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BNIP1 inhibits cell proliferation, migration and invasion, and promotes apoptosis by mTOR in cervical cancer cells

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Abstract. – OBJECTIVE: BNIP1, a member of the BH3-only protein family, plays essential roles in a variety of biological processes. However, the mechanism and function of BNIP1 are still unknown in cervical cancer. We aim to explore the roles of BNIP1 on cervical cancer cell proliferation, apoptosis, migration, and invasion abilities by mTOR signaling pathway.

PATIENTS AND METHODS: qRT-PCR and Western blot assays were performed to assess BNIP, mTOR, and p70S6K1 expressions. CCK-8, transwell and flow cytometry assays were used to measure the representative proliferation, migration, invasion, and apoptosis abilities.

RESULTS: Our findings indicated that BNIP1 is down-expressed in cervical cancer tissues and cells, and was negatively associated with lymphatic metastasis. Overexpression of BNIP1 suppressed proliferation, migration and invasion, and promoted apoptosis of cervical cancer cells. Silence of BNIP1 by siRNAs accelerated proliferation, migration and invasion, and inhibited apoptosis of cervical cancer cells. In addition, we found that BNIP1 significantly inhibited mTOR, p70S6K1, and p-p70S6K1 expressions; BNIP1 affected the proliferation, apoptosis, migration, and invasion abilities of cervical cancer cells by regulating mTOR expression.

CONCLUSIONS: BNIP1 can be considered a marker for cervical carcinoma therapy.

Key Words:
BNIP1, mTOR, Migration, Invasion, Cervical cancer.

Abbreviations

BNIP1: BCL2 and adenovirus E1B 19-kDa-interacting protein 1; mTOR: mammalian target of rapamycin; p70S6K: p70 ribosomal protein S6 kinase; HPV: Human papillomavirus infection; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; qRT-PCR: quantitative reverse transcriptase polymerase chain reaction; CCK-8: Cell counting kit-8; FIGO: International Federation of Gynecology and Obstetrics; BCL2: B-cell lymphoma 2; ER: endoplasmic reticulum; BH3: homology domain 3; DMEM: Dulbecco's modified Eagle's medium; FBS: fetal bovine serum; siRNA: small interfering RNA; DMSO: dimethylsulfoxide; PI3K: phosphoinositide 3-kinase; AKT: protein kinase B.

Introduction

Cervical cancer is the second most common type of cancer among women worldwide, and is the fourth leading cause of cancer-related death, resulting in approximately 300,000 deaths per year[1]. Globally, there are approximately 500,000 new cases each year, with 85% of the pathologic types being squamous cell carcinoma[2]. Currently in China, cervical cancer screening is based on exfoliative cytological testing performed at regular intervals[3]. Considering the fact that cervical cancer is mainly caused by high-risk HPV infection, vaccination can be viewed as primary prevention, with screening being secondary[4]. The standard management for patients with early-stage (International Federation of Gynecology and Obstetrics, FIGO, stage IA-IB1) cervical cancer is radical hysterectomy and lymph node dissection and/or radiation with or without chemotherapy[5,6]. The standard management for individuals with locally advanced cervical cancer

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includes external beam radiotherapy with concurrent cisplatin-based chemotherapy and brachytherapy[7,8]. Despite the fact that strategies for the prevention and treatment of tumors have rapidly developed over the past decades and most patients receive standard radiotherapy and chemotherapy, the prognosis for patients with advanced or recurrent cervical cancer remains very poor, and clinical outcomes still vary depending on the patient. The one-year survival rate is only 10-20%[9]. Therefore, many researchers are engaged in finding more effective therapies for this disease. The BNIPs (BCL2 and adenovirus E1B 19-kDa-interacting proteins) comprise a subfamily of BCL2 family proteins, typically containing a single BCL2 homology 3 (BH3) domain[10]. Some researchers have confirmed that BNIPs are involved in two major degradation processes in cells, namely, apoptosis and autophagy. BNIP1 (BCL2 interacting protein 1) along with BNIP-3 and BNIP-3L are members of the BNIP family. BNIP1, predominantly localized to the endoplasmic reticulum (ER), is a pro-apoptotic Bcl-2 homology domain 3 (BH3)-only protein[11]. In addition, the gene coding BNIP1 is located on 5q35.1, and its overexpression results in moderate pro-apoptotic activity[12,13]. Although it is known that the function of BNIPs is transcriptionally regulated under hypoxic conditions in tumors, the association between BNIP1 expression and the migration and invasion of cancer cells such as cervical cancer is largely unknown. mTOR is the target of the molecule rapamycin (or sirolimus), which is a macrolide produced by *Streptomyces hygroscopicus*; it first gained attention because of its broad anti-proliferative properties[14]. This pathway regulates many major cellular processes, and several researchers have suggested the importance of the mTOR pathway for cancer pathogenesis. Also, enhanced expression of mTOR is observed in a wide range of tumors including hepatocellular carcinoma and breast cancer, among others[15,16]. It has firmly been established that high mortality rates in cancer patients are not only associated with primary tumor occurrence but, even more profoundly, with metastases[17-19]. The roles of BNIP1 in cervical cancer progression and metastasis have not been uncovered. To assess the relationship between BNIP1 expression and disease progression, we detected BNIP1 expression in 76 cervical cancer patients. Moreover, we modulated the expression level of BNIP1 in HeLa cells to assess the proliferation, apoptosis, migration and invasion abilities of tumor cells. In this study, we confirmed the inhibitory effects of BNIP1 on the proliferation, migration and invasion, and the stimulating effect of BNIP1 on the apoptosis of cervical cancer cells. We also confirmed that these functions were affected by mTOR signaling in cervical cancer cells.

Patients and Methods

Patients

Seventy-six cervical cancer tissue samples were obtained from the Department of Gynecology and Obstetrics of the Affiliated Hospital of Guizhou Medical University. All samples were obtained with patient informed consent. All research protocol was approved by the Ethics Committee of the Second Affiliated Hospital of Soochow University. Patients with cervical cancer were independently diagnosed by two experienced doctors. All specimens were cervical squamous cell carcinoma, with 56 samples of stage I and 20 samples of stage II, according to the FIGO standard. Lymphatic metastasis was present in 31 patients.

Cell Culture and Group Assignment

The HeLa cell line was cultured in Dulbecco's modified Eagle's medium (DMEM, Cat. # SH30243, HyClone, GE Healthcare Life Sciences, South-Logan, UT, USA) with 10% fetal bovine serum (FBS, Cat. #S1810, Biowest, Nuaillé, France), 100 U/ml penicillin, and 100 microg/mL streptomycin (Invitrogen, Carlsbad, CA, USA). Cells were incubated in a 5% CO_2 incubator at 37°C. At 70-80% confluence, cells were split according to the standard procedures. HeLa cells were randomly assigned to one of five groups: (A) HeLa cells in Group A were treated with BNIP1 small interfering RNA (siRNA); (B) HeLa cells in Group B were transfected with pcDNA3.0 vector expressing BNIP1; (C) HeLa cells in Group C were treated with control siRNA; (D) HeLa cells in Group D were transfected with empty pcDNA3.0 vector; (E) Group E received no treatment. Subsequently, for detecting the molecular mechanism associated with BNIP1, HeLa cells were randomly assigned to one of four groups: (A) HeLa cells in Group A were treated with pcDNA3.0 vector expressing BNIP1 and dimethyl sulfoxide (DMSO); (B) HeLa cells in Group B were transfected with pcDNA3.0 vector expressing BNIP1 and mTOR activator (MHY1485, Sigma-Aldrich, St. Louis, MO, USA, SML0810); (C) HeLa cells

in Group C were transfected with BNIP1 small interfering RNA (siRNA) and DMSO (cat no. 2225; Ajax Finechem, Australia); (D) HeLa cells in Group D were transfected with BNIP1 small interfering RNA (siRNA) and mTOR inhibitor (Rapamycin, Cat. #V900930, Sigma-Aldrich; St. Louis, MO, USA).

BNIP1 Knockdown and Overexpression

cDNA encoding BNIP1 was amplified by PCR and later sub-cloned into the pcDNA3.0 vector. The empty pcDNA3.0 vector was used as a negative control. siRNA targeting BNIP1 was obtained commercially. Transfections were performed using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

RNA Extraction and Real-Time PCR (qPCR)

For qPCR analysis, the total RNA was extracted from different cell lines (Siha, HeLa, Caski, and C4-1, C-33a) using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The first-strand cDNA was synthesized from 1 µg of total RNA using the Reverse Transcription System Bestar qPCR RT kit (Bestar, Shanghai, China) according to the manufacturer's instruction. Each assay was performed in triplicate, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the endogenous control gene. The primer sequences used were as follows: BNIP1, 5'-CCAGTTTCTCTATCAAAGGGC-3' (forward) and 5'-ACTGAAGGTAACAGGT-3' (reverse); mTOR, 5'-AAAACCTCTGCCAGTGCTAT-3' (forward) and 5'-ACTGTCCTGGGAACCAAATC-3' (reverse); GAPDH, 5'-TGTTCGTCATGGGTGTGAAC-3' (forward) and 5'-ATGGCATGGACTGTGGTCAT-3' (reverse). The mRNA level of BNIP1 and mTOR, relative to GAPDH levels, was calculated using the $2^{-\Delta\Delta Ct}$ method and normalized using GAPDH cDNA as an internal control[20]. Finally, a cell line with moderate BNIP1 expression was selected for the following experiments.

Western Blotting

Western blotting was performed to determine BNIP1/mTOR protein expression. All proteins were resolved on 10% SDS-denatured polyacrylamide gels and transferred onto a polyvinylidene difluoride (PVDF) membrane (cat# IPVH00010, Merck Millipore, Billerica, MA, USA). Membranes were incubated with blocking buffer for 80 min at room temperature and then incubated with an antibody against BNIP1 (Dilution, 1:1000; Abcam, ab151551, Cambridge, MA, USA), mTOR (Dilution, 1:1000; Abcam, ab2732, Cambridge, MA, USA), p70S6K1 (Dilution, 1:1000; Abcam, ab32529, Cambridge, MA, USA), p-p70S6K1 (Dilution, 1:1000; Abcam, ab2571, Cambridge, MA, USA) and GAPDH (Dilution, 1:3000; Abcam, ab8245, Cambridge, MA, USA) overnight at 5°C. The membranes were washed and incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody (AuGCT, Inc., Beijing, China). Protein expression was assessed by enhanced chemiluminescence and exposure to chemiluminescent film. Lab Works Image Acquisition and Analysis Software (UVP) were used to quantitate band intensities.

Cell Counting Kit-8 (CCK-8) Assay

Cell proliferation was measured using the CCK-8 (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions. The treated HeLa (2×10^3 cells/well) were seeded into a 96-well plate and maintained at 37°C for 12, 24, 48, and 72 hrs. Then, 20 µl of CCK-8 solution was added into each well. After 3 hrs, a microplate reader was used to measure the absorbance at 450 nm.

Flow Cytometric Analysis

For cell apoptosis assay, cell apoptosis was measured with Annexin V-FITC/PI apoptosis detection kit (BestBio, Shanghai, China) according to the experiment instruction. The treated HeLa cells (1×10^6 cells/mL) were digested with trypsin, centrifuged (1000 rpm, 5 min, 4°C), washed with PBS, re-suspended using 1 × binding buffer (100 µL). Then, the cells were double stained with Annexin V-FITC/PI in the dark for 15 minutes at room temperature. Cell apoptosis was detected by flow cytometry using a FACS Calibur Flow Cytometer (BD Biosciences, Franklin Lakes, NJ, USA).

Cell Migration and Invasion Assays

For transwell migration assays, 1×10^5 HeLa cells in 200 ml of DMEM without fetal bovine serum (FBS) were seeded in the upper part of each transwell chamber (pore size, 8 µm; Corning Costar, Corning, NY, USA) containing a non-coated membrane. For the invasion assay, 1×10^5 HeLa cells were seeded in the upper chamber of each insert, which was coated with 50 µl of 2 microg/mL Matrigel growth factor, and 500 µl of DMEM with 20% FBS was added to the lower part of the chamber. After incubating for several hours, the chambers were disassembled, and the membranes were stained with a 2% crystal violet solution for

Table I. Relationship between BNIP1 expression and clinicopathological features in 76 cervical cancer cases (***$p < 0.001$).

Variance	No. of patients (%)	BNIP-1 (related expression)	*p*-value
Stage			
I B1	34 (44.7)	2.5	
I B2	22 (28.9)	2.1	***
II A	20 (26.4)	0.5	
Diameter			
< 4	40 (52.6)	2.3	***
≥ 4	36 (47.4)	0.6	
Grade			
Low	44 (57.9)	3.3	
Moderate	26 (34.2)	1.2	0.485
High	6 (7.9)	0.8	
Depth of muscle invasion			
< 1/2	41 (53.9)	1.9	***
≥ 1/2	35 (46.1)	0.5	
Intravascular tumor thrombus			
Negative	22 (28.9)	1.2	0.295
Positive	54 (71.1)	0.8	
Lymph node metastasis			
Negative	45 (59.2)	4.8	***
Positive	31 (40.8)	0.6	

15 min and placed on a glass slide. Cells that had migrated across the membrane were then counted from five random fields using a light microscope. All assays were performed three independent times in triplicate.

Statistical Analysis

The results are expressed as mean ± standard deviation (SD). Statistical significance was evaluated using the SPSS software package (standard V.19.0, SPSS Inc., Chicago, IL, USA). The comparisons of the clinicopathological variables and BNIP1 expression were conducted by Spearman analysis. The comparisons between two groups were conducted by performing a Student's *t*-test or one-way analysis of variance (ANOVA) with post-hoc Tukey test, and two-way ANOVA for comparison between different groups. A *p*-value less than 0.05 was considered statistically significant.

Results

BNIP1 Expression is Associated with Clinicopathological Features

To investigate the association between BNIP1 expression and clinicopathological features including stage, diameter, grade, depth of muscle invasion, intravascular tumor thrombus, and lymph node metastasis, we collected tissue samples from 76 cervical cancer patients with established clinicopathological data (Table I). The association between BNIP1 expression and stage, diameter, depth of muscle invasion, and lymph node metastasis was considered statistically significant ($p < 0.001$). However, the association between BNIP1 and grade, as well as intravascular tumor thrombus, did not achieve statistical significance. We found that the increasing stage, specifically I B1 (0.5), I B2 (2.1), and II A (2.5), corresponded with higher BNIP1 expression. The lower diameter group (0.6) exhibited less BNIP1 expression than the larger diameter group (2.3). We detected that, when the depth of muscle invasion was less than 1/2, relative BNIP1 expression was decreased by 0.5-fold; in contrast, when invasion was greater than 1/2, expression was increased 1.9-fold. In other words, deeper muscle invasion was associated with higher BNIP1 expression. We also found that relative expression of BNIP1 was 0.6 *vs.* 4.8 among patients with node metastasis-positive versus node metastasis-negative disease, respectively. Taken together, these findings suggest that BNIP1 might be involved in the suppression of malignant properties in cervical cancer cells.

BNIP1 is Down-Expressed in Cervical Cancer Tissues and Cells

We sorted out the non-tumor tissues (n = 30), stage I B1 (n = 34), stage I B2 (n = 22), and stage II A (n = 20) cervical cancer tissues. The expres-

sion level of BNIP1 was analyzed by qRT-PCR and Western blot assays. Our results showed that the expression level of BNIP1 was downregulated in cervical cancer tissues compared to non-tumor tissues, and BNIP1 expression was related to the grading of tumor ($p < 0.01$, $p < 0.001$, Figure 1A and B). In addition, we performed qRT-PCR and Western blot assays to test the relative expression of BNIP1 in different cell lines including C-33a, Caski, HeLa, SiHa, and C4-1 cells. As shown in Figure 1C and 1D, it was evident that the relative expression of BNIP1 is moderate in HeLa cells, as compared to that in the other cell lines. Thus, HeLa cells were selected for the following experiments.

BNIP1 Suppresses Proliferation, Migration and Invasion, and Promotes Apoptosis of Cervical Cancer Cells

To explore the roles of BNIP1 in cervical cancer cells, we augmented or inhibited BNIP1 levels using BNIP1 expression vectors or siRNAs, and subsequently assessed the migration, invasion, and apoptosis abilities of cervical cancer cells by performing transwell and flow cytometry assays. The results indicated that the number of migrated cells per field was significantly diminished in the BNIP1 overexpression group compared to that in the control group. Conversely, suppression of BNIP1 significantly promoted cell migration ability compared to that in control cells ($p < 0.01$,

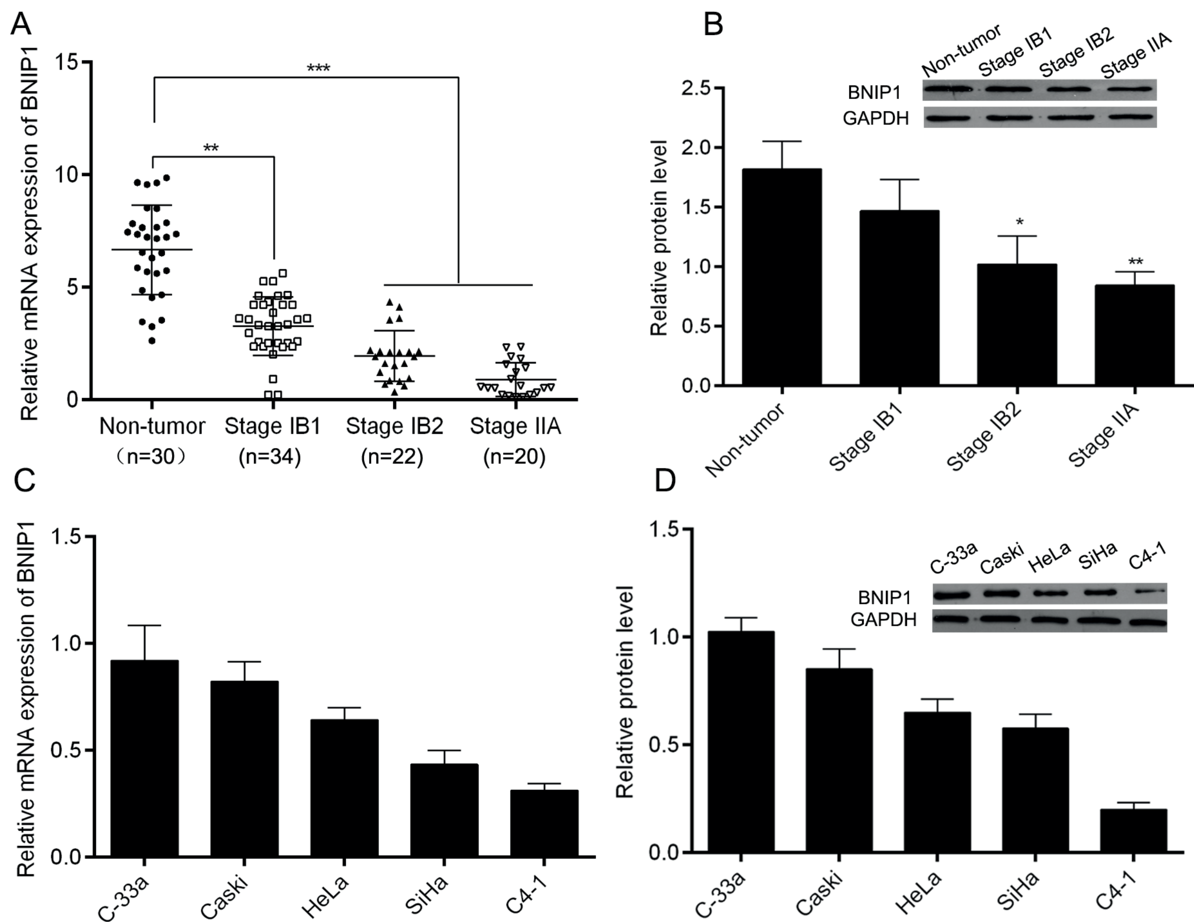

Figure 1. BNIP1 is downexpressed in cervical cancer tissues and cells. ***A,*** The mRNA expression level of BNIP1 was detected by qRT-PCR in non-tumor (n = 30), stage I B1 (n = 34), stage I B2 (n = 22), and stage II A (n = 20) tissues (**$p < 0.01$, ***$p < 0.001$). ***B,*** The protein expression level of *BNIP1* was detected by Western blot assay in non-tumor, stage I B1, stage I B2, and stage II A tissues (*$p < 0.05$, **$p < 0.01$), GAPDH was used as internal reference. ***C,*** The relative mRNA expression level of BNIP1 was detected by qRT-PCR in C-33a, Caski, HeLa, SiHa, and C4-1 cells. ***D,*** The protein expression level of BNIP1 was analyzed by Western Blot assay. GAPDH was used as loading control. Quantitative proteins were analyzed according to the protein gray values.

$p < 0.001$, Figure 2A and B). Based on the cell invasion assay results, HeLa cell invasion was significantly inhibited with BNIP1 overexpression, and was markedly increased in the BNIP1 knockdown group, compared to that in the respective negative control groups. This was in accordance with the results of the migration assays ($p < 0.01$, $p < 0.001$, Figure 2A and C). Together, these results indicate that BNIP1 inhibits the migration and invasion of cervical cancer cells. We also found that overexpression of BNIP1 significantly increased the apoptosis rates of HeLa cells, and knockdown of BNIP1 significantly decreased the apoptosis rates of HeLa cells ($p < 0.01$, $p < 0.001$, Figure 2A and D). The CCK-8 assay also indicated that overexpression of BNIP1 significantly inhibited the proliferation capacity of HeLa cells, and knockdown of BNIP1 significantly promoted the proliferation ability of HeLa cells ($p < 0.05$, $p < 0.01$, Figure 2E).

BNIP1 Significantly Inhibits mTOR, p70S6K1, and p-p70S6K1 Expressions

To further explore the regulatory mechanism of BNIP1 in cervical cancer, we then analyzed the influences of BNIP1 on mTOR, p70S6K1, and p-p70S6K1 expressions. As shown in Figure 3A and B, overexpression of BNIP1 significantly inhibited the mRNA expression levels of mTOR and p70S6K1 in HeLa cells, and knockdown of BNIP1 markedly accelerated the mRNA expression levels of mTOR and p70S6K1 in HeLa cells ($p < 0.05$). Western blot assay also proved that overexpression of BNIP1 dramatically decreased mTOR, p70S6K1, and p-p70S6K1 expressions, and knockdown of BNIP1 markedly increased mTOR, p70S6K1, and p-p70S6K1 expressions ($p < 0.05$, $p < 0.01$, Figure 3C and D). Collectively, these data indicated that BNIP1 negatively regulates endogenous mTOR, p70S6K1, and p-p70S6K1 expressions.

BNIP1 Affects Cervical Cancer Cell Proliferation, Apoptosis, Migration and Invasion by mTOR Signaling Pathway

To confirm the effects of BNIP1 on HeLa cells by mTOR, HeLa cells were transfected with BNIP1 + DMSO, BNIP1 + mTOR activator (MHY1485), BNIP1 siRNAs + DMSO, and BNIP1 siRNAs + mTOR inhibitor (Rapamycin), respectively. The migration, invasion, and apoptosis abilities were detected by transwell and flow cytometry assays. The results demonstrated that overexpression of BNIP1 significantly inhibited cell migration and invasion, mTOR activator (MHY1485) treatment then blocked this inibition mediated by BNIP1; silence of BNIP1 by siRNAs markedly increased cell migration and invasion, mTOR inhibitor (Rapamycin) treatment then held back this increasion mediated by BNIP1 knockdown ($p < 0.01$, $p < 0.001$, Figure 4A-C). Flow cytometry assay then certified that overexpression of BNIP1 observably promoted cell apoptosis, mTOR activator (MHY1485) treatment then blocked this promotion mediated by BNIP1; silence of BNIP1 by siRNAs markedly inhibited cell apoptosis, mTOR inhibitor (Rapamycin) treatment held back this inhibition mediated by BNIP1 knockdown ($p < 0.001$, Figure 4A and D). Furthermore, we further detected that whether BNIP1 inhibits the proliferation ability of cervical cancer cells via mTOR. The CCK-8 results indicated that BNIP1 suppressed the proliferation ability of HeLa cells via mTOR; silence of BNIP1 promoted the proliferation ability of A2780 cells through mTOR ($p < 0.05$, $p < 0.01$, Figure 4E). Together, we concluded that BNIP1 inhibited cell proliferation, migration and invasion, and promoted apoptosis by mTOR signaling in cervical cancer cells.

Discussion

Cervical cancer, as the second most common cancer in women, has become a major health care problem in developing countries[21]. Persistent infection with high-risk human papillomaviruses (HPV), particularly HPV16 and HPV18, is associated with an elevated high for this disease[22]. Invasion and metastasis through the bloodstream and lymph vessels are critical steps in the progression of cervical cancer[23]. Currently, chemotherapy is a widely-used treatment for patients with advanced or recurrent cervical cancer, because this disease is extremely chemo-sensitive[24]. Considering the existence of drug-resistance, a more effective, reliable therapeutic method is needed to delay cancer progression and even cure the disease. BNIP1 is known to involved in two major degradation processes in cells, namely, apoptosis and autophagy. It has been reported[10] that following the induction of starvation-induced autophagy, BNIP1 mRNA is selectively increased in cultured neurons. However, there is no related research clarifying the role of BNIP1 in cancer. According to the previously reported[10,11,24] function of BNIP1, we sought to determine whether

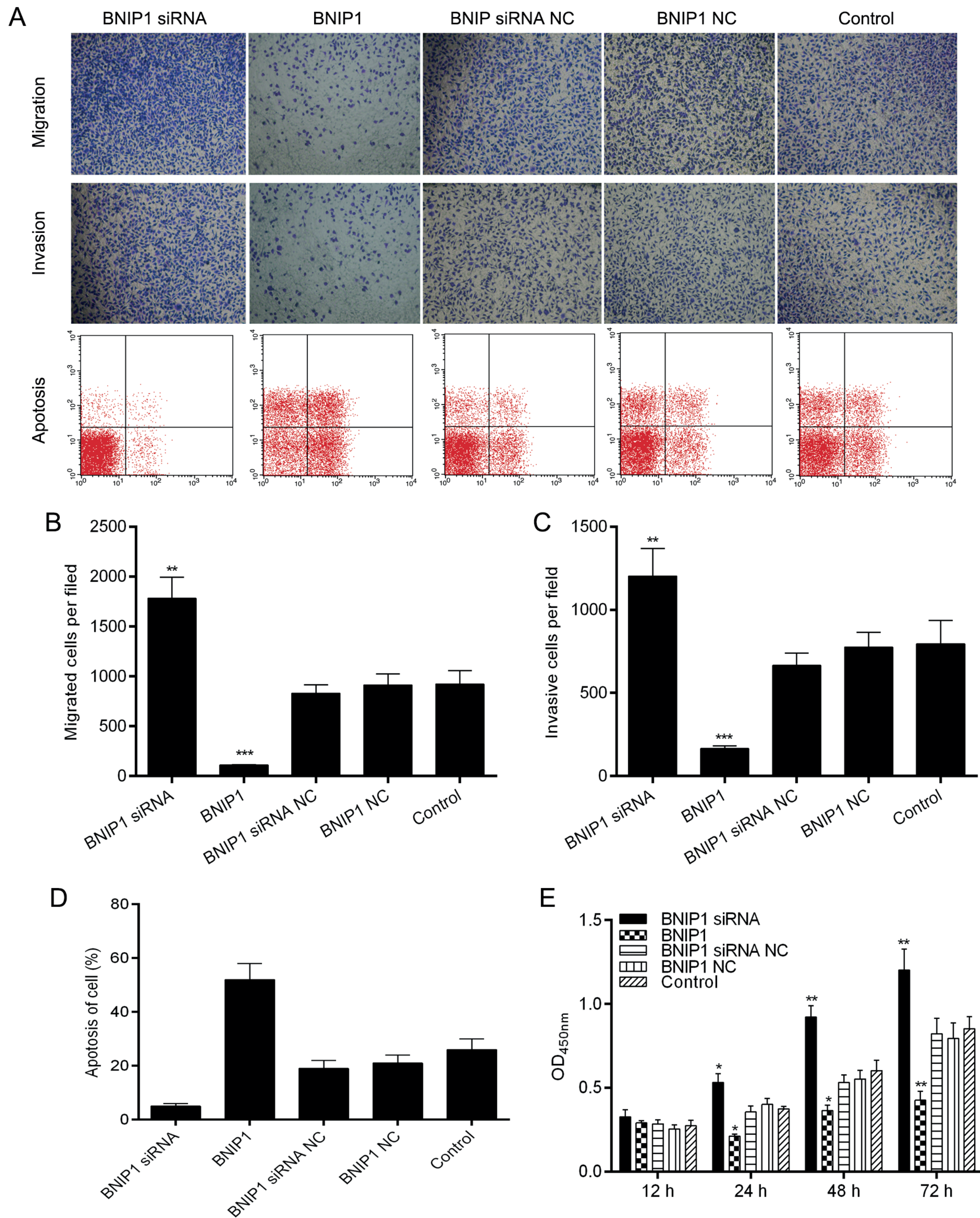

Figure 2. BNIP1 suppresses proliferation, migration and invasion, and promotes apoptosis of cervical cancer cells. HeLa cells were transfected with BNIP1 siRNAs, BNIP1 plasmid, BNIP1 siRNAs NC, BNIP1 NC, and control, respectively. NC, negative control. ***A,*** The migration, invasion and apoptosis abilities were measured by transwell and flow cytometry assays in treated HeLa cells. ***B,*** The migrated cells were counted in per filed (**$p < 0.01$). ***C,*** The invasive cells were counted in per filed (**$p < 0.01$). ***D,*** The apoptosis rates were analyzed in different groups. The right lower quadrant represents the relative proportions of early apoptosis; the right upper quadrant represents the relative proportions of late apoptosis. ***E,*** CCK-8 assay was performed to detect the proliferation ability of the treated HeLa cells (*$p < 0.05$, **$p < 0.01$).

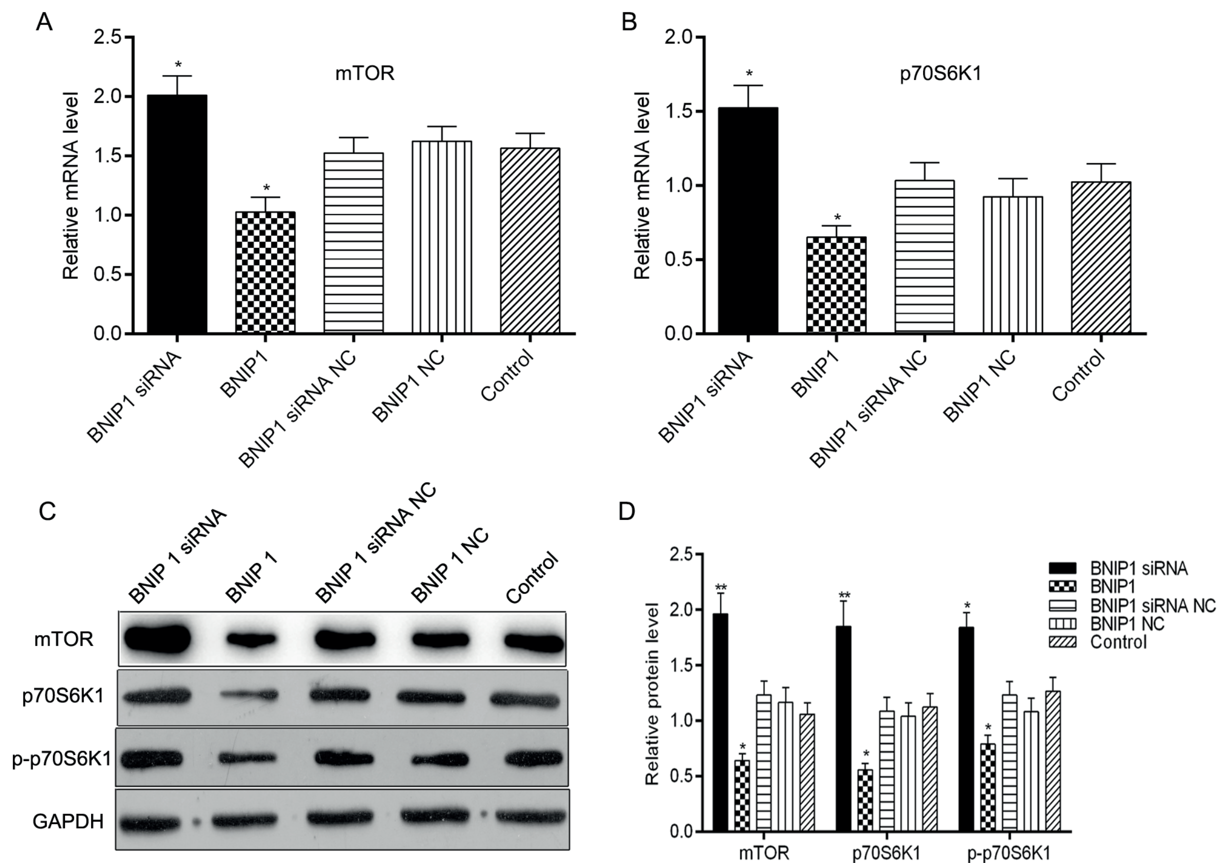

Figure 3. BNIP1 significantly inhibits mTOR, p70S6K1, and p-p70S6K1 expressions. ***A-B,*** qRT-PCR was used to analyze the mRNA expression levels of mTOR and p70S6K1 in treated HeLa cells (*$p < 0.05$). ***C,*** Representative images of Western blotting for mTOR expression after modulating BNIP1 expression. ***D,*** The quantitative analysis of Western blotting data (*$p < 0.05$, **$p < 0.01$).

there is a significant association between BNIP1 expression and clinicopathological features of tumors. In recent years, our group has focused also on the effect of BNIP1 expression on cervical cancer development, and especially growth and metastasis. We proved that BNIP1 also affects the proliferation, apoptosis, migration, and invasion of cervical cancer cells. mTOR is a kinase that is critical for the regulation of many cellular events such as cell proliferation, growth, survival, differentiation, adhesion, motility, angiogenesis, and metastasis[25,26]. Furthermore, the PI3K/AKT/mTOR pathway is dysregulated in a large proportion of human cancers, including cervical carcinoma, and it regulates the apoptotic response through its ability to interact with a number of key players in the apoptotic process[27]. This indicates that it might be a potential therapeutic target for the treatment of this malignancy. Moreover, the mTOR pathway is involved in chemosensitivity to cisplatin in cervical cancer[28]. There are many products that have been shown to prevent cell proliferation, induce apoptosis, suppress metastasis, and inhibit angiogenesis in cervical cancer by regulating the PI3K/AKT/mTOR signaling pathway[29]. Meanwhile, we found that BNIP1 could inhibit the mTOR levels. Herein, we considered that BNIP1 might inhibit cell proliferation, migration and invasion, and promote apoptosis by mTOR signaling pathway in cervical cancer cells. The 70 kDa ribosomal S6 kinase 1 (p70S6K1), a downstream target of mammalian target of mTOR, is an important regulator of cell cycle progression, cell proliferation, and cell survival, and plays a vital role in cell signal networks[30,31]. Previous investigations[32] have also shown that mTOR/P70S6K1 signaling pathway is involved in cisplatin resistance of ovarian cancer cells; mTOR/P70S6K1 signaling pathway participates in the processes of cervical cancer cell apoptosis[33]. In our study, we proved that BNIP1 significantly inhibited the levels of mTOR, p70S6K1, and p-p70S6K1.

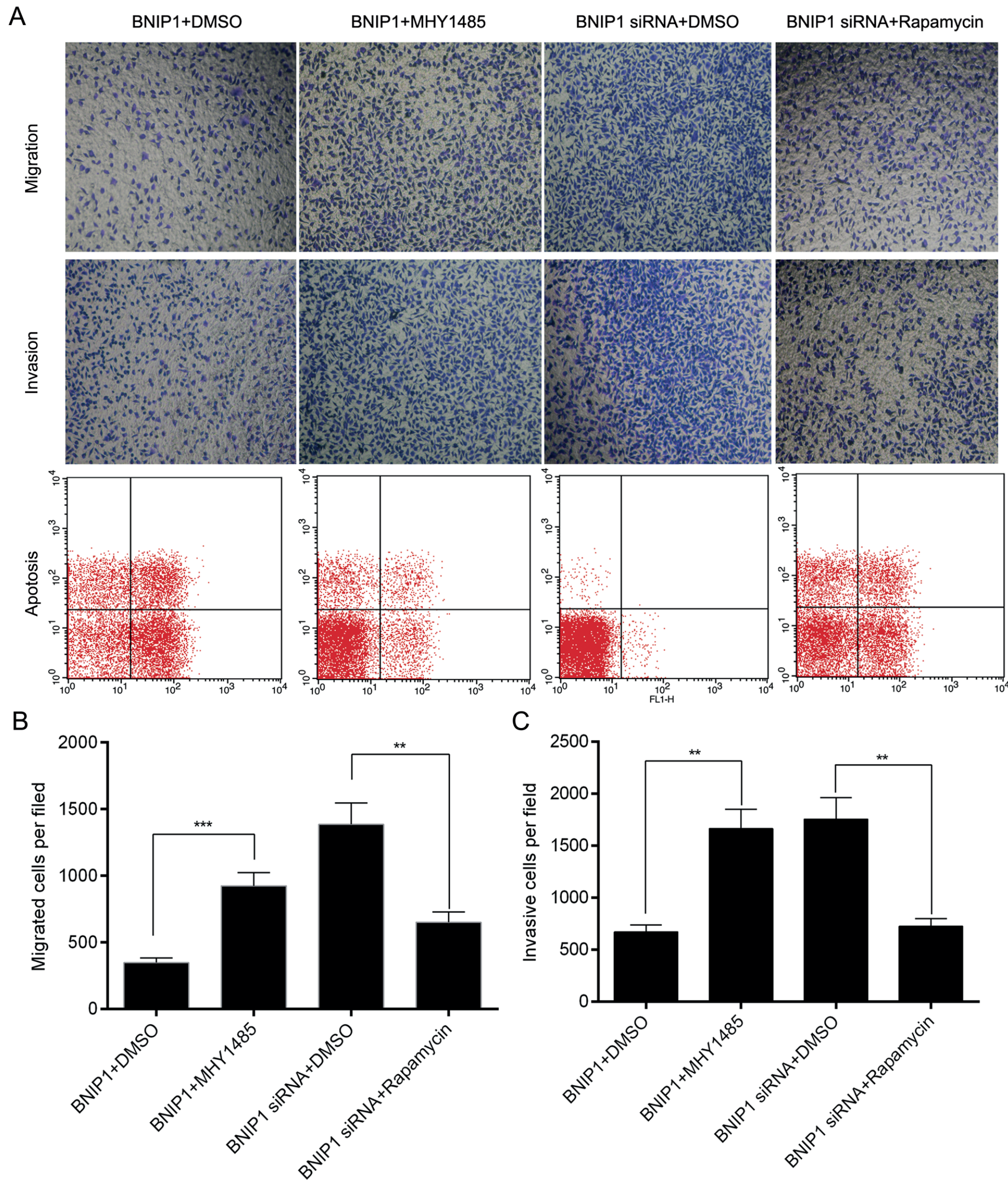

Figure 4. Silence of BNIP1 accelerates proliferation, migration and invasion, and suppresses apoptosis of cervical cancer cells by mTOR. HeLa cells were transfected with BNIP1 + DMSO, BNIP1 + mTOR activator (MHY1485), BNIP1 siRNAs + DMSO, and BNIP1 siRNAs + mTOR inhibitor (Rapamycin), respectively. ***A,*** Representative migration, invasion, and apoptosis images were shown in HeLa cells after modulation of BNIP1 and mTOR expressions. The statistical analysis of cell migration ***(B),*** invasion ***(C)***, and flow cytometry.

Figure continued

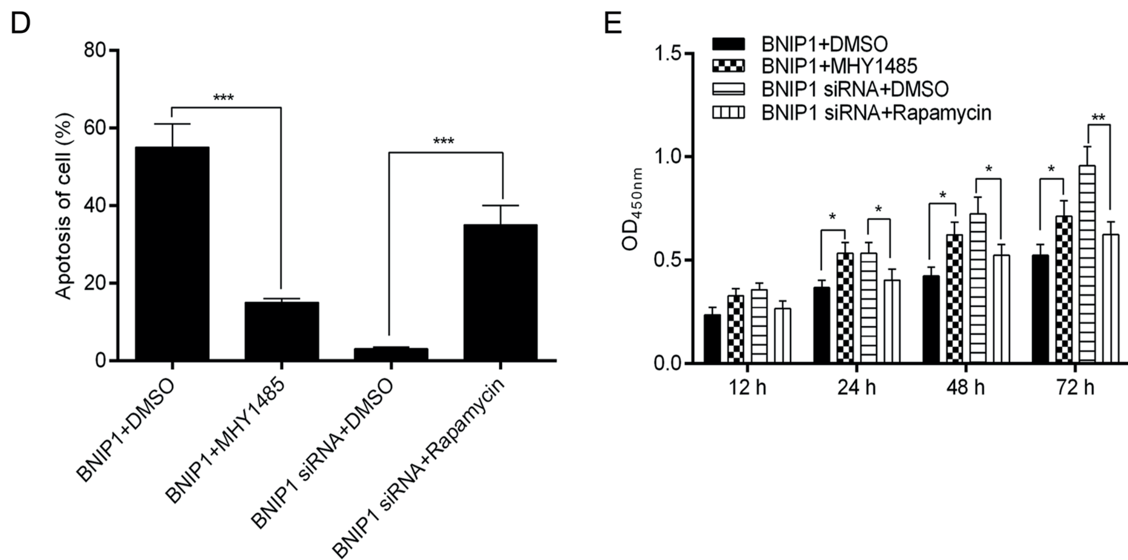

Figure 4. *(Continued). (**D**)* assays (** $p < 0.01$, *** $p < 0.001$). ***E,*** The proliferation results were detected by CCK-8 assay (* $p < 0.05$, ** $p < 0.01$).

Conclusions

We found that BNIP1 has a pivotal tumor suppressor role in cervical cancer. It can inhibit proliferation, migration and invasion, and accelerate apoptosis by mTOR signaling pathway. However, the molecular and cellular mechanisms underlying this association have not been determined completely. Further researches will be necessary to uncover this integral mechanism. Animal studies are also needed to confirm our results *in vivo*. In brief, we provide the basis for future clinical assessment of BNIP1 targeting strategies; however, *in vivo* experiments are needed.

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Conflict of Interest

The Authors declare that they have no conflict of interest.

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