Concomitant modulation of PTEN and Livin in gastric cancer treatment

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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 concomitantly 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...
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 above transfections were selected following multiple rounds of culture and selection. The 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 media (Fisher Scientific, Inc., Pittsburgh, PA, USA) for 5 min. The culture medium was discarded and 150 µl of 5% CO2 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 measured in an automatic plate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Western blot analysis. The cells or tissue samples 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).

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. RR047A; Takara Biotechnology Co., Ltd., Dalian, China). The RNA sample was then reverse transcribed into cDNA using a high capacity cDNA reverse transcription kit (cat no. RR047A; Takara Biotecnology Co., Ltd., Dalian, China). RT-qPCR analysis was performed using a real-time PCR kit (Biochemp 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’-TCCATCAGGACATTGAGTTG-3’ and reverse 5’-ACACGGCAGGCTACTCAT-3’; PTEN, forward 5’-TGCGGAACTGCAATCC-3’ and reverse 5’-GCTGAGAACTCAAGTAC-3’; Livin, forward 5’-TCCGCTCCTCATAAAAGG-3’ and reverse 5’-GCTGGCTCTTCGCGTTCTT-3’. cDNA was normalized to β-actin expression and relative expression was calculated using the 2-ΔΔcq method (21).

Cell proliferation assay. Cells in the exponential phase were collected and cultured in a 96-well culture plate at a concentration of 1×104 cells in a volume of 200 µl per well at 37˚C with 5% CO2 for 1-5 days. Each day, 20 µl of 3-(4,5-dimethyl-azol-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.).
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 37ºC 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 37ºC with 5% CO₂. 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, or empty control. 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 pRNAT-U6.1-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.

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.
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). Figure 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
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, (R²=0.7286 and R²=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.

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
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 and inhibited 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 protease 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)-κB 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-κB 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-κB 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.

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Competing interests

The authors declare that they have no competing interests.

References


