

Knockdown of serpin peptidase inhibitor clade C member 1 inhibits the growth of nasopharyngeal carcinoma cells

JIN XU¹, YIN YING², GAOYUN XIONG¹, LIQIN LAI³, QINGLIANG WANG¹ and YUE YANG³

Departments of ¹ENT, ²Pharmacy and ³Pathology, Tongde Hospital of Zhejiang Province, Hangzhou, Zhejiang 310012, P.R. China

Received May 28, 2018; Accepted March 1, 2019

DOI: 10.3892/mmr.2019.10021

Abstract. Nasopharyngeal carcinoma (NPC) is a type of cancer originating in the nasopharynx. There are no NPC-specific treatments available at present. Serpin peptidase inhibitor clade C member 1 (SERPINC1) serves roles in anticoagulation and anti-inflammation. The aim of the present study was to investigate the role of SERPINC1 in the proliferation and apoptosis of NPC cells. Tumor and adjacent healthy tissue samples were collected from patients with NPC. Additionally, the SERPINC1 gene was silenced in the HNE3 cell line using short interfering RNA targeted against SERPINC1 (SERPINC1-siRNA). Cell viability was determined via a Cell Counting Kit-8 assay; furthermore, proliferation and apoptosis were investigated via flow cytometry. Western blotting and reverse transcription-quantitative polymerase chain reaction analysis were performed to determine the expression levels of protein and mRNA. It was revealed that the expression levels of SERPINC1 mRNA and protein were increased in NPC tumor tissues compared with in adjacent healthy tissues. The expression of SERPINC1 mRNA and protein in HNE3 cells decreased following SERPINC1-siRNA transfection. Furthermore, knockdown of SERPINC1 promoted apoptosis and inhibited proliferation. It was also demonstrated that silencing SERPINC1 upregulated the expression of B-cell lymphoma-2 (Bcl-2)-associated X protein and p53 mRNA and protein, and downregulated that of Bcl-2, survivin and cyclin D1. Downregulation of SERPINC1 reduced the phosphorylation of phosphatidylinositol 3-kinase (PI3K), protein kinase B (Akt) and mammalian target of rapamycin (mTOR). Thus, SERPINC1 knockdown may promote the apoptosis of HNE3 cells and inhibit proliferation via the suppression of the PI3K/Akt/mTOR signaling pathway.

Introduction

Nasopharyngeal carcinoma (NPC) is a malignancy of the head and neck that exhibits a unique geographical distribution (1,2). NPC usually develops in the nasopharyngeal mucosa; the majority of NPCs are squamous cell carcinomas (3). NPC is more common in males than in females (4); however, the incidence of nasopharyngeal cancer has increased in previous decades (5).

Chemotherapy, radiotherapy and surgery are three main methods for treating NPC (6). As NPC is more sensitive to chemotherapy, a combined therapy, including induction, concurrent and adjuvant chemotherapy is commonly used to treat late-stage NPC (7-9). Clinical studies reported that combined chemotherapy demonstrated beneficial short-term effects on NPC (10,11); however, the incidences of acute recurrence and toxicity following chemotherapy are high (12,13). A novel anticancer approach, targeted therapy, has effectively treated lung cancer, melanoma, leukemia and other tumors (14-17). Therefore, it is important to investigate the mechanisms underlying the pathogenesis of NPC to aid the identification of potential novel targets in the treatment of NPC.

The gene encoding serpin peptidase inhibitor clade C member 1 (SERPINC1), also known as antithrombin III (ATIII), is located on chromosome 1q23-25.1 (18). ATIII regulates coagulation via the inhibition of coagulation factors, and exhibits anti-inflammatory effects on epithelial cells (19-21). Studies have previously reported that the occurrence and development of certain cancers was also closely associated with SERPINC1; Zietek *et al* (22,23) reported increased levels of ATIII in the serum of patients with kidney cancer and the tumor tissues of patients with bladder cancer. Other studies observed that SERPINC1-encoded proteins exhibited inhibitory effects on angiogenesis and suppressed proliferation (24,25). To the best of our knowledge, the role of SERPINC1 in the occurrence and development of NPC has not been investigated.

RNA interference is commonly used to study the role of genes by targeting the mRNA, and it allows the selective silencing of one or several genes (26). The present study investigated the role of SERPINC1 in the proliferation of NPC cells via determining the expression of the SERPINC1 gene in tissues from patients with NPC and silencing the gene in NPC cells. The results may provide novel insight and targets for the treatment of NPC.

Correspondence to: Dr Yue Yang, Department of Pathology, Tongde Hospital of Zhejiang Province, 234 Gucui Road, Xihu, Hangzhou, Zhejiang 310012, P.R. China
E-mail: yangyue_yy223@163.com

Key words: nasopharyngeal carcinoma, serpin peptidase inhibitor clade C member 1, phosphatidylinositol 3-kinase/protein kinase B/mammalian target of rapamycin, proliferation, apoptosis

Materials and methods

NPC samples. From September 2016 to September 2017, 41 NPC tumor and adjacent healthy tissues were collected from 19 males and 22 females (aged 49-80 years, with a mean of 58.53 ± 6.36 years) at the Department of Pathology, Tongde Hospital of Zhejiang Province (Hangzhou, China). The samples were stored at -80°C prior to subsequent experimentation. Patients included in the present study exhibited NPC that was histologically confirmed by biopsy, and no history of previous head and neck cancer. Patients with incomplete clinical data were excluded. In addition, NPC patients were staged according to the criteria of the 8th edition of The Union for International Cancer Control/American Joint Committee on Cancer staging system (27). Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was performed to determine the expression levels of SERPINC1 mRNA in each tissue sample. The patients were divided into low- and high-expression groups according to the median value of SERPINC1 mRNA expression. A total of 6 cases were randomly selected from the 41 patients for the analysis of SERPINC1 protein via western blotting. All patients provided signed informed consent, and the experiment was approved by the Ethics Committee of Tongde Hospital of Zhejiang Province.

Cell culture. The poorly differentiated squamous cell carcinoma NPC cell line HNE3 was purchased from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum, 100 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin at 37°C in an incubator with 5% CO_2 . All culture reagents were purchased from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Transfection of short interfering RNA (siRNA). In total, 2×10^5 cells/well were seeded in 24-well culture plates, and 0.5 ml medium without antibiotic was added in each well. Cells at 90% confluence were transfected. siRNA targeted against SERPINC1 (SERPINC1-siRNA) was purchased from Shanghai GenePharma Co., Ltd. (Shanghai, China). siRNA (50 nM) transfection was performed using Lipofectamine[®] 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) to generate the SERPINC1-siRNA group. Negative-siRNA (Thermo Fisher Scientific, Inc.) was inserted into an empty vector (pcDNA3.1; Thermo Fisher Scientific, Inc.), which served as the empty vector control group; a non-transfected control group was also established. The sequences of the siRNAs used in the present study were as follows: SERPINC1-siRNA forward, AUCACAUGGAAUACAUGGCC and reverse, CCAUGUAUCCAAUGUGAUAG; negative-siRNA forward, CAUGUGUCUGUCGCAUAAUA and reverse, CGGUACACCAGA CAGCGUAUU. Following transfection for 48 h, cells were harvested, and the efficiency of transfection was determined via RT-qPCR and western blot analysis.

Cell viability. A Cell Counting Kit-8 (CCK-8) assay was used to determine cell viability. Transfected cells at a density of 3×10^3 cells/well were inoculated in a 96-well plate and incubated at 37°C with 5% CO_2 for 12, 24 and 48 h following transfection. CCK-8 reagent (10 ml; Dojindo Molecular Technologies, Inc.,

Kumamoto, Japan) was then added to each well and cultured together at 37°C with 5% CO_2 for 4 h. The absorbance of each well at 450 nm was measured using a microplate reader (ELx800; BioTek Instruments Inc., Winooski, VT, USA), and cell viability was calculated according to the standard curve.

Flow cytometry for cell apoptosis and proliferation. Flow cytometry was used to evaluate cell apoptosis. Cells (1×10^6) were washed with PBS at 4°C and resuspended to a concentration of 4×10^5 cells/ml. Phycoerythrin-Annexin V Apoptosis Detection kit (5 μl ; BD Pharmingen; BD Biosciences, San Jose, CA, USA) was added to cell culture (200 μl), and then 10 μl 7-aminoactinomycin D (20 $\mu\text{g}/\text{ml}$; BD Biosciences) was added. The samples were incubated at room temperature in the dark for 10 min. The BD FACSCanto flow cytometer (BD Biosciences) was used to analyze apoptosis at 488 nm, analysis of data was performed using the FSC Express software (version 3; De Novo Software, Glendale, CA, USA). Living cells are presented in the lower left quadrant, necrotic cells in the upper left quadrant, advanced apoptotic cells in the upper right quadrant, and early apoptotic cells in the lower right quadrant. The proliferation of HNE3 cells was also analyzed via flow cytometry. Click-iT[®] Plus EdU Pacific Blue flow cytometry kit (Thermo Fisher Scientific, Inc.) was applied to detect the cell proliferation.

RT-qPCR. RT-qPCR was performed to investigate the expression levels of SERPINC1, B-cell lymphoma-2 (Bcl-2)-associated X protein (Bax), Bcl-2, survivin, cyclin D1 and p53 mRNA; GAPDH was used as a reference gene. RNA was extracted from NPC tissues and cells using TRIzol[®] (Thermo Fisher Scientific, Inc.) at 0°C for 5 min, isolated with CHCl_3 (Aladdin Shanghai Biochemical Technology Co., Ltd., Shanghai, China) and then dissolved in diethyl pyrocarbonate-treated water (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). RNA concentration was determined using a NanoDrop One Microvolume UV-Vis Spectrophotometer (NanoDrop Technologies; Thermo Fisher Scientific, Inc., Wilmington, DE, USA). RT was performed on RNA samples using the PrimeScript first Strand cDNA synthesis kit (Takara Bio, Inc., Otsu, Japan) to synthesize cDNA. The RT reaction was performed at 37°C for 15 min, followed by reverse transcriptase inactivation at 85°C for 15 sec. RT-qPCR reactions were performed using an ABI 7500 real-time PCR system (Thermo Fisher Scientific, Inc.), using the SYBR Prellix Ex Taq[™] Real-Time PCR Kit (Takara Bio, Inc.). qPCR was performed by activating the DNA polymerase at 95°C for 5 min, followed by 40 cycles of two-step PCR (at 95°C for 10 sec and at 60°C for 30 sec) and a final extension at 75°C for 10 min prior to holding at 4°C . DNase- and RNase-free water were used as negative control templates. All primers used were purchased from Genewiz, Inc. (Suzhou, China) and are presented in Table I. The $2^{-\Delta\Delta\text{C}_q}$ method (28) was applied to analyze relative expression levels of target genes normalized to GAPDH. Each experiment was performed in triplicate.

Western blotting. The expression of SERPINC1, apoptosis-, cell cycle-proteins, and phosphatidylinositol 3-kinase/protein kinase B/mammalian target of rapamycin (PI3K/Akt/mTOR) signaling pathway-associated proteins were determined via

Table I. Sequences of the primers used for reverse transcription-quantitative polymerase chain reaction.

Primer name	Sequence (5'-3')	Product size (bp)
SERPINC1, forward	GCCTGAAGGTAGCAGCTTGT	313
SERPINC1, reverse	CCCACACTCCCTCACTCTTC	
Bax, forward	TCCACCAAGAAGCTGAGCGAG	345
Bax, reverse	TTCTTTGAGTTCGGTGGGGTC	
Bcl-2, forward	CTGGTGGACAACATCGC	317
Bcl-2, reverse	GGAGAAATCAAACAGAGGC	
Survivin, forward	CCCTTTCTCAAGGACCACCGCATC	133
Survivin, reverse	GCCAAGTCTGGCTCGTTCTCAGT	
Cyclin D1, forward	CTGGCCATGAACTACCTGGA	245
Cyclin D1, reverse	GTCACACTTGATCACTCTGG	
p53, forward	CTGAGGTCGGCTCCGACTATACCACTATCC	360
p53, reverse	CTGATTCACTCTCGGAACATCTCGAAGCG	
GAPDH, forward	CCATCTTCCAGGAGCGAGAT	222
GAPDH, reverse	TGCTGATGATCTTGAGGCTG	

Bcl-2, B-cell lymphoma-2; Bax, Bcl-2-associated X protein; SERPINC1, serpin peptidase inhibitor clade C member 1.

western blot analysis; phosphorylation levels were also determined. Tissues and cells were lysed using liquid nitrogen and radioimmunoprecipitation assay buffer (Abmole Bioscience, Inc., Houston, TX, USA), and subsequently subjected to cleavage and lysis using 1% phenylmethane sulfonyl fluoride protease and phosphatase inhibitors (Abmole Bioscience, Inc.), for 30 min at 4°C. The supernatant was collected by centrifugation at 12,000 x g at 4°C for 15 min. Total protein concentration was determined using the bicinchoninic acid method. In total, 10 µg protein was loaded in each lane. Proteins were separated by 10% SDS-PAGE. The separated proteins were transferred onto a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Inc., Hercules, CA, USA) using a Trans-Blot Transfer Slot (Bio-Rad Laboratories, Inc.) and blocked with 5% skimmed milk for 2 h at room temperature. The membranes were incubated with the following primary antibodies obtained from Abcam (Cambridge, UK): Anti-SERPINC1 (1:800; ab126598); anti-Bax (1:700; ab32503); anti-Bcl-2 (1:900; ab32124); anti-survivin (1:700; ab76424); anti-cyclin D1 (1:800; ab134175); anti-p53 (1:600; ab26); anti-PI3K (1:700; ab125633); anti-phosphorylated (p)-PI3K (1:600; ab138364); anti-Akt (1:800; ab8805); anti-p-Akt (1:800; ab38449); anti-mTOR (1:800; ab2732); anti-p-mTOR (1:600; ab109268) and anti-GADPH (1:800; ab8245). Following the application of primary antibodies, membranes were agitated at room temperature for 2 h, and then incubated at 4°C for 12 h. Subsequently, membranes were incubated at room temperature for 1.5 h with the following secondary antibodies: Fluorescein isothiocyanate-conjugated goat anti-mouse IgG (1:8,000; ab6785; Abcam); horseradish peroxidase (HRP)-conjugated mouse anti-rabbit IgG (1:9,000; ab99697; Abcam); mouse anti-rabbit IgG (1:7,000; BA1034; Invitrogen; Thermo Fisher Scientific, Inc.); NL557-conjugated donkey anti-rabbit IgG (1:5,000; NL004; R&D Systems, Inc., Minneapolis, MN, USA); HRP-conjugated rabbit anti-human IgG (1:10,000; ab6759; Abcam). Protein bands were visualized

using an enhanced chemiluminescence reagent (Thermo Fisher Scientific, Inc.). The optical density was quantified using ImageJ software (version 1.46; National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. Experimental data were presented as the mean ± standard deviation. Data were analyzed using SPSS version 20.0 (IBM Corporation, Armonk, NY, USA). All experiments were performed three times. Differences between two groups were analyzed using paired Student's t-tests. One-way analyses of variance were performed to analyze differences between experimental groups, with Tukey's multiple comparison test used as a post hoc test. Associations between clinicopathological data and SERPINC1 expression were analyzed using χ^2 tests. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

SERPINC1 is upregulated in NPC tissue. RT-qPCR and western blotting were performed to analyze the levels of SERPINC1 expression in tumor and adjacent healthy tissues from patients with NPC. It was revealed that SERPINC1 protein and mRNA expression levels were significantly increased in NPC tissues compared with in adjacent tissues ($P < 0.05$; Fig. 1A-C). As presented in Table II, patients were separated into low- and high-expression groups according to the median value of SERPINC1 mRNA expression, and associations with clinicopathological features were investigated. It was demonstrated that increased SERPINC1 expression was significantly associated with the metastasis of NPC. The results suggested that the SERPINC1 gene was overexpressed in NPC tissues.

Transfection with SERPINC1-siRNA reduces SERPINC1 expression and the proliferation of HNE3 cells. The human poorly-differentiated squamous cell carcinoma NPC cell

Table II. Associations between clinicopathological data of patients with nasopharyngeal carcinoma and SERPINC1 expression.

Clinicopathological factor	N	SERPINC1 expression		P-value
		Low	High	
Gender				0.756
Female	23	11	12	
Male	18	10	8	
Age				0.756
<50	18	10	8	
>50	23	11	12	
TNM stage				0.536
I-II	19	11	8	
III-IV	22	10	12	
Metastasis				0.015 ^a
Yes	11	2	9	
No	30	19	11	

^aP<0.05. SERPINC1, serpin peptidase inhibitor clade C member 1; TNM, tumor, node and metastasis.

line HNE3 was used to study the role of SERPINC1 in NPC; si-SERPINC1 was transfected into HNE3 cells. The efficiency of SERPINC1-siRNA transfection was determined via RT-qPCR and western blot analysis. It was demonstrated that the expression levels of SERPINC1 mRNA and protein were significantly decreased in HNE3 cells following SERPINC1 knockdown compared with the negative siRNA-transfected (empty vector) control group ($P<0.01$; Fig. 2A-C). The proliferation of HNE3 cells was determined via a CCK-8 assay; cell proliferation was significantly reduced at 12, 24 and 48 h following transfection with SERPINC1-siRNA compared with the empty vector control ($P<0.05$, $P<0.05$ and $P<0.01$; Fig. 2D). The results indicated that transfection of the SERPINC1-siRNA were successfully conducted, and that downregulation of SERPINC1 expression induced a decrease in the proliferation of NPC cells.

SERPINC1 gene silencing inhibits cell proliferation and promotes apoptosis. Flow cytometry was performed to investigate the apoptosis and proliferation of HNE3 cells following SERPINC1-siRNA transfection. No significant differences in apoptosis or proliferation were reported between the empty vector and non-transfected control groups. Conversely, the apoptotic rate of the SERPINC1-siRNA group was significantly increased compared with the empty vector group ($P<0.01$; Fig. 3A and B), whereas proliferation was reduced following SERPINC1 knockdown compared with the empty vector group ($P<0.01$; Fig. 3C and D). The results indicated that SERPINC1 gene silencing inhibited the proliferation and promoted the apoptosis of NPC cells.

Downregulation of SERPINC1 gene affects cell cycle- and apoptosis-associated proteins. To further investigate the

effects of SERPINC1 knockdown on the proliferation and apoptosis of HNE3 cells, RT-qPCR and western blot analyses were conducted to determine the expression of apoptosis-associated (Bax, Bcl-2 and survivin) and cell cycle-associated (cyclin D1 and p53) genes and proteins, respectively. It was revealed that the expression levels of Bax mRNA were significantly upregulated in SERPINC1-siRNA-transfected cells compared with the empty vector group, whereas those of Bcl-2 and survivin mRNA were downregulated ($P<0.01$; Fig. 4A-C). Additionally, the expression levels of cyclin D1 mRNA were significantly decreased and those of p53 increased following silencing of the SERPINC1 gene compared with the empty vector group ($P<0.01$; Fig. 4D-G). Similar alterations in the expression levels of the genes were also reported at the protein level ($P<0.01$; Fig. 4F and G), indicating that the suppression of SERPINC1 expression promoted the expression of proapoptotic factors, inhibited the expression of antiapoptotic factors and suppressed proliferation via regulation of cell cycle-associated genes in NPC cells.

Knockdown of SERPINC1 inhibits the phosphorylation of proteins involved in the PI3K/Akt/mTOR signaling pathway. The mechanisms underlying the effects of SERPINC1 on the proliferation and apoptosis of NPC cells were investigated by evaluating the expression and phosphorylation levels of PI3K/Akt/mTOR-associated proteins. It was revealed that the phosphorylation levels of PI3K, Akt and mTOR were significantly decreased in HNE3 cells following transfection with SERPINC1-siRNA compared with the empty vector control group ($P<0.01$; Fig. 5A-D), suggesting that SERPINC1 knockdown inhibited the activity of the PI3K/Akt/mTOR pathway.

Discussion

NPC is a malignant tumor prevalent in Southeast Asia that occurs at the top of the nasopharyngeal cavity, and seriously affects the survival and quality of life of patients (29). In addition, >50% of patients with NPC are diagnosed at an advanced stage as the initial clinical symptoms of NPC and the site of lesions are difficult to identify (30). NPC is sensitive to chemotherapy and radiotherapy, but surgery is an effective method for treating NPC (31); however, treatment outcomes are usually poor due to late diagnosis. It was reported that the recurrence rate of NPC was 10-15% and the 5-year survival rate was ~60% (30,31). NPC exhibits high sensitivity to radiotherapy; however, patients commonly experience strong adverse reactions and side effects following treatment. Radiation brain injury was reported in 28.5% of patients treated with radiotherapy, whereas 38.1% of patients exhibited severe hearing loss, 40.6% patients possessed nasal pharyngeal mucosal injuries and >50% patients succumbed to mortality following severe radiation damage (32-34). Therefore, identifying a safer and more effective treatment of NPC is of great importance.

The SERPINC1 gene encodes ATIII, a serine protease inhibitor involved in coagulation cascades (18). The main roles of ATIII have been associated with the regulation of coagulation and hemostasis, and the induction of anti-inflammatory processes (35). A previous study reported that the activity of ATIII in the serum of patients with renal and bladder cancer was significantly increased compared with in healthy

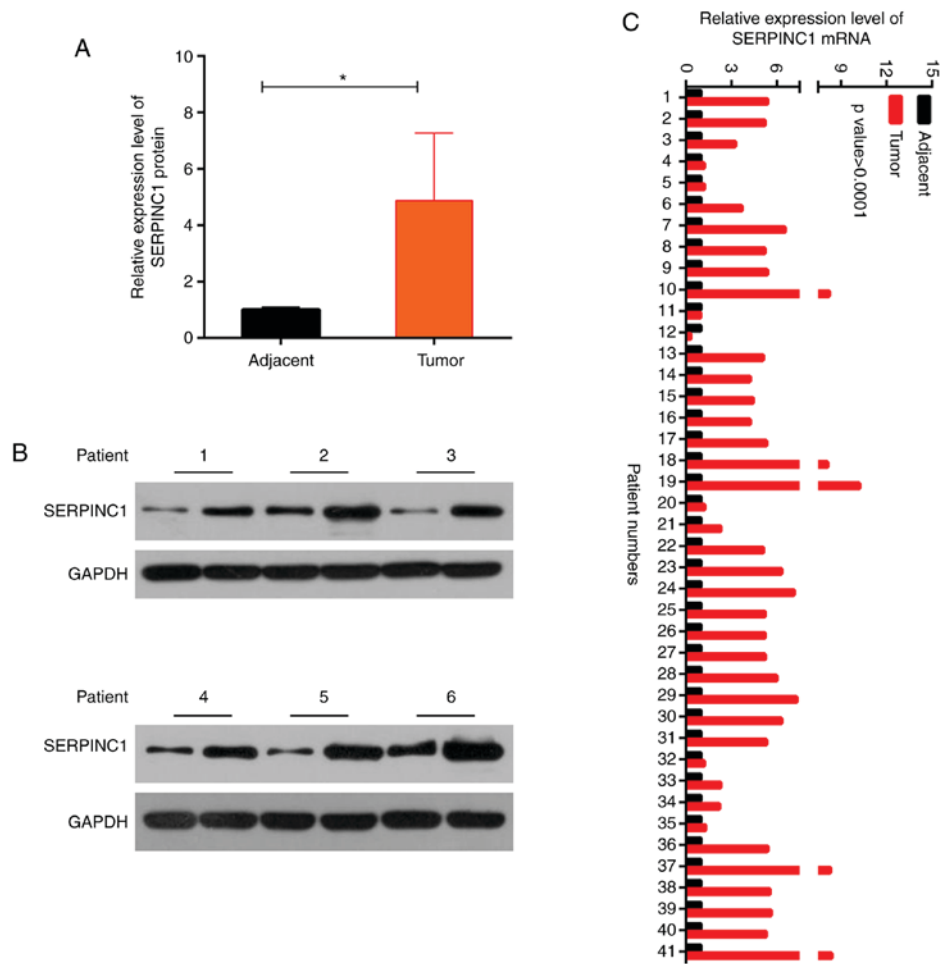


Figure 1. Expression of SERPINC1 in NPC tissues. (A and B) Expression levels of SERPINC1 protein were determined in six paired tumor and healthy adjacent tissue samples collected from patients with NPC by performing western blot analysis. (C) Expression of SERPINC1 mRNA was analyzed in 41 patients by reverse transcription-quantitative polymerase chain reaction. Data are presented as the mean \pm standard deviation. * $P < 0.05$. SERPINC1, serpin peptidase inhibitor clade C member 1.

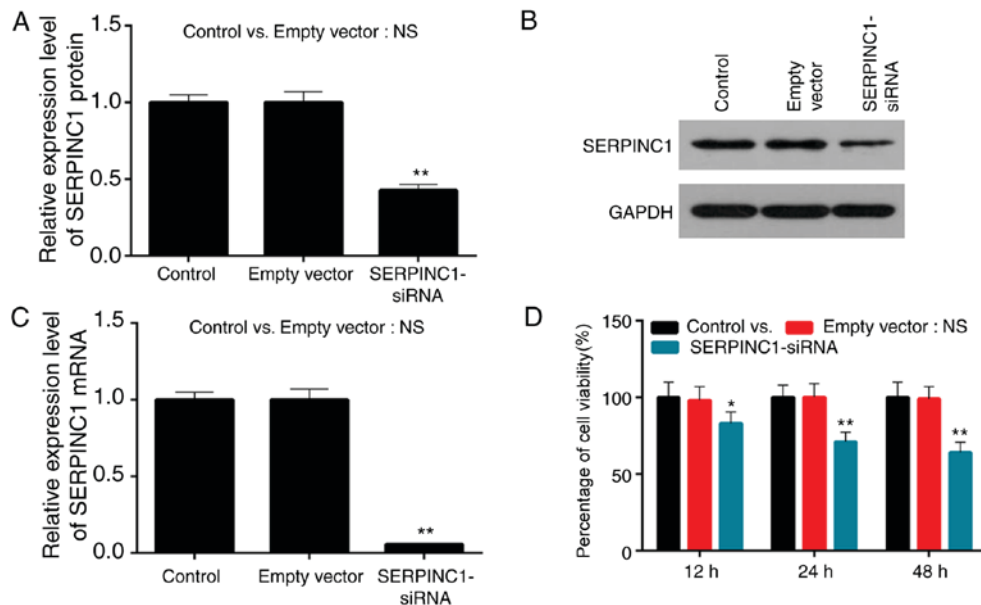


Figure 2. Effects of SERPINC1-siRNA transfection on the expression of SERPINC1 and the viability of HNE3 cells. HNE3 cells were treated with PBS, transfected with empty vector or SERPINC1-siRNA. (A and B) Western blot analysis was performed to determine the expression levels of SERPINC1 protein in each group. (C) Reverse transcription-quantitative polymerase chain reaction was performed to determine the mRNA expression of SERPINC1 in each group. (D) Cell viability was analyzed using a Cell Counting Kit-8 assay. Data are presented as the mean \pm standard deviation. * $P < 0.05$, ** $P < 0.01$ vs. the empty vector group. Empty vector, negative control siRNA; NS, not significant; SERPINC1, serpin peptidase inhibitor clade C member 1; siRNA, small interfering RNA.

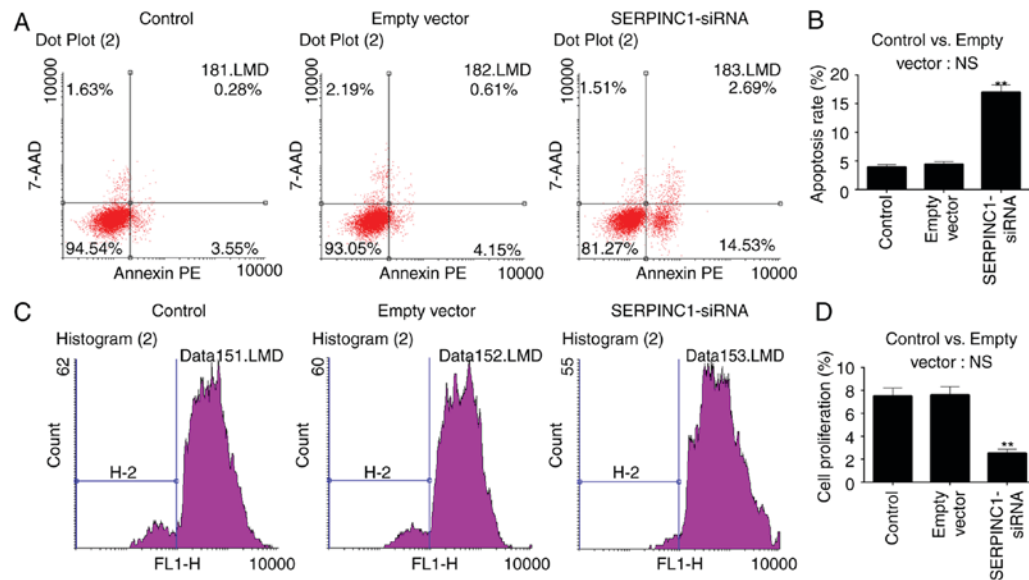


Figure 3. Effects of SERPINC1-siRNA transfection on apoptosis and proliferation of HNE3 cells. (A and B) Flow cytometry was performed to evaluate apoptosis in non-transfected, empty vector-transfected and SERPINC1-siRNA-transfected cells. (C and D) Flow cytometry was performed to analyze the proliferation of each group following transfection. Data are presented as the mean \pm standard deviation. ** $P < 0.01$ vs. the empty vector group. 7-AAD, 7-amino-actinomycin D; empty vector, negative control siRNA; NS, not significant; PE, phycoerythrin; SERPINC1, serpin peptidase inhibitor clade C member 1; siRNA, small interfering RNA.

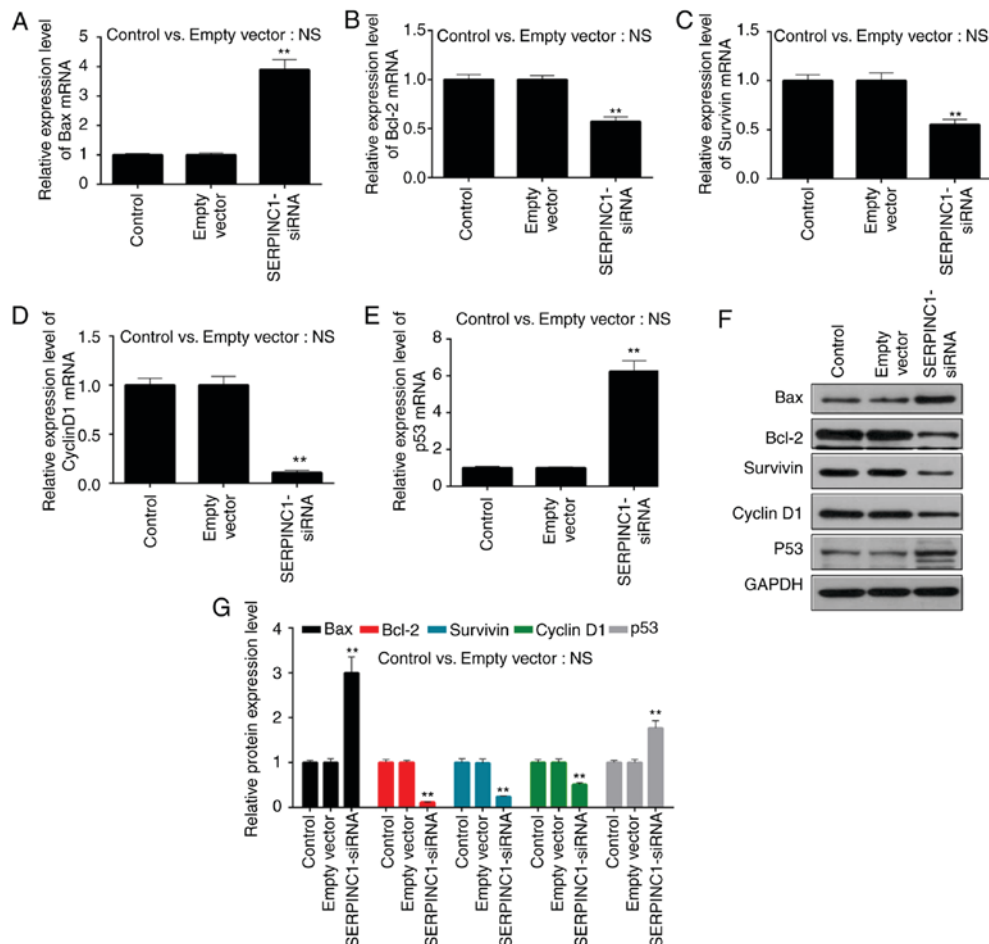


Figure 4. Effects of SERPINC1-siRNA transfection on the expression of apoptosis and cell cycle-associated genes in HNE3 cells. Reverse transcription-quantitative polymerase chain reaction was performed to determine the expression levels of (A) Bax, (B) Bcl-2, (C) survivin, (D) cyclin D1 and (E) p53 mRNA in non-transfected, empty vector-transfected and SERPINC1-siRNA-transfected cells. (F and G) Expression levels of Bax, Bcl-2, survivin, cyclin D1 and p53 proteins were determined via western blot analysis. Data are presented as the mean \pm standard deviation. ** $P < 0.01$ vs. the empty vector group. Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-associated X protein; empty vector, negative control siRNA; NS, not significant; SERPINC1, serpin peptidase inhibitor clade C member 1; siRNA, small interfering RNA.

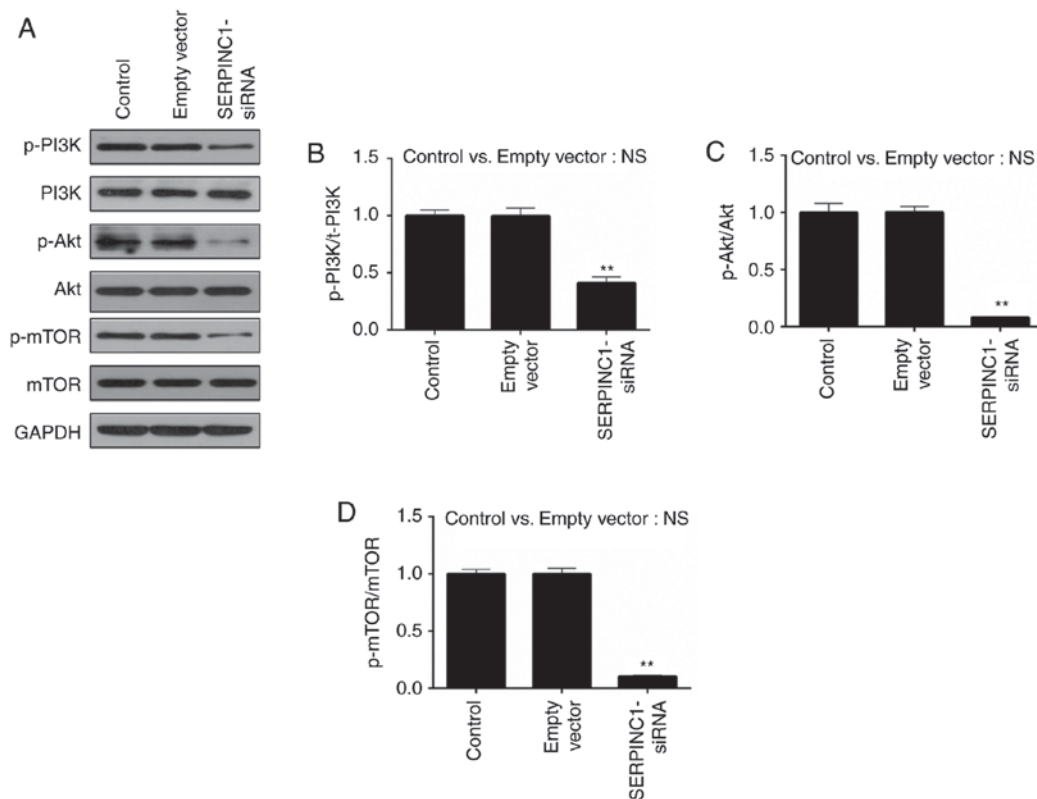


Figure 5. Effects of SERPINC1-siRNA transfection on PI3K/Akt/mTOR pathway. (A) Western blot analysis was performed to determine the expression and phosphorylation of PI3K, Akt and mTOR proteins. (B-D) p-PI3K/PI3K, p-Akt/Akt and p-mTOR/mTOR ratios were calculated for non-transfected, empty vector-transfected and SERPINC1-siRNA-transfected cells. Data are presented as the mean \pm standard deviation. **P<0.01 vs. the empty vector group. Akt, protein kinase B; empty vector, negative control siRNA; mTOR, mammalian target of rapamycin; NS, not significant; p, phosphorylated; PI3K, phosphatidylinositol 3-kinase; SERPINC1, serpin peptidase inhibitor clade C member 1; siRNA, small interfering RNA.

individuals (23). ATIII promotes the inhibition of proteases via interactions with heparin-like substances on the surface of endothelial cells (36). As NPC is a tumor that derives from a malignant lesion of epithelial cells (37), the expression of SERPINC1 in NPC was investigated. The results of the present study revealed that SERPINC1 was significantly upregulated in NPC tissues and the metastasis of NPC was highly associated with elevated expression of SERPINC1.

The present study proposed that knockdown of SERPINC1 may protect against the progression of NPC. The data revealed that the proliferation of the NPC cell line HNE3 was suppressed by SERPINC1-siRNA. To further investigate the effects of SERPINC1 on NPC, flow cytometry analysis was performed on cells following transfection with SERPINC1-siRNA. The results demonstrated that silencing SERPINC1 promoted the apoptosis and inhibited the proliferation of HNE3 cells. Therefore, the expression of apoptosis- and cell cycle-associated genes following SERPINC1-siRNA transfection was investigated. Cyclin D1 binds to and activates cyclin-dependent kinase 4 during G1 to regulate G1/S cell cycle transition, thereby promoting cell proliferation (38). Conversely, the tumor suppressor gene p53 inhibits cell proliferation (39). Bax directly regulates the activity of proapoptotic target proteins to induce apoptosis (40), whereas Bcl-2 exhibits antiapoptotic effects; overexpression of Bcl-2 is common in NPC (41). Survivin is expressed only in tumors and embryonic tissues, and is associated with cellular immortality (42). It was observed that the downregulation of SERPINC1 increased the

expression of Bax and p53, but decreased that of Bcl-2, survivin and cyclin D1. Collectively, SERPINC1 silencing promoted the apoptosis and inhibited the proliferation of NPC cells by regulating the expression of apoptosis-associated proteins and cyclin D1, suggesting that SERPINC1 may contribute to the pathogenesis of NPC.

The PI3K/Akt/mTOR signaling pathway contributes to the pathogenesis of numerous tumors (43,44). Similar to SERPINC1, the PI3K/Akt pathway serves roles in coagulation and anti-inflammatory processes (45,46). Therefore, the activity of the PI3K/Akt/mTOR signaling pathway was investigated following silencing of SERPINC1 in the present study. The results revealed that the expression levels of PI3K, Akt and mTOR proteins were markedly unaltered by transfection with SERPINC1-siRNA; however, the levels of phosphorylation of each protein were significantly downregulated. It was recently reported that the PI3K-dependent activation of Akt induced the phosphorylation of the Ser136/Ser112 residues of Bcl-2-associated death promoter (Bad), promoting apoptosis via the separation of Bad/Bcl-2 heterodimers (47). Furthermore, activation of the PI3K-Akt pathway promotes the phosphorylation of the Ser184 residue of Bax, inhibiting the antiapoptotic effects of the protein (48). Additionally, PI3K/Akt/mTOR promotes cell proliferation via regulation of cell cycle-associated proteins (49-52). Collectively, it was proposed that downregulation of SERPINC1 in NPC cells may suppress proliferation and induce apoptosis by inhibiting the activation of the PI3K/Akt/mTOR signaling pathway;

however, the association between this signaling pathway, and the proliferation and apoptosis of cells require further investigation. Furthermore, due to the crosstalk that occurs between signaling pathways, the possibility that other pathways may also be involved in the effects of SERPINC1 downregulation cannot be excluded. Additionally, the findings of the present study were obtained from a single cell line *in vitro*; thus, performing similar experiments in additional NPC cell lines or animals models may provide insight into the roles of SERPINC1 in the pathogenesis of NPC. Finally, the use of specific inhibitors of the PI3K/Akt/mTOR signaling pathway is required to further demonstrate the role of this pathway in the effects of SERPINC1 on NPC cells.

In conclusion, SERPINC1 was upregulated in tumor tissues from patients with NPC. Knockdown of SERPINC1 suppressed the proliferation and promoted the apoptosis of HNE3 cells by regulating the expression levels of cell cycle- and apoptosis-associated proteins. The activation of the PI3K/Akt/mTOR signaling pathway was suppressed by silencing of the SERPINC1 gene. Collectively, these findings suggested that SERPINC1 may be a potential target for the treatment of patients with NPC.

Acknowledgements

Not applicable.

Funding

No funding was received.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JX and YYi conceived and designed the present study. GX, LL, QW, YYa acquired, analyzed and interpreted data. YYi, JX, YYa drafted the article or critically revised it for important intellectual content. All authors read and approved the final manuscript. All authors agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

All patients provided signed informed consent. The present study was approved by The Ethics Committee of Tongde Hospital of Zhejiang Province (Hangzhou, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Chua MLK, Wee JTS, Hui EP and Chan ATC: Nasopharyngeal carcinoma. *Lancet* 387: 1012-1024, 2016.
2. Hsu MM and Tu SM: Nasopharyngeal carcinoma in Taiwan. Clinical manifestations and results of therapy. *Cancer* 52: 362-368, 1983.
3. Qi X, Li J, Zhou C, Lv C and Tian M: MicroRNA-320a inhibits cell proliferation, migration and invasion by targeting BMI-1 in nasopharyngeal carcinoma. *FEBS Lett* 588: 3732-3738, 2014.
4. Qin DX, Hu YH, Yan JH, Xu GZ, Cai WM, Wu XL, Cao DX and Gu XZ: Analysis of 1379 patients with nasopharyngeal carcinoma treated by radiation. *Cancer* 61: 1117-1124, 1988.
5. Jenkin RD, Anderson JR, Jereb B, Thompson JC, Pyesmany A, Wara WM and Hammond D: Nasopharyngeal carcinoma-a retrospective review of patients less than thirty years of age: A report of Children's cancer study group. *Cancer* 47: 360-366, 1981.
6. Geara FB, Glisson BS, Sanguineti G, Tucker SL, Garden AS, Ang KK, Lippman SM, Clayman GL, Goepfert H, Peters LJ and Hong WK: Induction chemotherapy followed by radiotherapy versus radiotherapy alone in patients with advanced nasopharyngeal carcinoma: Results of a matched cohort study. *Cancer* 79: 1279-1286, 1997.
7. OuYang PY, Xie C, Mao YP, Zhang Y, Liang XX, Su Z, Liu Q and Xie FY: Significant efficacies of neoadjuvant and adjuvant chemotherapy for nasopharyngeal carcinoma by meta-analysis of published literature-based randomized, controlled trials. *Ann Oncol* 24: 2136-2146, 2013.
8. Chen L, Hu CS, Chen XZ, Hu GQ, Cheng ZB, Sun Y, Li WX, Chen YY, Xie FY, Liang SB, *et al*: Adjuvant chemotherapy in patients with locoregionally advanced nasopharyngeal carcinoma: Long-term results of a phase 3 multicentre randomised controlled trial. *Eur J Cancer* 75: 150-158, 2017.
9. Chen YP, Tang LL, Yang Q, Poh SS, Hui EP, Chan ATC, Ong WS, Tan T, Wee J, Li WF, *et al*: Induction chemotherapy plus concurrent chemoradiotherapy in endemic nasopharyngeal carcinoma: Individual patient data pooled analysis of four randomized trials. *Clin Cancer Res* 24: 1824-1833, 2018.
10. Chua DT, Sham JS, Kwong DL, Choy DT, Au GK and Wu PM: Prognostic value of paranasopharyngeal extension of nasopharyngeal carcinoma. A significant factor in local control and distant metastasis. *Cancer* 78: 202-210, 1996.
11. Yan M, Kumachev A and Chan KKW: Is there any benefit to adding adjuvant chemotherapy after concurrent chemoradiotherapy for nasopharyngeal carcinoma? *Eur J Cancer* 56: 186-187, 2016.
12. Nieder C: Influence of dose and fractionation in intensity modulated re-irradiation of patients with relapse of nasopharyngeal carcinoma: A randomized phase II study. *Strahlenther Onkol* 191: 203-204, 2015 (In German).
13. Setton J, Han J, Kannarunimit D, Wu YR, Rosenberg SA, DeSelm C, Wolden SL, Jillian Tsai C, McBride SM, Riaz N and Lee NY: Long-term patterns of relapse and survival following definitive intensity-modulated radiotherapy for non-endemic nasopharyngeal carcinoma. *Oral Oncol* 53: 67-73, 2016.
14. Solomon B, Wilner KD and Shaw AT: Current status of targeted therapy for anaplastic lymphoma kinase-rearranged non-small cell lung cancer. *Clin Pharmacol Ther* 95: 15-23, 2014.
15. Sullivan RJ and Flaherty KT: Resistance to BRAF-targeted therapy in melanoma. *Eur J Cancer* 49: 1297-1304, 2013.
16. Knoechel B, Roderick JE, Williamson KE, Zhu J, Lohr JG, Cotton MJ, Gillespie SM, Fernandez D, Ku M, Wang H, *et al*: An epigenetic mechanism of resistance to targeted therapy in T cell acute lymphoblastic leukemia. *Nat Genet* 46: 364-370, 2014.
17. Riquelme I, Saavedra K, Espinoza JA, Weber H, Garcia P, Nervi B, Garrido M, Corvalán AH, Roa JC and Bizama C: Molecular classification of gastric cancer: Towards a pathway-driven targeted therapy. *Oncotarget* 6: 24750-24779, 2015.
18. Caspers M, Pavlova A, Driesen J, Harbrecht U, Klamroth R, Kadar J, Fischer R, Kemkes-Matthes B and Oldenburg J: Deficiencies of antithrombin, protein C and protein S-practical experience in genetic analysis of a large patient cohort. *Thromb Haemost* 108: 247-257, 2012.
19. Choay J, Petitou M, Lormeau JC, Sinay P, Casu B and Gatti G: Structure-activity relationship in heparin: A synthetic pentasaccharide with high affinity for antithrombin III and eliciting high anti-factor Xa activity. *Biochem Biophys Res Commun* 116: 492-499, 1983.

20. Fukui H, Taniguchi A, Sakamoto S, Kawahara S, Matsunaga T, Taira K, Tanaka S and Kamitsuji H: Antithrombin III in children with various renal diseases. *Pediatr Nephrol* 3: 144-148, 1989.
21. Levy JH, Sniecinski RM, Welsby IJ and Levi M: Antithrombin: Anti-inflammatory properties and clinical applications. *Thromb Haemost* 115: 712-728, 2016.
22. Zietek Z, Iwan-Zietek I, Kotschy M, Wiśniewska E and Tyloch F: Antithrombin III activity in blood of patients with renal cancer. *Pol Merkur Lekarski* 2: 191-192, 1997 (In Polish).
23. Zietek Z, Iwan-Zietek I, Kotschy M, Wiśniewska E and Tyloch F: Activity of antithrombin III in the blood of patients with bladder cancer. *Pol Merkur Lekarski* 2: 268-269, 1997 (In Polish).
24. Pal N, Kertai MD, Lakshminarasimhachar A and Avidan MS: Pharmacology and clinical applications of human recombinant antithrombin. *Expert Opin Biol Ther* 10: 1155-1168, 2010.
25. Maeda A, Ohta K, Ohta K, Nakayama Y, Hashida Y, Toma T, Saito T, Maruhashi K and Yachie A: Effects of antithrombin III treatment in vascular injury model of mice. *Pediatr Int* 53: 747-753, 2011.
26. Gonzalez-Rodriguez A and Valverde AM: RNA interference as a therapeutic strategy for the treatment of liver diseases. *Curr Pharm Des* 21: 4574-4586, 2015.
27. Amin MB, Edge S, Greene F, Byrd DR, Brookland RK, Washington MK, Gershenwald JE, Compton CC, Hess KR, Sullivan DC, *et al* (eds): American joint committee on cancer staging manual. 8th edition. Springer, New York, NY, 2017.
28. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods* 25: 402-408, 2001.
29. Bei JX, Li Y, Jia WH, Feng BJ, Zhou G, Chen LZ, Feng QS, Low HQ, Zhang H, He F, *et al*: A genome-wide association study of nasopharyngeal carcinoma identifies three new susceptibility loci. *Nat Genet* 42: 599-603, 2010.
30. Su SF, Han F, Zhao C, Huang Y, Chen CY, Xiao WW, Li JX and Lu TX: Treatment outcomes for different subgroups of nasopharyngeal carcinoma patients treated with intensity-modulated radiation therapy. *Chin J Cancer* 30: 565-573, 2011.
31. Lin S, Lu JJ, Han L, Chen Q and Pan J: Sequential chemotherapy and intensity-modulated radiation therapy in the management of locoregionally advanced nasopharyngeal carcinoma: Experience of 370 consecutive cases. *BMC Cancer* 10: 39, 2010.
32. Vargas C, Swartz D, Vashi A, Blasser M, Kasareian A, Cesaretti J, Kiley K and Terk M: Long-term outcomes and prognostic factors in patients treated with intraoperatively planned prostate brachytherapy. *Brachytherapy* 12: 120-125, 2013.
33. Tao CJ, Lin L, Zhou GQ, Tang LL, Chen L, Mao YP, Zeng MS, Kang TB, Jia WH, Shao JY, *et al*: Comparison of long-term survival and toxicity of cisplatin delivered weekly versus every three weeks concurrently with intensity-modulated radiotherapy in nasopharyngeal carcinoma. *PLoS One* 9: e110765, 2014.
34. Feng HX, Guo SP, Li GR, Zhong WH, Chen L, Huang LR and Qin HY: Toxicity of concurrent chemoradiotherapy with cetuximab for locoregionally advanced nasopharyngeal carcinoma. *Med Oncol* 31: 170, 2014.
35. Mekaj Y, Lulaj S, Daci F, Rafuna N, Miftari E, Hoxha H, Sllamniku X and Mekaj A: Prevalence and role of antithrombin III, protein C and protein S deficiencies and activated protein C resistance in Kosovo women with recurrent pregnancy loss during the first trimester of pregnancy. *J Hum Reprod Sci* 8: 224-229, 2015.
36. Absher E, Labarrere CA, Carter C, Haag B and Faulk WP: The endothelial heparan sulfate-antithrombin III natural anticoagulant pathway in normal and transplanted human kidneys. *Transplantation* 53: 828-834, 1992.
37. Huang PY, Zeng TT, Li MQ, Ban X, Zhu YH, Zhang BZ, Mai HQ, Zhang L, Guan XY and Li Y: Proteomic analysis of a nasopharyngeal carcinoma cell line and a nasopharyngeal epithelial cell line. *Tumori* 101: 676-683, 2015.
38. Harper JW, Adami GR, Wei N, Keyomarsi K and Elledge SJ: The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. *Cell* 75: 805-816, 1993.
39. Yin Y, Tainsky MA, Bischoff FZ, Strong LC and Wahl GM: Wild-type p53 restores cell cycle control and inhibits gene amplification in cells with mutant p53 alleles. *Cell* 70: 937-948, 1992.
40. Nomura M, Shimizu S, Sugiyama T, Narita M, Ito T, Matsuda H and Tsujimoto Y: 14-3-3 Interacts directly with and negatively regulates pro-apoptotic Bax. *J Biol Chem* 278: 2058-2065, 2003.
41. Pan LL, Wang AY, Huang YQ, Luo Y and Ling M: Mangiferin induces apoptosis by regulating Bcl-2 and Bax expression in the CNE2 nasopharyngeal carcinoma cell line. *Asian Pac J Cancer Prev* 15: 7065-7068, 2014.
42. Satoh K, Kaneko K, Hirota M, Masamune A, Satoh A and Shimosegawa T: Expression of survivin is correlated with cancer cell apoptosis and is involved in the development of human pancreatic duct cell tumors. *Cancer* 92: 271-278, 2001.
43. Xu G, Zhang W, Bertram P, Zheng XF and McLeod H: Pharmacogenomic profiling of the PI3K/PTEN-AKT-mTOR pathway in common human tumors. *Int J Oncol* 24: 893-900, 2004.
44. Ciruelos Gil EM: Targeting the PI3K/AKT/mTOR pathway in estrogen receptor-positive breast cancer. *Cancer Treat Rev* 40: 862-871, 2014.
45. Schabbauer G, Tencati M, Pedersen B, Pawlinski R and Mackman N: PI3K-Akt pathway suppresses coagulation and inflammation in endotoxemic mice. *Arterioscler Thromb Vasc Biol* 24: 1963-1969, 2004.
46. Xu YQ, Long L, Yan JQ, Wei L, Pan MQ, Gao HM, Zhou P, Liu M, Zhu CS, Tang BS and Wang Q: Simvastatin induces neuroprotection in 6-OHDA-lesioned PC12 via the PI3K/AKT/caspase 3 pathway and anti-inflammatory responses. *CNS Neurosci Ther* 19: 170-177, 2013.
47. Bao RK, Zheng SF and Wang XY: Selenium protects against cadmium-induced kidney apoptosis in chickens by activating the PI3K/AKT/Bcl-2 signaling pathway. *Environ Sci Pollut Res Int* 24: 20342-20353, 2017.
48. Sui Y, Zheng X and Zhao D: Rab31 promoted hepatocellular carcinoma (HCC) progression via inhibition of cell apoptosis induced by PI3K/AKT/Bcl-2/BAX pathway. *Tumour Biol* 36: 8661-8670, 2015.
49. Liu W, Ren H, Ren J, Yin T, Hu B, Xie S, Dai Y, Wu W, Xiao Z, Yang X and Xie D: The role of EGFR/PI3K/Akt/cyclinD1 signaling pathway in acquired middle ear cholesteatoma. *Mediators Inflamm* 2013: 651207, 2013.
50. Li Y, Qu X, Qu J, Zhang Y, Liu J, Teng Y, Hu X, Hou K and Liu Y: Arsenic trioxide induces apoptosis and G2/M phase arrest by inducing Cbl to inhibit PI3K/Akt signaling and thereby regulate p53 activation. *Cancer Lett* 284: 208-215, 2009.
51. Dey JH, Bianchi F, Voshol J, Bonenfant D, Oakeley EJ and Hynes NE: Targeting fibroblast growth factor receptors blocks PI3K/AKT signaling, induces apoptosis, and impairs mammary tumor outgrowth and metastasis. *Cancer Res* 70: 4151-4162, 2010.
52. Prasad SB, Yadav SS, Das M, Modi A, Kumari S, Pandey LK, Singh S, Pradhan S and Narayan G: PI3K/AKT pathway-mediated regulation of p27(Kip1) is associated with cell cycle arrest and apoptosis in cervical cancer. *Cell Oncol (Dordr)* 38: 215-225, 2015.



This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International (CC BY-NC-ND 4.0) License.