Inhibition of APLN suppresses cell proliferation and migration and promotes cell apoptosis in esophageal cancer cells in vitro, through activating PI3K/mTOR signaling pathway

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Esophageal cancer is the sixth leading cause of cancer mortalities globally with a high incidence rate. Apelin (APLN) plays regulatory roles in different organs. However, its role in esophageal cancer remains unknown. Therefore, our study aims to explore the effect of APLN on esophageal cancer. One hundred and eighty-four (184) esophageal tumor tissues samples from patients with esophageal cancer, and 11 esophageal tissues samples from healthy volunteers were analyzed for the expression of APLN. APLN was highly expressed in the tumor of patients with esophageal cancer and esophageal cancer cells. Patients with high expressions of APLN had a lower survival rate than the ones with low to medium expressions of APLN. Human esophageal carcinoma cell lines, TE-1 and ECA-109 cells were transfected with APLN siRNA to knockdown APLN, or transfected with pcDNA-APLN to overexpress APLN. Inhibition of APLN by siRNA-APLN reduced proliferative, migrative, and invasive abilities of esophageal cancer cells and promoted cell apoptosis, which could be all restored by pcDNA-APLN. Moreover, knocking down APLN by siRNA-APLN suppressed the PI3K/mTOR signaling pathway. These findings identify that APLN inhibition might ameliorate esophageal cancer through activating the PI3K/mTOR signaling pathway, thus APLN could be a potential target for esophageal cancer.

Key words: APLN; proliferation; migration; apoptosis; esophageal cancer.
Introduction

Esophageal cancer usually begins in the cells that line the inside of the esophagus and can occur anywhere along the esophagus. While esophageal cancer is the sixth most common cause of cancer deaths globally, its incidence rates vary within different geographic locations. In some regions, usage of tobacco and alcohol or special nutritional habits and obesity contribute to the high rates of esophageal cancer. Therefore, we aimed to explore a new candidate therapeutic drug for esophageal cancer to improve this situation. Apelin, also known as APLN, is one of two endogenous ligands for the G-protein-coupled APJ receptor. Different functions of APLN were identified in various organs, including brain, heart, bone, and muscles. APLN was also reported to promote hepatocellular carcinoma through the PI3K/Akt pathway, and APLN was also suggested as a druggable target. However, its role in esophageal cancer remains unknown. Therefore, our study aimed to explore whether APLN is involved in esophageal cancer.

Materials and Methods

Patients’ samples

One hundred and eighty-four (184) esophageal tumor tissue samples from patients with esophageal cancer, and 11 esophageal tissue samples from healthy volunteers were collected. Specimens were taken directly from the operating room within 15 min of surgery, transported for less than 30 min on dry ice and frozen rapidly and stored at -80°C until used for subsequent gene expression level analysis. All the volunteers were well-informed and signed the consent.

Cell culture

Normal esophageal epithelial cells, human esophageal carcinoma cell line (TE-1) and three different human esophageal squamous carcinoma cell lines (KYSE150, ECA-109, EC9706) were purchased from ATCC (Rockville, MD, USA). These cells were cultured in DMEM (Gibco, Scoresby, Australia) containing with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, Waltham, MA, USA) and passaged using 0.25% Trypsin (Thermo Fisher Scientific).

Gene silencing and overexpression

Cells were seeded into 6-well plates and transfected with scramble siRNA or APLN siRNA (siAPLN) using lipofectamine RNAiMax (Thermo Fisher Scientific) in serum-free Opti-MEM (Gibco). Briefly, siRNA-lipid complexes were prepared in 500 µL Opti-MEM media by mixing 20 µL transfection reagent and 20 µM siRNAs (APLN and negative control) for 5 min at room temperature. The mixtures were added to the cells in 10 mL culture medium. After 6 h of transfection, the medium was replaced with the complete culture media.

After being transfected with siAPLN, cells were transfected with pcDNA APLN plasmid, which is commercially available from GenePharma (Shanghai, China), using lipofectamine 2000 (Thermo Fisher Scientific) to overexpress APLN in cells. Before, cells were cultured in 6-well plate to 40% confluence. For each well, 5 µL pcDNA (20 µM) was added into 250 µL Opti-MEM medium, 5 µL of lipofectamine 2000 into 250 µL Opti-MEM medium, and then mixed pcDNA with lipofectamine 2000. The mixture was added to cells and incubated for 6 h before replacing the medium. Total RNAs and proteins were prepared 48 h after transfection.

The sequence of siRNA was as following: siRNA-APLN 1: F, 5’-AACAUUCUGAUUCGUUGCA-3’; R, 5’-CCAGAAGUCACGAAAGUUAG-3’. siRNA-APLN 2: F, 5’-AGCAAAAGGGGAGAAAGCCC-3’, R, 5’-GCUUUCUCCCUU-UGCUUGA-3’.

qRT-PCR

Total RNA was extracted from tissues and cells using the TRIzol Plus RNA purification system (Thermo Fisher Scientific) per manufacturer’s instructions, and transcribed into cDNA using the high-capacity RNA to cDNA synthesis kit by Applied Biosystems (Foster City, CA, USA). qRT-PCR was performed as previously described and was performed on a QuantiStudio 6 Flex system (Life Technologies, Gaithersburg, MD, USA). The sequence of primers was as following: APLN: F, 5’-ACTTITGGAGACC-TGCCCA-A3’; R, 5’-CACAGAAGGGAAGACTTTCCA-3’.

Cell counting kit-8 (CCK-8)

Treated cells were seeded into 96-well plates at a density of 5,000 cells per well. Plates were frozen after following incubation for the indicated time (24, 48, 73 and 96 h); 10 µL CCK-8 solution was added in each well 2 h before each of the indicated timepoints, and incubated for 2 h. Data was measured at absorbance of 450 nm using a microplate reader.

Colony formation assay

Treated cells were seeded into 24-well plates at a density of 500 cells/well for colony formation. After 14-21 days, 500 µg/mL MTT (3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide; Sigma-Aldrich, St. Louis, MO, USA) was added and cell were counted under microscope. Single clones containing over 100 cells was counted as one colony.

Transwell migration and invasion assay

Digested cells were resuspended with serum-free DMEM and seeded in 8 µm-pore transwell chambers coated with or without Matrigel for cell invasion and migration assay respectively, which were placed on the upper parts of 24-well plates. The lower parts of 24-well plates were filled with DMEM (Gibco) with 10% FBS (Thermo Fisher Scientific). Transwell chambers were removed from 24-well plates after 24 h, fixed in 4% paraformaldehyde (Sigma-Aldrich), stained with crystal violet (Sigma-Aldrich), and viewed under microscope.

To calculate the number of migrated and invaded cells, the non-invasive cells were cleaned by scrubbing with a cotton swab after incubated for indicated time. The cells that adhered to the outside of the membrane were fixed and dyed with crystal violet (Sigma-Aldrich). The number of migrated and invaded cells were quantified by counting in the pictures taken with ImageJ.

Western blot

Protein was extracted from TE-1 and ECA-109 cells using RIPA lysis buffer (Sigma-Aldrich), and protein concentration was determined using BCA protein kit (Boster, Wuhan, China). Then the same amounts of protein were loaded into 4%-15% polyacrylamide gels and transferred onto nitrocellulose (Bio-Rad, Hercules, CA, USA) and Western blot was performed as previously described. Membrane was blocked with blocking solution with protein antibodies related to the PI3K/mTOR signaling pathway including PI3K antibody (1:1,000), PDK1 antibody (1:1,000), mTOR (1:2,000), AKT antibody (1:1,000), EIF4G antibody (1:1,000) or GAPDH antibody (1:1,000). All primary antibodies were identified with appropriate horseradish peroxidase-conjugated secondary antibodies (1:1,000) and signals were visualized with ECL (Enhanced Chemiluminescence, Amersham).

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are commercially available from Abcam (Cambridge, MA, USA). After being washed with TBST 3 times, the membranes were incubated with HRP-conjugated secondary Abs (1:1000, Bio-Rad) for 1 h. Blots were washed again, and target proteins were visualized using the ECL detection system (Bio-Rad).

**Cell apoptosis assay**

Cell apoptosis was performed as previously described.14 Flow cytometric analysis was applied with Annexin V-FITC/PI Apoptosis Detection Kit (KeyGEN Biotech, Nanjing, China) according to the manufacturer’s instructions. Briefly, after relevant treatment or transfection, cells were seeded into 6-well plate with 2 × 10^4 cells per well and incubated in humidified incubator at 37 °C for 6 h. Then, cells in each group were collected, washed with PBS for twice and stained using 5 μl FITC-Annexin V and 5 μL PI solution for 30 min at 37°C in the dark. Followed by washing with PBS for twice, the rate of apoptotic cells in each group was recorded using flow cytometer (Beckman Coulter, Nyon, Switzerland). Data were analyzed using FCS Express software.

**Statistical analysis**

Data was presented as means ± SEM from at least three independent experiments. Data was analyzed using Student’s t-test or one-way ANOVA. Kaplan-Meier statistic allows us to estimate the survival rates; p<0.05 was regarded as significant difference.

**Results**

**APLN was highly expresses in the tumor tissue of patients with esophageal cancer**

We first evaluated APLN expression in esophageal cancer. We collected 184 esophageal tumor tissue samples from patients with esophageal cancer, and 11 esophageal tissue samples from healthy volunteers. Compared to the normal tissue, APLN mRNA was highly expressed in the primary tumor of esophageal cancer (Figure 1A). Then we divided these 184 primary tumors into two groups based on the expression of APLN and followed up with these patients after 3000 days. We found that patients with high expression of APLN had a lower survival rate than the ones with low or medium expression of APLN (Figure 1B). These data suggested that APLN might be involved in the development of esophageal cancer.

**APLN was highly expressed in esophageal cancer cells**

Then we assessed APLN expressions in esophageal cancer cells. Compared to normal esophageal epithelial cells, APLN expressions were significant in three different human esophageal cancer cell lines, including TE-1, KYSE150 and ECA-109 compared with normal esophageal epithelial cell (Figure 2A). Among the three different human esophageal carcinoma cell lines, TE-1 had the highest level of APLN (Figure 2A). We knocked down APLN in TE-1 and another relatively high APLN expressed human esophageal carcinoma cell line ECA-109 using two different APLN siRNA, and both of siRNA-APLN significantly reduced the mRNA levels of APLN in TE-1 and ECA-109 cells (Figure 2B).

**Inhibition of APLN suppressed the proliferation of esophageal cancer cells**

Next, we evaluated the role of APLN on the proliferation of esophageal cancer cells. CCK-8 data showed that APLN siRNA significantly suppressed cell proliferation both in TE-1 and ECA-109 cells from 48 to 96 h in comparison with scramble siRNA (Figure 3 A,B). Furthermore, when compared to scramble siRNA, inhibiting APLN expression by siRNA dramatically reduced colony formation both in TE-1 and ECA-109 cells (Figure 4 A,B). Altogether, these two experiments both revealed that cell proliferation was significantly reduced by inhibiting the expression of APLN in two different esophageal cancer cells, which suggests that inhibition of APLN might suppress the proliferation of esophageal cancer cells.
Inhibition of APLN suppressed the migration of esophageal cancer cells

Next, we assessed the role of APLN on cell migration. Migrated cells were dramatically reduced after knocking down the expression of APLN both in TE-1 and ECA-109 cells (Figure 5 A,B), which suggested that APLN inhibition significantly suppressed the migrative ability of esophageal cancer cells.

Inhibition of APLN reduced the invasion of esophageal cancer cells

We also explored the effect of APLN on cell invasion. Compared to scramble siRNA, APLN siRNA significantly decreased invading cells both in TE-1 and ECA-109 cells (Figure 6 A,B), which suggested that the inhibition of APLN significantly suppressed the invasive ability of esophageal cancer cells.

Inhibition of APLN induced the apoptosis of esophageal cancer cells

Furthermore, the effect of APLN on cell apoptosis was also evaluated. Flow cytometry data observed that cell apoptosis rates both in TE-1 and ECA-109 cells were remarkably promoted by APLN siRNA (Figure 7 A,B), which suggests that knocking down APLN in cells effectively promotes the apoptosis of esophageal cancer cells.

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**Figure 2.** APLN was highly expressed in esophageal cancer cells. A) RT-PCR analyzed the mRNA levels of APLN in three human esophageal carcinoma cells (TE-1, KYSE150, and ECA-109). *p<0.05, compared to esophageal epithelial cells. B) RT-PCR analyzed the efficacy of two APLN siRNAs in TE-1 and ECA-109 cells; *p<0.05 compared to scramble.

**Figure 3.** Inhibition of APLN promoted cell proliferation in esophageal cancer cells. APLN was knocked down in TE-1 and ECA-109 cells using APLN siRNA. Then CCK-8 assay was used to determine cell proliferation in TE-1 (A) and ECA-109 cells (B) at 24, 48, 72, and 96 h; *p<0.05 compared to scramble.

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Figure 4. Inhibition of APLN suppressed colony formation in esophageal cancer cells. APLN was knocked down in TE-1 and ECA-109 cells using APLN siRNA, then cells were seeded in 24-well plates at a density of 500 cells/well. Plates were photographed under microscope (A), and colonies were quantified (B); *p<0.05 compared to scramble.

Figure 5. Inhibition of APLN reduced migrative ability of esophageal cancer cells. TE-1 and ECA-109 cells were transfected with APLN siRNA to knockdown APLN, then seeded in Transwell chambers. After 24 h, migrated cells were stained and photographed under the microscope (A). Migrated cells were counted (B); *p<0.05 compared to scramble.
Overexpression of APLN restored si-APLN-altered cell apoptosis and cell proliferation in esophageal cancer cells

To further explore the role of APLN in esophageal cancer, we also transfected pcDNA-APLN in cells to overexpress APLN. Flow cytometry data demonstrated that cell apoptosis, which was induced by APLN siRNA, was dramatically increased by overexpression of APLN (Figure 8 A,B). Additionally, overexpression of APLN also restored cell proliferation, which was reduced by APLN siRNA (Figure 8C). This data suggests that APLN mediated cell apoptosis and cell proliferation in esophageal cancer.

APLN regulated PI3K/mTOR signaling pathway in esophageal cancer cells

We observed that knocking down APLN in esophageal cancer cells, could significantly decrease the expressions of phosphoinositi3-kinase (PI3K) and mechanistic target of rapamycin (mTOR) signal-related protein, including PI3K, Phosphoinositide-dependent kinase-1 (PDK-1), mTOR, Akt and Eukaryotic translation Initiation Factor 4G (EIF4G). Especially in comparison with scramble siRNA-treated cells (Figure 9 A,B), suggesting that inhibition of APLN in esophageal cancer cells suppressed PI3K/mTOR signaling pathway. Altogether, APLN might regulate cell migration, cell proliferation and cell apoptosis in esophageal cancer cells through PI3K/mTOR signaling pathway.

Discussion

The goal of our study was to explore the effect of APLN in the development of esophageal cancer for the first time. We discovered that patients with esophageal cancer had a higher expression of APLN in tumor tissues than the healthy ones. After that, we also demonstrated that patients with high expression of APLN had a lower survival rate than the ones with low or medium expression of APLN, which suggested that APLN might play an important role in the development of esophageal cancer, and decreasing the expression of APLN might improve survival rates of patients with esophageal cancer. Next, we observed that inhibition of APLN in esophageal cancer cells played a protective role in esophageal cancer cells through suppressing cell proliferation, colony formation, cell migration and invasion as well as promoting cell apoptosis. On the contrary, APLN rescue eliminated the protective effect of APLN inhibition on esophageal cancer cells by promoting cell proliferation and reducing cell apoptosis. Furthermore, inhibition of APLN could suppress PI3K/mTOR signaling pathway in esophageal cancer cells.

APLN is one of two APJ receptors that belong to the G protein-coupled receptor family. APJ and APLN are widely expressed in different organs and play a cytoprotective effect of various organs. APLN was reported to be involved in neurological, metabolic, hypertension, respiratory, gastrointestinal, hepatic, kidney, and cancerous diseases. APLN was identified as a positive protective effect on non-cancerous diseases and might serve as a therapeutic drug in many diseases. But APLN has also appeared as a tumor growth stimulator as well as a marker in the diagnosis of many
Figure 7. Inhibition of APLN increased cell apoptosis of esophageal cancer cells. TE-1 and ECA-109 cells were transfected with APLN siRNA to knockdown APLN, and stained with FITC-Annexin V. Then cells were collected by flow cytometry. Representative images of flow cytometry (A) and quantitative (B) were shown; *p<0.05, compared to scramble.

Figure 8. APLN reduced cell apoptosis and promoted cell proliferation of esophageal cancer cells. TE-1 and ECA-109 cells were transfected with APLN siRNA without or with pcDNA-APLN to knockdown APLN or rescue APLN. Cells were stained with FITC-Annexin V and collected by flow cytometry. Representative images of flow cytometry were shown (A) and quantitative (B). Cell proliferation was shown (C); *p<0.05 compared to scramble.
However, there is no publication about the role of APLN in esophageal cancer. Therefore, our study is the first to demonstrate the stimulatory effect of APLN in esophageal cancer cells.

PI3k/mTOR signaling pathway is the active pathway in the development of many cancers. Previous publications indicated that PI3k/mTOR signaling pathway was involved in the APLN-regulated cancer, therefore our study also evaluated PI3k/mTOR signaling pathway in APLN-regulated esophageal cancer. Our data demonstrated that inhibition of APLN by siRNA-APLN significantly suppressed PI3k/mTOR signal. However, because our study aimed to explore the protective effect of APLN inhibition, we did not explore whether overexpression of APLN in APLN-knockdown cells could activate this signaling pathway suppressed by APLN inhibition.

In summary, our study clearly demonstrated that inhibition of APLN could effectively promote cell apoptosis, and suppress cell proliferation and migration in vitro through the PI3k/mTOR signaling pathway, which suggests that APLN is a potential molecular target for esophageal cancers. It also suggests that APLN inhibition by siRNA or small inhibitors might serve as a therapeutic strategy to treat patients with esophageal cancer.

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