

Long non-coding RNA plasmacytoma variant translocation 1 gene promotes the development of cervical cancer via the NF- κ B pathway

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Abstract. The long noncoding RNA plasmacytoma variant translocation 1 gene (LncRNA PVT1) has an important role in tumor occurrence and development, yet the role and underlying molecular mechanisms of this RNA in cervical cancer have not yet been elucidated. In the present study, three cervical cancer cell lines (HeLa, Ca Ski and SiHa) were used to verify how LncRNA PVT1 mediates cervical cancer development, and the H8 cell line was used as a control. A LncRNA PVT1 overexpression vector or small interfering RNAs targeting LncRNA PVT1 were transfected into cervical cancer cells to generate LncRNA PVT1 overexpression and silencing in these cells. LncRNA PVT1 overexpression accelerated the growth of cervical cancer cells by advancing the cell cycle and inhibiting cellular apoptosis; increases in Cyclin D1 (CCND1) mRNA and activated Bcl-2 protein expression levels also supported this finding. Furthermore, NF- κ B activation and expression was increased by LncRNA PVT1 overexpression. In addition, NF- κ B activation or inhibition induced changes in cell viability, accompanied by changes in CCND1 and Bcl-2 expression. Increases or decreases in microRNA-16 (miR-16) expression (using miR mimics and inhibitors) also corresponded to changes in LncRNA PVT1 expression, *in vitro*. miR-16 mimics and inhibitor had opposite effects to those of NF- κ B activity, and miR-16 was demonstrated to directly interact with the NF- κ B gene as measured using the dual-luciferase assay. In summary, LncRNA PVT1 inhibits the effect of miR-16, promoting the cell cycle and inhibiting cellular apoptosis

of cervical cancer cells, potentially via the NF- κ B pathway. The data from the present study will contribute to the current knowledge surrounding the theoretical basis of cervical cancer and provide a new perspective for the treatment of cervical cancer.

Introduction

There were ~500,000 people with cervical cancer worldwide in 2012, and in women, cervical cancer ranks second in cancer occurrence and 15th in cancer mortality (1-3). Currently, the pathogenesis of cervical cancer is not fully clear, but human papillomavirus (HPV) infection is established to be the most closely associated risk factor for cervical cancer occurrence. However, high-risk HPV infection alone is not enough to induce tumor progression (4).

Previous studies have revealed that HPV activates the NF- κ B pathway to promote expression of different anti-apoptosis genes that induce cervical cancer development through inhibition of cellular apoptosis (5-8). NF- κ B an essential nuclear transcription factor composed of RelA/p65, p50, p52 and NF- κ B family members (9). NF- κ B pathway activation is associated with the development of various tumors and can promote tumor cell growth, proliferation and anti-apoptotic effects to promote malignant transformation and tumor cell metastases (10,11). NF- κ B pathway activation is a common mechanism used to promote tumor survival; the activated NF- κ B induces expression of various anti-apoptotic genes, such as the cellular inhibitor of apoptosis protein (12), the X-linked inhibitor of apoptosis protein (13) and Bcl-2 family proteins (14). In addition, NF- κ B activation was shown to be present in cervical cancer (15), suggesting that NF- κ B may have a role in the development and progression of cervical cancer.

MicroRNAs (miRs) serve as markers of cervical cancer progression (16), and it was previously reported that miR-16 has an important role in the regulation of cell apoptosis, proliferation, migration and invasion of cervical cancer (17-19). In addition, miR-16 targets inhibitor of nuclear factor κ B kinase subunit β (I κ B α) in hepatocellular carcinoma (20), and decreases NF- κ B

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gene expression; thus, reducing the levels of activated NF- κ B (21). Therefore, miR-16 has the important role of regulating the NF- κ B signaling pathway. On the other hand, long non-coding RNAs (lncRNAs) are involved in different tumor processes (22) through regulation of target gene expression at the transcriptional, post-transcriptional and epigenetic levels (23-25). lncRNAs also serve as precursors of small interfering RNAs (siRNAs), miRs and piwi-interacting RNAs (26,27), and can compete with miR as competing endogenous RNAs (ceRNAs) to achieve inter-communication and regulation (28-33). Furthermore, the lncRNA-plasmacytoma variant translocation 1 (lncRNA PVT1) gene was reported to be a biomarker of tumorigenesis and associated with the development of cervical cancer (34-37) by competitively inhibiting miRs (38,39).

Although no evidence currently exists to support the hypothesis that the interaction between lncRNA PVT1 and miR-16 induces cervical cancer development, it is speculated that the lncRNA PVT1 gene promotes cervical cancer development by inhibiting miR expression and activating the NF- κ B pathway. In the present study, three known cervical cancer cell lines (HeLa, Ca Ski and SiHa cells) and the H8 cell line (as an external control of three cervical cancer cell lines) were selected as the experimental models for *in vitro* experiments to investigate the effect and molecular mechanisms of lncRNA PVT1 on cervical cancer development.

Materials and methods

Cell culture. As it involved the use of human materials, the present study was approved by the Ethics Committee of the Affiliated Hospital of Qingdao University (Qingdao, China) according to the experimental requirements of the school. HeLa, Ca Ski, SiHa and H8 cells were purchased from the National Infrastructure of Cell Line Resources of China. H8 cell line (the human cervical epithelial immortalized cell line) was used as the external control of the three cervical cancer cell lines, HeLa, Ca Ski and SiHa. Each cell line was cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) containing 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) in 100 mm culture dishes (Corning Inc.) and incubated in a 5% CO₂ incubator at 37°C. The media was changed every 3 days, and the cells were passaged once they reached 80% confluence. Additionally, 293T cells (purchased from the National Infrastructure of Cell Line Resources of China) were used in co-transfection experiment and cultured with the same aforementioned treatment. To ensure cell activity, the cells from passage 2-4 were used for further experiments.

Construction of lncRNA PVT1 and 3'-untranslated region (3'UTR) of NF- κ B overexpression vectors and siRNA of lncRNA PVT1. According to database sequences of the human lncRNA PVT1 (National Center for Biotechnology Information reference sequence: NR_003367.3), the pEGFP-N3 eukaryotic expression vector (Beijing Tianyi Huiyuan Bioscience & Technology Inc.) carrying the lncRNA PVT1 sequence was constructed via artificial nucleotide synthesis at Tianyi Huiyuan Biotechnology Co. Ltd. Similarly, according to the information on lncRNA PVT1 in the Ensembl genome browser 96 database (reference number, ENSMUSG00000173039). The sequences of the 3'UTR and the mutant of the 3'UTR of NF- κ B

gene were obtained via artificial nucleotide synthesis at Beijing Tianyi Huiyuan Bioscience & Technology Inc. and the expression plasmid constructed. The siRNA (5'-GAGCUGCGAGCAAGAUGU-3') and control siRNA (si-NC; 5'-GAUCGUACUAUAGCUUGUA-3') of lncRNA PVT1 were purchased from Guangzhou RiboBio Co., Ltd.

Cell transfection. Eugene6 transfection reagent (Roche Diagnostics) was used according to the manufacturer's protocol. The overexpression vector (final concentration 1 μ g/ml) or siRNA of lncRNA PVT1 (final concentration 50 nm) were transfected into HeLa, Ca Ski and SiHa cells in 96-well plates or 100 mm dishes with 1x10⁵ cells/ml and cultured for 48 or 60 h, respectively, and the pEGFP-N3 empty vector or si-NC were also transfected with the corresponding internal controls. Additionally, HeLa cells were respectively treated with 5 μ g/ml lipopolysaccharides (LPS; Beyotime Institute of Biotechnology) plus the lncRNA PVT1 siRNA, with pyrrolidinedithiocarbamate (PDTC; 50 μ mol/l; Beyotime Institute of Biotechnology) alone, or with PDTC (50 μ mol/l) on the basis of the lncRNA PVT1 overexpression treatment for 8 h to activate or inhibit the NF- κ B pathway, and then the cells were continuously cultured for 60 h. In addition, using the Eugene6 transfection reagent, HeLa cells with 60% confluency were transiently transfected with the miR-16 mimics (final concentration 100 nm) or miR-16 inhibitor (final concentration 50 nm). All cells were collected for the experiments below following 60 h treatment.

MTT assay. Following culture of the cells from the four cell lines in 96-well plates with different treatments or no treatment for 60 h, cell numbers were determined using the MTT assay. The assay was conducted by adding 20 μ l MTT (5 mg/ml) to each well of the plates and incubating the plates for 4 h at 37°C. Following removal of the supernatant, formazan crystals were dissolved in 200 μ l dimethyl sulfoxide, and the absorbance was measured at 490 nm by microplate reader (Thermo Fisher Scientific, Inc.). There were eight replicate wells for each group to ensure the accuracy of the experiments.

Flow cytometry (FCM). Following treatment with or without the lncRNA-PVT1 siRNA for 48 h, HeLa cells were collected to use for cell cycle or cell apoptosis analyses using a FACSCanto II flow cytometer (Becton, Dickinson and Company). For the cell cycle analysis, cells were centrifuged at 300 x g for 5 min at 4°C and washed with ice-cold PBS following digestion with trypsin. The cells were then mixed with 0.25% Triton X-100 and 5 μ l propidium iodide (PI) and incubated for 30 min at room temperature in the dark. Cells were resuspended in 0.5 ml PBS and analyzed immediately.

For cellular apoptosis analysis, cells were digested with trypsin, centrifuged at 300 x g for 5 min at 4°C, and washed with ice-cold PBS. The cell pellets were resuspended in 100 μ l of Annexin V binding buffer, transferred to a 5 ml culture tube containing 5 μ l Annexin V-FITC, and mixed with 10 μ l PI by using the Annexin V-FITC Apoptosis Detection kit (Beyotime Institute of Biotechnology). The tubes were gently vortexed and incubated for 15 min at room temperature in the dark. Subsequently, 300 μ l binding buffer was added and the cells were analyzed immediately.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from three cervical cancer cells and H8 cells with different treatments using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. First-strand cDNA was synthesized using a PrimeScript RT Master kit (Takara Biotechnology Co., Ltd.) according to the manufacturer's protocol (37°C for 15 min, 85°C for 5 sec and then stored at 4°C). The qPCR was performed using SYBR Green reagents (Takara Biotechnology Co., Ltd.) and a 7500 Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). Following initial denaturation for 30 sec at 95°C, amplification was performed for 40 cycles (95°C for 5 sec and 60°C for 32 sec). The specific primer sequences were as follows: miR-16, forward 5'-TAGCAGCACGTAATATTGGCG-3'; LncRNA PVT1, forward 5'-TGCTCTAGAATCTGATGCACGTTCCACC-3', reverse 5'-CCGGAATTCCTTAATTCTCCAATCTCAAAA TAC-3'; NF-κB, forward 5'-GCATCCAGACCAACAACA ACC-3', reverse 5'-AGAGTTTCGGTTCCTCGGC-3'; and cyclin D1 (CCND1), forward 5'-CAGATCATCCGCAAA CACG C-3', reverse 5'-AAGTTGTTGGGGCTCCTCAG-3'. Expression was normalized using β-actin or U6 using the 2^{-ΔΔC_q} method (40). Primer sequences were as follows: β-actin, forward 5'-GCTCGTCGTCGACAACGGCTC-3', reverse 5'-CAAACATGATCTGGGTCATCTTCTC-3'; U6, forward 5'-CTCGCTTCGGCAGCAC-3', reverse 5'-GCGAGCACA GAATTAATACGAC-3'. A melting curve was constructed to verify the amplification of a single PCR product. Experiments were performed in triplicate with standard deviations of the C_q values not exceeding 0.5 on a within-run basis.

Dual-luciferase reporter assay. Following the sequence alignment analysis of NF-κB-3'UTR and miR-16, the pGL3-NF-κB-3'UTR reporter plasmid (binding sites, UUAUAA), the corresponding mutant plasmid (AAGGCC) and the empty plasmid (Beijing Tianyi Huiyuan Bioscience & Technology Inc.) were constructed. Using the Eugene6 transfection reagent, 293T cells with 60% confluency were transiently transfected with the pGL3-NF-κB-3'UTR reporter plasmid (final concentration 1 μg/ml) and miR-16 mimics (final concentration 100 nm) in the dual-luciferase reporter system, the corresponding mutant plasmid and the empty plasmid were transfected as the internal controls. After incubation for 36 h, firefly and *Renilla* luciferase activities were measured and compared using the dual-luciferase reporter assay kit (Promega Corporation) by Thermo Scientific™ Fluoroskan Ascent™ FL microplate reader (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol.

Western blotting. Total cell lysates were extracted from HeLa cells with the different treatments using a radioimmunoprecipitation assay (RIPA) buffer (Beijing Solarbio Science & Technology Co., Ltd.) to detect the pNF-κB (p65), NF-κB (p65), Iκ-Bα, Bcl-2 and CCND1 protein levels, and total protein levels in all samples were assessed using a BCA Protein Assay kit (Beijing Solarbio Science & Technology Co., Ltd.). After denaturation, equal amounts of protein were loaded into each well of a 10-12.5% polyacrylamide gel and subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were then transferred

onto polyvinylidene difluoride (PVDF) membranes, which were blocked with 5% skimmed milk (Sigma-Aldrich, Merck KGaA) at room temperature for 1 h on a shaker. Next, the membranes were incubated with primary antibodies overnight at 4°C and subsequently washed three times for 30 min before being incubated with goat anti-rabbit IgG-HRP (dilution 1:5,000, cat. no. ab205718; Abcam) at room temperature for 1 h. The bound antibodies were visualized with the ECL Plus Western Blotting Detection System (GE Healthcare Life Sciences). The following monoclonal antibodies were used: Rabbit anti-pNF-κB (p65) (65 kDa, dilution 1:10,000, cat. no. ab76302), rabbit anti-NF-κB (p65) (65 kDa, dilution 1:1,000, cat. no. ab32536), rabbit anti-Iκ-Bα (17 kDa, dilution 1:1,000, cat. no. ab178847), rabbit anti-Bcl-2 (26 kDa, dilution 1:1,000, cat. no. Ab32124), rabbit anti-CCND1 (34 kDa, dilution 1:10,000, cat. no. ab134175) and rabbit anti-Tubulin antibody (55 kDa, dilution 1:5,000, cat. no. ab59680) served as an internal protein loading control (Abcam).

Statistical analysis. All statistical analyses were performed using one-way ANOVA followed by a Student-Newman-Keuls test (the treatments with siRNA, siRNA plus LPS, overexpression, PDTC, or PDTC plus overexpression, respectively) with the Statistical Analysis Systems software (version 8.2; SAS Institute, Inc.) to determine significance. The data are expressed as the mean ± SD. P<0.05 was considered to indicate a statistically significant difference.

Results

LncRNA PVT1 induces an increase in cervical cancer cell number. Compared with H8 cells, LncRNA PVT1 expression levels were significantly increased in the three cervical cancer cells (Fig. 1A), and the cell numbers had also significantly increased (Fig. 1B). To further identify the effect of LncRNA PVT1 on cervical cancer, HeLa, Ca Ski and SiHa cells were transfected with either the LncRNA PVT1 overexpression vector or siRNA for 60 h, and then cells were counted. Following transfection with the overexpression vector, expression of the LncRNA PVT1 was significantly increased in the three cervical cancer cells (9-12-fold changes), and the knockdown efficiency of the LncRNA PVT1 siRNA was also apparent (75-90%; Fig. 1C). The results also revealed that cell numbers were significantly (P<0.05 or P<0.01) higher in the three cervical cancer cell lines treated with the LncRNA PVT1 overexpression vector and significantly lower (P<0.01) in those treated with siRNA compared with that of the corresponding internal control cells (empty vector or si-NC; Fig. 1D). Given that LncRNA PVT1 had similar effects on the cell numbers of the three cervical cancer cell types, HeLa cells were used for subsequent further analysis in the present study.

Furthermore, the FCM results revealed that cell percentages in S phase were significantly lower (P<0.05) and in the G₁ phase were significantly higher (P<0.05; Fig. 2A), and the apoptosis rate was significantly higher (P<0.05; Fig. 2B) in HeLa cells with the interference vector compared with that of untreated HeLa cells. CCND1 mRNA and Bcl-2 protein expression was decreased in HeLa cells treated with siRNA of LncRNA PVT1 compared with untreated HeLa cells (P<0.01). However, CCND1 mRNA and Bcl-2 protein expression was

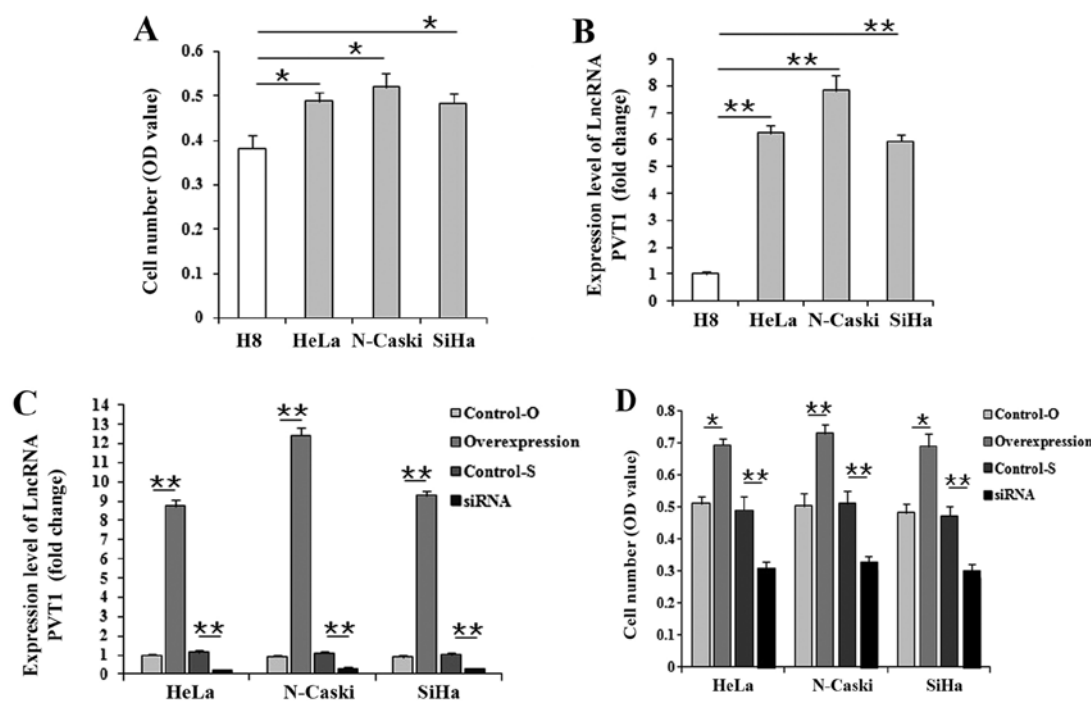


Figure 1. Effect of LncRNA PVT1 on the cell number of cervical cancer cell lines. After culturing for 60 h *in vitro*, (A) the comparison of cell number, assessed with MTT, and (B) LncRNA PVT1 expression, assessed with qPCR, between normal cervical epithelial cells (H8) and cervical cancer cells (HeLa, Ca Ski and SiHa) was performed. After culturing for 60 h *in vitro*, (C) cell numbers and (D) LncRNA PVT1 expression of three cervical cancer cells treated with LncRNA PVT1 overexpression or siRNAs was performed. Data are presented as the mean \pm standard deviation; n=3 (qPCR) or 8 (MTT) *P<0.05; **P<0.01. OD, optical density; LncRNA PVT1, long non-coding RNA plasmacytoma variant translocation 1; qPCR, quantitative PCR; Control-O, vector control; Control-S, negative control siRNA; siRNA, small interfering RNA.

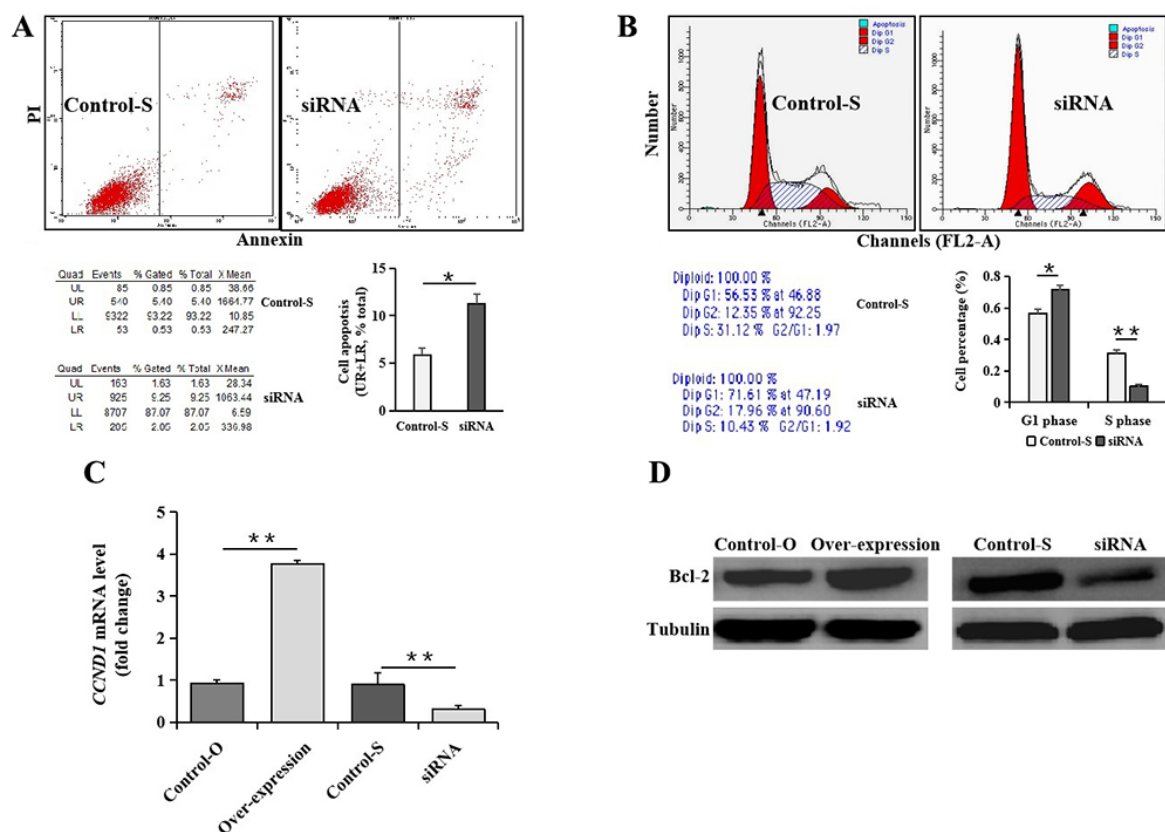


Figure 2. Effect of LncRNA PVT1 on the cell cycle and cell apoptosis of cervical cancer cells. (A) Apoptosis or (B) cell cycle analyses performed using flow cytometry were compared between HeLa cells with or without LncRNA PVT1 interference. (C) CCND1 mRNA and (D) Bcl-2 protein expression in HeLa cells treated with LncRNA PVT1 overexpression or siRNA. Data are presented as the mean \pm standard deviation; n=3. *P<0.05, **P<0.01. LncRNA PVT1, long non-coding RNA plasmacytoma variant translocation 1; PI, propidium iodide; Control-S, negative control siRNA; siRNA, small interfering RNA; CCND1, cyclin D1; Control-O, vector control.

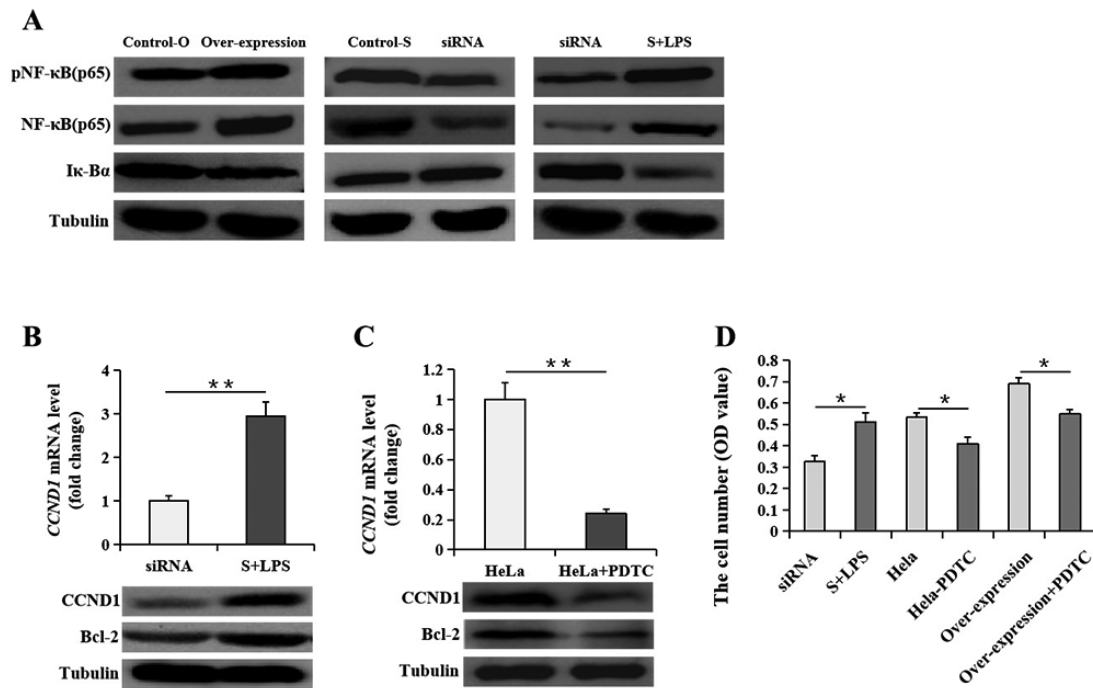


Figure 3. NF-κB signaling pathway is involved in the regulation of LncRNA PVT1 on cervical cancer cells. (A) The changes of NF-κB (p65) expression and activity and Iκ-Bα expression using western blotting in HeLa cells given different treatments. (B) CCND1 mRNA and protein expression and Bcl-2 protein expression in HeLa cells with LncRNA PVT1 siRNAs with or without LPS treatment. (C) CCND1 mRNA and protein expression and Bcl-2 protein expression in HeLa cells with PDTC. (D) HeLa cell number changes in cultures with treatments of LncRNA PVT1 siRNAs with LPS, PDTC or PDTC with LncRNA PVT1 overexpression were analyzed using an MTT assay. Data are presented as the mean ± SEM; n=3 or 8. *P<0.05; **P<0.01. LncRNA PVT1, long non-coding RNA plasmacytoma variant translocation 1; Control-O, vector control; Control-S, negative control siRNA; siRNA, small interfering RNA; S + LPS, siRNA + lipopolysaccharide; p, phosphorylated; Iκ-Bα, inhibitor of nuclear factor κB kinase subunit β; CCND1, cyclin D1; PDTC, pyrrolidinedithiocarbamate; OD, optical density.

increased in HeLa cells with LncRNA PVT1 overexpression compared with those of untreated HeLa cells (Fig. 2C and D).

LncRNA PVT1 activates the NF-κB pathway in HeLa cells. As the NF-κB pathway has a role in cell apoptosis and proliferation, whether LncRNA PVT1 could activate the NF-κB pathway was investigated. The results revealed that both NF-κB expression and activity levels were lower in HeLa cells with the siRNA targeting LncRNA PVT1, and higher in HeLa cells with the overexpression vector compared with that of untreated HeLa cells. Similarly, Iκ-Bα levels changed in a manner opposite to that of NF-κB activity in HeLa cells given the different treatments. Furthermore, HeLa cells were co-treated with 5 μg/ml LPS (as an activator of NF-κB) following transfection with siRNA LncRNA PVT1. After 8 h of co-treatment, NF-κB activity recovered in the untreated HeLa cell levels, but Iκ-Bα levels decreased again, demonstrating an opposite effect to that NF-κB (Fig. 3A), which was accompanied by corresponding changes in CCND1 and Bcl-2 expression (Fig. 3B). Furthermore, PDTC was used as a specific inhibitor of NF-κB to investigate the specific mediating action of NF-κB in the present study. When HeLa cells were treated with PDTC for 8 h, the CCND1 and Bcl-2 expression levels exhibited opposite effects to those observed following LPS treatment (Fig. 3C). In addition, the cell number of HeLa cells treated with both siRNA and LPS was significantly increased (P<0.05) compared with that of the HeLa cells treated with siRNA targeting LncRNA PVT1 only (Fig. 3D). However, the cell number of HeLa cells was significantly decreased by treatment with PDTC (P<0.05) compared with that in HeLa cells without

PDTC, and a significant decrease (P<0.05) was also observed in HeLa cells treated with both PDTC and transfected with the LncRNA PVT1 overexpression vector compared with that in HeLa cells transfected with the overexpression of LncRNA PVT1 only (Fig. 3D).

miR-16 is downregulated by LncRNA PVT1 and targets the NF-κB gene. According to the target regulation of miR-16 to NF-κB, miR-16 expression levels were assessed in the present study. Firstly, compared with H8 cells, miR-16 expression levels were significantly lower in the three cervical cancer cell lines (Fig. 4A). Subsequently, miR-16 expression levels were significantly reduced (P<0.01) in HeLa cells treated with the LncRNA PVT1 overexpression vector compared with HeLa cells transfected with the vector control (Fig. 4B). On the contrary, miR-16 expression levels were significantly upregulated (P<0.01) in HeLa cells transfected with LncRNA PVT1 siRNA compared with the control siRNA (Fig. 4B). Subsequently, when the HeLa cells were transfected with miR-16 mimics and an miR-16 inhibitor (Fig. 4C), NF-κB expression levels were reduced by miR-16 mimics and increased by miR-16 inhibitor (Fig. 4D). Considering the target regulation of miR-16 to NF-κB as previous reported (41), additional experiments were performed in order to investigate the regulatory association between miR-16 and NF-κB in the present study. The results of the dual-luciferase reporter assay revealed that luciferase activity significantly increased in 293T cells (P<0.01) following co-transfection with the miR-16 mimics and the pEGFP-N3-3'UTR vector of the NF-κB gene (Fig. 4E), providing direct evidence that miR-16 targets NF-κB mRNA.

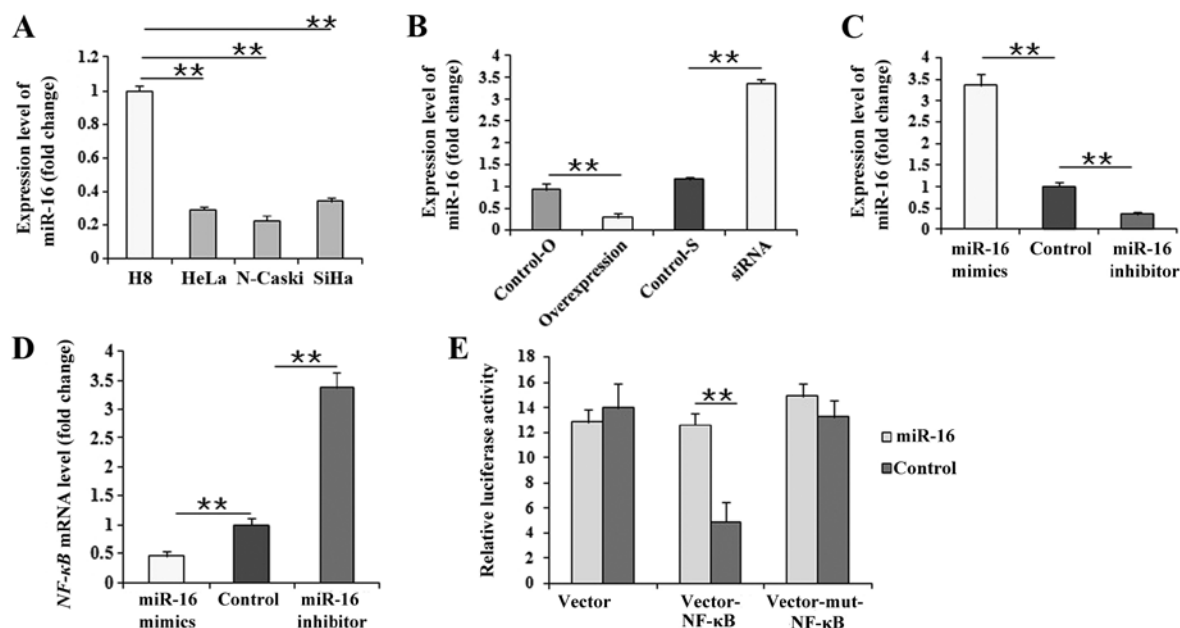


Figure 4. miR-16 regulation of NF- κ B (p65) expression in cervical cancer cells. (A) The comparisons of miRNA-16 expression levels between normal cervical epithelial cells and cervical cancer cells as measured using qPCR. (B and C) miR-16 expression levels in HeLa cells with different treatments as measured using qPCR. (D) The changes in NF- κ B expression levels in the HeLa cells with treatments with miR-16 mimics or an inhibitor using qPCR. (E) The relative luciferase activity between the miRNA-16 and NF- κ B genes as measured with the dual luciferase system. Data are presented as the mean \pm SD; $n=3$. ** $P<0.01$. qPCR, quantitative PCR; miR, microRNA; Control-O, vector control; Control-S, negative control siRNA; siRNA, small interfering RNA; mut, mutant.

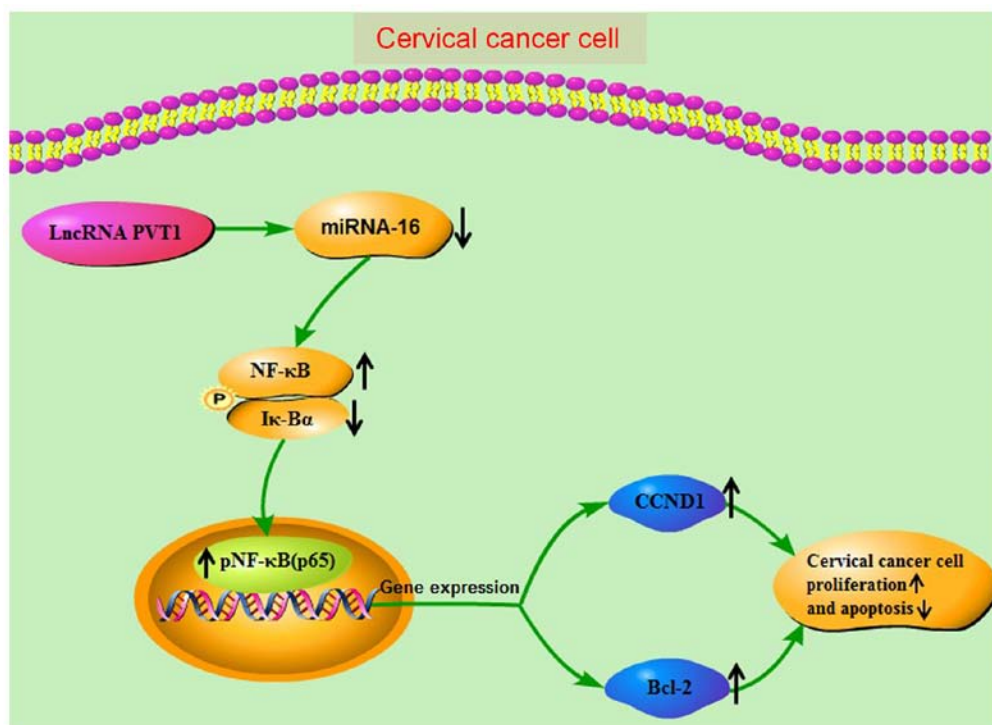


Figure 5. Underlying LncRNA PVT1 regulatory mechanisms may promote cervical cancer development. LncRNA PVT1 competitively downregulated miRNA-16 expression, which targeted the NF- κ B gene to upregulate NF- κ B expression and activity causing inhibition of apoptosis and advancement of the cell cycle in cervical cancer cells. LncRNA PVT1, long non-coding RNA plasmacytoma variant translocation 1; miRNA, microRNA; p, phosphorylated; I κ -B α , inhibitor of nuclear factor κ B kinase subunit β ; CCND1, cyclin D1.

Discussion

Cervical cancer causes considerable morbidity and mortality in patients afflicted with this disease (1-3); however, the

pathogenesis of cervical cancer remains unclear. It is known that the HPV infection is closely associated with the occurrence of cervical cancer. However, simple high-risk HPV infection is not enough to induce tumor progression (4).

Considering previously reported studies, it was speculated that lncRNA PVT1 may promote the occurrence and development of cervical cancer via the NF- κ B pathway. As such, HeLa cells, a cervical cancer cell line, were used to perform *in vitro* experiments that investigated the effect of lncRNA PVT1 on the occurrence and development of cervical cancer.

Using HeLa, Ca Ski and SiHa cells transfected with lncRNA PVT1 overexpression or siRNAs, changes in cell numbers of the three different cervical cancer cell lines were identified, which were similar to that of lncRNA PVT1 expression. Given that lncRNA PVT1 had a similar effect on cell numbers in all three cell lines, HeLa cells were used for subsequent experiments in the present study. Because of the change in cell numbers during the cell cycle or as a result of apoptosis, FCM analysis was also performed to define the effect of lncRNA PVT1 on HeLa cells, and revealed that lncRNA PVT1 siRNA inhibited the G₁/S phase transition and promoted cellular apoptosis. CCND1 and Bcl-2 are the important factors that positively regulate the cell cycle or cell apoptosis (42,43). In the present study, the CCND1 mRNA levels and Bcl-2 protein levels revealed results consistent with that of the FCM results, which supported positive regulation of the cell cycle and cellular apoptosis via these two factors. Therefore, it can be assumed that lncRNA PVT1 promotes HeLa cell proliferation by advancing the transition of the G₁/S phase and inhibiting cellular apoptosis. In order to assess the regulatory role of the NF- κ B pathway on preventing apoptosis and promoting proliferation of the tumor cells (10,11,44), an additional experiment was performed to detect NF- κ B (p65) activity in HeLa cells. As expected, the NF- κ B (p65) activity in HeLa cells was altered, which was similar to the change seen with lncRNA PVT1 expression; however, Ik-B α activity exhibited the opposite change. LPS is a known activator of the NF- κ B signaling pathway, and PDTC is the key inhibitor of the NF- κ B signaling pathway, and so it was verified that NF- κ B pathway mediated lncRNA PVT1 regulation in cervical cancer cells (45). HeLa cells were treated with LPS based on the lncRNA PVT1 interference studies, for which both NF- κ B (p65) activity and cell numbers recovered. In addition, the viability of HeLa cells overexpressing lncRNA PVT1 was reduced by treatment with PDTC. These results confirmed that lncRNA PVT1 promoted the increase of cervical cancer cells via the NF- κ B pathway.

Based on a previous study regarding miR-16 in tumor regulation (46), miR-16 expression was detected and significantly downregulated by lncRNA PVT1. As lncRNAs can also compete with miRs, as ceRNA, to achieve intercommunication and regulation (28-31), it was hypothesized that a competitive relationship exists between lncRNA PVT1 and miR-16 in HeLa cells. Thus, miR-16 mimics and an inhibitor were used to investigate the regulatory relationship between miR-16 and NF- κ B. It was revealed that miR-16 and NF- κ B expression levels were contrary to one another, in that NF- κ B levels were significantly decreased after HeLa cells were transfected with the miR-16 mimics, but were significantly increased after being transfected with the miR-16 inhibitor. These results indicated that miR-16 downregulated NF- κ B expression. It was previously reported that NF- κ B activity was directly regulated by miR-16, which targets the NF- κ B gene (38). Therefore, a dual-luciferase reporter assay was performed to provide direct evidence that miR-16 targets the NF- κ B gene.

Together, these findings expand the current understanding of the underlying regulatory mechanism of lncRNA PVT1 in promoting increases in HeLa cell numbers. Finally, Fig. 5 presents a proposed a molecular regulatory network mediated by lncRNA PVT1 HeLa cells.

In summary, the data obtained in the present study demonstrate that lncRNA PVT1 competitively inhibits miR-16, causing an increase in the number of cervical cancer cells by promoting the cell cycle and inhibiting cellular apoptosis via the NF- κ B pathway, thus promoting cervical cancer development. These results will add to the theoretical basis of cervical cancer and provide a new perspective for the treatment of cervical cancer.

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Availability of data and materials

All data generated or analyzed during the present study are included in this published article.

Authors' contributions

CW and HZ contributed to the design of the study and performed the experiments, the interpretation of data and writing of the manuscript. HY, LW, HC and JJ contributed to the interpretation of data and writing of the manuscript. YW and AC contributed to the design of the study. All authors submitted comments on drafts and read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Joo YH, Jung CK, Sun DI, Park JO, Cho KJ and Kim MS: High-risk human papillomavirus and cervical lymph node metastasis in patients with oropharyngeal cancer. *Head Neck* 34: 10-14, 2012.
2. Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J and Jemal A: Global cancer statistics, 2012. *CA Cancer J Clin* 65: 87-108, 2015.
3. den Boon JA, Pyeon D, Wang SS, Horswill M, Schiffman M, Sherman M, Zuna RE, Wang Z, Hewitt SM, Pearson R, *et al*: Molecular transitions from papillomavirus infection to cervical precancer and cancer: Role of stromal estrogen receptor signaling. *Proc Natl Acad Sci USA* 112: E3255-E3264, 2015.

4. Dalstein V, Riethmuller D, Pr  tet JL, Le Bail Carval K, Sauti  re JL, Carbillet JP, Kantelip B, Schaal JP and Mougin C: Persistence and load of high-risk HPV are predictors for development of high-grade cervical lesions: A longitudinal French cohort study. *Int J Cancer* 106: 396-403, 2003.
5. Nees M, Geoghegan JM, Hyman T, Frank S, Miller L and Woodworth CD: Papillomavirus type 16 oncogenes downregulate expression of interferon-responsive genes and upregulate proliferation-associated and NF-kappaB-responsive genes in cervical keratinocytes. *J Virol* 75: 4283-4296, 2001.
6. Spitkovsky D, Hehner SP, Hofmann TG, M  ller A and Schmitz ML: The human papillomavirus oncoprotein E7 attenuates NF-kappa B activation by targeting the Ikappa B kinase complex. *J Biol Chem* 277: 25576-25582, 2002.
7. James MA, Lee JH and Klingelutz AJ: Human papillomavirus type 16 E6 activates NF-kappaB, induces cIAP-2 expression, and protects against apoptosis in a PDZ binding motif-dependent manner. *J Virol* 80: 5301-5307, 2006.
8. Jia QP, Yan CY, Zheng XR, Pan X, Cao X and Cao L: Upregulation of MTA1 expression by human papillomavirus infection promotes CDDP resistance in cervical cancer cells via modulation of NF-kB/APOBEC3B cascade. *Cancer Chemother Pharmacol* 83: 625-637, 2019.
9. Gilmore TD: Introduction to NF-kappaB: Players, pathways, perspectives. *Oncogene* 25: 6680-6684, 2006.
10. Jiang N, Xie F, Guo Q, Li MQ, Xiao J and Sui L: Toll-like receptor 4 promotes proliferation and apoptosis resistance in human papillomavirus-related cervical cancer cells through the Toll-like receptor 4/nuclear factor-kB pathway. *Tumour Biol* 39: 1010428317710586, 2017.
11. Perkins ND: The diverse and complex roles of NF-kB subunits in cancer. *Nat Rev Cancer* 12: 121-132, 2012.
12. Deveraux QL, Roy N, Stennicke HR, Van Arsdale T, Zhou Q, Srinivasula SM, Alnemri ES, Salvesen GS and Reed JC: IAPs block apoptotic events induced by caspase-8 and cytochrome c by direct inhibition of distinct caspases. *EMBO J* 17: 2215-2223, 1998.
13. Ono H, Iizumi Y, Goi W, Sowa Y, Taguchi T and Sakai T: Ribosomal protein S3 regulates XIAP expression independently of the NF-kB pathway in breast cancer cells. *Oncol Rep* 38: 3205-3210, 2017.
14. Chen QM and Tu VC: Apoptosis and heart failure: Mechanisms and therapeutic implications. *Am J Cardiovasc Drugs* 2: 43-57, 2002.
15. Prusty BK, Husain SA and Das BC: Constitutive activation of nuclear factor-kB: Preferential homodimerization of p50 subunits in cervical carcinoma. *Front Biosci* 10: 1510-1519, 2005.
16. Pardini B, De Maria D, Francavilla A, Di Gaetano C, Ronco G and Naccarati A: MicroRNAs as markers of progression in cervical cancer: A systematic review. *BMC Cancer* 18: 696, 2018.
17. Liu J, Yang L, Zhang J, Zhang J, Chen Y, Li K, Li Y, Li Y, Yao L and Guo G: Knock-down of NDRG2 sensitizes cervical cancer Hela cells to cisplatin through suppressing Bcl-2 expression. *BMC Cancer* 12: 370, 2012.
18. Zubillaga-Guerrero MI, Alarc  n-Romero Ldel C, Illades-Aguir B, Flores-Alfaro E, Berm  dez-Morales VH, Deas J and Peralta-Zaragoza O: MicroRNA miR-16-1 regulates CCNE1 (cyclin E1) gene expression in human cervical cancer-cells. *Int J Clin Exp Med* 8: 15999-16006, 2015.
19. Liu Z, Wu M, Shi H, Huang C, Luo S and Song X: DDN-AS1-miR-15a/16-1-TCF3 feedback loop regulates tumor progression in cervical cancer. *J Cell Biochem* 120: 10228-10238, 2018.
20. Huang Y, Chen G, Wang Y, He R, Du J, Jiao X and Tai Q: Inhibition of microRNA-16 facilitates the paclitaxel resistance by targeting IKBKB via NF-kB signaling pathway in hepatocellular carcinoma. *Biochem Biophys Res Commun* 503: 1035-1041, 2018.
21. Yang TQ, Lu XJ, Wu TF, Ding DD, Zhao ZH, Chen GL, Xie XS, Li B, Wei YX, Guo LC, *et al*: MicroRNA-16 inhibits glioma cell growth and invasion through suppression of BCL2 and the nuclear factor-kB1/MMP9 signaling pathway. *Cancer Sci* 105: 265-271, 2014.
22. Qi F, Liu X, Wu H, Yu X, Wei C, Huang X, Ji G, Nie F and Wang K: Long noncoding AGAP2-AS1 is activated by SP1 and promotes cell proliferation and invasion in gastric cancer. *J Hematol Oncol* 10: 48, 2017.
23. Bai Y, Dai X, Harrison AP and Chen M: RNA regulatory networks in animals and plants: A long noncoding RNA perspective. *Brief Funct Genomics* 14: 91-101, 2015.
24. Chen LL and Carmichael GG: Decoding the function of nuclear long non-coding RNAs. *Curr Opin Cell Biol* 22: 357-364, 2010.
25. Wierzbicki AT: The role of long non-coding RNA in transcriptional gene silencing. *Curr Opin Plant Biol* 15: 517-522, 2012.
26. Ben Amor B, Wirth S, Merchan F, Laporte P, d'Aubenton-Carafa Y, Hirsch J, Maizel A, Mallory A, Lucas A, Deragon JM, *et al*: Novel long nonprotein coding RNAs involved in Arabidopsis differentiation and stress responses. *Genome Res* 19: 57-69, 2009.
27. Ma X, Shao C, Jin Y, Wang H and Meng Y: Long non-coding RNAs: A novel endogenous source for the generation of dicer-like 1-dependent small RNAs in Arabidopsis thaliana. *RNA Biol* 11: 373-390, 2014.
28. Salmena L, Poliseno L, Tay Y, Kats L and Pandolfi PP: A ceRNA hypothesis: The Rosetta Stone of a hidden RNA language. *Cell* 146: 353-358, 2011.
29. Karreth FA, Tay Y, Perna D, Ala U, Tan SM, Rust AG, DeNicola G, Webster KA, Weiss D, Perez-Mancera PA, *et al*: In vivo identification of tumor-suppressive PTEN ceRNAs in an oncogenic BRAF-induced mouse model of melanoma. *Cell* 147: 382-395, 2011.
30. Tay Y, Kats L, Salmena L, Weiss D, Tan SM, Ala U, Karreth F, Poliseno L, Provero P, Di Cunto F, *et al*: Coding-independent regulation of the tumor suppressor PTEN by competing endogenous mRNAs. *Cell* 147: 344-357, 2011.
31. Cesana M, Cacchiarelli D, Legnini I, Santini T, Sthandier O, Chinappi M, Tramontano A and Bozzoni I: A long noncoding RNA controls muscle differentiation by functioning as a competing endogenous RNA. *Cell* 147: 358-369, 2011.
32. Wang C, Han C, Zhang Y and Liu F: LncRNA PVT1 regulate expression of HIF1   via functioning as ceRNA for miR-199a-5p in non-small cell lung cancer under hypoxia. *Mol Med Rep* 17: 1105-1110, 2018.
33. Guo D, Wang Y, Ren K and Han X: Knockdown of LncRNA PVT1 inhibits tumorigenesis in non-small-cell lung cancer by regulating miR-497 expression. *Exp Cell Res* 362: 172-179, 2018.
34. Yang JP, Yang XJ, Xiao L and Wang Y: Long noncoding RNA PVT1 as a novel serum biomarker for detection of cervical cancer. *Eur Rev Med Pharmacol Sci* 20: 3980-3986, 2016.
35. Iden M, Fye S, Li K, Chowdhury T, Ramchandran R and Rader JS: The lncRNA PVT1 contributes to the cervical cancer phenotype and associates with poor patient prognosis. *PLoS One* 11: e0156274, 2016.
36. Shen CJ, Cheng YM and Wang CL: LncRNA PVT1 epigenetically silences miR-195 and modulates EMT and chemoresistance in cervical cancer cells. *J Drug Target* 25: 637-644, 2017.
37. Wang X, Wang G, Zhang L, Cong J, Hou J and Liu C: LncRNA PVT1 promotes the growth of HPV positive and negative cervical squamous cell carcinoma by inhibiting TGF-  1. *Cancer Cell Int* 18: 70, 2018.
38. Zhang S, Zhang G and Liu J: Long noncoding RNA PVT1 promotes cervical cancer progression through epigenetically silencing miR-200b. *APMIS* 124: 649-658, 2016.
39. Gao YL, Zhao ZS, Zhang MY, Han LJ, Dong YJ and Xu B: Long Noncoding RNA PVT1 facilitates cervical cancer progression via negative regulating of miR-424. *Oncol Res* 25: 1391-1398, 2017.
40. 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.
41. Wang F, Yang L, Sun J, Zheng J, Shi L, Zhang G and Cui N: Tumor suppressors microRNA-302d and microRNA-16 inhibit human glioblastoma multiforme by targeting NF-kB and FGF2. *Mol Biosyst* 13: 1345-1354, 2017.
42. Wong L, Power N, Miles A and Tropepe V: Mutual antagonism of the paired-type homeobox genes, *vsx2* and *dmbx1*, regulates retinal progenitor cell cycle exit upstream of *ccnd1* expression. *Dev Biol* 402: 216-228, 2015.
43. Valentin R, Grabow S and Davids MS: The rise of apoptosis: Targeting apoptosis in hematologic malignancies. *Blood* 132: 1248-1264, 2018.
44. Hsieh SC, Hsieh WJ, Chiang AN, Su NW, Yeh YT and Liao YC: The methanol-ethyl acetate partitioned fraction from Chinese olive fruits inhibits cancer cell proliferation and tumor growth by promoting apoptosis through the suppression of the NF-kB signaling pathway. *Food Funct* 7: 4797-4803, 2016.
45. Xie S, Liu B, Fu S, Wang W, Yin Y, Li N, Chen W, Liu J and Liu D: GLP-2 suppresses LPS-induced inflammation in macrophages by inhibiting ERK phosphorylation and NF-kB activation. *Cell Physiol Biochem* 34: 590-602, 2014.
46. Reid G, Pel ME, Kirschner MB, Cheng YY, Mugridge N, Weiss J, Williams M, Wright C, Edelman JJ, Vallely MP, *et al*: Restoring expression of miR-16: A novel approach to therapy for malignant pleural mesothelioma. *Ann Oncol* 24: 3128-3135, 2013.