

## Original Article

# Long non-coding RNA MALAT1 regulates cell proliferation, invasion and apoptosis by modulating the Wnt signaling pathway in squamous cell carcinoma

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**Abstract:** Objective: To explore the mechanisms by which long non-coding RNA, (lncRNA) metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) regulates cell proliferation, invasion and apoptosis through signaling axes in cutaneous squamous cell carcinoma (CSCC) cells. Methods: A total of 60 CSCC samples and 15 normal skin tissue samples were collected. qRT-PCR was used to determine MALAT1 expression. After knockdown of MALAT1 expression in A431 cells, Transwell assay was performed to detect cell migration and invasion, CCK8 assay was used to detect cell proliferation, and Western blotting was used to detect EMT-related protein expression. Results: Compared with the normal group, the MALAT1 positive expression rate was significantly higher in the low, moderate, and high differentiation groups ( $P < 0.05$ ). The expression of MALAT1 in A431 cells in the siMALAT1-1 and siMALAT1-2 groups was lower than that in siNC group ( $P < 0.05$ ). A431 cell proliferation, invasion and apoptosis at 24 h, 48 h and 72 h in the siMALAT1-1 and siMALAT1-2 groups were all lower and the apoptosis rate of A431 cells were all higher than that of the siNC group ( $P < 0.05$ ). E-cadherin expression was higher while the expression of  $\beta$ -catenin, vimentin, and Bcl-2 was lower in the siMALAT1-1 and siMALAT1-2 groups than those of the siNC group ( $P < 0.05$ ). Conclusion: Down-regulation of lncRNA MALAT1 expression may promote apoptosis of CSCC cells and inhibit cell migration, invasion and proliferation by regulating the Wnt signaling pathway.

**Keywords:** Cutaneous squamous cell carcinoma, long non-coding RNA, cell proliferation, invasion, apoptosis

## Introduction

Cutaneous squamous cell carcinoma (CSCC) is a common malignant tumor originating from squamous epithelial tissue of the skin, and it is characterized by strong invasive ability, frequent recurrence and drug resistance. The pathogenesis has not been fully elucidated so far, but it is mostly believed to be related to immunosuppression, UV exposure, human papillomavirus infection and other skin diseases [1, 2]. The development and occurrence of malignant tumors are related to the inactivation of tumor-suppressor genes, the external environment, genetic factors and abnormal signaling pathways, caused by a combination of multi-pathway, multi-gene and multi-stage factors. Therefore, it is particularly important to understand the pathogenesis and early diagnostic targets of CSCC and search for mole-

cules that play a key role in the development of the disease in order to formulate treatment plans and improve prognosis [3, 4].

Long non-coding RNA (lncRNA) belongs to a class of RNA molecules with a length of about 200 nucleotides that can regulate gene expression. Although lncRNA cannot encode proteins, it can regulate protein transcription and expression indirectly [5, 6]. So far, many lncRNAs have been proven to regulate gene expression by maintaining mRNA stability, chromosomal modification, preventing miRNA degradation of mRNA, and epigenetic mechanisms to regulate gene expression, thereby regulating tumor growth, invasion and metastasis [7-9]. Metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) is one of the first lncRNAs that was discovered. It can participate in the biological processes of tumor growth, invasion,

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proliferation and apoptosis through alternative splicing or transcriptional regulation [10]. It has been found that the Wnt/ $\beta$ -catenin signaling pathway plays an important role in skin development, mainly promoting the division and proliferation of immature cells and promoting the proliferation and differentiation of normal skin stem cells into hair cells. Xiang et al [11] found that the positive expression of  $\beta$ -catenin in CSCC was significantly higher than that in normal skin tissue, and it played an important role in promoting the occurrence and development of CSCC. However, there are no clinical studies on the role and mechanism of MALAT1 in the biological behavior of CSCC cells. This study analyzed the mechanism by which lncRNA MALAT1 regulates cell proliferation, invasion and apoptosis through regulating the signaling pathway axis in CSCC cells, aiming to provide new targets for the diagnosis and treatment of CSCC.

### Material and methods

#### Materials

Sixty CSCC samples (20 highly differentiated, 29 moderately differentiated, and 11 low differentiated) were collected from January to December 2018 at our hospital and another 15 normal skin tissue samples were collected from the department of plastic surgery in our hospital. The CSCC cell line (A431) was obtained from the American Typical Culture Collection. Inclusion criteria: patients with complete relevant clinical data and postoperative pathological and diagnosed with CSCC were included. Exclusion criteria: patients with incomplete clinical medical records; patients with relapse at the first visit; patients who had received surgical treatment, preoperative radiotherapy and chemotherapy in other hospitals; patients with metastases from other organs; patients with chronic kidney disease, liver cirrhosis, heart failure, and immune system deficiency; and patients complicated with other confirmed malignant tumors. This study has been approved by the Ethics Committee of Shanghai Baoshan Hospital of Integrated Traditional Chinese and Western Medicine. All study participants provided written informed consent before participating in the study.

#### Main reagents

Fetal bovine serum, DMEM high glucose medium and trypsin (Hyclon, USA), CCK-8 reagent

(Fuyuan (Shanghai) Biotechnology Co., Ltd., China), goat anti-rabbit IgG-horseradish peroxidase (Shanghai Yujin Biotechnology Co., Ltd., China), Transwell dx room (Millipore, United States), DAB color development kit (Shanghai Jizhi Biochemical Technology Co., Ltd., China), Total RNA extraction kit, Lipofectamine<sup>TM</sup> 2000 liposome transfection kit (Invitrogen, USA), and primers were synthesized by Invitrogen Biotech, USA.

#### Main instruments

Ultra-clean workbench (Beijing Jiayuan Industrial Technology Co., Ltd., China), 1-5K desktop high-speed low-temperature centrifuge (Haiyihui Biotechnology Co., Ltd., China), -80°C ultra-low temperature freezer (Shanghai Fuze Trading Co., Ltd., China), cell culture plate, EP tube (Gibco, USA), flow cytometer (Beckman coulter, USA), and a real-time fluorescent quantitative PCR instrument (Roch, Switzerland).

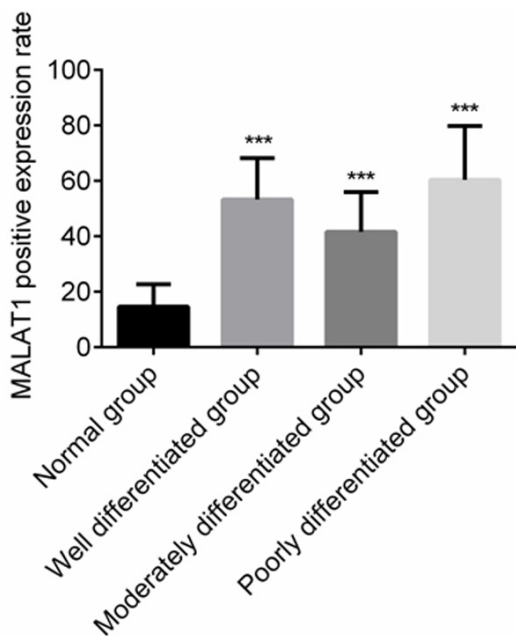
#### Cell culture

A431 cells were cultured in DMEM high sugar medium (containing 100 U/ml penicillin + 100 mg/L streptomycin + 10% fetal bovine serum) and placed in an incubator at 5% CO<sub>2</sub>, 37°C, with liquid exchange every 2 d. Cell morphology was observed under an inverted microscope, and passages were performed according to 1:3.

#### Cell transfection

MALAT1 was knocked down in A431 cells using Lipofectamine 2000 and small interfering RNAs (siNC, siMALATI-1, siMALATI-2), and the cells were laid out 1 d earlier. The next day, when the confluence reached 70-80%, Lipofectamine 2000 and small interfering RNAs (siNC, siMALATI-1, siMALATI-2) were used to co-transfect the cells.

MALAT1 expression was detected by reverse transcription RT-PCR, and total RNA was extracted with Total RNA and reverse transcribed with M-MLT reverse transcriptase, with a reaction system of 20  $\mu$ L, and the cDNA was stored in an ultra-low temperature freezer. GAPDH was used as an internal reference, upstream: 5'-GGTGAAGGTCGGAGTCAACGG-3', downstream: 5'-GAGGTCATGAAGGGGTCATTG-3'. MALAT1 upstream: 5'-AAAGCAAGGTCTCCCA CAAG-3', downstream: 5'-GGTCTGTGCT-



**Figure 1.** Comparison of MALAT1 positive expression rates ( $\bar{x}$  %). Note: Compared to the normal group, \*\*\* $P < 0.001$ .

AGATCAAAAGG CA-3'. PCR conditions: MALAT1: 95°C for 5 min, 95°C for 10 s, 60°C for 45 s, over 40 cycles.  $\Delta C(t)$  values were calculated using Opticon 3 software, and target gene expression was expressed as  $2^{-\Delta\Delta Ct}$ .

*Transwell assay was used to detect cell migration and invasion*

When the confluence of cells reached 70%-80%, the cells were transfected with Lipofectamine 2000 and small interfering RNAs and were harvested 24 hours later, cultured in DMEM high sugar medium without serum for 24 hours.  $1 \times 10^5$ /mL cells (100  $\mu$ L) were spread on the surface of the upper chamber, 700  $\mu$ L DMEM high sugar medium containing serum was added to the lower chamber and incubated for 12-16 h at 5% CO<sub>2</sub> and 37°C. Transwell inserts were removed and the cells were fixed in the transwell insert with formaldehyde for 10 min, followed by staining. The number of permeabilized cells was observed under  $\times 200$  microscope. Five high magnification fields were selected to count the average number of migrating cells.

*Detection of cell proliferation by CCK8 method*

A density of  $5 \times 10^5$  cells were seeded in 96-well plates at 100  $\mu$ L/well, and the cells were incu-

bated for 24 h, 48 h and 72 h in 20  $\mu$ L of CCK8 solution, and placed in an incubator in the dark.

*Determination of apoptosis by flow cytometry*

A431 cells were collected 48 h after transfection, centrifuged for 5-10 min, rinsed 3 times with pre-cooled PBS, fixed in 70% ethanol for 30 min, rinsed 3 times with pre-cooled PBS. The cells were precipitated by centrifugation again, the supernatant was removed, and the binding buffer was added to resuspend the cells to prepare the single-cell suspension with the concentration of  $5 \times 10^6$ /mL. Then 100  $\mu$ L of the single-cell suspension was added into a 1.5 mL centrifuge tube. After adding 5  $\mu$ L of Annexin V-FITC, the cells were incubated at room temperature for 5 min in the dark, followed by adding 10  $\mu$ L of 20  $\mu$ g/mL PI solution and 400  $\mu$ L of binding buffer and mixed well. Next, 10,000 cells were measured by flow cytometry after filtration into flow tube with 300-mesh screen, and apoptosis was analyzed by CellQuest software.

*Western blotting for changes in EMT-related protein expression*

Cells were collected, lysed and extracted for total protein. The protein concentration was quantified by BCA according to kit instructions. ECL development was used to analyze the intensity of the bands. Expression of E-cadherin,  $\beta$ -catenin, vimentin, and Bcl-2 was determined using GAPDH as an internal reference.

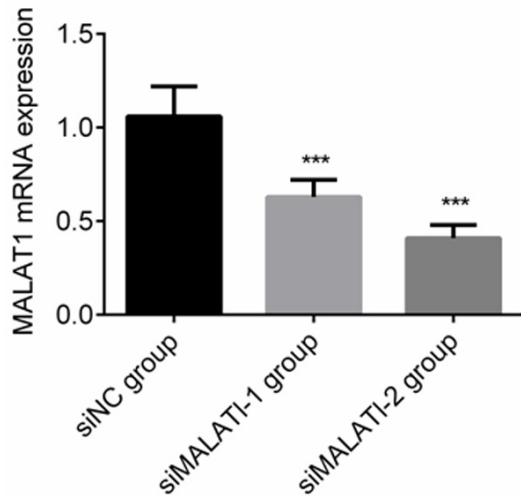
*Statistical analysis*

The SPSS 23.0 was the analytic tool. The measurement data was examined by the normal distribution test. Data conforming to a normal distribution was expressed as  $\bar{x} \pm s$ . The comparison between multiple groups was examined using one-way ANOVA with post hoc LSD test.  $P < 0.05$  indicated the difference is significant.

**Results**

*Comparison of MALAT1 positive expression rate*

The positive expression rate of MALAT1 was significantly higher in the high, moderate, and low differentiation groups than in the normal group ( $P < 0.05$ ), indicating that MALAT1 was highly expressed in CSCC tissues compared to normal tissues (**Figure 1**).



**Figure 2.** Comparison of MALAT1 expression of A431 cells after down-regulation of MALAT1 expression. Note: Compared to the siNC group, \*\*\* $P < 0.001$ .

#### *Establishment of MALAT1 silencing model*

The expression of MALAT1 of A431 cells in the siMALATI-1 and siMALATI-2 groups was lower than that in the siNC group ( $P < 0.05$ ), indicating that small interfering RNAs could reduce the expression of MALAT1 in A431 cells and MALAT1 silencing model was successfully established (**Figure 2**).

#### *Comparison of migration and invasion ability of A431 cells after down-regulation of MALAT1 expression*

The number of migratory cells and invasive cells in siMALATI-1 and siMALATI-2 groups were all lower than those in siNC group ( $P < 0.05$ ), suggesting that down-regulation of MALAT1 expression could inhibit the migration and invasive ability of A431 cells (**Figure 3**).

#### *Comparison of changes in proliferation of A431 cells after down-regulation of MALAT1 expression*

Compared with the siNC group, the number of A431 cells proliferating in the siMALATI-1 and siMALATI-2 groups was significantly lower ( $P < 0.05$ ), indicating that down-regulation of MALAT1 expression could cause a decrease in A431 cell proliferation and inhibits their proliferative capacity (**Figure 4**).

#### *Comparison of apoptosis rate of A431 cells after down-regulation of MALAT1 expression*

The apoptosis rate of A431 cells was higher in the siMALATI-1 group and the siMALATI-2 group than in the siNC group ( $P < 0.05$ ), indicating that down-regulation of MALAT1 expression could enhance the apoptosis rate of A431 cells (**Figure 5**).

#### *Comparison of EMT-related protein expression after down-regulation of MALAT1 expression*

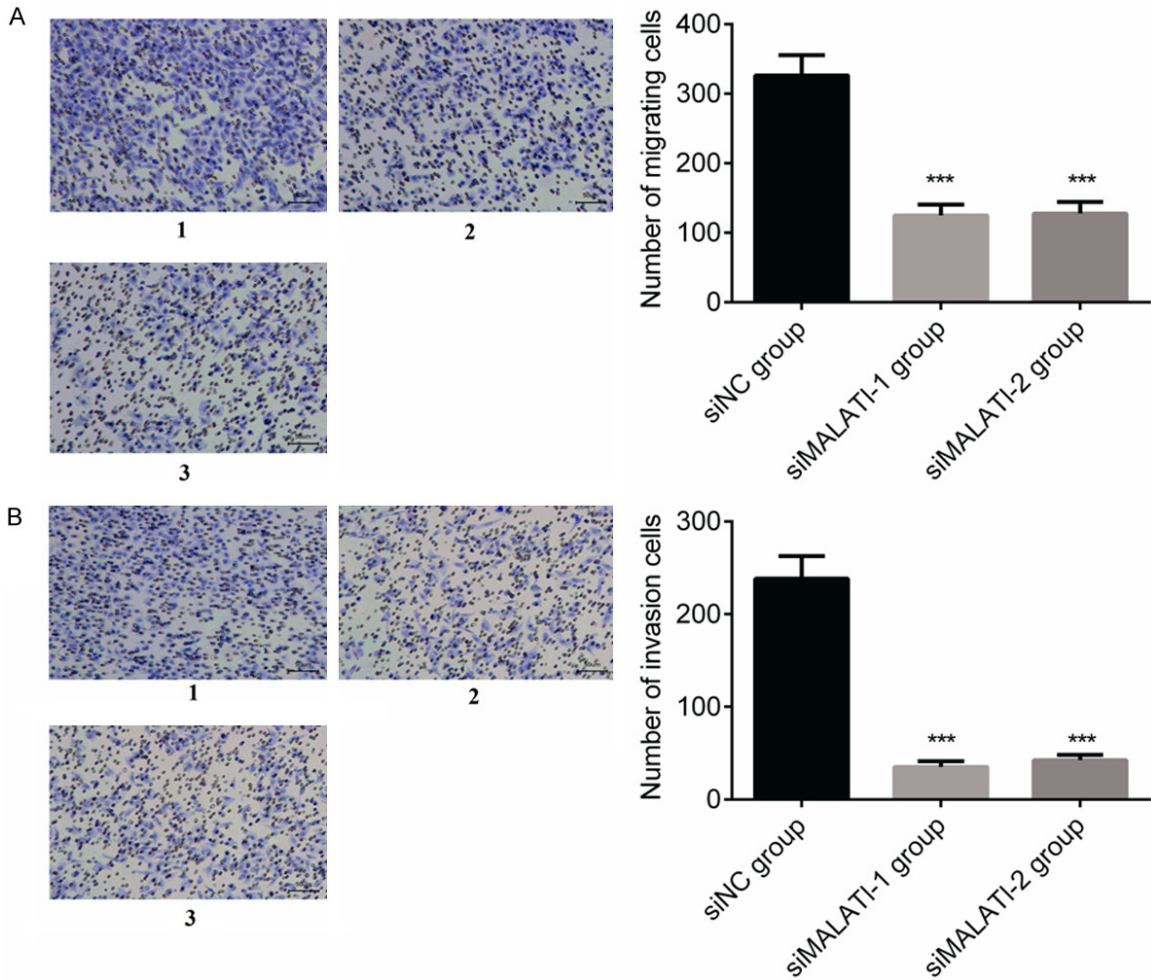
The expression of E-cadherin was higher, and the expression of  $\beta$ -catenin, vimentin, and Bcl-2 was lower in the siMALATI-1 and siMALATI-2 groups than that in the siNC group ( $P < 0.05$ ), showing that down-regulation of MALAT1 expression could promote EMT progression (**Figure 6**).

### **Discussion**

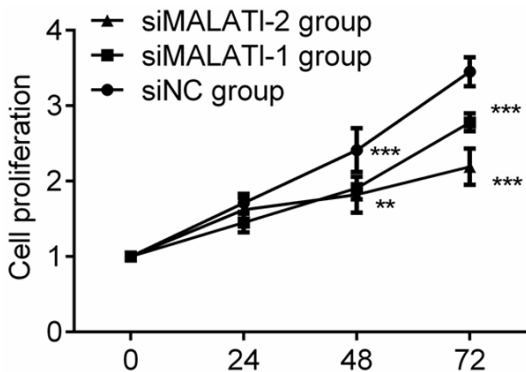
The pathogenesis of CSCC has not been fully elucidated, but an imbalance in cell proliferation and apoptosis has been shown to be involved in the progression of the disease [12, 13]. Li et al. [14] have shown that the abnormal expression of MALAT1 is closely related to the proliferation and apoptosis of many tumor cells, and it is expected to become a potential biomarker for tumor diagnosis and prognosis. This study analyzed the pathogenesis of CSCC from the perspective of LncRNA and found that MALAT1 can regulate many biological behaviors of cells by regulating the Wnt signaling pathway in CSCC cells.

Lai et al. [15] found that MALAT1 was highly expressed in hepatocellular carcinoma patients, and after inhibiting its expression, the motility, survival and invasion ability of hepatocellular carcinoma cells were suppressed. In this study, MALAT1 expression in A431 cells in the siMALATI-1 and siMALATI-2 groups was lower than that in the siNC group, indicating that small interfering RNAs could reduce MALAT1 expression in A431 cells. After knocking down MALAT1 in CSCC A431 cells, the number of A431 cell migration, invasion and proliferation decreased, indicating that knocking down MALAT1 expression could inhibit the migration, invasion and proliferation of CSCC cells.





**Figure 3.** Comparison of migration and invasion ability of A431 cells after down-regulation of MALAT1 expression. Note: A: Number of A431 cell migration. B: Number of A431 cell invasion. Compared with the siNC group, \*\*\* $P < 0.001$ .



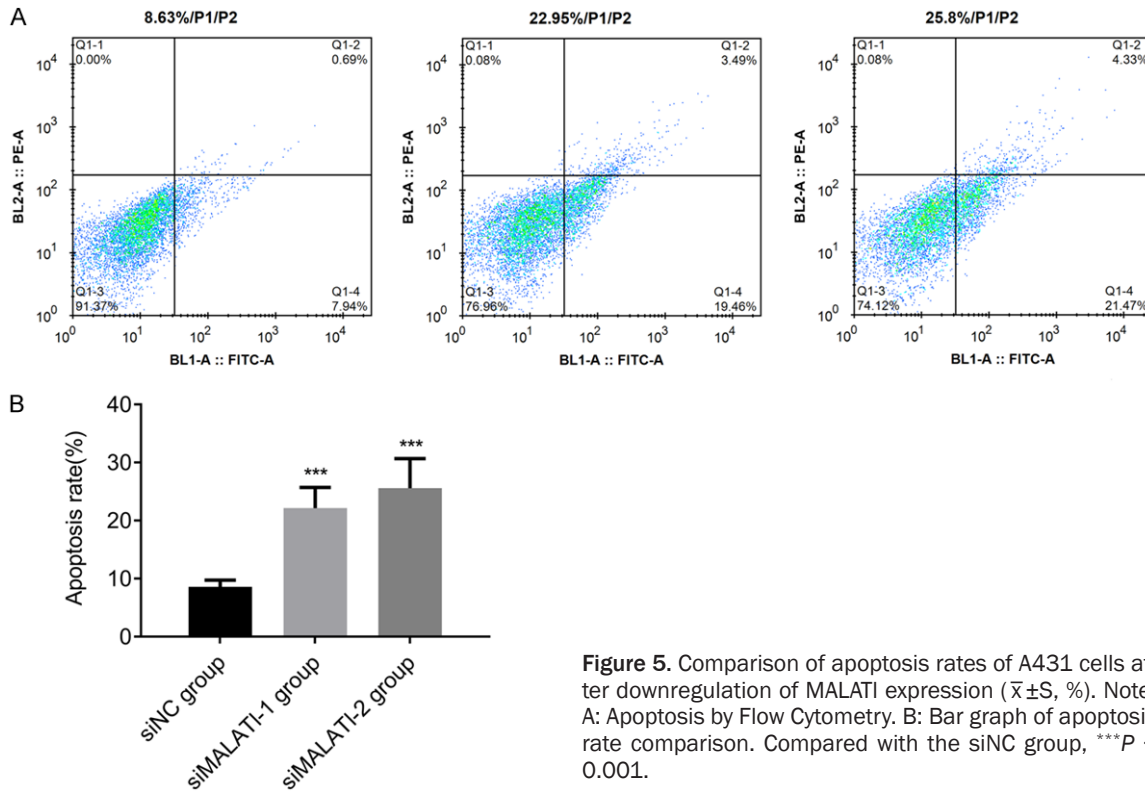
**Figure 4.** Comparison of the changes in A431 cell proliferation after down-regulation of MALAT1 expression ( $\times 10^3$ ). Note: Compared to the siNC group, \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

Inhibition of the degradation of  $\beta$ -catenin protein is the key to the activation of the Wnt/ $\beta$ -

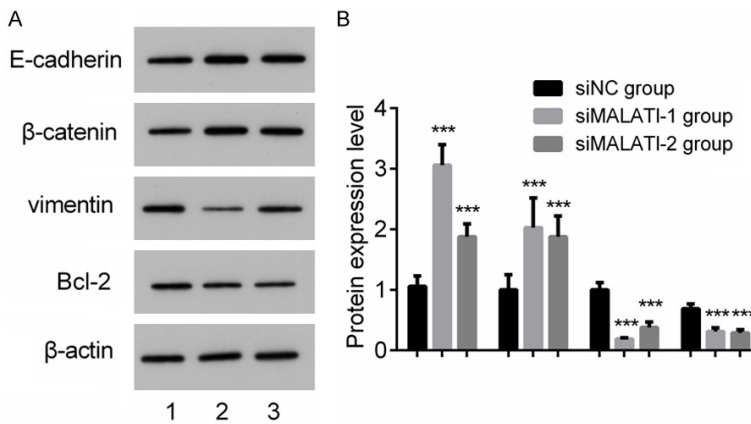
catenin signal transduction pathway. Abnormal expression of  $\beta$ -catenin can promote the abnormal activation of Wnt/ $\beta$ -catenin signal transduction pathway, leading to tumorigenesis [16, 17]. Hu et al. [18] found that the abnormal expression rate of  $\beta$ -catenin in CSCC was as high as 96.00%, and the Wnt signaling pathway may play a certain role in the process of epidermal tumor formation.

Li et al. [19] used RT-qPCR to detect the content of lncRNA MALAT 1 in the arterial tissue of mice on a high-fat diet. After knocking out MALAT 1, they found that overexpression of MALAT1 enhanced the effect of Wnt/ $\beta$ -catenin signal activation. Liang et al. [20] found that lncRNA MALAT 1 is up-regulated in TSCC tissues, and it induces cell migration and invasion by regulating the Wnt/ $\beta$ -catenin signaling path-

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**Figure 5.** Comparison of apoptosis rates of A431 cells after downregulation of MALAT1 expression ( $\bar{x} \pm S$ , %). Note: A: Apoptosis by Flow Cytometry. B: Bar graph of apoptosis rate comparison. Compared with the siNC group, \*\*\* $P < 0.001$ .



**Figure 6.** Comparison of EMT-related protein expression after down-regulation of MALAT1 expression. Note: A: Protein band. B: Bar graph of protein expression. Compared with the siNC group, \*\*\* $P < 0.001$ .

way, and inhibits cell apoptosis, while inhibiting Wnt/ $\beta$ -catenin signaling pathway can reduce the effect of exogenous MALAT1. We can conclude that up-regulating MALAT1 in tumor cells can affect cell apoptosis, migration and other biological behaviors by regulating the Wnt/ $\beta$ -catenin signaling pathway. In this study, in order to further explore the specific mechanism of cell proliferation, invasion, and apoptosis

induced by Lipofectamine 2000 and small interfering RNAs silencing lncRNA MALAT1 expression, the expression of E-cadherin,  $\beta$ -catenin, vimentin, Bcl-2 and other proteins were detected by Western blotting, and down-regulation was found. The expression of MALAT1 can promote the increase of E-cadherin, and the expression of  $\beta$ -catenin, vimentin, and Bcl-2 decrease. It can be seen that MALAT1 can participate in the proliferation and migration of CSCC cells through the Wnt signaling pathway axis, which

is basically consistent with the above research results.

This study has some shortcomings. As a preliminary study, only the changes in the role of  $\beta$ -catenin, the core protein of the Wnt signaling pathway, were observed, and changes in the corresponding downstream genes have not been observed. In the next study, further exper-

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iments will be carried out to observe the specific actions of MALAT1 in relationship to the Wnt signaling pathway.

In summary, down-regulating the expression of lncRNA MALAT1 may promote CSCC cell apoptosis and inhibit cell migration, invasion and proliferation by regulating the Wnt signaling pathway. It may be used as a new clinical target for the diagnosis, treatment and prognosis of CSCC. Since the role and mechanism of MALAT1 in the biological behavior of CSCC cells have not yet been reported at this stage, this study is of high value. However, the molecular mechanism of MALAT1 regulating the biological behavior of CSCC, related regulatory links and clinical applications are still to be further clarified.

### Disclosure of conflict of interest

None.

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