

Adenovirus with p16 gene exerts antitumor effect on laryngeal carcinoma Hep2 cells

ZHENGANG YANG^{1,2}, JINGXIA HU³, DAJUN LI² and XINLIANG PAN¹

¹Department of Otolaryngology, Qilu Hospital, Shandong University, Jinan, Shandong 250012;

²Department of Otolaryngology, Tai'an Central Hospital, Tai'an, Shandong 271000; ³Institute of Immunology, Taishan Medical University, Tai'an, Shandong 271100, P.R. China

Received June 12, 2015; Accepted May 18, 2016

DOI: 10.3892/mmr.2016.5355

Abstract. Laryngeal cancer is an uncommon form of cancer. The tumor suppressor P16, known to be mutated or deleted in various types of human tumor, including laryngeal carcinoma, is involved in the formation and development of laryngeal carcinoma. It has been previously reported that the inactivation or loss of P16 is associated with the acquisition of malignant characteristics. The current study hypothesized that restoring wild-type P16 activity into P16-null malignant Hep2 cells may exert an antitumor effect. A recombinant adenovirus carrying the P16 gene (Ad-P16) was used to infect and express high levels of P16 protein in P16-null Hep2 cells. Cell proliferation and invasion assays and polymerase chain reaction were performed to evaluate the effects of the P16 gene on cell proliferation and the antitumor effect on Hep2 cells. The results demonstrated that the Hep2 cells infected with Ad-P16 exhibited significantly reduced cell proliferation, invasion and tumor volume compared with untreated or control adenovirus cells. Furthermore, the expression of laryngeal carcinoma-associated genes, EGFR, survivin and cyclin D1, were measured in Ad-P16-infected cells and were significantly reduced compared with control groups. The results of the current study demonstrate that restoring wild-type P16 activity into P16-null Hep2 cells exerts an antitumor effect.

Introduction

Cases of cancer arising in the larynx (voice box) are predominantly squamous cell carcinoma, which is a rare form of cancer that develops inside the tissue of the larynx. It has been reported that laryngeal cancer accounts for ~200,000 mortalities

worldwide each year, which represents 2-5% of all malignant tumors (1). However, laryngeal cancer is particularly detrimental to the life quality of patients due to the effects on the voice and swallowing abilities of patients. It is estimated that >12,000 cases are diagnosed each year in the United States, and the incidence is increasing whilst it is decreasing in other types of cancer, thus, the research and treatment of laryngeal cancer is crucial (2).

Tumor suppressor genes, which protect cells from cancer, are critical for regulating tumor formation and development. When these genes mutate leading to reduction or loss of their function, cells may progress to a cancerous state, usually in combination with other genetic changes. The loss of tumor suppressor genes may be of greater importance than proto-oncogene/oncogene activation for the formation of numerous types of human cancer (3). Tumor suppressor genes can be divided into a variety of categories, including caretaker genes, gatekeeper genes and landscaper genes. Among them, the tumor suppressor gene, P16, has previously been reported to be altered in a variety of human tumors (4). It was reported that P16 is involved in the progression of multiple types of human cancer, including skin, lung, brain and laryngeal cancer, and the P16 protein was demonstrated to be deleted or mutated in these tumors (5).

It has been previously reported that P16 expression was commonly decreased in laryngeal tumors. Yuen *et al* (6) reported that P16 expression was decreased in 48% of breast cancer tumors, and that there was a higher frequency of reduced P16 expression in tumors of the larynx compared with those from the pharynx and oral cavity. Kalfert *et al* (7) demonstrated that P16 overexpression in glottic laryngeal squamous cell carcinoma may be associated with a low risk of disease recurrence.

The current study aimed to assess the importance of P16 expression on cell apoptosis and the antitumor effect on Hep2 laryngeal carcinoma cells. A recombinant adenovirus carrying the P16 gene was used to infect cells and result in expression of high levels of P16 protein in P16-null Hep2 laryngeal carcinoma cells. Cell proliferation, invasion assays and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) were used to evaluate the effect of the P16 gene on cell apoptosis and the antitumor effect on Hep2 cells.

Correspondence to: Professor Xinliang Pan, Department of Otolaryngology, Qilu Hospital, Shandong University, 107 West Wenhua Road, Jinan, Shandong 250012, P.R. China
E-mail: drpanxinliang@126.com

Key words: adenovirus, p16, antitumor, laryngeal carcinoma cells, proliferation

Materials and methods

Cell culture. Hep2 (P16 null) cells were purchased from the Chinese Academy of Medical Sciences (Beijing, China) in March 2014. This cell line is known to be contaminated, but it was tested in the present study and no bacterial or cell line contamination was identified, thus the correct identity of the cells were confirmed. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), penicillin (100 U/ml) and streptomycin (100 µg/ml; Gibco; Thermo Fisher Scientific, Inc.) and incubated at 37°C in 5% CO₂.

Adenovirus infection. Adenoviral vectors containing wild-type P16 cDNA and a cytomegalovirus promoter (Agilent Technologies, Inc., Santa Clara, CA, USA) were inserted into the E1-deleted region of modified adenovirus (Ad-P16) as described previously (8). Monolayer cells were cultured in DMEM with 10% FBS and were infected with P16 cDNA-adenoviral vectors as the experimental group. Cells receiving empty vector adenovirus and untreated cells were used as control groups.

Western blot analysis. After 1 week of infection with Ad-P16 and control adenovirus (Ad-control), the expression levels of P16 in Ad-P16-treated cells, Ad-control-treated cells and untreated cells were examined by western blotting as previously described (9).

Briefly, protein was extracted from ~10⁷ cells by lysis in extraction buffer [50 mM Tris-HCl, pH=8; 150 mM NaCl; 1% NP-40; 0.1% sodium dodecyl sulfate (SDS), 1 mM phenylmethylsulfonyl fluoride; Thermo Fisher Scientific, Inc.]. Bio-Rad protein assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used to determine the concentration of protein. Equivalent amounts of protein (100 µg) were fractionated by electrophoresis in 10% SDS-polyacrylamide gels. The proteins were subsequently transferred to Immobilon-P nitrocellulose membrane (Bio-Rad Laboratories, Inc.) under semi-dry conditions and blocked with 5% nonfat dried milk for 1 h. Primary antibodies were incubated for 1 h at room temperature and were as follows: Monoclonal rabbit anti-P16 (Bioscience International, Inc., Saco, MA, USA; cat. no. K59470R) and mouse monoclonal anti-β-actin (dilution, 1:2,000; Sigma-Aldrich, St. Louis, MO, USA; cat. no. A1978) were used. The blots were incubated in horse anti-mouse (cat. no. 7076) or goat anti-rabbit (cat. no. 7074) IgG horseradish peroxidase-conjugated secondary antibody (dilution, 1:5,000; Cell Signaling Technology, Inc., Danvers, MA, USA) for 1 hour at room temperature. The expression of P16 and β-actin proteins were detected by an enhanced chemiluminescence system (Amersham ECL Western Blotting Detection reagent; GE Healthcare Life Sciences, Chalfont, UK) according to the manufacturer's instruction and visualized using Quantity One 1-D software version 4.2.1 (Bio-Rad Laboratories, Inc.).

Cell proliferation. The cells were seeded in a 96-well flat-bottomed microplate (1,000 cells/well) and cultured in 100 µl DMEM at 37°C and 5% CO₂. At days 1, 3, 5 and 7,

the cell culture medium in each well was then replaced with 100 µl of cell growth medium containing 10 µl cell counting kit-8 (CCK-8) solution (Dojindo Molecular Technologies, Inc., Kumamoto, Japan). After incubation for 4 h at 37°C, the absorbance at 450 nm were measured on a SpectraMax Plus 384 microplate reader (Molecular Devices, LLC, Sunnyvale, CA, USA) and presented as the mean ± standard deviation from triplicate wells (10).

Animal models and in vivo experiments. Hep2 cells (0.1 ml, 1x10⁸ cells/ml) were subcutaneously injected into 18 male BALB/c (nu/nu) mice (age, 6 weeks; weight, 20 g) obtained from the Experimental Animal Center of Shandong (Jinan, China). The mice were maintained at 18-23°C, 35-65% humidity, under a 12-h light/dark cycle with access to food and water *ad libitum*. The present study was approved by the ethics committee of Shangong University (Jinan, China). When the tumor xenografts reached 50 mm³ in size, mice were divided randomly into 3 groups (n=6 per group) as follows: Adenovirus-P16 (Ad-P16); Ad-control; and phosphate-buffered saline (PBS). The mice received 3 intratumoral injections every other day for 3 weeks at a total dosage of 10⁹ pfu virus per mouse in the virus-treated groups and 100 µl PBS per mouse in the control group. Tumor size was measured at weeks 1, 2 and 3, and tumor volume was estimated with the following formula: $a \times b^2 \times 0.5$ (a, maximal diameter; b, minimal diameter) (11). The mice were sacrificed by CO₂ inhalation.

Cancer cell invasion assay. Cancer cell invasion *in vitro* was measured using an invasion assay according to a previously described protocol (12). Briefly, Transwell inserts with 8 mm pore size were coated with a final concentration of 0.78 mg/ml of Matrigel (Corning Incorporated, Corning, NY, USA) in cold serum-free DMEM. Cells were trypsinized (Thermo Fisher Scientific, Inc.) and 200 µl cell suspension (1x10⁶ cells/ml) from each treatment was added in triplicate wells. After 48 h incubation, the cells that passed through the filter into the lower wells were quantified by CCK-8 assay, and expressed as a percentage of the sum of the cells in the upper and lower wells.

RT-qPCR. The expression levels of epidermal growth factor receptor (EGFR), survivin and cyclin D1 after 48 h were determined by RT-qPCR as previous described (13). Total RNA was extracted using the phenol-chloroform method (Ambion; Thermo Fisher Scientific, Inc.) and treated with DNase I. The RNA was reverse transcribed using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols. The cDNA was diluted to ~1 ng/µl and qPCR was conducted with SYBR® Green PCR Master mix (Applied Biosystems; Thermo Fisher Scientific, Inc.) in a ABI 7300 Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). Specific primers with the following sequences were used: EGFR, forward 5'TATTGATCGGGAGAGCCGGA and reverse 5'GCGCAGTGATCTCAAGGAA; survivin, forward 5'GCCAGTGTCTTCTCTGCTT and reverse 5'CCG GACGAATGCTTTTATG; cyclin D1, forward 5'AAGCG GAGGAGACCTGCGCG and reverse 5'ATCGTGCGGCAT TGCGGC; and GAPDH, forward 5'AGCCACATCGCTCAG

ACAC and reverse 5'GCCCAATACGACCAAATCC. The qPCR cycling conditions were as follows: Initial denaturation at 95°C for 10 min; 45 cycles of amplification at 95°C for 10 sec, 60°C for 30 sec and 72°C for 1 sec; and a single fluorescence acquisition. GAPDH was used as an internal control. Relative gene expression was calculated using the $2^{-\Delta\Delta C_q}$ method (14,15).

Statistical analysis. All analysis was performed with SPSS software (version 17.0; SPSS, Inc., Chicago, IL, USA). Differences between groups were tested using two-way analysis of variance and Fisher's least significant difference test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Western blot analysis of P16 protein expression. Western blotting was performed to detect the P16 protein expression level in Hep2 cells. The results demonstrated that the level of expression of P16 was barely detectable in untreated cells and Ad-control cells, whereas P16 was markedly increased following infection with Ad-P16 (Fig. 1).

Cell proliferation and tumor growth. After 3 days of infection, Ad-P16 cells exhibited reduced levels of proliferation compared with the untreated cells and Ad-control cells ($P < 0.05$), indicating that P16 exerts a significant inhibitory effect on Hep2 cell growth (Fig. 2).

In order to determine the effect of elicited by P16 overexpression on tumor growth, the tumor formation was measured at different times following viral infection to evaluate the antitumor effects. Tumors injected with Ad-P16 exhibited a reduced growth rate compared with those injected with PBS or Ad-control (Fig. 3; $P < 0.05$).

P16 inhibits laryngeal cancer cell invasion. An important hallmarks of malignant laryngeal cancer cells is invasion. Cells (1×10^6 cells/ml density) were seeded in the upper chamber of a Transwell insert and cells that invaded through the Matrigel were detected by CCK-8 assay. Compared with untreated cells (47%) and Ad-control cells (42%), the number of invasive Ad-P16 Hep2 cells (14%) was decreased significantly (Fig. 4; $P < 0.05$).

Expression of laryngeal cancer-associated genes. High expression of EGFR, survivin and cyclin D1 are commonly observed in the development of laryngeal cancer, thus, the expression patterns of the 3 genes were analyzed to evaluate the effect of Ad-P16 on Hep2 cells. Compared with untreated cells and Ad-control cells, the mRNA expression levels of EGFR, survivin and cyclin D1 were significantly decreased in Ad-P16 cells ($P < 0.05$; Fig. 5).

Discussion

Laryngeal cancer is a disease in which malignant cells form in the tissues of the larynx. P16 has been previously demonstrated to be important in the development of laryngeal cancer. P16 is a cyclin-dependent kinase-4 inhibitor expressed in certain normal tissues and tumors (16). P16 is frequently inactivated in multiple types of human cancer and the major biochemical effect of P16

is to halt cell-cycle progression at the G1/S boundary. Loss of P16 function may lead to cancer progression by allowing unregulated cellular proliferation (4). Cyclin-dependent kinases 4/6/2 (Cdk4/6/2) are proteins that lead progression through the G1-S transition and are regulated in the process of cell proliferation. P16 binds to Cdk4/6 and inhibits phosphorylation of the retinoblastoma protein, forcing cells to remain in the G1 phase and therefore, arresting cell division (17). Thus, the overexpression and restoration of P16 in cancer cells may be able to inhibit cancer development. The current study transfected P16 cDNA into Hep2 cells to restore the expression of P16. The results of western blot analysis demonstrated that P16 cDNA was successfully transfected into the cell line.

Cell proliferation is an important process to evaluate the effect of P16 on cancer cells. The current study demonstrated that after 3 days of infection with Ad-P16, the cell proliferation in the experimental group was significantly reduced compared with the Ad-control-treated cells and untreated cells, indicating that P16 inhibits cell growth. Nalabothula *et al* (18) demonstrated that restoration of P16 reduces glioma growth in nude mice and downregulates $\alpha\beta 3$ integrin receptor expression. Furthermore, they demonstrated that a sense P16 and anti-sense plasminogen activator urokinase receptor bicistronic construct significantly inhibited angiogenesis, induced apoptosis by deregulation of the phosphatidylinositol-4,5-bisphosphate 3-kinase/ ν -akt murine thymoma viral oncogene homolog 1 pathway and down-regulated $\alpha\beta 5$ integrin receptor expression. The result was further corroborated by the tumor formation assay performed in the current study. The tumor injected with Ad-P16 exhibited reduced growth compared with those injected with PBS or Ad-control virus. By contrast, the loss of nuclear P16 protein expression has been demonstrated to be correlated with increased tumor cell proliferation. Straume *et al* (19) demonstrated that the loss of nuclear P16 protein expression in vertical growth phase melanomas is associated with increased tumor cell proliferation and independently predicts decreased patient survival.

Cell invasion assays, based on the invasive and metastatic abilities of cancer cells, have been widely used to study the interactions between tumor cells and the extracellular matrix. In the current study, Matrigel was used to provide structural support for the cells to grow and move. Cells secrete enzymes that degrade certain components of the Matrigel to migrate towards chemoattractants. Due to increased motility and/or enzymatic activity degrading Matrigel components, metastatic tumor cells often exhibit increased invasiveness through the Matrigel. The results of the current study demonstrated that the Ad-P16 transfection inhibited Hep2 cell invasion. The results are consistent with those from a study by Chintala *et al* (12), which demonstrated that restoring wild-type P16 activity into P16-null SNB19 glioma cells significantly inhibited tumor cell invasion, thus, suggesting the P16 gene may inhibit the invasion of multiple tumor cell types. Liang *et al* (20) also demonstrated that reduced glioma cell invasion due to decreased BMI-1 proto-oncogene was rescued by P16 downregulation, and that BMI-1 enhances to the motility of glioma cells by regulating the expression of P16, indicating that P16 is important during tumor cell invasion (20).

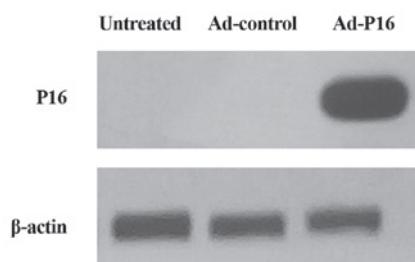


Figure 1. P16 protein expression. The p16 protein was detected in the laryngeal cancer cells infected with Ad-p16, while there was no expression of p16 in untreated or Ad-control cells. Ad, adenovirus.

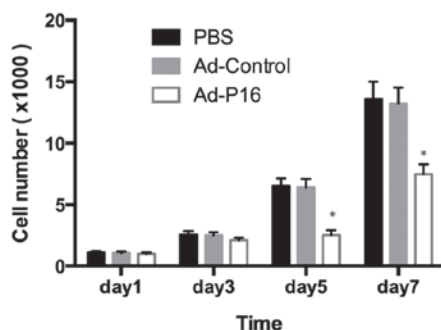


Figure 2. Cell proliferation. After 3 days of infection, a significant difference was observed between the Ad-P16 group and the other two groups ($P < 0.05$), indicating P16 exerts a significant growth inhibition effect on laryngeal cells. * $P < 0.05$ vs. the Ad-control group. PBS, phosphate-buffered saline; Ad, adenovirus.

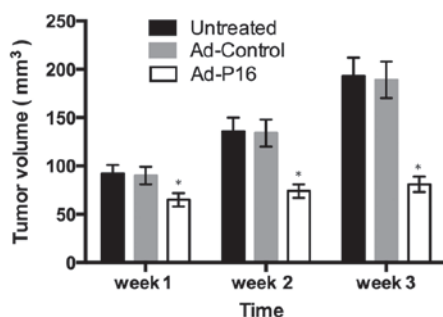


Figure 3. Tumor growth. In order to determine the effect of growth inhibition elicited by p16 overexpression in tumor growth, the tumor formation assay was analyzed at different times after viral infection to evaluate the antitumor effects. Tumor injected with Ad-p16 demonstrated a lower growth rate than those injected with phosphate-buffered saline and Ad-control. * $P < 0.05$ vs. the Ad-control group. Ad, adenovirus.

Numerous genes contribute to the development of cancer. EGFR is a transmembrane tyrosine kinase involved in cell transformation and tumor progression. High EGFR levels are associated with poor prognosis in patients with laryngeal cancer (21,22). Survivin, an anti-apoptotic protein, is highly expressed in numerous types of cancer and is associated with chemotherapy resistance, increased tumor recurrence and shorter patient survival, making anti-survivin therapy an attractive cancer treatment strategy (23). Cyclin D1 is an important regulator of cell cycle progression and functions as a transcriptional co-regulator. The overexpression of cyclin D1 has previously been associated with the development and

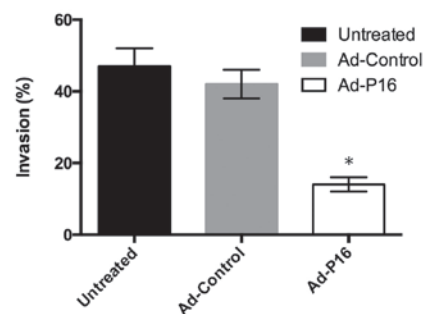


Figure 4. Tumor cell invasion assay. Quantitative analysis of the number of cells demonstrated that 14% of Ad-P16-treated laryngeal cancer cells invaded to the lower side of the membrane after 48 h, while 47% of untreated cells and 42% of Ad-control cells were observed on the lower side of the membrane. * $P < 0.05$ vs. the Ad-control group. Ad, adenovirus.

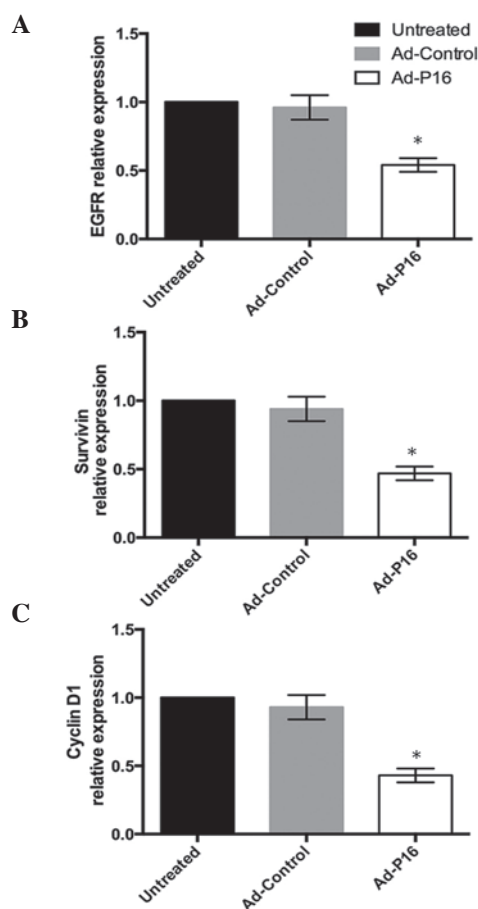


Figure 5. Reverse transcription-quantitative polymerase chain reaction results. Compared with untreated cells and Ad-control cells, the expression level of (A) EGFR, (B) survivin and (C) cyclin D1 in Ad-p21-treated cells demonstrated a significant decrease. * $P < 0.05$ vs. the Ad-control group. EGFR, epidermal growth factor receptor; Ad, adenovirus.

progression of cancer (24). Cyclin D1 genotype and protein expression are important risk markers for laryngeal cancer, and future trials targeting upstream regulators of cyclin D1 transcription may be useful (25). The present study evaluated the effect of P16 on 3 genes in tumor cells. After 72 h infection, Ad-P16-infected Hep2 cells demonstrated reduced expression of EGFR, survivin and cyclin D1 compared with Ad-control and untreated cells.

In conclusion, a recombinant adenovirus carrying the P16/gene was used to infect and express high levels of P16 protein in P16-null Hep2 cells. Cell proliferation, invasion assays and RT-qPCR were used to evaluate the antitumor effect of P16. The results demonstrate that restoring wild-type P16 activity into P16-null carcinoma cells exerts an antitumor effect. This may be useful in the development of novel anti-tumor therapeutic agents or gene therapy.

Acknowledgements

The present study is supported by Shandong Provincial Natural Science Foundation, China (nos. ZR2013HM107 and ZR2013HQ055).

References

- Jemal A, Clegg LX, Ward E, Ries LA, Wu X, Jamison PM, Wingo PA, Howe HL, Anderson RN and Edwards BK: Annual report to the nation on the status of cancer, 1975-2001, with a special feature regarding survival. *Cancer* 101: 3-27, 2004.
- Siegel RL, Miller KD and Jemal A: Cancer statistics, 2015. *Ca: Cancer J Clin* 65: 5-29, 2015.
- Weinberg RA (ed.): pRb and Control of the Cell Cycle Clock. In: *The Biology of Cancer*. 2nd edition. Garland Science, New York, NY, pp275-329, 2014.
- Liggett WH Jr and Sidransky D: Role of the p16 tumor suppressor gene in cancer. *J Clin Oncol* 16: 1197-1206, 1998.
- Attri J, Srinivasan R, Majumdar S, Radotra BD and Wig J: Alterations of tumor suppressor gene p16INK4a in pancreatic ductal carcinoma. *BMC Gastroenterol* 5: 22, 2005.
- Yuen PW, Man M, Lam KY and Kwong YL: Clinicopathological significance of p16 gene expression in the surgical treatment of head and neck squamous cell carcinomas. *J Clin Pathol* 55: 58-60, 2002.
- Kalfert D, Celakovsky P, Laco J and Ludvikova M: The role of protein p16 (INK4a) in glottic laryngeal squamous cell carcinoma. *Pathol Oncol Res* 20: 909-915, 2014.
- Craig C, Kim M, Ohri E, Wersto R, Katayose D, Li Z, Choi YH, Mudahar B, Srivastava S, Seth P and Cowan K: Effects of adenovirus-mediated p16INK4A expression on cell cycle arrest are determined by endogenous p16 and Rb status in human cancer cells. *Oncogene* 16: 265-272, 1998.
- Piao CQ, Zhao YL and Hei TK: Analysis of p16 and p21 (Cip1) expression in tumorigenic human bronchial epithelial cells induced by asbestos. *Oncogene* 20: 7301-7306, 2001.
- Li X, Zhu J, Man Z, Ao Y and Chen H: Investigation on the structure and upconversion fluorescence of Yb³⁺/Ho³⁺ co-doped fluorapatite crystals for potential biomedical applications. *Sci Rep* 4: 4446, 2014.
- Ma J, He X, Wang W, Huang Y, Chen L, Cong W, Gu J, Hu H, Shi J, Li L and Su C: E2F promoter-regulated oncolytic adenovirus with p16 gene induces cell apoptosis and exerts antitumor effect on gastric cancer. *Dig Dis Sci* 54: 1425-1431, 2009.
- Chintala SK, Fueyo J, Gomez-Manzano C, Venkaiah B, Bjerkvig R, Yung WK, Sawaya R, Kyritsis AP and Rao JS: Adenovirus-mediated p16/CDKN2 gene transfer suppresses glioma invasion in vitro. *Oncogene* 15: 2049-2057, 1997.
- Knight BB, Oprea-Ilie GM, Nagalingam A, Yang L, Cohen C, Saxena NK and Sharma D: Survivin upregulation, dependent on leptin-EGFR-Notch1 axis, is essential for leptin-induced migration of breast carcinoma cells. *Endocr Relat Cancer* 18: 413-428, 2011.
- 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.
- Huang YP, Lin IJ, Chen CC, Hsu YC, Chang CC and Lee MJ: Delivery of small interfering RNAs in human cervical cancer cells by polyethylenimine-functionalized carbon nanotubes. *Nanoscale Res Lett* 8: 267, 2013.
- O'Neill CJ and McCluggage WG: p16 expression in the female genital tract and its value in diagnosis. *Adv Anat Pathol* 13: 8-15, 2006.
- Villacañas O, Pérez JJ and Rubio-Martínez J: Structural analysis of the inhibition of Cdk4 and Cdk6 by p16 (INK4a) through molecular dynamics simulations. *J Biomol Struct Dyn* 20: 347-358, 2002.
- Nalabothula N, Lakka SS, Dinh DH, Gujrati M, Olivero WC and Rao JS: Sense p16 and antisense uPAR bicistronic construct inhibits angiogenesis and induces glioma cell death. *Int J Oncol* 30: 669-678, 2007.
- Straume O, Sviland L and Akslen LA: Loss of nuclear p16 protein expression correlates with increased tumor cell proliferation (Ki-67) and poor prognosis in patients with vertical growth phase melanoma. *Clin Cancer Res* 6: 1845-1853, 2000.
- Liang J, Wang P, Xie S, Wang W, Zhou X, Hu J, Shi Q, Zhang X and Yu R: Bmi-1 regulates the migration and invasion of glioma cells through p16. *Cell Biol Int* 39: 283-290, 2015.
- Maurizi M, Almadori G, Ferrandina G, Distefano M, Romanini ME, Cadoni G, Benedetti-Panici P, Paludetti G, Scambia G and Mancuso S: Prognostic significance of epidermal growth factor receptor in laryngeal squamous cell carcinoma. *Br J Cancer* 74: 1253-1257, 1996.
- Lionello M, Lovato A, Staffieri A, Blandamura S, Turato C, Giacomelli L, Staffieri C and Marioni G: The EGFR-mTOR pathway and laryngeal cancer angiogenesis. *Eur Arch Otorhinolaryngol* 271: 757-764, 2014.
- Fukuda S and Pelus LM: Survivin, a cancer target with an emerging role in normal adult tissues. *Mol Cancer Ther* 5: 1087-1098, 2006.
- Alao JP: The regulation of cyclin D1 degradation: Roles in cancer development and the potential for therapeutic invention. *Mol Cancer* 6: 24, 2007.
- Papadimitrakopoulou V, Izzo JG, Liu DD, Myers J, Ceron TL, Lewin J, William WN Jr, Atwell A, Lee JJ, Gillenwater A, et al: Cyclin D1 and cancer development in laryngeal premalignancy patients. *Cancer Prev Res (Phila)* 2: 14-21, 2009.