Original Article
IKKα/IKKβ/NFκB/SURVIVIN expression regulated by E2F1 in esophageal cancer cell line ECA109

Hongbo Lv*, Huiwu Li*, Xiaohong Sun, Zuoliang Pang

Department of Thoracic Surgery, The Affiliated Tumor Hospital of Xinjiang Medical University, Urumqi, Xinjiang, China. * Equal contributors.

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Abstract: Background: To investigate the regulation of SURVIVIN by E2F1 through the classical NFκB activation pathway and to elucidate the effects of E2F1 on the biological functions of esophageal cancer cells. Materials and methods: An E2F1-overexpression plasmid, pGV142-E2F1, was constructed and transfected into the esophageal cancer cell line ECA109 by electroporation. Transfection efficiency was determined by observing the intensity of green fluorescent protein (GFP) under an inverted fluorescence microscope. Cell cycle and apoptosis of transfected cells were measured by flow cytometry. Total RNA and protein were extracted from transfected cells to determine changes in transcript and protein levels, respectively, of E2F1, IKKα, IKKβ, NFκB, and SURVIVIN. Results: GFP expression in ECA109 cells transfected with the E2F1 overexpression plasmid showed a needle-like shape. The proportion of pGV142-E2F1-transfected ECA109 cells in S-phase was elevated compared to the number of S-phase cells in the empty plasmid and negative control groups (P < 0.05). The proportion of early apoptotic cells following pGV142-E2F1 transfection was significantly reduced compared to that with the empty plasmid and negative control groups (P < 0.05). Expression of E2F1 mRNA and protein were markedly elevated 48 h post-transfection (P < 0.05). Compared to the negative control group, expression levels of NFκB and SURVIVIN mRNA and proteins were significantly increased with overexpression of E2F1. The expression level of IKKα was markedly reduced, and IKKβ mRNA and protein levels were markedly increased on E2F1 overexpression compared to the negative control (P < 0.05). Conclusion: E2F1 and SURVIVIN share a regulatory relationship in ECA109 cells. E2F1 overexpression has a cis-effect on the regulation of IKKβ, NFκB and SURVIVIN, but has a trans-effect on IKKα regulation. Regulation of the expression of SURVIVIN by E2F1 in ECA109 cells may be conducted through the activation of IKKβ, which subsequently activates the classical NFκB activation pathway, leading to the transcriptional activation of SURVIVIN.

Keywords: Esophageal cancer cells, NFκB activation pathway, SURVIVIN, E2F1

Introduction

Esophageal cancer is a common malignant gastrointestinal cancer that results in approximately 300,000 deaths each year worldwide. Currently, SURVIVIN is known to be one of the strongest apoptosis inhibitors that are highly expressed in malignant tissues. This protein is expressed in about 80% of the esophageal cancer tissues and plays an important anti-apoptotic role in tumor cells by promoting cell mitosis and proliferation, and inhibiting apoptosis. In addition, SURVIVIN plays a vital role in the development, invasion, and metastasis of esophageal cancer cells. The IKKα/IKKβ/NFκB signaling pathway is one of the factors that regulate cell apoptosis in concert with the upregulation of SURVIVIN. E2F1 is an important member of the cell cycle-related transcription factor E2F family and it plays a key role in the regulation of cell proliferation and apoptosis [1, 2]. A number of studies have shown that E2F1 is involved in the regulation of apoptosis in various tumor cells [3, 4]. It has been reported that overexpression of E2F1 was found on 59.8% of esophageal cancers, and an increase in expression was associated with post-operation tumor progression, lymph node metastasis, and poor prognosis [5]. Overexpression of E2F1 mRNA was also found in 40% of gastric cancers and 60% of colorectal cancers, also associated with poor prognosis, demonstrating that E2F1 plays a unique role in the development of gastrointestinal cancer. To understand the involvement of
E2F1 in cancer, we have constructed the E2F1 overexpression plasmid pGV142-E2F1 and transfected it into ECA109 cells via electroporation. Real-time PCR and Western blot were used to detect changes in the transcription and protein levels of E2F1, IKKα, IKKβ, NFκB, and SURVIVIN in order to determine whether they share a regulatory relationship in esophageal squamous cell carcinoma.

**Materials and methods**

**Object of study**

Esophageal cancer cell line ECA109 was provided by the Research Center of Xinjiang Medical University and the cells were used in our laboratory following recovery.

**Electroporation**

One day prior to electroporation, cells were passaged to an appropriate density in order to be in the logarithmic growth phase during transfection. Cells were collected by 0.25% trypsin digestion, transferred into a 15 mL tube for centrifugation at 1000 rpm for 5 min, and supernatant was discarded. Cells were washed twice with serum-free opti-MEM medium and supernatant was discarded. Plasmid (10 µg) was added into 400 µL electroporation buffer and mixed well. The optimal cell concentration was between 2×10^6 and 2×10^7 cells/mL. Cell pellets were fully re-suspended in 400 µL electroporation buffer containing the plasmid, and then the mixture was placed in a 0.4-cm electroporation cuvette. The cuvette was electroporated with a 200 V pulse for 15 ms using square waves to obtain optimal results. The cuvette was immediately removed; mixture was transferred to a culture flask containing complete culture medium and cultured in an incubator. Since electroporation causes relatively high damage to cells, cell medium was changed 6 h following electroporation and fluorescence was observed and photographed using an inverted fluorescence microscope at 48 h. Total RNA was then extracted.

**Extraction and detection of total RNA concentration**

Trizol (0.5 mL) was added (or pre-added) to the cells, vigorously mixed for 15 s and incubated at room temperature for 5 min. 100 µL was added, vigorously mixed for 15 s and placed at room temperature for 3 min. The mixture was centrifuged at 4°C, 12000 rpm for 15 min. The upper colorless aqueous phase containing RNA was transferred to a centrifugation tube to avoid drawing proteins in the interface. If extraction was incomplete, extraction was repeated by adding 200 µL chloroform. Isopropyl alcohol (800 µL) was added to the aqueous phase, mixed well, placed at room temperature for 10 min (cell RNA placed at -20°C for 20 min), and centrifuged at 4°C, 12000 rpm for 10 min to pellet RNA. Supernatant was carefully decanted and EP tube was inverted on an RNase Free absorbent paper for 2-3 min to remove residual alcohol. RNA pellet was washed by adding 0.7 mL anhydrous ethanol and 200 µL DEPC-treated water, and centrifuged at 4°C, 8000 rpm for 5 min. After removing the supernatant, EP tube was inverted to dry on RNase Free absorbent paper for 3-5 min at room temperature. DEPC-treated water (20 µL; RNase Free water) was added to dissolve the dried pellet at 50°C for 10 min and the solution was stored at -80°C. DEPC-treated water was used as blank. RNA sample was diluted in DEPC-treated water (e.g. 1:20 dilution) and mixed well. OD260, OD280 and OD260/OD280

**Table 1. Primer sequence of each gene**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (5’–3’)</th>
<th>Annealing temperature (°C)</th>
<th>Fragment length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E2F1</td>
<td>F: 5-CCCAACTCCCTACCCCTT-3 R: 5-CTCCCACTCTCATCCACCT-3</td>
<td>54</td>
<td>271</td>
</tr>
<tr>
<td>IKKα</td>
<td>F: 5-CATTGGCTTCAGCTTGGT-3 R: 5-ATAGAATTGGTAGGACTTT-3</td>
<td>60</td>
<td>197</td>
</tr>
<tr>
<td>IKKβ</td>
<td>F: 5-GTTCCTTGGGTGTGGA-3 R: 5-TGCTTCCGGGTTGTTATTT-3</td>
<td>60</td>
<td>245</td>
</tr>
<tr>
<td>NFκB-P65</td>
<td>F: 5-TCACCCAGGGTTTGAGGG-3 R: 5-AAAGCACTGGAAAGGAGG-3</td>
<td>60</td>
<td>168</td>
</tr>
<tr>
<td>SURVIVIN</td>
<td>F: 5-CCCTGCTGGCAGCGCTTC-3 R: 5-CTGGCTCCAGCGCTTC-3</td>
<td>60</td>
<td>188</td>
</tr>
<tr>
<td>GAPDH</td>
<td>F: 5-GGGAACCTGCGCCGGTAT-3 R: 5-AAAGTTGAGGAGATGTT-3</td>
<td>60</td>
<td>309</td>
</tr>
</tbody>
</table>
values of the diluted sample were determined. After each sample was measured, the cuvette was washed twice with DEPC-treated water before measuring the next sample. Real-time PCR analysis showed in Table 1.

**Western blot analysis**

Western blot analyses were performed. Briefly, proteins were extracted from cells, and their concentrations were determined using a protein assay. Equal amounts of protein were separated using 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Resolved proteins were transferred onto polyvinylidene fluoride (PVDF) membranes, which were incubated with primary antibodies (1:1000), followed by incubation with HRP-linked secondary antibodies (1:2000). The blots were developed using Immobilon Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA, USA).

**Cell cycle detection by flow cytometry**

Cells were digested by 0.25% trypsin and collected through centrifugation. After removal of supernatant, cells were washed twice by pre-chilled PBS. Pre-chilled 70% ethanol was added to fix cells at 4°C overnight or -20°C for 1 h. Cell staining: cells were centrifuged at 3000 rpm and washed once with 1 mL PBS. PBS (500 µL) containing 50 µg/mL ethidium bromide (PI), 100 µg/mL RNase A, and 0.2% Triton X-100 was added to the cells and the mixture was incubated at 4°C for 30 min in the dark. A Beckman Coulter flow cytometer was used to determine cell cycle distribution.

**Statistical analysis**

The SPSS 16.0 software was used for data analysis. T-test for two independent samples and ANOVA were used to analyze data from two groups and multiple groups respectively. \( P < \)
Results

Observation of transfection efficiency using inverted fluorescent microscope

As shown in Figure 1, GFP expression was needle-like in ECA109 cells transfected with the

**Table 3.** Apoptosis of ECA109 at 48 h post-transfection

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>ECA109 early apoptotic rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>3</td>
<td>31.46±2.73</td>
</tr>
<tr>
<td>Empty plasmid</td>
<td>3</td>
<td>31.70±5.56</td>
</tr>
<tr>
<td>E2F1 overexpression</td>
<td>3</td>
<td>16.50±3.21</td>
</tr>
<tr>
<td><strong>F value</strong></td>
<td></td>
<td>24.22</td>
</tr>
<tr>
<td><strong>P</strong></td>
<td></td>
<td>0.000</td>
</tr>
</tbody>
</table>
E2F1 overexpression plasmid. As a transcription factor, E2F1 enters the nucleus following its synthesis and this leads to the appearance of needle-like fluorescence.

Cell cycle changes in transfected cells

Changes in cell cycle of the transfected cells were determined by flow cytometry. As shown in Table 2 and Figure 2, the proportion of pGV142-E2F1 transfected ECA109 cells in S-phase was elevated (35.66±2.99%), which was significantly different ($P < 0.05$) compared to the number of S-phase cells in the empty plasmid group (28.86±1.80%) and the negative control group (29.83±1.84%). No significant difference was detected between the empty plasmid and negative control group.

Cell apoptosis changes in transfected cells

Table 3 and Figure 3 shows that the proportion of early apoptotic ECA109 cells was significantly reduced ($P < 0.05$) in the pGV142-E2F1 transfected group (16.50±3.21%) compared to those in the empty plasmid (31.70±5.56%) and negative control groups (31.46±2.73%). No significant difference was found between the empty plasmid and negative control group ($P > 0.05$).
Changes in E2F1, IKKα, IKKβ, NFκB, and SURVIVIN gene expression 48 h after E2F1 overexpression plasmid transfection

As shown in Table 4, E2F1 mRNA was significantly elevated ($P < 0.05$) at 48 h following pGV142-E2F1 transfection of ECA109 cells, whereas there was no significant difference in mRNA level between the empty plasmid and negative control groups ($P > 0.05$). Expression levels of NFκB mRNA and SURVIVIN mRNA increased with the overexpression of E2F1 mRNA, and this result was significantly different than the expression pattern in the negative control ($P < 0.05$). In contrast, expression levels of the IKKα and IKKβ mRNAs were markedly reduced compared to the negative control ($P < 0.05$).

In order to study the changes in E2F1, IKKα, IKKβ, NFκB and SURVIVIN protein levels following pGV142-E2F1 transfection, we collected the proteins from ECA109 cells 48 h after transfection and performed a Western blot to detect changes in protein levels of each gene. Using the image lab 4.1 software, the relative expression of the target protein was determined as the ratio between the grey values of the target protein and the reference protein. Figure 4 and Table 5 demonstrate that the level of E2F1 protein was significantly increased in pGV142-E2F1 transfected ECA109 cells compared to the negative control ($P < 0.05$). There was no significant difference in protein levels between the negative control and the empty plasmid group ($P > 0.05$). The increase in IKKβ, NFκB, and SURVIVIN protein expression following overexpression of E2F1 was statistically significant ($P < 0.05$). While the IKKα protein expression was decreased, and there was statistically significant ($P < 0.05$) compared with the negative control group.

Discussion

In this study, the changes of cell cycle were detected by flow cytometry. Our results revealed that the proportion of ECA109 cells in S-phase following pGV142-E12F1 transfection was elevated (35.66±2.99%), indicating that E2F1 has a role in promoting the G1/S-phase transition and cell proliferation in ECA109 cells. E2F1 promotes cell cycle progression in esophageal squamous cell carcinoma cells, which was consistent with the findings of Blais A et al. [6]. Since E2F1 is involved in cell cycle progression in various tumor cells, many researchers have investigated in E2F1 as a potential therapeutic target [7-11].

Detection of cell apoptosis by flow cytometry showed that the proportion of early apoptotic ECA109 cells following pGV142-E12F1 was significantly reduced, suggesting that E2F1 sup-

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**Table 4.** Relative expression of 5 genes in pGV142-E2F1 transfected ECA109 cells after 24 hours and 48 hours

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Relative expression of gene mRNA (mean ± SD)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>E2F1</td>
</tr>
<tr>
<td>Negative control</td>
<td>3</td>
<td>1.00±0.00</td>
</tr>
<tr>
<td>Empty plasmid</td>
<td>3</td>
<td>0.95±0.03</td>
</tr>
<tr>
<td>24 h after GV142-E2F1 transfection</td>
<td>3</td>
<td>1.32±0.11</td>
</tr>
<tr>
<td>48 h after GV142-E2F1 transfection</td>
<td>3</td>
<td>1.79±0.18</td>
</tr>
<tr>
<td>F value</td>
<td></td>
<td>41.59</td>
</tr>
<tr>
<td>P</td>
<td></td>
<td>0.000</td>
</tr>
</tbody>
</table>
presses ECA109 cell apoptosis. Comprehensive analysis of our experimental results demonstrated that the biological functions of the transcription factor E2F1 in ECA109 cells include promoting cell G1/S-phase transition, promoting cell proliferation, and inhibiting cell apoptosis, which were consistent with the report of Johnson DG et al. [12]. At present, the SURVIVIN gene is known to be the strongest inhibitor of apoptosis and is associated with cell cycle regulation, angiogenesis, tumor invasion and metastasis, drug intolerance, and radiotherapy resistance. The expression of SURVIVIN is strictly cell cycle-dependent, and is G2/M-phase-specific [13-17]. E2F1 has an important role in inhibiting cell apoptosis in various tumors [18-23]. The biological function of E2F1 in suppressing apoptosis is similar to that of the apoptosis inhibitor SURVIVIN and the two proteins are similarly expressed during the regulation of gene expression.

The transcription factor E2F1 has an important role in promoting the G1/S-phase transition and cell proliferation, and the SURVIVIN gene is vital in promoting cell mitosis and proliferation, and inhibiting cell apoptosis. They both play an important role in promoting the cell cycle. When studying the participation of E2F in tumor cell proliferation, Araki et al. found that the IKKα/IKKβ/NFκB signaling pathway could directly inhibit the transcription of E2F1, E2F2, and E2F3 [24]. James Shaw et al. showed that NFκB can inhibit apoptosis by antagonizing the expression of the apoptosis-related target genes of E2F1 [25]. Results from our study demonstrated that E2F1 and SURVIVIN are cis-expressed. Thereby, we can speculate that E2F1 plays a role in suppressing tumor genes in esophageal squamous cell carcinoma and is involved in regulating the expression of SURVIVIN. Although SURVIVIN is currently the strongest apoptosis inhibitor, its regulatory mechanism in tumor cells is still being investigated. The use of SURVIVIN as a target for drug therapy is currently being developed and has already entered phase II clinical trials. We have found a relationship in gene expression regulation between E2F1 and SURVIVIN, and this result will provide new ideas for further studies of the molecular mechanism of the highly sustained expression of SURVIVIN in tumor tissues.

E2F1 is oncogenic under certain conditions. A study by Jiang et al. [26] showed that the human and murine SURVIVIN promoter contains CDE/CHR (cell cycle gene homology region) elements and E2F1 binding sites. Using ChIP (chromosome immunoprecipitation) technology, they found that E2F1, E2F2, and E2F3 could directly bind to the promoter of SURVIVIN to promote its transcription, and this effect could be abrogated by mutating the E2F1 binding site on the SURVIVIN gene. In this study, we have constructed a transcription factor E2F1-overexpression plasmid into ECA109 cells. After transfecting ECA109 cells with the E2F1-overexpression plasmid by electroporation, the transcript and protein expression levels of E2F1 were elevated, accompanied by an increase in the transcript and protein expression levels of NFκB and SURVIVIN. The expression of IKKα transcripts and proteins was reduced, which was opposite of the expected regulation of E2F1 expression. Our results suggested that E2F1 and SURVIVIN share a regulatory relationship in ECA109 cells. While E2F1 expression has a cis-effect on the regulation of IKKβ, NFκB and SURVIVIN, it has a trans-effect on IKKα regulation. Therefore, we speculate that the regulation of the expression of SURVIVIN by E2F1 in ECA109 cells may be conducted through the activation of IKKβ, which then activates the classical NFκB activation pathway, leading to the transcriptional activation of SURVIVIN.
Acknowledgements

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Disclosure of conflict of interest

None.

Address correspondence to: Zuoliang Pang, Department of Thoracic Surgery, The Affiliated Tumor Hospital of Xinjiang Medical University, Urumqi 830011, Xinjiang, China. Tel: +86 15022979845; E-mail: 454726435@qq.com

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