European Review for Medical and Pharmacological Sciences 2019; 23: 1397-1407

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

F.-H. LI[1,2], L. XIANG[3], L. RAN[2], S. ZHOU[3], Z. HUANG[3], M. CH[illegible], W.-F. Y[illegible]

[1]Department of Oncology, the Second Affiliated Hospital of Soochow U[illegible]ersity, [illegible]ou, C[illegible]na
[2]Department of Breast and Gynecologic Oncology, the Affiliated Hospit[illegible] [illegible]ou Me[illegible] University, Guizhou Cancer Hospital, Guiyang, China
[3]Department of Interventional Radiology, the Affiliated Hospital [illegible]uizhou M[illegible] U[illegible]ersity, Guiyang, China
[4]The Key Laboratory of Medical Molecular Biology, GuiZhou M[illegible] [illegible]iversity, Guiy[illegible]ng, China

Fenghu Li and Lei Xiang contributed equally

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 canc[illegible]ll proliferation, apoptosis, migration, and i[illegible] abilities by mTOR signaling pathway.

PATIENTS AND METHODS: qRT-PCR[illegible]d Western blot assays were performed to as[illegible] BNIP, mTOR, and p70S6K1 expressions. CCK[illegible] transwell and flow cytometry as[illegible] were use[illegible] to measure the representativ[illegible]tion, migration, invasion, and apopt[illegible] abil[illegible]

RESULTS: Our findings i[illegible]ated th[illegible]NIP1 is down-expressed in cerv[illegible]cancer[illegible] cells, and was negativ[illegible]si[illegible] with[illegible] phatic metastasis. [illegible]rexp[illegible] of BNIP1 suppressed prolif[illegible]on, mig[illegible]and invasion, and promot[illegible]poptosis of c[illegible] cancer cells. Silence[illegible] by siRNAs a[illegible]lerated proliferation, [illegible]ati[illegible] invasion, and inhibited apoptosis of cervic[illegible]cer cells. In addition, we f[illegible] that BNIP1 s[illegible]antly inhibited mTOR, [illegible]6K1, and p-p70S[illegible] expressions; BNIP1 [illegible]cted t[illegible] proliferation, apoptosis, migrati[illegible]and in[illegible]n abilities of cervical cancer ce[illegible]regul[illegible]g mTO[illegible]xpression.

CO[illegible]S: BN[illegible]can be considered a marker f[illegible]ical c[illegible]noma therapy.

Key[illegible]ds:
B[illegible] mTOR, Migration, Invasion, Cervical cancer.

Abbreviations

[illegible]1: BCL2 and adenovirus E1B 19-kDa-interacting p[illegible]1: mTOR: mammalian target of rapamycin; [illegible]S6K: p70 ribo[illegible]l protein S6 kinase; HPV: Human papillomaviru[illegible]fection; GAPDH: glyceraldehyde [illegible]phate dehyd[illegible]nase; qRT-PCR: quantitative reve[illegible]ascripta[illegible]olymerase chain reaction; CCK-8: Cell [illegible], FIGO: International Federation of Gynecology and Obstetrics; BCL2: B-cell lymphoma 2; ER: endoplasmic reticulum; BH3: homology domain [illegible]M: Dulbecco's modified Eagle's medium; FBS: [illegible]e 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

Corresponding Authors: Ming Chen, MD; e-mail: chenming@zjcc.org.cn
Wenfeng Yu, MD; e-mail: wenfengyu21@sohu.com

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 loca[illegible]n 5q35.1, and its overexpression results in n[illegible] pro-apoptotic activity[12,13]. Although it is [illegible]vn that the function of BNIPs is transcriptional[illegible] gulated under hypoxic conditions in tumors, [illegible] association between BNIP1 expr[illegible]ion and t[illegible] migration and invasion of ca[illegible] such as cervical cancer is largely un[illegible]wn. [illegible]R is the target of the molecule ra[illegible]cin (or [illegible]limus), which is a macrolide pr[illegible]d by *hygroscopicus*; it firs[illegible]ain[illegible]on because of its broad anti-p[illegible]erative [illegible]ties[14]. This pathway regulate[illegible]ny major c[illegible] proces-ses, and severa[illegible]hers have sug[illegible]ted the importance o[illegible] m1[illegible]thway for cancer pathogenesis. Also, enhanc[illegible]ression of mTOR is observ[illegible] a wide range[illegible]ors including hepato[illegible]lar carcinoma and breast cancer, amo[illegible]hers[15,1[illegible] has firmly been established tha[illegible] mor[illegible]y rates i[illegible]ancer patients are not only a[illegible] with r[illegible]ary tumor occurrence but even[illegible] prof[illegible]ly, with metastases[17-19]. [illegible]les o[illegible] cervical cancer progression[illegible] metasta[illegible]have not been uncovered. To asse[illegible]he relationship between BNIP1 expressi[illegible] progression, we detected BNIP1 [illegible]ression in 76 cervical cancer patients. Moreo-[illegible]we modulated the expression level of BNIP1 i[illegible] cells to assess the proliferation, apoptosis, [illegible]igration and invasion abilities of tumor cells. In this study, we confirmed the inhibitory effects of BNIP1 on the proliferation[illegible] and invasion, and the stimulating ef[illegible]of BNI[illegible] on the apoptosis of cervical canc[illegible]ls. We also confirmed that these functions[illegible] affected by mTOR signaling in cervical cance[illegible]

Patients a[illegible] Meth[illegible]ds

Patients

Seventy-six [illegible]ica[illegible] tissu[illegible]amples were obtained[illegible]m the [illegible]me[illegible] of Gynecology and [illegible]etrics of the [illegible]ted Hospital of Gui[illegible]ical Universi[illegible] All samples were ob[illegible]ed w[illegible]atient informed consent. All research protoco[illegible]pproved by the Ethics Co[illegible] of the Seco[illegible]filiated Hospital of S[illegible]ow University. Patients with cervical can-[illegible] were independ[illegible]ntly diagnosed by two experi-[illegible]ed doctors. A[illegible]ecimens were cervical squa-[illegible]s cell carcin[illegible], with 56 samples of stage I a[illegible]samples[illegible]age II, according to the FIGO stan[illegible]tic metastasis was present in 31 patients.

[illegible]ure and Group Assignment

The[illegible]eLa 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 manufacturer's instruction. Each assay was performed in triplicate, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous control gene. The primer sequences used were as follows: BNIP1, 5'-TTCTC-TATCAAAGGGC-3' (forward) and 5'-ACT-GAAGGTAACAGGT-3' (reverse); mTOR, 5'-AAAACCTCTGCCA-TTAT-and 5'-ACTGTCCTC-GA-TC-3' (reverse); GAPDH, GTTC-TGGGTGT-GAAC-3' (forward) and 5'-ATGC-GGACT-GTGGTCAT-3' (reverse). The mRNA level of BNIP1 and mTOR, relative to GAPDH levels, was calculated using the $2^{-\Delta\Delta C}$ method and normalized using GAPDH cDNA as an internal control[20]. Finally, a cell line with moderate BNIP1 expression was selected for following experiments.

Western Blotting

Western blotting was performed to determine mTOR/mTOR expression. All proteins were resolved on 10% SDS-denatured polyacrylamide gels and transferred onto a polyvinylidene difluoride (PVDF) membrane (cat# IPVH00010, 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, ab2[illegible] Cambridge, MA, USA) and GAPDH (Dilution, 1:5000; Abcam, ab8245, Cambridge, MA, USA) overnight at 5°C. The membranes were washed and incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody (EarthOX, Beijing, China). Protein expression was assessed by enhanced chemiluminescence and exposure to chemiluminescent film. UVP Works Image Acquisition and Analysis Software (UVP) were used to quantitate band intensity.

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 × 10³ cells/well) were seeded into a 96-well plate and maintained at 37°C for 12, 24, 48, and 72 hrs. Then, 10 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)	
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
High	6 (7.9)	0.8	
Depth of muscle invasion			
< 1/2	41 (53.9)	1	***
≥ 1/2	35 (46.1)		
Intravascular tumor thrombus			
Negative	22 (28.9)	.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 micro . All assays were performed three inde times in triplicate.

Statistical Analysis

The results are expressed as mean ± standa deviation (SD). Statistical sig was eva- luated using the SPSS soft pac (standard V.19.0, SPSS Inc., C go, IL, A). The comparisons of the clini logi and BNIP1 expressio wer d by Spearman analysis. Th mpari tween two groups were co ed by peric a Student's *t*-test o analysis o ariance (ANOVA) wi ost- ukey test, and two-way ANOV for compa etween different groups. A alue less than as considered statisti significant.

Results

BNIP1 Expression Is Associated with Clinicopathological Features

T vestigate the association between BNIP1 e clinicopathological features ading stage, diameter, grade, depth of mu- invasion, intravascular tumor thrombus, a ph node metastasis, we collected tissue sam from 76 cervical cancer patients with e hed cli pathological data (Table I). The between BNIP1 expression and stage, diameter, depth of muscle invasion, and ymph node metastasis was considered statistically significant ($p < 0.001$). However, the ssoci on 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.

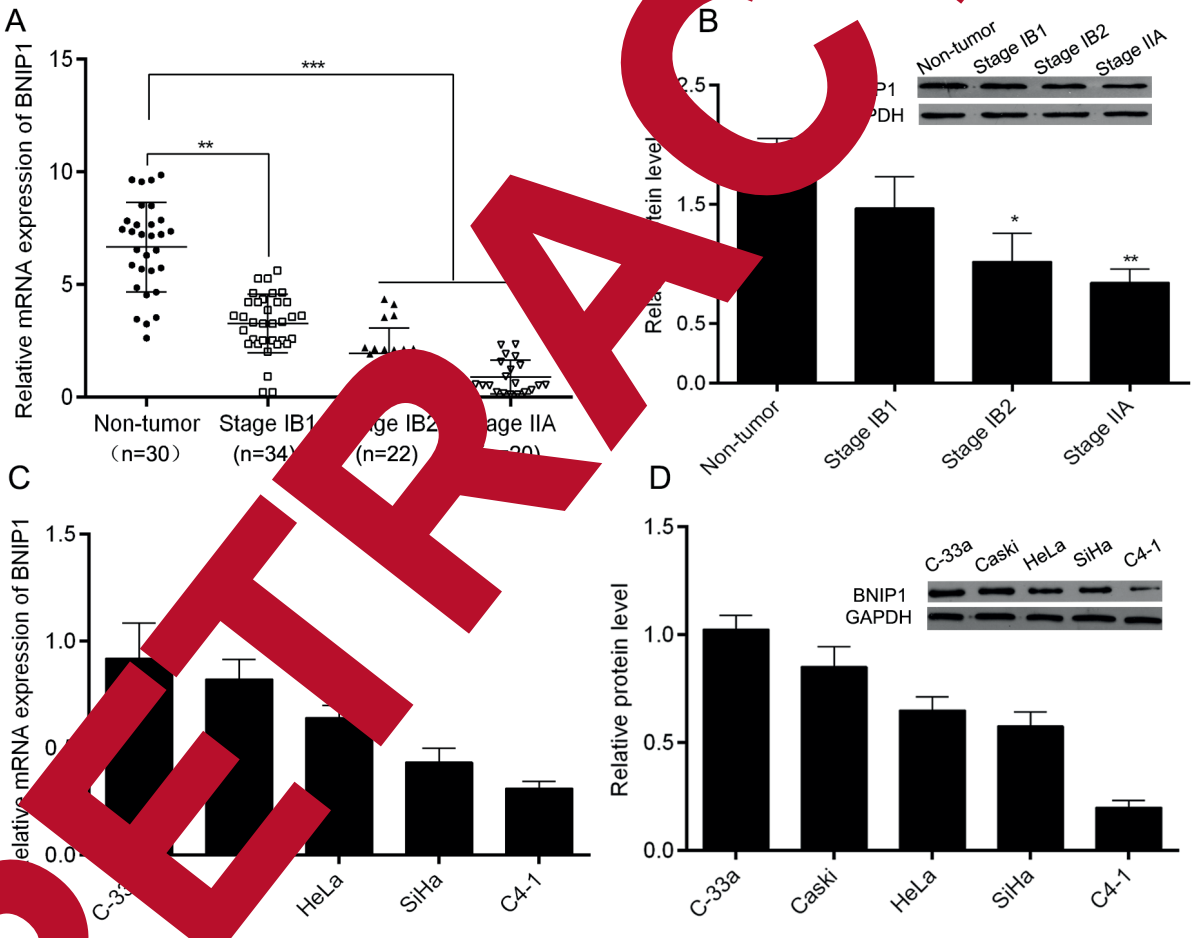
BNIP1 is Down-Expressed in Cervical Cancer Tissues and Cells

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 Pr… Apoptosis of Cervical Cancer …

To explore the roles of BNIP1 … cervical cancer cells, we augmented or inhib… BNIP1 levels using BNIP1 expression vectors … siRNAs, and subsequently assessed the … migration … sion and apoptosis abilities of … vical cancer … performing transwell … low cy…metry as… The results indicate… t the … ber of migrated cells per field w… ific…y dimi…ed in the BNIP1 overe… ssi… comp… to that in the control … p. Con… s… ession of BNIP1 signi…tly promoted … gration ability comp… t in control … s ($p < 0.01$,



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

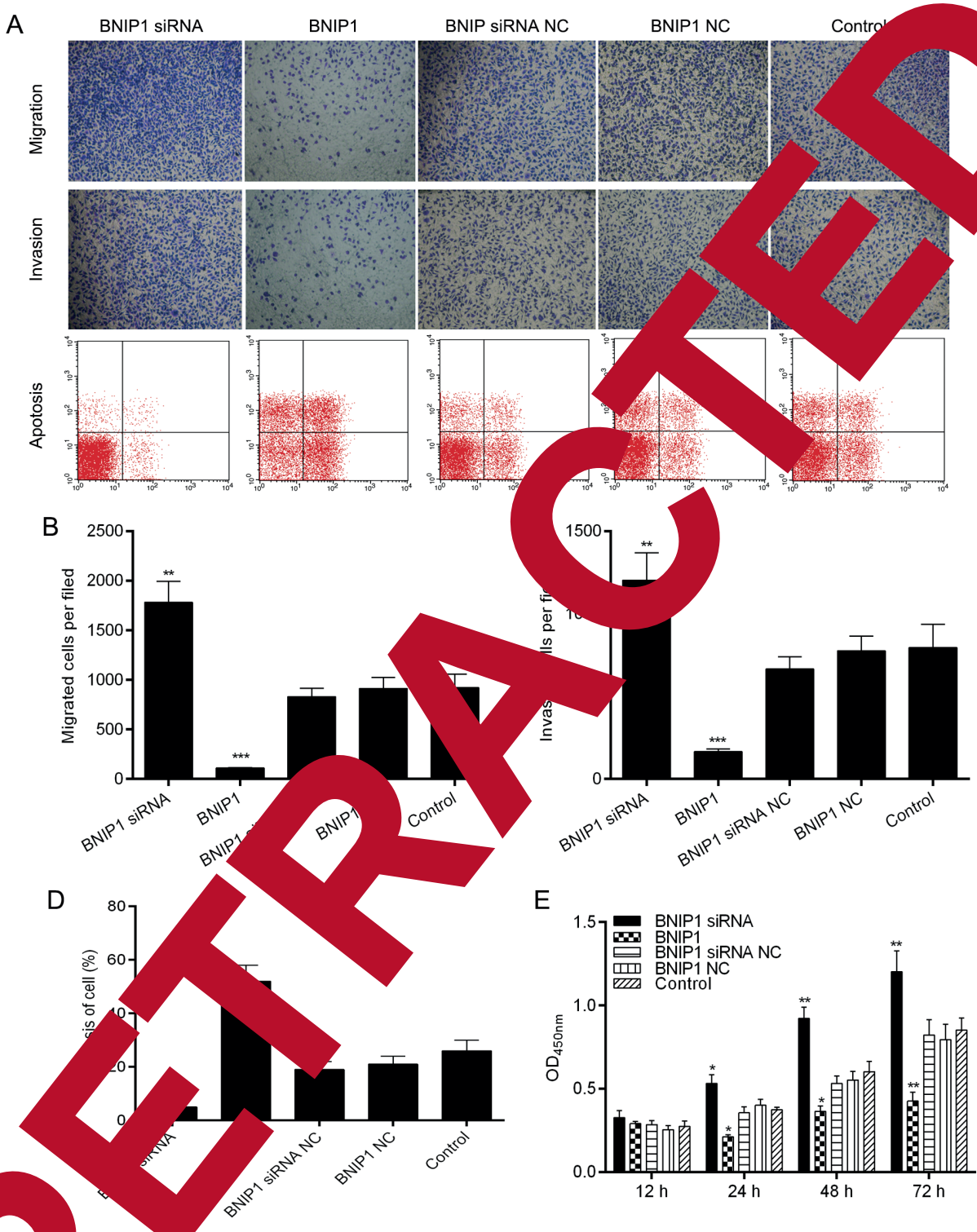
To further explore the regulatory mechanism of BNIP1 in cervical cancer, we then an[illegible] the influences of BNIP1 on mTOR, p[illegible] and p-p70S6K1 expressions. As shown in [illegible]re 3A and B, overexpression of BNIP1 signific[illegible] inhibited the mRNA expression levels of mT[illegible] and p70S6K1 in HeLa cells, and [illegible]nockdown [illegible] BNIP1 markedly accelerated t[illegible] expression levels of mTOR and p7[illegible]K1 in [illegible]a cells ($p < 0.05$). Western blot [illegible]y also [illegible]ed that overexpression of BNIP[illegible]atica[illegible] mTOR, p70S6K1, an[illegible]p-p[illegible]xpressions, and knockdown of [illegible]IP1 m[illegible] increased mTOR, p70S6K1 [illegible]p-p70S6K1 [illegible]sions ($p < 0.05$, $p < 0.0$[illegible]3C and D). C[illegible]ctively, these data ind[illegible]d th[illegible]P1 negatively regulates endoger[illegible]us mTOR, p7[illegible]1, and p-p70S6K1 expressio[illegible]

BNI[illegible]ffect[illegible]rvical Cancer Cell Pr[illegible]atio[illegible]popto[illegible], Migration and Inva[illegible]TOR[illegible]naling Pathway

To co[illegible] the [illegible]ts of BNIP1 on HeLa [illegible]y m[illegible]a cells were transfected wit[illegible]NIP1 + [illegible]O, BNIP1 + mTOR activator ([illegible]Y1485), BNIP1 siRNAs + DMSO, and B[illegible]+ mTOR inhibitor (Rapamycin), [illegible]ectively. The migration, invasion, and apop[illegible] abilities were detected by transwell and f[illegible]tometry assays. The results demonstrated that [illegible]rexpression of BNIP1 significantly inhibited cell migration and invasion, mTOR activator (MHY1485) treatment then block[illegible] bition mediated by BNIP1; silence[illegible]NIP1 siRNAs markedly increased cel[illegible]gration and invasion, mTOR inhibitor (R[illegible]ycin) treatment then held back this increas[illegible]diated by BNIP1 knockdown ($p < 0$[illegible], $p < 0$[illegible]igure 4A-C). Flow cytometry [illegible]y then certi[illegible] overexpression of BN[illegible]obser[illegible]bly pron[illegible] cell apoptosis, mTO[illegible]tivato[illegible]HY1485) treatment then blocke[illegible]pro[illegible]on me[illegible]d by BNIP1; silence[illegible]BN[illegible]RNAs[illegible]rkedly inhibited cell[illegible]ptosis, [illegible]i[illegible]tor (Rapamycin) tr[illegible]nt held back[illegible]ibition mediated by[illegible]ockdown (p[illegible].001, Figure 4A and[illegible] Fur[illegible]re, we further detected that whether BNIP[illegible]bits the proliferation abi[illegible]ervical canc[illegible]ls via mTOR. The C[illegible]8 results indicated that BNIP1 suppressed proliferation[illegible]ility of HeLa cells via mTOR; [illegible]nce of BNIP[illegible]omoted the proliferation abi[illegible]of A2780 ce[illegible]through mTOR ($p < 0.05$, p <[illegible] Figure[illegible] Together, we concluded that BNI[illegible] cell proliferation, migration and invasion, and promoted apoptosis by mTOR [illegible]naling 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



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

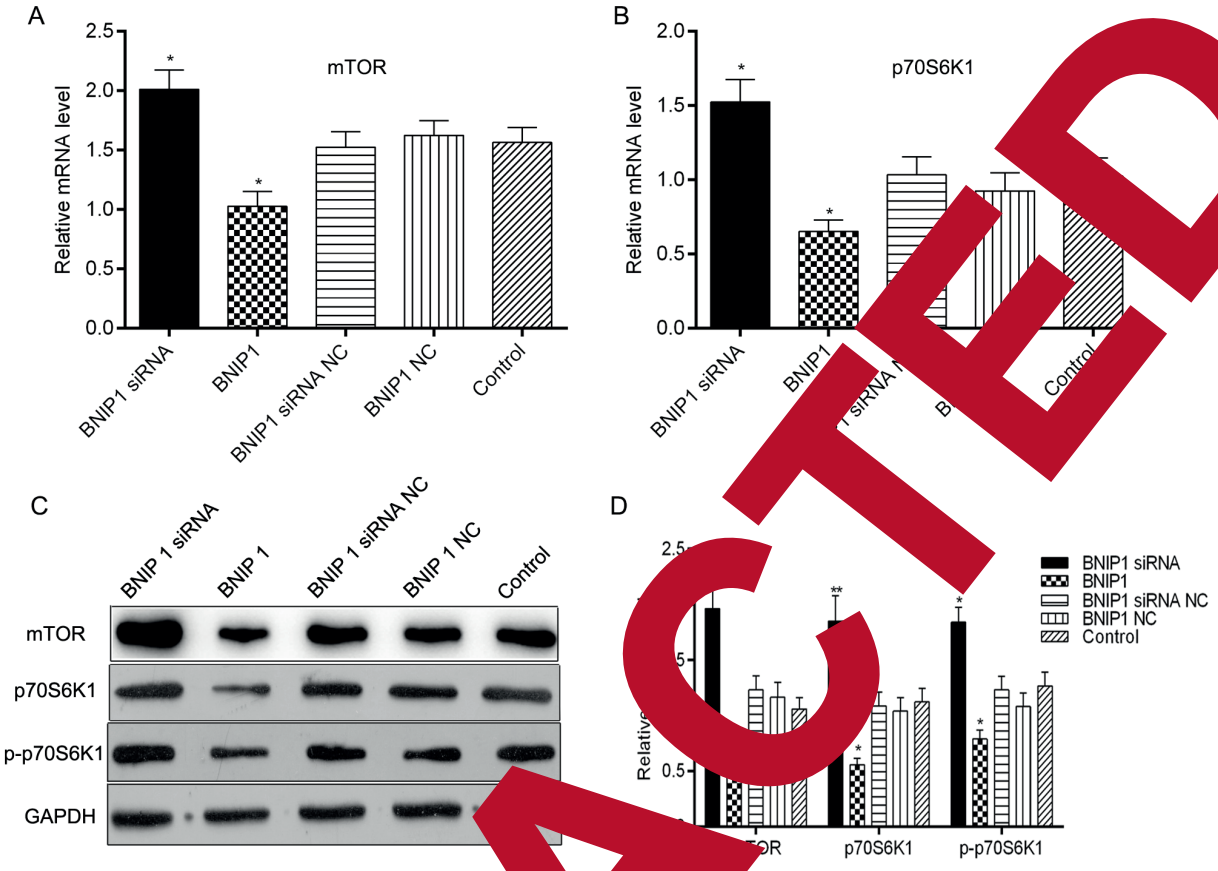
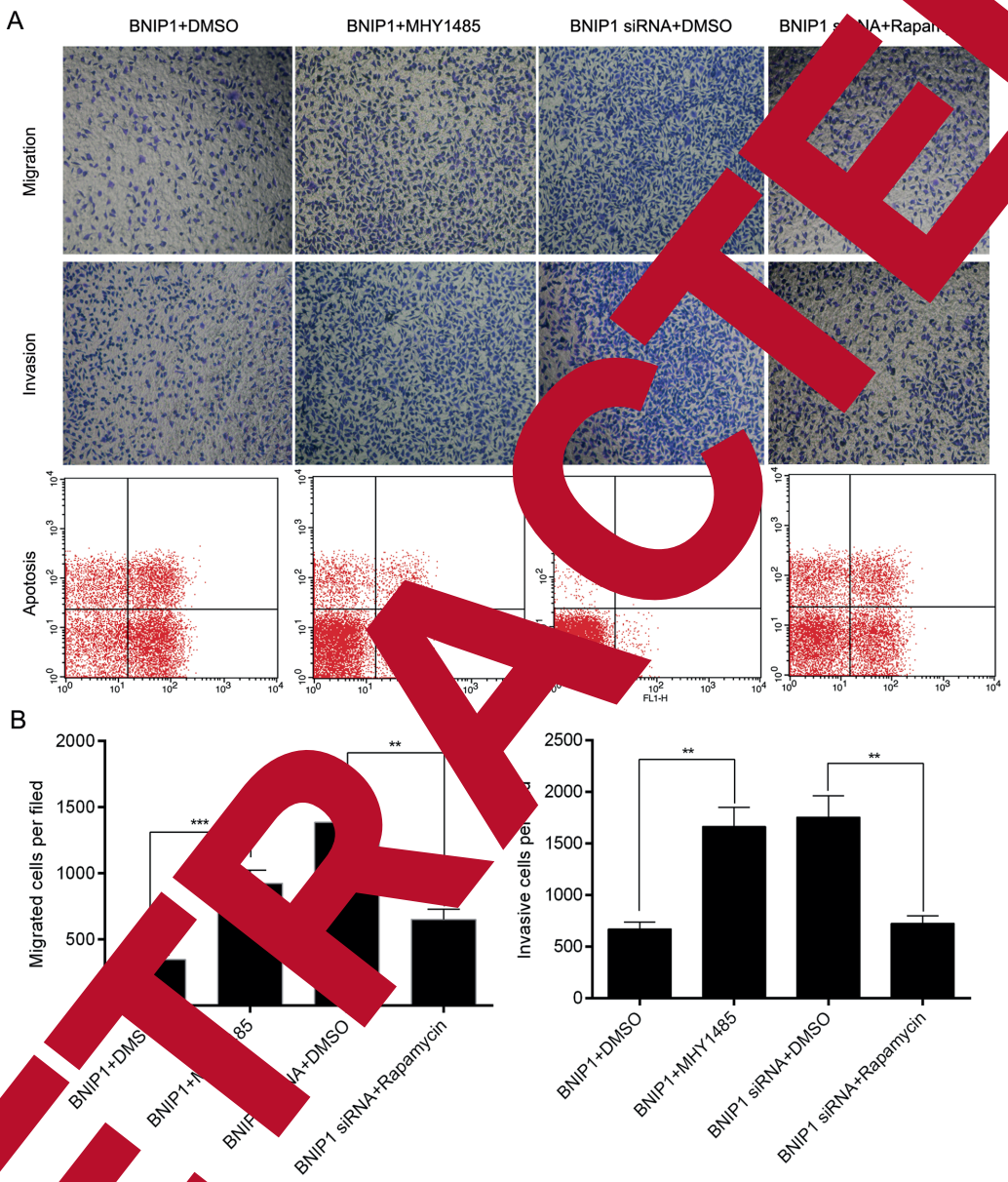


Figure 3. BNIP1 significantly inhibits mTOR, p70S[illegible]p70S6K1 expressions. ***A-B***, qRT-PCR was used to analyze the mRNA expression levels of mTOR and p70S6K1 in [illegible]HeLa cells (*$p < 0.05$). ***C***, Representative images of Western blotting for mTOR expression after m[illegible] BNIP1 ex[illegible]on. ***D***, The quantitative analysis of Western blotting data (*$p < 0.05$, **$p < 0.01$).

there is a significant ass[illegible]n be[illegible] expression and clinico[illegible]tho[illegible]tures of tumors. In recent yea[illegible]r grou[illegible]focused also on the effect of [illegible]P1 expressio[illegible]cervical cancer develop[illegible]d especially g[illegible]wth and metastasis. W[illegible]rove[illegible] BNIP1 also affects the prolife[illegible]tion, apoptos[illegible]ration, and invasion of c[illegible]al cancer cells[illegible]R is a kinase that is [illegible]al for the regulation of many cellular even[illegible]ch as proliferation, growth, survival, [illegible]renti[illegible], adh[illegible]n, motility, angiogenesis, [illegible]stasis[25]. Furthermore, the PI3K/AKT/m[illegible]thwa[illegible] dysregulated in a large [illegible]tion o[illegible]ancers, including cervical carc[illegible]na, and [illegible]gulates the apoptotic response thro[illegible]its ability to interact with a number of key pl[illegible]optotic process[27]. This indicates [illegible]t might be a potential therapeutic target for [illegible]reatment of this malignancy. Moreover, the [illegible]athway is involved in chemosensitivity to cispl[illegible]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.



Figu ile[illegible]f BNIP[illegible]elerates proliferation, migration and invasion, and suppresses apoptosis of cervical cancer cells by [illegible]eLa cell[illegible]e transfected with BNIP1 + DMSO, BNIP1 + mTOR activator (MHY1485), BNIP1 siRNAs + [illegible]SO, and [illegible]siR[illegible] mTOR inhibitor (Rapamycin), respectively. ***A,*** Representative migration, invasion, and apop-[illegible]ges w[illegible]n HeLa cells after modulation of BNIP1 and mTOR expressions. The statistical analysis of cell mig[illegible] ***(B),*** inva[illegible]***)***, and flow cytometry.

Figure continued

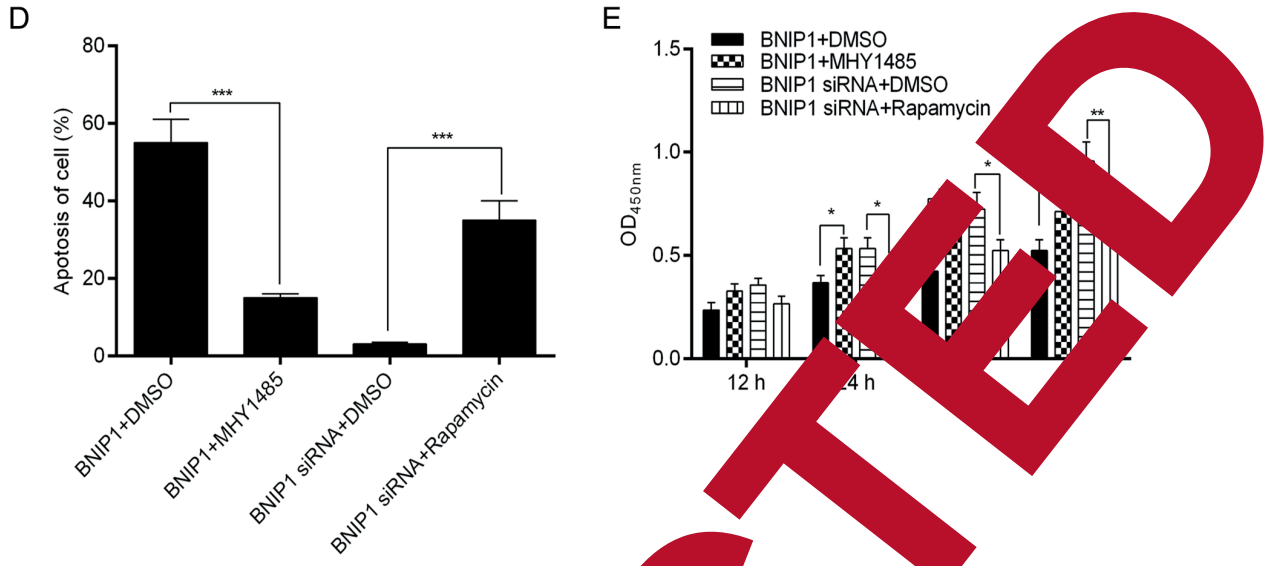


Figure 4. *(Continued).* ***(D)*** assays (**$p < 0.01$, ***$p < 0.001$). ***E,*** roliferation results were detected by CCK-8 assay (*$p < 0.05$, **$p < 0.01$).

Conclusions

We found that BNIP1 has a pivotal tumo pressor role in cervical cancer. It can inh liferation, migration and invasion, and acc te apoptosis by mTOR signaling pathway. How the molecular and cellular mechanisms un lying this association have not b determin completely. Further researches ecessary to uncover this integral mec sm. nal studies are also needed to co our res *in vivo*. In brief, we provide the for f assessment of BNIP1 gies; howe-ver, *in vivo* experim are nec

Funding

This study w nanced by izhou Province Science and T logy Fund (J tific in Guizhou) ([2016]73 015]7377).

Conf erest

The Author e that have no conflict of interest.

References

1) Ili CG, Brebi P, López J, García P, Leal P, Suarez E, a JC. Genotyping of human papillomavirus in cal intraepithelial neoplasia in a high-risk po-tion. J Med Virology 2011; 83: 833-837.

2) o S, Yao en J, Ding N, Ren F. MiR-20a pro- cancer proliferation and metastasis *in vivo*. PLoS One 2015; 10: e0120905.

3) Sun P, Shen Y, Gong JM, Zhou LL, Sheng JH, Duan FJ. A new MicroRNA expression signature for cervical Int J Gynecol Cancer 2017; 27: 339-343.

4) Small W Jr, Bacon MA, Bajaj A, Chuang LT, Fisher BJ, Harkenrider MM, Jhingran A, Kitchener HC, Mileshkin LR, Viswanathan AN, Gaffney DK. Cervical cancer: a global health crisis. Cancer 2017; 123: 2404-2412.

5) Landoni F, Maneo A, Colombo A, Placa F, Milani R, Perego P, Favini G, Ferri L, Mangioni C. Randomised study of radical surgery versus radiotherapy for stage Ib-IIa cervical cancer. Lancet 1997; 350: 535-540.

6) Gadducci A, Sartori E, Maggino T, Landoni F, Zola P, Cosio S, Pasinetti B, Alessi C, Maneo A, Ferrero A. The clinical outcome of patients with stage Ia1 and Ia2 squamous cell carcinoma of the uterine cervix: a Cooperation Task Force (CTF) study. Eur J Gynaecol Oncol 2003; 24: 513-516.

7) Sturdza A, Pötter R, Fokdal LU, Haie-Meder C, Tan LT, Mazeron R, Petric P, Šegedin B, Jurgenliemk-Schulz IM, Nomden C, Gillham C, McArdle O, Van Limbergen E, Janssen H, Hoskin P, Lowe G, Tharavichitkul E, Villafranca E, Mahantshetty U, Georg P, Kirchheiner K, Kirisits C, Tanderup K, Lindegaard JC. Image guided brachytherapy in locally advanced cervical cancer: Improved pelvic control and survival in RetroEMBRACE, a multicenter cohort study. Radiother Oncol 2016; 120: 428-433.

8) Pötter R, Georg P, Dimopoulos JC, Grimm M, Berger D, Nesvacil N, Georg D, Schmid MP, Reinthaller A, Sturdza A, Kirisits C. Clinical outcome of protocol based image (MRI) guided adaptive brachytherapy combined with 3D conformal radiotherapy

with or without chemotherapy in patients with locally advanced cervical cancer. Radiother Oncol 2011; 100: 116-123.

9) Diaz-Padilla I, Monk BJ, Mackay HJ, Oaknin A. Treatment of metastatic cervical cancer: future directions involving targeted agents. Crit Rev Oncol Hematol 2013; 85: 303-314.

10) Cho B, Choi SY, Park OH, Sun W, Geum D. Differential expression of BNIP family members of BH3-only proteins during the development and after axotomy in the rat. Mol Cells 2012; 33: 605-610.

11) Ryu SW, Choi K, Yoon J, Kim S, Choi C. Endoplasmic reticulum-specific BH3-only protein BNIP1 induces mitochondrial fragmentation in a Bcl-2- and Drp1-dependent manner. J Cell Physiol 2012; 227: 3027-3035.

12) Yasuda M, Chinnadurai G. Functional identification of the apoptosis effector BH3 domain in cellular protein BNIP1. Oncogene 2000; 19: 2363-2367.

13) Nakajima K, Hirose H, Taniguchi M, Kurashina H, Arasaki K, Nagahama M, Tani K, Yamamoto A, Tagaya M. Involvement of BNIP1 in apoptosis and endoplasmic reticulum membrane fusion. EMBO J 2004; 23: 3216-3226.

14) Cong J, Liu R, Wang X, Jiang H, Zhang Y. MiR-634 decreases cell proliferation and induces apoptosis by targeting mTOR signaling pathway in cervical cancer cells. Artif Cells Nanomed Biotechnol 2016; 44: 1694-1701.

15) Huang X, Taeb S, Jahangiri S, Emmenegger U, Bruce J, Mesci A, Korpela E, Vesprini D, Wo[illegible] S, Bristow RG, Liu FF, Liu SK. miRNA-95 mec[illegible] radioresistance in tumors by targeting the sp[illegible] golipid phosphatase SGPP1. Cancer Res 20[illegible] 73: 6972-6986.

16) Higgs G, Slack F. The multiple[illegible] of [illegible]RNA-155 in oncogenesis. J Clin Bio[illegible]na 2013; [illegible]7.

17) You Y, Li H, Qin X, Zh[illegible] Song [illegible] F. Decreased CDK10 e[illegible] on [illegible] lymph node metas[illegible] a[illegible] poor outcome in breast can[illegible] atients [illegible] report. Cell Oncol (Dordr) [illegible] 38: 485-491.

18) Cao Z, Shan[illegible] G, Miele L, Sark[illegible] H, Wang Z, Zhou Q[illegible] nor[illegible] diated neovascularization and ly[illegible]phangiog[illegible] contrive tumor progressi[illegible]d cancer meta[illegible] Biochim Biophys Acta [illegible]; 1836: 273-286.

19) W[illegible] Liu Y, W[illegible] Y, Wang W, Ren W, Fei C, Chen Y, [illegible] Z, Wa[illegible] Wang J, Jiang Y, Zhou L, He T, Zhang [illegible]MP9 [illegible]tes cro[illegible]alk between breast can[illegible] bone [illegible]w-derived mesenchymal stem[illegible]ell On[illegible]ordr) 2014; 37: 363-375.

[illegible]ak KJ, [illegible] D. Analysis of relative gene [illegible]ression [illegible]ing real-time quantitative PCR [illegible] the 2(-De[illegible] Delta C(T)) Method. Methods [illegible]402-408.

2[illegible] [illegible]en Y, He YX, Lei DM, Zhang Z, Li XF. New technology for cervical cancer screening. Int J Gynecol Cancer 2012; 22: 1564-1569.

22) Shen Y, Gong JM, Li YQ, Gong YM, Lei DM, Cheng GM, Li XF. Epidemiology and genotyp[illegible] tion of human papillomavirus (HPV[illegible] Henan Province, China. Clin Chim[illegible]t 2013; 4[illegible] 297-301.

23) Dai Y, Tong R, Guo H, Yu T, Wa[illegible] ssociation of CXCR4, CCR7, VEGF-C and VE[illegible] xpression with lymph node metast[illegible] in pati[illegible]h cer[illegible] vical cancer. Eur J Ob[illegible] Gynecol H[illegible] 2017; 214: 178-183.

24) Wang P, Wu Y, Li Y[illegible] J, Ta[illegible] A novel RI[illegible]G finger E3 ligase[illegible]86 rec[illegible] ER st[illegible]s-mediated apoptosis [illegible] action w[illegible]Nip1. Cell Signal 2[illegible] 25: [illegible] 33.

25) Bai H, Li H[illegible], Gui T, Ya[illegible] [illegible]hen K. The PI3K/AK[illegible]OR pathway is [illegible]ial predictor of disti[illegible] and migrator[illegible]apacities in human [illegible]ian [illegible]cell lines. Oncotarget 2015; 6: 25520-25532.

26) [illegible] Xu B, Liu L, [illegible]o Y, Chen X, Barzegar [illegible]ng J, Huang S. Bo[illegible]TORC1 and mTORC2 are involved in the regulation of cell adhesion. Oncotarget 2015; [illegible]7136-7150.

Will M, Qin A[illegible]oy W, Yao Z, Rodrik-Outmezguine V, Schneider C[illegible]ang X, Monian P, Jiang X, de Stanchina E, Bas[illegible], Liu N, Chandarlapaty S, Rosen N. [illegible] of apoptosis by PI3K inhibitors is dep[illegible]upon their transient inhibition of RAS-ERK signaling. Cancer Discov 2014; 4: 334-347.

[illegible]u H, Wu J, Zhang W, Luo H, Shen Z, Cheng H, Zhu [illegible]M2 enhances chemosensitivity to cisplatin th[illegible]gh interaction with the mTOR pathway in cervical cancer. Sci Rep 2016; 6: 30788.

29) Bahrami A, Hasanzadeh M, Hassanian SM, ShahidSales S, Ghayour-Mobarhan M, Ferns GA, Avan A. The potential value of the PI3K/Akt/mTOR signaling pathway for assessing prognosis in cervical cancer and as a target for therapy. J Cell Biochem 2017; 118: 4163-4169.

30) Zhang LJ, Lu R, Song YN, Zhu JY, Xia W, Zhang M, Shao ZY, Huang Y, Zhou Y, Zhang H, Guo L, Zhang M, Zhang H. Knockdown of anion exchanger 2 suppressed the growth of ovarian cancer cells via mTOR/p70S6K1 signaling. Sci Rep 2017; 7: 6362.

31) Zhang P, Guo Z, Wu Y, Hu R, Du J, He X, Jiao X, Zhu X. Histone deacetylase inhibitors inhibit the proliferation of gallbladder carcinoma cells by suppressing AKT/mTOR signaling. PLoS One 2015; 10: e0136193.

32) Xu S, Fu GB, Tao Z, OuYang J, Kong F, Jiang BH, Wan X, Chen K. MiR-497 decreases cisplatin resistance in ovarian cancer cells by targeting mTOR/P70S6K1. Oncotarget 2015; 6: 26457-26471.

33) Zhang D, Zhou XH, Zhang J, Zhou YX, Ying J, Wu GQ, Qian JH. Propofol promotes cell apoptosis via inhibiting HOTAIR mediated mTOR pathway in cervical cancer. Biochem Biophys Res Commun 2015; 468: 561-567.