

Silencing UBE4B induces nasopharyngeal carcinoma apoptosis through the activation of caspase3 and p53

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Aim: The human ubiquitination factor E4B (UBE4B) gene is frequently amplified in some solid cancers. However, the role of UBE4B in nasopharyngeal carcinoma (NPC) has not yet been investigated.

Methods: Firstly, we analyzed the expression of UBE4B in NPC samples using real-time quantitative PCR and Western blot analysis. After knocking down UBE4B using small interfering RNA technology, the functions of UBE4B on cell proliferation, apoptosis, and cell cycle, as well as underlying mechanism, were investigated.

Results: Compared with matched adjacent non-tumor tissues, both protein and mRNA levels of UBE4B were much higher in most NPC cancerous specimens. Deficiency of UBE4B could significantly inhibit tumor cell growth and induce cell apoptosis. Knocking down UBE4B could promote the expression of cleaved caspase3 and p53, and inhibition of caspase3 could prevent cell apoptosis induced by the deficiency of UBE4B.

Conclusion: These results indicate that expression of UBE4B was higher in most NPC tissues compared to adjacent non-tumoral tissues, and that knockdown of UBE4B inhibited the cell growth and induced apoptosis in NPC cells. This process was regulated by the activation of caspase3 and p53. Thus, UBE4B gene might act as a potential molecular target to develop novel strategy for NPC patients.

Keywords: UBE4B, nasopharyngeal cancer, apoptosis, caspase3, p53

Introduction

Nasopharyngeal carcinoma (NPC) is the most common cancer that originates from the nasopharynx and is endemic in South China and Southeast Asia. Annually, about 15–50 cases per 100,000 are newly diagnosed with NPC.¹ The incidence of NPC is particularly higher in Guangdong, accounting for 18% of all cancers in China.² So NPC is usually referred to as Cantonese cancer because of its high incidence in this region. According to WHO classification, the NPC could be histopathologically classified into three subtypes: keratinizing squamous cell carcinoma (WHO I), non-keratinizing differentiated carcinoma (WHO II), and non-keratinizing undifferentiated carcinoma (WHO III). Despite the recent advancements in diagnostic technologies and treatment strategies, distant metastasis and local relapse remain serious issues.^{3,4} In fact, in the areas with high incidence, about 95% of NPC patients are diagnosed as the poorly differentiated type (WHO types II and III), resulting in poor survival rate.^{5,6} A large amount of research needs to be carried out to understand the exact molecular and morphological targets and mechanisms involved in the strong aggressive behavior of NPC. Since there has been advancements in the development of the methods for early

detection and personalized therapeutic strategies, it would be of great value to explore novel biomarkers that can identify patients who may benefit from novel clinical interventions.

Ubiquitination factor E4B (UBE4B) is a member of ubiquitin conjugation factor E4 family. Ubiquitination takes part in a series of biological reactions catalyzed by several classes of enzymes, and is a process involving several enzymes and substrate factors. Firstly, ubiquitin-activating enzyme E1 could generate a high-energy thioester bond between a cysteine of its active site and the C terminus of ubiquitin with ATP hydrolysis. After being activated, ubiquitin is transduced to members of the family of ubiquitin-conjugating enzymes (E2s). Finally, ubiquitin is covalently attached to the substrate protein directly by the E2s or, alternatively, by ubiquitin–protein ligases (E3s).^{7–9} E4s play an important role in the degradation of target proteins through the ubiquitin fusion degradation pathway. E4s stimulate the elongation of a polyubiquitin chain, which results in the degradation of polyubiquitinated substrate proteins by the proteasome.¹⁰

There are a series of downstream substrate proteins which UBE4B targets, including p53,^{11–13} p63,¹⁴ p73,¹⁵ ataxin-3,¹⁶ fasciculation and elongation protein ζ -1,¹⁷ and epidermal growth factor receptor (EGFR).¹⁸ UBE4B regulates the proteasome-dependent degradation of certain substrates and is involved in several biological processes, including cell growth, protein trafficking, fetation, and senescence process.¹⁹

The human UBE4B gene is localized on chromosome 1p36, which is reported to be amplified in a bunch of tumors, including medulloblastoma,¹¹ promyelocytic leukemia,²⁰ colon cancer, breast cancer,²¹ and primary hepatocellular carcinoma.²² UBE4B has pro-apoptotic function in primary hepatocellular carcinoma cell lines such as BEL-7402 and Huh7.²² The research in breast cancer demonstrated that deficiency of UBE4B could suppress cell proliferation and induce cell apoptosis.²¹

However, roles of UBE4B in NPC have not been well studied, and it remains largely unknown whether UBE4B exerts any effect on NPC. This study is designed to first study the expression of UBE4B in NPC. Then, the expression of UBE4B is downregulated, and its functions and underlying mechanisms in cell proliferation and apoptosis studied. This study provides evidence for the possibility of applying UBE4B inhibition in clinical treatment of NPC.

Materials and methods

Clinical specimens

This project was approved by the Institutional Review Board of Guangzhou First People's Hospital, and written

informed consent was obtained from the patients prior to sample collection. Human NPC tumor samples and adjacent non-tumoral samples from 24 patients were obtained. All the tissue specimens were snap-frozen in liquid nitrogen and stored at -80°C in a refrigerator.

Cell culture

Normal nasopharyngeal epithelial cell line (NPEC) was purchased from American Type Culture Collection (ATCC) and cultured in keratinocyte serum-free medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with epithelial growth factor, bovine pituitary extract, 120 $\mu\text{g/mL}$ streptomycin, and 120 $\mu\text{g/mL}$ penicillin. Human NPC cell lines, including HK1, CNE1, CNE2, SUNE1, and Hone1, were purchased from ATCC and grown in RPMI 1640 (Thermo Fisher Scientific) medium supplemented with 10% FBS (HyClone), 100 $\mu\text{g/mL}$ streptomycin, and 100 $\mu\text{g/mL}$ penicillin.

RNA extraction and real-time quantitative PCR

Total RNA from fresh tissues was extracted by using TRIzol reagent (Thermo Fisher Scientific) according to manufacturer's instructions. The concentration and quality of total RNA was assessed on a Nanodrop spectrophotometer (ND-1000; Thermo Fisher Scientific) by measuring the absorbance at 260 nm. Reverse transcription was performed to synthesize the first-strand of cDNA according to the manufacturer's instructions. Resulting cDNA was subjected to real-time quantitative PCR (RT-qPCR) analysis. The expression of UBE4B at the mRNA level was examined in both NPC tumor tissues and paired non-tumoral tissues. RT-qPCR was carried out using an ABI 7900HT Real-time PCR system (Life Technologies, Carlsbad, CA, USA) with 15 μL of PCR mixture consisting of synthesized cDNA, oligonucleotide primers, and SYBR Green Master Mix (Thermo Fisher Scientific). All measurements were made in triplicate. Primers for real-time PCR were as follows: UBE4B, forward: 5'-CCAAAGAAGCTGTTGGACCAACTG-3' and reverse: 5'-GGGTGTCCATCAGAGGGTCTCTG-3'; and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), forward: 5'-GGAGATTGTTGCCATCAACG-3' and reverse: 5'-TTGGTGGTGCAGGATGCATT-3'.

The Ct (threshold cycle) value of each sample was measured during exponential amplification, and was calculated using the SDS 2.3 software. The relative expression of UBE4B was normalized to that of the internal control gene

(GAPDH). Data were calculated using the comparative threshold cycle ($2^{-\Delta\Delta CT}$) method.

Western blotting

Both tumor tissues and matched non-tumoral tissues were lysed by using RIPA lysis buffer supplemented with protease inhibitor cocktail (Hoffman-La Roche Ltd., Basel, Switzerland) to extract total protein. Western blot analysis was performed according to standard protocol. The following primary antibodies were applied: rabbit anti-UBE4B polyclonal antibody (1:500 dilution; Abnova), rabbit anti-caspase3 polyclonal antibody (1:500 dilution; Cell Signaling Technology), mouse anti-p53 monoclonal antibody (1:100 dilution; Santa Cruz Biotechnology Inc.), and rabbit anti-GAPDH polyclonal antibody (1:10,000 dilution; Proteintech).

siRNA-mediated silencing of UBE4B

To knockdown the expression of UBE4B, siRNA and negative control (NC) RNA synthesized by GenePharma (Shanghai, China) were transiently transfected into CNE2 and SUNE1 cells using Lipofectamine RNAi MAX reagent (Thermo Fisher Scientific) according to the manufacturer's protocol. The siRNA sequences were as follows: siUBE4B#2, sense: 5'CUGCAAUGCUGAACUUUAATT3', antisense: 5'UUAAGUUCAGCAUUGCAGTT3'; siNC, sense: 5'UUCUCCGAACGUGUCACGUTT3', antisense: 5'ACGUGACACGUUCGGAGAATT3'.

MTT assay

For cell proliferation analysis, CNE-2 and SUNE-1 cells transfected with siRNAs were plated in 96-well plates (1,000 cells/well). The cells were stained with 20 μ L of MTT dye (0.5 mg/mL; Sigma-Aldrich Co.) starting from day 1 to day 7. After removing the culture medium, dimethyl sulfoxide (Sigma-Aldrich Co.) was added. The absorbance of each well was measured on a spectrophotometric plate reader at a wavelength of 490 nm. All experiments were performed at least three times.

Cell apoptosis assay

For apoptosis analysis, tumor cells were harvested 48 hours after being transfected with siRNA. This was followed by staining with Annexin V-FITC and propidium iodide using the Annexin V-FITC Apoptosis Detection Kit (Keygen Co. Ltd., Guangzhou, China), the percentage of apoptotic cells was assessed using a flow cytometry cell sorter (Beckman Coulter Corp., Fullerton, CA, USA). The doublets were excluded using an FL3-A versus FL3-W gate. All experiments were repeated in triplicate.

Cell apoptosis under the stimulation of caspase3 inhibitor

Firstly, CNE2 and SUNE1 were transfected with siNC or siUBE4B#2. Then the tumor cells were cultured with or without caspase3 inhibitor (10 μ M) (Santa Cruz Biotechnology). Then the cell apoptosis was examined by flow cytometry analysis. All experiments were repeated in triplicate.

Cell cycle assay

To carry out cell cycle analysis, tumor cells transfected with siNC or siUBE4B#2 were harvested and fixed using 70% ethanol. After being washed, the cells were incubated with RNase A. Then propidium iodide (Keygen Co. Ltd.) was added to the cell suspension and the cell cycle was analyzed using flow cytometry (Beckman Coulter Corp). All experiments were carried out in triplicate.

Statistical analysis

All the data were analyzed with GraphPad Prism 6 software (GraphPad Software Inc., La Jolla, CA, USA). Data are shown as mean \pm SD. Student's *t*-test was used to compare the data between two groups. $P < 0.05$ was considered to be statistically significant.

Results

Increased expression of UBE4B in NPC

To investigate the relationship between UBE4B and NPC, we detected UBE4B expression in NPC tissues and adjacent non-tumor tissues from NPC patients both at protein and mRNA levels. The mRNA levels of UBE4B were higher in 24 NPC tissues than in adjacent tissues ($P = 0.0055$, Figure 1A). In accordance with the qRT-PCR results, our Western-blot results showed that the protein levels of UBE4B were much higher in tumor tissues compared with adjacent non-tumoral tissues ($P = 0.015$, Figure 1B and C). Additionally, we also found that the protein level of UBE4B was remarkably high in the tested five tumor cell lines compared with normal NPEC (Figure 2A).

Silencing of UBE4B inhibits the proliferation of CNE2 and SUNE1 cells

To further study the biological function of UBE4B in NPC proliferation, the expression of UBE4B was knocked down in CNE2 or SUNE1 cells with UBE4B-specific siRNA for 48 hours. CNE2 or SUNE1 cells subjected to control small siRNA (siNC) were used as a negative control. The UBE4B protein was significantly downregulated in CNE2-siUBE4B#2 and SUNE1-siUBE4B#2 cells when

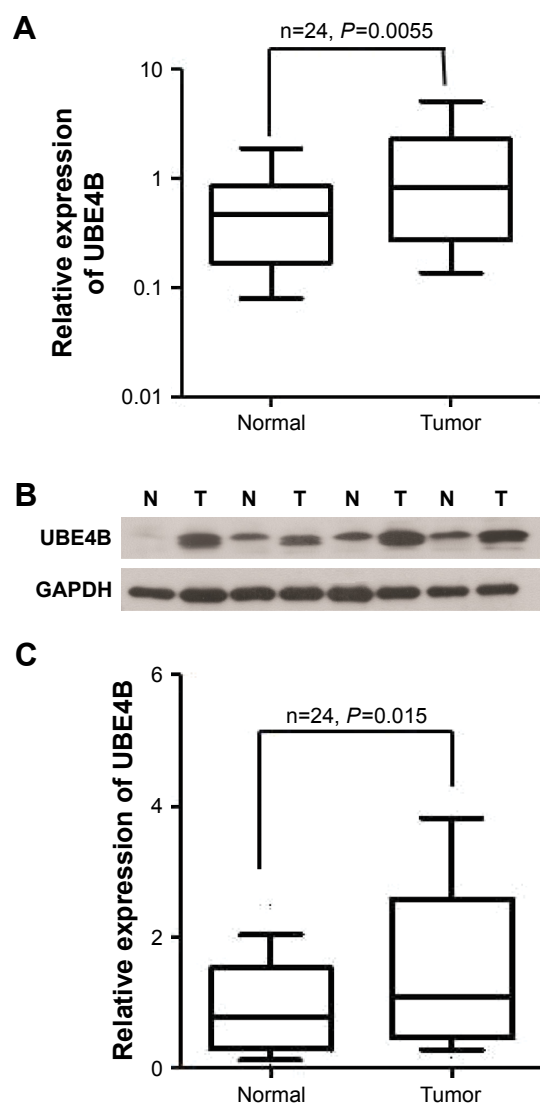


Figure 1 Characteristics of UBE4B expression in 24 clinical samples. **(A)** Real-time PCR was performed to detect UBE4B mRNA in nasopharyngeal carcinoma (NPC) cancerous tissues and their surrounding tissues. Data are shown as mean \pm SD ($n=3$). **(B)** Protein levels of UBE4B in NPC cancerous tissues and surrounding tissues were examined by Western blotting (N, adjacent non-tumor tissues; T, NPC cancerous tissues). **(C)** Quantitative analysis of UBE4B protein in NPC cancerous tissues and their matched adjacent non-tumor tissues.

compared to siNC after 48 hours (Figure 2B and C). The cell growth (viability) of CNE2 subjected to siUBE4B#2 was significantly decreased compared to control cells ($P<0.01$, Figure 2D). Similar results were generated from SUNE1 cells ($P<0.01$, Figure 2E). These results demonstrated that knockdown of UBE4B could suppress NPC cell proliferation.

Deficiency of UBE4B induces apoptosis of NPC cells

It is generally accepted that one important cause of tumor suppression is cell apoptosis. To determine the mechanism

by which deficiency of UBE4B inhibited cell growth, the apoptosis of CNE2 cells subjected to siNC or siUBE4B#2 was analyzed. Percentage of necrosis in CNE2-siUBE4B#2 or CNE2-siUBE4B#2 cells was increased by $\sim 12.3\%$ when compared to that in CNE2-siNC cells (Figure 3A). Similar results were obtained for SUNE1 cells (Figure 3B). The discrepancy in early apoptosis (Annexin V positive/PI negative) between SUNE1-siUBE4B#2 and SUNE1-siNC cells was $\sim 8.2\%$ (Figure 3B). Thus, deficiency of UBE4B could significantly induce apoptosis of CNE2 and SUNE1 cells.

Knockdown of UBE4B increases the expression of cleaved caspase3 and p53

Caspase3, as a crucial executioner of cell apoptosis, is involved in the proteolytic cleavage of many critical proteins.²³ The caspase3 zymogen gains no activity until it is cleaved by an initiator caspase after the stimulation of apoptotic signaling.²⁴

The activation of caspase3 requires proteolytic processing of its inactive zymogen into activated p17 or p12 fragments.²⁵ Our results showed that knockdown of UBE4B could induce NPC cell apoptosis. Thus, we further detected the expression of cleaved caspase3 in CNE2 and SUNE1 cells transfected with siUBE4B#2 or siNC. The expression levels of cleaved caspase3 were significantly upregulated in CNE2 and SUNE1 cells treated with siUBE4B#2 (Figure 3C and D). P53, as a tumor suppressor, is also involved in cell apoptosis.²⁶ UBE4B could interact with the p53 DNA-binding domain and enhance the nuclear export of p53 through promoting p53 ubiquitination, thus inhibiting the transcriptional activities of p53.¹¹ Our data demonstrated increased expression of p53 in CNE2 cells subjected to siUBE4B#2 as compared with siNC group (Figure 3C). There was increased expression of p53 in SUNE1-siUBE4B#2 cells compared to SUNE1-siNC cells (Figure 3D).

Bcl-2, as an important anti-apoptotic protein, plays an important part in cell apoptosis. Herein, we further detected the expression of Bcl2 in CNE2 and SUNE1 cells with siUBE4B#2 transfection. The expression level of Bcl2 protein was much lower in both CNE2-siUBE4B#2 and SUNE1-siUBE4B cells compared with tumor cells transfected with siNC (Figure 3C and D). The data showed that knocking down of UBE4B using siRNA technology resulted in decreasing the protein levels of cleaved caspase3 and p53 (Figure 3C and D). These data indicated that the mechanism of UBE4B-induced apoptosis depended on increased production of cleaved caspase3 and p53.

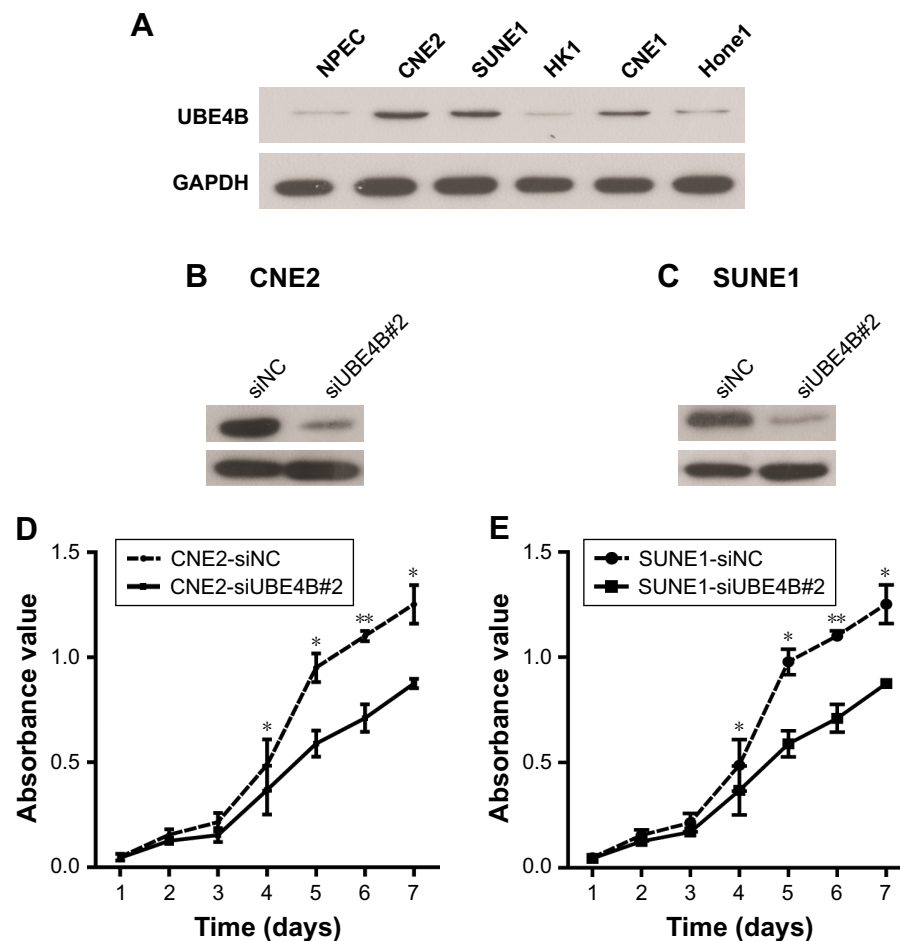


Figure 2 The impact of silencing UBE4B on the proliferation of nasopharyngeal carcinoma (NPC) cells. **(A)** Expression of UBE4B protein in NPC cell lines and normal nasopharyngeal epithelial cell line (NPEC). UBE4B protein was upregulated in HK1, CNE1, CNE2, SUNE1, and Hone1 cells (particularly in CNE2 and SUNE1 cells) compared with the normal NPEC. **(B and C)** Representative protein expression of UBE4B in **(B)** CNE2 or **(C)** SUNE1 cells treated with control siRNA (siNC) or UBE4B siRNA (siUBE4B#2) for 48 hours. GAPDH was used as a loading control. **(D and E)** Cell growth of **(D)** CNE2 or **(E)** SUNE1 cells treated with siNC or siUBE4B#2 for 1, 2, 3, 4, 5, 6, and 7 days. Data are shown as mean \pm SD ($n=3$). * $P<0.05$, ** $P<0.01$.

Inhibition of caspase3 prevents cell apoptosis induced by the silencing of UBE4B

To further understand whether UBE4B deficiency-induced apoptosis depends on caspase3 activation, the apoptosis of NPC tumor cells transfected with siUBE4B was analyzed after co-culturing with caspase3 inhibitor. After being transfected with siNC or siUBE4B#2, NPC cells were cultured with or without caspase3 inhibitor. Then the cell apoptosis was analyzed using a flow cytometer. As shown in Figure 4A and B, the apoptosis percentage of CNE2-siUBE4B#2 cells subjected to caspase3 inhibitor was reduced by about 15.6% as compared with CNE2-siUBE4B#2 without the stimulation of caspase3 inhibitor. Also, there was no difference in the apoptosis percentage between CNE2-siUBE4B#2 cells and CNE2-siNC cells stimulated with caspase3 inhibitor.

Similar results were generated in SUNE1, another NPC cell line (Figure 4C and D). These data indicated that the caspase3 inhibition could reverse apoptosis induced by the silencing of UBE4B.

Silencing of UBE4B exerted no effect on cell cycle of NPC cells

The cell cycle distribution of both CNE2 and SUNE1 cells was analyzed after being transfected with siUBE4B#2. There were no changes in the percentage of cells at the G0/G1 phase (48.2% vs 52.3%), G2/M phase (31.2% vs 34.2%), and S-phase (15.2% vs 17.2%) in CNE 2 cells (Figure 5A and B). Same results were confirmed in SUNE1-siUBE4B#2 cells, where percentages of cell distribution were unchanged at the G0/G1 phase (51.9% vs 57.2%), G2/M phase (29.2% vs 34.5%), and S-phase (13.5% vs 15.2%) (Figure 5C and D).

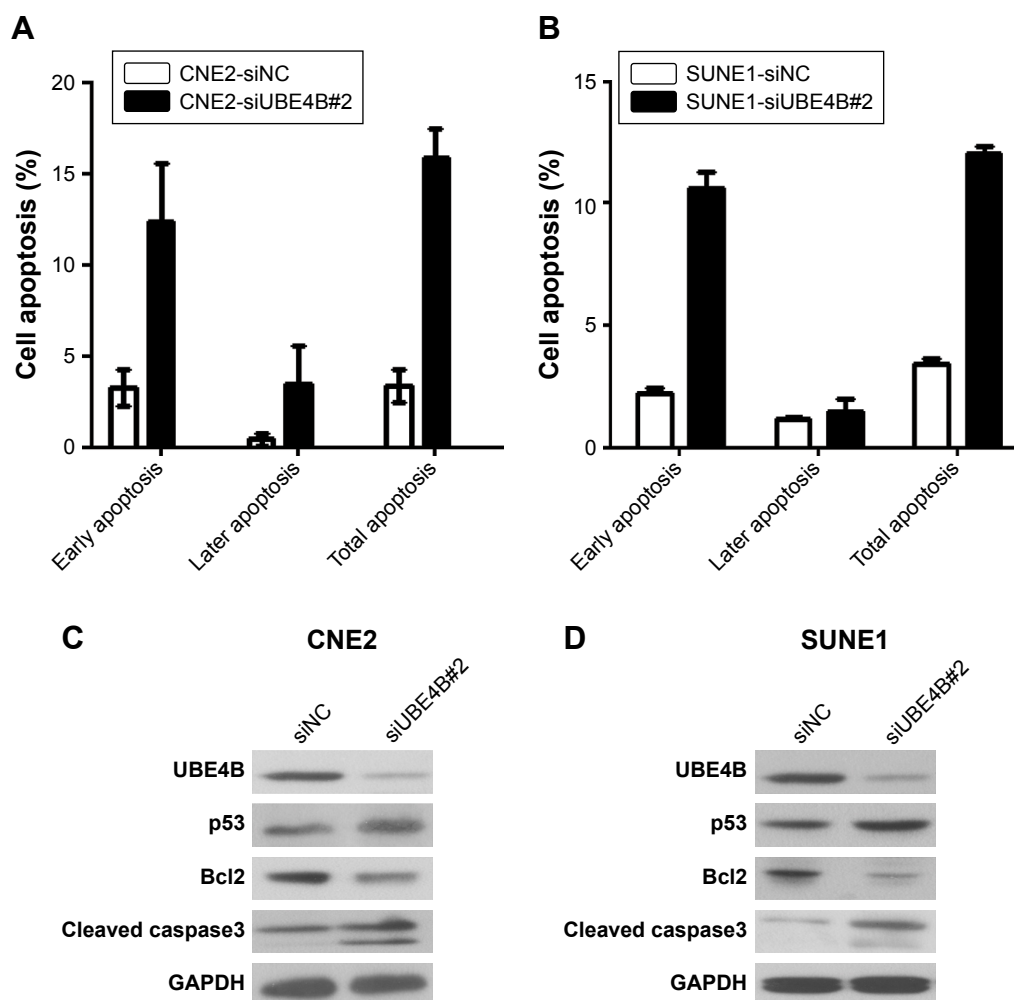


Figure 3 Knockdown of UBE4B induces cell apoptosis in nasopharyngeal carcinoma (NPC) cells; downregulation of UBE4B promotes cleaved caspase3 and p53 expression and decreases Bcl2 expression. (A and B) Knockdown of UBE4B expression with siUBE4B#2 induces cell apoptosis of (A) CNE2 or (B) SUNE1 cells. (C and D) Downregulation of UBE4B with siUBE4B#2 promotes protein levels of cleaved caspase3 in (C) CNE2 and (D) SUNE1 cells. Downregulation of UBE4B promotes p53 expression in (C) CNE2 and (D) SUNE1 cells. Downregulation of UBE4B decreases Bcl2 expression in (B) CNE2 and (C) SUNE1 cells.

These results demonstrated that silencing of UBE4B exerted no significant effect on the changes of cell cycle.

Discussion

The genesis and development of NPC is a complicated process that involves several parameters including oncogenes. Here, we detected increased expression of UBE4B in both NPC tissues and cell lines. Silencing of UBE4B using siRNA technology inhibited the proliferation capabilities of NPC cells. In addition, knocking down of UBE4B induced cell apoptosis which was mediated through activating caspase3 and p53.

The amplification of UBE4B has been detected in a bunch of human cancers.^{27,28} However, there was no research concerning the expression of UBE4B in human NPC. Here, we examined the UBE4B expression in NPC tissues. Both RT-qPCR and Western blot analysis showed that the expression levels of UBE4B in tumor tissues were much higher than their

adjacent non-tumoral tissues. These results are consistent with that obtained by Zhang et al, who found that increased gene copy number of UBE4B was detected in human primary hepatocellular carcinoma.²²

To further investigate whether UBE4B is a promising molecular target for NPC, the biological functions of UBE4B were studied by silencing the expressing of UBE4B in NPC cell lines. The proliferation capabilities of both CNE2 and SUNE1 cells were inhibited significantly with siUBE4B transfection. It is generally accepted that apoptosis is an important cause of proliferation inhibition. To determine the mechanism by which silencing of UBE4B inhibits tumor cell growth, the apoptosis assay was performed. The apoptosis percentage of CNE2 cells was significantly increased after siUBE4B transfection. These results were verified in another cell line (SUNE1). Consistent with this, Zhang et al found that downregulation of UBE4B promoted the apoptosis of human

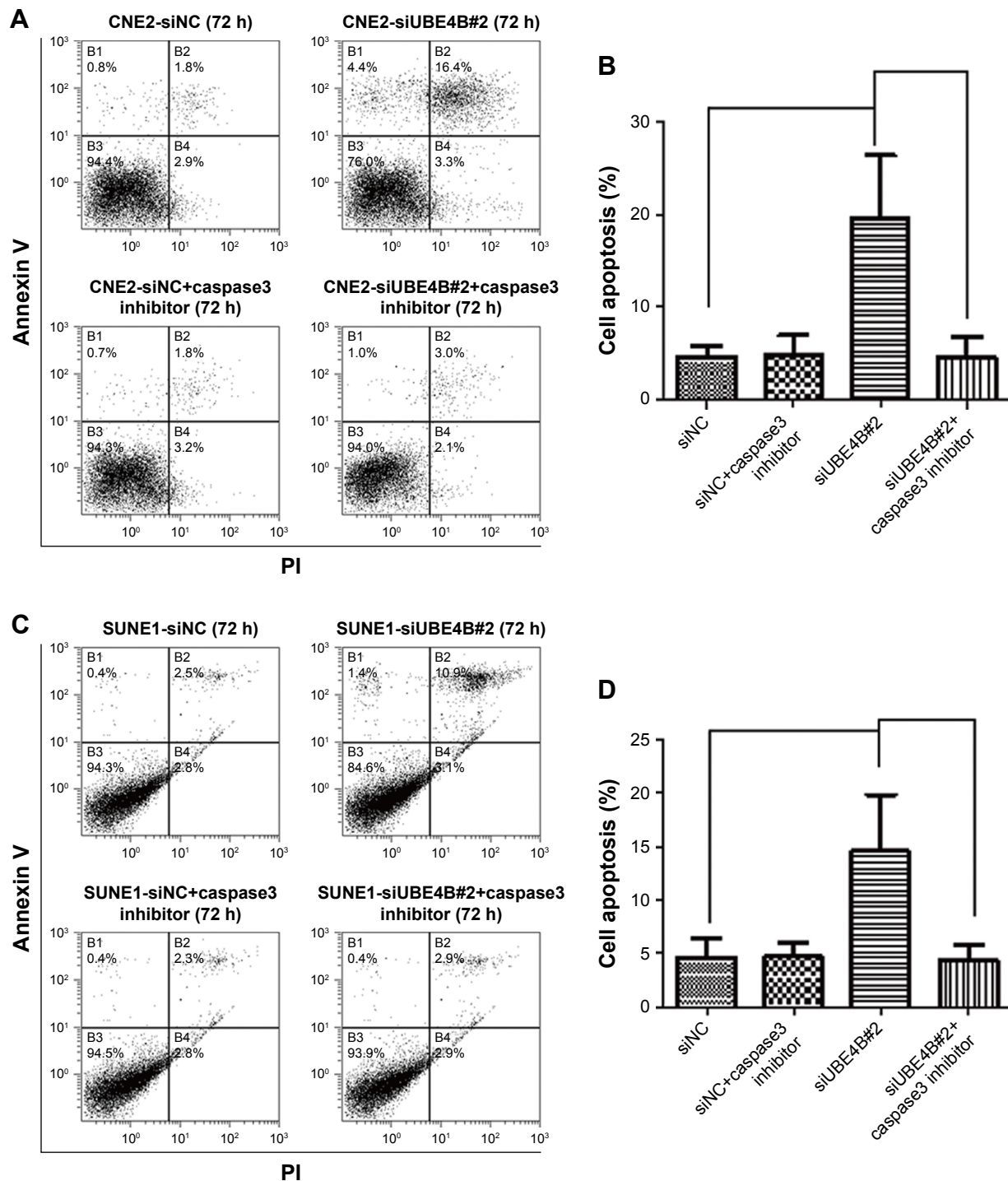


Figure 4 Caspase3 inhibition could prevent cell apoptosis induced by the knockdown of UBE4B. **(A and C)** The cell apoptosis levels of CNE2-siNC, CNE2-siUBE4B#2, and SUNE1-siNC, SUNE1-siUBE4B#2 cells cultured in the presence of caspase3 inhibitor. FITC-labeled Annexin V-positive cells (upper right and lower right) were considered apoptotic cells: **(A)** CNE2 cell, **(C)** SUNE1 cell. **(B and D)** Quantitative analysis of apoptotic percentages of CNE2-siNC cells, CNE2-siUBE4B#2, and SUNE1-siNC, SUNE1-siUBE4B#2 cells cultured in the presence or absence of caspase3 inhibitor: **(B)** CNE2 cell, **(D)** SUNE1 cell.

Abbreviation: FITC, fluorescein isothiocyanate.

breast cancer cells.²¹ However, cell cycle assay showed that deficiency of UBE4B failed to change the cell phases of CNE2 and SUNE1 cells with siUBE4B transfection.

There are many factors that are involved in the process of cell apoptosis. Among them, caspase3 is well studied and

considered as a crucial executioner of apoptosis.²⁵ Caspase-3 is activated in apoptotic cells after being stimulated by both intrinsic and extrinsic signals and pathways.^{29,30} To further understand the potential mechanisms underlying siUBE4B-induced apoptotic process, the expression of cleaved caspase3

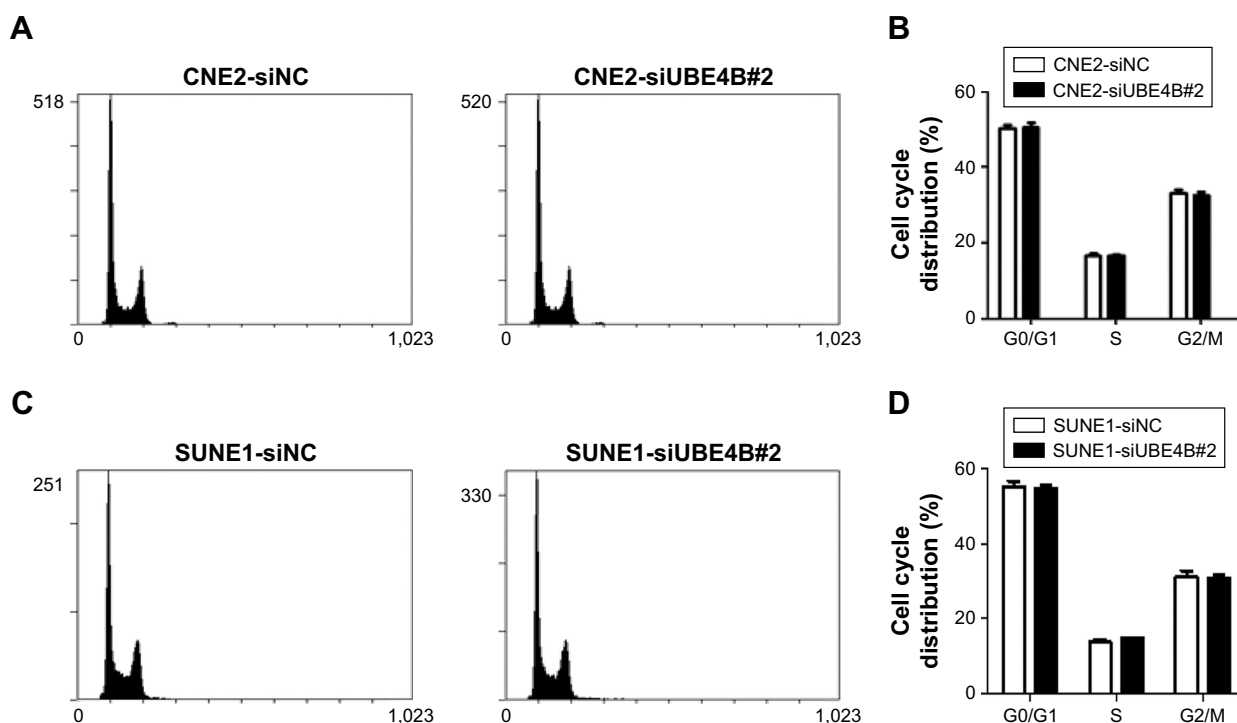


Figure 5 Effect of UBE4B on the cell cycle in nasopharyngeal carcinoma (NPC) cells. Cell cycle distribution was not changed significantly between CNE2 (**A** and **B**) and SUNE1 (**C** and **D**) cells transfected with siNC and siUBE4B#2.

in CNE2-siUBE4B and SUNE1-siUBE4B cells was examined by Western blot analysis. The results demonstrated that there was overexpression of cleaved caspase3 in tumor cells transfected with siUBE4B. Moreover, we also found that application of the caspase3 inhibitor reversed cell apoptosis mediated by UBE4B silence. Few reports have confirmed the correlation between UBE4B and caspase3.²² In this study, we found that silencing of UBE4B could significantly activate caspase3.

There is another factor that is involved in the process of apoptosis, which is the well known tumor suppressor p53.¹¹ Hence, we studied the impact of UBE4B levels on the expression of p53 in CNE2 and SUNE1 cells. The results demonstrated that the protein levels of p53 were upregulated after siUBE4B transfection. These findings were in accordance with those reported by Zhang et al, whose study demonstrated that UBE4B could induce the ubiquitination of p53 and promote the nuclear export of p53, resulting in reduced p53 transcriptional activities.²²

Conclusion

In summary, our research indicated that UBE4B was overexpressed in most NPC cancerous specimens compared to their corresponding non-tumoral tissues, and that deficiency of UBE4B could activate caspase3 and p53 expression resulting in suppression of cell proliferation and induction of apoptosis in NPC cells. Although we preliminarily studied the

expression and mechanism of UBE4B action in NPC patients, future work is needed with increased number of specimens to validate our results. This study may provide important research evidence for developing a novel NPC strategy.

Ethical conduct of research

This study was approved by the Ethics Committee of Guangzhou First People's Hospital, and written informed consent was obtained from all patients. All procedures performed in this study involving human specimens were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

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Author contributions

All authors contributed toward data analysis, drafting and critically revising the paper, gave final approval of the version

to be published, and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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