DACT2 is a candidate tumor suppressor and prognostic marker in esophageal squamous cell carcinoma.

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Running title: Tumor-suppressorgene DACT2 in esophageal cancer

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Abstract

In animals ranging from fish to mice, the function of DACT2 as a negative regulator of the TGF-beta/Nodal signal pathway is conserved in evolution, indicating it might play an important role in human cancer. In this study, we demonstrated that tumors with higher DACT2 protein level were correlated with better differentiation and better survival rate in esophageal squamous cell carcinoma (ESCC) patients. Restored expression of DACT2 significantly inhibited growth, migration and invasion of ESCC cells in vitro, and reduced tumorigenicity in vivo. Furthermore, when DACT2 expression was restored, the activity of TGF-beta/SMAD2/3 was suppressed via both proteasome and lysosomal degradation pathways, leading to F-actin rearrangement that might depend on the involvement of cofilin and ezrin-redixin-moesin (ERM) proteins. Taken together, we propose here that DACT2 serves as a prognostic marker that reduces tumor cell malignancy by suppressing TGF-beta signaling and promotes actin rearrangement in ESCC.
Introduction

Esophageal squamous cell carcinoma (ESCC) is one of the most aggressive carcinomas in China, due to its markedly poor prognosis despite significant advancements in multimodal therapies (1-3). In ESCC, the TGF-beta pathway is altered, with TGF-beta expression being up-regulated, resulting in enhanced invasive capability by affecting a series of growth- or invasiveness-related genes (4-8). Thus, blocking TGF-beta’s function might be an effective therapy for ESCC.

Recently, DACT2, a member of Dapper family, has been identified as an antagonist of TGF-beta/Nodal signaling and an important factor involved in normal vertebrate development (9-12). In zebrafish, dpr2 (DACT2) suppresses Nodal-mediated mesoderm induction by promoting the lysosomal degradation of TGF-beta receptors ALK4 and ALK5 (9). Interestingly, zebrafish dpr2 is essential for convergent extension movement while acting as an enhancer of non-canonical Wnt signaling (13). Unlike zebrafish DACT2, murine DACT2 is expressed in all three germ layers during gastrulation, and targets the TGF-beta type I receptor ALK5 for degradation (12). Knockdown of mDACT2 expression in collecting duct cells enhances sensitivity to TGF-beta to change cell behavior (10). Dpr2(DACT2)-deficient (Dpr2−/−) mice showed accelerated re-epithelialization during cutaneous wound healing, which is associated with enhanced response to TGF-beta signaling (11). Due to these important functions and the fact that DACT2 gene is conserved in evolution (14-16), DACT2 is believed to play an important role in the development of human cancers. However, little is known about the roles of DACT2 in
tumorigenesis, although it has been predicted to have function in a series of cancers (15). In this study, we investigated the DACT2 expression pattern, the functional mechanism, and its prospect as a potential prognostic in ESCC.

**Materials and methods**

**Cell lines and culture**

Human kidney 293T cells and human ESCC cell lines (EC171, EC8712, KYSE150, KYSE180 and KYSE510) were used. 293T and KYSE cell lines were provided by professor Dong Xie (Institute for Nutritional Sciences of Chinese Academy of Sciences, China). EC171 and EC8712 cell lines were provided by professor Sai-Wah Tsao (Department of Anatomy, University of Hong Kong, China). KYSE cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (HYCLONE, USA). EC cell lines were cultured in 199 medium (HYCLONE, USA). The 293T cell line was cultured in Dulbecco’s modified Eagles’ medium (DMEM) (HYCLONE, USA). All medium were supplemented with 10% fetal bovine serum and antibiotics (100 U/ml penicillin and 100 mg/l streptomycin). Cells were grown under a 5% CO2 atmosphere at 37°C. All 5 human ESCC and 293T cell lines were tested and authenticated by short tandem repeat (STR) DNA profiling (Land Huagene Biosciences Co., LTD) and the authentication results are shown in Supplementary Fig. S1.

**Vector construction and transfection**

For DACT2 expression vector construction, the DACT2 coding region was amplified from EC8712 cells by using the following primers:
5’-AAGCTTATGTGGACGCCGGACGAC-3’ and 5’-GGTACCAGTGCGTCACCATGGTCATGACCTTCAGGGC-3’. The PCR product was then subcloned into the pEGFP-N1 plasmid (Clotech, Palo Alto, CA, USA) or pcDNA3 (Invitrogen, CA) plasmid vector.

For transient transfection assays, plasmids were transfected with lipofectamine 2000, and cells were harvested after 48h for further analysis.

Stable transfection process was performed as described (17). For stable cell line generation, KYSE150 cells were plated into 35 mm dishes at least 24h before transfection to achieve 60% to 80% confluence per well. pEGFP/DACT2 or control vectors were transfected with lipofectamine 2000 (Invitrogen, CA) following the manufacturer’s instructions. Transfected cells were selected in G418 (600 μg/ml) for 1 month. Pooled transfectants were obtained and expanded for further analysis.

**Western blotting**

The technique used was described previously (18). The primary antibodies used in this study are summarized in supplementary Table S1. Antigen-antibody complexes were detected by western blotting luminol reagent (Santa Cruz Biotechnology, Santa Cruz, CA).

**Colony formation assay**

Transfected cells were trypsinized, counted with a cell counter (Bio-Rad, Hercules, CA), and then 500 cells were inoculated in each well of 6-well plates. Cultures were maintained for 3 weeks, and cells were then fixed, stained and photographed.
Cell cycle analysis

For cell cycle analysis by flow cytometry, transfected cells were fixed with 70% ethanol overnight at 4°C. Cell pellets were incubated in PBS containing 0.1% Triton-X 100 for 10 min at room temperature, and then were treated with RNase (50 μg/ml) for 10 min and PI (5 μg/ml) for 30 min, respectively. DNA content was analyzed by flow cytometry (BD FACS Aria II). Flowjo 7.6 software was used to determine the cell cycle phases.

Cell migration and invasion assay

The experimental protocols of cell migration and invasion assays were described previously (7). Transfected cells were counted with a cell counter (Bio-Rad, Hercules, CA), and 3×10^4 cells were seeded for the cell migration assay and 5×10^4 cells for the invasion assay.

Tumorigenicity assays in nude mice

Stably transfected pEGFP/DACT2 and pEGFP/Vector cells (3.0 × 10^6 cells/flank) were injected into 8-week-old male nude mice. The resulting tumors were measured twice a week, and tumor volume (mm^3) was calculated using the formula: 1/2 × length × width × width. Tumors were harvested eighteen days after injection and individually weighed before fixation. Data are presented as tumor volume (mean ± SD) and tumor weight (mean ± SD). Statistical analysis was performed via SPSS using the Student’s t test. Tumor tissue was analyzed by HE, and DACT2 (1:100; Abcam, Cambridge, UK) and cytokeratins 5/6 (ready to use, clone numer, CK5/6. 007; ZSGB-Bio, Beijing, China) were detected by immunohistochemistry.
**Tissue specimens and immunohistochemical staining**

The tumor specimens used in this study were described previously (19). Briefly, tumor specimens were obtained from 168 patients with primary ESCC at Shantou Central Hospital from 2000 to 2006. The follow-up study was performed up to 31 December 2012. We retrieved information, including sex, age, stage of disease, and histopathological factors, from the patient hospital charts. Patient data are summarized in supplementary Table S2. The patients who experienced severe post-operative complications, who died of other tumors or other causes were excluded. The pathologic features of the specimens were classified based on the 7th edition of the TNM classification of the International Union against Cancer. The local ethics committee approved the study.

Tissue microarray (TMA) construction was as described previously (19). The primary antibody was rabbit polyclonal antibody against DACT2 (1:100; Abcam, Cambridge, UK). In assays of negative controls, the primary antibody was omitted.

The evaluation of immunostaining was also described (19). A score range of ≤ 6, or >6 was marked as negative or positive, respectively. The correlations between DACT2 protein expression levels and clinicopathologic variables were analyzed using Kendall’s tall-b test. Survival curves were estimated by the Kaplan-Meier method and compared by the log-rank test.

**Cancer pathway reporter array**

Cignal Finder cancer 10-pathway Reporter Array (SABiosciences, Frederick, MD) was used based on the manufacturer’s instructions. Briefly, cells were inoculated
in 96-well plates and grown to 50–80% confluence. Then, cells were transfected with 100 ng of each dual luciferase Cell Signaling transcription factor-responsive reporter constructs and 200 ng of pcDNA3/DACT2 or pcDNA3 constructs by using lipofectamine 2000 (Invitrogen, CA) following the manufacturer’s instruction. After transfection, cells were incubated for 48h and harvested in Passive Lysis Buffer (Promega Madison, WI). The luciferase reporter activity of the lysates was measured using Dual-Luciferase Reporter Assay System (Promega, Madison, WI) according to the manufacturer’s recommendations. Statistical analysis was performed with SPSS using the Student’s t test.

Quantitation of F-actin levels

For F-actin analysis via flow cytometry, cells were fixed with 70% ethanol overnight at 4°C. Cell pellets were incubated in PBS containing 0.1% Triton-X 100 for 15 min at room temperature, and then were treated with phalloidin-Coumarin (2 μg/ml) (Sigma–Aldrich, St. Louis, Missouri, USA) for 30 min. F-actin content was analyzed by flow cytometry (BD FACS Aria). Flowjo 7.6 software was used to analyze the F-actin content of each group.

Analysis of F/G-actin content ratio

The F-actin and G-actin ratio was determined using the G/F-actin in vivo assay kit (Cytoskeleton, Inc., Denver, CO) according to the manufacturer’s instructions. In brief, cells were homogenized in the lysis and F-actin stabilization buffers followed by centrifugation for 1h at 100,000 ×g at 37°C for F-actin separation. The supernatants containing G-actin were collected, and the pellets containing F-actin
were resuspended in F-actin depolymerizing solution. Equal amounts of both supernatant (G-actin) and the resuspended pellet (F-actin) were subjected to Western blotting with the use of anti-actin antibody.

Confocal fluorescence microscopy

Transfected cells grown on coverslips were treated with or without TGF-beta1 (5 ng/ml, Invitrogen) for 10 min after starvation, and then were fixed with 3.7% paraformaldehyde in PBS for 10 min, rinsed with PBS, permeabilized in 0.1% Triton X-100 for 5 min. After washing, nonspecific binding was blocked by incubation of cells with blocking solution (10% donkey serum in PBS) for 30 min. Next, cells were incubated with a primary antibody overnight at 4°C, followed by donkey anti-mouse IgG (DyLight 488) or donkey anti-rabbit IgG (DyLight 488) (Jackson, Germany) for 30 min at 37°C. Finally, the cells were incubated with 100 nM Acti-stainTM 555 phalloidin (Cytoskeleton, Inc) and then with DAPI (Sigma, St. Louis, MO, USA). Cells were analyzed by Olympus FV1000 confocal microscope (Olympus, Tokyo, Japan).

Results

Effects of DACT2 on ESCC cell behavior in vitro

First, we assessed the expression level of DACT2 in ESCC cell lines, using 293T cells transfected with DACT2 plasmid as positive control. The results showed that DACT2 was weakly detected in the SHEE and KYSE180 cell lines, but rarely detected in EC171, KYSE150, and cell lines, as well as 293T cell (Fig. 1 A). Then, for functional investigation of DACT2, the low expressing cell lines, such as
KYSE150 and KYSE180, were selected for high exogenous DACT2 expression (Fig. 1 B) and further analysis. Next, we monitored the effect of DACT2 on the capacity of ESCC cell colony formation. In contrast to the control, DACT2-transfected cells displayed dramatically fewer colonies (Fig. 1 C). Furthermore, we examined the effects of DACT2 on cell cycle progressions in ESCC cells (Fig. 1 D). Enhanced DACT2 expression blocked ESCC cell cycle progression at the G2/M phase. These results above suggested that DACT2 could inhibit the proliferation of ESCC cells.

To determine whether DACT2 may play a role in cell migration and invasion, a transwell migration assay and matrigel invasion chambers were used in this study. Our results showed that DACT2-transfected cells had lower migration and invasion rates compared with control cells (Fig. 1 E and F), suggesting that DACT2 inhibits the invasiveness of ESCC cells.

**DACT2 suppression of tumorigenicity in vivo**

We further tested if DACT2-expressing ESCC cells could show attenuated tumorigenesis in an *in vivo* model. Control and DACT2-transfected KYSE150 cells were injected into ten nude mice in each group. Seven days after injection, tumor development in both control and DACT2 groups was measured. Tumors in the control group (mean size: 74±37 mm³) were much larger than those in the DACT2-expressing group (mean size: 47±22 mm³). In addition, DACT2-expressing tumors displayed significantly slower growth rates. At the end of observation (18 days), the tumors of DACT2-expressing tumors grew to 159±131 mm³ in size (0.145±0.099 g in weight) whereas tumors in the control group grew to 953±315 mm³ (0.600±0.222 g in weight)
Similar results were observed when the experiment was terminated 8 days later (Fig S2). HE staining of the xenograft tumor tissue showed that the tumor cells of control group were undifferentiated with a higher nuclear to cytoplasmic ratio compared with DACT2-expressing tumors. Furthermore, we detected CK5/6, a marker for poor differentiation(20), using immunohistochemistry. CK5/6 immunohistochemistry gave diffuse positive staining in the control tumors, but no staining in the DACT2-expressing tumors (Fig 2D). These data demonstrate that DACT2 contributes to growth inhibition and enhanced differentiation of tumor cells.

**DACT2 expression correlates with advanced differentiation and better survival rate in ESCC**

To explore the clinical significance of DACT2 expression profiles in ESCC, we immunohistochemically stained for DACT2 in patient ESCC tumor tissue. DACT2 expression was detected in non-tumor esophageal epithelium (Fig. 3 A and B). In cancerous tissues, poorly differentiation tumors (Fig. 3 C and D) displayed less intense DACT2 staining than those of advanced differentiation (Fig. 3 E and F). Further statistic analysis showed that DACT2 expression significantly correlated with differentiation in ESCC (Table 1, $P=0.008$). Moreover, the 5-year survival rate in patients with DACT2-positive tumors was much higher than that in patients with DACT2-negative tumors (54.9% for DACT2-positive patients and 45.1% for DACT2-negative patients, $P=0.045$) (Fig. 4). These results indicated that DACT2 might be a potential prognostic marker of ESCC.

**DACT2 suppresses activation of the TGF-beta pathway in ESCC**
Previous studies demonstrated that DACT2 inhibits TGF-beta activity via lysosomal inhibitor-sensitive degradation of the TGF-beta receptor (9, 12). However, whether a similar mechanism exists in ESCC remains unclear. We used a Cignal Finder 10-pathway Reporter Array to examine cellular signaling mechanisms that may be affected by DACT2 in ESCC cells. Only TGF-beta signaling was altered by DACT2 over-expression (Fig. 5 A, The Ratio of Vector vs. DACT2 =2.42, \( P<0.05 \)).

Further western blotting analysis also showed that elevation of DACT2 expression reduced p-Smad 2/3, an index of TGF-beta activity, but not other signaling pathway factors in ESCC cells (Fig. 5 B). To further confirm the antagonism of TGF-beta signaling by DACT2, cells were treated with TGF-beta1 after co-transfection with a SMAD response element reporter gene and the DACT2/Vector plasmid. The results showed that TGF-beta1 stimulated the expression of the SMAD reporter gene in ESCC cells, and DACT2 interfered with TGF-beta1-mediated induction of the SMAD-dependent reporter gene (Fig. 5 C). Moreover, when treated with proteasome inhibitor such as MG132 and lysosomal inhibitors such as NH\(_4\)Cl and chloroquine, the TGF-beta-dependent activity recovered (Fig. 5 D). These results suggested that DACT2 remarkably suppressed the activity of TGF-beta signaling in ESCC via both proteasome and lysosomal pathways.

**DACT2 induced F-actin rearrangement**

TGF-beta signaling was found to alter a series of cytoskeleton-related genes expressions, which could induce cytoskeleton recombination that could subsequently promote/inhibit cell migration (21-26). In cytoskeleton rearrangement, F-actin...
reorganization is the essential step (27, 28). Thus, we investigated if DACT2 affects F-actin rearrangement. Phalloidin-Coumarin staining indicated that DACT2 restored expression increased the F-actin contents (Fig. 6 A). The subsequent F-actin isolation and immunoblot analysis also demonstrated that DACT2 increased F-actin and decreased G-actin molecules (Fig. 6 B). In comparing with the control group, cells with up-regulated DACT2 displayed fewer filopodia. When treated with TGF-beta1, the filopodia were induced in control cells but not in the DACT2-expressing cells (Fig. 6 C). These results indicated that DACT2 was involved in breaking the balance between F-actin and G-actin content, and inhibiting the filopodia formation induced by TGF-beta1.

To explore the molecular mechanism of F-actin rearrangement induced by DACT2, we firstly tested the presence of cofilin and ezrin-redixin-moesin (ERM), which played a crucial role in F-actin rearrangement (29, 30). Our results showed that DACT2 could reduce ERM, phosphorylated ERM (pERM) and phosphorylated cofilin (p-cofilin), and increased cofilin (Fig. 6 D). Further immunofluorescence results showed that restored DACT2 expression could increase cofilin and decrease p-cofilin. In addition, DACT2 could cause the rearrangement of both cofilin and p-cofilin location, in accord with F-actin distribution (Fig. 6 E). These results suggested that DACT2 alters the level or/and the location of a series of cytoskeletal-related proteins, resulting in F-actin accumulation.

Discussion

DACT2 has been found to have play important functions in embryo development
and wound healing in animal (9-12). However, roles for DACT2 in humans have rarely been known. In keratinocytes, down-regulation of DACT2 enhances cell migration but has no effect on proliferation(11). In collecting duct-derived cells, DACT2 down-regulation causes a migration defect and partial epithelial-mesenchymal transition(10). In lung cancer cells, restoration of DACT2 expression causes cell arrested in the G0/1 phase(31). We show elevation of DACT2 expression inhibits not only cell proliferation, in which the cells arrest in G2/M phase, but also inhibits the invasiveness of ESCC cells (Fig. 1). Various reports show that the DACT2 has distinct roles in different cells, indicating the tissue type- or species-specific roles of DACT2 function. These actions of DACT2 were also confirmed in vivo. Restored-expression of DACT2 significantly reduces the ability of tumor cells to grow as xenograft tumors in nude mice (Fig. 2). In ESCC, increased expression of DACT2 correlates with well differentiation status (Fig. 2 D, Fig. 3, Table 1); and a higher survival rate is observed in patients with DACT2-expressing tumor (Fig. 4). We therefore propose that DACT2 serves as a protective factor against the progression of ESCC. The mechanism by which decreased expression of DACT2 in poorly differentiated ESCC tissue occurs is unknown. In this process, epigenetic regulations might be involved as reflected in methylation of the DACT2 promoter in colorectal cancer cells and lung cancer cells (31, 32).

In animals ranging from fish to mice, the function of DACT2 as a negative regulator of TGF-beta/Nodal signal pathway is conserved in evolution (9, 11, 12). In the mouse model, DACT2 has no detectable effects on canonical Wnt signaling and
has minor effects on non-canonical Wnt signaling only when DACT2 is massively over-expressed (12). However, little is known of its roles in human cancer, such as ESCC. Recent studies showed that DACT2 could suppress Wnt signaling by inhibiting TCF/LEF in lung cancer (31). However, in our study, DACT2 significantly decreases pSmad2/3 binding to its response element, but has no effect on the activators or transcriptional regulatory elements of the canonical Wnt pathway or other pathways (Fig. 5 A, B and C, supplement Fig. S3), suggesting possible tissue-type specificity of function. Furthermore, DACT2 inhibits TGF-beta activity via lysosomal inhibitor-sensitive degradation of ALK4 and/or ALK5, the TGF-beta receptors (9, 12). However, as shown in our study, TGF-beta signaling activity could be recovered by treating with both proteasome and lysosomal inhibitors (Fig. 5 D), suggesting that DACT2 might affect TGF-beta signaling activity by both proteasome and lysosomal pathways in ESCC. Based on recent studies, both proteasome and lysosomal pathways are involved in TGF-beta signaling regulation, which targets not only the receptors but also the important mediators of TGF-beta signaling (33-36). Thus, in ESCC system, new mechanism by which DACT2 suppresses TGF-beta signaling pathway might exist. However, it’s still unclear which receptor or/and mediator of TGF-beta signaling pathway is involved.

TGF-beta can cause the morphologic changes of cell via actin cytoskeleton reorganization (37-39). In mIMCD3 cells, knockdown of DACT2 altered actin organization to change the morphogenetic behavior (10). Therefore, we became curious as to whether there was any connection between DACT2 and F-actin. In
ESCC cells, DACT2 could alter the equilibrium between F-actin and G-actin, disturb actin depolymerization, and inhibit filopodia formation (Fig. 6 A, B and C), consistent with an ability of DACT2 to modify actin cytoskeleton organization. In actin cytoskeleton rearrangement, a series of molecular events are involved. The TGF-beta signaling pathway has an essential role in regulation of cofilin and ERM (25, 40, 41). Cofilin can determine the cytoskeleton dynamics by its phosphorylation status. Phosphorylation of cofilin by TGF-beta/RhoA/ROCK/LIMK pathway could inhibit ability of cofilin to promote filament disassembly (29, 40, 41). Nevertheless, cofilin can also promote actin polymerization, indicating that it serves as a dynamic component of the steering wheel of cells (42). Besides cofilin, ERM family members play an important role in actin rearrangement. Members of the ERM family, which can be activated by Rho kinase/phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2), act both as linkers between actin cytoskeleton and plasma membrane proteins, and as signaling transducers in responses involving cytoskeletal remodeling (30, 43, 44). Our data indicate that DACT2 might alter the conversion between cofilin and p-cofilin, and decrease both ERM and pERM in ESCC (Fig. 6 D). In addition, DACT2 could rearrange cofilin and p-cofilin (Fig. 6 E). These data suggests that DACT2 might cause the disruption of F-actin/G-actin equilibrium by altering the level or/and location of TGF-beta dependent cytoskeletal-related proteins in ESCC cells.

In summary, we show that decreased DACT2 protein expression correlate with poor differentiation status and poor survival of ESCC patients. Restoration of DACT2 expression significantly inhibits not only proliferation and invasiveness of ESCC cells.
in vitro, but also the tumorigenicity in vivo. DACT2 expression suppresses TGF-beta signaling, and leads to F-actin rearrangement induced by coflin and ERM proteins. Taken together, we propose that DACT2 served as a prognostic marker by inhibiting tumor cell malignancy in ESCC.

Acknowledgements

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Figure Legends

Fig. 1 Inhibitory effects of DACT2 on ESCC cell behavior in vitro.

(A) DACT2 expression level in ESCC cell lines. 293T cells transiently transfected with a DACT2 expression plasmid was the positive control. (B) Establishment of DACT2 over-expressing cell lines. KYSE150 cells were used for stable transfection, and KYSE180 cells for transient transfection. (C, D, E and F) These two cell lines were used for following analysis. (C) Cell growth was determined in clony formation assays. 500 transfected cells were inoculated in each well of the 6-well plates. Cultures were maintained for 3 weeks, and cells were then fixed, stained and photographed. (D) Cell cycle distribution was determined using flow cytometry. The cell DNA was stained by PI (5 \( \mu \)g/ml), and the content of DNA was analyzed using flow cytometry (BD FACSARia II). Transwell (E) and invasiveness (F) assays were used to determine the effects of DACT2 on cell migration and invasion. Migrating and invading cells were fixed and stained, and representative fields were photographed. For quantification, the cells were counted in 10 random fields under a light microscope (\( \times 400 \)). Each experiment was performed in triplicate, and results represent the Sum \( \pm \) SD of three experiments. * \( P<0.05 \).

Fig. 2 DACT2 suppression of ESCC cells in vivo.

KYSE150 cells stably transfected with DACT2 (KYSE150/DACT2) or vector (KYSE150/Vector) were injected s.c. into nude mice (\( 3 \times 10^6 \) cells/flank). (A) Eighteen days after injection with either KYSE150/Vector (up) or KYSE150/DACT2 (down). (B) Time course of tumor growth. Tumor volumes were measured every week. Each point represents the mean volume \( \pm \) SD of ten tumors. * \( P<0.05 \). (C) Tumor weights
at autopsy. At day 18 after injection, tumors were removed and weighed. Results are shown as mean ± SD of tumor weights. (D) Representative photographs of HE staining, DACT2 and CK5/6 immunohistochemical staining of the tumor tissue are displayed. Scale bars, 50 μm.

**Fig. 3 Immunohistochemical analysis of DACT2 expression in ESCC.**

DACT2 expression was detected in non-tumor esophageal epithelium (A, ×200; B, ×400). The DACT2 was moderately expressed in poorly differentiated ESCC tissues (C, ×200; D, ×400) but intensely in ESCC tissues of advanced differentiation status (E, ×200; F, ×400). Scale bars: 50 μm

**Fig. 4 Overall survival of patients with ESCC in relation to DACT2.**

**Fig. 5 Effects of DACT2 on TGF-beta signaling in ESCC.**

(A) Reporter gene assays of key response elements in major cancer-related pathways. KYSE150 cells were inoculated in 96-well plates and grown to 50~80% confluency, and then cells were transiently co-transfected with 100 ng of each dual luciferase Cignal transcription factor-responsive reporter constructs and 200 ng of pcDNA3/DACT2 or pcDNA3 constructs. The luciferase reporter activity of the lysates was measured using a Dual-Luciferase Reporter Assay System (Promega, Madison, WI). The reporter signals of KYSE150 cells transfected with DACT2 or Vector plasmid are shown. Each value represents the mean ± SD. * P<0.05. (B) Western blotting analysis was used to show a series of signaling activity mediated by DACT2 in stably transfected KYSE150 cells. (C) KYSE150 cells were transiently co-transfected with a reporter gene, driven by a SMAD response element and
DACT2/Vector plasmid, and then treated with or without TGF-beta 1 (5 ng/ml, Invitrogen). The reporter signals from the cells are shown. Each value represents the mean ± SD. * P<0.05. (D) Stably transfected cells were treated with or without MG132 (MG, 20μM, Merck), NH₄Cl (NC, 25mM, Sigma), or chloroquine (Chlq, 100μM, Sigma), and then harvested for examination.

**Fig. 6 Effects of DACT2 on F-actin rearrangement in ESCC.**

(A) Stably (KYSE150) and transiently (KYSE180) transfected cells were stained with phallloidin-Coumarin (2 μg/ml) and the F-actin content was analyzed using flow cytometry. (B) F-actin (pellet, p) and G-actin (supernatant, s) content was determined in transiently transfected KYSE150 and KYSE180 cells. (C) KYSE150 cells were transiently transfected with or without pEGFP/DACT2 expression plasmids, and then transfected cells were treated with or without TGF-beta1 (5 ng/ml). Subsequently, cells were fixed, and F-actin organization was analyzed by phallloidin staining. (D) Western blotting analysis was used to show the effect of DACT2 on a series of cytoskeleton-related genes in stably transfected cells. (E) KYSE150 cells were transiently transfected with pcDNA3.0/DACT2 or pcDNA3.0 expression plasmids, and then the cofilin and p-cofilin location in transfected cells was detected by immunofluorescence, and the F-actin organization was also analyzed.
Table 1 Association between DACT2 expression and clinical pathological parameters in ESCC

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<th>Clinical Parameters</th>
<th>DACT2 expression</th>
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<td>Age (years)</td>
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<tr>
<td>Invasive depth</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1+T2</td>
<td>5(6.8%)</td>
<td>5(6.7%)</td>
</tr>
<tr>
<td>T3+T4</td>
<td>69(93.2%)</td>
<td>85(93.3%)</td>
</tr>
<tr>
<td>LN metastasis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N0</td>
<td>37(50.0%)</td>
<td>52(57.8%)</td>
</tr>
<tr>
<td>N1+N2+N3</td>
<td>37(50.0%)</td>
<td>38(42.2%)</td>
</tr>
<tr>
<td>TNM classification</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I+II</td>
<td>41(55.4%)</td>
<td>52(57.8%)</td>
</tr>
<tr>
<td>III+IV</td>
<td>33(44.6%)</td>
<td>38(42.2%)</td>
</tr>
</tbody>
</table>

Note: Statistical analysis: the Kendall’s tau-b test  
L N: lymph node  
Negative: 0~6; positive: 7~12
**Figure 1**

(A) Cell composition of different cell lines.

(B) Western blot analysis showing levels of DACT2 and Beta-actin in KYSE150 and KYSE180 cells with and without DACT2/GFP expression.

(C) Immunofluorescence images of KYSE150 and KYSE180 cells transfected with Vector or DACT2, showing the localization of DACT2 and Beta-actin.

(D) Histograms showing cell cycle composition of KYSE150 and KYSE180 cells transfected with Vector or DACT2, with quantification of G2/M, S, and G1 phases.

(E) Immunohistochemical staining of KYSE150 and KYSE180 cells transfected with control or DACT2 vectors, showing the expression of Beta-actin.

(F) Flow cytometry analysis comparing cell population of KYSE150 and KYSE180 cells treated with Vector or DACT2, with asterisks indicating significant differences.

**Legend:**
- EC71, SHE, KYSE150, KYSE180, KYSE510, 293T, control
- DACT2, Beta-actin
- Vector, DACT2
- KYSE150, KYSE180
- Cell cycle composition: G2/M, S, G1
- Cell count
- Control, DACT2
Figure 2

A. Photographs showing tumor growth for Vector and DACT2 groups.

B. Graph showing tumor size (mm$^3$) over days post tumor cell injection. The x-axis represents days post injection, and the y-axis represents tumor size. Data points for each group are connected by line segments. The Vector group starts with a lower tumor size and shows a slower increase compared to the DACT2 group, which has a more rapid increase in tumor size.

C. Bar graph depicting tumor weight (g). The Vector group has a higher tumor weight compared to the DACT2 group.

D. Images showing HE and IHC staining for Vector and DACT2 groups. The HE images show the general structure of the tumors, while the IHC images highlight specific markers. The images indicate differences in tumor morphology and marker expression between the two groups.
Figure 3
Figure 1

Cum survival

- Positive (scores of 7-12; n=90; 54.9%)
- Negative (scores of 0-6; n=74; 45.1%)

P=0.045
Figure 4

A) Relative Luciferase Activity

B) Western Blot

C) Relative Luciferase Activity

D) Western Blot
Figure 6
Cancer Prevention Research

DACT2 is a candidate tumor suppressor and prognostic marker in esophageal squamous cell carcinoma.


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