Concomitant modulation of *PTEN* and *Livin* in gastric cancer treatment

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Received September 28, 2017; Accepted February 2, 2018

DOI: 10.3892/ijmm.2018.3475

Abstract. Phosphatase and tensin homolog deleted on chromosome 10 (PTEN) and Livin are important in the development of gastric cancer (GC). PTEN and Livin are involved in the regulation of tumor cell proliferation, migration and apoptosis. The modulation of PTEN or Livin has been investigated extensively in various cancer models. However, no studies have been performed to evaluate the combined effect of concurrently modulating these two genes on the development of GC. In the present study, the BGC823 human gastric carcinoma cell line was transfected with a dual gene modified vector (pCL-neo-PTEN-siLivin) in parallel with single gene modified vectors (pCL-neo-PTEN or pRNAT-U6.1-siLivin), and an empty control vector. Dual gene modulation (pCL-neo-PTEN-siLivin) had a more marked effect on the inhibition of cell proliferation, induction of apoptosis, and reduction of cell penetration in Matrigel, compared with either single gene alone or empty vector transfection. In a xenograft nude mouse model, the inoculation of pCL-neo-PTEN-siLivin-transfected BGC823 cells led to a markedly reduced tumor burden, compared with that in all other inoculation groups. In conclusion, the overexpression of *PTEN* concomitant with *Livin* gene silencing was confirmed as a feasible and effective *in vitro* and *in vivo* gene modulation method, which may represent a potential therapeutic strategy for the treatment of GC.

Introduction

Gastric cancer (GC) is a malignant tumor arising from the lining of the stomach. GC is the fifth most common type of cancer in the world, and remains the second most common cause of cancer-associated mortality worldwide (1,2). The development of GC may result from complex interactions between multiple factors, including genetic and epigenetic alterations of oncogenes and tumor-suppressor genes, cell-cycle regulators, cell adhesion molecules and DNA repair genes (3,4).

Phosphatase and tensin homology deleted on chromosome 10 (*PTEN*) is a tumor suppressor gene belonging to the phosphatase family, and is one of the most frequently mutated tumor suppressors in several types of human cancer (5). PTEN is important in cell proliferation, migration and apoptosis (6). In GC, a significant reduction in the expression of PTEN has been reported. In addition, the expression of PTEN negatively correlates with tumor size, Borrmann classification, lymph node metastasis and tumor staging (7,8). Loss of a *PTEN* allele has been identified in 70% of patients with prostate cancer at the time of diagnosis (9). The inactivation of PTEN can be attributed to gene mutation, hypermethylation, microRNA-mediated regulation and post-translational phosphorylation (10). Previous studies have shown that the functional inactivation of PTEN has been detected in multiple cases of GC and other tumors (11-13). These reports suggest that the inactivation of PTEN is closely associated with the incidence, progression and prognosis of GC.

Livin, a member of the inhibitor-of-apoptosis protein (IAP) family is expressed in a variety of tumors, including GC, melanoma, neuroblastoma, mesothelioma and osteosarcoma (14-16). The expression of Livin is increased in human GC, and correlates with tumor differentiation and lymph node metastases. The underlying mechanism of its effect indicates that Livin protein binds directly to caspases, thereby inhibiting apoptosis and promoting tumor growth. By contrast, the knockdown of Livin inhibits cell growth and invasion (17,18). Therefore, the results reported to date suggest that the down-regulation of Livin may be a potential therapeutic strategy for GC.

PTEN and Livin are involved in the regulation of tumor cell proliferation, migration and apoptosis, in which PTEN suppresses and Livin promotes cancer development. Although the modulation of PTEN or Livin has been investigated

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Key words: Livin, phosphatase and tensin homolog deleted on chromosome 10, plasmid, gastric cancer, overexpression, silencing

extensively in various cancer models (7,8,19), no studies have been performed to evaluate the combined effect on the development of GC of concurrently modulating these two genes. Therefore, the present study focused on evaluating the biological effect of this dual gene modulation on the development of GC by employing recombinant vectors with *PTEN* and/or *Livin* gene modulation capacity, previously constructed in the laboratory (20).

Materials and methods

Mice. All animal experiments were performed in accordance with protocols approved by the Animal Ethics Committee of Zhengzhou University (Zhengzhou, China). The 6-8-week-old BALB/c male mice were purchased from the Laboratory Animal Center of Zhengzhou University. All animal experimental procedures were approved by the Institutional Animal Care and Use Committee of Zhengzhou University. The mice were bred in the animal room with continuous air circulation at 25-27˚C, 40-60% humidity and a regular 12 h light/dark cycle. Sterile food and water containing multi-vitamins was provided to the mice.

BGC823 cell transfection. The following vectors were used in the present study: pCL-neo-PTEN vector for the overexpression of *PTEN*; pRNAT-U6.1-siLivin vector for silencing of the Livin gene; pCL-neo-PTEN-siLivin vector for the simultaneous overexpression of *PTEN* and silencing of the *Livin* gene; empty vector as a transfection control. The vectors were constructed as described previously (20). These vectors were transfected into BGC823 cells (Culture Collection of the Chinese Academy of Sciences, Shanghai, China) using a Lipofectamine™2000 transfection kit (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The BGC823 cells were cultured in a 6-well plate in RPMI-1640 culture medium at a concentration of $1x10^6$ cells/ml at 37˚C and in the presence of 5% CO_2. The stable transfection clones from the above transfections were selected following multiple rounds of culture and selection.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Tumor tissue (20-30 mg) from each mouse was ground in liquid nitrogen. Total RNA was extracted and purified using an RNA extraction kit (cat no. 15596026; Invitrogen; Thermo Fisher Scientific, Inc.). The RNA sample was then reverse transcribed into cDNA using a high capacity cDNA reverse transcription kit (cat no. RR047A; Takara Biotechnology Co., Ltd., Dalian, China). RT-qPCR analysis was performed using a real-time PCR kit (Baocheng Biotech Co., Ltd., Dalian, China). Subsequently, qPCR was performed using the SYBR Green Master mix (cat no. 4472908; Thermo Fisher Scientific, Inc.) on a 7500 Fast Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). Thermocycling conditions were as follows; 50˚C for 2 min; pre-denaturation at 95˚C for 2 min; followed by 15 sec at 95˚C then 1 min at 60˚C for 40 cycles; melt curve was at 95˚C for 15 sec, 1 min at 60˚C, 15 sec at 95˚C and 15 sec at 60˚C.

RNA was normalized to the expression levels of β2-microglobulin (*B2M*) and relative expression was calculated. The sequences of the PCR primers used were as follows: B2M, forward 5'-TCCATCCGACATTGAAGTTG-3' and reverse 5'-ACACGGCAGGCATACTCAT-3'; PTEN, forward 5'-TGGCGGAACTYGCAATCC-3' and reverse 5'-GCTGAGGAACTCAAAGTAC-3'; Livin, forward 5'-TCC TGCTCCGGTCAAAAGG-3' and reverse 5'-GCTGCGTCT TCCGGTTCTT-3'. RNA levels were normalized to β-actin expression and relative expression was calculated using the $2^{-\Delta\Delta Cq}$ method (21).

Western blot analysis. The cells or tumor tissues were harvested and extracted using radioimmunoprecipitation assay lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% deoxycholate, 1% Triton X-100, 2 mM EDTA, 0.1% SDS and 50 mM NaF) containing a protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany). Protein concentrations were determined using the bicinchoninic acid method (Pierce; Thermo Fisher Scientific, Inc.). The extracts were heated for 5 min in loading buffer, and equal quantities (40 μg) of cell extracts were separated on 12% SDS-PAGE gels. The separated protein bands were transferred onto polyvinylidene fluoride membranes and then blocked in 10% skim milk for 1 h at room temperature. Primary antibodies against PTEN (cat no. sc-133242; Santa Cruz Biotechnology, Inc., California, CA, USA), Livin (cat no. ab-127979; Abcam, Cambridge, MA, USA) and β-actin (cat no. sc-130301, Santa Cruz Biotechnology, Inc.) were diluted 1:1,000 according to the manufacturer's protocol and incubated overnight at 4˚C. The membranes were then incubated with the corresponding horseradish peroxidase-conjugated secondary antibodies (1:5,000; cat no. sc-2005; Santa Cruz Biotechnology, Inc.) at room temperature for 1 h. Chemiluminescent solution (NEN Life Science, Boston, MA, USA) was used to visualize the blot following exposure onto hyper film (GE Healthcare Life Sciences, Pittsburgh, PA, USA) for 5 min. Blots were analyzed and quantified by Quantity One software (4.6.2 version; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Cell proliferation assay. Cells in the exponential phase were collected and cultured in a 96-well culture plate at a concentration of $1x10^4$ cells in a volume of 200 μl per well at 37˚C with 5% CO_2 for 1-5 days. Each day, 20 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg/ml) was added to the culture and incubated for another 4 h at 37˚C. The culture medium was discarded and 150 μl of DMSO was added for 10 min to dissolve the crystals. The absorption at 570 nm was detected in an automatic plate reader (Bio-Rad Laboratories, Inc.).

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) apoptosis assay. A TUNEL assay was used to detect apoptosis following transfection with different vectors. The cells were deposited onto glass slides, and cell climbing slides were produced and dried at room temperature. Following washing three times with phosphate-buffered saline (PBS), the cells on slides were fixed by adding 4% paraformaldehyde and then washed twice with PBS. The apoptotic cells were detected using a TUNEL kit (R&D Systems, Inc., Minneapolis, MN, USA) according to the manufacturer's protocol. PBS was used as a negative control. Cells presenting

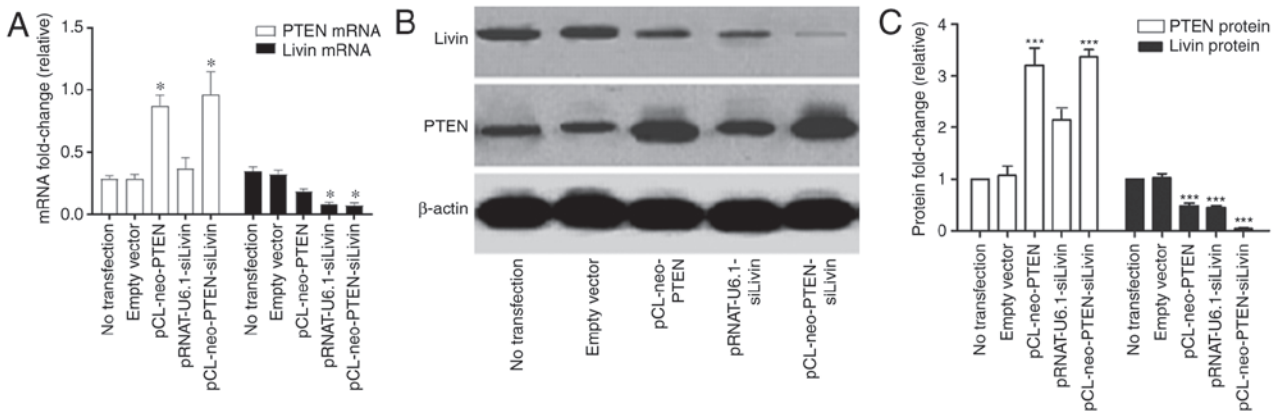

Figure 1. Characterization of *PTEN* and *Livin* gene expression in transfected BGC823 cells. BGC823 cells were transfected with vectors pCL-neo-PTEN, pRNAT-U6.1-siLivin, pCL-PTEN-siLivin, or empty control. mRNA and protein levels were evaluated by reverse transcription-quantitative polymerase chain reaction and western blot analyses, respectively. (A) mRNA levels of *PTEN* and *Livin*. β2-microglobulin was used as an endogenous control. (B) Blots and (C) quantification of protein levels of PTEN and Livin. β-actin was used as an endogenous control. *P<0.05 and ***P<0.01 compared with the empty vector transfection group. n=6/group. Data are representative of three experiments. PTEN, phosphatase and tensin homolog deleted on chromosome 10.

with brown granules in the nuclei were classified as apoptotic with an inverted microscope (X-71; Olympus Corporation, Tokyo, Japan). The percentage of apoptotic cells and the apoptotic indices were calculated by randomly counting 500 cells selected from five spots in the slides.

Detection of caspase-3 and -9 activity. Following transfection, 2x10^6 BGC823 cells were collected, and active caspase-3 and -9 were examined using a caspase activity detection kit (GenMed Scientifics, Wilmington, DE, USA) according to the manufacturer's protocol. The fluorescence of each sample was detected using a spectrophotometer with absorption/emission maxima of ~511/533 nm (Bio-Rad Laboratories, Inc.).

Cell migration assay. *In vitro* cell invasion was evaluated using the Boyden Chamber Assay (Cell Biolabs, Inc., San Diego, CA, USA) as previously reported (22). Briefly, serum-free RPMI-1640 containing 3.9 μg/μl of Matrigel™ (BD Biosciences, San Jose, CA, USA) was added to the upper chamber above the filter membrane, and incubated at 37˚C for 2 h. Subsequently, 200 μl of serum-free cell culture supernatant from NIH3T3 cells (purchased from the Shanghai Institute of Pharmaceutical Industry, Shanghai, China) was added into the lower chamber as the chemoattractant factor. The cells (400 μl) transfected with different vectors were added to the upper chamber at a concentration of 1x10^6 cells/ml and cultured for 24 h at 37˚C with 5% CO_2. The cells that had migrated into the Matrigel were detected by hematoxylin and eosin (HE) staining and those from five randomly selected spots were counted under an inverted microscope (X-71; Olympus Corporation). Five chambers were assessed for each cell line transfected by different vectors. At least three independent experiments were performed.

In vivo mouse models of GC. *In vivo* tumorigenesis was investigated in 6-8-week-old BALB/c nude mice (Shanghai SLAC Laboratory Animal Co. Ltd.). The 25 nude mice were divided into five groups (n=5/group) for xenograft transplantation of non-transfected and transfected BGC823 cells. The vectors used for transfection were pCL-neo-PTEN, pRNAT-U6.1-siLivin, pCL-neo-PTEN-siLivin, and control. Briefly, 1x10^7 (1.0 ml) BGC823 cells were inoculated subcutaneously. Tumor size was carefully measured from day 7 post-inoculation. The tumors were removed and weighed on day 28.

Statistical analysis. Data were processed and analyzed using SPSS 13.0 (SPSS, Inc., Chicago, IL, USA). Results are shown as the mean ± standard error of the mean. One-way analysis of variance was used to detect differences between groups. If a significant difference was found by analysis of variance, the Fisher LSD test was used to detect specific differences between the study groups. P<0.05 was considered to indicate a statistically significant difference.

Results

Characterization of the expression of PTEN and Livin in transfected BGC823 cells. The constructs, which were previously constructed in the laboratory (pCL-neo-PTEN, pRNAT-U6.1-siLivin and pCL-neo-PTEN-siLivin) and the empty vector control were transfected into BGC823 cells. To evaluate the quality of transfection, the mRNA and protein expression levels were characterized. The results showed that the cells transfected with either pCL-neo-PTEN or pCL-neo-PTEN-siLivin vectors exhibited a significant increase in mRNA and protein expression levels of *PTEN*, compared with cells transfected with empty vector alone (Fig. 1A-C). In addition, a significant decrease in the mRNA and protein expression levels of *Livin* were observed in the cells transfected with either the pRNAT-U6.1-siLivin or pCL-neo-PTEN-siLivin vector (Fig. 1A and C). pCL-neo-PTEN transfection also resulted in a partial decrease in the mRNA and protein expression levels of *Livin*, although the inhibitory effect was not as pronounced as that observed in the *Livin* silencing groups. By contrast, transfection with the pRNAT-U6.1-siLivin vector had minimal effect on the expression of *PTEN*. These data suggested that PTEN may be involved in regulating the gene expression of *Livin*.

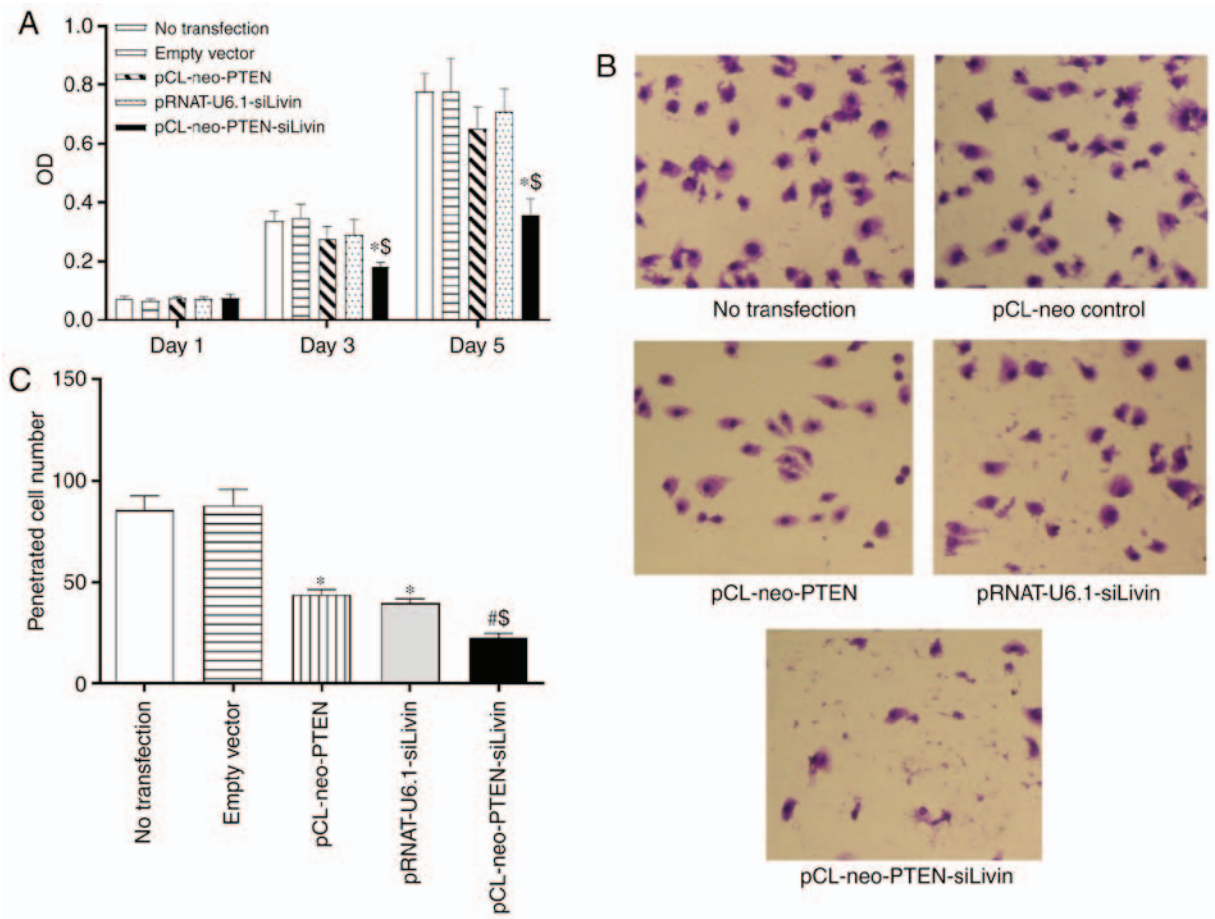

Figure 2. Characterization of the effect of modulated expression of *PTEN* and *Livin* on tumor cell proliferation and migration *in vitro*. (A) Proliferation of transfected BGC823 cells was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay on days 1, 3 and 5. (B) Matrigel was used to characterize the migration ability of transfected BGC823 cells. Representative random spots were viewed under a microscope (magnification, x200 and x400). (C) Numbers of transfected cells penetrated through the Matrigel were analyzed. *P<0.05 and $^{\#}$P<0.01, compared with empty vector transfection; $^{\$}$P<0.05, compared with pCL-neo-PTEN or pRNAT-U6.1-siLivin transfection. n=6/group. Data are representative of three experiments. PTEN, phosphatase and tensin homolog deleted on chromosome 10.

Effect of PTEN and Livin transfection on tumor cell proliferation and migration. Subsequently, the present study characterized whether the modulation of PTEN and Livin had any effect on BGC823 cell proliferation. Following transfection of the BGC823 cells with the various constructs, cell proliferation was monitored on days 1, 3, and 5 using the MTT assay. The data demonstrated that neither pCL-neo-PTEN nor pRNAT-U6.1-siLivin vector transfection alone significantly affected cell proliferation at the monitored time-points. By contrast, pCL-neo-PTEN-siLivin vector transfection, in which *PTEN* and *Livin* were modulated concurrently, significantly inhibited cell proliferation on days 3 and 5, compared with either empty vector transfection or PTEN or Livin monovector transfection (P<0.05, Fig. 2A).

To evaluate whether the modulation of *PTEN* and *Livin* exerted any effect on the metastasis of transfected cells, an *in vitro* Matrigel assay was performed. The migrated cells were detected in the Matrigel by HE staining and the numbers of cells in five randomly selected spots were counted under the microscope. Compared with the cells transfected with the empty vector, the single (pCL-neo-PTEN or pRNAT-U6.1-siLivin transfection) and dual (pCL-neo-PTEN-siLivin transfection) gene modulation resulted in a significant inhibition of cell migration (P<0.05 and P<0.01 for single and dual modulation, respectively; Fig. 2B and C). Furthermore, compared with either pCL-neo-PTEN or pRNAT-U6.1-siLivin single gene transfection, dual gene modulation resulted in the maximal level of migration inhibition (P<0.05; Fig. 2B and C). The data suggested that PTEN and Livin are critical in cell migration, and concurrent PTEN and Livin gene modulation may result in the most marked inhibition of tumor cell migration.

Modulation of PTEN and Livin induces apoptosis by activating the caspase-signaling pathway. The present study used TUNEL staining to evaluate whether modulation of the *PTEN* and *Livin* genes affected apoptosis. Single (pCL-neo-PTEN or pRNAT-U6.1-siLivin transfection) and dual (pCL-neo-PTEN-siLivin transfection) gene modulation resulted in a significant increase in the percentage of apoptotic cells, compared with that in the control (Fig. 3A). The percentages of apoptotic cells observed following pCL-neo-PTEN, pRNAT-U6.1-siLivin, and pCL-neo-PTEN-siLivin transfections were 8.67±1.27, 10.39±1.31 and 16.72±1.84, respectively. Furthermore, compared with either pCL-neo-PTEN or pRNAT-U6.1-siLivin single gene transfection, dual gene

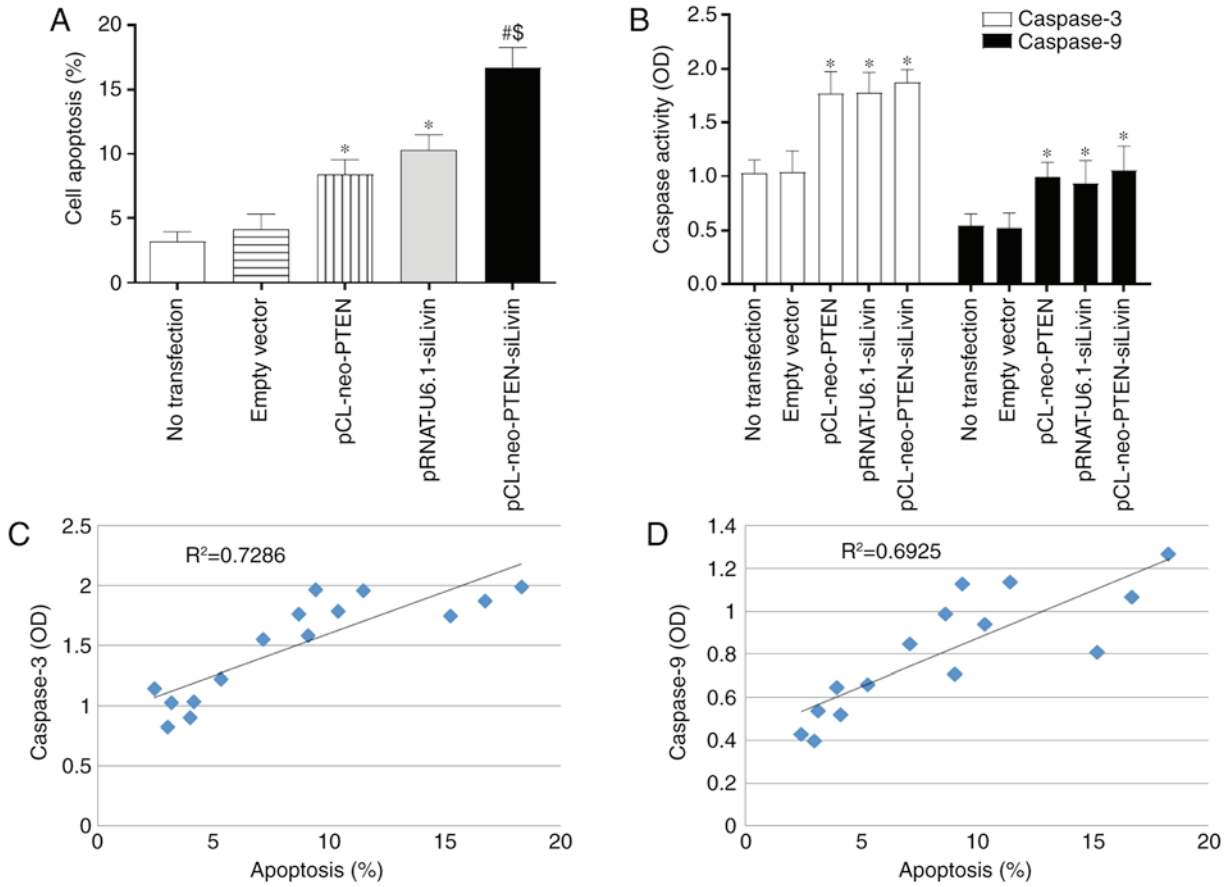

Figure 3. Modulation of the expression of *PTEN* and *Livin* induces apoptosis by activating the caspase-signaling pathway. (A) Terminal deoxynucleotidyl transferase dUTP nick end labeling staining was used to assess the level of apoptosis in transfected BGC823 cells. (B) Caspase-3 and -9 activity was determined using a caspase activity detection kit. (C) Correlation between apoptosis and caspase-3 activity. (D) Correlation between apoptosis and caspase-9 activity. *P<0.05 and #P<0.01, compared with empty control vector transfection; $P<0.05, compared with pCL-neo-PTEN or pRNAT-U6.1-siLivin transfection. n=6/group. Data are representative of three experiments. PTEN, phosphatase and tensin homolog deleted on chromosome 10.

modulation resulted in the highest level of cell apoptosis (P<0.05; Fig. 3A).

Caspase activity is a marker of apoptosis. Therefore, the present study examined the activities of caspase-3 and caspase-9 in the transfected BGC823 cells. Single (pCL-neo-PTEN or pRNAT-U6.1-siLivin transfection) and dual (pCL-neo-PTEN-siLivin transfection) gene modulation induced significant increases in caspase-3 and caspase-9 activities (P<0.05; Fig. 3B). However, there were no significant differences between the three transfection groups. Further examination revealed that the observed apoptosis was positively correlated with caspase 3 and caspase 9 activity, (R^2=0.7286 and R^2=0.6925, respectively; Fig. 3C and D). These data suggested that the modulation of *PTEN* and *Livin* induced apoptosis via activation of the caspase signaling pathway.

Modulation of PTEN and Livin inhibits tumor growth in vivo. To determine the effect of *PTEN* and *Livin* gene modulation on tumor growth *in vivo*, various vector-transfected BGC823 cells were injected into nude mice. Tumor growth was monitored weekly between days 0 and 28, when the mice were sacrificed. The tumors grew continuously from day 0 to day 28 in the non-transfected and empty vector transfection groups (Fig. 4A). By contrast, tumor growth was suppressed in the single gene (pCL-neo-PTEN or pRNAT-U6.1-siLivin) and dual gene (pCL-neo-PTEN-siLivin) transfected groups, with the dual gene modulation group demonstrating the most marked suppression of tumor growth (Fig. 4A). The percentage tumor growth inhibition (% TGI) in the pCL-neo-PTEN, pRNAT-U6.1-siLivin and pCL-neo-PTEN-siLivin transfection groups was 45%, 51 and 72%, respectively (Fig. 4B).

Characterization of the gene expression of PTEN and Livin in tumors. To verify whether the observed inhibitory effects on tumor growth were specific to *PTEN* or *Livin* gene modulation, the expression levels of *PTEN* and *Livin* in the tumor tissues were examined. The mice were sacrificed 28 days following establishment of the xenograft model and the tumors were collected. Tumor tissues from the mice with cells expressing pCL-neo-PTEN or pCL-neo-PTEN-siLivin expressed significantly higher mRNA and protein levels of *PTEN*, compared with those with control or empty vector cells (P<0.05; Fig. 5A-C). Tumor tissues from mice with cells expressing pRNAT-U6.1-siLivin or pCL-neo-PTEN-siLivin expressed significantly lower mRNA and protein levels of *Livin*, compared with those with control or empty vector cells (P<0.05; Fig. 5A-C). These data indicated that the transfected cells maintained stable expression of the target genes in the tumors *in vivo*. These results showed that the *in vivo*

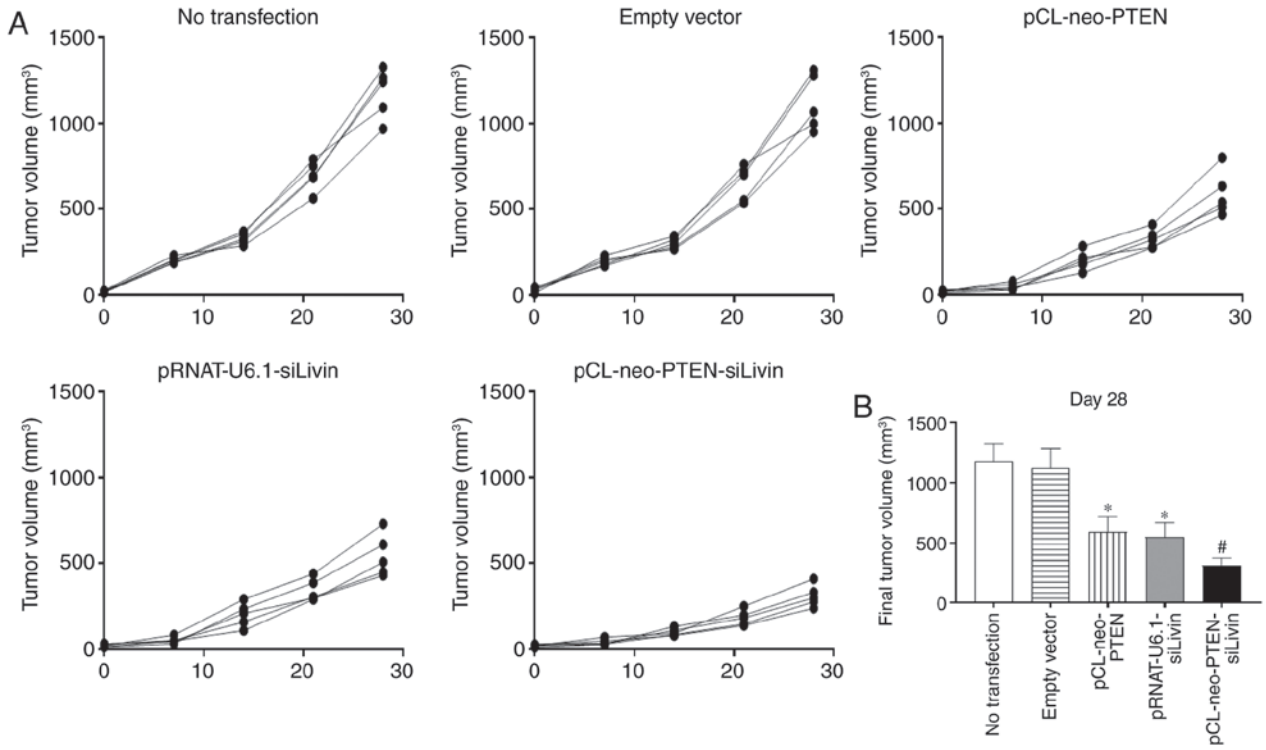

Figure 4. Modulation of the gene expression of *PTEN* and *Livin* inhibits tumor growth *in vivo*. Transfected BGC823 cells were inoculated subcutaneously into nude mice and tumor growth was monitored weekly. (A) Tumor volume was measured weekly between day 0 and 28. (B) The volume of tumors from each group was measured on day 28. *P<0.05 and $^{\#}$P<0.01 compared with pCL-neo control vector transfection. n=5/group. PTEN, phosphatase and tensin homolog deleted on chromosome 10.

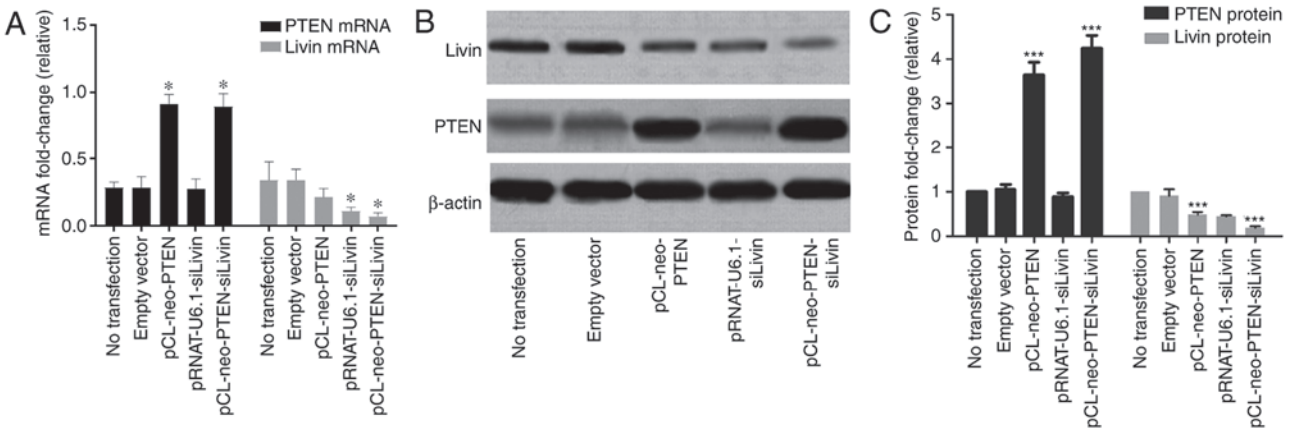

Figure 5. Characterization of the expression of *PTEN* and *Livin* in tumors. Tumor tissues were excised and total RNA and protein were measured. (A) mRNA expression of *PTEN* and *Livin* was detected by reverse transcription-quantitative polymerase chain reaction analysis, with β2-microglobulin as an internal control. (B) Blots and (C) quantification of PTEN and Livin proteins detected by western blot analysis, with β-actin as a control. *P<0.05 and ***P<0.01 compared with the empty vector transfection group. n=5/group. PTEN, phosphatase and tensin homolog deleted on chromosome 10.

experiments on tumor tissues and *in vitro* experiments using BGC823 cells produced comparable results.

Discussion

Several studies have demonstrated an association between the expression of PTEN and Livin and the malignancies of renal cell carcinoma (23,24), breast cancer (25) and retinocytoma (26). Specifically, a low expression of PTEN and high expression of Livin were significantly associated with the clinical stage and lymph node metastases of patient malignancies. However, no studies have investigated a direct correlation between *PTEN* and *Livin* gene expression and the malignancy of GC.

In the present study, it was shown that the overexpression of *PTEN* or gene silencing of *Livin* promoted apoptosis in BGC823 cells and inhibited cell proliferation. The data demonstrated that the combined effect of *PTEN*/*Livin* dual gene modulation on apoptosis and proliferation was more marked, compared with that of either single gene alone. The functional effects of *PTEN* and *Livin* gene regulation on GC apoptosis and proliferation were further supported by the xenograft experiments in nude mice. The results suggested that the

overexpression of *PTEN* concomitant with the gene silencing of *Livin* may represent a potential therapeutic strategy for gene therapy in the treatment of GC.

Caspase-3 is a proteinase with a crucial role in the apoptotic pathway. Caspase-3 can be detected in almost all cell types, emphasizing its importance in modulating cell survival and death (23). The increased expression of PTEN in cultured neonatal rat primary cardiomyocytes leading to increased caspase-3 activity and apoptosis has been reported (24), which suggests that caspase-3 is the major effector of PTEN. PTEN has been shown to inhibit cell proliferation and apoptotic function by downregulating the AKT/PKB pathway (25). By contrast, Livin demonstrates anti-apoptotic activity. Livin contains a unique Baculovirus IAP repeat (BIR) domain and a Really Interesting New Gene (RING) finger motif domain (26). The BIR domain forms a novel zinc-fold, which is the critical motif for the anti-apoptotic activity of the parent protein, its interaction with caspase 3, -7 and -9, and its E3 ubiquitin ligase (27). The RING domain is critical in tumor necrosis factor-α-mediated nuclear factor (NF)-κβ activation, thereby providing an additional mechanism for the anti-apoptotic activity of Livin (28). Livin also promotes the degradation of the inhibitor of apoptosis antagonist SMAC/DIABLO (29,30). These data suggest that PTEN and Livin exert opposite effects on the caspase signaling pathway. Consistent with this, the present study observed significantly increased caspase-3 and caspase-9 activity in BGC823 cells overexpressing *PTEN*, with silenced *Livin* gene expression, or with dual modulation. Therefore, the results are consistent with those previously published and showed that the *PTEN* and *Livin* genes are important in apoptosis by regulating caspase-3/9 activity. Although dual gene modulation led to the most marked effect on apoptosis, no significant difference in caspase activity was identified between the three transfection groups (Fig. 3B). This suggests that additional mechanisms, including Fas/Fasl signaling and cytochrome *c* release, may trigger cell death. Further investigations are warranted to address which signaling cascades are involved and the underlying mechanisms through which they are contributing to apoptosis in BGC823 cells.

PTEN is important in the regulation of tumor cell metastasis. Hwang *et al* showed that PTEN enhanced tumor metastasis through vascular endothelial factor and matrix metalloproteinases (31). In other studies, the overexpression of PTEN inhibited glioblastoma cell migration (32), and PTEN-knockdown enhanced cell migration in fibroblasts by regulating FAK, a cytoplasmic phosphoprotein activated by integrin (33). Livin was shown to regulate tumor cell invasion, the first step of metastasis, through the NF-κβ signaling pathway (34,35). Furthermore, knockdown of the *Livin* gene inhibited tumor invasion by inhibiting the mitogen-activated protein kinase (MAPK) signaling (17,36). In the present study, the migration of BGC823 cells was significantly inhibited following transfection with a vector overexpressing *PTEN*, silencing *Livin*, or modulating the two genes, with dual transfection having the highest inhibitory effect. Further experiments are required to characterize whether molecules, including FAK, NF-κβ and MAPK, are involved in metastasis in GC; however, they are presently beyond the scope of the present study.

The results of the present study are consistent with those of previous reports showing that either the overexpression of *PTEN* or silencing of the *Livin* gene significantly inhibited cell proliferation and invasion, and induced apoptosis in GC. The present study successfully established *in vitro* and *in vivo* models with the simultaneous overexpression of *PTEN* and silencing of *Livin* for the first time, to the best of our knowledge. The results demonstrated that dual gene modulation produced more marked antitumor and antimetastatic effects, compared with either single gene modulation alone, *in vitro* and *in vivo*. Therefore, the simultaneous overexpression of *PTEN* and silencing of *Livin* may represent a novel therapeutic approach for the treatment of GC.

Acknowledgements

This study was supported by the Major Public Interest Foundation of Henan Province, China (grant no. HNZB2010N91).

Competing interests

The authors declare that they have no competing interests.

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