Abstract. Esophageal cancer is the eighth most common malignant tumor in the world and is a common cause of tumor-related death. The development of esophageal cancer is a complex process involving many pathogenic factors, multiple stages, and accumulation of multiple gene mutations and interactions. This study aimed to investigate the effects of Raf kinase inhibitor protein (RKIP) on the proliferation, apoptosis, and invasion of TE-1 esophageal cancer cells. Surgical specimens from esophageal cancer patients were classified into esophageal cancer tissues, tumor-adjacent tissues, and normal esophageal tissues. The tissues were fixed in 4% paraformaldehyde solution for hematoxylin and eosin and immunohistochemical staining. RKIP expression in esophageal tissues was detected by immunohistochemical staining. The esophageal cancer cell line TE-1 was exposed to four different viruses: RKIP-RNAi-AD, NC-RNAi-GFP-AD, RKIP-AD, and GFP-AD. Cell proliferation was detected by MTT assay, and cell apoptosis was detected by flow cytometry. Cell invasion was determined by a Transwell coated with Matrigel. RKIP, phospho-RKIP, Raf-1, phospho-Raf-1, ERK1/2, phospho-ERK1/2, GRK-2, and GAPDH expression was assayed by western blotting. LIN28 and MMP-14 mRNA was assayed by qPCR. The results showed that RKIP expression was reduced in esophageal cancer tissues in comparison with expression in normal esophageal epithelium tissues and tumor-adjacent tissues. Reduced RKIP expression was associated with lymph node or distant metastasis in esophageal cancer. RKIP inhibited the invasive and metastatic abilities of esophageal cancer cell line TE-1 by downregulating mRNA expression of LIN28 and MMP-14. RKIP had no effect on the MAPK signaling pathway in the esophageal cancer cell line TE-1, but was involved in the G protein-coupled signaling pathway. Our findings clearly demonstrate that RKIP inhibits esophageal cancer cell invasion by downregulating the expression of GRK-2, LIN28, and MMP-14.

Introduction

Esophageal cancer is a malignant tumor of the esophageal epithelial tissues, which occurs more frequently in males than in females and is traditionally more prevalent in subjects older than 40 years. However, in recent years there has been an increased tendency for the disease to appear at a younger age (1,2). The pathological classification predominantly includes squamous cell carcinoma and adenocarcinoma, which account for over 95% of all cases. Squamous cell carcinoma is the more common of the two types. It has been estimated that ~80% of all esophageal cancer cases occur in developing countries, with the majority of the cases being squamous cell carcinoma (2). However, the incidence of esophageal adenocarcinoma has been shown to be increasing in Western industrialized countries (3).

The development of esophageal cancer is a complex process involving many pathogenic factors, multiple stages, and accumulation of multiple gene mutations and interactions. These factors cause dysregulation of oncogenes, tumor-suppressor genes, and signaling protein molecules at the molecular level (4). Raf kinase inhibitor protein (RKIP) is a family of small, cysteolic phosphatidylethanolamine-binding proteins originally purified from bovine brain. This protein family is highly conserved and is rarely homologous to other types of proteins. RKIP is widely distributed in many tissues in various
mammals such as mouse, monkey and human. It acts as a signal regulator, which not only inhibits Raf-mediated MAPK and ERK activities, but also inhibits the NF-κB signaling pathway and regulates the activity of G protein-coupled receptors (5-7).

Reduced RKIP expression has been shown to affect cell growth, angiogenesis, apoptosis and gene integrity (8). Our previous study found that RKIP may prevent liver fibrosis, which is a precancerous lesion, through its inhibition of hepatic stellate cell (HSC) proliferation (9). Increased RKIP expression was shown to suppress invasion and reduce metastasis to basilar membranes in mouse models of prostate cancer. It has also been shown to inhibit growth and invasion of ovarian cancer. Reduced RKIP expression is closely associated with progression and prognosis of hepatocellular carcinoma, colorectal cancer, gastric cancer and gastrointestinal stromal tumors (10-21). It has recently been proposed that reduced RKIP expression is also related to progression and pathological staging of esophageal cancer (22-24). However, the underlying mechanisms of RKIP in esophageal cancer cells remain unclear. The present study was, therefore, designed to investigate the mechanisms involved in RKIP esophageal cancer progression.

Materials and methods

Subjects. Surgical specimens from esophageal cancer patients were classified into esophageal cancer tissues, tumor-adjacent tissues (2 cm from the lesion) and normal esophageal tissues (5 cm from the lesion). The specimens were collected from the Department of Thoracic Surgery, The Second Hospital of Hebei Medical University. Patient gender and age, post-surgical pathological staging, lymph node status and distant metastasis were documented. The tissues were either fixed in 4% paraformaldehyde solution for hematoxylin and eosin (H&E) and immunohistochemical staining, or were stored at -80°C for western blotting analysis.

The study was approved by the Ethics Committee of The Second Hospital of Hebei Medical University. Informed consent was obtained from all participants following a detailed description of the purpose and potential benefits of the study.

Immunohistochemical staining. The tissues were cut into paraffin sections (4-μm), dewaxed in xylene, dehydrated and high-pressure hot repaired in citrate buffer. The sections were incubated in 3% hydrogen peroxide and methanol at room temperature, and incubated in 1 μg/200 μl of RKIP polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) [phosphate-buffered saline (PBS) was used in the negative controls] at 4°C overnight. The sections were then incubated in 50 μl of polymer reinforcing agent at 37°C for 20 min, incubated with 50 μl of rabbit-on-mouse HRP-polymer (Santa Cruz Biotechnology, Inc.) at 37°C for 30 min and visualized using 3,3′-diaminobenzidine (DAB) for 3-5 min. Visualization was terminated with tap water. The sections were stained with hematoxylin, with a tan color indicating positive staining. Five fields of view at high magnification (x400) were randomly selected and observed, and the area of positive staining was measured. Image-Pro Plus version 6.0 software was used for analysis. The RKIP staining results were scored by a previously described method (23). The IHC score (0-300) was calculated as the product of the staining intensity (1, weak; 2, moderate or 3, strong expression) and the staining rate (percentage of positive cells in tissues, 0-100%). Tissues with final scores exceeding the median score were determined to have high RKIP expression; tissues equal or below the median were determined as having downregulated RKIP expression. Correlations between RKIP expression and lymph node or distant metastases were investigated.

Cell culture and viral infection. The esophageal cancer cell line TE-1 was purchased from the Cell Resource Center, Shanghai Institutes for Biological Science of the Chinese Academy of Sciences (Shanghai, China). The TE-1 cells were incubated in RPMI-1640 medium containing 10% fetal bovine serum (FBS), 100 μg/ml streptomycin, 100 IU/ml penicillin, 1 mol/l HEPES and 4 mmol/l glucose in 5% CO₂ at 37°C. When 90% single-layer saturation density was achieved, the cells were digested with trypsin containing 0.5% EDTA and passaged at a ratio of 1:2. The cell culture solution was replaced after 24 and 48 h later the cells were re-passaged.

Recombinant adenovirus was reconstructed by Shanghai GeneChem Co. Ltd. (Shanghai, China). The TE-1 cells were exposed to four different viruses: RKIP-RNAi-AD (which carried adeno viral RNAi vector targeting RKIP); NC-RNAi- GFP-AD (a control viral vector for RNAi without a specific target); RKIP-AD (a recombinant adenosine carrying the RKIP gene and expressing GFP); GFP-AD (a control vector RKIP-AD expressing GFP). RPMI-1640 medium without serum and antibiotics was used as a control. The TE-1 cells were seeded onto plates at a 70% saturation density. They were synchronized with RPMI-1640 medium without serum and antibiotics for 24 h prior to treatment. The number of virus particles required was calculated as the cell count x multiplicity of infection (MOI). The cell surface was covered with RPMI-1640 medium without serum and antibiotics, and incubated at 37°C for 2 h, with a further incubation for 46 h after addition of complete medium. GFP expression was observed under an inverted fluorescence microscope (Leica DMI3000 B microscope), and determined by flow cytometry (FCM) to identify the viral infection.

Determination of cell proliferation. TE-1 cells in an exponential growth phase were seeded onto 96-well plates at a concentration of 5x10⁴/ml/200 μl well. The cells were infected with adenovirus as detailed above. Six replicate wells were used for each group, with RPMI-1640 medium containing 2% FBS being used as a negative control. After infection for 24, 48, 72 and 96 h, 20 μl of 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT; Sigma Chemical Co., St. Louis, MO, USA) was added to each well and incubated for 4 h, followed by the addition of 150 μl of dimethyl sulfoxide (DMSO). The absorbance of each well at 492 nm (A₄₉₂ value) was determined using a microplate reader, and the negative control well was adjusted to 0. The results were expressed as the A₄₉₂ value in the experimental group/A₄₉₂ value in the control group x 100%.

Determination of apoptosis. TE-1 cells in an exponential growth phase were seeded onto 6-well plates at a concentration of 5x10⁴/ml. The cells were infected with virus for 48 h, digested with trypsin without EDTA, and re-suspended in PBS.
Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with 80 µg of protein per concentration was determined using the Bradford method. For 10 min at 4˚C. The supernatant was removed, and 100 µl of modified RIPA lysis buffer was added to the sediment. The Transwells were removed, air-dried, and rinsed three times with blocking solution, supplemented with 1 µl of Annexin V-PE, was diluted with blocking solution, and slightly mixed. Apoptosis was determined after incubation for 5 to 15 min at room temperature.

Detection of cell invasiveness. Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) and 24-well Transwells were stored at 4˚C overnight. A total of 50 µl of dissolved Matrigel was evenly coated onto the bottom of the upper chamber of each Transwell. The coated Transwells were incubated at 37˚C for at least 1 h in 5% CO₂. After interference for 48 h, the cells were digested with pancreatin, centrifuged and re-suspended in RPMI-1640 medium containing 1% bovine serum albumin (BSA), adjusting the cell concentration to 5x10⁶/ml. The cell suspension (200 µl) was added to the upper chamber of the Transwell. The coated Transwells were incubated at 37˚C for 48 h in 5% CO₂. The non-invasive cells on the upper chamber were lightly removed with cotton swabs, and the Transwell was inverted, and air-dried. Anhydrous ethanol (600 µl) was added to the 24-well plates, and the membranes were immersed in the well. After 30 min, the Transwells were removed, air-dried, and rinsed three times in PBS for 10 min. The cells were stained with crystal violet, counted and photographed.

Western blot analysis. Frozen tissue samples (100 mg) were rinsed twice in iced PBS, transferred to the ampulla of the homogenate tubes, and cut into pieces. RIPA lysis buffer (1 ml) was added to the tubes, and the tissues were prepared in the homogenate. The TE-1 cells were cultured at a concentration of 1x10⁶/ml, rinsed with iced PBS and centrifuged at 3,000 x g for 10 min at 4˚C, and the supernatant was removed. TRizol (1 ml) was immediately added to the cell sediment and shaken. RNA was extracted following the manufacturer's instructions. In brief, 2 µl of RNA was added to 500 µl of diethylpyrocarbonate (DEPC)-treated water, and the OD of the RNA samples was measured at 280 and 260 nm on an ultraviolet spectrophotometer. RNA was reverse transcribed into cDNA, and quantitative RT-PCR was performed on an ABI StepOne Plus real-time PCR system device. GAPDH was used as an internal reference for RKIP, and RKIP served as an internal reference for phospho-RKIP. Results were expressed as the percentage of the optical density (OD) of the target band in relation to the OD of the internal reference.

Quantitative RT-PCR assay. Approximately 1x10⁷ of the cell suspension was used for quantitative RT-PCR assay, and the ΔCt system was used for quantitative RT-PCR assay, and the ΔCt

Table I. Concentration of the primary antibodies.

<table>
<thead>
<tr>
<th>Primary antibodies</th>
<th>Dilution</th>
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<tbody>
<tr>
<td>RKIP</td>
<td>1:300</td>
</tr>
<tr>
<td>Phospho-RKIP (Ser 153)</td>
<td>1:300</td>
</tr>
<tr>
<td>GRK2</td>
<td>1:300</td>
</tr>
<tr>
<td>Raf-1</td>
<td>1:500</td>
</tr>
<tr>
<td>Phospho-Raf-1 (Ser 338/Tyr 341)</td>
<td>1:600</td>
</tr>
<tr>
<td>ERK1/2</td>
<td>1:2000</td>
</tr>
<tr>
<td>Phospho-ERK1/2 (Thr 202/Tyr 204)</td>
<td>1:2000</td>
</tr>
<tr>
<td>GAPDH</td>
<td>1:1000</td>
</tr>
</tbody>
</table>

RKIP, Raf kinase inhibitor protein; GRK2, G-protein-coupled receptor kinase-2.

Table II. RNA oligonucleotides and qRT-PCR primers.

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer sequence (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>RKIP</td>
<td>AGACCCCAACGACATTTGCTG</td>
</tr>
<tr>
<td>Sens</td>
<td>GCTGATGTCAAGCCCTTCA</td>
</tr>
<tr>
<td>Antisense</td>
<td>GCTGAGATCAAGGCCAATGT</td>
</tr>
<tr>
<td>MMP-14</td>
<td>ATGTAGCGCATGGGCACCTC</td>
</tr>
<tr>
<td>Sens</td>
<td>TCGGACTTTTCGCGGCCAG</td>
</tr>
<tr>
<td>Antisense</td>
<td>GCTGGTTGGACACGGAGCCC</td>
</tr>
<tr>
<td>LIN28</td>
<td>GAACGGGAAGCTACTGGCATGGC</td>
</tr>
<tr>
<td>Sens</td>
<td>TGAGGTCACACACCCTGGCTG</td>
</tr>
</tbody>
</table>

ΔCt
value was calculated. Each reading was carried out in triplicate. The difference in mRNA gene expression was evaluated by relative quantification using the $2^{-\Delta\Delta Ct}$ method. The mRNA expression of the gene in the blank vector control group was defined as 1 (the blank vector served as controls). The $2^{-\Delta\Delta Ct}$ value indicated the relative mRNA expression of the target gene in the RKIP-RNAi-AD or RKIP-AD group.

**Statistical analysis.** Statistical analyses were performed using SPSS version 13.0. Data are expressed as the means ± standard deviations (SD). The Chi-square, Kruskal-Wallis, Spearman, one-way analysis of variance (ANOVA), and Fisher’s least significant difference (LSD) tests were used when appropriate. Values of $P<0.05$ were considered statistically significant.

**Results**

Reduced RKIP expression in esophageal cancer tissues in comparison with normal esophageal epithelium tissues and tumor-adjacent tissues. H&E staining of the specimens from 40 patients with esophageal cancer was able to distinguish the specimens of esophageal squamous cell carcinoma, tumor-adjacent tissues and normal squamous epithelium of the esophagus.

Immunohistochemical staining using the RKIP polyclonal antibody showed high RKIP expression in normal esophageal epithelia and tumor-adjacent tissues with abundant yellowish-brown particle sediments noted in the cytoplasm. RKIP expression was significantly lower in the esophageal squamous cell carcinoma cells with no obvious yellowish-brown particle sediments observed. The frequency of RKIP-positive expression was 70, 55 and 20% in the normal esophageal epithelia, tumor-adjacent tissues and esophageal squamous cell carcinoma cases, respectively (Fig. 1). These results indicate that the RKIP expression was significantly lower in the esophageal cancer tissues than that in the normal esophageal epithelia ($P<0.05$, Chi-square test).

Western blot analysis estimates of RKIP expression in the esophageal squamous cancer tissues, tumor-adjacent tissues and normal esophageal epithelia were 0.32±0.05, 0.56±0.08 and 0.70±0.11, respectively, also indicating significantly reduced RKIP expression in the cancer tissues in comparison with the tumor-adjacent tissues and the esophageal epithelia ($P<0.05$; Kruskal-Wallis test) (Fig. 2).

**RKIP expression is associated with lymph node and distant metastasis of esophageal cancer.** Immunohistochemical staining was performed to measure the mean density of the RKIP-positive staining in the cancer tissues. The mean rank of each parameter was calculated.

No correlation was observed between RKIP expression and gender, age or pathological tumor staging ($P>0.05$). Eleven of the 40 cases were well-differentiated carcinoma,
21 were moderately differentiated carcinoma and 8 were poorly differentiated carcinoma. Multiple independent sample nonparametric testing resulted in mean ranks of 25.18, 19.69 and 16.19, respectively (P>0.05), demonstrating that RKIP expression was not associated with the degree of differentiation of esophageal cancer tissues (Table III). These results indicated low levels of RKIP expression in all 3 categories of tumor differentiation.

Fifteen of the 40 subjects had lymph node or distant metastasis. Independent two sample Mann-Whitney tests provided mean ranks of 11.4 and 15.96, respectively for patients with and without lymph node involvement or metastases (P<0.01), indicating that lower RKIP expression was associated with esophageal cancer metastasis (Table III). These results indicated low levels of RKIP expression in all 3 categories of tumor differentiation.

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**RKIP has no effects on proliferation and apoptosis of the esophageal cancer cell line TE-1.** The adenovirus infection efficiencies in the different groups were determined using flow cytometry. Adenovirus-mediated gene transfection at a MOI of 400 was employed for the subsequent experiments. MTT assay demonstrated that adenoviral infection for 24, 48, 72 and 96 h had no effect on the viability of TE-1 cells (P>0.05) (Fig. 3A).

Flow cytometry revealed that there was no significant difference in the apoptotic rate of TE-1 cells between the RKIP-RNAi-AD (0.83±0.12%) and NC-RNAi-GFP-AD group (0.70±0.10%; P>0.05) (Fig. 3B).

**RKIP inhibits the invasive ability of the esophageal cancer cell line TE-1.** The effect of RKIP expression on the invasive ability of esophageal cancer TE-1 cells was investigated (Fig. 4). The results showed that there were no significant differences in the invasive ability of TE cells among the GFP-AD (80.25±10.87), NC-RNAi-GFP-AD (67.75±13.30) and control (75.50±17.48; P<0.05) groups. However, invasive potential was significantly higher in the RKIP-RNAi-AD group (127.25±16.62) than that in the NC-RNAi-GFP-AD group (67.75±13.30; P<0.05) and the invasive ability was significantly lower in the RKIP-AD group (9.50±7.14) than that in the GFP-AD group (80.25±10.87; P<0.05) (Fig. 4).

**RKIP has no effect on the MAPK signaling pathway in esophageal TE-1 cells, but is involved in the G protein-coupled signaling pathway.** Western blot analysis was performed to determine the expression of RKIP and its phosphorylation (p-RKIP) level in TE-1 cells following treatment with the adenoviruses. The results showed that RKIP expression was significantly higher in the RKIP-AD group (2.31±0.36) than that in the GFP-AD group (0.43±0.09; P<0.05), and was significantly lower in the RKIP-RNAi-AD group (0.83±0.12%) and NC-RNAi-GFP-AD group (0.70±0.10%; P>0.05) (Fig. 3B).

**Table III. Analysis of the clinicopathological parameters and RKIP expression.**

<table>
<thead>
<tr>
<th>Clinicopathological parameters</th>
<th>No.</th>
<th>Mean of rank</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>27</td>
<td>20.15</td>
<td>0.404</td>
</tr>
<tr>
<td>Female</td>
<td>13</td>
<td>21.13</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;60</td>
<td>22</td>
<td>19.97</td>
<td>0.388</td>
</tr>
<tr>
<td>≤60</td>
<td>18</td>
<td>20.93</td>
<td></td>
</tr>
<tr>
<td>Pathological stage</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Stage I-II</td>
<td>16</td>
<td>24.41</td>
<td>0.084</td>
</tr>
<tr>
<td>Above stage II</td>
<td>24</td>
<td>17.90</td>
<td></td>
</tr>
<tr>
<td>Tumor differentiation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Well differentiated</td>
<td>11</td>
<td>25.18</td>
<td>0.228</td>
</tr>
<tr>
<td>Moderately differentiated</td>
<td>21</td>
<td>19.69</td>
<td></td>
</tr>
<tr>
<td>Poorly differentiated</td>
<td>8</td>
<td>16.19</td>
<td></td>
</tr>
<tr>
<td>Lymph node or distant metastasis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Metastasis</td>
<td>15</td>
<td>11.40</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Without metastasis</td>
<td>25</td>
<td>15.96</td>
<td></td>
</tr>
</tbody>
</table>

RKIP, Raf kinase inhibitor protein.
Figure 4. Invasive ability of TE-1 cells at 48 h after recombinant adenoviral infection as detected by the invasion assay (magnification, x200). TE-1 cells were infected with (A) RKIP-RNAi-AD, (B) NC-RNAi-GFP-AD, (C) RKIP-AD and (D) GFP-AD, respectively. (E) TE-1 cells not infected with any vectors were used as the control. (F) The result showed that Raf kinase inhibitor protein (RKIP) inhibited the invasive ability of TE-1 cells. *P<0.05 compared with the NC-RNAi-GFP-AD group. #P<0.05 compared with the GFP-AD group.

Figure 5. Western blot analysis was used to detect Raf kinase inhibitor protein (RKIP), p-RKIP, Raf, p-Raf, ERK, p-ERK, GRK2 and GAPDH protein expression in TE-1 cells at 48 h after adenoviral infection. Lane 1, RKIP-RNAi-AD; lane 2, NC-RNAi-GFP-AD; lane 3, RKIP-AD; lane 4, GFP-AD and lane 5, control group. In the RKIP-AD group, a large amount of RKIP was expressed as an exogenous protein (**P<0.05 compared with the GFP-AD group). At the same time, RKIP expression was decreased in the RKIP-RNAi-AD group (*P<0.05 compared with the NC-RNAi-GFP-AD group). However, there were no differences in p-RKIP expression among the groups (A and B). GRK2 expression was increased in the RKIP-RNAi-AD group and decreased in the RKIP-AD group (##P<0.05 compared with the NC-RNAi-GFP-AD group; #P<0.05 compared with the GFP-AD group). No differences in ERK, p-ERK, Raf and p-Raf were noted among the groups (C and D).
and the mRNA expression of MMP-14, RKIP, LIN28 and GAPDH in the different groups was compared using the relative quantitative method. RKIP mRNA expression was significantly higher in the RKIP-AD group (166.46±0.09) than that in the GFP-AD group (P<0.01), and was significantly lower in the RKIP-RNAi-AD group (0.17±0.09) than that in the NC-RKIP-RNAi-AD group (1.00±0.00; P<0.01). The mRNA expression of MMP-14 was significantly lower in the RKIP-AD group (0.26±0.20) than that in the GFP-AD group (1.00±0.00; P<0.01), and the mRNA expression of MMP-14 was significantly higher in the RKIP-AD group (6.89±0.84) than that in the GFP-AD group (1.00±0.00; P<0.01). In addition, mRNA expression of LIN28 was significantly lower in the RKIP-AD group (0.28±0.06) than that in the GFP-AD group (1.00±0.00; P<0.01), and was increased in the RKIP-RNAi-AD group (1.86±0.12) when compared with that in the NC-RKIP-RNAi-AD group (1.00±0.00; P<0.01) (Fig. 6).

Discussion

Esophageal cancer is the eighth most common malignant tumor in the world and the sixth most common cause of tumor-related death. The incidence of esophageal cancer has continued to increase during the last three decades (1,25). In addition, there is regional variation in the incidence of esophageal cancer with an incidence of ~4-10 cases/100,000 in Western countries compared with an incidence of 50-100 cases/100,000 in China and South Africa (26,29).

RKIP is a regulator protein which is involved in many signaling pathways where it mediates cell proliferation, apoptosis and metastasis in order to regulate cell proliferation balance. The present study demonstrated that RKIP expression was reduced in esophageal cancer tissues in a manner that was independent of gender, age, degree of tumor differentiation and pathological tumor stage. However, we also demonstrated that reduced RKIP expression was associated with an increased occurrence of lymph node and distant metastases, suggesting that it may be closely related with the invasive capacity of esophageal cancer cells. Other studies have also shown that downregulation of RKIP expression is related to regional lymph node metastasis and tumor stage (22-24). The critical role of RKIP in tumor metastasis was first identified in metastatic prostate cancer tissues. It was shown that RKIP expression was lower in metastatic prostate cancer than in primary tumor cells. Based on these findings, blood levels of RKIP are used as a prognostic marker for the malignant potential of prostate cancer. Other studies have shown that RKIP expression is downregulated in metastatic lymph nodes associated with human breast and metastatic colon cancer, further suggesting that downregulation of RKIP expression is involved in tumor metastasis. It has also been shown that overexpression of RKIP is associated with reduced vascular invasion in prostate cancer and melanoma. RKIP has also been reported to inhibit tumor invasion, and significantly reduced RKIP expression has been detected in insulinoma, colorectal carcinoma, hepatocellular carcinoma and ovarian cancer (30-34).

In the present study, an adenovirus overexpressing RKIP and a viral RNAi vector of RKIP was used to interfere with RKIP expression in esophageal cancer cells in order to investigate the role of RKIP in proliferation, apoptosis and invasion...
of esophageal cancer cells. RKIP expression had no effect on the proliferation and apoptosis of esophageal cancer cells. However, in vitro overexpression of RKIP significantly inhibited the invasive capacity of esophageal cancer cells.

Invasion is the most important phenotype of malignant tumors, and it is the major factor that determines the prognosis of malignant tumors. Specific microRNAs (miRNAs), including Let-7, have been reported to play a role in the maintenance of the invasiveness of cancer cells. It has been demonstrated that Let-7 degrades target genes and inhibits their transcription. In this way it functions at both the gene and protein levels to promote tumor metastasis.

Increased RKIP expression has been shown to promote Let-7 expression by inhibiting the MAPK signaling transduction pathway, and thereby preventing cell invasion and metastasis (31,35). When cells are stimulated by extracellular growth factors, activated Ras activates Raf-1 which in turn activates the MAPK signaling pathway. Subsequent activation of ERK1/2 promotes Myc expression and further promotes LIN28 expression. LIN28 inhibits Let-7 expression resulting in elevated expression of its target gene HMGAI, thereby activating genes such as Snail, Twist and Slug which promote cell invasion and metastasis. In the present study, we showed that RKIP overexpression inhibited the invasiveness of esophageal cancer cells and significantly reduced LIN28 transcription, suggesting that RKIP inhibited esophageal cancer cell invasion by downregulating LIN28 expression.

For tumors to develop, the normal cellular homeostasis of the microenvironment is destroyed and is replaced by one that facilitates tumor cell growth. The presence of inflammatory cytokines in the tumor microenvironment also plays a critical role in tumor invasion (36-39). Thus, maintaining the tumor microenvironment is a prerequisite for protecting or promoting the occurrence and development of tumors.

It has been shown that RKIP prevents the invasion of cancer cells by controlling the expression of matrix metalloproteinases (MMPs), particularly MMP-1 and MMP-2 (40). Silencing RKIP expression results in a highly invasive phenotype of the cancer cells with dramatically elevated levels of MMP-1 and MMP-2 expression, whereas overexpression of RKIP decreases cancer cell invasion in vitro and reduces MMP-1 and MMP-2 expression. In accordance with this finding, MMP-1 or MMP-2 in RKIP-knockout cells reverts their invasiveness and metastatic potential to normal levels (40). In the present study, we demonstrated that overexpression of RKIP significantly inhibited MMP-14 expression, indicating that it reduced the invasiveness of esophageal TE-1 cancer cells by downregulating MMP-14 expression. RKIP is a natural suppressor of Raf, which inhibits Raf phosphorylation, thereby inhibiting the MAPK signaling transduction pathway. It has been found that RKIP is highly expressed in Merkel cell carcinoma cells, but the MAPK signaling transduction pathway is not activated. These results indicate that RKIP does not affect the ERK/MAPK signaling transduction pathway or proliferation or apoptosis in Merkel cells.

In our studies with esophageal TE-1 cancer cells, overexpression or low expression of RKIP for 24 h resulted in stabilization of the MAPK signaling pathway, and either inhibition or activation of the G protein-coupled signaling pathway. G protein is a transmembrane protein, which acts as a second messenger, converting extracellular information to intracellular information. G-protein-coupled receptor kinase-2 (GRK-2) is therefore a negative feedback inhibitory protein for G protein-coupled receptors (GPCRs). GRK-2 has been shown to phosphorylate GPCRs, and separate G protein from GPCRs.

Protein kinase C (PKC) has been shown to phosphorylate RKIP at serine 153. Phosphorylated RKIP dissociates from Raf-1, binds to GRK-2 and inhibits its activity (6). The binding conversion from Raf-1 to GRK-2 suggests that RKIP serves as a signal regulator. In other words, after PKC is activated, RKIP is phosphorylated, becomes dissociated from Raf-1, and thereby prevents the inhibition of the ERK signaling pathway and associated increases in cell permeability. In this way it facilitates the binding of RKIP to GRK-2, resulting in activation of the G protein-coupled signaling pathway. It can, therefore, be hypothesized that RKIP acts as a key that switches from one signaling pathway to another, depending on its degree of phosphorylation.

In conclusion, our findings clearly demonstrate that reduced RKIP expression is related to the development of lymph nodes or distant metastasis associated with esophageal cancer. We also provide evidence to suggest that RKIP inhibits esophageal cancer cell invasion by downregulating the expression of GRK-2, LIN28 and MMP-14.

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