

URG4-silencing suppresses cell proliferation in nasopharyngeal carcinoma through induction of apoptosis

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Abstract. – **OBJECTIVE:** To investigate the expression and function of up-regulator of gene-4 (URG4) in nasopharyngeal carcinoma (NPC).

PATIENTS AND METHODS: Fresh NPC tumor tissue samples with paired adjacent normal nasopharyngeal tissues samples of 9 NPC patients were collected from NPC curative resection surgery. NPC cell lines (CNE1, CNE2 and HONE1) were cultured. Lentivirus-mediated URG4-specific short hairpin RNA (shRNA) stable transfection was done. The effect of URG4 on CNE4 and HONE1 cells viability was determined via 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). The plate-colony-formation assay was performed. Apoptosis analysis was done by flow cytometry. The expression levels of protein and RNA were detected by Western blotting and quantitative polymerase chain reaction (qPCR).

RESULTS: We determined the expression of URG4/URGCP in NPC tissues and cell lines using qPCR analysis and found it was significantly upregulated in NPC. After that, stable URG4-silencing NPC cells were constructed by transfection with lentivirus-mediated shRNA. Functionally analyses indicated that knockdown of URG4 significantly impaired cell viability and colony formation ability, as confirmed by MTT and colony formation assays. Furthermore, URG4-silencing NPC cells showed more cells in the stage of early and late apoptosis compared with controls by flow cytometry assay. Western blot analysis further confirmed that knockdown of URG4 enhanced the expression of cleaved caspase-3, cleaved PARP and Bax, while decreased the expression of Bcl-2 and survivin.

CONCLUSIONS: URG4/URGCP might play an essential role in NPC cell growth and proliferation and its silencing might be as a potential therapeutic target for NPC.

Key Words:

NPC, URG4, Cell proliferation, Apoptosis, Caspase-3, PARP.

Abbreviations

URG4: up-regulator of gene-4; NPC: nasopharyngeal carcinoma; shRNA: short hairpin RNA; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; qPCR: quantitative polymerase chain reaction; FBS: fetal bovine serum. GAPDH: glyceraldehyde 3-phosphate dehydrogenase; TBS-T: Tris-buffered saline-tween.

Introduction

Nasopharyngeal carcinoma (NPC) is one of the most common and leading causes of cancer-related death malignant tumors in southeast of Asia, especially in Southern China¹. The occurrence and progression of NPC are mainly ascribed to viral infection, genetic alterations and environmental factors²⁻⁵. Currently, generally therapies for NPC are radiation therapy and chemical drug therapy, but the five-year survival rate of NPC patients remains relatively low for radio-chemo-resistance⁶. Moreover, local recurrence and metastasis are the main reasons that limit the efficacy of chemotherapy or radiotherapy⁷. Therefore, a better understanding of the molecular mechanisms underlying the initiation of NPC will help us to develop novel therapeutic targets for the treatments of NPC.

Up-regulated gene-4 (URG4), also known as upregulator of cell proliferation (URGCP), is located in the long arm of the chromosome 7p13, which has been identified as an oncogene associated with cell cycle regulation⁸. Tufan et al⁹ firstly found that URG4 was up-regulated in hepatocellular carcinoma (HCC) tissues and played a positive role in hepatocellular growth and survival. Concretely, overexpression of URG4 could reduce cell cycle protein p21Cip1 and p27Kip1, and

upregulate cyclin D1 in HepG2 cells¹⁰. What's more, elevated URG4 expression has been also observed in ovarian cancer¹¹, gastric cancer¹² and prostate cancer¹³. Additionally, URG4 is involved in activating NF- κ B signaling in apoptotic resistance of leukemia¹⁴ and invasiveness of non-small cell lung cancer cells¹⁵. Of note, previous studies demonstrated that URG4 is upregulated in human NPC tissue¹⁶. However, the function of URG4 on NPC and the underlying mechanisms have not yet been elucidated.

Therefore, the objective of this study was to investigate the biological function of URG4 in NPC cells *in vitro*. We firstly demonstrated that URG4 was upregulated in NPC tissues and cell lines. Loss-of-function assays were performed on NPC cells and showed knockdown of URG4 could inhibit cell viability and proliferation. Furthermore, knockdown of URG4 significantly promoted cell apoptosis in NPC cells by activating cell apoptotic signaling pathways. Based on these data, we could conclude URG4 might have potential as therapeutic target in NPC.

Patients and Methods

NPC Specimens

Fresh NPC tumor tissue samples with paired adjacent normal nasopharyngeal tissues (located > 5 cm away from the tumor) samples of 9 NPC patients were collected from NPC curative resection surgery in the First Affiliated Hospital of Xiamen University. These tissues samples were immediately stored at -80°C until use for experimental purposes. All the patients enrolled in this study provided their consents and none of these patients had received radio-therapy or chemotherapy prior to surgical treatment. This study was approved by the Ethics Committee of The First Affiliated Hospital of Xiamen University.

Cell Lines

NPC cell lines (CNE1, CNE2 and HONE1) were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) (Gibco Rockville, MD, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA). Non-malignant nasopharyngeal epithelial cell line NP69 was cultured in keratinocyte-serum-free medium (Invitrogen, Carlsbad, CA, USA). All cells were maintained at 37°C in a moist atmosphere containing 5% CO₂.

Lentivirus-mediated URG4-specific shRNA Stable Transfection

For silencing of URG4 in HONE1 and CNE2 cells, the specific shRNA (Cat no. 163812) for URG4 (shURG4: 5'-CCGGCGCGTTGTAACATCATGTGTTTCTCGAGAAACACATGAGTTA-CAACGCGTTTTTTG') and negative control shRNA (shCtrl: 5'-TTCTCCGAACGTGTCACGT-3') were synthetically purchased from OriGene Technologies, Inc. (Rockville, MD, USA). The oligos were annealed and ligated into the pFH-L vectors (Shanghai Hollybio, Shanghai, China), which were confirmed by DNA sequencing. Recombinant lentiviruses were produced by transfecting 293 T cells using Lipofectamine 2000 reagent. For transfection, two target cell lines, HONE1 and CNE2 cells, were seeded in six-well plates and then transfected with shURG4 or shCtrl at a multiplicity of infection of 40. After transfection for 96 h, cells were filtered and screened in medium containing puromycin (Sigma-Aldrich, St. Louis, MO, USA). The expression of silencing URG4 was confirmed by quantitative PCR (qPCR) and Western blot analysis.

qPCR Assay

CNE2 and HONE1 cells were collected after 4 days of lentivirus infection and total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA Cat No. 15596-026) according to the manufacturer's protocol. The purity and the RNA concentration were assessed by NanodropTM spectrophotometer (Thermo Scientific, Waltham, MA, USA). First-strand cDNA was synthesized and then PCR primers were designed to amplify fragments. The primer sequences were as following: URG4 (forward: 5'-CGCAATCATCTCCTTCCATT-3' and reverse: 5'-GATTTGGGAGAAGTAGCCCC-3'). The qPCR was carried out in the linear range using the SYBR Green Core Reagents kit on a BioRad CFX96 TouchTM Real-time PCR system according to the following procedure: pre-denaturation 95°C for 60 s, denaturation at 95°C for 5 s and 40 cycles, annealing and extension 60°C for 20 s.

MTT Assay

The effect of URG4 on CNE4 and HONE1 cells viability was determined once a day for consecutive 5 days depended on growth curves using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Briefly, cells were seeded at 96 well plates after 96 h of lenti-

virus infection at a density of 2500 cells/well in RPMI-1640 medium containing 10% fetal bovine serum (FBS). Then, each well was added with 20 μ L MTT solution (5 mg/mL in PBS) following by incubation for 4 h at 37°C. After removing the supernatant of each well, we added 100 μ L of dimethyl sulfoxide (DMSO) and determined the absorbance value on Epoch Microplate Spectrophotometer (Santa Cruz, CA, USA) at a wavelength of 490 nm.

Colony Formation Assay

The plate-colony-formation assay was performed on CNE4 and HONE1 cells after transfection with shURG4 or shCtrl. Briefly, cells were washed in PBS twice, and incubated in 4% polyformaldehyde for 30 min at room temperature. The colonies were stained with 1% crystal violet for 30 s. The number of colonies (>50 cells/colony) was manual counted under a light microscope. Image analysis was conducted using Image-Pro Plus 6.0 software (Media Cybernetics, Silver Springs, MD, USA).

Apoptosis Analysis by Flow Cytometry

Cell apoptosis of CNE4 and HONE1 cells was analyzed by flow cytometry with Annexin V-APC/7-AAD Apoptosis Detection Kit (Keygen Biotech., Nanjing, China, Cat No. KGA1026). Briefly, CNE4 and HONE1 cells were fostered for 48 h in 6-cm dishes at 3×10^5 cells/well. After that, cells were collected and washed twice with PBS (pH=7.4), and resuspended in 100 μ L Annexin-binding buffer. Subsequently, cells were subjected to Annexin V-APC/7-AAD double staining according to the manufacturer's protocol. All flow cytometry data were acquired using an LSRFortessa Analyzer (BD Biosciences, Franklin Lakes, NJ, USA) and analyzed using Flowjo Software (Treestar Inc., San Carlos, CA, USA).

Western Blot Analysis

After 4 days of lentivirus infection, CNE2 and HONE1 cells were harvested and washed with pre-cold PBS three times; then, cells were lysed in radioimmunoprecipitation assay (RIPA) buffer on ice for 30 min. Total protein concentrations were quantified by bicinchoninic acid (BCA) Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA). A total of 30 μ g cellular protein was separated on 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercu-

les, CA, USA Cat No.162-0177). The membrane was incubated with primary antibodies, including anti-URG4 (1:1000, HPA020134, Sigma-Aldrich, St. Louis, MO, USA), cleaved-Caspase-3 (1:500, #9661, Cell Signaling Technology, Danvers, MA, USA), cleaved-PARP (1:1000, #9542, Cell Signaling Technology, Danvers, MA, USA), Bax (1:1000, #6547, Cell Signaling Technology, Danvers, MA, USA), Bcl-2 (1:500, #2876, Cell Signaling Technology, Danvers, MA, USA), survivin (1:500, #8697, Cell Signaling Technology, Danvers, MA, USA) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, 1:100000, #9876, Cell Signaling Technology, Danvers, MA, USA) overnight at 4°C. Next, the membrane was washed three times with Tris-buffered saline and Tween (TBST) and incubated with secondary antibody horseradish peroxidase (HRP)-conjugated goat anti-rabbit (1:5000, Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 2 h at room temperature. GAPDH was used as the internal standard. Signals were detected using the ECL-PLUS/Kit (Amersham, Cat No. RPN2132, Little Chalfont, UK).

Statistical Analysis

The statistical analyses were performed using the Graphpad Prism 5.0 (La Jolla, CA, USA) and SPSS 19.0 software (IBM, Armonk, NY, USA). The values are expressed as the mean of at least three different experiments \pm S.D. The results were evaluated by Student's *t*-test and *p* < 0.05 was considered statistically significant.

Results

URG4 was Upregulated in NPC Tissues and Cell Lines

To investigate the role of URG4 in NPC *in vitro*, we first analyzed its expression at the protein level in 9 different NPC tissues (indicated as T) compared with non-cancerous tissues (indicated as N) by Western blot. The clinicopathologic characteristics of 9 patients were shown in Table I. Our results revealed URG4 expression levels were significantly higher in tumor tissues compared with adjacent normal nasopharyngeal tissues (Figure 1 A and B). Meanwhile, Western blot was performed in three NPC cell lines (CNE1, CNE2 and HONE1) and one normal nasopharyngeal epithelial cell line (NP69). As shown in Figure 1 C, the level of URG4 protein in NPC cell lines was much higher than that in NP69

Table I. The clinicopathologic characteristics of patients with NPC.

Characteristics	Cases
Age (years)	
< 45	3
≥ 45	6
Gender	
Male	5
Female	4
Clinical stagea	
I, II	7
III, IV	2
Lymph node metastasis	
Yes	8
No	1

a: According to the International Union Against Cancer (UICC).

cells. These data suggested URG4 was increased in NPC, suggesting that URG4 protein might have a putative correlation with NPC progression. Given that URG4 protein in CNE2 and HONE1 cells was relatively higher, the two cell lines were chosen for subsequent analysis.

The expression of URG4 was Efficiently Suppressed in NPC Cells

In order to deeply investigate the function of URG4 protein in NPC, NPC cell lines (CNE2 and HONE1) with higher URG4 expression were transfected with lentiviral shRNA (shCtrl and shURG4). The silencing efficiency of URG4 of these recombinants was confirmed by both qPCR and Western blot. URG4 mRNA level was reduced

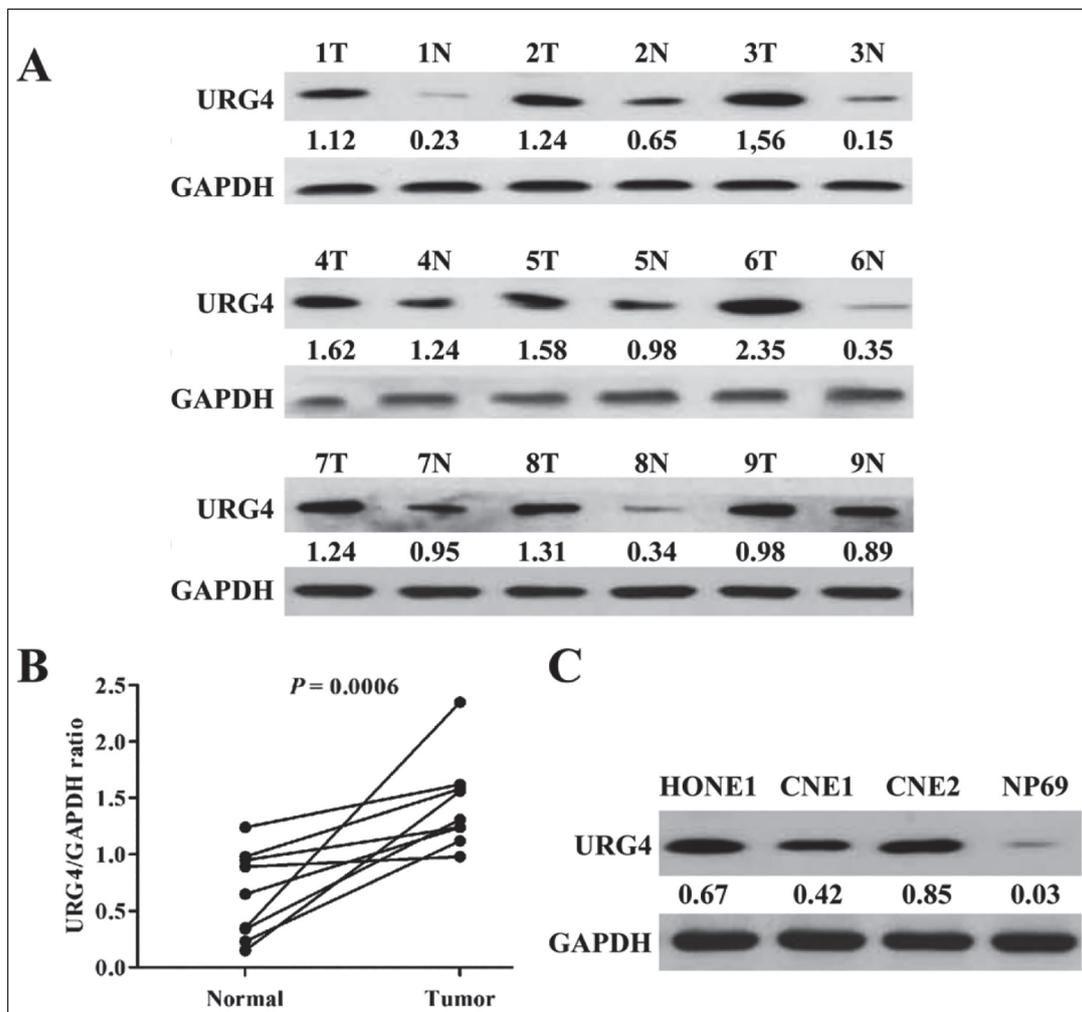


Figure 1. Expression levels of URG4 in NPC tissues as well as NPC cell lines. **A**, The expression levels of URG4 protein in 9 paired NPC tissues, assessed by Western blotting. NPC: nasopharyngeal carcinoma; N: Nonmalignant nasopharyngeal tissues; T: NPC tissues. **B**, Quantitative analysis of URG4 protein in 9 paired NPC tissues. **C**, Western blot analysis of URG4 protein expression in NPC cell lines (CNE1, CNE2 and HONE1) and one nasopharyngeal epithelial cell line NP69.

significantly following infection of shURG4 in CNE2 and HONE1 cells, compared with the shCtrl groups (Figure 2A and C, $p < 0.001$). Consistently, Western blot analysis showed that the protein level of URG4 was also significantly downregulated in CNE2 and HONE1 cells infected with shURG4, respectively (Figure 2B and D). These results suggested that shURG4 construction efficiently suppressed URG4 expression at both mRNA and protein level.

Silencing URG4 Observably Suppressed the NPC Cells Proliferation

To assess the effects of silencing URG4 on the inhibition ability of cell proliferation, MTT assay and the colony proliferation assay were performed in CNE2 and HONE1 cells. As shown in Figure 3A and B, shURG4 exhibited marked inhibition in both CNE2 and HONE1 cells compared to shCtrl from day 4 to day 5 ($p < 0.001$). Compared with those cells infected with shCtrl,

colonies of CNE2 and HONE1 cells were significantly reduced (Figure 3C and D, $p < 0.001$). These results indicated that silencing of URG4 dramatically inhibited the cell proliferation of NPC cell lines (CNE2 and HONE1).

Silencing URG4 Significantly Promoted the NPC Cells Apoptosis

To reveal whether knockdown the URG4 could affect apoptotic rates, flow cytometry assay was carried out in CNE2 and HONE1 cells. As shown in Figure 4A and B, we found knockdown of URG4 in CNE2 cells increased the percentage of early apoptotic cells (Annexin V⁺/7-AAD⁻) from $7.00 \pm 0.2\%$ to $15.45 \pm 0.5\%$ ($p < 0.001$) and late apoptotic cells (Annexin V⁺/7-AAD⁺) from $3.25 \pm 0.0\%$ to $7.84 \pm 0.5\%$ ($p < 0.001$). Results exhibited a 2-fold increase in total cell apoptotic population of shURG4 group compared to shCon group ($p < 0.001$). Similar results were also observed in HONE1 cells (Figure 4C and D, $p < 0.001$). The

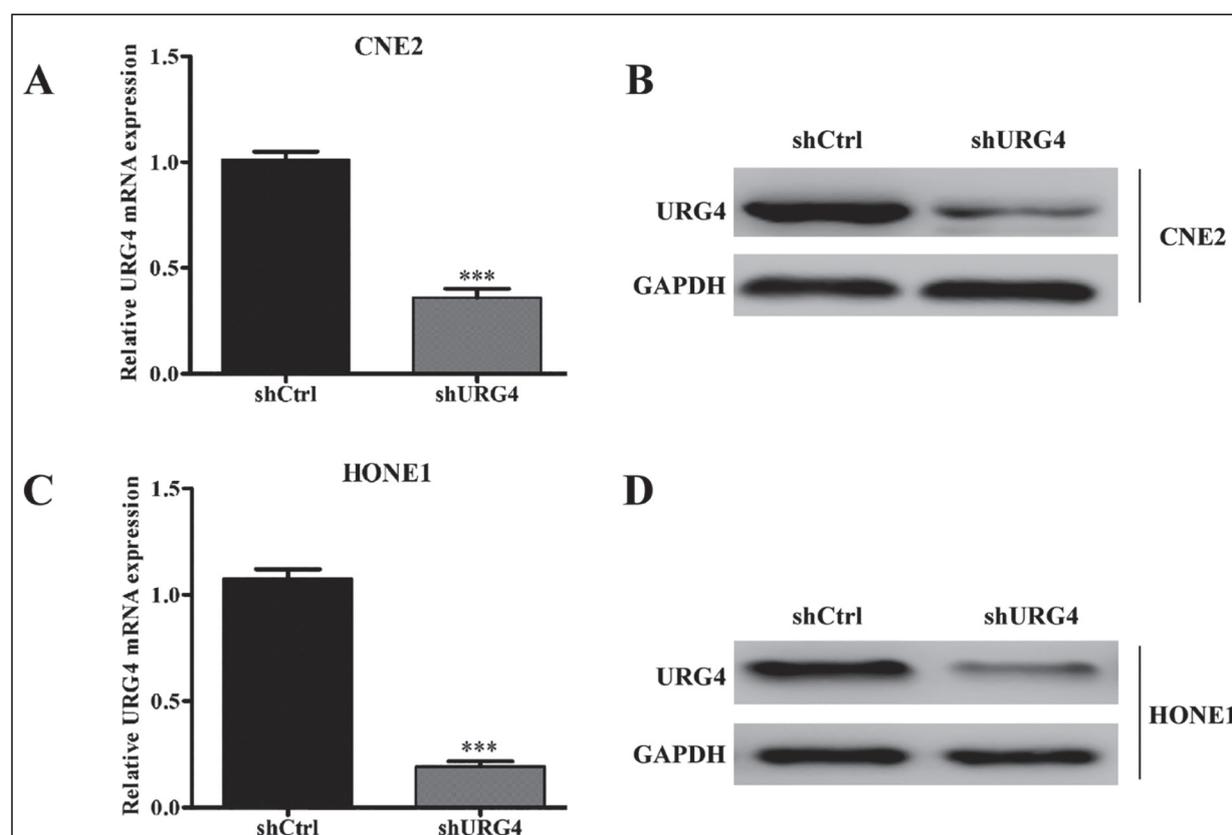


Figure 2. Silencing effect of URG4 in NCP cell lines. **A**, The expression level of URG4 mRNA when silencing URG4 gene by qRT-PCR in CNE2 cells. **B**, Western blotting analysis the expression of URG4 protein levels in CNE2 cells between shURG4 and shCtrl. **C**, QRT-PCR of URG4 mRNA expression in HONE1 cells, shURG4 is lower than shCtrl. **D**, The URG4 protein level of HONE1 cells between shURG4 and shCtrl by Western blotting, the level in shCtrl is increased than shURG4.

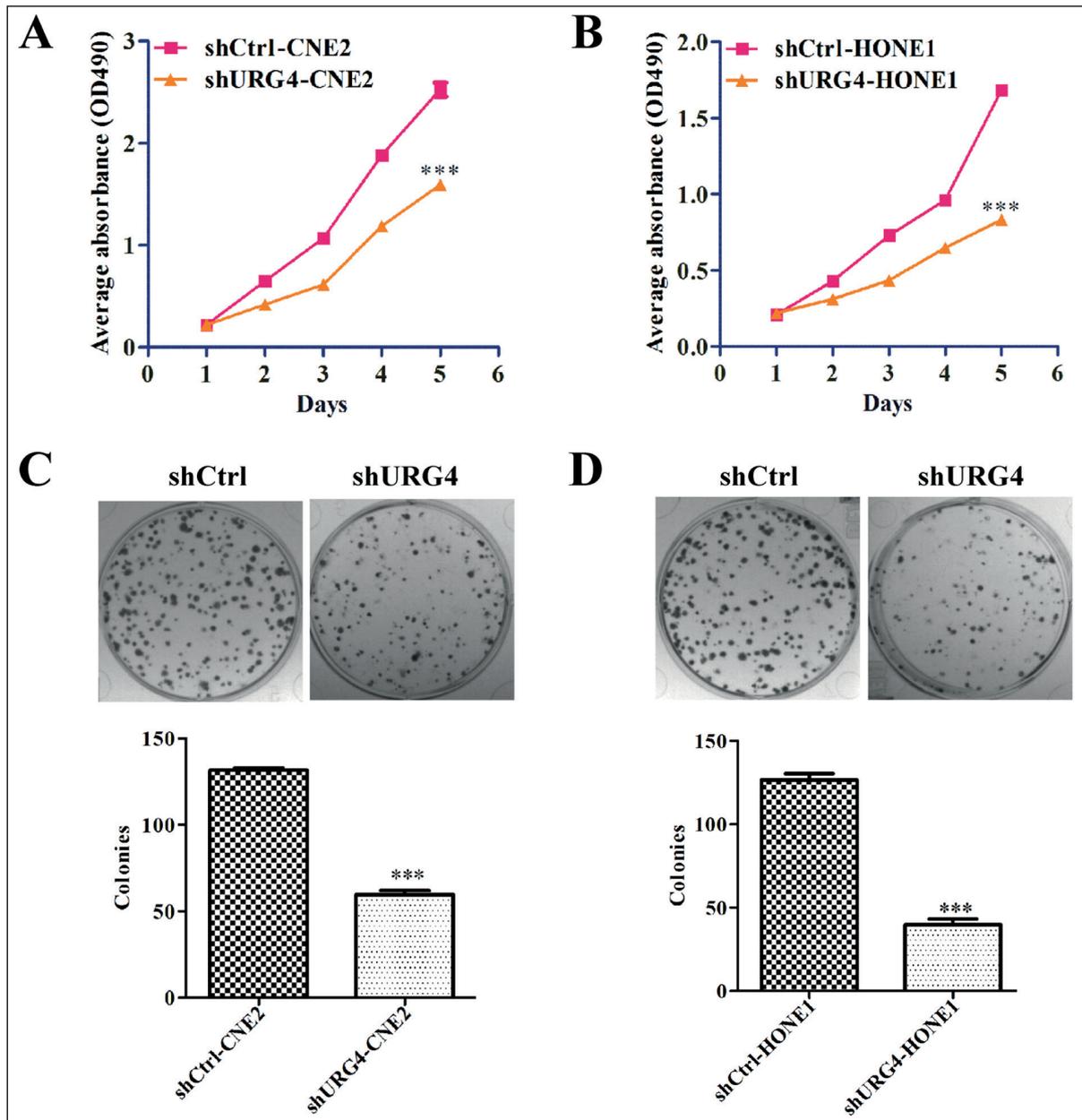


Figure 3. Silencing of URG4 was efficiently suppressed the NPC cells proliferation. **A, B**, MTT detected the growth curve of CNE2 and HONE1 cells, shURG4 CNE2 cell and HONE1 cell growth curve show slowness compared with shCtrl. $***p < 0.001$. **C, D**, Silencing URG4, colonies of CNE2 cells and HONE1 cells are dramatically reduced in six-well plates, Bars denote S.E.M. $***p < 0.001$.

results revealed that URG4 silencing enhanced cell apoptosis in NPC cells.

Silencing URG4 Efficiently Promoted the Apoptosis Proteins Expression and Decreased the Anti-Apoptotic Protein Expression in NPC Cells

Finally, we examined the expression levels of apoptosis and anti-apoptosis proteins by Western

blot. As shown in Figure 5, apoptotic markers (cleaved-caspase3 and cleaved-PARP), and pro-apoptotic protein Bax levels were up-regulated, while anti-apoptotic protein Bcl-2 and survivin were down-regulated in HONE1 cells after URG4 silencing. The results further demonstrated that silencing URG4 could promote NCP cells apoptosis by regulating apoptosis related signaling pathways.

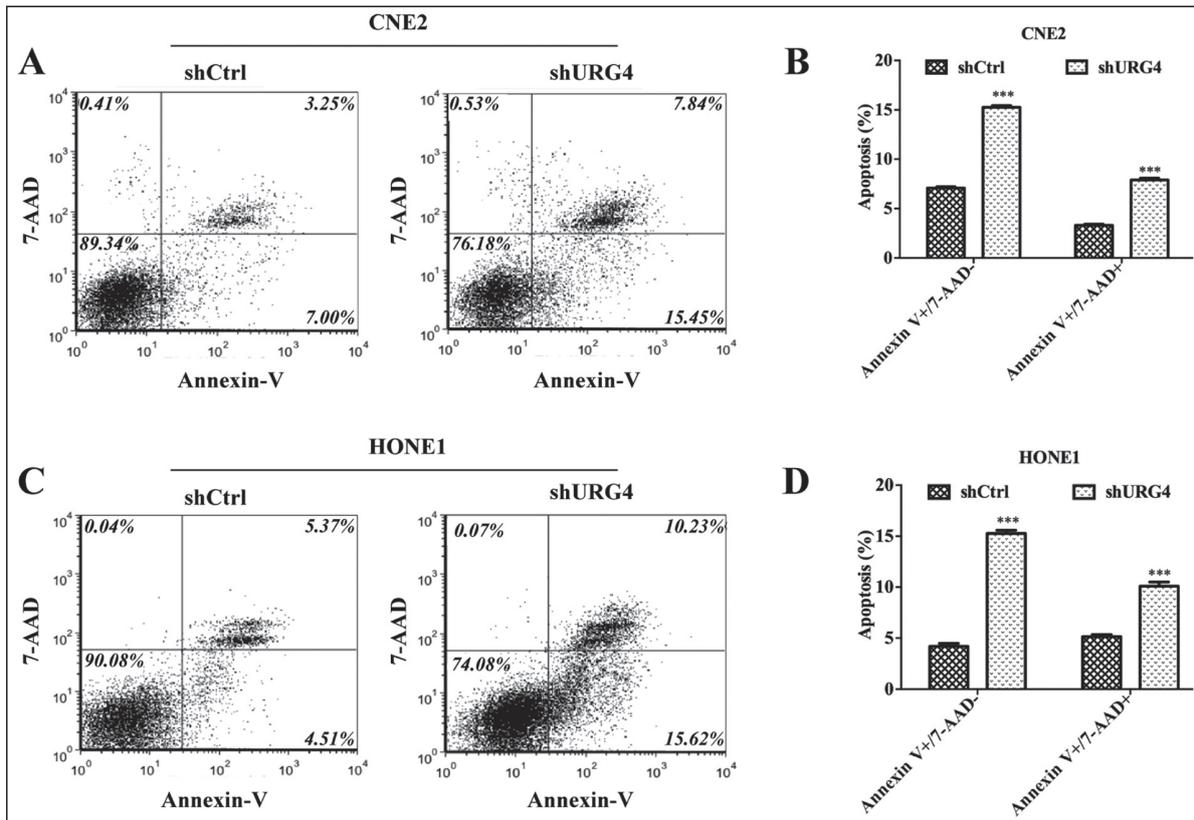


Figure 4. Silencing of URG4 was promoted the apoptosis in NPC cell lines. **A, C,** Flow cytometry analysis apoptosis in shURG4 CNE2 and HONE1 cells, shURG4 apoptosis rates increased significantly compared with shCtrl in CNE2 and HONE1 cells. **B, D,** Percentage of apoptosis in CNE2 and HONE1 cell lines, shURG4 could promote apoptosis dramatically. *** $p < 0.001$.

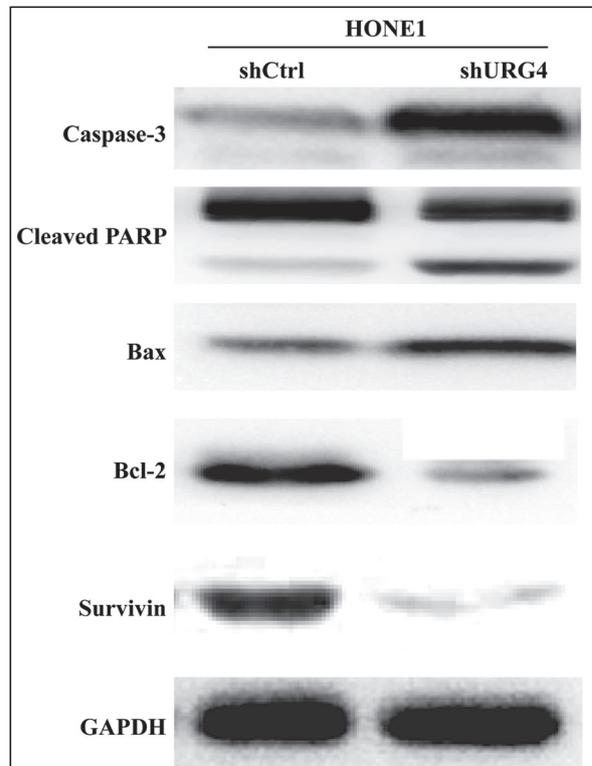


Figure 5. Expression of apoptosis and anti-apoptosis protein in NCP cell lines. Western blotting detected the expression levels on apoptosis protein caspase-3, PARP and BAX in HONE1 cells. When silencing URG4, apoptosis protein is increase compared with shCtrl in HONE1 cells. The levels of expression of anti-apoptosis protein such as Bcl-2, Survivin were decreased observably.

Discussion

Recent evidence found that URG4 was over-expressed in NPC and significantly correlated with poor clinical outcome in patients with higher URG4 expression¹⁶; however, the biological function of URG4 in NPC still remains unclear. We demonstrated that URG4 was upregulated in NPC tissues and cells. Silencing expression of URG4 suppressed the cell viability and proliferation ability of NPC *in vitro*, which suggested that URG4-silencing had a tumor suppressive potential in NPC cells. In fact, many recent studies have found that URG4 is overexpressed in various human tumor types, including hepatocellular carcinoma¹⁷, cervical cancer⁸, and bladder cancer¹⁴.

URG4 is a natural product of hepatitis Bx antigen and also a downstream gene of HBx, which has previously been found to stimulate HepG2 cell growth and survival¹⁸. In addition, URG4 could significantly enhance cell survival in gastric cancer and ovarian cancer¹⁹, but such potential oncogenic function of endogenous URG4 in NPC remains unclear. As our best knowledge, apoptosis is a process of programmed cell death and characterized as cell shrinkage and nuclear DNA fragmentation^{20,21}. Thus, we speculated that activation of apoptosis might be a possible mechanism by which URG4 silencing inhibits NPC cell survival. As expected, our results of Annexin-V assay revealed a significant increase of apoptotic cells number after URG4 knockdown.

Consistently, pro-apoptosis proteins, including cleaved-caspase-3, Bax and cleaved-PARP were increased, while anti-apoptosis proteins Bcl-2 and surviving were decreased in shURG4 group in HONE1 cells. As we know, a combination of accumulation of apoptotic activators or loss of apoptotic inhibitors may cause mitotic cell death, of which caspases, BCL-2 family of proteins²² and survivin²³ are main players of apoptotic pathway. Caspase-3 is the key enzyme involved in central part of cell apoptosis²⁴ and could cut its specificity substrate such as PARP to induce apoptosis^{25,26}. Bcl-2 and BAX belong to members of BCL-2 family, which both contribute to pro-apoptotic signaling²⁷. Therefore, we inferred that activating cell apoptotic related signaling is the mechanism of URG4 inducing cell apoptosis.

Conclusions

According to the above results, we found silencing URG4 could inhibit proliferation of NPC cells by inducing apoptosis. Its induction of apoptosis may activate the caspase, upregulate the expression of Bax, and downregulate the expression of Bcl-2 and survivin. These findings will provide an experiment basis for URG4 as a potentially therapeutic target in NPC. Additionally, further research is still needed to expose the further molecular mechanism of the oncogenic function of URG4 in NPC *in vivo*.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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