

Role and mechanism of Dvl3 in the esophageal squamous cell carcinoma

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Abstract. – OBJECTIVE: The purpose of this project was to investigate the expression of Dishevelled-3 (Dvl3) in esophageal squamous cell carcinoma and cultured cells, and to determine the consequence of *Dvl3* silencing in the tumorous properties of esophageal squamous cell carcinoma cells.

PATIENTS AND METHODS: The expression of *Dvl3* mRNA and protein in 50 cases of esophageal squamous cell carcinoma was detected. The expression of *Dvl3* mRNA and protein was significantly elevated in esophageal squamous cell carcinoma tissues compared with atypical hyperplasia and normal esophageal mucosa.

RESULTS: *Dvl3* promoted the proliferation of esophageal squamous cell carcinoma cells and cell migration of cells expressing *Dvl3* siRNA was significantly lower than that of the non-transfected cells. Flow cytometry showed that silencing *Dvl3* promoted apoptosis of esophageal squamous cell carcinoma. *Dvl3* overexpression cells in the subcutaneous tissue of nude mice promoted the formation of tumors. The expression of *Dvl3* was associated with invasion and metastasis of the esophageal squamous cell carcinoma.

CONCLUSIONS: Overall, down-regulation of *Dvl3* expression can control the progression of esophageal squamous cell carcinoma, inhibit the growth and promote the apoptosis of tumor cells. Thus, *Dvl3* has potential applications for early diagnosis, prognosis and therapeutics in the esophageal squamous cell carcinoma.

Key Words:

Esophageal squamous cell carcinoma, Immunohistochemistry, RT-PCR, apoptosis.

Introduction

Esophageal cancer is one of the most common malignant tumors, fifth in incidence rate but se-

cond in mortality rate. The incidence of esophageal cancer is fourth in China and 90% of those tumors are esophageal squamous cell carcinoma. Unfortunately, most esophageal cancers are diagnosed in advanced stages, following invasion and metastasis, which are the main causes of death and poor prognosis^{1,2}. Invasion and metastasis of malignant tumors are regulated by many factors, including the Wnt signaling pathway, which is closely related to the infiltration and metastasis of tumor cells.

The Dishevelled (Dvl) protein family are key regulators of the Wnt pathway. The Dvl protein family was first identified in *Drosophila melanogaster*³. At present, there are three human orthologues, Dvl-1, Dvl-2, and Dvl-3, that are highly conserved to each other in sequence^{4,5}. The importance of Dvl proteins in the Wnt signaling pathway and embryonic development is widely recognized^{6,7}. In recent years, it has been reported that Dvl proteins are highly expressed in malignant tumor tissues, which may be related to the malignant behavior of tumors⁸. Dvl-3 can effectively enhance the sensitivity to IGFIR inhibition *in vitro* and *in vivo*⁹. Dvl-1 and Dvl-3 can affect the cell infiltration by classical and non-classical Wnt pathways, and are closely related to the poor prognosis of non-small cell lung cancer^{10,11}. In primary cervical cancer, upregulation of *Dvl3* was associated with the rise of β -catenin and the downstream regulatory gene Cyclin D1 in the Wnt signaling pathway¹².

These results suggest that the Dvl proteins may play an important role in the development of tumors and may have potential roles in tumor invasion and metastasis. However, no relationship between *Dvl3* and invasion and metastasis of esophageal squamous cell carcinoma has been described in public databases. This paper will focus on the role of *Dvl3*

in the occurrence and development of esophageal squamous cell carcinoma and its effect on invasion and metastasis. The study will provide new experimental evidence for the use of Dvl3 as a marker for early diagnosis and screening of esophageal squamous cell carcinoma.

Materials and Methods

Main Materials

BALB/c (nu-nu) nude mice were purchased from the Shanghai Institute of Materia Medica of the Chinese Academy of Sciences, certificate number: Shanghai Composite Certificate No. 122. Mice were 28-32 days and weighed 16-20 g. Feeding was provided under SPF environment. The lentiviral vector was purchased from Shanghai Keygen.

Main Reagents

RPMI-1640 media was purchased from Thermo-Fisher (Waltham, MA, USA). Fetal bovine serum was purchased from CellMax Cell Technology (Beijing, China). The MK1030 immunohistochemistry kit was purchased from Boster Bioengineering (Wuhan, China). The high sensitivity ECL chemiluminescence, MTT, and S-P kits were purchased from Shanghai BioEngineering (Shanghai, China). The Annexin V-Fluorescein isothiocyanate (FITC)/propidium iodide (PI) cell apoptosis detection kit was purchased from Beyotime Biotechnology (Shanghai, China). The Lipofectamine 2000 transfection kit was purchased from Invitrogen (Carlsbad, CA, USA). The HRP-labeled rabbit anti-mouse and fluorescent-labeled goat anti-mouse secondary antibodies, Dimethyl Sulfoxide (DMSO), Triton X-100, Bovine Serum Albumin (BSA), and polyvinylidene difluoride (PVDF) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti-DVL3 and β -DVL3-p antibodies were purchased from Orgentec (Mainz, Germany).

Collection of Tissue Samples

50 surgical specimens of suspected esophageal squamous cell carcinoma were collected and frozen in liquid nitrogen in the First Affiliated Hospital of Henan University of Traditional Chinese Medicine from January 2014 to December 2015. Esophageal squamous cell carcinoma was confirmed by pathology. No case was treated with radiotherapy or chemotherapy before the surgery. 30 specimens were male and 20 were female. Ages were 27-70 years old. 18 cases were classified as

Class I + II, 32 as Class III + IV. Infiltration to the surface (superficial muscle layer and above) was found in 14 cases, deep infiltration (deep muscle layer and below) in 36 cases. The collection of specimens was approved by the Hospital Ethics Committee.

In Situ Hybridization

Frozen sections and RNA probes were prepared by Shanghai Bioengineering (Shanghai, China). The method and procedure of *in situ* hybridization were performed according to the instructions in the Peroxidase (POD) detection kit (MK1030 type) from Boster Bioengineering (Wuhan, China).

Immunohistochemistry

Immunohistochemistry was performed following the instructions in the S-P Kit, with DAB color rendering at the end. A case of anaplastic large cell lymphoma was used as a positive control and PBS was used as a negative control instead of primary antibody. The judgment criteria were determined by positive staining intensity and percentage of positive cells. Each slice was randomly observed in 10 fields at 400 x magnification. The average proportion of positive cells in the slice and the depth of coloring was scored 0-4 points. No positive cells in the slice: 0 points, < 30% positive cells; 1 point, 30%-60% positive cells; 2 points, 60%-75%; and >75% positive cells: 3 points.

Cell Culture and Transfection

The esophageal squamous cell carcinoma cell lines ECA109 and ECA9706 were purchased from Cell Institute of the Shanghai Chinese Academy of Sciences. Roswell Park Memorial Institute (RPMI)-1640 medium, fetal bovine serum (Gibco, Grand Island, NY, USA), 100 IU/ml penicillin, and 100 μ g/ml streptomycin were used for cell culture in a 5% CO₂ incubator. After culturing the cells for 2-3 days at 37°C, a subculture was taken. Transfection experiments were performed when the cells were in the logarithmic growth phase. Lipofectamine 2000 was used for transfection according to the instructions. Cells transfected with pCS2+ and pMyc-cyto were used as controls. Transfection efficiency was measured by indirect immunofluorescence¹³.

RT-PCR

Total RNA was extracted from esophageal squamous cell carcinoma samples and subjected to RT-PCR. PCR primers, amplification conditions and product length are shown in Table I. The

Table 1. Dvl3 mRNA expression in esophageal squamous cell carcinoma.

| Groups | No. | Dvl 3 | | | Positive rate (%) | χ^2 | P |
|----------------------------------|-----|-------|----|-----|-------------------|----------|-------|
| | | + | ++ | +++ | | | |
| Normal esophageal mucosa tissues | 50 | 1 | 6 | 2 | 18.0 | 21.001 | 0.000 |
| Tumor-adjacent tissues | 50 | 6 | 10 | 8 | 48.0 | | |
| ESCC tissues | 50 | 9 | 19 | 12 | 80.0 | | |

sequences of primers for qPCR were: forward primer: 5' GGCTGCTGATGGACGCATCG 3'; reverse primer: 5' GGGAAGGGTGCCGGTCA-TGG 3'. qPCR cycle was as follows: 94°C, 15 s; 68°C, 30 s; 20 cycles. The PCR products were resolved in 1% agarose gel electrophoresis and the grayscale ratio of the bands was quantified relative to β -actin.

Western Blotting

Protein homogenates were generated and electrophoresed on 15% SDS-PAGE. After electrophoresis, the proteins were transferred to a PVDF membrane and blocked with 5% skim milk at 4°C for 1 h. Anti-DVL3 (1:500) and -GAPDH (1:1000) were added and incubated at 4°C overnight. Primary antibodies were washed twice with PBS, and then the secondary antibodies were incubated at 4°C overnight. The membrane was washed twice with PBS, the signal was developed by chemiluminescence and the relative expression levels were analyzed by Bio-Rad molecular imaging system (Hercules, CA, USA).

Cell Proliferation

When the stably transfected ECA9706 and ECA109 cells were cultured to logarithmic growth phase, 10^5 cells were inoculated in 96-well plates, in 6 replicates. At 0, 24, and 48 h each well received 10 μ l MTT, and the values were detected after 4 h culture. Absorbance at 490 nm was measured on a microplate reader. The experiment was repeated 3 times. The cell inhibition rate was calculated according to the formula: cell growth inhibition rate = (1- Light absorption value of the test group/ light absorption value of the control group) \times 100.

Cell Scratch Test

10^5 stably transfected ECA9706 and ECA109 cells were inoculated in 6-well plates. Each group had three wells, after 2 h culture a pipette tip was used to scratch the culture well and washed three times with PBS. Removed the cells, added serum-free medium, placed it in 37°C 5% CO₂ in-

cubator and 0, 6, 12 and image at 24 h to observe the cell migration.

Transwell Experiment Protocol

The polycarbonate membrane of a transwell chamber was coated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) and cured at 37°C for 1 h. After 48 h, stably transfected esophageal squamous cell carcinoma cells were washed with 1% serum RPMI-1640 medium and the cell concentration was adjusted to 5×10^4 cells /100 μ l. Cells were added to the transwell upper chamber and the lower chamber was filled with complete medium containing 10% serum. After 24 h of culture, the cells of the upper chamber were discarded and cells in the bottom were fixed with 4% paraformaldehyde for 30 min, stained with crystal violet for 20 min and then washed with PBS 3 times. The number of transmembrane cells in five fields was then counted under a microscope. Cell invasion inhibition rate = [(number of invasive cells in the control group – number of invasive cells in the experimental group)/number of invasive cells in the control group] \times 100.

Flow Cytometry

The stably transfected ECA9706 and ECA109 cells at logarithmic growth phase were trypsin digested. The cell density was adjusted to 10^6 cells in 6-well plates. Blank control wells were also prepared only with culture medium. After incubating for 24 h, the cells were washed twice with PBS and the cells were collected for detection. The procedure was carried out according to the instructions in the Annexin V-FITC/PI double staining cell kit. Flow cytometry was used to detect apoptosis.

Grouping and Treatment of Laboratory Animals

The mice were divided into two groups, experimental and control, with five mice in each group. The control group was inoculated esophageal squamous cell carcinoma cells transfected

Table II. Expression of Dvl3 protein in esophageal squamous cell carcinoma.

| Groups | No. | Dvl 3 | | | Positive rate (%) | χ^2 | P |
|----------------------------------|-----|-------|----|-----|-------------------|----------|-------|
| | | + | ++ | +++ | | | |
| Normal esophageal mucosa tissues | 50 | 1 | 7 | 2 | 20.0 | 19.002 | 0.000 |
| Tumor-adjacent tissues | 50 | 6 | 10 | 7 | 46.0 | | |
| ESCC tissues | 50 | 9 | 18 | 12 | 78.0 | | |

with empty vector and the experimental group was inoculated with esophageal squamous cell carcinoma cells overexpressing Dvl3. Stably transfected esophageal squamous cell carcinoma cells were cultured to 70-90% fusions, trypsin digested them and counted them. Nude mice were inoculated subcutaneously in the density of $10^5/0.5$ ml into the back to establish orthotopic esophageal squamous cell carcinoma models. The tumor growth status was observed and recorded. After 6-8 weeks of inoculation, the changes in tumor volume, tumor size and other proliferation indexes were measured.

Statistical Analysis

The differences between the experimental results were tested by Student's *t*-test. Statistical processing was performed on SPSS 19.0 software

(IBM, Armonk, NY USA). $p < 0.05$ indicated that the differences were statistically significant.

Results

Distribution of Dvl 3 in Esophageal Squamous Cell Carcinoma and Normal Esophagus

To determine the potential role of Dvl3 in the esophageal squamous cell carcinoma, we first examined *Dvl3* mRNA and protein distribution in cancerous and normal tissues. *In situ* hybridization and immunohistochemistry results showed that *Dvl3* mRNA and protein expression were elevated in 50 cases of esophageal squamous cell carcinoma (Figure 1 and Figure 2). The positive rates were 80% for *Dvl3* mRNA and 78%

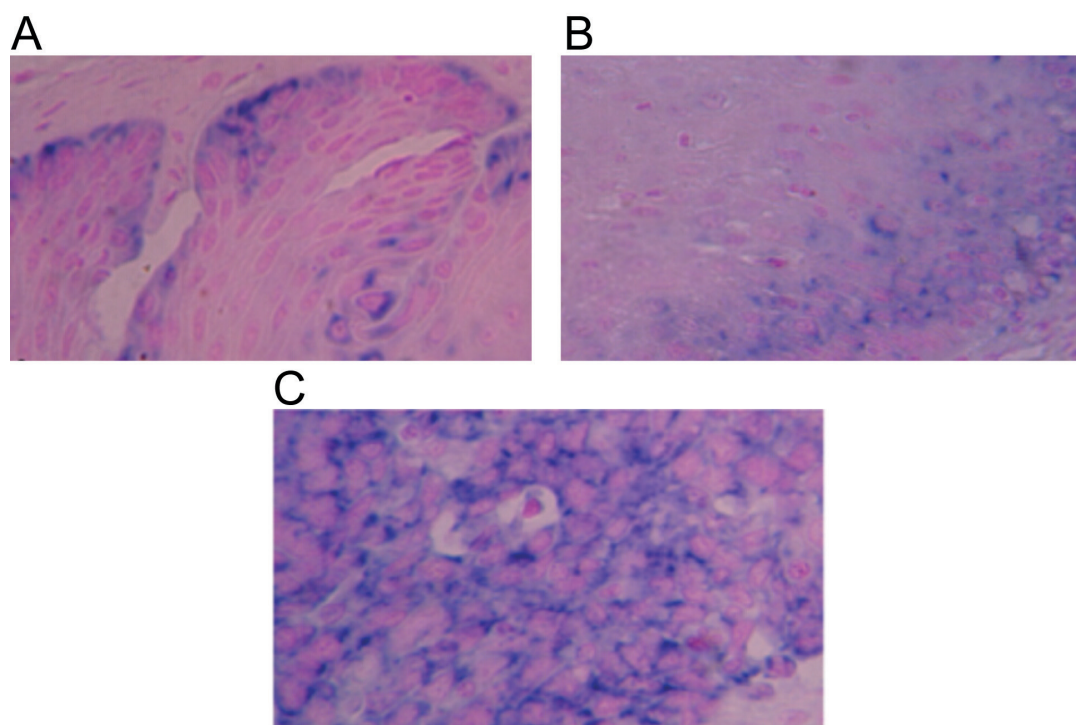


Figure 1. Dvl3 mRNA expression in esophageal squamous cell carcinoma, paracarcinoma and normal esophageal mucosa (ISH 400 \times). *A*, Normal esophageal mucosa. *B*, Esophageal cancer tissue. *C*, Esophageal squamous cell carcinoma.

for Dvl3 protein. We found significant differences compared with atypical hyperplasia and normal esophageal mucosa tissues (Tables I and II) ($\chi^2 = 21.001, 19.002, p < 0.01$).

Relationship of Dvl3 with Pathologic Grade, Invasion, and Metastasis of Esophageal Cancer

The expression of *Dvl3* mRNA and protein in esophageal squamous cell carcinoma increased with the depth of invasion. The positive expression rate of Dvl3 when the infiltration reached the deep and outer membrane was significantly higher compared to the cases in which the infiltration was superficial. The positive expression rate of *Dvl3* mRNA and protein when the lymph node was metastatic was significantly higher than that in the cases in which the lymph node was not affected. The expression of *Dvl3* mRNA and protein in esophageal squamous cell carcinoma correlated with clinical stage, and the positive expression rate of stages III-IV was significantly higher than that of stages I-II. The analysis of the relationship between the abnormal expression of Dvl3 and clinical pathological features in esophageal squamous cell carcinoma showed that high

expression of Dvl3 was not associated with age and sex, but was correlated with the degree of differentiation, lymph node metastasis, depth of invasion and TNM staging (Tables III and IV).

Detection of Dvl3 by RT-PCR and Western Blot

In the ECA109 and ECA9706 cell lines, *Dvl3* mRNA and protein were highly expressed in both well differentiated and poorly differentiated cell lines by RT-PCR and Western blot (Figure 3).

Impact of Dvl3 on Cell Proliferation

To determine the role of Dvl3 to regulate cell proliferation, we stably transfected cells with *Dvl3* siRNA and detected the cell growth curve of each group by the MTT method. There was a significant difference between the *Dvl3* siRNA transfected cells and the untransfected cells. The absorbance of cells in the *Dvl3* siRNA stably transfected group at each time point were: 0.328 ± 0.024 , 0.374 ± 0.027 , 0.496 ± 0.032 , and 0.562 ± 0.028 at 0, 24, 48, and 72 h, respectively. Down-regulation of *Dvl3* can inhibit the proliferation of esophageal cancer ECA109 cells. The

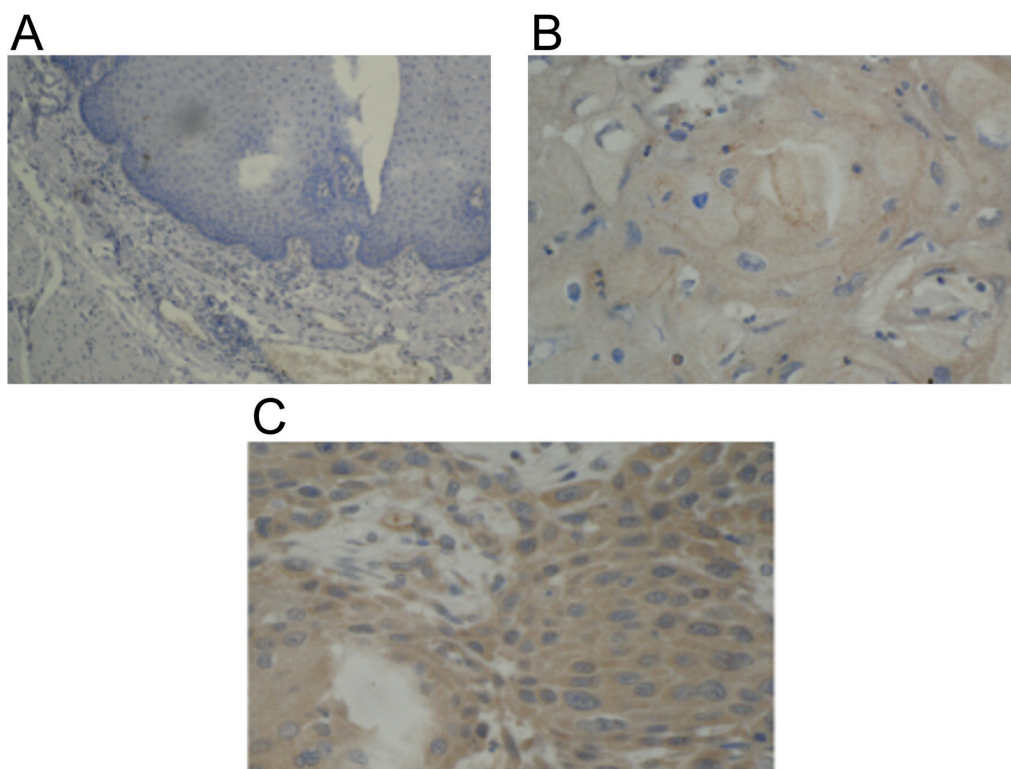


Figure 2. Dvl 2 protein expression in esophageal squamous cell carcinoma, paraneoplastic and normal esophageal mucosa (SP method 400 \times). *A*, Normal esophageal mucosa. *B*, Esophageal cancer tissue. *C*, Esophageal squamous cell carcinoma.

Table III. Relationship between the expression of Dvl 3 mRNA and clinicopathological features in esophageal squamous cell carcinoma.

| Characteristics | No. | Dvl 3 | | Positive (%) | χ^2 | P |
|---------------------------------|-----|-------|----|--------------|----------|-------|
| | | - | + | | | |
| ≤ 60 years old | 29 | 6 | 23 | 79.3 | 0.495 | 0.482 |
| > 60 years old | 21 | 4 | 17 | 80.9 | | |
| Male | 30 | 5 | 25 | 83.3 | 0.210 | 0.650 |
| Female | 20 | 4 | 16 | 80.0 | | |
| Differentiation degree | | | | | | |
| Well to moderate | 36 | 8 | 28 | 77.8 | 4.125 | 0.040 |
| Poor differentiation | 14 | 0 | 14 | 100.0 | | |
| Regional lymph nodes metastasis | | | | | | |
| Yes | 26 | 1 | 25 | 96.1 | 5.753 | 0.015 |
| No | 24 | 7 | 17 | 70.8 | | |
| Infiltration depth | | | | | | |
| Superficial layer | 14 | 5 | 9 | 64.3 | 4.165 | 0.041 |
| Deep layer | 36 | 4 | 32 | 88.9 | | |
| Clinical stage | | | | | | |
| I+II | 18 | 6 | 12 | 66.7 | 3.965 | 0.040 |
| III-IV | 32 | 3 | 29 | 90.6 | | |

Table IV. Relationship between Dvl3 expression protein in esophageal squamous cell carcinoma and clinicopathological features.

| Characteristics | No. | Dvl 3 | | Positive (%) | χ^2 | P |
|-----------------------------------|-----|-------|----|--------------|----------|-------|
| | | - | + | | | |
| ≤ 60 years old | 29 | 6 | 23 | 79.3 | 0.476 | 0.475 |
| > 60 years old | 21 | 7 | 16 | 76.2 | | |
| Male | 30 | 7 | 23 | 76.7 | 0.204 | 0.670 |
| Female | 20 | 4 | 16 | 80.0 | | |
| Differentiation degree | | | | | | |
| Well and moderate differentiation | 36 | 8 | 28 | 77.8 | 4.265 | 0.042 |
| Poor differentiation | 14 | 1 | 13 | 92.9 | | |
| Regional lymph nodes metastasis | | | | | | |
| Yes | 26 | 2 | 24 | 92.3 | 5.552 | 0.020 |
| No | 24 | 7 | 17 | 70.8 | | |
| Infiltration depth | | | | | | |
| Superficial layer | 14 | 5 | 9 | 64.3 | 4.265 | 0.043 |
| Deep layer | 36 | 5 | 31 | 86.1 | | |
| Clinical stage | | | | | | |
| I+II | 18 | 6 | 12 | 66.7 | 4.025 | 0.040 |
| III-IV | 32 | 4 | 28 | 87.5 | | |

highest inhibitory rate of 48 h *Dvl3* siRNA stably transfected group was $23.26 \pm 0.18\%$ (Figure 4) ($p < 0.05$).

Effect of *Dvl3* on Invasion and Migration of Esophageal Squamous Cell Carcinoma

Cell scratch test showed that after 24 h culture, the relative distance of the cells in the control

group ($12.40 \pm 2.04 \mu\text{m}$) was significantly smaller than that in the *Dvl3* siRNA stably transfected cells ($43.80 \pm 7.63 \mu\text{m}$) (Figure 5). The results of transwell chamber showed that the ability of invasion and migration of *Dvl3* siRNA stable transfection cells was significantly lower than that of the control group ($p < 0.01$), and the *Dvl3* overexpression group had higher ability of invasion

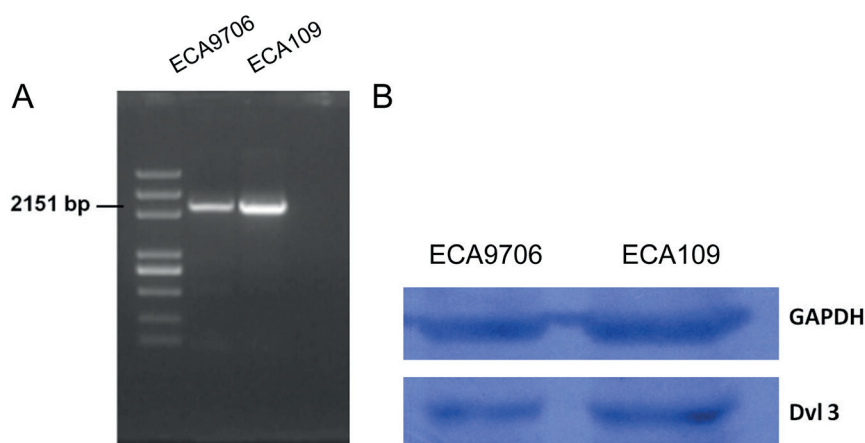


Figure 3. Expression of *Dvl3* mRNA (A) and protein (B) in esophageal squamous cell carcinoma cell lines ECA9706 and ECA109.

and migration compared with the control group ($p < 0.01$) (Table V).

Effect of *Dvl3* in Cell death by Flow Cytometry

The apoptosis of ECA109 and ECA9706 esophageal squamous carcinoma cells was detected by Annexin-V-FITC and PI double staining after stable transfection. The apoptotic rate of ECA109 cells was 46.35%, the apoptosis rate of the control group was 10.92%, the apoptosis rate of the experimental group was significantly higher than that of the control group ($p < 0.05$)

(Figure 6-A, C). The apoptotic rate of ECA9706 cells was 40.75%, and that of the control group was 9.20%. The apoptosis rate of the experimental group was significantly higher than that of the control group ($p < 0.05$) (Figure 6-B,C). These data suggest that the down-regulation of *Dvl3* expression can induce apoptosis of esophageal squamous cell carcinoma.

Effect of *Dvl3* on Tumor Formation

To determine the ability of *Dvl3* to influence the cancerous behavior of cells, we inoculated nude mice with control cells or cells overexpress-

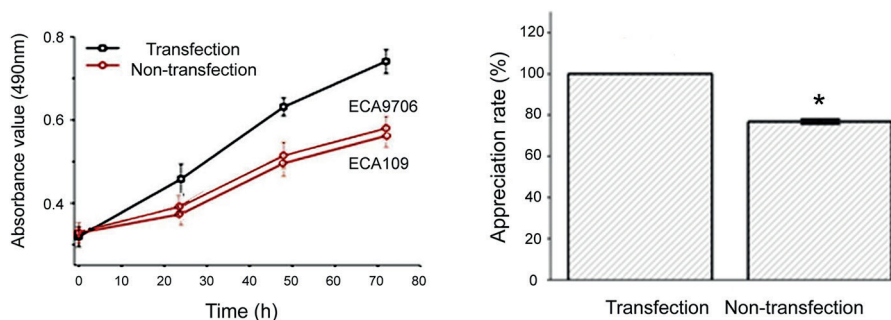


Figure 4. Effect of *Dvl3* on proliferation of esophageal squamous cell carcinoma cell line ECA109 by MTT assay ($*p < 0.05$).

Table V. Effect of overexpression of miR-185 on migration inhibition rate and invasion inhibition rate of SMMC-7721 cells ($n=3$, $x \pm s$).

| Group | Cell erosion | Cell migration | Erosion inhibition rate (%) | Migration inhibition rate (%) |
|----------------|--------------|------------------------|-----------------------------|-------------------------------|
| DVL3 siRNA | 75.2±3.5 | 81.2±5.9 ^a | 32.8±5.4 ^a | 42.6±3.7 ^a |
| Overexpression | 125.4±4.2 | 161.6±5.1 ^a | -15.8±5.6 ^a | -9.2±3.8 ^a |
| Control | 106.5±4.9 | 142.5±4.6 | 0 | 0 |

^aCompared with the control group $p < 0.01$.

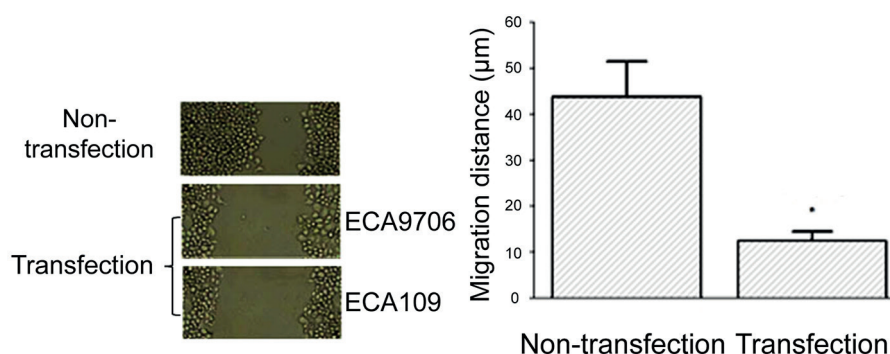


Figure 5. Effect of Dvl3 on migration of esophageal squamous cell carcinoma cell line ECA109 by scratch test (* $p < 0.05$).

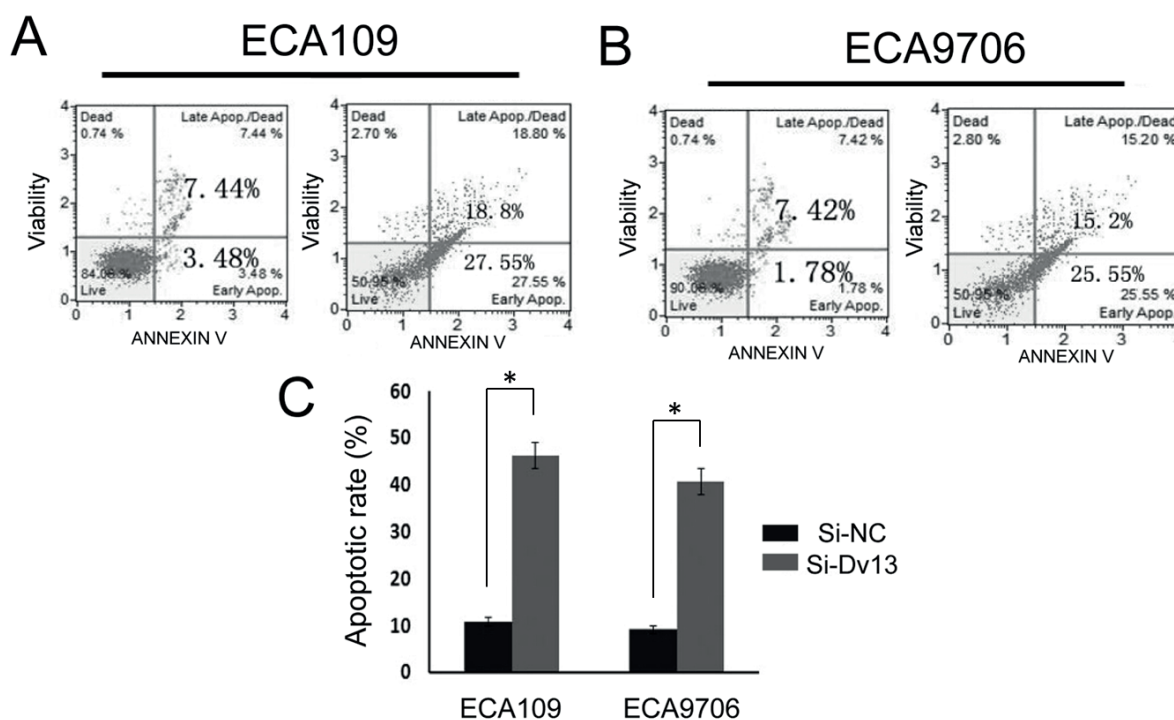


Figure 6. *A* and *C* show a significant difference between the experimental group and the control group in the apoptotic rate of ECA109 cells ($p < 0.05$). *B* and *C* show a significant difference between the experimental group and the control group in the apoptotic rate of ECA9706 cells.

sing Dvl3. The tumor cells inoculated with Dvl3 produced significantly larger tumors in nude mice than those in the control group (Figure 7). We removed the tumors from the nude mice and measured their weight. The mean tumor mass of the cells expressing Dvl3 was 258.8 ± 70.6 mg and the weight of tumors in the control group was 65.5 ± 32.5 mg. The weight of the tumors overexpressing Dvl3 siRNA was 53.8 ± 28.6 mg, significantly different from the other groups.

Discussion

The Wnt signaling pathway plays a key role in normal cell proliferation, cell differentiation and cell invasion and metastasis. Not surprisingly, this pathway plays an important role in the tumor development and progression¹⁴⁻¹⁶. The Dvl protein family is a key component of the Wnt receptor complex and is the most critical regulator of the Wnt signaling pathway¹⁷. At present, the role of Dvl pro-

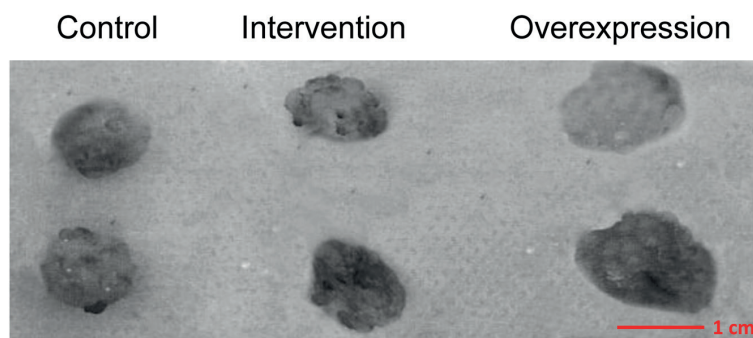


Figure 7. Size of tumor tissues from 3 different groups. It shows statistically significant differences among 3 groups in mean tumor size ($p < 0.05$). The larger size of tumor tissue was 1.5 cm in the overexpression group.

teins in tumor development has been reported, including Dvl upregulation in cervical, breast, liver, prostate, and lung cancer. However, the expression of Dvl proteins may differ in different tumor cells¹⁸⁻²¹. In particular, the relationship between Dvl3 and the occurrence and development of esophageal squamous cell carcinoma is unclear at this time.

We found that *Dvl3* mRNA and protein is overexpressed after studying 50 cases of esophageal squamous cell carcinoma. Additionally, abnormal expression of *Dvl3* was closely related to the invasion and metastasis of esophageal squamous cell carcinoma. Our manipulation of *Dvl3* expression shows that its down-regulation inhibits the proliferation of esophageal squamous carcinoma cells and accelerates the apoptosis of tumor cells. On the other hand, *Dvl3* overexpression in esophageal squamous carcinoma cells promotes tumorigenesis in nude mice. Thus, *Dvl3* not only is overexpressed in esophageal squamous cell carcinoma but also plays a role in the tumor development through the classic Wnt signaling pathway.

Conclusions

This study provides the basis for screening for a new therapeutic target of the esophageal squamous cell carcinoma. *Dvl3* can also be a new marker for the early diagnosis of the esophageal squamous cell carcinoma and provides new prognostic indicators to the esophageal squamous cell carcinoma.

Acknowledgements

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Conflict of Interest

The Authors declare that they have no conflict of interest.

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