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Effects of PTEN overexpression and/or livin silencing in gastric cancer

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Summary. *Aims and Background:* Gastric cancer (GC) remains one of the major causes of cancer-related mortality worldwide. Loss of function of PTEN, a tumor suppressor, and a requirement for livin, a member of the inhibitors of protein apoptosis family, have been implicated in the development of gastric cancer. However, the combined effects of modulation of PTEN and livin on GC development have not been studied. In the present study, we investigated the biological effects of gene modulation by concomitant PTEN overexpression and Livin silencing on GC development *in vitro* and *in vivo*. *Methods:* In this study, we transfected pCL-neo-PTEN-siLivin, pCL-neo-PTEN, pRNAT-U6.1-siLivin, or an empty control vector, into human gastric carcinoma cell line BGC823. The levels of mRNA and protein expression of PTEN and Livin in each transfected group were quantitated by real time PCR and Western immunoblotting. Cell proliferation, apoptosis, caspase-3/9 activity, and cell invasion were examined *in vitro*. Finally, transfected BGC823 cells were injected into nude mice, and tumor growth was evaluated *in vivo*. *Results:* Concomitant transfection with PTEN and Livin (pCL-neo-PTEN-siLivin) vectors significantly decreased cell proliferation, induced cell apoptosis and decreased cell penetration in Matrigel compared with the individual transfection groups. Injection of concomitant transfected (pCL-neo-PTEN-siLivin) BGC823 cells into nude mice suppressed tumor growth much more than the injection of single gene transfected cells. *Conclusions:* PTEN overexpression concomitant with Livin silencing is a feasible and effective gene modulation method both in vitro and in vivo, which may represent a potential therapeutic strategy for gene therapy in the treatment of GC.

Key words: PTEN, Livin, gastric cancer, BGC823

Introduction

Gastric carcinoma (GC) is a malignant tumor arising from the lining of the stomach. GC is the fifth most common cancer in the world, and remains the second most common cause of cancer-related 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 recognized as one of the most frequently mutated tumor suppressors in a variety of human cancers (5). PTEN is known to play an important role in cell proliferation, migration, and apoptosis, via negatively regulating the PI3K signaling pathway. PTEN has also been shown to inhibit the activation of downstream components AKT/protein kinase B (PKB) and transcription factors including nuclear factor B (NF-κB) (6-8). In GC, a significant reduction in the expression of PTEN has been

reported and the expression negatively correlates with tumor size, Borrmann classification, lymph node metastasis, and tumor staging (9, 10). Studies with PTEN in prostate cancer have indicated loss of a PTEN allele in 70% of men at the time of diagnosis (11). Inactivation of PTEN can be attributed to gene mutation, hypermethylation, microRNA-mediated regulation, and post-translational phosphorylation (12). Recent studies have indicated that functional inactivation of the tumor suppressor protein PTEN has been detected in multiple cases of GC and other tumors (13). These reports suggest that PTEN inactivation is closely related to 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). Expression of Livin has been shown to be increased in human GC and correlated with tumor differentiation and lymph node metastases. The underlying mechanism behind its effect suggests that Livin protein binds directly to caspases, thus inhibiting cell apoptosis and promoting tumor growth. In contrast, knockdown of Livin inhibited cell growth and invasion (17, 18). These studies reported to date imply that down-regulation of Livin may be a potential therapeutic strategy for GC.

Although studies with regard to PTEN overexpression or knock-down of Livin have shown an inhibitory effect on cell proliferation and induction of cell apoptosis in a variety of cancers (9, 10, 19), the combined effect of modulating both PTEN and Livin has not been studied in relation to GC. Thus the present study focused on evaluating the biological effects of this concomitant gene modulation on GC development by employing previously constructed recombinant vectors with PTEN and Livin from our laboratory (20).

Materials and methods

Construction of experimental vectors

To construct the pCL-neo-PTEN vector, briefly, the full length of PTEN cDNA was successfully cloned from E. coli containing pCMV6-PTEN plasmid (OriGene). The PTEN cDNA product was further amplified using the following primers: PTEN forward 5'-TAGAATTCATGACAGCCATCATCAAAG AGATCG-3' and PTEN reverse 5'-CTTGTCGACT CAGACTTTTGTAATTTGTGTATGCTG-3'. The cDNA product was purified of the agarose gel using a DNA purification kit (Cat# 28104, QIAGEN). The purified PTEN cDNA and the pCL-neo vector (Cat# E1841 Promega) were digested using the restriction endonucleases EcoRI and SalI and then ligated with T4 DNA ligase (Promega) (Supplemental Figure, 1A). Recombinant plasmids were transformed into E. coli DH5α competent cells and then selected. The selected plasmids were further confirmed by sequencing. For the pRNAT-U6.1-siLivin vector, the Ambion siRNA design system was used to scan the Livin cDNA sequence (NM_139317). Homolog analysis was performed through BLAST, and the 19bp gene sequence (GAGGTGCTTCTTCTGCTAT) of the Livin DNA was selected as the target of the siRNA. Single chain DNA with a short hairpin RNA structure designated upon the targeting gene was synthesized as: siLivin forward 5'GATCCGAGGTGCTTCTTCT-GCTATTTCAAGAGAATAGCAGAAGA AG-CACCTCTTTTTTA-3',andsiLivinreverse5'-AGCT TAAAAAAGAGGTGCTTCTTCTGC-TATTCTCTTGAAATAGCAGAAGAAGCAC-CTCG-3' (Sangon Biotech, Shanghai, China). These DNA chains were double-digested with BamHI and HindIII and blunt end-ligated it to pRNAT-U6.1 vector (GeneScript) using PCR (Supplemental Figure, 1B) The recombinant plasmids were transformed into E. coli DH5α competent cells, and the plasmids with successful insertion of the targeted gene were selected and sequenced.

To construct the pCL-neo-PTEN-siLivin vector, we first cut the pRNAT-U6.1-siLivin vector with HindIII, and blunted the ends with DNA polymerase I, large (Klenow) fragment. Next, the siLivin fragment was cut by BglII. In parallel, the pCL-neo-PTEN vector was cut and blunted by BstX I and Klenow fragments respectively. Another sticky end was made by BamHI. Next, the siLivin fragment was ligated into the linear pCL-neo-PTEN to construct the pCL-neo-PTEN-siLivin vector (Supplemental Figure, 1C). Successful construction was further confirmed

by EcoRI digestion, and DNA sequencing (CyberSyn, Beijing, China).

BGC823 cell transfection

In this study, the following vectors were used: pCL-neo-PTEN vector for PTEN overexpression, pRNAT-U6.1-siLivin vector for Livin silencing, pCL-neo-PTEN-siLivin vector for both PTEN overexpression and Livin silencing, and pCL-neo or pRNAT-U6.1 empty vector as the transfection control. These vectors were constructed as described above and as previously reported (20). The vectors mentioned above were transfected into BGC823 cells (Culture Collection of the Chinese Academy of Sciences, Shanghai, China), respectively, using a LipofectamineTM 2000 transfection kit (Invitrogen, Grand Island, NY). BGC823 cells were cultured in 6-well plates at a concentration of 1X10^6 cells/mL in RPMI 1640 culture medium at 37°C in the presence of 5% CO_2. Successful transfection was screened by G418 for 4 weeks, after which the levels of mRNA and protein expression were confirmed by real-time PCR and Western blot as reported (20).

Cell proliferation assay

Cells in an exponential phase were collected and cultured in a 96-well culture plate at a concentration of 1X10^4 cells/200 μl /well at 37°C with 5% CO_2 for one to five 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 hours at 37°C. Culture medium was discarded and 150 μL of DMSO was added for 10 min to dissolve the crystals. Absorption at 570 nm was detected in an automatic plate reader (Bio-Rad, Hercules, CA).

Detection of activities of caspase-3 and -9

After transfection, 2X10^6 of BGC823 cells were collected and active caspase-3 and -9 were examined using a caspase activity detection kit (GenMed Scientifics, Wilmington, DE) following the manufacturer's protocol. Fluorescence of each sample was detected using a spectrometer with absorption/emission maxima of ~511/533 nm (Bio-Rad).

TUNEL cell apoptosis assay

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was used to detect cell apoptosis after transfection with different vectors. Cells were added onto glass slides, and cell climbing slides were produced and dried at room temperature. After washing 3 times with PBS, cells on slides were fixed by adding 4% paraformaldehyde and then washed twice with PBS. Apoptotic cells were detected using a TUNEL kit (R&D system, Minneapolis, MN) following the manufacturer's protocol. PBS was used as a negative control. Cells stained with brown granules in the nuclei were classified as apoptotic cells. Percentages of apoptotic cells and the apoptosis index (AI) were calculated by randomly counting 500 cells selected from 5 spots in the slides.

In vitro cell invasion assay

In vitro cell invasion was evaluated using the Boyden Chamber Assay (Cell Biolabs, San Diego, CA) as previously reported (21). Briefly, serum free RPMI 1640 containing 3.9 μg/μL of Matrigel™ (BD, San Jose, CA) was added to the upper chamber above the filter membrane, and incubated at 37°C for 2 hours. 200 μL of serum-free cell culture supernatant of NIH3T3 cells was added into the lower chamber as the chemoattractant factor. Cells (400 μL) at a concentration of 1X10^6 cells/mL, transfected with different vectors, were added to the upper chamber and cultured for 24 h at 37°C with 5% CO_2. The migrated cells in the matrigel gel were detected by hematoxylin and eosin (HE) staining and counted at 5 random spots under a microscope. Five chambers were repetitively detected for each cell line transfected by different vectors At least three independent experiments were performed.

In vivo mouse models of GC

Animal work was conducted in accordance with protocols approved by the institutional care and use committee for animal research at the First Affiliated

Hospital of Zhengzhou University, China. *In vivo* tumorigenesis was investigated in BALB/c nude mice at 5 weeks (Shanghai SLAC Laboratory Animal Co. Ltd, Shanghai, China). Mice were bred in the animal room with continuous air circulation at room temperature maintained at 25°C -27°C and humidity of 40-60%. Sterilized food and water containing multivitamins were provided for the mice. Twenty-five nude mice were equally divided into 5 groups (n=5/group) for xenograft transplantation of non-transfected and transfected BGC823 cells. The vectors used for transfection included pCL-neo-PTEN, pRNAT-U6.1-siLivin, pCL-neo-PTEN-siLivin, and control. Briefly, $1X10^7$ BGC823 (0.1 ml) cells were inoculated subcutaneously. Tumor size was carefully measured from day 7 after inoculation. Tumors were removed and weighted at day 28.

Quantitative Real-time PCR

Tumor tissue (20-30 mg) from each mouse was ground in liquid nitrogen. Total RNA was extracted and purified by an RNA extraction kit (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Real-time PCR was performed using a real-time PCR kit (Baocheng Biotech Co. Ltd, Dalian, China). RNA was normalized to expression levels of β2-microglobulin and the relative expression was calculated. The PCR primer sequences were as follows: B2M: 5'- TCCATGAGACATTGAAGTTG-3' and 5'- ACACGGCAGGCATACTCAT-3'; PTEN: 5'-TGGCGGAAGTYGCAATAC-3'and5'-GCTGAGGAACTCAAAGTAC-3'; Livin: 5'-TCCTGCTCCGGTCAAAAGG-3'and5'-GCTGCGTCTTCCGGTTCTT-3'

Western Blot

Cells or tumor tissue were harvested and extracted using lysis buffer (Tris-HCl, SDS, mercaptoethanol, glycerol). Extracts were heated for 5 min in loading buffer, and then equal amounts of cell extract were separated on 12% SDS-PAGE gels. Separated protein bands were transferred into polyvinylidene fluoride (PVDF) membranes and the membranes were blocked in 10% skimmed milk for 1 hour at room temperature. The primary antibodies against PTEN (catalog# sc-133242), LIVIN (catalog# sc-71592), and β-actin (catalog# sc-130301, Santa Cruz, CA) were diluted according to the manufacturer's instructions and incubated overnight at 4°C. The membranes were then incubated with the corresponding horseradish peroxidase-conjugated secondary antibody (Santa Cruz) at room temperature for one hour. Chemiluminescent solution (NEN, MA) was used to visualize the blot, followed by exposure of the blot onto hyper film (GE, Pittsburgh, PA) for 5 min.

Statistical analyses

Data were processed and analyzed using SPSS 13.0. Results were shown as mean ± standard error. One-way ANOVA was used to detect differences among groups. If a significant difference was found by ANOVA, the Fisher LSD test was used to detect specific differences between the study groups. A value of $P<0.05$ was considered statistically significant.

Results

Effects of PTEN overexpression and/or Livin silencing in BGC823 cells

In order to determine the effects of PTEN overexpression and Livin silencing we transfected the previously constructed recombinant vectors (20) into BGC823 cells and evaluated their mRNA and protein expression. Our data showed that cells transfected with pCL-neo-PTEN or pCL-neo-PTEN-siLivin resulted in a significant increase in PTEN mRNA and protein expression, compared to those transfected with pCL-neo empty vector. However, we did not observe any significant differences in PTEN expression between pCL-neo-PTEN and pCL-neo-PTEN-siLivin transfection groups (Figures 1A and 1B). Furthermore, cells transfected with either pRNAT-U6.1-siLivin or pCL-neo-PTEN-siLivin resulted in a significant decrease in Livin mRNA and protein expression (Figures 1C and 1D). Interestingly, pCL-neo-PTEN transfection also resulted in a significant decrease in Livin mRNA and protein expression, although the inhibitory effect

was not as pronounced as the effects observed in the Livin silencing groups. Our data suggested that inserting Livin siRNA into the PTEN overexpression vector has no effect on PTEN expression but suppresses Livin expression significantly. Thus, PTEN overexpression might play a role in regulating Livin gene expression.

Transfection of pCL-neo-PTEN-siLivin inhibited tumor cell proliferation and migration

We next examined the effects of PTEN and/or Livin transfection on BGC823 cell proliferation using the MTT assay. BGC823 cells were transfected and monitored from Day 1 to Day 5. Our data demonstrated that of the five experimental groups only the pCL-neo-PTEN-siLivin transfection group resulted in a significant inhibition of cell proliferation on days 3, 4, and 5. In contrast, modulation of PTEN or Livin individually had no significant effect on cell proliferation from days 1 to day 5 (Table 1).

To evaluate the metastasis of the transfected cells, we performed a Matrigel assay *in vitro*. The migrated cells in the matrigel gel were detected by hematoxylin and eosin (HE) staining and counted in 5 random spots under the microscope. As shown in Figures 2A and 2B, both single (pCL-neo-PTEN or pRNAT-U6.1-siLivin transfection) and concomitant (pCL-neo-PTEN-siLivin transfection) gene modulation resulted in a dramatic inhibition of cell migration, compared with empty vector (pCL-neo) transfection. Our data demonstrated that concomitant gene modulation resulted in maximal inhibition among all groups.

Transfection of pCL-neo-PTEN-siLivin induced cell apoptosis via activating the Caspase signaling pathway

In a parallel study, we evaluated cell apoptosis by TUNEL staining. Our data showed that both single (pCL-neo-PTEN or pRNAT-U6.1-siLivin transfection) and concomitant (pCL-neo-PTEN-siLivin transfection) gene modulation resulted in a significant increase in the percentage of apoptotic cells, compared with empty vector (pCL-neo) transfection (Figure 3A). The percentages of the apoptotic rate for pCL-neo-PTEN transfection, pRNAT-U6.1-siLivin transfection, and pCL-neo-PTEN-siLivin transfection were 8.67 ± 1.27, 10.39 ± 1.31, and 16.72 ± 1.84, respectively.

To characterize the underlying mechanism of cell apoptosis, we determined the activity of caspase-3 and caspase-9 in these transfected BGC823 cells. As shown in Figure 3B, both single and concomitant gene modulation induced significant increases in the activities of both caspase-3 and caspase-9, although no significant differences were identified among these three modulated groups.

Transfection of pCL-neo-PTEN-siLivin inhibited tumor growth in vivo

To determine the effect of PTEN and/or Livin modulation on tumor growth *in vivo*, we injected various vector-transfected BGC823 cells to nude mice. The tumor growth was monitored weekly from day 7 to day 28, when the mice were sacrificed. As shown in Figure 4A, the tumors grew continuously from day 7 to day 28 in both 'no transfection' and pCL-neo control transfection groups. However, the tumor growth was suppressed significantly in both single gene (pCL-neo-PTEN or pRNAT-U6.1-siLivin) and concomitant gene (pCL-neo-PTEN-siLivin) transfected groups, with the concomitant gene transfection group demonstrating the better efficacy.

Tumors were collected and weighed at day 28. As shown in Figure 4B, the tumor weights for pCL-neo-PTEN-siLivin transfection, pCL-neo-PTEN transfection, and pRNAT-U6.1-siLivin transfection were 215.42 ± 35.15 mg, 461.73 ± 58.17 mg, and 368.23 ± 53.72 mg, respectively, which was significantly smaller than those in the pCL-neo transfection group (968.88 ± 194.39 mg). No significant difference was identified between the 'no transfection' group (1051.29 ± 175.95 mg) and the pCL-neo transfection group.

Expression of PTEN and Livin in tumors

To verify if the inhibitory effects on tumor growth are specific to PTEN and/or Livin gene modulation, we examined the gene expression of PTEN and Livin in the tumor tissues. Our data indicated that tumor tissues from pCL-neo-PTEN or pCL-neo-PTEN-siLivin mice resulted in a significant increase in PTEN

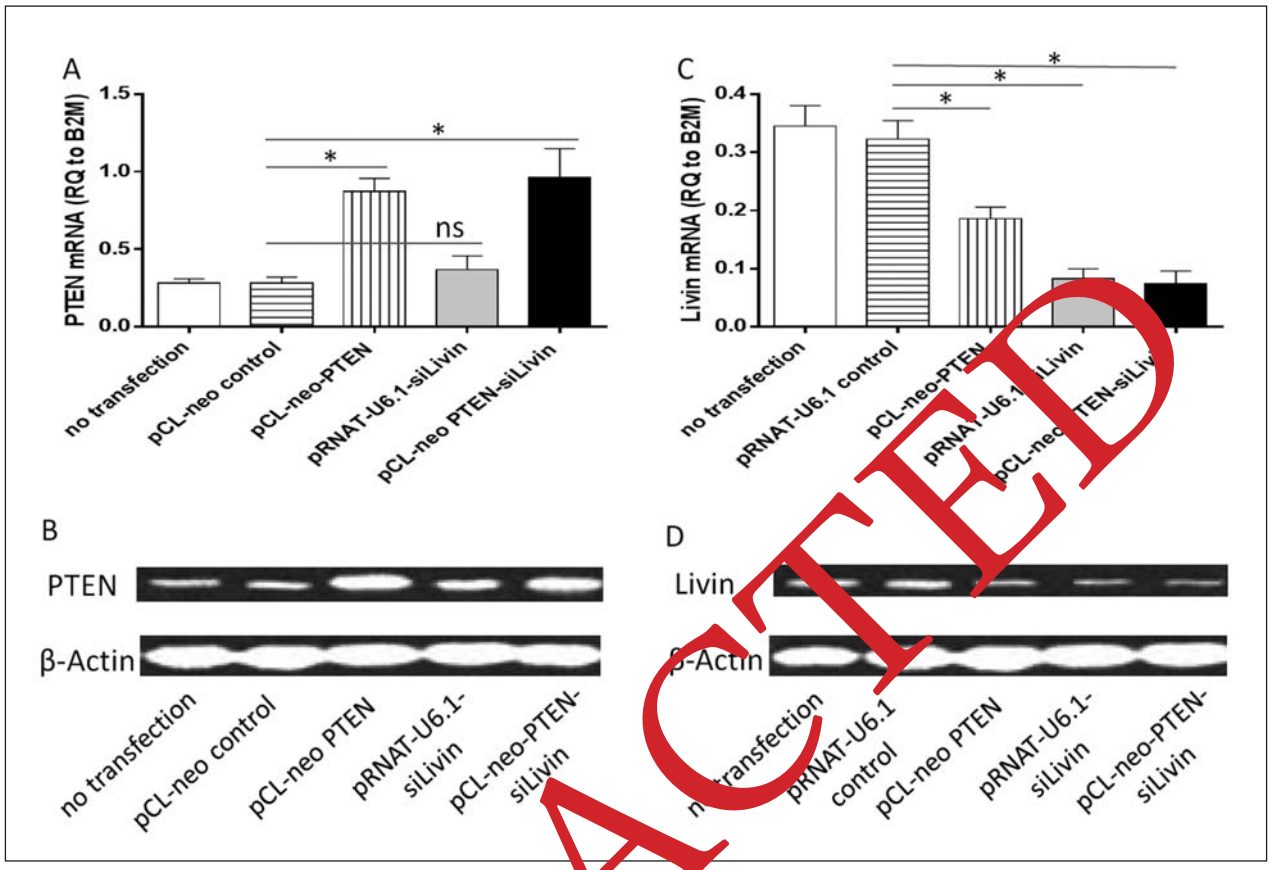

Figure 1. PTEN Overexpression and Livin silencing in BGC823 cells. BGC823 cells were transfected with various constructed vectors, which included pCL-neo control, pRNAT-U6.1 control, pCL-PTEN, pRNAT-U6.1-siLivin, and pCL-PTEN-siLivin vectors. Successful transfection was screened by G418 for 4 weeks followed by mRNA and protein quantitation. PTEN mRNA (A) and Livin mRNA (C) were detected by real-time PCR, with β-actin as an endogenous control. PTEN protein (B) and Livin protein (D) were detected by Western Blot, with β-actin as the endogenous control. * P<0.05, compared to pCL-neo control vector transfection or pRANT-U6.1 control vector transfection, respectively. N=6/group. Data are representative of 3 experiments.

Table 1. Effect of PTEN and/or Livin transfection on BGC823 cell proliferation. BGC823 cells were transfected with various vectors as shown in the table. Cell proliferation was measured by MTT assay from Day 1 to Day 5. Absorption at 570 nm was detected. The absorption values are plotted as a mean ± standard error. N=6: each group includes 6 samples. *p<0.05, compared to pCL-neo control transfection.

Group	N	Day 1	Day 2	Day 3	Day 4	Day 5
No transfection	6	0.07±0.01	0.182±0.03	0.36±0.05	0.51±0.07	0.79±0.11
pCL-neo control	6	0.065±0.01	0.184±0.02	0.34±0.05	0.49±0.06	0.76±0.09
pCL-neo-PTEN	6	0.075±0.01	0.172±0.02	0.29±0.04	0.47±0.04	0.69±0.09
pRNAT-U6.1-siLivin	6	0.07±0.01	0.181±0.01	0.30±0.04	0.46±0.05	0.71±0.08
pCL-neo-PTEN-siLivin	6	0.07±0.01	0.139±0.02	0.181±0.03*	0.251±0.03*	0.358±0.05*

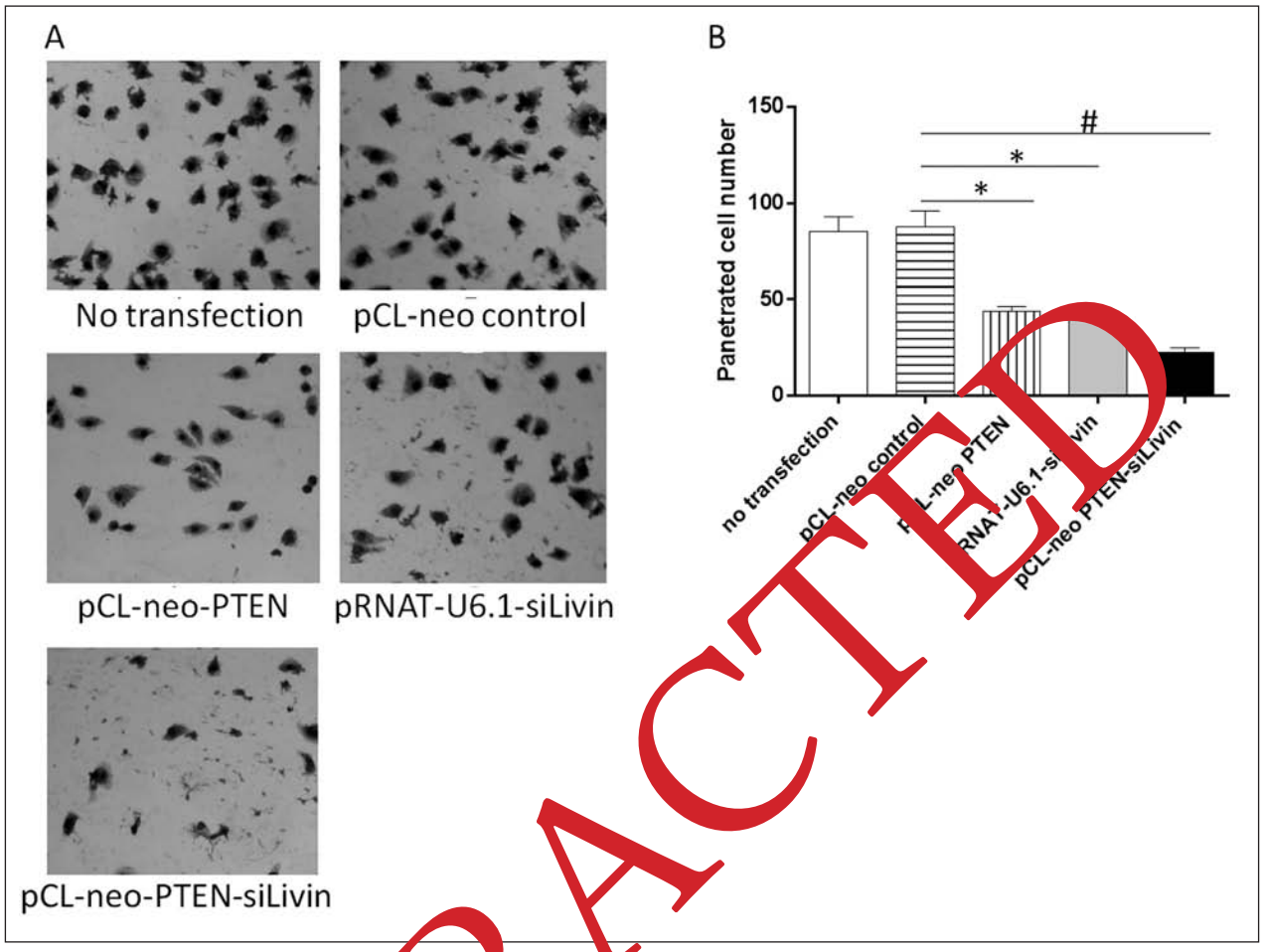

Figure 2. Transfection of pCL-neo-PTEN-siLivin inhibited BGC823 cell migration. BGC823 cells were transfected with various constructed vectors, which included pCL-neo control, pCL-PTEN, pRNAT-U6.1-siLivin, and pCL-PTEN-siLivin vectors. The penetration ability of transfected BGC823 cells was characterized by Matrigel assay. The migrated cells in the matrigel gel were detected by hematoxylin and eosin (HE) staining and counted in 5 random spots under the microscope. (A) HE staining showed the migrated cells in the matrigel. Representative random spots under the microscope were viewed. (B) The number of transfected cells penetrating through the Matrigel was analyzed. *P<0.05; # P<0.01, compared to pCL-neo control vector transfection. N=6/group. Data are representative of 3 experiments.

mRNA and protein expression as compared to pCL-neo control (Figures 5A and 5B) In contrast, tumor tissues from pRNAT-U6.1-siLivin or pCL-neo-PTEN-siLivin mice resulted in a significant decrease in Livin mRNA and protein expression, compared to pCL-neo control (Figure 5C and 5D). Our data demonstrated that the in *vivo* studies on tumor tissues are comparable to the *in vitro* studies on BGC823 cells. Therefore our data indicate that the transfected cells maintained a stable expression of target genes in the tumors *in vivo*.

Discussion

Normal tissue cells maintain a dynamic equilibrium between cell proliferation and cell death. Apoptosis is an active mechanism of cell death. It plays an essential role in embryo development, differentiation and tissue morphogenesis (22, 23). Deregulation of apoptosis leads to a variety of diseases including cancer. PTEN has been shown to promote apoptotic cell death, which in turn suppresses tumor growth (24, 25).

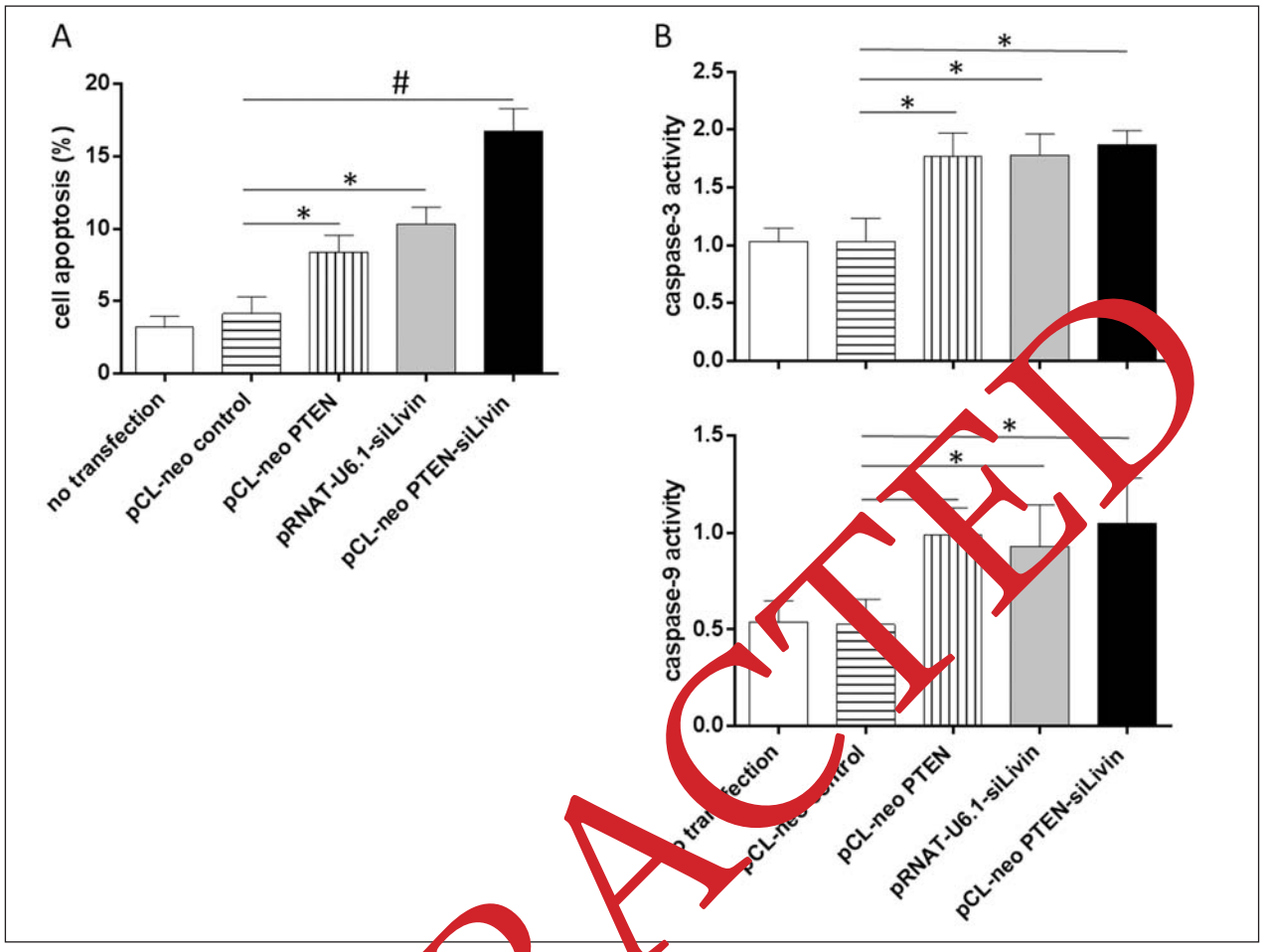

Figure 3. Transfection of pCL-neo-PTEN-siLivin induced cell apoptosis via activation of caspase signaling. BGC823 cells were transfected with various constructed vectors which included pCL-neo control, pCL-PTEN, pRNAT-U6.1-siLivin, and pCL-PTEN-siLivin vectors. Cell apoptosis and caspase activity were evaluated. (A) The number of apoptotic cells was determined by TUNEL staining. (B) The activity of caspase-3 (upper panel) and caspase-9 (lower panel) was determined by a caspase activity detection kit. * P<0.05; #P<0.01, compared to pCL-neo control vector transfection. N=6/group. Data are representative of 3 experiments.

Puc et al. demonstrated that loss of PTEN resulted in a breakdown of DNA double strands, thus inducing cell cycle arrest at G2/S phase (26). In contrast, Livin was shown to inhibit apoptosis and promote cell proliferation via transition of cells from G0/G1 into the active cell cycle (27, 28). Overexpression of Livin makes the cells more resistant to apoptotic stimuli than normal cells (29, 30). These reports are consistent with the results of the current study, indicating that PTEN overexpression or Livin silencing can promote BGC823 cell apoptosis concurrent with inhibition of cell proliferation. Most importantly, our data demonstrate a novel effect of PTEN/Livin concomitant modulation on cell apoptosis and proliferation in comparison to the single gene modulations tested. The functional effects of PTEN and Livin regulation on GC cell apoptosis and proliferation were further supported by our xenograft study in nude mice. Currently, no study has investigated whether there is a direct correlation between PTEN and Livin expression and the malignancy of gastric cancer. However, studies have demonstrated a relationship between PTEN and Livin expression and the malignancies of renal cell carcinoma (31, 32), breast cancer (33), and Retinocytoma (34). These studies demonstrated that low expression of PTEN and high expression of Livin were significantly correlated

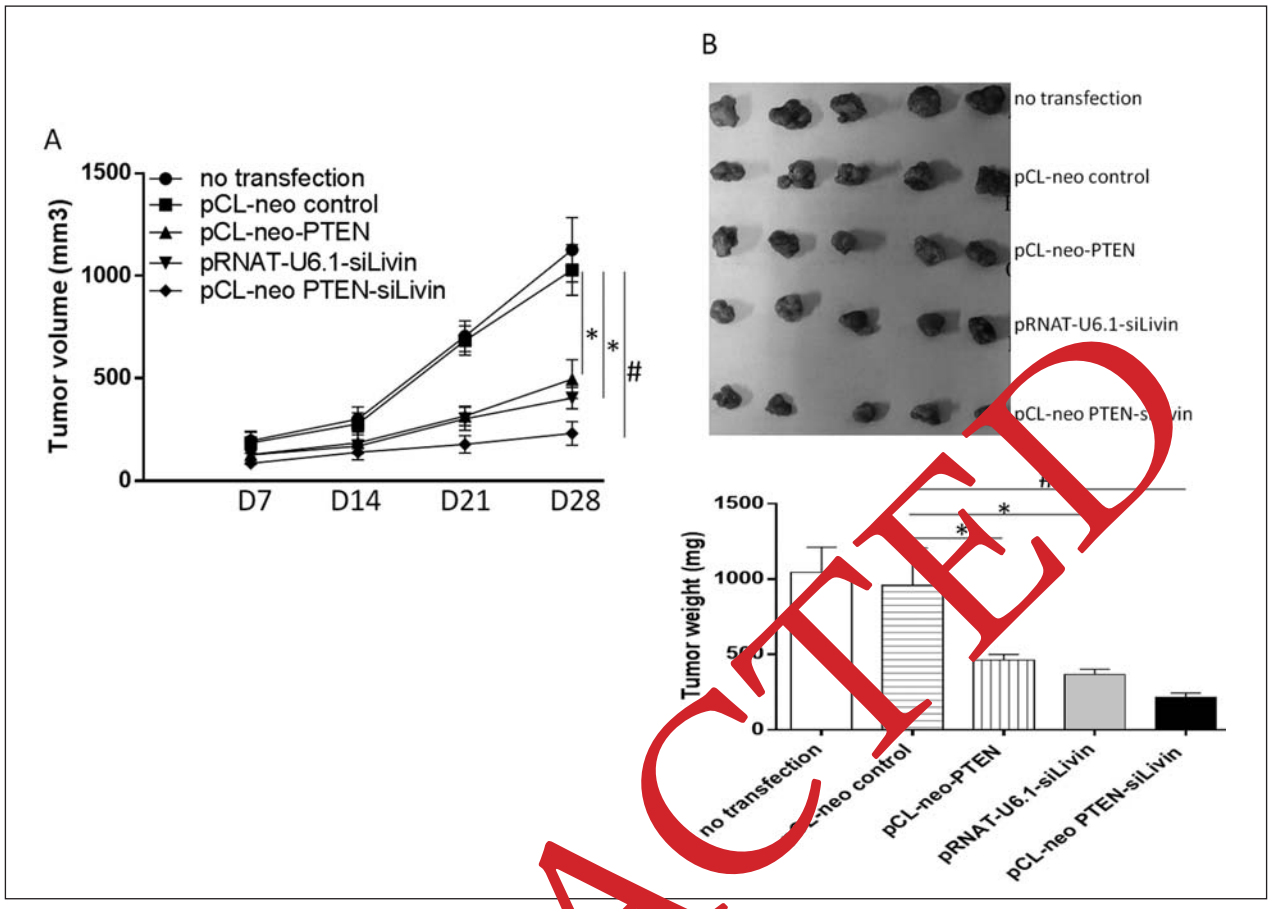

Figure 4. Transfection of PTEN and Livin vectors inhibited tumor growth *in vivo*. BGC823 cells were transfected with various constructed vectors, which included pCL-neo control, pCL-PTEN, pRNAT-U6.1-siLivin, and pCL-PTEN-siLivin vectors. The transfected BGC823 cells were then inoculated into nude mice s.c. The tumor growth was monitored weekly. (A) Tumor volume was measured on D7, D14, D21, and D28. (B) Tumors from each group were dissected on D28 (upper panel) and weighed (lower panel). * P<0.05; # P<0.01 compared to pCL-neo control vector transfection. N=5/group.

with the clinical stage and lymph node metastases of patients' malignancies. Our current study suggests that PTEN overexpression concomitant with Livin silencing may represent a potential therapeutic strategy for gene therapy in the treatment of gastric cancer.

Caspase-3 is a protease and plays a crucial role in the apoptotic pathway. Caspase-3 can be detected in almost all cell types, emphasizing its role in modulating cell survival and death (35). Increased PTEN expression in cultured neonatal rat primary cardiomyocytes leading to increased caspase-3 activity and cell apoptosis has been reported (36), which suggests that caspase-3 is the major effector of PTEN. In contrast, Livin demonstrates an anti-apoptotic activity via binding to caspase3, -7 and -9, and its E3 ubiquitinligase. Livin also promotes the degradation of IAP antagonist SMAC/DIABLO (37, 38). These data suggest that PTEN and Livin share a caspase signaling pathway with opposite effects. Our studies are comparable with the above studies, as we also observed significantly increased activity by caspase-3 and its initiator caspase-9 in BGC823 cells with PTEN overexpression, Livin silencing or concomitant modulation. Thus, our study delineates an important role for PTEN and Livin on cell apoptosis via regulation of caspase-3/9 activity in gastric cancer consistent with earlier reported studies. Interestingly, though, concomitant gene modulation led to better effects on cell apoptosis than single

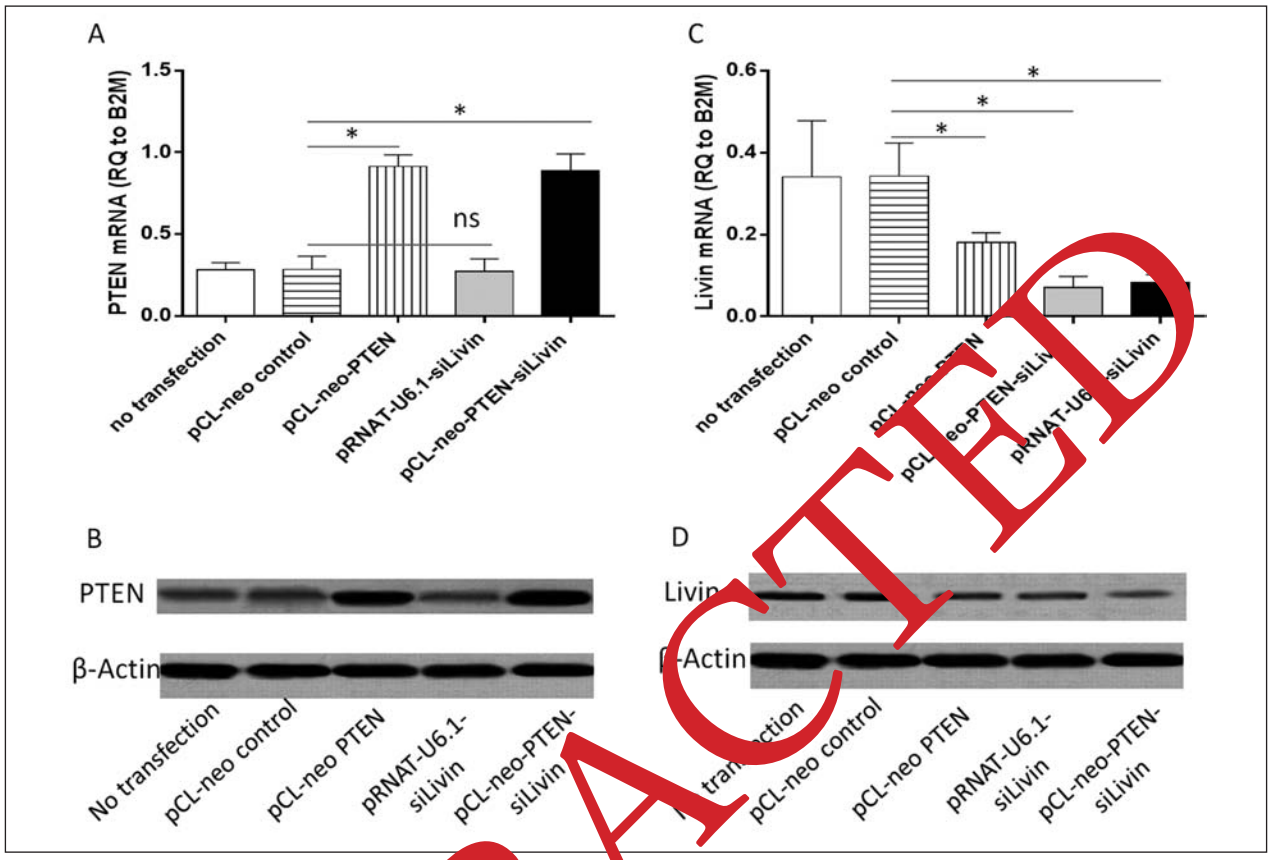

Figure 5. Expression of PTEN and Livin in tumors. Tumor tissues were excised and Total RNA and protein were measured. PTEN mRNA (A) and Livin mRNA (C) were detected by real-time PCR, with β2M as an endogenous control. PTEN protein (B) and Livin protein (D) were detected by Western Blot with β-actin as the endogenous control. * P<0.05, compared to pCL-neo control vector transfection. N=5/group.

gene modulation, while no significant difference was identified across these three transfected groups regarding caspase activity (Figure 3B). This result suggests that in addition to caspase signaling, other potential mechanisms may trigger cell death, including Fas/Fasl signaling and cytochrome c release. Further studies are warranted to address the underlying mechanism in signaling cascades.

PTEN plays an important role in the regulation of tumor cell metastasis. Hwang *et al.* showed that PTEN enhanced tumor metastasis by acting on VEGF and matrix metalloproteinases (MMPs) (39). In other studies, PTEN overexpression inhibited the migration of glioblastoma cells (40), whereas PTEN knockdown enhanced cell migration in fibroblasts via regulation of focal adhesion kinase (FAK), a cytoplasmic phosphoprotein activated by integrin (41). Livin was shown to regulate tumor cell invasion, which is vital and the first step for metastasis, through the NFKB signaling pathway (42, 43). Further, knockdown of Livin inhibited tumor invasion through blocking MAPK signaling (17, 44). In our study, the migration of BGC823 cells was significantly inhibited after transfection of PTEN, siLivin, or both genes, with concomitant transfection demonstrating the highest inhibitory effects. Further studies are needed to characterize whether molecules like FAK, NFKB, and MAPK are involved in metastasis, which is beyond the research scope of the current study.

The results from the current study are consistent with previous reports that either PTEN overexpression or Livin silencing significantly inhibited cell proliferation and invasion, and induced cell apoptosis in GC Most importantly, for the first time, we successfully established this in both *in vitro* and *in vivo* mod-

els with simultaneous PTEN overexpression and Livin silencing. Our models demonstrated that concomitant gene modulation resulted in more beneficial effects than single gene regulation both *in vitro* and *in vivo*.

Conclusion

PTEN overexpression and Livin silencing resulted in potent effects on regulation of cell proliferation, apoptosis, migration, and tumor growth compared to single gene modulation. Thus, a combination of PTEN overexpression and Livin silencing may represent a novel therapeutic approach for GC treatment or gene therapy.

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