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Mechanism of decorin protein inhibiting invasion and metastasis of non-small cell lung cancer

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Abstract. – OBJECTIVE: To detect the protein level of Decorin in non-small cell lung cancer (NSCLC) patients, and to study the mechanism of Decorin inhibiting invasion and metastasis of non-small cell lung cancer from the perspective of *in vitro* cells, and provide some theoretical support for the treatment of non-small cell lung cancer.

MATERIALS AND METHODS: Immunohistochemical staining was used to detect the expression of Decorin protein in 332 cases of stage I-IIIA clinical NSCLC and 70 cases of adjacent tissues. Then, *in vitro* cell experiments (cell scratch assay and transwell assay) were used to further study the effects of Decorin on migration and invasion of human lung cancer cell line A549; the effect of transforming growth factor-β on the expression of Decorin and Ets-1 protein was verified by Western blotting. The binding sites of Ets-1 and Decorin promoter were analyzed by bioinformatics.

RESULTS: Exogenous Decorin inhibited invasion and metastasis of lung adenocarcinoma cells *in vitro*. Immunohistochemical staining showed that Decorin was lowly expressed in non-small cell lung cancer and Decorin had a certain effect on lung fibroblast activation. Western blotting results showed that TGF-β affects the expression of Decorin and Ets-1. Bioinformatics results showed that Ets-1 and Decorin gene DNA promoter regions have 18 binding sites.

CONCLUSIONS: Decorin inhibits invasion and metastasis of non-small cell lung cancer through the TGF-β signaling pathway.

Key Words:
Decorin, Non-small cell lung cancer, TGF-β signaling pathway.

Introduction

Lung cancer is one of the most malignant tumors in the world, which seriously threatens the survival and health of human beings. In recent years, its morbidity and mortality have shown a sharp upward trend. Non-small cell lung cancer (NSCLC) accounts for about 80% of all lung cancers. It is the most common histological type in lung cancer. In recent years, its incidence has been increasing, and has become the tumor with the highest mortality. Decorin is a leucine-rich low-protein proteoglycan (PG) present in the extracellular matrix (ECM), which is mainly found in mammalian connective tissue with various biological activity, including participation in the inhibition of collagen fiber formation, regulation of cell proliferation, migration and adhesion[1]. In recent years, with the in-depth study of Decorin, its expression is extremely important for the occurrence and development of tumors, and it is very likely to be a tumor suppressor[2]. Goldoni et al[3] found that Decorin can effectively inhibit the growth and metastasis of breast cancer cells, and proposed Decorin as one of the drug candidates for breast cancer targeted therapy. Horvath et al[4] found that Decorin is lowly expressed in hepatocarcinoma tissues and lacking Decorin promotes liver cancer. It is speculated that Decorin may exert its anti-tumor effect by inhibiting cell proliferation, arresting the cell cycle and increasing Caspase-3 enzyme activity, inhibiting the growth of liver cancer cells. Bi et al[5] showed that Decorin gene expression was low in colorectal cancer tissues, and by increasing the expression level of Decorin in cancer tissues, the growth and metastasis of colorectal cancer cells was inhibited. There may be two mechanisms of action: (1) Decorin binds to transforming growth factor-β (TGF-β), thereby inhibiting the biological activity of TGF-β; (2) Decorin increases p21 expression and inhibits tumor cell growth[6]. It has been well reported that TGF-β has a certain inhibitory effect on lung cancer, but how Decorin inhibits lung cancer by TGF-β is still unclear.

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In the early stage, we have used cDNA microarray technology and Reverse Transcription-Polymerase Chain Reaction (qRT-PCR) technology to identify the specific gene expression of radiation-resistant NSCLC cell lines. The results showed that Decorin gene was included in 43 low-expression genes[7]. At the same time, the expression of Decorin was down-regulated by small interfering RNA (siRNA), which was found to promote the proliferation, migration and invasion of human lung adenocarcinoma A549[8]. At present, there are relatively few studies on the mechanism of Decorin gene on non-small cell lung cancer (NSCLC). This work used immunohistochemical staining, Western blotting, transwell assay and bioinformatics analysis to analyze the expression of Decorin in lung cancer tissues and mechanism of its related effects on NSCLC, and lay a certain theoretical foundation for the treatment of the late stage non-small cell lung cancer.

Materials and Methods

Cell Migration Experiment (Scratch Assay)

Human lung cancer cell A549 (purchased from the American Type Culture Collection cell bank (ATCC, Manassas, VA, USA) was cultured to the logarithmic phase of growth; the original medium was aspirated; the cells were washed three times with sterile Phosphate-Buffered Saline (PBS; Gibco, Grand Island, NY, USA). 1 mL of 0.25% trypsin (HyClone, South Logan, UT, USA) was added to digest the cells; all cells were observed under microscope until became rounded, and then, the complete medium was added for termination, centrifuged at 1000 rpm for 5 min. Cells were plated at a cell density of 2 x 10^4 cells/mL in a 24-well plate and placed in an incubator overnight until adherent. A 200 uL sterile tip was used to align the cell surface from one end of the well to the other. The medium was aspirated and washed 3 times with sterile PBS; the medium containing 1% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) was added as a negative control group, and 25 μg/mL exogenous recombinant Decorin was added as an experimental group, and each group had 3 duplicate wells. This study was approved by the Ethics Committee of Harbin Medical University Cancer Hospital.

Cell Invasion Assay (Transwell Assay)

Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) was placed on a transwell chamber filter and placed in a 37°C incubator overnight. The cell suspension was prepared according to the method of 1.1, and the cells were resuspended in serum-free medium to adjust the cell density to 1×10^5 /mL. 100 uL of the cell suspension was slowly added to the transwell chamber along the inner wall of the chamber, and 500 uL of complete medium containing 10% fetal bovine serum was added to the lower chamber of the 24-well plate and placed in a 37°C incubator for cultivation. After culturing for 24 hours, the medium was discarded, and the cells in Matrigel and the chamber were gently wiped with a cotton swab, and stained with 500 uL of 0.1% crystal violet; the mixture was washed with PBS several times at room temperature for 15 min, and photographed under a microscope.

Immunohistochemical Staining and Semi-Quantitative Analysis of Results

Immunohistochemical staining was performed on 332 cases of stage I-IIIA NSCLC and 70 cases of paracancerous lung tissues. The streptavidin method (SP method) and the mouse Decorin antibody (Abcam, purchased from Shanghai Yanjing Co., Ltd., China) were diluted by 1:200; paraffin sections were dewaxed with xylene, gradual ethanol (100%, 95%, 80%, 70%) hydration, 3% H_2O_2 methanol aqueous solution at room temperature for 15 min, washed with PBS 3 times; the serum was closed at room temperature for 10 min, after removing the serum, and the diluted primary antibody was added overnight at 4°C; after being washed 3 times with PBS, biotin-labeled secondary antibody (Abcam, purchased from Shanghai Yanjing Co., Ltd., China) was incubated at 37°C for 30 min; after being washed with PBS 3 times, Streptomyces avidin-catalase (Shanghai Qiaoyu Biotechnology Co., Ltd., QY-MY601J, China) was added, incubated at 37°C for 30 min; DAB color development, hematoxylin counterstaining, slice gradient dehydration (70%, 80%, 95%, 100% ethanol), xylene transparent for 5 min, and the neutral resin was sealed and observed under an optical microscope.

In this work, the results of immunohistochemical staining were analyzed by semi-quantitative scoring method. The specifics were as follows: the tissue sections were divided into negative staining, light yellow, light brown, dark brown by 0-3 under the light microscope. The positive range was scored (1-4 points were 0-25%, 26-50%, 51-75%, 76-100%), and the final scores were added and compared.

Western Blotting

Plates were plated at a cell density of 2 x 10^4 cells/mL in 24-well plates and placed in a 37°C, 5% CO_2 cell culture incubator overnight. Adherent lung fibroblasts were then treated with different concentrations of TGF-β (0, 0.1, 1 ng/mL, respectively) for 24 h. After discarding the medium, it was washed 3 times with PBS, and then 300 μL of radioimmunoprecipitation assay (RIPA; Beyotime, Shanghai, China) cell lysate was added and lysed on ice for 30 min. The cells were gently scraped and centrifuged at 12,000 rpm for 10 min at 4°C. The protein concentration was determined by the bicinchoninic acid (BCA) method. Then, 500 μg total protein was taken for SDS-PAGE electrophoresis, then transferred to polyvinylidene difluoride (PVDF) membrane, added 5% skim milk powder for 90 min at 37°C, added primary antibody (Decorin, Ets-1, α-tubulin; diluted at 1:1000), incubated overnight at 4°C, rinsed with Tris-Buffered Saline and Tween (TBST; Sigma-Aldrich, St. Louis, MO, USA) 3 times for 10 min each time, added secondary antibody (Abcam, purchased from Shanghai Yanjing Co., Ltd., China, diluted 1:1000) for 4 h, and developed by electrochemiluminescence (ECL; Thermo Fisher Scientific, Waltham, MA, USA). Finally, the Grayscale calculation was performed on the conditions in the Western blotting results using Image-Pro Plus software.

Bioinformatics Analysis

PROMO software (TRANSFAC version 8.3) was adopted, the target gene DCN was entered as a promoter region, finding its transcription factor site and further predicting whether Ets-1 has a binding site with the DCN promoter region. The detailed steps were as follows: first open http://alggen.lsi.upc.es/cgi-bin/promov3/promo/promoinit.cgi?dirDB=TF 8.3, then step 1: select species: select human, select factors: select Ets-1[T00112]; Step 2: search Sites: enter the promoter area of the DCN.

Statistical Analysis

The experimental data were analyzed by SPSS 13.0 statistical software (SPSS Inc., Chicago, IL, USA), using mean ± standard deviation. The two groups were compared by *t*-test, and the mean of both groups was analyzed by Analysis of Variance (ANOVA). $p<0.05$ for the difference was considered statistically significant.

Results

Effects of Exogenous Decorin on Migration and Invasion of A549 Cells

The scratch test is shown in Figure 1. It can be seen from Figure 1A that the scratches disappeared after 72 hours of treatment in the con-

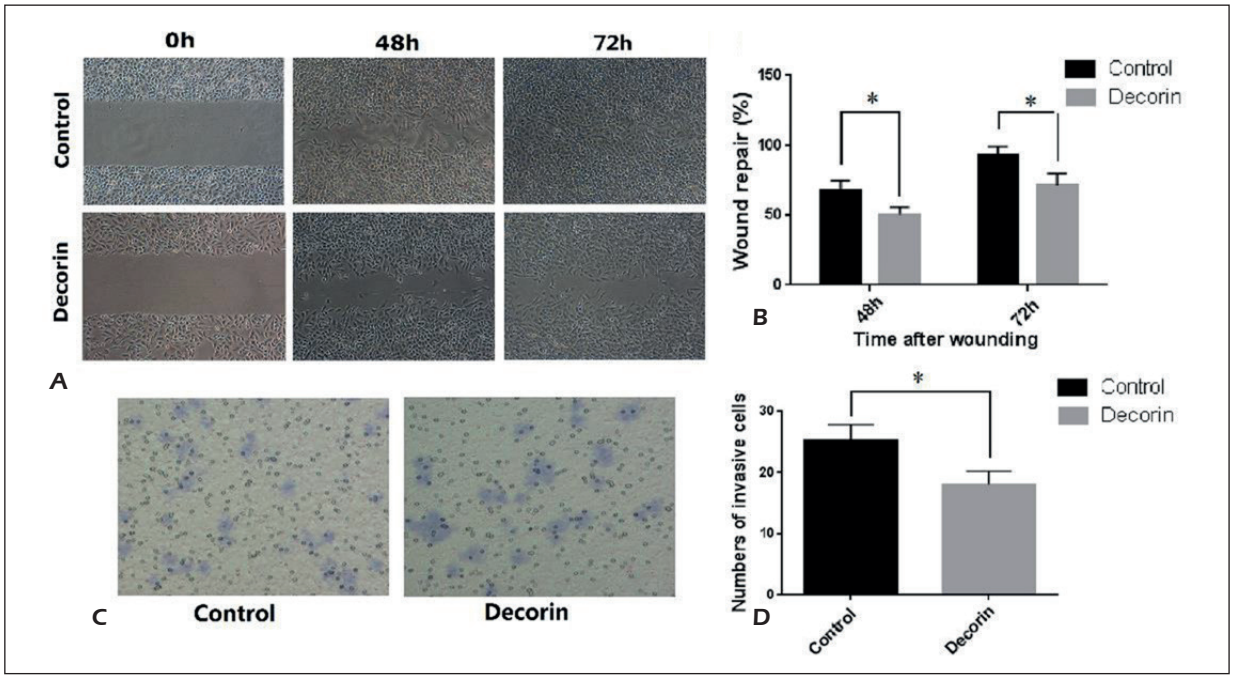

Figure 1. Effect of exogenous Decorin on migration and invasion of lung adenocarci-noma cell line A549 (*indicates $p<0.05$).

trol group, and there were certain scratches in the experimental group. The area of the scratch was calculated by Image Plus software, and the scratch repairability was calculated. The ability shown in Figure 1B showed significant differences in the repairability between the control group and the experimental group at 48 h and 72 h, indicating that exogenous Decorin has a certain inhibitory effect on the migration of A549 cells and reduces the migration ability of lung adenocarcinoma cells.

As can be seen from Figures 1C and 1D, after the Decorin treatment of lung adenocarcinoma A549 cells, the invasive ability of the cells was inhibited.

Immunohistochemical Staining Results Immunohistochemical Detection of Decorin Expression in NSCLC Tissues

Immunohistochemical staining showed that Decorin was lowly expressed in the interstitial tissues of NSCLC. The positive rate of Decorin in the tumor stroma was 64%, while the positive rate in normal tissues was as high as 95%, and the difference was statistically significant ($p<0.001$). By staining results, the degree of Decorin-positive staining in the tumor stroma was generally weaker than that in the normal interstitial tissue (Figure 2). Comparing the semi-quantitative immunohistochemical scores,

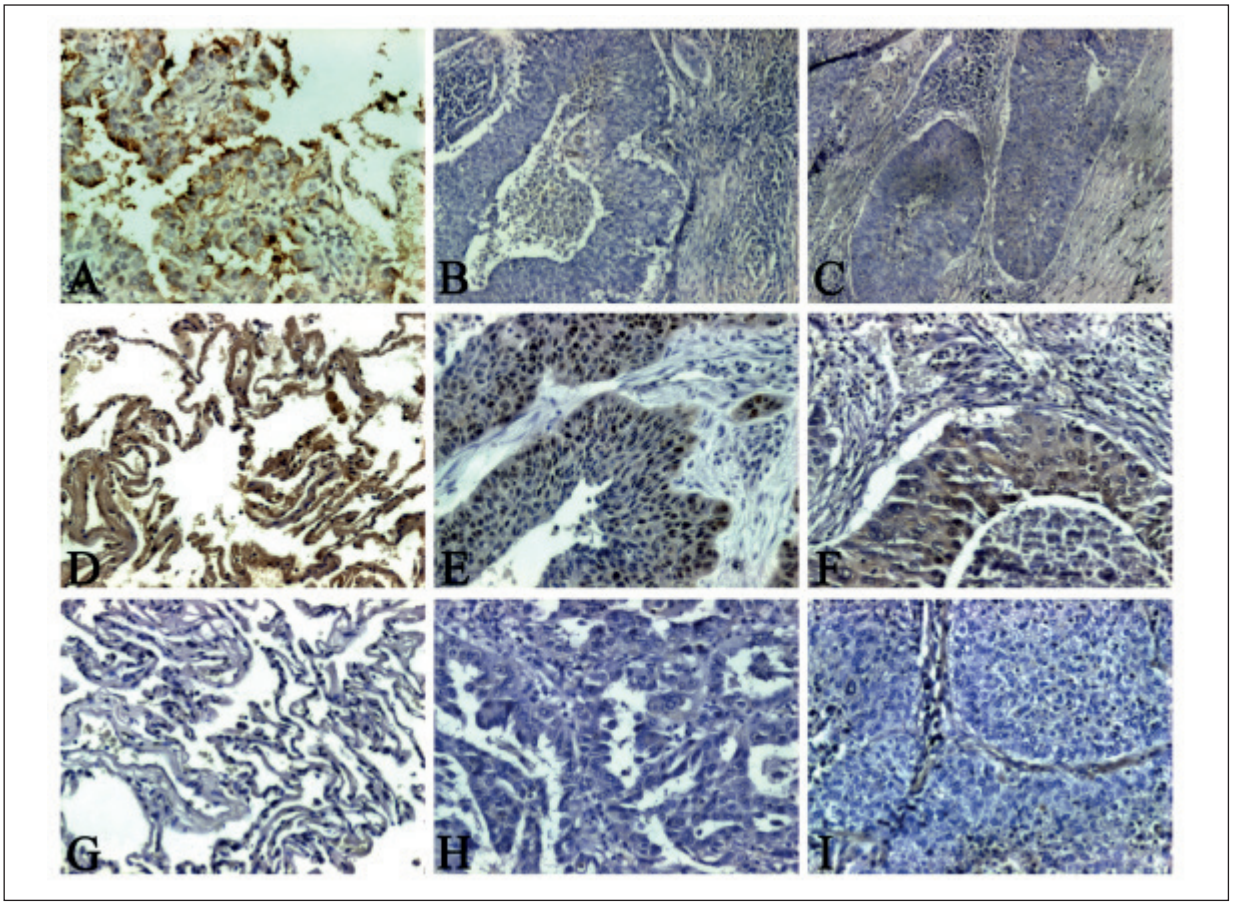

Figure 2. Immunohistochemical staining for Decorin expression in normal lung tissue, lung adenocarcinoma, and non-small cell lung cancer (NSCLC). ABC Decorin expres-sion in the stroma (×200). **A**, Decorin is positively expressed in the adjacent bronchial epithelial stroma. **B**, Decorin is positively expressed in the lung adenocarcinoma. **C**, Decorin is positively expressed in the squamous cell carcinoma of the lung. **D-F**, Decorin expression in different tissue cells (×200). **D**, Decorin is positively expressed in normal alveolar epithelium. **E**, Decorin is positively expressed in lung adenocarcinoma cells. **F**, Decorin is positively expressed in lung squamous carcinoma cells. **G-I**, Decorin negative expression (×200). **G**, Decorin is negatively expressed in normal alve-olar epithelium. **H**, Decorin is negatively expressed in lung adenocarcinoma cells and stroma. **I**, Decorin is negatively expressed in lung squamous cell carcinoma and inter-stitium.

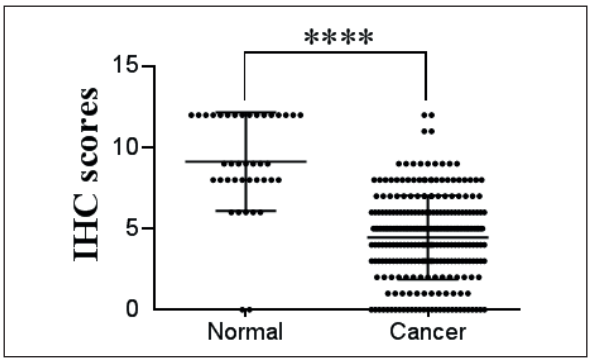

Figure 3. Decorin expression IHC score in NSCLC interstitial is significantly lower than normal tissue interstitial.

the Decorin expression score in the tumor stroma was significantly lower than that in the normal interstitial tissue ($p<0.001$; Figure 3). The correlation analysis between Decorin interstitial expression and clinical features showed that the Decorin positive rate in adenocarcinoma was significantly lower than that in squamous cell carcinoma (58.3% *vs.* 70.4%, $p = 0.041$), and compared with that in high-grade differentiated adenocarcinoma, the positive expression rate of Decorin in differentiated adenocarcinoma was lower (51.3% *vs.* 67.8%, $p=0.037$).

Study of Decorin and α-SMA in the Interstitial of NSCLC

Immunohistochemistry was used to detect the expression of α-SMA in tumor-associated fibroblasts (CAFs) markers in NSCLC tissues. It was found that Decorin interstitial expression was negatively correlated with α-SMA ($p=0.009$) (Figure 4), suggesting that there is a certain correlation between the decrease in Decorin expression and the activation of lung fibroblasts.

TGF-β Enhances the Expression of Ets-1 and Inhibits the Expression of Decorin in Lung Fibroblasts

Western blotting revealed that TGF-β-treated lung fibroblasts (TIG-7) enhanced the expression of transcription factor Ets-1, inhibited Decorin expression and increased with increasing of TGF-β concentration, indicating a concentration-dependent effect. (Figure 5). The DNA binding sites of Ets-1 and DCN (Decorin gene) promoter region were predicted by bioinformatics software, and the results showed that there were 18 potential DNA binding sites (Figure 6).

Discussion

Decorin is one of the important components of the extracellular matrix, which regulates and controls biological processes such as cell proliferation, differentiation and collagen fiber formation[9]. In recent years, it has been found that Decorin has a certain inhibitory effect on various types of tumors, and may be an important inhibitor of tumor development and metastasis.

McDoniels et al[10] detected the gene expression in lung adenocarcinoma and squamous cell carcinoma by gene chip and quantitative PCR, and found that Decorin gene was lowly expressed in lung adenocarcinoma and squamous cell carcinoma. In this study, the expression of Decorin in non-small cell lung cancer and normal tissues was detected by immunohistochemical staining. The Decorin positive rate in the tumor stroma was 64%, and the positive rate in normal tissues was as high as 95%. Decorin is lowly expressed in NSCLC, which is consistent with the findings of McDoniels et al[10]. Decorin may have a certain relationship with the occurrence and development of tumors. The addition of exogenous Decorin was

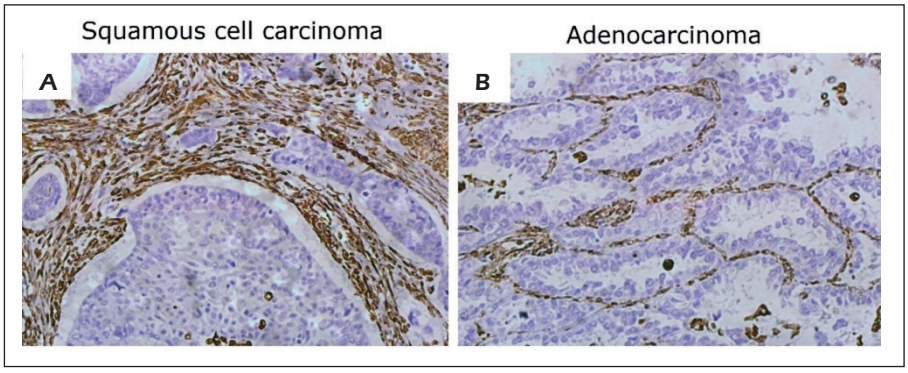

Figure 4. A, Positive expression of α-SMA in the squamous cell carcinoma of the lung (×200). **B**, Positive expression of α-SMA in lung adenocarcinoma (×200).

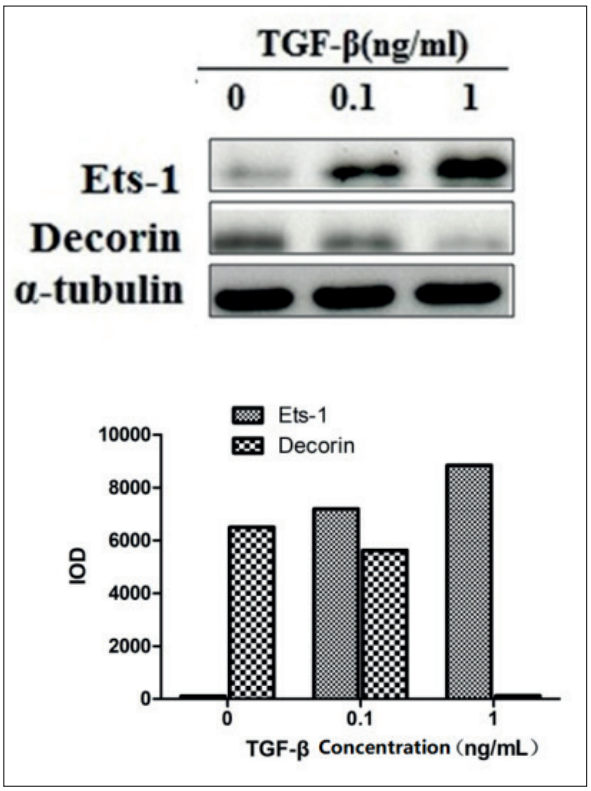

Figure 5. TGF-β enhances Ets-1 expression and inhibits Decorin expression in human lung fibroblasts TIG-7.

found to inhibit invasion and migration of lung adenocarcinoma A549 cells *in vitro*. Previously, Goldoni et al[11] found that exogenous Decorin protein can inhibit the growth of breast cancer cells.

In the study of the molecular mechanism of non-cellular lung cancer, the TGF-β signaling pathway has attracted the attention of researchers. According to the literature, Decorin has a functional binding domain of TGF-β, which binds to TGF-β as a heteromeric complex and then to the collagen of the extracellular matrix, thereby inhibiting the expression of the TGF-β signaling pathway[12,13]. However, it is unclear how the mechanism of the TGF-β signaling pathway affects the metastasis and development of non-small cell lung cancer.

With the deepening of research on tumor biology and immunology, more and more researchers believe that the occurrence of tumors is closely related to the tumor microenvironment. CAF is an important component of primary or metastatic tumor stroma and plays an important role in tumor formation and progression. α-SMA is generally used as a marker molecule for fibroblasts. This study found that Decorin interstitial expression was negatively correlated with α-SMA, indicating a decrease in Decorin expression is correlated with activation of lung fibroblasts. Later, it was found that TGF-β treatment of lung fibroblasts (TIG-7) enhanced the expression of transcription factor Ets-1 and inhibited Decorin expression. Bioinformatics analysis revealed that there are 18 potential DNA binding sites in the DNA binding site of the promoter region of Ets-1 and DCN (Decorin gene).

Conclusions

Decorin may inhibit the invasion and metastasis of non-small cell lung cancer through the TGF-β signaling pathway. TGF-β acts on TFG-βRI/II in the cytoplasm of lung fibroblasts, and then promotes Ets-1 gene transcription; Ets-1

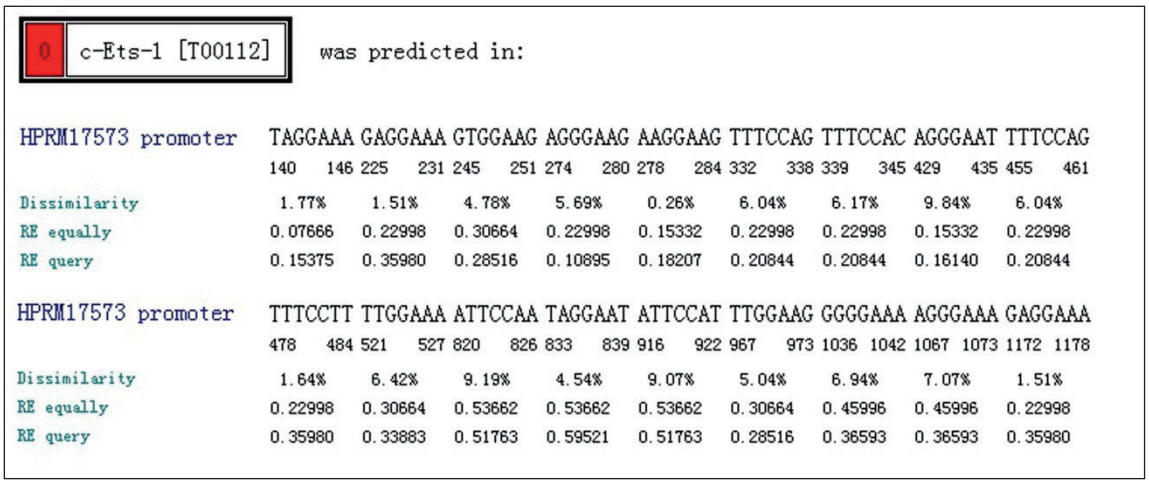

Figure 6. Bioinformatics tools predict the presence of 18 potential DNA binding sites in the promoter region of Ets-1 and DCN.

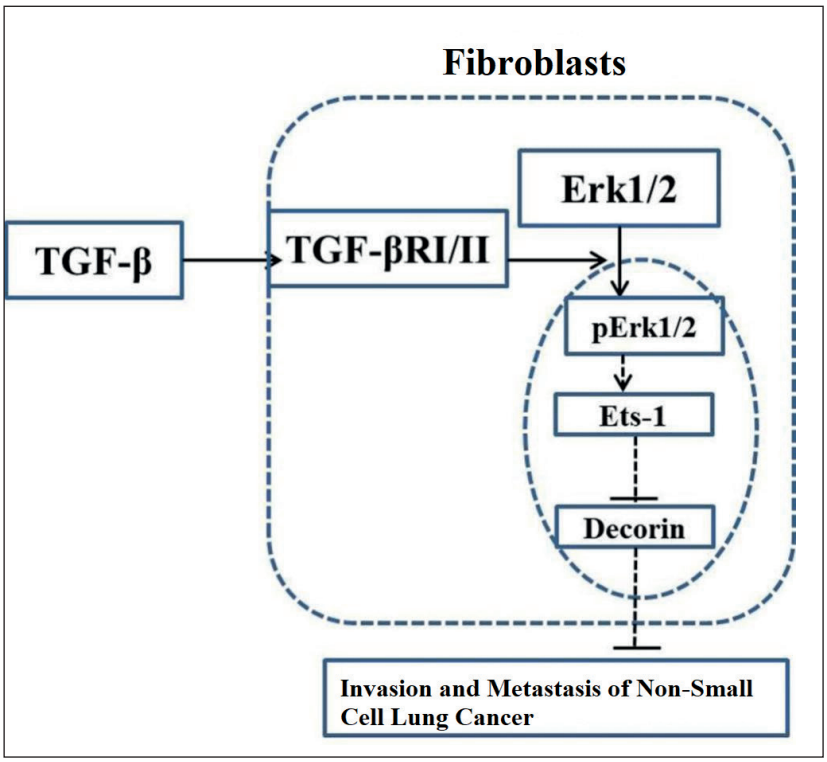

Figure 7. Research hypothesis mechanism diagram.

gene binds to the promoter region of DCN and inhibits the expression of Decorin protein, thereby inhibiting the invasion and metastasis of non-small cell lung cancer. The hypothesis is shown in Figure 7, which provides a theoretical basis for the clinical application of Decorin gene therapy.

Acknowledgments

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Conflict of Interests

The authors declare that they have no conflict of interest.

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