion assay, the transwell chambers were coated with basement membrane Matrigel (Corning, Acton, MA) at 60 μL per filter. In brief, cells (at a density of 2×10^5 (cells/mL)) with serum-free medium

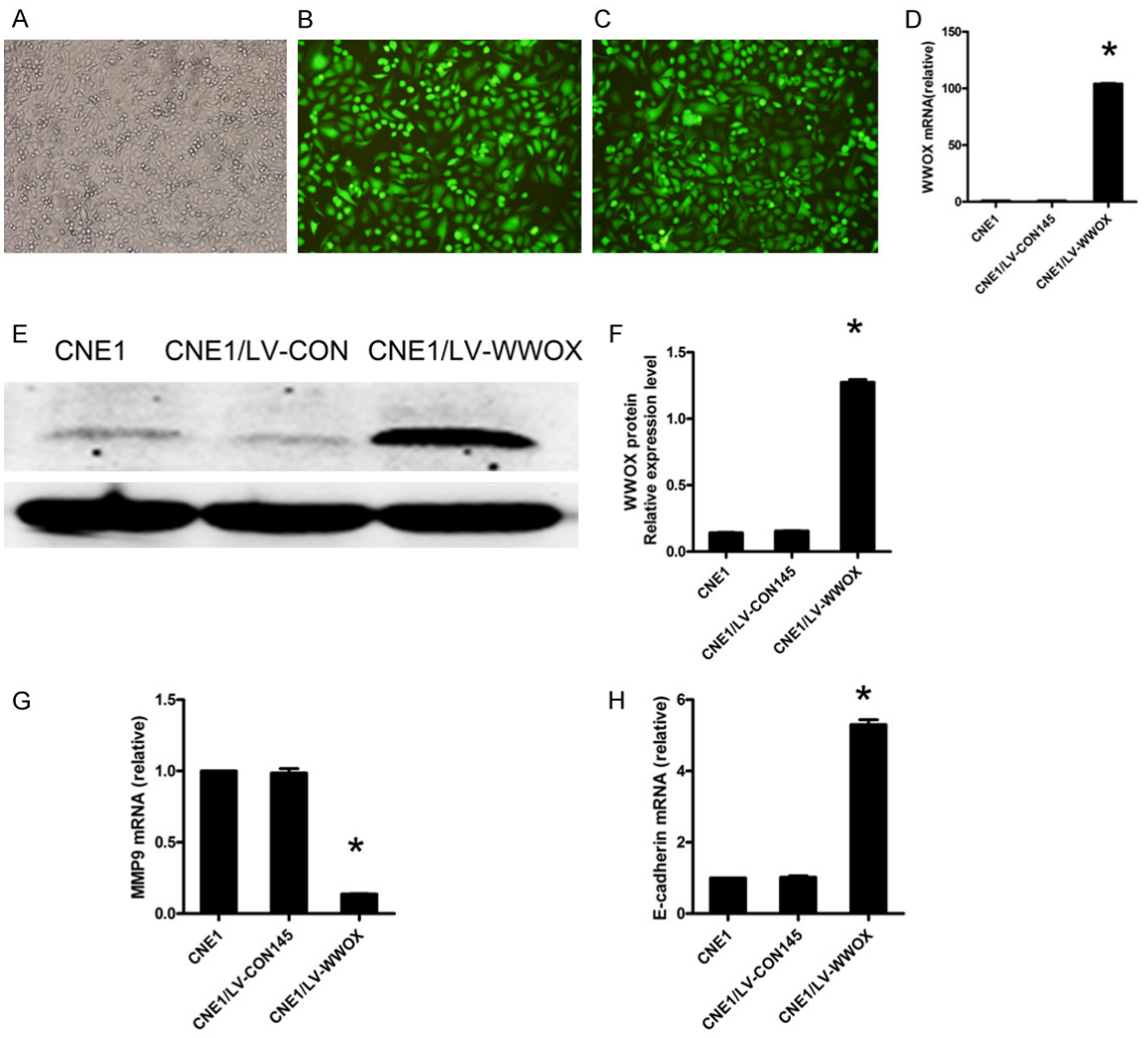

Figure 1. WWOX overexpression by transfection of lentiviral expression vector containing the WWOX gene in NPC CNE1 cells. The transfection efficiency was determined by fluorescent microscopy in CNE1 (A), CNE1/LV-CON145 (B) and CNE1/LV-WWOX (C) cells. The mRNA and protein expression of WWOX were evaluated using RT-PCR (D) and western blotting (E, F), respectively. The mRNA expressions of MMP9 (G) and E-cadherin (H) were evaluated using RT-PCR. The data are presented as the mean ± SD (*$P < 0.05$).

were seeded onto the upper chamber of the transwell membrane of a 24-well culture plate. Meanwhile, medium containing 10% FBS was added to the lower chamber as a chemoattractant. The assays were performed in a 5% CO_2 atmosphere at 37°C. After 24 h of incubation, the cells on the top of the membrane were removed by wiping with a cotton swab. The migrated cells on the lower surface of the membrane were fixed with 4% paraformaldehyde for 30 min and stained with crystal violet for 20 min at room temperature. Then, five random fields were counted under an inverted microscope.

Flow cytometry analysis of the cell cycle and apoptosis analysis

The logarithmically growing cells were harvested and digested by 0.25% trypsin-EDTA (Solarbio, Beijing, China). Briefly, cells were washed thrice with cold PBS, centrifuged at 1500 × g for 8 min and fixed with ice-cold 70% ethanol to incubate at 4°C for at least 4 h. After washing with PBS, the cells were suspended in PBS containing 100 μL RNase A (10 μg/mL) (KeyGEN Biotech, Nanjing, China) incubation at 37°C for 30 min, and resuspended in 400 μL propidium iodide (50 μg/mL) (KeyGEN) incubation in the dark for 30 min. The distribution of

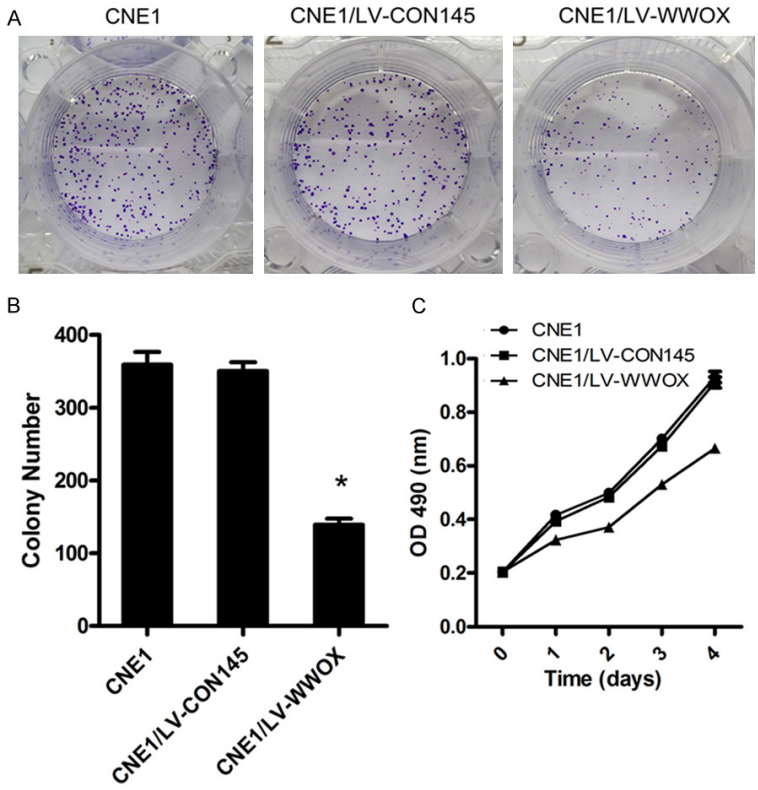

Figure 2. WWOX overexpression inhibits the proliferation of CNE1 cells. The proliferative capacity of three groups was detected by plate colony formation assay (A, B) and MTT assay (C). The data are presented as the mean ± SD (**P* < 0.05).

G1, S, and G2 phase was measured using a FACS Calibur flow cytometer (BD Biosciences, USA). Data were analyzed using ModFit LT software (Verity Software House, Topsham, ME).

Apoptosis assay was double stained with Annexin V-ADD and APC according to the manufacturer's instructions. The cells were treated in the same way as in the cell cycle analysis.

Statistical analysis

Statistical analyses were executed using SPSS version 22.0 (SPSS, Inc., Chicago, IL, USA). The results of experiments were presented as mean ± standard deviation (SD). One-way ANOVA test and student's t-test were performed for statistical analysis, where appropriate. $P < 0.05$ was considered statistically significant.

Results

Production of WWOX overexpression cell strain by lentiviral transfection in CNE1 cells

To investigate the role of WWOX in NPC, we successfully established stable strains CNE1/LV-WWOX and empty vector CNE1/LV-CON45 by lentiviral transfection. A transfection efficiency of 96.6 ± 1.7 and 97.2 ± 0.8% was observed using fluorescence microscopy in CNE1/LVWWOX and CNE1/LV-CON145 cells, respectively ($P < 0.05$; **Figure 1A-C**). The mRNA expression of WWOX, MMP9, E-cadherin and WWOX protein were evaluated using RT-PCR and western blotting, respectively. WWOX expression in CNE1/LV-WWOX cells was significantly increased at both the mRNA and protein levels compared with control groups (CNE1 and CNE1/LV-CON145) ($P < 0.05$; **Figure 1D-F**). The expression of MMP9 was reduced remarkably and the expression of E-cadherin was increased notably in comparison with control ($P < 0.05$; **Figure 1G**, **1H**).

Overexpression of WWOX inhibits cell proliferation in vitro

To study the effect of WWOX on growth of the CNE1 NPC cell line, colony formation and cell proliferation were assayed. The colony formation assay indicates that CNE1/LV-WWOX group forms significantly fewer and smaller colonies compared with control groups ($P < 0.05$) (**Figure 2A**, **2B**). As shown, cell proliferation is significantly inhibited in the CNE1/LV-WWOX group compared with control (**Figure 2C**). These results suggest that WWOX overexpression significantly inhibits colony formation and proliferation of NPC cells.

Overexpression of WWOX suppresses migration and invasion of NPC cells

To study the effect of WWOX on the migration and invasion capability of the CNE1 cells, a wound healing assay was performed, as well as a transwell migration assay and a transwell invasion assay *in vitro*. The migration ability of CNE1 cells is suppressed by WWOX overexpression, as indicated by the results of the wound healing assay (**Figure 3A**) and transwell migration assay (**Figure 3B**). Consistent with these data, WWOX overexpression inhibits the invasion ability of CNE1 cells (**Figure 3C**).

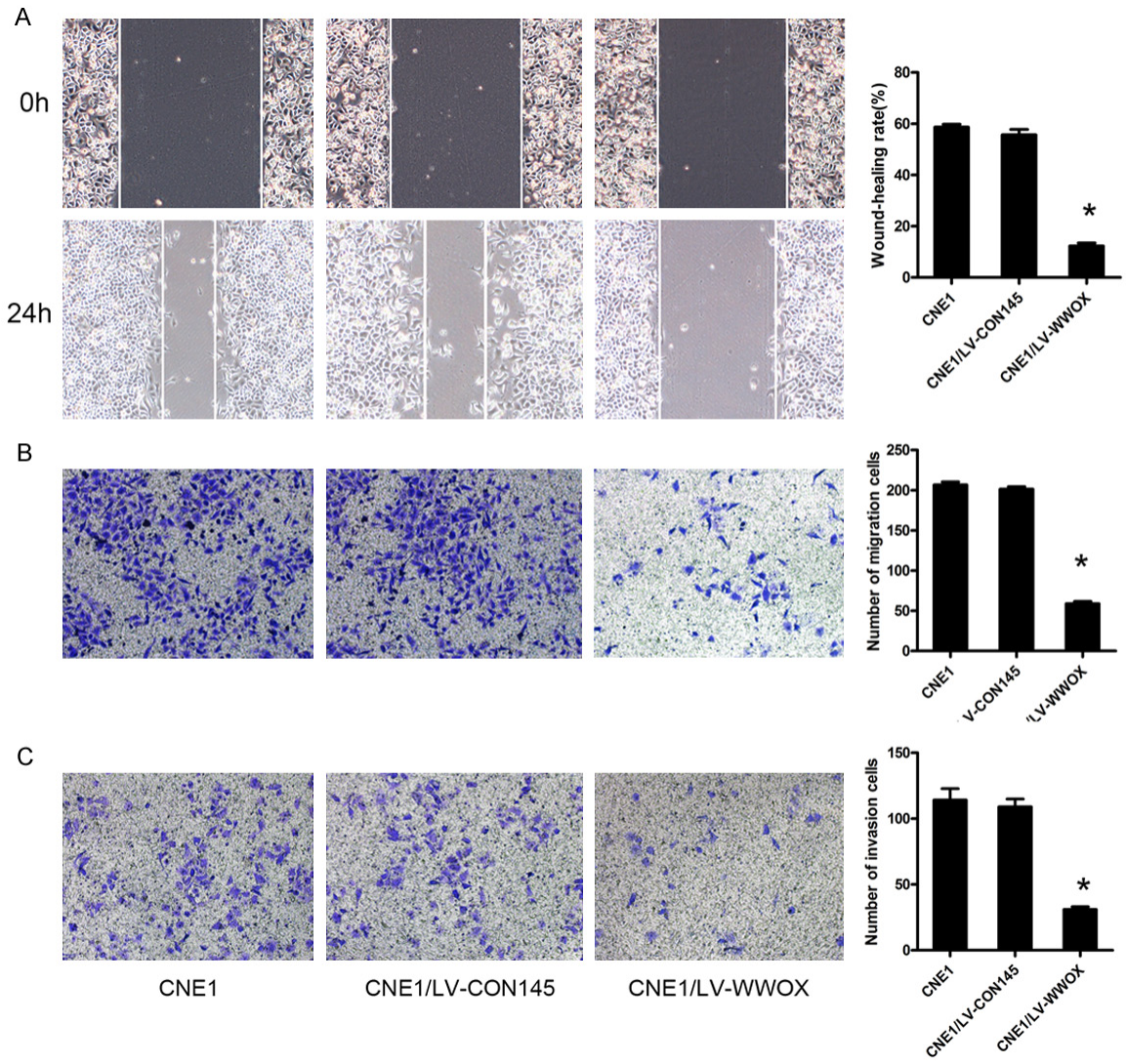

Figure 3. Overexpression of WWOX inhibits migration and invasion of NPC cells. The migration of CNE1 cells was examined by wound healing experiments (A) and transwell migration (B). The invasion of CNE1 cells was detected by transwell invasion assays (C). The data are presented as the mean ± SD (*$P < 0.05$).

Overexpression of WWOX arrests NPC cell cycle progression at G2/M and induces cell apoptosis

To further explore the cell cycle effects of overexpressing WWOX, cell cycle progression was analyzed by flow cytometry. A reduced number of cells was observed in G1 phase (62% to 41%), an increased number of cells in G2/M phase (8% to 22%), and little effect was observed in the S phase population (**Figure 4A**, **4B**, $P < 0.05$). These observations suggest that WWOX is required for cell cycle progression and survival and could induce cell cycle arrest in G2/M phase. Furthermore, the results of the apoptosis assay indicate a significant increase in the number of apoptotic cells after WWOX overexpression. These results demonstrate that CNE1/LV-WWOX cells undergo apoptosis at significantly higher rates than control groups (**Figure 4C**, $P < 0.05$). With regard to apoptosis signaling, the abundance of cleaved caspase-3 protein was measured by western blotting (**Figure 5A**, $P < 0.05$). The data show cleaved caspase-3 is elevated in cells transfected with CNE1/LV-WWOX, indicating that WWOX promotes apoptosis in NPC cells.

Caspase-3 activation and downregulation of phosphorylation of AKT (p-AKT) by WWOX

The effect of WWOX overexpression on apoptosis-related caspase-3 was evaluated by western blotting. The abundance of caspase-3 de-

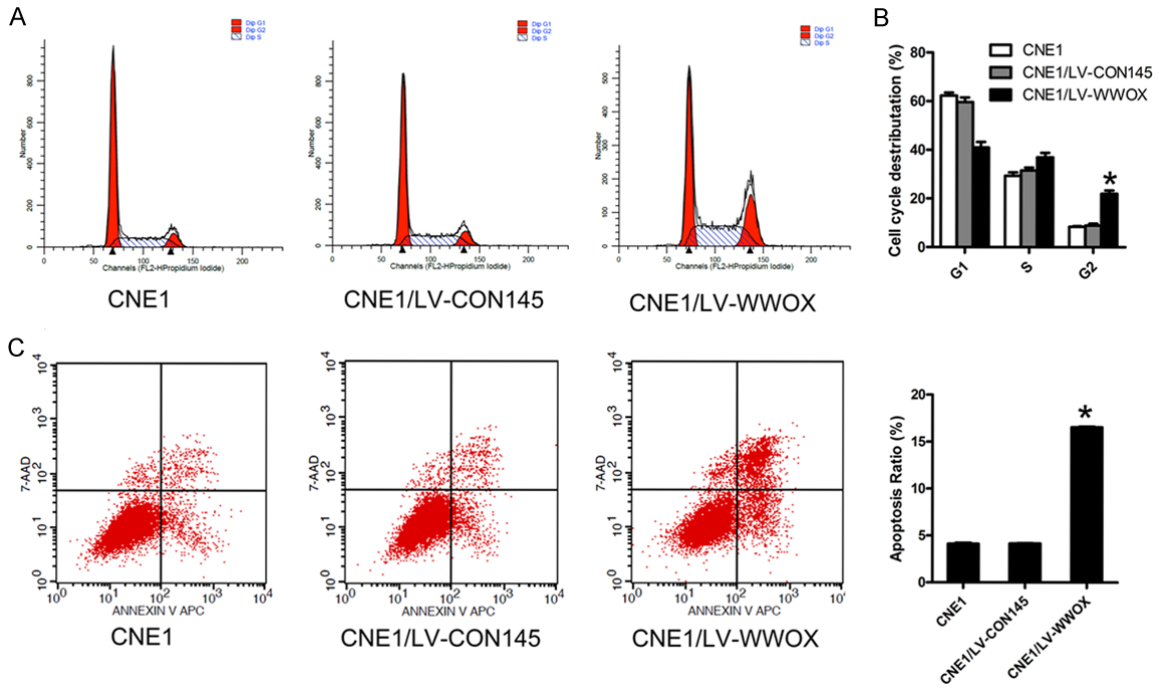

Figure 4. Overexpression of WWOX inhibits cell progression through inducing G2 arrest. (1) Cell cycle progression was analyzed by flow cytometry (A). The number of WWOX-overexpressing cells is reduced in G1 and increased in G2 (B). (2) CNE1/LV-WWOX cells undergo apoptosis at significantly higher rates than control groups (C, $*P < 0.05$). The data are presented as the mean ± SD ($*P < 0.05$).

creases after WWOX overexpression, while the abundance of cleaved caspase-3 increases in cells overexpressing WWOX (**Figure 5A**). To evaluate the effect of WWOX on AKT/PKB activity, AKT and p-AKT abundances were measured. AKT abundance showed no significant difference in three groups, while p-AKT (Ser-473) abundance significantly decreased in cells overexpressing WWOX, compared with cells transfected with a control vector (**Figure 5B**).

Discussion

Due to the significantly higher incidence of NPC among the Chinese, especially in South China, NPC is regarded as the third most common malignant tumor in southern China [26]. Low expression of WWOX is associated with more aggressive tumors and poorer prognosis [27]. To our knowledge, WWOX is absent or reduced in multiple malignant cancers. Here, for the first time, WWOX overexpression has been demonstrated to play crucial roles in various biological functions of NPC cells.

Cell proliferation, migration, and invasion capabilities are closely related to carcinogenesis and cancer development, which are crucial aspects of cancer metastasis. Cell proliferation is a fairly complex process which closely correlates with cell growth and death. Our study indicates that WWOX can be involved in the development of NPC by regulating the proliferation, migration, and invasion capacities. The expression of MMP9 was significantly decreased and the expression of E-cadherin was notably increased. The results indicate that the adhesion and invasion capacity of NPC cells decreases after WWOX overexpression. These results suggest that as an anti-cancer reagent, WWOX might participate in the development of NPC, in part by affecting migration and invasion.

Cell cycle deregulation is a common phenomenon of human cancer, and can lead to unscheduled proliferation and also accumulation of mutations in cancer cells [28]. Cell number can be reduced by either suppressing the cell cycle progression, or by increasing the rate of cell death via the process of apoptosis. Cell cycle deregulation and apoptosis are closely connected, and disturbance of the cell cycle progress may ultimately lead to apoptotic death in the case of serious DNA damage [23]. The current findings suggest that WWOX could inhibit

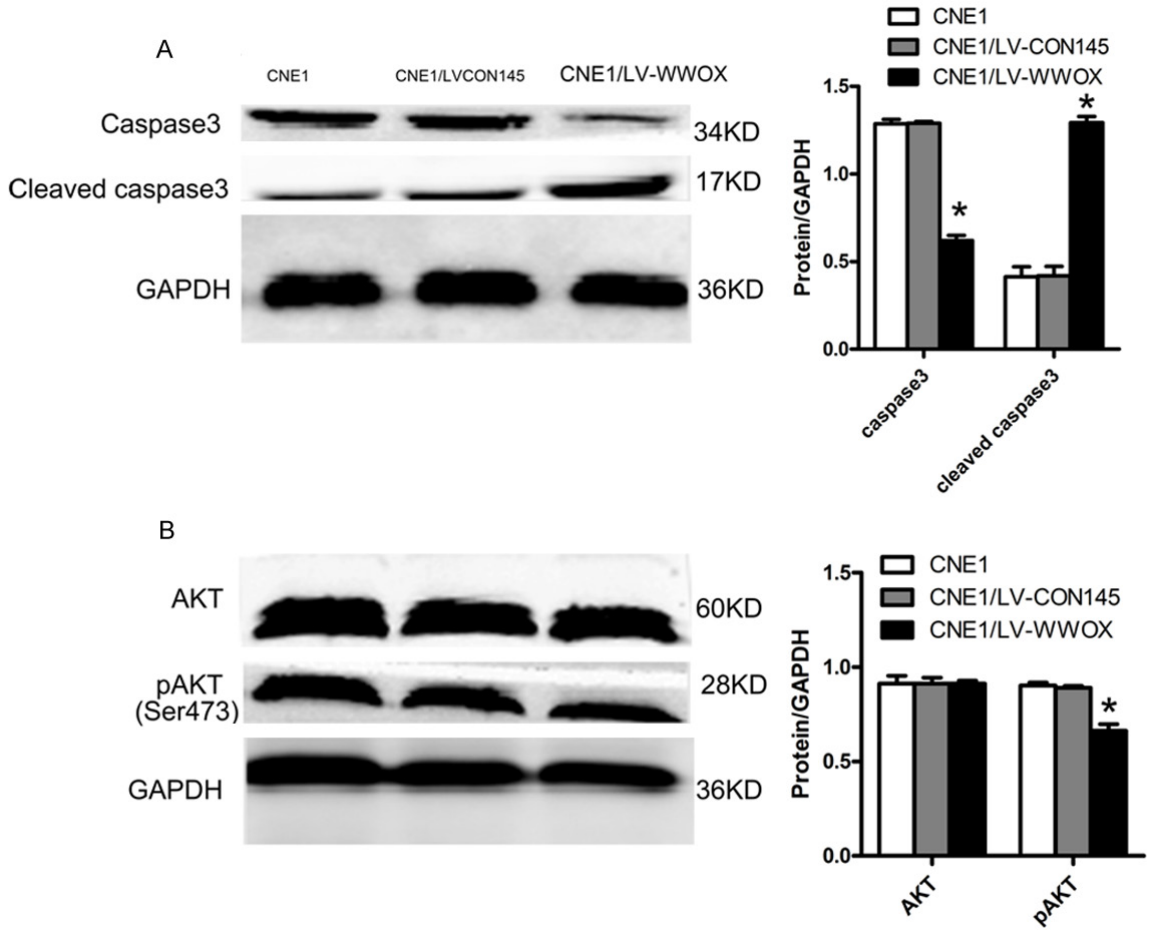

Figure 5. Overexpression of WWOX elevates the expression of caspase-3 protein and decreases the phosphorylation of p-AKT (Ser473). Cell lysates were western blotted for caspase-3, cleaved caspase-3 (A), and AKT, p-AKT (Ser473) (B). The data are presented as the mean ± SD (*$P < 0.05$).

cell proliferation via control of cell cycle progression, which induces G2 phase arrested cells to apoptotic cell death.

In addition to cell cycle arrest, WWOX overexpression also exhibits pro-apoptotic properties. Previous studies showed that WWOX may induce apoptosis by regulating the endogenous channel [20, 29, 30]. Our findings suggest that caspase-3 and Akt are involved in the mechanism by which WWOX induces cell apoptosis. The caspase family plays a crucial role in the apoptosis-signaling pathway. Furthermore, caspase-3 has been recognized as the crucial executive caspase in apoptosis [31]. Caspase-3 activity is the most important and reliable determinant for apoptosis [32]. We analyzed caspase-3 and cleaved caspase-3 through western blotting. In the current study, our data reveals that the expression of cleaved caspase-3 increases remarkably after WWOX overexpression, further suggesting that WWOX might regulate apoptosis of NPC cells through the caspase-3 pathway. Phosphorylation of AKT is necessary for the activation of a cascade of multiple protein targets that are involved in cell growth, proliferation, and invasion, and hence promote tumorigenesis. Our investigation demonstrates that WWOX overexpression significantly downregulates the phosphorylation of p-AKT at Ser473 but exerts no significant effect on the total abundance of AKT among the groups. AKT/PKB, acting as important downstream effectors of the PI3-kinase is also a Ser/Thr protein kinase that plays a vital role in the regulation of various cellular signaling pathways [20]. AKT/PKB can regulate cell survival and apoptosis, and its activation in various cells can protect against apoptosis. WWOX has been reported to sup-

press tumorigenicity through induction of apoptosis in cervical cancer and prostate cancer [30, 33]. Our research shows that WWOX overexpression has a multipoint blocking effect on PI3K/AKT, the tumor signaling network. Therefore, the blocked PI3K/AKT/mTOR signal path may be one of the reasons for cell growth inhibition after WWOX overexpression. Furthermore, it is currently unknown how WWOX affects AKT phosphorylation, as it does not directly influence PI3-kinase activity [34]. This topic remains to be explored in future work.

Conclusion

In conclusion, our results indicate that WWOX inhibits the growth of NPC cells, providing strong evidence for the mechanisms of G2/M cell cycle arrest, which targets the AKT/PKB pathway, and apoptosis that targets caspase-3 pathway. Therefore, the present study provides preclinical proof-of-concept for future development of WWOX as a novel chemotherapeutic agent against NPC.

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Disclosure of conflict of interest

None.

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