Influence of smoothened siRNA on human esophageal cancer cell line EC9706 proliferation and apoptosis

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Abstract: Apply siRNA technology to take Smoothened (Smo) genetic locus, the possibility of Hh signaling pathway, and also inhibits esophageal squamous carcinoma cell proliferation and induction of apoptosis. Utilizing Smo siRNA transfected esophageal squamous carcinoma EC9706 cells, adopting RT-PCR and Western blot technology to detect Smo, Gli1 mRNA and protein expression in each group of cells, employing MTT and FCM to detect the influence of Smo siRNA on cell proliferation and apoptosis. Compared with each control group, after transfected Smo siRNA transfected cell for 24h, 48h and 72h, Smo siRNA and Gli1 mRNA expression was evenly reduced obviously, Smo and Gli1 protein expression level was also reduced clearly. Smo siRNA could inhibit cell proliferation, and even promote cell apoptosis. Smo gene played a role in regulating and controlling esophageal squamous carcinoma cell proliferation and apoptosis and Smo was likely to be an effective target spot for tumor gene therapy.

Keywords: esophageal cancer; Smo gene; cell Proliferation; cell Apoptosis; RNA interference

INTRODUCTION

Hedgehog (Hh) is a highly conservative signaling pathway. It plays an important role in human embryonic development, organ formation and tissue differentiation, and also in tissue repair. Smo protein is a membrane protein on Hh signaling pathway and also a converter of Hh signaling pathway, which can convert extra cellular Hh signal into intracellular glihomolog (Gli) signal, triggers intracellular signal to download, activate transcription factor Gli1, and have mobilizing function on Sonic hedgehog signaling pathway (Raju, 2011). Numerous studies have found, transduction pathway where Hh signal mediated by human Smo is activated unusually, this is closely related to the occurrence and the development of many malignant tumors, for instance melanoma, basal cell carcinoma, pancreatic cancer, nasopharyngeal carcinoma, gastric cancer, prostate cancer, breast cancer, colorectal cancer etc (Tao et al., 2011; Agyeman, Ding et al., 2012; Atwood, Li and Wang et al., 2013). Researches on the relations between Smo, Gli1 and tumors have become hot issues in recent years (Robbins et al., 2012), but rare literature reports are on the role of Gli1 gene expression in the generating process of esophageal squamous carcinoma and in the abnormal activation of Hh signaling pathway. This study intends to make use of Smo siRNA transfected esophageal squamous carcinoma EC9706 cells, and further to explore the influence of Smo gene on esophageal cancer cell proliferation and apoptosis, in add to provide theoretical basis for gene therapy of human esophageal carcinoma.

MATERIALS AND METHODS

Materials

Cell lines

Human esophageal squamous carcinoma EC9706 Cell Line was presented as a gift by state key Laboratory of Molecular Oncology in Cancer Research of Chinese Academy of Medical Sciences.

Main reagents

Transfected reagent Lipofectamine™ 2000 and TRN zol reagent were purchased from Invitrogen Company. Go Taq® Green Master Mix was purchased from Takara Biotechnology (Dalian) Co., Ltd. Revert Aid TM First Stranded cDNA Synthesis Kit purchased from Fermentas company. Smo, Gli1 and β-actin primers synthesized by Shanghai Sangon Biotech Co., Ltd. PVDF membrane purchased from Peking Suo Lai Bao Science and Technology Co., Ltd. Prestained protein marker, Smo and Gli1 Primary Antibody was purchased from American Santa Cruz Company. β-actin Primary Antibody, Smo and Gli1 Secondary Antibody purchased from Beijing Biosynthesis Biotechnology Co. Ltd. MTT powder purchased from Beijing Comwin Biotech Co., Ltd. Dimethyl sulfoxide (DMSO) purchased from American Santa Cruz Company. Samoa siRNA kit (including 3 different kinds as Smo siRNA, negative control siRNA, negative control FAM - siRNA) purchased from the Shanghai Genepharma company.

Methods

Cell culture and transfection

EC9706 cell added complete culture solution, and put in 37°C, 5% CO₂ incubator for culture. Selected the cells in the good growth before being transfected, inoculated in 6
orifices was cell density of $4.5 \times 10^5$, prepared for the transaction until cell coverage reached 30%–50%. Added 10µl siRNA in 250µl culture solution; added 5µL Lipofectamine™ 2000 in another 250µl culture solution, after 5 min when incubated at room temperature, mixed these two things gently and evenly, and then after further 20 min when incubated at room temperature, added the mixture liquor droplets in 6-well plates, and mixed them evenly. Continued to culture in the incubator, and renewed complete culture solution after 5h.

**Screening Smo siRNA transfected density**

Used FAM-siRNA transfected human esophageal cancer cell EC9706 in three densities of 33 nm, 66 nm and 99 nm, and after transfection 24h, observed under the fluorescence microscope, the transfection efficiency in 99nM is the highest (fig. 1), screening 99 nm is experimental density for the follow-up study.

**Experiment groups**

Six groups: Smo siRNA-1 group, Smo siRNA-2 group, Smo siRNA-3 group, irrelevant sequence group, transfected reagent group and cell control group. Screened out sequence which inhibited efficiency was the highest for the follow-up experiment. Follow-up experiment was still divided into four groups: Smo siRNA-3 group, irrelevant sequence group, transfected reagent group and cell control group, and three groups are as well in different transfected time points: 24 h group, 48 h group and 72 h group.

**MTT assay detects cell growth inhibition ratio**

Vaccinated cells was in 96-well plates, the cell density is $(3~6) \times 10^3$/well, set up control well and apoptosis well; MTT assay detects cell growth inhibition ratio. Cell growth inhibition ratio= ($X_{\text{treatment group} }- X_{\text{control group}}$)/$X_{\text{control group}} \times 100%$.

**Flow cytometry detects cell apoptosis**

Collected transfected cells of each group, washed thricers by PBS, resuspended cells in 100µl 1×binding Buffer, added 5µl FITC Annexin V and 5µl PI was incubated for 15min without light and then added 400µl 1×binding Buffer and mixed evenly, detected the proportion of apoptotic cells in each group on flow cytometry.

**RT-PCR detects Smo, Gli1 mRNA expression**

Extracted total RNA in each group and each time point after transfected by TRNzol, measured OD value by UV spectrophotometer, all the OD260/OD280 values were between 1.9–2.1. Synthesized cDNA in the instructions on cDNA kit with reverse transcription. Smo upstream primer sequence was 5’-CGCTACCTCGTCTTATCT-CT-3’, the downstream primer sequence was 5’-CAGGT-GGAATGAGGAGTCTTG-3’; Gli1 upstream primer sequence was 5’- TGGGAGAAGCGGAGGCTATC-3’, the downstream primer sequence was 5’- GAGTA-CAGAGTTGGAGGTAAGG-3’; β-actin upstream primer sequence was 5’-CTCTAAGAAGCTTTGCGGTG- G-3’, the downstream primer sequence was 5’-GAGCTA-CGAGCTGCTGCTGAG-3’. The Conducted RT- PCR amplification. The reaction conditions of Smo/Gli1/β-actin were as the follow: pre modified for 5 min at 95°C; modified for 30 s at 95°C, and annealed for 45 s at 63°C /68°C /67°C, extended for 45 s at 72°C, 30 cycles totally; eventually extended for 5 min at 72°C, the sizes of Smo/Gli1/β-actin amplification products were 306 bp/201 bp/416 bp. RT-PCR products were taken through 2% agarose gel electrophoresis, imaged by Alpha multifunctional gel-imaging system, analyzed the imaging by Alpha View software, expressed the relative transcript level of Smo and Gli1 mRNA by the comparison of the absorbance values between electrophoresis bands of Smo and β-actin and between electrophoresis bands of Gli1 and β-actin.

**Western blot detects Smo and Gli1 protein expression for cells of each group**

Collected respectively transfected cells of each group and compared to non-transfected cells of each group, obtained the total protein of the samples in each group by splitting, and furthermore detected protein density, separated by 10% SDS-PAGE gel electrophoresis, transferred onto PVDF membrane after protein electrophoresis, diluted First Resistance by TBST, the dilution rate of Smo and Gli1 was 1: 300 and for β-actin was 1:1000, incubated overnight in the refrigerator at 4°C, incubated for 1h at room temperature when TBST diluted Second Resistance (1: 5000), took the semi-quantitative analysis by using Alpha multi-function imaging system and photographic system in internal reference to β-actin. Repeated the experiment for 5 times.

**Statistical analysis by using SPSS 17.0 statistical software**

Measurement data for which the results conformed to normal distribution adopted mean ± standard deviation (x ±s), the comparison of the means in many sets used by one-way analysis of variance (ANVOA), for the measurement data with significant differences, compared the means in multiple sets pair wise by using LSD-t test, test level adopted $α=0.05$.

**RESULTS**

**Screening the best segments of Smo siRNA specificity**

Smo and Gli1 mRNA expression had no effect in the cells of each control group (EC9706 cell control group,
transfected reagent group and irrelative sequence group), and also pair wise differences had no statistical significance between each group (P>0.05). Compared with each control group, Smo and Gli1 mRNA levels of three chemosynthetic Smo siRNA transfected cells were equally lower than those of each control group (P<0.05), of which the inhibition efficiency of Smo siRNA-3 was the highest (P<0.05), took the subsequent experiments after screening Smo siRNA-3 sequence, as shown in fig. 1, fig. 2.

**Fig. 1**: Influence of siRNA on EC9706 cell Smo mRNA expression in each group

M: DNA Maker; 1: cell control group; 2: transfected reagent group; 3: Irrelevant sequence group; 4: Smo siRNA-1 group; 5: Smo siRNA-2 group; 6: Smo siRNA-3 group

**Fig. 2**: Influence of siRNA on EC9706 cell Gli1 mRNA expression in each group

M: DNA Marker; 1: cell control group; 2: transfected reagent group; 3: Irrelevant sequence group; 4: Smo siRNA-1 group; 5: Smo siRNA-2 group; 6: Smo siRNA-3 group

Influence of Smo siRNA-3 on Smo and Gli1 mRNA expression in EC9706 cells

Compared with the cells of control group, Smo and Gli1 mRNA expression quantities after Smo siRNA-3 transfected for 24h group, 48h group and 72h group were evenly reduced, of which transfected 72h group was reduced the most prominently, and also pair wise differences had statistical significance between each group (P<0.05). Shown as table 1, fig. 3, fig. 4 and fig. 5.

**Fig. 4**: Influence of Smo siRNA on Smo mRNA expression in esophageal cancer EC9706 cells

M: DNA Maker; 1: cell control group; 2: After transfected Smo siRNA-3 for 24h group; 3: after transfected Smo siRNA-3 for 48h group; 4: after transfected Smo siRNA-3 for 72h group

**Fig. 5**: Influence of Smo siRNA on Gli1 mRNA expression in esophageal cancer EC9706 cells

M: DNA Marker; 1: cell control group; 2: Transfected reagent group; 3: Irrelevant sequence group; 4: Transfected Smo siRNA-3 group

**Fig. 6**: Influence of Smo siRNA-3 on Smo and Gli1 protein expression in esophageal cancer EC9706 cells

**Fig. 3**: Influence of Smo siRNA-3 on Smo and Gli1 mRNA expression in esophageal cancer EC9706 cell
**Western blot results**

Detection by Western blot method, each treatment factor had no influence on Smo and Gli1 protein expression quantities in esophageal cancer EC9706 cells, cell control group, transfected reagent group and irrelative sequence group had no influence on the protein expression in esophageal cancer EC9706 cells, Smo protein expressions of each control group were respectively 0.624±0.017, 0.622±0.031 and 0.628±0.037 and even pair wise differences had no statistical significance between each group; Gli1 protein expressions of each control group were respectively 0.636±0.011, 0.628±0.036 and 0.622±0.028, and even pair wise differences had no statistical significance between each group. After Smo siRNA-3 group transfected esophageal cancer EC9706 cells for 72h, Smo protein expression quantity was 0.330±0.016, Gli1 protein expression was 0.324±0.021, compared with each control group, Smo and Gli1 protein levels of esophagus carcinoma EC9706 cells were evenly lower than those of each control group (equally P<0.05), as shown in table 2, fig. 6.

**Fig. 7**: Influence of Smo siRNA-3 on Smo and Gli1 protein expression in esophageal cancer EC9706 cells

After Smo siRNA-3 transfected EC9706 cells for 24h group, 48 h group and 72h group were respectively 0.540±0.035, 0.430±0.023 and 0.324±0.021, compared with Gli1 protein expression of 0.632±0.019 in cell control group was evenly rather lower, of which transfected 72h group reduced most obviously and also pair wise differences had statistical significance between each group(P<0.05), as shown in table 3 and fig. 7.

**Fig. 8**: Comparison of cell growth inhibition ratios that Smo siRNA transfected each group of cells

**Smo siRNA-3 inhibited cell proliferation**

After Smo siRNA-3 transfected cells for 24h, 48h and 72h, the cell growth inhibition ratio in Smo siRNA-3 group was obviously higher than that in each control group (evenly P<0.05), the cell growth inhibition ratio increased with the extension of transfected time, and even pair wise differences had statistical significance at different time points (evenly P<0.05), as shown in table 4 and fig. 8.

**Smo siRNA promoted cell apoptosis**

Compared transfected 72h with each control group, the proportions of non-viable apoptotic cells and viable apoptotic cells in Smo siRNA-3 transfected group were both significantly increased, the differences had equal statistical significance (evenly P<0.05), as shown in table 5 and fig. 9.

**DISCUSSIONS**

RNA interference (RNAi) refers to a phenomena of gene silence after specific transcription induced by double-stranded RNA which is homologous to target gene. It combines with homologous target RNA according to the principle of base pairing, specific enzymes can degrade target RNA, and thereby inhibit reducing target gene expression. Small interfering RNA (siRNA) is a micro molecular RNA, that consists of 21–25 nucleotides, can block gene expression effectively and peculiarly, and has already become a strong biological tool for tumor gene therapy (Haussecker, 2010).
Table 1: Influence of Smo siRNA-3 on Smo and Gli1 mRNA expression in esophageal cancer EC9706 cells (x ±s)

<table>
<thead>
<tr>
<th>Group</th>
<th>Smo mRNA Expression Quantity</th>
<th>Gli1 mRNA Expression Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell control group</td>
<td>0.644±0.023</td>
<td>0.642±0.025</td>
</tr>
<tr>
<td>transfected Smo siRNA-3 for 24 h group</td>
<td>0.524±0.011</td>
<td>0.518±0.008</td>
</tr>
<tr>
<td>transfected Smo siRNA-3 for 48 h group</td>
<td>0.422±0.008</td>
<td>0.434±0.025</td>
</tr>
<tr>
<td>transfected Smo siRNA-3 for 72 h group</td>
<td>0.332±0.019</td>
<td>0.330±0.019</td>
</tr>
<tr>
<td>F</td>
<td>327.87</td>
<td>205.33</td>
</tr>
<tr>
<td>P</td>
<td>P&lt;0.01</td>
<td>P&lt;0.01</td>
</tr>
</tbody>
</table>

Note: Compared with cell control group, each transfected group was evenly P<0.05, pair wise comparison at different time points, evenly P<0.05.

Table 2: Influence of Smo siRNA-3 on Smo and Gli1 protein expression in esophageal cancer EC9706 cells (x ±s)

<table>
<thead>
<tr>
<th>Group</th>
<th>Smo Protein Expression Quantity</th>
<th>Gli1 Protein Expression Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>cell control group</td>
<td>0.624±0.017</td>
<td>0.636±0.011</td>
</tr>
<tr>
<td>transfected reagent group</td>
<td>0.622±0.031</td>
<td>0.628±0.036</td>
</tr>
<tr>
<td>Irrelative sequence group</td>
<td>0.628±0.037</td>
<td>0.622±0.028</td>
</tr>
<tr>
<td>transfected Smo siRNA-3 for 72 h group</td>
<td>0.330±0.016</td>
<td>0.324±0.021</td>
</tr>
<tr>
<td>F</td>
<td>151.31</td>
<td>178.76</td>
</tr>
<tr>
<td>P</td>
<td>P&lt;0.01</td>
<td>P&lt;0.01</td>
</tr>
</tbody>
</table>

Note: compared with each control group, after transfected Smo siRNA-3 for 72 h group, P < 0.05.

Table 3: Influence of Smo siRNA-3 on Smo and Gli1 protein expression in esophageal cancer EC9706 cells (x ±s)

<table>
<thead>
<tr>
<th>Group</th>
<th>Smo Protein Expression Quantity</th>
<th>Gli1 Protein Expression Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>cell control group</td>
<td>0.628±0.015</td>
<td>0.632±0.019</td>
</tr>
<tr>
<td>transfected Smo siRNA -3 for 24 h group</td>
<td>0.534±0.034</td>
<td>0.540±0.035</td>
</tr>
<tr>
<td>transfected Smo siRNA -3 for 48 h group</td>
<td>0.426±0.021</td>
<td>0.430±0.023</td>
</tr>
<tr>
<td>transfected Smo siRNA -3 for 72 h group</td>
<td>0.330±0.016</td>
<td>0.324±0.021</td>
</tr>
<tr>
<td>F</td>
<td>164.97</td>
<td>137.26</td>
</tr>
<tr>
<td>P</td>
<td>P&lt;0.01</td>
<td>P&lt;0.01</td>
</tr>
</tbody>
</table>

Note: Compared with cell control group, each transfected group was evenly P<0.05, pair wise comparison at different time points, (evenly P < 0.05).

Table 4: Comparison of cell growth inhibition ratio for Smo siRNA transfected cells in each group (x ±s)

<table>
<thead>
<tr>
<th>Group</th>
<th>Cell inhibition ratio ( % )</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24h</td>
<td>48h</td>
<td>72h</td>
</tr>
<tr>
<td>Cell control group</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
<td>0.00±0.00</td>
</tr>
<tr>
<td>Transfected reagent group</td>
<td>13.93±2.67</td>
<td>16.01±3.69</td>
<td>15.05±4.35</td>
</tr>
<tr>
<td>Irrelative sequence group</td>
<td>15.32±5.18</td>
<td>16.68±5.28</td>
<td>14.72±4.36</td>
</tr>
<tr>
<td>Transfected Smo siRNA-3 group</td>
<td>53.55±3.25</td>
<td>68.50±7.33</td>
<td>88.06±7.56</td>
</tr>
</tbody>
</table>

Smo protein is composed of 1024 amino acids (approximately 86 KD), Smo plays the role of G-protein-coupled receptors, Smo protein has three domains: one intracellular carboxyl terminus domain, one extracellular amino terminal domain and seven lyophobic transmembrane domains (Haussecker et al, 2010; Raju, 2011). When there is no Hh protein, receptor protein Ptc inhibits the activity of Smo protein, and thereby blocks Hh signaling pathway; and while there is Hh protein, Hh combines with Ptc protein, removes from the inhibition for Smo protein, and even activated Smo protein can convert extracellular Hh signals into intracellular Gli signals, and then activate the transcription of Hh corresponding gene, including Hhip, ptc, Gli1, cyclin D, Myc, Bmi1, Bcl2, VEGF and etc. (Robbins.,2012). Hh signal transduction pathway has already become a hot for malignant tumor gene therapy in recent years. Related research results show that, siRNA inhibits Smo and Gli1
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expressions of gastric carcinoma MGC803 cells, breast cancer MCF-7 cells, liver cancer Huh-7 cells, pancreatic cancer PANC-1 cells and inhibits tumor cell proliferation, induces cell apoptosis (Tao et al., 2011; Agyeman and Ding et al., 2012; Li and Wang et al., 2013), enhances the sensitivity to chemotherapy drugs, even thus indicates that Smo is likely to be an effective target spot for tumor gene therapy (Karhadkar et al., 2004; Mori et al., 2006; Inghan 2008; Watson et al., 2010). In this study, we used use of subsequent experiments as well, Smo siRNA-3 inhibits Smo and Gli1 gene expressions significantly on mRNA and protein level, Smo siRNA inhibits human esophageal cancer EC9706 cell gene expression, downgrades Gli1 protein and mRNA expression with downstream transcription factor. Smo siRNA inhibits human esophageal cancer EC9706 cell proliferation and also induces its apoptosis. The above research results points out that, the abnormal activation of Hh signaling pathway

Table 5: Influence of Smo siRNA on EC9706 cell apoptosis (%) (x ±s)

<table>
<thead>
<tr>
<th>Group</th>
<th>Injured cell (Q1 domain)</th>
<th>Non-viable apoptotic cell (Q2 domain)</th>
<th>viable cell (Q3 domain)</th>
<th>viable apoptotic cell (Q4 domain)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell control group</td>
<td>6.40±1.25</td>
<td>1.08±0.50</td>
<td>84.26±4.83</td>
<td>3.40±1.18</td>
</tr>
<tr>
<td>Transfected reagent group</td>
<td>5.52±1.45</td>
<td>1.60±0.28</td>
<td>88.96±0.95</td>
<td>2.26±0.32</td>
</tr>
<tr>
<td>Un-related sequence group</td>
<td>6.62±2.56</td>
<td>1.68±0.51</td>
<td>86.06±1.23</td>
<td>2.20±0.77</td>
</tr>
<tr>
<td>Smo siRNA-3 group</td>
<td>5.48±1.83</td>
<td>8.78±0.44</td>
<td>77.22±1.90</td>
<td>11.32±1.31</td>
</tr>
<tr>
<td>F</td>
<td>221.61</td>
<td>355.08</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P</td>
<td>P&lt;0.05</td>
<td>P&lt;0.05</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: The differences had statistical significance, pair wise comparison adopted by LSD-t, evenly P <0.05.

Fig. 9: Influence of Smo siRNA on the proportion of esophageal cancer EC9706 cell apoptosis
A: Cell control group; B: Transfected reagent group; C: Un-related sequence group; D: After transfected Smo siRNA-3 for 72 h group

siRNA transfected esophageal cancer EC9706 cells, RT-PCR detected results show that, compared with cell control group, Smo and Gli1 mRNA expressions in transfected reagent group and irrelevant sequence group have not significant changes, and that Smo and Gli1 mRNA expressions in Smo siRNA-3 group reduce obviously and also screen out Smo siRNA-3 sequence as the best segments for specificity. We proves in the plays an important role in the occurrence of esophageal cancer, Smo plays an important role in esophageal cancer cell proliferation and apoptosis, detecting Smo and Gli1 protein expression is likely to provide a reference basis for clinical diagnosis and treatment of esophageal cancer, and is also an effective target spot for gene therapy (Watson et al., 2010).
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