The Adenovirus-Mediated Transfer of PTEN Inhibits the Growth of Esophageal Cancer Cells in Vitro and in Vivo

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The development and progression of esophageal cancer is associated with multiple alterations in the genome, including loss of the tumor suppressor phosphatase and tensin homolog deleted from the chromosome 10 (PTEN) gene. The purpose of this study was to determine the effects of adenovirus-mediated MMAC/PTEN expression on the growth and survival of human esophageal cancer cells in vitro and in vivo. We found that compared to control cells, overexpression of PTEN significantly suppressed growth and induced apoptosis in esophageal cancer cell lines Eca-109 and TE-1 via downregulation of Bcl-2 expression and changes in cell-cycle progression. Adenovirus PTEN also inhibited the growth of subcutaneous tumor xenografts by significantly reducing tumor size in vivo. Thus our results confirm the proposed functional role of MMAC/PTEN as a regulator of esophageal cancer progression in vivo and in vitro. PTEN might be an important biological marker and potential therapeutic target in the treatment of human esophageal cancer.

Key words: PTEN; human esophageal cancer; adenovirus; gene therapy

Many studies have implicated the tumor suppressor gene MMAC/PTEN as critical to the biological effects associated with deletions or mutations of chromosome 10q23 in a variety of tumors, including glioblastomas and endometrial, prostate, breast, and non-small cell lung cancers.1–3) MMAC/PTEN has intrinsic lipid and protein phosphatase activity that dephosphorylates certain phospholipids and proteins, thereby inhibiting cell growth, promoting apoptosis, and regulating cell adhesion, migration, diffusion, and differentiation.4) As an example, MMAC/PTEN activity downregulates the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway. Specifically, PTEN acts as a phosphatidylinositol phosphatase with a possible role in PI3K-mediated signal transduction, which transduces extracellular growth regulatory signals to intracellular mediators of growth and cell survival.5) Loss of PTEN expression has been linked to poor prognoses in patients with gliomas and endometrial, prostate, gastric, and colorectal carcinomas. Recently, it was found that transfection of wild-type PTEN into a number of different cancer cells inhibits their growth and survival.6,7)

Esophageal cancer (EC) is one of the most common fatal cancers worldwide and it occurs at very high frequency in certain areas of China. Unfortunately, even with the use of modern surgical techniques combined with various adjuvant treatment modalities such as radiotherapy and chemotherapy, the overall 5-year survival rate of EC patients remains low (10%–40%) due to advanced disease, metastasis, and the resistance of the tumor to radiotherapy and chemotherapy.8–10) This reinforces the pressing need for improvements in the treatment of esophageal cancer. Recently, gene therapy has been explored for the treatment of human cancers. Tumor suppressor gene therapy aims to restore the function of a tumor suppressor gene that has been lost or functionally inactivated in cancer cells. PTEN is a logical candidate for gene therapy, since decreased expression of PTEN has been observed in patients with EC.11)

In this study, we found that PTEN gene therapy in two EC cell lines mediated by adenovirus (Ad-PTEN) inhibited proliferation and induced apoptosis via downregulation of Bcl-2 expression and inhibition of cell-cycle progression. The PTEN gene therapy also significantly reduced tumor size in vivo. Our study indicates that PTEN might be an important biological marker, and that PTEN gene therapy might be an important strategy in the treatment of esophageal cancer.

Materials and Methods

Cell lines and recombinant adenovirus vector. Human esophageal cancer cell lines Eca-109 and TE-1 were obtained from the Cell Bank of Shanghai Institute, CAS (Shanghai, China). The cell lines were grown in RPMI 1640 medium supplemented with 10% FCS and 1% penicillin/streptomycin. The cells were passaged every 2–3 d to maintain exponential growth prior to experimental use. Recombinant adenovirus vector Ad-PTEN, which expresses human wild-type PTEN cDNA under the control of the human cytomegalovirus immediate-early promoter/enhancer, was provided by Introgen (Houston, TX). Control adenovirus vector, derived from the same vector, lacks the PTEN transgene but expresses luciferase (Ad-Luc). Prior to the start of the experiment, transduction efficiency following in vitro infection of the cell lines was determined using a recombinant adenoviral vector carrying green fluorescent protein (GFP).

Western blot analysis. Subconfluent Eca-109 and TE-1 cells were infected with Ad-PTEN or Ad-Luc, or were mock infected. At 24 h after infection the medium was changed to serum-free medium. At 72 h
after infection the cells were harvested and cell lysates were prepared in a buffer containing 0.1 M NaCl, 0.01 M Tris-HCl (pH 7.6), 1 mM EDTA (pH 8.0), 1 mg/ml aprotinin, 100 mg/ml PMSF, and 1% NP-40. Protein was quantified by a Lowry protein concentration assay, and equal amounts of protein were electrophoretically separated and electrically transferred onto a PVDF membrane. After blocking with 5% nonfat dry milk in PBST, the membranes were incubated with antibodies against PTEN or Bcl-2 (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C, followed by horseradish peroxidase–conjugated secondary antibody. Protein-antibody complexes were detected using enhanced chemiluminescence system ECL-Plus. The blots were stripped and reprobed using antibodies against β-actin (Sigma-Aldrich, St. Louis, MO) to ensure equal loading and transfer of proteins.

Cell proliferation assay. Briefly, cells were seeded in 96-well tissue culture plates at a density of 5 × 10^4 cells/well and then infected with Ad-PTEN or Ad-Luc at the indicated multiplicity of infection (MOI) by directly adding the diluted vectors to the growth medium. Other cells were mock infected (no virus). The following day, cells in each treatment group were harvested by trypsinization at different time points. The cells were stained with trypan blue to label dead cells, and viable cells were counted using a hemacytometer.

Apoptotic staining. The cells were seeded in six-well tissue culture dishes at a density of 1 × 10^5 cells/well and then infected with Ad-PTEN or Ad-Luc, or were mock infected. At 72 h after treatment, a Hoechst staining kit (Sigma Chemicals, St. Louis, MO) was used to stain apoptotic cells. The apoptotic cells were identified by the presence of an apoptotic body or by chromosome condensation.

Cell-cycle analysis. To analyze cell-cycle distribution, cells were seeded in six-well tissue culture dishes at a density of 1 × 10^5 cells/well and then infected with Ad-PTEN or Ad-Luc, or mock infected. At 48 h after infection, the cells were harvested by trypsinization, washed once with ice-cold PBS, fixed with 70% ethanol/1% paraformaldehyde, and stored at 4°C. Following overnight incubation, they were washed twice with ice-cold PBS and treated with RNase for 30 min. DNA was stained with 50 μg/ml of propidium iodide. Cell-cycle distribution was determined using a FACScan flow cytometer.

In vivo tumor xenograft studies. Subcutaneous tumor xenografts were established in nude mice by injecting Eca-109 or TE-1 tumor cells (1 × 10^6 cells/animal) into the left dorsal flank. When the tumor had reached a size of 70–100 mm^2, the animals were divided randomly into three groups and mock treated or transfused with Ad-Luc or Ad-PTEN (3 × 10^10 viral particles/dose). All intratumoral injections were performed under anesthesia. Tumor dimensions were measured with calipers every week. Mean tumor volume was calculated as width^2 × length × 0.52.12 All measurements were performed in a coded and blinded fashion. The therapeutic effect was determined by statistical analysis. All procedures had the approval of the Animal Ethics Committee of Fourth Military Medical University.

Results

PTEN expression in esophageal cancer cells

Western blot analysis showed that both EC cell lines, whether infected with Ad-PTEN or Ad-Luc or mock infected, expressed an endogenous level of PTEN 72 h after treatment. However, higher levels of PTEN were seen in Ad-PTEN-infected cells, presumably due to expression of the PTEN transgene (Fig. 1A). Infection with Ad-PTEN also caused a pronounced decrease in Bcl-2 expression in Eca-109, TE-1 cells as compared to Ad-Luc- or mock-infected cells (Fig. 1B). Thus the function of the MMAC1/PTEN gene appears to vary in different cell types, and depend on interactions with various signal transduction pathways.

PTEN effects on cell proliferation in vitro

Esophageal cancer cells were infected with Ad-PTEN or Ad-Luc or mock infected to determine the effects of exogenous PTEN expression on cell proliferation at various time points. Daily analysis of cell viability on days 1–5 indicated that adenoviral transduction of PTEN significantly inhibited cell proliferation (p < 0.01) in both Eca-109 and TE-1 cells as compared with mock-infected or mock infected with Ad-Luc (Fig. 2). The numbers of Eca-109 and TE-1 cells 4 d after infection with Ad-PTEN were reduced by more than 70% and 60% respectively as compared with cells infected with the same amount of control Ad-Luc. These results suggest that Ad-PTEN effectively inhibits the proliferation of esophageal cancer cells.

PTEN effects on cell cycle in vitro

Next we determined the effects of Ad-PTEN and Ad-Luc treatment on cell cycle progression in Eca-109 and
TE-1 cells by fluorescence-activated cell sorting (FACS) analysis. FACS analysis that overexpression of PTEN for 48 h and downregulation of Bcl-2 produced an 18% (Eca-109 cells) and 15% (TE-1 cells) increase in the number of cells in the G1 phase as compared to control cells infected with Ad-Luc, and a concurrent decrease in cells in the S and G2M phases (Fig. 3). Thus treatment with Ad-PTEN can influence the G2M cell-cycle arrest caused by overexpression of Bcl-2 in esophageal cancer cells. There was no significant difference in G1 population between the mock-infected cells and the cells infected with Ad-Luc.

**PTEN effects on cell apoptosis in vitro**

To determine whether treatment with Ad-PTEN would induce apoptosis in vitro, Eca-109 and TE-1 cells were analyzed 72 h after treatment with Ad-PTEN or Ad-Luc using a Hoechst staining kit. After Ad-PTEN treatment for 72 h, Eca-109 and TE-1 cells had condensed and fragmented nuclei, an indicator that the cells were undergoing apoptosis. No changes in apoptotic morphology were observed in any of the cells treated with Ad-Luc (Fig. 4).

**PTEN effects on gene therapy in vivo**

Next we evaluated the ability of Ad-PTEN to inhibit the growth of subcutaneous tumor xenografts. Subcutaneous esophageal tumor xenografts were established in nude mice, which were then divided into three groups (n = 6 per group) and mock treated or infected with Ad-Luc or Ad-PTEN. Intratumoral injections of Ad-PTEN into Eca-109 and TE-1 tumor xenografts resulted in
significant \((p < 0.01)\) inhibition of tumor growth as compared to mock or Ad-Luc control tumors (Fig. 5), but complete tumor regression was not observed. We also noted treatment-related toxicity that did not occur during the course of the experiment.

**Discussion**

The tumor suppressor gene PTEN, which encodes a multifunctional phosphatase, is mutated in a variety of human cancers. PTEN participates in regulating apoptosis and growth through its lipid phosphatase activity, and also contributes to the regulation of cell adhesion, migration, and invasion. Several reports have indicated that PTEN has growth-suppressing and apoptosis-promoting properties and displays an altered expression pattern during human oncogenesis.\(^{13,14}\)

Identification of the mechanisms of action of MMAC1/PTEN lead to a better understanding of the mechanisms of esophageal cancer progression and might suggest novel targets for therapy. Here we investigated the effects of adenovirus-mediated MMAC/PTEN expression on the behavior of human esophageal cancer cells both \textit{in vitro} and \textit{in vivo} using the cell lines Eca-109 and TE-1.\(^{15,16}\) We found that overexpression of PTEN significantly suppressed the growth of and

![Fig. 4. Analysis of Apoptosis in Eca-109 and TE-1 Cells by Hoechst Staining after Treatment with Ad-Luc or Ad-PTEN. Magnification is 40× for all panels. Cell apoptosis analysis was performed 3 d after infection.](image)

**Fig. 4.** Analysis of Apoptosis in Eca-109 and TE-1 Cells by Hoechst Staining after Treatment with Ad-Luc or Ad-PTEN. Magnification is 40× for all panels. Cell apoptosis analysis was performed 3 d after infection.

![Fig. 5. Therapeutic Effects of Ad-PTEN on Subcutaneous Human Esophageal Tumor Xenografts. Tumors treated with Ad-PTEN showed significant inhibition of tumor growth as compared to tumors that were not treated or treated with Ad-Luc. Each time point represents the mean tumor volume for a group.](image)

**Fig. 5.** Therapeutic Effects of Ad-PTEN on Subcutaneous Human Esophageal Tumor Xenografts. Tumors treated with Ad-PTEN showed significant inhibition of tumor growth as compared to tumors that were not treated or treated with Ad-Luc. Each time point represents the mean tumor volume for a group.
induced apoptosis in these cells as compared to the controls.

Esophageal cancer cells were infected with Ad-PTEN to determine the effects of exogenous PTEN expression on cell proliferation at various time points. At 4 d after infection, the numbers of Eca-109 and TE-1 cells were reduced with Ad-PTEN by about 70% and 60% respectively as compared to mock-infected cells and cells infected with Ad-Luc. These results indicate that Ad-PTEN can effectively inhibit the growth of esophageal cancer cells as compared to cells treated with Ad-Luc or mock-infected cells. After treatment with Ad-PTEN, we confirmed overexpression of PTEN and also observed a pronounced downregulation of Bcl-2 expression.17,18) Endogenous PTEN expression was observed in all controls, indicating that both cell lines are of the wild type for PTEN. The expression of PTEN counteracted the anomalies of Bcl-2 expression and the decrease in Bcl-2 expression in the TE-1 cells was much more than it in the Eca-109 cells after infection with Ad-PTEN.

The Eca-109 and TE-1 cells underwent apoptosis following Ad-PTEN infection. No changes were observed in any cells treated with Ad-Luc. We found that the population of apoptotic cells increased remarkably and that cell proliferation decreased significantly in the Ad-PTEN treated cells but there was no significant difference between Eca-109 and TE-1 cells. Cell cycle analysis by flow cytometry demonstrated that overexpression of PTEN and downregulation of Bcl-2 in esophageal cancer cells can induce G1 cell-cycle arrest and G2/M block. Compared to the controls, the percentages of cells in the G1 phase increased by approximately 18% and 15% in Ad-PTEN-infected Eca-109 cells and TE-1 cells respectively, with a concurrent decrease in the number of cells in the S and G2/M phases. We also found that there were no differences between Eca-109 and TE-1 cells. The changes in the population of cells in the G1 phase were not significantly different as compared to either the mock-treated cells or the cells infected with Ad-Luc. Finally, we investigated the ability of Ad-PTEN to suppress the growth of Eca-109 and TE-1 xenograft tumors. Ad-PTEN infection significantly inhibited tumor growth, consistent with our in vitro findings. And after treatment with Ad-PTEN, the decrease in tumor size was much more in the TE-1 cells than in the Eca-109 cells. Regardless of the underlying mechanism, it is clear that PTEN plays an important role in the process of cell-cycle arrest and inhibition of tumor growth. The ability of Ad-PTEN to inhibit human esophageal tumor growth has not been documented previously.

In conclusion, we confirmed the ability of Ad-PTEN to inhibit esophageal cancer cell growth significantly and to induce apoptosis, possibly by downregulation of Bcl-2 expression. Ad-PTEN infection also altered cell-cycle progression and significantly reduced tumor size in vivo. These results confirm the proposed role of MMAC/PTEN as a regulator of esophageal cancer progression in vivo and in vitro. Additional investigation is needed to assess the potential of MMAC/PTEN as a biological marker and therapeutic target in the treatment of patients with esophageal cancer.

References