Original Article

Expression and role of HSP90α in esophageal squamous cell carcinoma

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Abstract: Objective: This study aims to examine the expression of HSP90α in esophageal carcinoma, and explore the effects of siRNA-HSP90α on the biological behaviors of esophageal cancer cell CE81T-4. Methods: The expression of HSP90α in esophageal squamous cell carcinoma (ESCC) of Kazakh was detected by IHC. The relationship between this expression and clinical pathological parameters was analyzed. The expression of HSP90α in CE81T-4 cells was downregulated by siRNA and changes of HSP90α were detected by qRT-PCR and western blot. Its impact on CE81T-4 cell proliferation was analyzed by MTT assay, while cell cycle and apoptosis were analyzed by flow cytometry. Moreover, cell migration and invasion changes were detected by scratch and Transwell assays. Results: The expression of HSP90α in ESCC was significantly higher than that in paracancerous tissues (P<0.05). Its expression was associated with tumor invasion, differentiation and lymph node metastasis (P<0.05). After siRNA-HSP90α was expressed in CE81T-4 cells, mRNA and protein levels decreased (P<0.01). Furthermore, cell proliferation was inhibited by HSP90α downregulation, cell cycle was arrested at the G0/G1 phase, apoptosis was enhanced, and cell migration and invasion were decreased (P<0.01). Conclusions: HSP90α was highly expressed in ESCC of Kazakh, and was found to be associated to the invasion, metastasis and differentiation of ESCC. The downregulation of HSP90α could inhibit the proliferation, migration and invasion of esophageal cancer cells.

Keywords: Esophageal carcinoma, HSP90α, cell proliferation, invasion and metastasis, cell cycle

Introduction

Esophageal carcinoma (EC) is one of the most common malignant tumors of the digestive tract, which is divided into two histological types: esophageal squamous cell carcinoma (ESCC), and esophageal adenocarcinoma (EAC). Among malignant tumors, EC ranks eighth in terms of incidence, and sixth in terms of mortality rate [1-4]. The distribution of EC has obvious regional differences. In China, Kazakh people who live in Xinjiang have been reported to have the highest incidence of EC; and the adjusted mortality was 68.88/100 thousands [5]. This rate is more than ten times of that in other nations (5.13/100 thousands), and 90% of these morbidities are due to ESCC. Furthermore, the five-year survival rate of esophageal cancer is less than 20% [3, 6], in which invasion and metastasis are the main causes of death. The invasion and metastasis of EC are both complex biological processes that involve multiple factors. Currently, it has been found that a variety of factors, such as the matrix metalloproteinase (MMP) family [7-9], EGFR [10], VEGF [11] and CXCR [12], participate in the invasion and metastasis of EC. In order to further explore the invasion and metastasis of EC, we performed proteome and gene chip analysis on cell lines with high and low metastatic potentials in the preliminary study. HSP90α was highly expressed in EC cells with high metastatic potential in the two kinds of screening. Previous studies have found that HSP90α is highly expressed in many kinds of malignant tumors such as lung cancer, bladder epithelial cancer, liver cancer and breast cancer; and participates in the invasion and metastasis of tumors [13-16]. In this study, we observed the expression of HSP90α in EC of
Kazakh people living in Xinjiang, and its relationship with clinical pathological parameters. RNA interference technology was used to silence the expression of HSP90α in EC cell line CE81T-4, which has high metastatic potential. Then, effects of the downregulation of HSP90α in the proliferation, migration and other biological behaviors of EC cells were observed. All of the above aimed at investigating the expression and function of HSP90α in EC, providing theoretical basis for searching for diagnostic targets, prognosis judgment and the treatment of EC.

Materials and methods

Materials

Tissue samples: ESCC specimens obtained from Kazakh people living in Xinjiang including the paired cancer tissues and paracancerous tissues (esophageal mucosal tissue with normal appearance, which was 2-5 cm distant to the edge of the tumor body) were paraffin embedded. A total of 86 cases were enrolled in each group. Surgical resection specimens were stored by the Department of Pathology of the First Affiliated Hospital of Xinjiang Medical University between 2001 and 2013. The clinical pathological data were intact: 55 cases of male, and 31 cases of female; patient age ranged within 35-77 years old, with an average age of 59.86 years old; depth of tumor invasion: 5, 23, 55 and 3 cases, in T1, T2, T3 and T4 respectively. According to the degree of histological differentiation, these cases were divided into three groups: high differentiation (20 cases), moderate differentiation (37 cases), and poor differentiation (29 cases). Furthermore, according to lymph node metastasis, these cases were divided into two groups: no metastasis (36 cases), and metastasis (50 cases). No radiotherapy, chemotherapy, or biological therapy was given to patients prior to surgery. Sample acquisition and application, were signed informed consent by patients and approved by Ethics Committee of Xinjiang Medical University.

Esophageal cancer cell line: The CE81T-4 high invasion esophageal squamous carcinoma cell line was offered by Professor Ming-Tsang Wu, Kaohsiung Medical University Hospital, Kaohsiung, Taiwan [17].

Reagent: Rabbit anti-human HSP90α polyclonal antibody was purchased from Cell Signal Technology; DMEM medium and fetal bovine serum (FBS) was purchased from Gibco, USA; prime ScriptTM one-step RT-PCR kit, SYBR Green PCR premix, and the DNA Marker were purchased from Takara Biotechnology Co. Ltd.; RIPA lysate and the PCR product purification kit was purchased from Beijing Baitaike, China; LipofectamineTM 2000, Western blot second antibody kit, methylthiotetrazole (MTT), Western blot, Opti-MEM, and Trizol were purchased from Invitrogen; glycine was purchased from Biovision, USA; diethylpyrocarbonate (DEPC) was purchased from Amresco, USA; DMSO, TEMED and SDS were purchased from Sigma, USA; glycine was purchased from Biovision, USA; anhydrous ethanol, NaCl, KCl, NaHPO₄, KH₂PO₄ and methanol reagent were domestic products.

Methods

HSP90α expression in ESCC and adjacent tissues were detected by immunohistochemistry in 86 cases of Kazakh people in Xinjiang: Samples were detected by SP method, according to kit instructions. The first antibody was replaced with PBS liquid as the negative control, and took the known positive reaction section as the positive control. Tissue sections were routinely deparaffinized, dehydrated, re-paired with citric acid sodium EDTA antigen retrieval buffers for 20 minutes, and incubated with 3% H₂O₂ for 10-15 minutes to block the endogenous peroxidase. Then, the first antibody HSP90α (diluted at a ratio of 1:250) was added and incubated at 4°C overnight. Next, horseradish peroxidase-labeled secondary antibody biotin was added, washed with PBS in each step, and incubated at room temperature. Then, the secondary antibody was discarded, DAB developing liquid was added, re-satined with hematoxylin to make the section bluing, dehydrated in graded alcohol, and the section was sealed with neutral gum. Immunohistochemistry (IHC) staining scores: The Formowitz comprehensive scoring method was adopted [18]. Ten high power fields (HPFs, ×200) were randomly selected in each section, counted at least one thousand cells, and staining intensity and positive cell percentage scores were evaluated in each field. Scores were evaluated
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HSP90α mRNA expression in cells in each group were detected by real-time fluorescence quantitative polymerase chain reaction (qRT-PCR): Cells were collected, total RNA was extracted according to the proportion that 1 ml of Trizol was added into the cells at the density of 2.0×10^5, the whole operation process was carried out on ice, and optical density (OD) values of the extracted RNA was measured. The concentration and purity of RNAs were detected, and purity of the RNA was considered qualified if absorbance ratio at OD 260/280 was within 1.8-2.0. The reverse transcription reaction system was 20 μl, and these reactions were carried out with reverse transcription primers of HSP90α and β-actin. The upstream primer of HSP90α was 5'-CTGCTTATTTGGTTCGAGAACTT-3' and the downstream primer was 5'-TCCTTTATTTCTGTTCTCCACATG-3', while the upstream primer of β-actin was 5'-TggCACCCAgCACAATgAAA-3' and the downstream primer was 5'-CTAAGTATAgTCTCgCCTAgAAgC-3'. The obtained 2 μl cDNA was taken as the template, and qRT-PCR reaction was carried out, with a total reaction system of 20 μl. Reaction conditions were: pre-degeneration at 95°C for three minutes → degeneration at 95°C for 3S → annealed at 55°C for 30 seconds (for β-actin)/57°C for 30 seconds (for HSP90α), and reacted for 40 cycles. Next, the dissolution curve drawn, and the difference in HSP90α mRNA expression was compared by using the relative quantitation method of the standard curve.

Detection of HSP90α protein expression in each group by western blot: The BCA method was used to determine the protein concentration. Total protein of the sample was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Sampling quantity was 30 μg. Electrophoresis was performed at 120 V for 1.5-2 hours, and the protein was transferred onto the polyvinylidene fluoride (PVDF) film. Then, the film was blocked at 37°C for 60 minutes. Next, rabbit anti-human HSP90α polyclonal antibody diluted at a ratio of 1:250 was added. The film was incubated at 4°C overnight, incubated with the second antibody for 30 minutes, incubated with a luminescence agent until the bands were clear, and terminated the developing reaction with distilled water. The film was scanned by a gel imaging instrument, and the

Cell culture and transfection: After recovery, CEB81T-4 cells were cultured in 8% FBS and DMEM medium at 37°C with 5% CO₂, and digested by trypsin for cell passage. Twenty-four hours before transfection, CEB81T-4 cells in the logarithmic growth period were inoculated into six-well culture plate at a density of 2.0×10^5; and transient transfection was performed when the abundance approximately reached 70%-80%. The experiment was divided into three groups: experimental group (siRNA-HSP90α group), negative control group (blank vector group) and blank control group (normal growth group). HSP90α siRNA interference sequences were designed and synthesized by Ji Ma Pharmaceutical Technology Co., Ltd., Shanghai, which are as follows: 5'-GGAGGAGGAACGUGAUAATT-3' and 5'-UUUAUCACGUUCUCCCTT-3'. The interference sequence for the negative control group was: 5'-UUCUCCGACCCAgCACATTgAAA-3' and 5'-CTAAGTATAgTCTCgCCTAgAAgC-3'. The obtained 2 μl cDNA was taken as the template, and qRT-PCR reaction was carried out, with a total reaction system of 20 μl. Reaction conditions were: pre-degeneration at 95°C for three minutes → degeneration at 95°C for 3S → annealed at 55°C for 30 seconds (for β-actin)/57°C for 30 seconds (for HSP90α), and reacted for 40 cycles. Next, the dissolution curve drawn, and the difference in HSP90α mRNA expression was compared by using the relative quantitation method of the standard curve.

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Based on staining strength: 0 point, no staining; 1 point, light yellow staining; 2 points, pale-brown staining; 3 points, brown staining. Furthermore, scores were also given based on the number of positive cells: <5%, 0 point; 5%-25%, 1 point; 26%-50%, 2 points; 51%-75%, 3 points; >75%, 4 points. Next, the following scores were given based on the sum of the staining intensity score and the positive cell proportion score: 2 points, the result is negative (-); 2-3 points, the result is weak positive (+); 4-5 points, the result is moderately positive (++); 6-7 points, the result is strong positive (+++). Negative and weak positive were assigned as the low expression group, while positive and strong positive were assigned as the high expression group. Immunohistochemical staining results were evaluated by two pathologists.
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Table 1. Expression of HSP90α in ESCC and its relationship with clinical pathological parameters

<table>
<thead>
<tr>
<th>Pathological parameters</th>
<th>Sample number</th>
<th>HSP90α expression</th>
<th>P value</th>
</tr>
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<tbody>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>55</td>
<td>Low expression</td>
<td>8</td>
</tr>
<tr>
<td>Female</td>
<td>31</td>
<td>High expression</td>
<td>47</td>
</tr>
<tr>
<td>P value</td>
<td>0.516</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;60 old</td>
<td>36</td>
<td>Low expression</td>
<td>5</td>
</tr>
<tr>
<td>&gt;=60 old</td>
<td>50</td>
<td>High expression</td>
<td>31</td>
</tr>
<tr>
<td>P value</td>
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<td></td>
<td></td>
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<td>Depth infiltration</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>5</td>
<td>Low expression</td>
<td>2</td>
</tr>
<tr>
<td>T2</td>
<td>23</td>
<td>High expression</td>
<td>6</td>
</tr>
<tr>
<td>T3</td>
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<td></td>
<td>3</td>
</tr>
<tr>
<td>T4</td>
<td>3</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>P value</td>
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<td></td>
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<tr>
<td>Degree of differentiation</td>
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</tr>
<tr>
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<td>20</td>
<td>Low expression</td>
<td>6</td>
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<tr>
<td>Middle differentiation</td>
<td>37</td>
<td>High expression</td>
<td>4</td>
</tr>
<tr>
<td>Poorly differentiated</td>
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</tr>
<tr>
<td>P value</td>
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<td>Lymph node metastasis</td>
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<td>50</td>
<td>Low expression</td>
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<tr>
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<tr>
<td>P value</td>
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</table>

*p<0.05.

Gray value ratio was calculated as the relative protein expression level.

Cell proliferation detection: The MTT method was adopted. Three groups of cells were inoculated into 96-well plate at a cell density of approximately 1×10^3-10^4/ml. Then, 20 μl of MTT solution (5 mg/ml) was added into wells prior to inoculation (zero-hour), and at 24, 48, 72 and 96 hours after transfection, respectively. Subsequently, cells were incubated at 37°C for four hours, and the supernatant was discarded. Then, cells were washed with pre-cooled PBS twice, centrifuged at 1,000 rpm for five minutes, and the supernatant was discarded. Then, cells were fixed with 75 mL/L frozen ethanol, and incubated at 4°C overnight. Cell concentration was adjusted to a density of 1×10^5/ml, 2 μl (25 mg/ml) of RNaseA was added into the cells and mixed evenly, the mixture was placed in a water bath at 37°C for 30 minutes, and cells were filtered with a 400 mesh sieve. Next, 400 μl of propidium iodide (PI) dying solution was added, incubated at 4°C for 30-60 minutes, and kept in the dark. The fluorescence intensity of DNA-PI was detected by flow cytometry. The longest excitation wavelength was 488 nm.

Cell apoptosis detection: At 48 hours after transfection, three groups of cells were collected, washed with pre-cooled PBS twice, centrifuged at 1,000 rpm for five minutes, and the supernatant was discarded. Then, cells were suspended with 400 μl of 1 X Annexin-V combination solutions, 5 μl of Annexin V-FITC staining liquid was added, cells were incubated at 4°C, and was kept in the dark. Next, 10 μl of PI staining solution was added, incubated at 4°C for five minutes, and kept in the dark. The distribution of cells in these four quadrants were detected by flow cytometry within 30 minutes.

Cell migration ability detection by cell scratch test: Cells were inoculated into 6-well plate at a density of 1×10^5/well and cultured until the cell growth coverage rate reached 100%. At 24 hours after transfection, cells in these three groups were taken; and a straight line was drawn with a 10 μl sterile micro pipette tip at the bottom of the well, ensuring that the thickness of the line was even for each well. Under an inverted microscope, 5-6 different dots were marked at positions where the scratch width and cell density was consistent, and photos were taken to measure the scratch width at zero, 24, 48 and 72 hours, respectively; then, healing speed of the scratch was observed.
Detection of cell invasion ability by transwell invasion assay in vitro: At 24 hours after transfection, cells in the three groups were proceeded cultured for 12 hours withdrawal serum; and the cell suspensions were prepared. The Matrigel in the Transwell chamber was rehydrated, cells suspensions were added into the chamber, 500 μl of culture medium containing 10% FBS was added to the bottom, and incubated for 36 hours. The Matrigel and cells that did not invade was wiped-off at the bottom of the upper chamber. Then, cells were stained with crystal violet, and observed and counted under an inverted microscope.

Statistical analysis

SPSS 17.0 statistical software was used for statistical analysis. Measurement data were expressed as mean ± standard deviation (±SD), conformed to normal distribution, and t-test was used for evaluating the results. Variance analysis was performed among multiple groups. If measurement data conformed to the skewed distribution, the rank sum test was adopted. Wilcoxon test was used for comparison between two groups, and chi-square test was used for count data. P<0.05 was considered statistically significant.

Results

HSP90α expression in ESCC and paracancerous tissues in Kazakh people living in Xinjiang

Results revealed that HSP90α was positively expressed mainly in the cytoplasm of EC cells and paracarcinoma tissues, and a small part had a weak positive expression in the nucleus of EC cells. The high expression rates of HSP90α in 86 cases of Kazakh people living in Xinjiang with ESCC and adjacent tissues were 75 cases (87.2%) and 16 cases (18.6%) (P=0.0000) respectively. HSP90α expression was positively correlated to the invasion depth of EC, and was negatively correlated to the differentiation degree of ESCC. Furthermore, HSP90α expression was higher in patients with lymph node metastasis, compared with patients without lymph node metastasis (P<0.05); and it was not correlated with the age and gender of patients (Table 1 and Figure 1).

Effects of RNA interference silencing on HSP90α expression in CE81T-4 cells detected by qRT-PCR and western blot

The relative mRNA expression of HSP90α was detected by qRT-PCR after siRNA-HSP90α transfection. These results revealed that HSP90α mRNA expression in the experimental group was significantly reduced than in the other two groups (P<0.01). Furthermore, there was no difference in HSP90α mRNA expression between the negative control group and blank control group (P>0.05, Figure 2). After siRNA-HSP90α transfection for 72 hours, western blot was applied to detect the expression of HSP90α. Results revealed that the relative expression quantity of HSP90α protein among
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The expression and role of HSP90α in esophageal squamous cell carcinoma (ESCC) were studied. Three groups (siRNA-HSP90α group, blank vector group, and normal growth group) were compared. The expression of HSP90α mRNA in the siRNA-HSP90α group was significantly lower than in the other two groups (P<0.01). Western blot analysis showed that HSP90α protein in the siRNA-HSP90α group was significantly decreased compared to the other two groups (P<0.01, Figure 3). These results indicate that HSP90α was successfully silenced by RNA interference.

**Effect of HSP90α silencing on CE81T-4 cell proliferation**

CE81T-4 cells were transfected with siRNA-HSP90α, and the proliferation activity of these three groups was detected by MTT assay at 0, 24, 48, 72, and 96 hours post-transfection. There were no significant differences among these groups at zero-hour and 24 hours post-transfection (P>0.05). At 48 hours, cell proliferation in the HSP90α silenced group was inhibited, in comparison with the negative control group and blank control group. The same trends were found at 72 hours and 96 hours, and the differences were statistically significant (P<0.05, Figure 4). These results suggest that the downregulation of HSP90α significantly inhibited the proliferation of CE81T-4 cells.

**Effect of HSP90α downregulation on CE81T-4 cell cycle and apoptosis**

Cell cycle was detected by flow cytometry. Results revealed that there were no significant changes in the percentage of each phase in the negative control group, compared with the blank control group. Furthermore, cells in the G0/G1 phase in the siRNA-HSP90α group significantly increased (P<0.01), and no obvious changes were found in the other phases (Table 2). Using fluorescein FITC-labeled Annexin-V as a probe, apoptosis was detected by flow cytometry. Apoptosis rates in the siRNA-HSP90α group, negative control group and blank control group were 27.30±4.33%, 5.63±1.12%, 10.20±2.04%, respectively (P<0.01, Figure 5). These indicate that the downregulated expression of HSP90α in CE81T-4 cells, which led to cell cycle arrest at the G0/G1 phase and promoted cell apoptosis.

**Effect of HSP90α inhibition on the migration ability of CE81T-4 cells**

Effect of HSP90α on the migration ability of CE81T-4 cells was analyzed by cell scratch test. Results revealed that cell migration was inhibited in the siRNA-HSP90α group at 24, 48 and 72 hours post-scratch, and scratch healing was retarded, when compared with the other two groups (Figure 6). This suggests that the downregulated expression of HSP90α could inhibit the migration of CE81T-4 cells.

**Effect of HSP90α silencing on the invasion ability of CE81T-4 cells**

The invasion ability of CE81T-4 cells was detected by Transwell invasion test. When HSP90α expression in cells was downregulated, six visu-
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al fields were randomly selected in each group; and cells were observed and counted under an inverted microscope. Results revealed that the number of invasive migrated cells in the experimental group was (73.83±3.65), which was significantly lower than in the blank control group (183.50±7.66) and negative control group (163.33±10.03); and the difference was statistically significant (P<0.01).

Discussion

HSP90 is a highly conserved special protein that belongs to the molecular chaperone family. In addition to its participation in the processes of folding, transportation and synthesis of protein, it plays important roles in the development and progression of tumors. At present, as a novel anticancer therapeutic target, HSP90 has attracted much concern, because it has more than one hundred client proteins [19] such as TGF-receptor, Raf, HIF-1, P53, EGFR, Akt, and ERK1/2. These are involved in a variety of cellular signal transduction pathways such as cell proliferation, cycle regulation and apoptosis. HSP90 has two isoforms, Hsp90α and Hsp90β; and the similarity in amino acid sequences between these two isoforms is 93%, which a consistency of 86% [20]. Furthermore, substrate proteins that can specifically combine with these have been recently found, and HSP90α has a more close relationship with tumors. Studies have shown that HSP90α is highly expressed in multiple tumors and is associated with malignancy degree, and that it can be used to judge the prognosis of the disease [13-16, 20]. The most important role of HSP-90α in cancer is to stabilize oncogenes and some over expressed factors in an activated state [21], and provide a basic growth environment for the development of tumors, in order to adapt to the needs of cell proliferation. It can also regulate the number of proto-oncogenes expression and transformation growth factors, as well as inhibit the transcription for regulating genes related to cell growth; which would lead to changes in cell growth and differentiation, and induce cell carcinogenesis [22].

By IHC, we found in this study that among the 86 cases of Kazakh people living in Xinjiang with ESCC, 75 (87.2%) cases were high expression of HSP90α, which was significantly higher than that in the adjacent tissues of cancer that the high expression of HSP90α was 16 cases (18.6%), HSP90α is mainly expressed in the cytoplasm and partly expressed in the nucleus. This was basically consistent with the result of previous studies on HSP90α in EC conducted by X Wu [23]. We also found that the more deeper the EC infiltrated, the higher the expression level of HSP90α was. However, the

Figure 4. The proliferation and viability of CE81T-4 cells transfected with siRNA-HSP90α cells were detected by MTT. *It indicates that compared with the control group, the difference is statistically significant, p<0.01.

Table 2. Down-regulation of HSP90α induced an accumulation of G1-phase cells compared to blank vector and normal growth group

<table>
<thead>
<tr>
<th>Group</th>
<th>Phase distribution of cell cycle</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>G0/G1</td>
</tr>
<tr>
<td>SIRNA-HSP90α group</td>
<td>74.16±1.17*</td>
</tr>
<tr>
<td>Blank vector group</td>
<td>66.83±0.45</td>
</tr>
<tr>
<td>Normal growth group</td>
<td>66.93±0.97</td>
</tr>
</tbody>
</table>

All data are presented as the means SD from at least three independent experiments performed in triplicate. *P<0.01 versus “blank vector/normal” group.
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Figure 5. Flow cytometry detected apoptosis rate of CE81T-4 after transfectied. A: Experimental group; B: Negative control group; C: Blank control group; *P<0.01.

Figure 6. Images of Inverted microscope in Wound healing assay. A: Blank control group; B: Negative control group; C: Experimental group.

In summary, we found that HSP90α was highly expressed in ESCC in Kazakh people evaluated in this study, and confirmed at histological and cellular levels that HSP90α was involved in EC invasion and metastasis processes. Furthermore, it was also found that HSP90α could affect the proliferation and apoptosis of EC cells. Some studies have suggested that HSP90α inhibitor 17-AGG can inhibit HGF/SF mediated tumor cell invasion [23, 24]. In a recent study, Suzuki R found that in multiple myeloma cells, HSP90α/β inhibitors can affect...
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the proliferation and apoptosis of tumor cells by acting on the pathway of RAS-RAF-MEK-ERK [25]. Whether HSP90α is involved in the biological behaviors of tumor cells in ESCC through the same mechanisms described above, it still needs further research.

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Disclosure of conflict of interest

None.

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