Abstract. – OBJECTIVE: To investigate the effect of endostatin on the proliferation, invasion and epithelial-mesenchymal transition (EMT) of human basal cell carcinoma (BCC) cells (A431).

PATIENTS AND METHODS: CCK-8 assay and transwell chamber assay were performed to detect cell proliferation and invasion abilities, respectively. Western blot was performed for the detection of the expressions of EMT-related proteins levels. The therapeutic effect of endostatin on tumor formation was tested using a mouse xenograft model.

RESULTS: After endostatin treatment, transwell assay showed that the number of invasive cells in the observation group and control group were (38.25±8.13) and (98.25±9.14), respectively; the relative expression level of E-cadherin protein in the observation group was (0.34±0.03), which was significantly higher than that in the control group (0.14±0.01); the relative expression levels of N-cadherin protein in the observation group was (0.18±0.05), which was significantly lower than that in the control group (0.43±0.03), (all \( p < 0.05 \)).

CONCLUSIONS: The expression levels of Vimentin and Fibronectin proteins were significantly lower, while the expression levels of \( \alpha \)-smooth muscle Actin (\( \alpha \)-SMA) were significantly higher in the observation group than those in the control group. Treatment with endostatin significantly inhibited tumor growth in the mouse xenograft model. Therefore, endostatin can inhibit the proliferation, invasion and EMT in BCC.

Key Words: Endostatin, Basal cell carcinoma cell, Proliferation, Invasion, Epithelial-mesenchymal transition.

Introduction

Basal cell carcinoma (BCC) originates from the basal cells of the skin or its accessory organs, so BCC is also called basal cell epithelial tumor. BCC accounts for about 60% of malignant tumors in the skin that occurs in light-colored races. BCC mainly affects men and elderly patients, and the affected regions are mainly face, neck and hand skin. Studies have shown that ultraviolet (UV) exposure is closely related to the pathogenesis of BCC that may also be induced by other non-environmental factors such as ionizing radiation, dust and high-fat diet. In addition, HPV infection may also induce the occurrence of BCC. Studies have shown that mutations in oncogenes and tumor suppressor genes (such as p53 and PTCH) frequently occur in BCC patients. BCC has the potential to differentiate in multiple directions, with a total of nine histological types such as superficial and pigmented types. At present, surgical resection is the first choice for the treatment of BCC, but it will affect the patient’s appearance and related functions. Radiotherapy and chemotherapy have certain toxic and side effects, which are not conducive to the long-term survival of patients.

Endostatin is an inhibitor of angiogenesis that inhibits tumor neovascularization and cell proliferation, and induces apoptosis. Various studies have shown that it has an inhibitory effect on lung cancer, rectal cancer and other cancers, but its role in BCC has not been clarified. This work aimed to reveal the function of endostatin in BCC by investigating its effects on the proliferation, migration, invasion and epithelial-mesenchymal transition (EMT) of A431 cells, to provide a new theoretical basis for the treatment of BCC.

Materials and Methods

Materials and Reagents

Human BCC cell strain A431 (Jennio Biotech Co., Ltd., Guangzhou, China), fetal bovine serum

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(FBS) and Roswell Park Memorial Institute-1640 culture medium (RPMI-1640; HyClone, South Logan, UT, USA); endothelial cell inhibitant endostatin (Shenzhen Biotechnology Co., Ltd., Guangzhou, China); TRIZol reagent, radioimmunoprecipitation assay (RIPA) lysate and sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) buffer solution (Shanghai Yisheng Biotechnology Co., Ltd, Shanghai, China); rabbit anti-human E-cadherin (Cat nos. ABP51220, Abbkine, Wuhan, China)/N-cadherin (Cat no. ABP51903, Abbkine, Wuhan, China)/vimentin (Cat no. ABP50223, Abbkine, Wuhan, China)/fibronectin (Cat no. ABM40137, Abbkine, Wuhan, China)/α-smooth muscle Actin (α-SMA) (Cat no. LS-C169737, LSBio, Seattle, WA, USA) polyclonal antibody, rabbit anti-human GAPDH polyclonal antibody and horseradish peroxidase labeled goat anti-rabbit secondary antibody (Cat nos. A12004-1, EpiGentek, Farmingdale, NY, USA); Cell Counting Kit-8 (CCK-8) cell proliferation assay kit (Wuhan Biotechnology Co., Ltd., Wuhan, China); transwell chamber and Matrigel (Shanghai Ziqi Biotechnology Co., Ltd, Shanghai, China).

CCK-8 Assay to Detect the Effects of Endostatin on A431 Cell Proliferation

A431 cells were cultured in RPMI-1640 complete medium with 10% fetal bovine serum (FBS) in an incubator at 37°C, 5% CO₂ and humidity of 60%. Cells were harvested at logarithmic growth phase (fusion rate was 90%) to prepare single cells suspensions (1×10⁵/ml). Cells were transferred to a 96-well plate with 1×10⁴ cells/100 μl/well. Endostatin was added at doses of 0 (control), 20, 40, 80 and 160 μl/mg. Three replicates were set for each dose. 10 μl of CCK-8 solution was added 24 h, 48 h, 72 h later. Cells were cultured for an additional 3 hours and the absorbance at 450 nm was measured using a microplate reader. The proliferation inhibition rate = 1 - (endostatin well)/(control well).

CCK-8 Assay of Cell Viability

Based on the results of inhibitory concentration (IC) 50 of A431 cells after endostatin treatment, the action time and concentration of endostatin in the subsequent experiment were determined. The observation group was treated with endostatin, while the control group was treated with an equal amount of RPMI-1640 medium. CCK-8 was used to detect cell viability after cell culture for 12 h, 24 h, 48 h, 72 h, and 96 h. The specific procedures were the same as described in 2.1. Three replicates were set for each sample. Detection was repeated 3 times for each well.

Transwell Invasion Experiment

Trypsin-digested each group of cells was starved for 12 h and single cell suspensions (1×10⁵/ml) were prepared with the serum-free RPMI-1640 medium. The cell suspensions were transferred to the upper chamber of a 24-well transwell with 100 μl/well. 600 μl 1640 medium supplemented with 10% fetal bovine serum was added into the lower chamber. Cells were incubated at 37°C and 5% CO₂ for 24 h. After that, polycarbonate membrane was fixed with 5% glutaraldehyde for 10 min, followed by crystal violet staining for 10 min. After being washed with phosphate-buffered saline (PBS) two times, a sterile cotton swab was used to gently wipe off Matrigel. Five visual fields were randomly selected under the microscope (100 times) to count invading cells. The 8 μm chamber was used in the invasive experiment, and the matrix adhesive was Matrigel of BD Biosciences (Franklin Lakes, NJ, USA).

Western Blot

Cells were lysed with RIPA lysate, and the concentration of total protein was determined using the bicinchoninic acid (BCA) method. After denaturing, 10% SDS-PAGE electrophoresis was performed, followed by gel transfer to polyvinylidene difluoride membrane. After that, membranes were blocked in 5% skim milk for 2 h at room temperature. Rabbit anti-human E-cadherin/N-cadherin/vimentin/fibronectin/α-SMA polyclonal antibody was added at a ratio of 1:1000, rabbit anti-human endogenous control GAPDH polyclonal antibody was added at a ratio of 1:3000. After incubation at room temperature for 2 h, membranes were washed with Tris-Buffered Saline and Tween (TBST) and labeled horseradish peroxidase goat anti-rabbit secondary antibody was added at a ratio of 1:9000, followed by incubation at room temperature for 1 h. After washing with TBST 5 times, signals were developed and the gray value of each protein band was analyzed using Quantity One software (Bio-Rad, Hercules, CA, USA). The relative expression level of protein = the gray value of the target protein band/the gray value of GAPDH protein band. Repeating 3 rounds of experiments, each round of experiments was repeated 3 times for each sample.
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Nude Mouse Xenograft Model
Fifteen Specific Pathogen Free (SPF) female nude mice (4-6 weeks old) were raised in SPF animal rooms. A431 cells were harvested at logarithmic growth phase and were digested with 0.25% trypsin to prepare single cell suspensions. 0.3 mL of the cell suspension (1.0×10^7/ml) was subcutaneously injected into the left side of the nude mouse. On the 10th day when tumor diameter reached 6 mm, mice were randomly divided into control group (administered with the same amount of normal saline), ES1 group (administered with the 5 mg/kg endostatin) and ES2 group (administered with the 15 mg/kg endostatin). Endostatin treatment was performed for 14 days and tumors were measured every two days to calculate tumor volume according to the following formula: tumor volume = long diameter×short diameter^2/2. Tumor growth curves were plotted. Animals were sacrificed on the 15th day after treatment. Tumors were weighed and tumor inhibition rate (TIR) was calculated: TIR = (mean tumor volume of control group - mean tumor volume of endostatin group)/mean tumor volume of control group x 100%. This study was approved by the Ethics Committee of Henan Luoyang Orthopaedic Hospital (Henan Orthopaedics Hospital).

Statistical Analysis
SPSS 20.0 (Asia Analytics Formerly SPSS China) software statistical package was used for the analysis of all data. Measurement data were expressed as mean ± standard deviation (X ± s), and the t-test was used for comparisons between the two groups. The comparisons among multiple groups were analyzed by univariate factor ANOVA, followed by LSD test. The repeated measures analysis of variance was used for comparisons between different time points in the same group. GraphPad Prism 5 (Shanghai Cabot Information Technology Co., Ltd., Shanghai, China) was used to calculate IC50. The significance level is α=0.05.

Table I. Proliferation inhibition rate of A431 treated with different concentrations of endostatin at different times (%).

<table>
<thead>
<tr>
<th></th>
<th>0 μl/mg endostatin</th>
<th>20 μl/mg endostatin</th>
<th>40 μl/mg endostatin</th>
<th>80 μl/mg endostatin</th>
<th>160 μl/mg endostatin</th>
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<th>p</th>
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<tr>
<td>24h</td>
<td>(2.24±1.01)</td>
<td>(12.43±1.45)</td>
<td>(21.41±2.12)</td>
<td>(34.21±3.58)</td>
<td>(46.29±4.16)</td>
<td>18.58</td>
<td>0.00</td>
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<tr>
<td>48h</td>
<td>(11.53±1.92)</td>
<td>(19.62±1.23)</td>
<td>(33.71±2.11)</td>
<td>(44.68±3.94)</td>
<td>(59.31±4.91)</td>
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</tr>
<tr>
<td>72h</td>
<td>(16.42±1.93)</td>
<td>(29.91±2.19)</td>
<td>(46.72±2.98)</td>
<td>(61.93±4.61)</td>
<td>(85.92±5.93)</td>
<td>12.90</td>
<td>0.01</td>
</tr>
<tr>
<td>F</td>
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<td>0.00</td>
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</tr>
</tbody>
</table>

Note: *: the treatment time is the same, compared with the previous low concentration gradient, p<0.05. #: the concentration gradient is the same, compared with the previous treatment time, p<0.05.

Results
Determination of IC 50 of A431 Cells After Endostatin Treatment
After A431 cells were treated with different dosages of endostatin for 24 h, 48 h and 72 h, cell proliferation rate was measured by the CCK-8 assay. It was found that endostatin inhibited cell proliferation in a time- and dose-dependent manner (see Table I and Figure 1 for details). The IC50 values at 24 h, 48 h and 72 h were 168.31 μg/ml, 93.44 μg/ml and 53.33 μg/ml, respectively. Treatment with endostatin at 40 μl/mg for 72 h was used for subsequent experiments.

Figure 1. Endostatin on A431 proliferation inhibition rate. After A431 was treated with endostatin with different concentrations for 24 h, 48 h and 72 h, the cell proliferation inhibition rate was detected by CCK8 assay. It was observed that endostatin inhibited cancer cell proliferation in a dose and time-dependent manner. Note: *: compared with the previous low concentration gradient at the same time point, p<0.05. #: compared with the previous treatment time at the same concentration of endostatin, p<0.05.
Effect of Endostatin on Proliferation Ability of A431 Cells

After endostatin treatment, as shown in Table II, there was no significant difference in OD values (450) at 24 h between the observation group and the control group ($p>0.05$). After 24 h, at each time point, OD values of the observation group were significantly lower than those of the control group ($p<0.05$) at each time point; the OD values (450) in both groups were increased with prolonged time. The CCK-8 proliferation curve is shown in Figure 2.

**Table II.** CCK-8 assay for detection of proliferation activity OD values in each group.

<table>
<thead>
<tr>
<th>Culture time (h)</th>
<th>Observation group</th>
<th>Control group</th>
<th>t</th>
<th>p</th>
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<tr>
<td>12</td>
<td>0.44±0.11</td>
<td>0.41±0.08</td>
<td>0.66</td>
<td>0.52</td>
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<tr>
<td>24</td>
<td>0.99±0.14*#</td>
<td>1.21±0.15#</td>
<td>3.22</td>
<td>0.00</td>
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<td>48</td>
<td>1.37±0.22#</td>
<td>1.81±0.24#</td>
<td>4.05</td>
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</tr>
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<td>72</td>
<td>1.59±0.22#</td>
<td>2.09±0.21#</td>
<td>4.93</td>
<td>0.00</td>
</tr>
<tr>
<td>96</td>
<td>1.67±0.21*</td>
<td>2.34±0.23#</td>
<td>6.45</td>
<td>0.00</td>
</tr>
<tr>
<td>F</td>
<td>66.67</td>
<td>146.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p</td>
<td>0.00</td>
<td>0.00</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: *: at the same time point, compared with the control group, all $p<0.05$. #: in the same group, compared with the previous time point, $p<0.05$.

Effect of Endostatin on Invasion Ability of A431 Cells

After endostatin treatment, transwell experiment showed that the number of invading cells in the observation group and control group were (38.25±8.13) and (98.25±9.14), respectively. The number of invading cells in the observation group was significantly lower than that in the control group ($p<0.01$) (see Figure 3 for details).

Effect of Endostatin on Expression of EMT Related Markers in A431 Cells

The relative expression level of E-cadherin protein in the observation group was (0.34±0.03), which was significantly higher than that in the control group (0.14±0.01). The relative expression levels of N-cadherin protein in the observation and control groups were (0.18±0.05) and (0.43±0.03), respectively, and the expression level of N-cadherin protein in the observation group was significantly lower than that in the control group ($p<0.05$). The relative expression levels of Vimentin protein in the observation and control groups were (0.23±0.08) and (0.56±0.12), respectively. The expression level of Vimentin protein in the observation group was significantly lower than that in the control group ($p<0.05$).
relative expression level of Fibronectin protein in the observation group was (0.42±0.16), which was significantly lower than that in the control group (0.78±0.24) (p<0.05). The relative expression levels of α-SMA protein in the observation and control groups were (0.38±0.12) and (0.16±0.07), respectively. The relative expression of α-SMA protein in the observation group was significantly higher than that in the control group (p<0.05) (see Figures 4 and 5 for details).

**Effects of Endostatin on Tumor Formation in Mouse Xenograft Model**

No significant differences in tumor volume among the three groups at day 0 (p>0.05). Differences begin to show up on day 3. Tumor volume of the three groups increased with prolonged time (Figure 6). Tumor volume in the ES1 and ES2 group was smaller than that in the control group at each time point. After day 6, tumor volume in ES2 group was smaller than that in the ES1 group (p<0.05). The average tumor weight of ES1 group and ES2 group was lower than that of the control group, and the average tumor weight of ES2 group was lower than that of ES1 group (p<0.05) (see Figure 7 for details).

**Discussion**

As a chronic progressive disease with a low metastasis rate, BCC is an invasive and destructive cancer that can spread to deep soft tissue and bone tissue after the necrosis in the center, resulting in invasive necrosis of the skin and severe damage to the function of the corresponding tis-
Chen et al. found that endostatin could reverse EMT in esophageal cancer. The proliferation and invasion of solid tumor cells depend on the angiogenesis, which can be inhibited by endostatin. Therefore, this work investigated the effect of endostatin on the proliferation, migration, invasion and EMT of A431 cells, to understand the role of it in BCC and provide new suggestions for the treatment of it.

After endostatin treatment, the OD values detected by CCK-8 in the observation group were significantly lower than those in the control group at each time point after 24 h, indicating that the cell proliferation ability of the observation group significantly decreased compared with that in the control group, and endostatin inhibited the proliferation of A431 cells. Byekova et al. investigated the effect of endostatin on the resistance genes mdr 1 and gst-π of A431 cells and found that endostatin could inhibit the proliferation of A431, down-regulate expression of mdr 1.
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and gst-π, and increase the sensitivity of cancer cells to chemotherapy. Huang et al.21 found that endostatin could inhibit the proliferation and invasion of gastric cancer cells. This is consistent with the results of this study. After endostatin treatment, the relative expression levels of E-cadherin protein in the observation group was significantly higher than that in the control group, but the expression level of N-cadherin protein in the observation group was significantly lower than that in the control group, suggesting that endostatin could inhibit the N-cadherin expression and promote the E-cadherin expression in A431 cells, to reverse EMT.

In this work, we determined the concentration and action time of endostatin IC50. It was found that the proliferation inhibition of endostatin on A431 was closely related to the concentration and action time. According to the calculated IC50 at 24 h, 48 h and 72 h, the minimum concentration of IC50 and its corresponding action time were used in subsequent experiments. It can be seen from Table I that the inhibition rate of 40 μl/mg treatment for 72 h in the observation group was (46.72±2.98), close to 50%. However, this work also has some deficiencies. The specific pathway and mechanism involved in the endostatin-mediated regulation of the proliferation, invasion and EMT inhibition of A431 cells remain to be studied.

Figure 7. The average tumor weight of ES1 group and ES2 group was lower than that of the control group, and the average tumor weight of ES2 group was lower than that of the ES1 group (p<0.05). Group ES2 was significantly higher than group ES1 (p<0.05). Note: * compared with the control group, p<0.05. #: compared with group ES1, p<0.05.

Conclusions

We found that endostatin can inhibit the proliferation, invasion and EMT of BCC cells. Our study provided new insights to the treatment of BCC.

Conflict of Interest

The Authors declare that they have no conflict of interest.

References


