

Long non-coding RNA DUXAP8 regulates proliferation and invasion of esophageal squamous cell cancer

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Abstract. – **OBJECTIVE:** The purpose of this research was to detect the expression of long non-coding RNA DUXAP8 in esophageal cancer, and to explore its underlying mechanism in the development of esophageal squamous cell carcinoma (ESCC).

PATIENTS AND METHODS: We collected 78 pairs of esophageal cancer tissues and normal adjacent tissues. The mRNA level of DUXAP8 in these esophageal cancer tissues and corresponding adjacent tissues was detected by quantitative Real-time polymerase chain reaction (qRT-PCR). The relationship between DUXAP8 expression and the prognosis of esophageal cancer was analyzed. Small interfering RNA (siRNA) was applied to reduce the expression of DUXAP8 in ESCC cell lines (TE-1 and KYSE520). Meanwhile, the specific effect of DUXAP8 on the biological functions of ESCC cells was analyzed by CCK-8 assay (cell counting kit-8), colony formation assay and transwell assay, respectively. Furthermore, the regulatory effect of DUXAP8 on Wnt/ β -catenin pathway was detected by Western blot.

RESULTS: DUXAP8 was overexpressed in ESCC tissues than that of normal adjacent tissues. DUXAP8 expression was positively correlated to tumor stage and lymph node metastasis, whereas negatively correlated to the survival rate of ESCC patients. Cell proliferation, colony formation and invasion abilities were significantly decreased after knockdown of DUXAP8 in ESCC cells. Western blot results showed that DUXAP8 could regulate the occurrence of ESCC via Wnt/ β -catenin pathway.

CONCLUSIONS: DUXAP8 expression was significantly correlated with tumor stage, lymph node metastasis and poor prognosis of ESCC patients. DUXAP8 may promote the occurrence of ESCC via Wnt/ β -catenin pathway.

Key Words

Long non-coding RNA, DUXAP8, ESCC, Prognosis, Wnt/ β -catenin.

Introduction

Esophageal cancer is one of the most common gastrointestinal malignancies in the world. Globally, there are 455,800 new cases and 400,200 dead cases each year^{1,2}. Based on the histological stage, esophageal cancer could be divided into esophageal squamous cell carcinoma (ESCC) and adenocarcinoma³. In China, more than 90% of esophageal cancer belongs to ESCC. Although great progress has been made in improving diagnosis and treatment of ESCC, the long-term survival rate of ESCC is still unsatisfied⁴⁻⁶. Currently, there are still many difficulties in the early diagnosis, recurrence control and prognosis evaluation of ESCC^{7,8}. It is of great significance to search for biomarkers of ESCC, so as to better predict and treat ESCC patients.

Long non-coding RNAs (lncRNAs) are a class of RNAs that do not possess the protein-coding function. lncRNAs mediate transcriptional synthesis by RNA polymerase III⁹. Functionally, lncRNA plays a very important regulatory role in protecting chromosome integrity, maintaining genome structure, gene marking, transcription, translation and epigenetic regulation^{10,11}. Accumulating researches^{12,13} have also confirmed the crucial role of lncRNA in tumorigenesis. However, the potential molecular mechanisms of lncRNAs in tumors are still not fully elucidated. To our best knowledge, few researches have been conducted on exploring the role of lncRNA in ESCC. Differentially expressed lncRNAs are rarely reported in the process of proliferation, invasion and metastasis of ESCC cells^{13,14}. This work aims to investigate the role of DUXAP8 in ESCC tissues and its underlying mechanism.

Patients and Methods

Patients and Tumor Samples

A total of 78 pairs of surgically resected ESCC tissues and normal adjacent tissues were collected. All included patients were pathologically confirmed as ESCC. ESCC patients did not receive any preoperative anti-tumor treatments. This study was approved by the Ethics Committee of Huai'an First People's Hospital. Signed written informed consents were obtained from all participants before the study.

Cell Lines and Reagents

Four ESCC cell lines (TE-1, ESCCA109, ESC-CA9706 and KYSE520) and one normal esophageal epithelial cell line (HEESCC) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). All cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Thermo Fisher Scientific, Waltham, MA, USA) containing 10% inactivated fetal bovine serum (FBS), 10% penicillin and streptomycin (100 µg/mL), and maintained in a 5% CO₂ incubator at 37°C, relative humidity 90%.

Transfection

Negative control (si-RNA) and small interference sequence of DUXAP8 (si-DUXAP8) were purchased from GenePharma, Shanghai, China. The cells were seeded in 6-well plates. Corresponding siRNAs were transfected by Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) when the cell confluence was up to 70%. After transfection for 48 h, quantitative Real-time polymerase chain reaction (qRT-PCR) was performed to verify the transfection efficacy.

CCK-8 Assay

After transfection for 48 h, cells were inoculated into 96-well plates at a density of 2.5×10^3 per well. 10 µL of CCK-8 solution (Dojindo Laboratories, Kumamoto, Japan) were added in each well. Next, cells were cultured for 0, 24, 48, and 72 h, respectively. Optical density (OD) value of each well in the wavelength of 490 nm was detected by the microplate reader after cell culture for 0, 24, 48, and 72 h, respectively. Each experiment was performed in triplicate.

Colony Formation Assay

The cells were seeded into 6-well plates after transfection for 48 h at a density of 500 cells per well, with 3 wells per group. Cells were then

cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) for 2 weeks. Subsequently, cells were washed with phosphate-buffered saline (PBS) for 3 times, fixed with methanol for 30 min, and stained by 0.1% crystal violet (purchased from Sigma-Aldrich, St. Louis, MO, USA) for 20 min. Finally, images were observed and captured.

Transwell Assay

The cells were resuspended in serum-free medium after transfection for 48 h. Transwell chamber was pre-covered with Matrigel. 4×10^4 cells and 500 µL of Dulbecco's Modified Eagle Medium (DMEM) containing 10% FBS were added in the upper and lower chamber, respectively. After cell culture for 48 h, transwell chamber was fixed with 4% paraformaldehyde for 30 min. Non-adherent cells were carefully cleaned, followed by crystal violet staining for 15 min. 5 randomly selected fields were captured and observed.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

The total RNAs in ESCC cell lines and tissues were extracted by TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and reversely transcribed into complementary Deoxyribose Nucleic Acid (cDNA) by Primescript RT Reagent (TaKaRa, Otsu, Shiga, Japan). The qRT-PCR reaction was processed according to the instructions of SYBR®Premix Ex Taq™ (TaKaRa, Otsu, Shiga, Japan), and the StepOne Plus Real-time PCR system (Applied Biosystems, Foster City, CA, USA). Data were analyzed by ABI Step One software and the relative expression levels of mRNAs were calculated by the $2^{-\Delta\Delta C_t}$ method. The DUXAP8 sequences were as the following: forward: AGACGCCATGGAA-CAT, reverse: AAGCGGAGACCTGAGGAG.

Western Blot

Lysis buffer were used to lyse the transfected cells, followed by gentle mixture on ice for 30 min and centrifuged at 14,000 rpm for 10 min at 4°C. Total protein concentration was calculated by the bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA). The extracted protein was transferred onto a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA) after separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel. Membranes were then incubated in 5% skim milk at room temperature for 1 h. Primary antibody was added for incubation at room temperature overnight,

followed by incubation of corresponding secondary antibody at 4°C for 1 h. Finally, membranes were exposed by the imaging system. Primary antibodies used in this experiment were β -catenin, cyclin D1 and c-myc. Secondary antibodies were anti-mouse and anti-rabbit (Cell Signaling Technology, Danvers, MA, USA).

Statistical Analysis

All data were analyzed by statistical product and service solutions (SPSS) 22.0 program (IBM, Armonk, NY, USA). Data were expressed as mean $\pm$ SD ($\bar{x} \pm s$). Kaplan-Meier was introduced for evaluating the prognosis of ESCC patients and differences between the curves was analyzed by the Log-rank test. Continuous variables were compared using *t*-test, and classification variables were compared using χ^2 -test or Fisher's test. Comparison between groups was performed using One-way ANOVA test followed by Least Significant Difference (LSD). $p < 0.05$ was considered as statistically significant.

Results

DUXAP8 was Overexpressed in ESCC Tissues and Cells

We detected DUXAP8 expressions in 78 pairs of ESCC tissues and adjacent normal tissues by qRT-PCR. The results showed that DUXAP8 expression in ESCC tissues was significantly higher

than that of normal adjacent tissues. The difference was statistically significant (Figure 1A). DUXAP8 was also significantly overexpressed in ESCC cells compared to that of normal esophageal epithelial cells (HEESCC) (Figure 2A). In particular, TE-1 and KYSE520 cells presented the highest DUXAP8 expression in the four ESCC cell lines, which were selected for the following experiments.

DUXAP8 Expression Was Closely Related to the Clinical Stage, Lymph Nodes Metastasis and Overall Survival of ESCC

According to the expression level of DUXAP8 in ESCC tissues, DUXAP8 patients were assigned into high and low expression groups. The relationship between DUXAP8 expression, gender, age, tumor size, clinical stage, grading, and lymph node metastasis of ESCC patients were analyzed. The data demonstrated that DUXAP8 expression was positively correlated with clinical stage and lymph node metastasis of ESCC patients, but not correlated with age, gender and tumor size (Table I). In addition, survival curves showed that DUXAP8 expression was negatively correlated with the prognosis of ESCC patients ($p = 0.032$, Figure 1B).

Knockdown of DUXAP8 Inhibited Cell Proliferation

The mRNA level of DUXAP8 was found to be upregulated in ESCC cell lines than that of normal esophageal epithelial cell line (Figure 2A). To

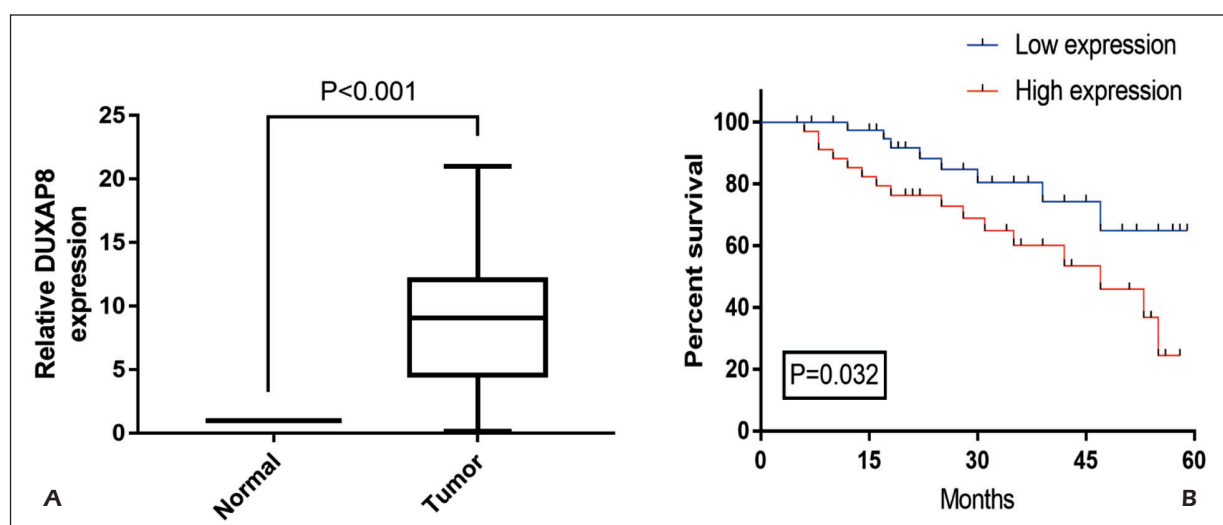


Figure 1. A, The expression of DUXAP8 in ESCC tissue was significantly increased than that of normal adjacent tissue; B, Kaplan-Meier survival curves of ESCC patients based on DUXAP8 expression. Patients in the high expression group had a significantly more unfavorable prognosis than those in low expression group.

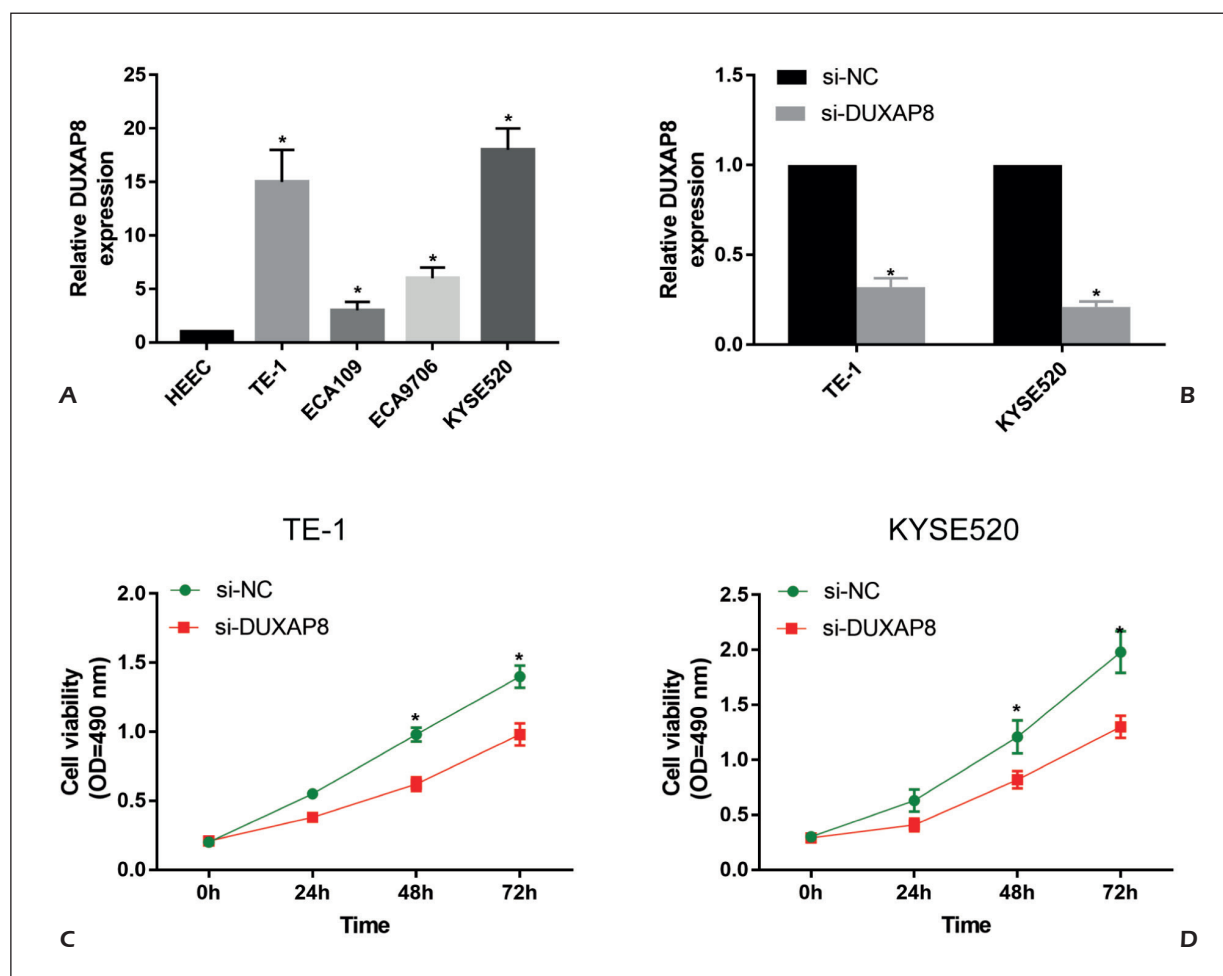


Figure 2. A, The mRNA level of DUXAP8 in ESCC cell lines; B, QRT-PCR was used to verify the efficiency of DUXAP8 knockdown. C-D, Growth curve analysis showed the cell growth of TE-1 and KYSE520 cells after DUXAP8 knockdown.

explore the effect of DUXAP8 on the proliferation of ESCC cells, we performed CCK-8 assay in ESCC cells after altering DUXAP8 expression. Transfection efficacy of si-DUXAP8 was verified by qRT-PCR (Figure 2B). Cell proliferation was significantly decreased in ESCC cells transfected with si-DUXAP8 (Figure 2C and 2D). Similar results were obtained in the colony formation assay (Figure 3A and 3B).

Knockdown of DUXAP8 Inhibited Cell Invasion

Transwell assay was conducted to explore the impact of DUXAP8 on the invasion of ESCC cells. The results showed that the number of penetrated cells was significantly reduced after knockdown of DUXAP8, suggesting that cell invasion was inhibited (Figure 3C and 3D).

Knockdown of DUXAP8 Inhibited Wnt/ β -Catenin Signaling Pathway

After DUXAP8 knockdown, we found that protein expressions of Wnt/ β -catenin pathway-related genes, including β -catenin, cyclin D1, and c-myc were significantly decreased. Our results suggested that DUXAP8 promotes the development of ESCC via Wnt/ β -catenin pathway (Figure 4).

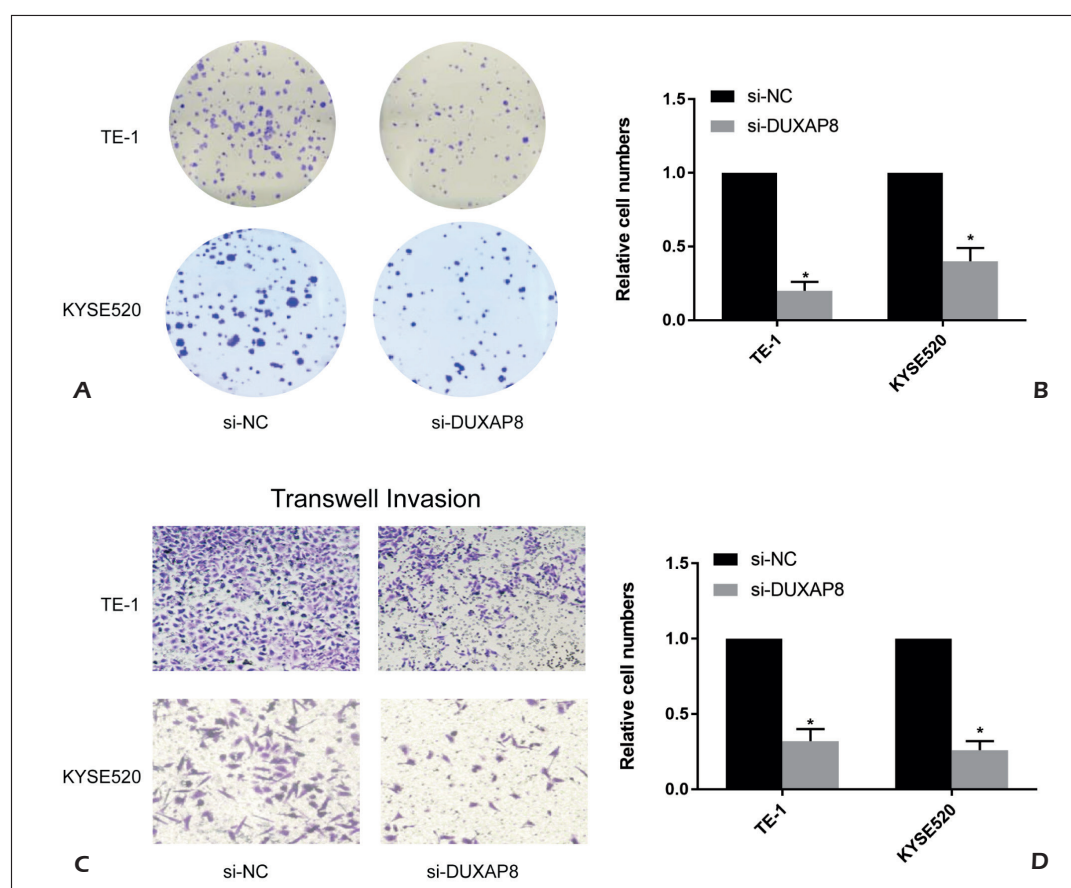
Discussion

ESCC is one of the most common malignancies. Current treatments of ESCC include surgery, neoadjuvant chemotherapy, radioactive, biological therapy and other combined treatments. The prognosis and 5-year overall sur-

Table I. Association of DUXAP8 expression with clinicopathologic characteristics of ESCC patients.

Parameters	Number of cases	DUXAP8 expression		<i>p</i> -value
		Low	High	
Gender				0.613
Male	48	26	22	
Female	30	18	12	
Age (years)				0.606
< 60	37	22	15	
≥ 60	41	22	19	
Tumor size (cm)				0.341
< 4	46	28	18	
≥ 4	32	16	16	
TNM stage				0.013
I-II	40	28	12	
III-IV	38	16	22	
Tumor Grade				0.004
Low	42	30	12	
Middle+High	36	14	22	
Lymph nodes metastasis				0.001
No	58	39	19	
Yes	20	5	15	

vival rate, however, are still unsatisfied. More seriously, ESCC is not sensitive to radiotherapy and chemotherapy, the prognosis of which is often worse than that of adenocarcinoma¹⁵⁻¹⁷. So far, the pathogenesis and prognosis of ESCC remain unclear. It is of great sense to explore the development of ESCC and to evaluate the prognosis of ESCC patients. A great number of researches¹⁸ have shown that lncRNA regulates the biological processes of tumors through various ways. In this study, we first demonstrated that DUXAP8 was significantly overexpressed in ESCC tissues and positively related to staging, lymph node metastasis and poor prognosis of ESCC patients. Therefore, we speculated that DUXAP8 may play a role in tumor promotion of ESCC. To further investigate the effect of DUXAP8 on the biological function of ESCC, DUXAP8 was knocked down using small interfering RNA. CCK-8, colony formation and transwell assay indicated that DUXAP8 can promote the proliferation and invasion of ESCC cells.

**Figure 3.** A-B, TE-1 and KYSE520 cells transfected with si-DUXAP8 displayed significantly lower colony formation capacity. C-D, TE-1 and KYSE520 cells transfected with si-DUXAP8 displayed significantly lower invasion capacity.

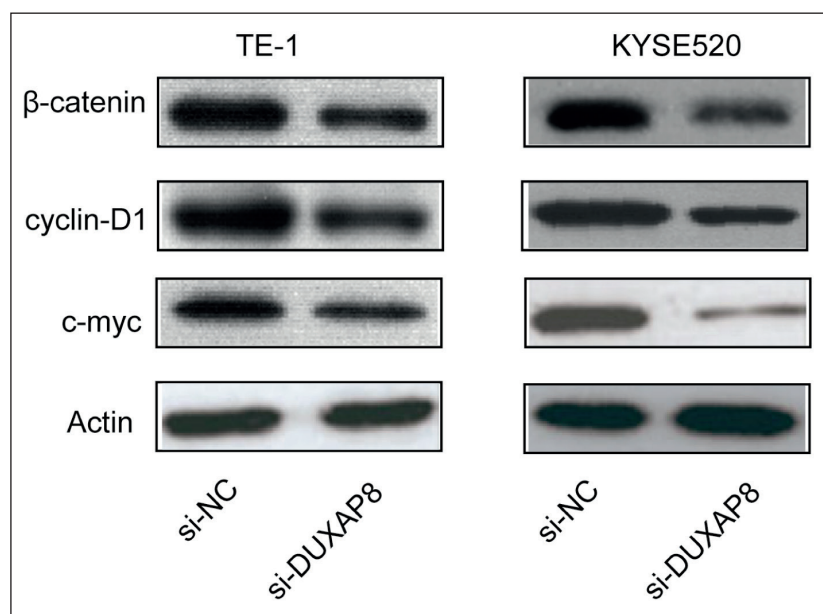


Figure 4. Knockdown of DUXAP8 significantly decreased the expressions of β -catenin, cyclin D1 and c-myc.

Wnt/ β -catenin signaling pathway plays a very important role in the occurrence and development of ESCC^{19,20}. Wnt signaling pathway is a network regulatory pathway that may be regulated by multiple key proteins. This pathway has also been studied in other types of cancers, such as colorectal cancer, breast cancer, gastric cancer and etc^{21,22}. In recent years, the study of Wnt/ β -catenin signaling pathway has become a hot spot in cancer research. The regulatory effect of Wnt/ β -catenin signaling pathway on physiological processes (cell differentiation, proliferation and apoptosis, etc.) and pathological processes (cell carcinogenesis, tumor invasion, etc.) have been well recognized^{23,24}. As a target of anti-tumor therapy, key genes in Wnt/ β -catenin signaling pathway have been served as biomarkers in predicting and treating different types of tumors^{25,26}.

To investigate whether DUXAP8 regulates ESCC development *via* Wnt/ β -catenin pathway, protein expressions of β -catenin, cyclin D1 and c-myc were detected by Western blot. The findings showed that expressions of β -catenin, cyclin D1 and c-myc in ESCC cells transfected with si-DUXAP8 were significantly decreased. Our results suggested that DUXAP8 promotes the development of ESCC *via* regulating Wnt/ β -catenin pathway.

Conclusions

We showed that DUXAP8 was overexpressed in ESCC and was significantly correlated with

clinical stage, lymph node metastasis and poor prognosis of ESCC patients. DUXAP8 promotes ESCC development *via* activating the Wnt/ β -catenin pathway.

Conflict of Interest

The authors declared no conflict of interest.

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