DPP3 Expression Promotes Cell Proliferation and Migration in Vitro and Tumor Growth in Vivo That Associates with Poor Prognosis of Esophageal Carcinoma

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Research Article

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Abstract

Background: Dipeptidyl peptidase III (DPP3) is a zinc-dependent metallopeptidase and elevated in a variety of malignant tumors, but the underlying mechanism is not well understood so far. Here we investigated the association of esophageal carcinogenesis with the regulation of DPP3 expression by tissue-based quantitative analysis and the depletion of DPP3 expression in esophageal cancer cells and xenograft model.

Methods: The expression level of DPP3 in esophageal cancer tissues and adjacent normal tissues was detected in 93 cases of tissue biopsies collected from patients diagnosed with esophageal carcinoma by immunohistochemistry. The effect of DPP3 expression on cell proliferation, migration or apoptosis was determined in DPP3-depleted esophageal cancer cells created by infection with the lentivirus containing the shRNA specific to human DPP3 mRNA sequence followed by cytometric detection using celigo cell count assay, flow cytometry, wound-healing assay and trans-well assay as well as chip screening with a Human Apoptosis Antibody Array kit, which enables the quantitative detection of 43 apoptosis-related genes. A xenograft model was applied to the detection of tumor growth and invasion of DPP3-depleted cancer cells in nude mice.

Results: DPP3 expression was elevated in esophageal cancer tissues compared with adjacent non-tumor tissues (normal controls) with statistical significance (P<0.05), and associated with poor prognosis of esophageal carcinoma. The DPP3-depletion resulted in a reduced cell proliferation and migration and enhanced cell-cycle arrest and apoptosis of esophageal cancer cells, and lead to the inhibition of tumor growth and invasion in xenograft model. In addition, DPP3-depletion was associated with the upregulation of pro-apoptotic proteins and the downregulation of anti-apoptotic proteins.

Conclusions: These findings suggest that DPP3 may promote cell proliferation, migration and survival of esophageal cancer cells in vitro, and tumor growth and invasion of esophageal carcinoma in vivo and this might serve as a molecular target for tumor therapy.

Introduction

Esophageal cancer has a very high prevalence and mortality worldwide [1-2]. Esophageal squamous cell carcinoma (ESCC) is the main histological type of esophageal cancer in developing countries and accounts for more than 90% of cancer-related death in China [3-4]. Early detection is the prerequisite for an effective cancer treatment, but when patients are diagnosed with ESCC, most of them have often tumors at medium or late stages with high metastatic potential that leads to poor prognosis and 5-years survival [5]. Thus, there is an urgent need to identify and characterize molecular candidates that can serve as biomarkers for early diagnosis and monitoring the treatment of ESCC.

Growth and survival are major events in the initiation and progression of human cancers. In this process, metallopeptidases may play an important role by promoting cell proliferation and reducing apoptosis due to their critical function in protein metabolism [6-8]. In fact, the deregulation of metallopeptidases is
causally associated with the development of various diseases ranging from cancer, inflammation, microbial infection, neurodegenerative diseases and cardiovascular disorders [9]. As a metallopeptidase, dipeptidyl peptidase III (DPP3) is involved in the progression of diseases such as cancer development, oxidative stress and inflammation [10-11]. A number of studies demonstrate the DPP3 elevation associated with carcinogenesis of the breast, lung, endometria, ovary and brain, and may become a potential target in tumor therapy [12-17]. In a genome-wide expression array analysis, we also found the elevation of DPP3 in ESCC tissues (data not shown). However, the role of DPP3 expression in ESCC is not fully understood. Here we evaluated the differential expression of DPP3 between tumor and normal tissues from ESCC patients, and studied the aberrant regulation of DPP3 expression associated with cellular functions and esophageal carcinogenesis using DPP3-depleted esophageal cancer cells and xenograft modeling for understanding of its role in ESCC progression and providing evidence for DPP3 as a molecular target in early diagnosis and treatment of ESCC.

**Materials And Methods**

**Human tissue specimens**

This study was approved and monitored by the Ethics Committee of the First Affiliated Hospital of Xinjiang Medical University. All procedures were followed in accordance with the Helsinki Declaration of 1975, as revised in 2000. Informed consent was obtained from all donors, and the data was analyzed anonymously throughout the study. A total of 93 patients diagnosed with esophageal cancer were enrolled in this study according to the criteria of World Health Organization and the Chinese Medical Association. The average age of patients was 62 (43–78). None of the patients received chemoradiotherapy or radiotherapy prior to surgery. The patients were followed-up by Outpatient, Inpatient, and telephonic monitoring. The average follow-up period accounted for 18 months and ended in September 2019.

For immunohistochemistry analysis, 156 cases of formalin-fixed and paraffin-embedded esophageal cancer and adjacent non-tumor specimens from the tumor periphery were obtained from the specimen bank in the pathology department after a case review by two experienced pathologists at the First Affiliated Hospital of Xinjiang Medical University, China. The clinical staging of the patients was based on the guideline established by the American Joint Committee on Cancer, as revised in 2009. Tumor specimens were collected from cancer patients who underwent radical thoracic surgery for esophageal cancer at clinical stages I–IV from December 2017 to June 2018.

**Immunohistochemistry**

Paraffin-embedded tissues were sectioned into 3 µm slices. The tissue slices were conventionally stained by the streptavidin peroxidase-conjugated (S-P) method using primary antibody against DPP3 (PA5-35038, 1:50, Thermo Fisher Scientific Inc., Waltham, MA, USA) and IHC Kits containing biotin-labeled secondary antibodies (Zhongshan Golden Bridge Biotechnology Co., Peking, China) in accordance with
manufacturer’s recommended procedures. The negative controls were stained with phosphate buffered saline (PBS) instead of the primary antibody. All tissue slices were counterstained with hematoxylin.

For evaluation, stained tissue sections were scored under a light microscope by two experienced pathologists. The positive-stained area and intensity were scored using the following criteria: Positively stained area: score 0 for negative signal, 1 for 0 to 30% positivity, 2 for 31% to 60%, and 3 for more than 60%. Positive intensity: score 0 for negative signal, 1 for weak signal intensity, 2 for moderate intensity, and 3 for high intensity. A consensus number between the two investigators was reached for each tissue slice. An overall score (0 to 12) was calculated by addition of both scores to evaluate the expression level of a protein in three categories: an overall score of 0 to 6 as total loss or weak expression and 7 to 12 as strong expression of the protein.

**Cell culture**

The EC9706, KYSE450, EC9706 and TE-1 human esophageal squamous cancer cell lines were obtained from Shanghai Cell Collection (Shanghai, China). The cells were grown in Dulbecco’s Modified Eagle Medium (DMEM) (BI, Cromwell, CT, USA) supplemented with 10% fetal bovine serum (FBS) (BI, Cromwell, CT, USA), and 1% penicillin/streptomycin (BI, Cromwell, CT, USA) in a 37 °C incubator filled with 5% CO₂.

**DPP3-depletion by lentiviral expression of shRNA**

For depletion of DPP3 expression in esophageal cancer cells, three different shRNA fragments targeting the mRNA sequence coding for DPP3 protein were designed, synthesized and ligated into the BR-V108 lentiviral vector (GeneChem, Shanghai, China), that expresses both an shRNA fragment and the enhanced green fluorescent protein (EGFP) as a reporter gene. DPP3-depleted esophageal cancer cells were created by liposomal transfection of 293-T cells with the constructs to produce virus for 24 hours in a 6-well plate and followed by infection of Eca-109 and TE-1 esophageal cancer cells with the virus at virus titers of at 1×10⁸ TU/ mL and 20μg/ mL polybrene for 72 hours in accordance with the manufacturer’s recommendation. The efficacy of viral expression was estimated by determining the EGFP-derived fluorescence with the flow cytometry (Millipore, Guava easyCyte HT, Massachusetts, USA).

**shRNA sequences of DPP3**

| DPP3-shRNA   | Sequences                       |
|--------------|--------------------------------|
| DPP3-shRNA-1 | CTTCAAGAGGTCGATGGAGA           |
| DPP3-shRNA-2 | CCGAGGAAGATTTGAAGGTTT          |
| DPP3-shRNA-3 | GCTGGAGAGGCGAAGCCAGCCTA        |

**RNA extraction and quantitative analysis by RT-PCR**
Total RNA was isolated from culture cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) followed by phenol/chloroform extraction and ethanol precipitation. The RNA quality and quantity were determined with Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, USA). The cDNA was synthesized from 1 mg of total mRNA by reverse transcription (RT) using the Revert Aid First Strand cDNA Synthesis kit (Thermo Fisher Scientific, USA) at 42°C for 60 min. The cDNA (20 ng) was analyzed in a 25 µL mixture by quantitative polymerase chain reaction (qPCR) using the QuantiNova SYBR Green PCR Kit (QIAGEN, Germany) and a primer pair specific to the mRNA sequence coding for DPP3 (forward primer: 5'-TGAGTGCCAAGTTTGAGCG-3'; reverse primer: 5'-AGCGAAGGTGAGAACATCCAG-3'), setting the mRNA coding for glyceraldehyde-3-phosphate dehydrogenate (GAPDH; forward primer: 5'-TGACTTCAACAGCGACACCCA -3'; reverse primer: 5'-CACCTGTGGCTGTAGCCAAA -3') as a control. The PCR amplification was performed on an Applied Biosystems® 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) using a program with a reaction condition suitable for DPP3 mRNA: initial denaturation at 95°C for 1 min, followed by 45 cycles of denaturation at 95°C for 10 s and synthesis at 60°C for 30 s. All reactions were performed in triplicate and the expression level of target genes was quantified by the \(2^{-\Delta\Delta Ct}\) method using the internal software of the PCR system, setting GAPDH as an internal control.

**Western blotting**

Protein extracts were prepared by cell lysis using radioimmunoprecipitation assay buffer (RIPA; 0.5M Tris-HCl, pH 7.4, 1.5M NaCl, 2.5% deoxycholic acid, 10% NP-40, 10mM EDTA.) followed by incubation at ice for 30 min prior to centrifugation at 10,000 g and 4°C for 10 min. Protein concentration was determined using the BCA protein assay kit (Thermo Fisher Scientific, USA). For western blotting analysis, 20 mg protein extract as separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by transfer onto polyvinylidene fluoride (PVDF) microporous membrane (Millipore Corp, Billerica, MA, USA). The membrane was blocked and incubated with rabbit primary antibodies against DPP3 (1:1000 dilution, PA5-35038, Invitrogen, Carlsbad, CA, USA), setting GAPDH (1:3000 dilution, Bioworld Technology, USA) as an internal control. After incubation with goat anti-rabbit secondary antibody (Beyotime Biotechnology Co. Ltd, Shanghai, China), the protein expression was detected by chemiluminescence on the WesternBreeze® Chromogenic Immunodetection System (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol.

**Cell growth assay**

The effect of DPP3-depletion on proliferation of esophageal cancer cells, the cells were inoculated in the logarithmic phase into 96-well plates with a culture volume of 100 µL each well and setting three wells as a group for testing. The cell growth was determined in a 5-days interval by counting cells using the Celigo Imaging (Nexcelom Cellometer, San Diego , CA, USA) per day.

**Wound healing assay**
The effect of DPP3-depletion on the wound closure rate of esophageal cancer cells was evaluated by a wound healing assay. Briefly, the cells were inoculated in the logarithmic phase into 96-well plates and cultured at 37 °C and 5% CO₂ for 24 hours. After achieving an appropriate cell attachment, scrape wounds were generated with 96 Wounding Replicator (VP Scientific, San Diego, CA, USA). The wound healing process was then captured by microscopy using an Inverted Fluorescence Microscope (IX73, Olympus, Tokyo, Japan) at intervals of 0, 24, and 48 hours. The wound closure rate was calculated as follows: \[ \frac{\text{cell-free distance (0h) - cell-free distance (24/48)}}{\text{cell-free distance (0 h)}} \].

**Cell migration**

Cell motility associated with DPP3 expression was determined by Transwell assay. The cells were suspended in 100 µL serum-free DMEM and transferred to the upper wells of the Transwell chamber (Corning Inc, Corning, NY, USA) at a density of 5´10⁵ cells per well. The bottom chamber was then filled with 600 µL DMEM containing 10% FBS. The cells were incubated for 40 hours for migration and invasion through the fibronectin-coated polycarbonate membrane of the chamber. The cells remained on top of the membrane were collected with a wet cotton swab and transferred into a fix solution containing 4% paraformaldehyde. The cells that migrated through the membrane of the chamber were collected and stained with Giemsa solution (Shanghai Dingguo, Shanghai, China) and counted under a light microscope (Olympus, Tokyo, Japan) within the scope of 5 random fields.

**Detection of cell cycle and apoptosis**

For cell cycle analysis, the DPP3-depleted cells and controls were collected in pre-cooled phosphate buffered saline (PBS) solution, then immobilized in 75% pre-cooled ethanol for at least 1 hour and stained with propidium iodide (PI, 2 mg/mL, Sigma, St. Louis, MO, USA) followed by analysis on flow cytometry (Millipore-Guava EasyCyte HT, Massachusetts, USA) in accordance with manufacturer’s protocols. Cellular apoptosis was detected using the Annexin V Apoptosis Detection Kit APC (Thermo Fisher Scientific, Waltham, MA, USA) on flow cytometry and the data was analyzed with Flowjo software.

**Animal model**

The animal experiment was approved and monitored by the Animal Ethics Committee of the First Affiliated Hospital of Xinjiang Medical University. Twenty female, 4 to 6-weeks-old, weighing 13-15 g, BALB/c nude mice were purchased from Charles River Laboratories (Beijing, China) and raised in a specific pathogen free (SPF) level environment. The mice were randomly allocated into two groups: as shDPP3 and shCtrl with 10 mice in each group. To investigate the effect of DPP3 depletion in vivo, the mice divided to shDPP3 and shCtrl groups were injected with DPP3-depleted Eca-109 esophageal cancer cells as an experimental group and the Eca-109 cells infected with lentivirus that doesn't contain shRNA for DPP3 as a control group, respectively. Tumor size was measured after one week of injection and the tumor dimensions were measured every three days till the end of the experiment in 18 days.
Tumor volume (TV) was calculated according to the formula: $TV (mm^3) = \pi/6 \times \text{length} \times \text{width}^2$, that is $TV (mm^3) = 3.14/6 \times \text{length} \times \text{width}^2$. The animals were anesthetized by intraperitoneal injection of D-luciferin (15 mg/mL) and sodium pentobarbital solution (70%) at a dose of 10uL/g prior to the disposition by cervical dislocation, and analyzed by fluorescence imaging using an in vivo imaging system (IVIS, Berthold Technologies, Germany). All subcutaneous tumor tissues were collected, weighted, fixed with 4% paraformaldehyde, and embedded, resected, stained with hematoxylin-eosin (HE) to confirm the histopathology and immunohistochemical analysis with antibody against Ki-67 (1:200 dilution, Ab16667, Abcam, Cambridge, UK).

**Antibody array analysis**

The expression of apoptosis-related proteins was detected in cell lysates from DPP3-depleted cells and controls using a Human Apoptosis Antibody Array kit (ab134001, Abcam, Cambridge, UK) that can detect 43 apoptosis-related proteins of human origin. Pixel densities of each protein spot were determined by using the Image J software.

**Statistical analysis**

Statistical analyses were performed using SPSS (version 21.0; SPSS, Inc. Chicago, IL, USA) and GraphPad Prism Version 8.0.1(GraphPad Inc, San Diego, CA, USA) software. Data are presented as the mean ± standard deviation (SD). The Student's t test and the Mann Whitney U test were used to analyze the differences between the two groups. ANOVA was used to compare statistical differences of more than three groups. Patient survival was analyzed using Kaplan-Meier survival analysis and log-rank test. Spearman correlation method was used to calculate the correlation between DPP3 and lymph node metastasis. P<0.05 was considered statistically significant.

**Results**

**The clinical significance of DPP3 expression in esophageal carcinogenesis**

To evaluate the role of DPP3 expression during the development of esophageal carcinoma, tumor tissues and their adjacent normal tissues from the tumor periphery were analyzed by immunohistostchemistry. The data showed a strong DPP3 staining in the cytoplasm and nucleus of esophageal cancer cells and a weak expression in most of the controls (Fig.1a). Statistical analysis confirmed a significantly high DPP3 expression level in tumor tissues compared with normal controls (Tab. 1, $P<0.05$). In addition, DPP3 expression was positively correlated with lymph node metastasis and decreased overall survival (Tab. 2 and 3, Fig. 1b, $P<0.05$), but no difference was found for age, gender, tumor size, T infiltrate, differential stage, lymphoid positive number, grade ($P>0.05$).

Table 1. Analysis of DPP3 expression in esophageal cancer by immunohistochemistry
Table 2. DPP3 expression associated with lymph node metastasis of esophageal cancer

| DPP3 expression | Tumor specimens | Normal controls | p   |
|-----------------|----------------|----------------|-----|
| weak            | 51 (54.8%)     | 57 (90.5%)     | <0.001 |
| strong          | 42 (45.2%)     | 6 (9.5%)       |     |
| Features                     | No. of patients | DPP3 expression | p value |
|------------------------------|-----------------|-----------------|---------|
|                              |                 | low  | high |     |
| All patients                 | 93              | 51   | 42   | 0.925 |
| Age (years)                  |                 | 0.925|      |     |
| <65                          | 46              | 25   | 21   |        |
| ≥65                          | 47              | 26   | 21   |        |
| Gender                       |                 | 0.326|      |     |
| Male                         | 75              | 43   | 32   |        |
| Female                       | 18              | 8    | 10   |        |
| Tumor size                   |                 | 0.900|      |     |
| ≤5cm                         | 48              | 26   | 22   |        |
| >5cm                         | 36              | 20   | 16   |        |
| T Infiltrate                 |                 | 0.564|      |     |
| T0                           | 1               | 1    | 0    |        |
| T1                           | 3               | 1    | 2    |        |
| T2                           | 14              | 8    | 6    |        |
| T3                           | 41              | 24   | 17   |        |
| T4                           | 12              | 5    | 7    |        |
| lymph node metastasis[N]     |                 | 0.028*|      |     |
| N0                           | 31              | 22   | 9    |        |
| N1                           | 19              | 8    | 11   |        |
| N2                           | 13              | 6    | 7    |        |
| N3                           | 8               | 3    | 5    |        |
| Stage                        |                 | 0.172|      |     |
| I                            | 3               | 2    | 1    |        |
| II                           | 30              | 20   | 10   |        |
| III                          | 35              | 14   | 21   |        |
| IV                           | 3               | 3    | 0    |        |
| Lymphoid positive number     |                 | 0.319|      |     |
| Grade |   N   | Pearson correlation | Significance (double-tail) |
|-------|-------|---------------------|---------------------------|
| I     | 43    | 26                  | 17                        |
| ≥1    | 48    | 24                  | 24                        |
| Grade | 0.474 |                     |                           |
| I     | 8     | 5                   | 3                         |
| II    | 50    | 26                  | 24                        |
| III   | 26    | 17                  | 9                         |

Table 3. Relationship between DPP3 expression and tumor characteristics in patients with esophagus cancer

DPP3 had an identical transcription pattern in four esophageal cancer cells, KYSE450, Eca-109, TE-1 and EC9706, with a relatively high expression level in Eca-109 and TE-1 cells compared with the others (Fig. 2a). Three shRNA fragments targeting DPP3 mRNA were then screened for the efficiency of DPP3-depletion after lentiviral expression in Eca-109 and TE-1 cells, and an almost identical pattern and level of DPP3-depletion was confirmed by quantitative RT-PCR (Fig. 2b). The transfection efficiency was confirmed by detection of the green fluorescent protein (GFP) simultaneously expressed by the shDPP3 construct that contains shRNA targeting DPP3 mRNA and the shCtrl as normal control (Fig. 2c). The qRT-PCR and western blotting analyses demonstrated a significant decrease in DPP3 expression after shRNA expression in Eca-109 and TE-1 cells (Fig. 2d and 2e).

Depletion of DPP3 in esophageal cancer cells by lentiviral expression of shRNA targeting DPP3 mRNA

The impact of DPP3-depletion on the cellular function of esophageal cancer

Fluorescent microscopy, flow cytometry, wound healing assay and Transwell assay were applied to detect changes in cellular function after expression of the shRNA targeting DPP3 mRNA in Eca109 and TE-1 esophageal cancer cells (Fig. 3). The data showed a remarkable decrease in cell proliferation, cell-cycle retention in the S and G2 phases, and increased apoptosis as well as the inhibition of cell migration and invasion after depletion of DPP3 in esophageal cancer cells.
Changes in protein expression profile related apoptosis after DPP3-depletion

As described above, cellular apoptosis was remarkably inhibited in esophageal cancer cells after depletion of DPP3 expression. Accordingly, we detected protein expression profile associated with apoptosis using an antibody array recognizing 43 human proteins functionally related apoptosis signaling. The data demonstrated a remarkable in the expression of p53 and SMAC, known as pro-apoptotic proteins, and the decrease in cIAP-2/IGFBP-2 and TRAILR-4 as anti-apoptotic proteins (Fig. 4a and 4b). These findings suggest that DPP3 expression may play an important role in the upregulation of proteins associated with apoptosis and cell survival signaling in esophageal cancer cells.

The impact of DPP3-depletion on the growth of tumor xenografts from esophageal cancer cells

The role of DPP3 expression in tumorigenesis in vivo was studied by analyses of tumor xenografts generated by intracutaneous injection of nude mice with Eca109 esophageal cancer cells before and after DPP3 depletion (shDPP3 vs. shCtrl). DPP3-depletion resulted in a rapid decrease in tumor weight, tumor growth, tumor size and fluorescent expression in animals injected with DPP3-depleted esophageal cancer cells compared with controls (Fig. 4 c and d). Immunohistochemical analysis confirmed a reduced expression of Ki-67 protein in tumor xenografts from DPP3-depleted esophageal cancer cells compared with controls (Fig. 4e). These results suggest that DPP3-depletion in esophageal cancer cells may not only inhibit cell proliferation and promote apoptosis \textit{in vitro}, but also inhibit tumor growth \textit{in vivo}.

Discussion

Mammalian dipeptidyl peptidases (DPPs) consist of eight members, DPP1 (cathepsin-C), DPP2 (DPP7), DPP3, DPP4 (CD26), DPP6, DPP8, DPP9, and DPP10, and play a role in oligopeptide N-terminal processing and degradation of bioactive peptides [18]. The research field of DPPs family has been guided by the study of DPP4 over the past 25 years, leading to the identification of diverse physiological bioactive substrates and inhibitors that indicate a role of DPPs in metabolism, cellular homeostasis, cell signaling and immunity [19-20]. Recent studies focused on the role DPP4 and other family members such as DPP1, DPP2 and DPP3 in the progression of metabolic disorders, inflammation, blood pressure regulation and cancer [21-23]. However, the role of most DPPs family members in physiological functions and pathological conditions remain largely unsolved.

In this study, we demonstrated the significant upregulation of DPP3 in esophageal cancer and its association with lymph node metastasis. This is in consistency with the previous reports on the elevation of DPP3 in several human cancers in particular in aggressive ovarian and endometrial cancers [14-15]. DPP3 is overexpressed in ER-positive breast cancer and associated with poor survival [24]. DPP3 is a member of M49 family of metallopeptidases that have a catalytic activity dependent on Zn$^{2+}$-binding motif conserved from bacterial to mammals [22]. DPP3 has substrate specificity for several bioactive peptides including angiotensins, proctolins, enkephalins, endorphins, and dipeptidyl derivatives, that play diverse physiological roles in cell signaling, pain modulation, inflammation, and blood pressure regulation.
[23][25]. Because of the specificity for short peptides, DPP3 may play a role in the turnover of peptides resulting from protein digestion by other proteases like proteosome system [26]. The degradation by DPP3 may prevent peptide leaking from necrotic cells and block antigen presentation to the immune system, which can be utilized by tumor cells for immune evasion [27]. Thus, this may provide evidence for the role of DPP3 expression in survival and metastasis of esophageal carcinoma.

By analyses of DPP3-depleted esophageal cancer cells and tumor xenografts, we showed that DPP3 elevation had an impact on cell functions during esophageal carcinogenesis including the inhibition of cell cycle arrest and apoptosis, and the increase in cell proliferation and migration in vitro, and the promotion of tumor growth and survival in vivo. This may further explain the role of DPP3 elevation in growth, invasion and metastasis of esophageal carcinoma. DPP3 is mainly localized in cytosol, but a few studies found its membranous activity [28-30]. The upregulation of DPP3 expression is positively correlated with increased expression of NRF2 in lung cancer indicating a possible link between DPP3 and NRF2 in esophageal cancer [13]. A few studies demonstrate an oxidative stress-induced binding of DPP3 to Keap1 that prevents Keap1-mediated degradation of NRF2 leading to increased NRF2 nuclear translocation [12]. It is suggested that DPP3 elevation may result in increased NRF2 downstream expression of cytoprotective genes associated with aggressive cancer phenotypes [31][13]. The role of NRF2 in cancer is controversially discussed due to its acting in both tumor suppression and promotion that depends largely on the context [32-33]. The induction of NRF2 in normal cells may activate a broad cellular defense against cancer, while constitutive elevation or increased release of NRF2 by binding of DPP3 to Keap1 may create a redox environment for tumor growth and resistance to chemotherapy [34-35]. Thus, increased DPP3 expression may support growth, survival, metastasis and resistance to therapy of esophageal carcinoma.

Antibody array analysis revealed that DPP3-depletion may cause the downregulation of pro-apoptotic genes such as cIAP-2 IGFBP-2 TRAILR-4, and the upregulation of anti-apoptotic genes P53 and SMAC. Apoptosis is a tightly regulated cellular process and faulty regulation of apoptosis is a hallmark of human cancers. TNF-related apoptosis-inducing ligand (TRAIL) is a type II transmembrane protein and binds its receptors TRAIL-R1 and TRAIL-R2, which in turn recruit downstream adaptor proteins via its intracellular death domain (DD) and activate the intrinsic apoptotic pathway triggering selective neoplastic cell apoptosis [36-37]. This is regulated by non-functional TRAIL receptors, TRAIL-R3 and TRAIL-R4 that are devoid of a cytoplasmic tail or carry a truncated intracellular DD, respectively, and block TRAIL-mediated apoptosis [38-39]. TRAILR4 can also induce non-apoptotic signaling mediated by NF-κB and AKT pathways that is correlated with its overexpression in malignant tumor phenotypes [40-42]. In human cancer cells, TRAIL-R4 expression level is also positively correlated with TRAIL resistance and its downregulation leads to reduced tumorigenic potential or apoptosis [43-45]. Cellular inhibitor of apoptosis (cIAP2) plays a role in degrading caspases by linking them to ubiquitin molecules and supports cell survival by preventing cellular apoptosis of cancer cells [46-48]. cIAP-2 is upregulated in malignant tumors, and promotes proliferation and invasion of tumor cells through the activation of NF-κB signaling pathway [49-50]. Second mitochondria-derived activator of caspases (SMAC) is an endogenous antagonist of cIAP1, cIAP2 and XIAP, and promotes apoptosis by binding IAPs and preventing the
inhibition of caspases 3, 7, and 9 [51]. SMAC is normally sequestered within the mitochondria and is released into the cytoplasm upon cell death stimuli, thereby overcomes anti-apoptotic actions caused by the IAPs [52]. In line with the pro-apoptotic role of SMAC, its expression is downregulated in esophageal cancer cells and tumor specimens from patients with esophageal cancer [53]. Insulin-like growth factor binding protein 2 (IGFBP2) is a member of IGF system and apoptosis suppressor [54]. IGFBP2 is elevated in tumor specimens from patients and plays a role in tumor cell proliferation, migration, invasion, angiogenesis, and epithelial to mesenchymal transition (EMT) by integrating a series of signaling pathways [55-56]. Among the members of IGFBP2-associated network, integrin functions as a receptor for collecting IGFBP2 extracellular signals; PTEN acts as a checkpoint in IGFBP2 signaling; STAT3 and NFκB serve as downstream transcriptional factors that direct tumorigenic intracellular signaling; nuclear IGFBP2 itself functions as a tumor enhancer by directly targeting multiple oncogene promoters [57]. P53 is a well-known tumor suppressor that negatively regulates cell proliferation and promotes cell differentiation by inducing cell cycle arrest and apoptosis [58]. These studies provide evidence for the role of DPP3 in the upregulation of pro-apoptotic genes clAP-2IGFBP-2 and TRAILR-4, and the downregulation of anti-apoptotic genes SMAC and p53 that contribute to tumor initiation and progression.

Based on these analyses, we suggest that the role of DPP3 in degrading short peptides might be utilized by tumor cells for inhibition of antigen-cross presentation and thus immune evasion associated with growth and metastasis of ESCC. DPP3 interacts with Keap1 and prevents the inhibition of NRF2 activity by Keap1-NRF2 interaction, which contributes to increased cell proliferation and migration, and decreased cell-cycle control and apoptosis of esophageal cancer cells in vitro, leading to the tumor growth and progression in vivo. The upregulation of pro-apoptotic genes and the downregulation of anti-apoptotic genes may also be attributed to DPP3 function to promote tumor growth and survival.

**Conclusions**

In this study, we demonstrated the aberrant regulation of DPP3 expression associated with lymph node metastasis. We assume that DPP3 function in degrading short peptides might be utilized by cancer cells for immune evasion and survival. In addition to this, the interaction of DPP3 with Keap1 enables Nfr2 to release from Keap1-NRF2 complex followed by nuclear translocation leading to the expression of cytoprotective genes that contribute to enhanced cell proliferation and migration, and inhibition of apoptosis and cell cycle control as shown by analyses of DPP3-depleted esophageal cancer cells and tumor xenografts. Moreover, DPP3 may contribute to reduced apoptosis of esophageal cancer cells by upregulation of pro-apoptotic genes clAP-2IGFBP-2 and TRAILR-4, and the downregulation of anti-apoptotic genes SMAC and p53.

**Declarations**

*Ethics approval and consent to participate*
Specimen collection and analysis processes were approved by the Ethics Committee of the First Affiliated Hospital of Xinjiang Medical University.

**Consent for publication**

Not applicable.

**Availability of data and materials**

All data generated or analysed during this study are included in this article.

**Competing interests**

The authors declare that they have no competing interests.

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**Authors' contributions**

JKL, A.A performed most experiments and wrote the manuscript; HTY analyzed the data; LXX participated in the in vivo study; A.T. participated in the in vitro study; GB revised the manuscript; and M. E. designed the overall study, supervised the experiments, and analyzed the results.

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Figures

Figure 1

DPP3 expression in esophageal cancer tissues and controls. (A) IHC staining in esophageal cancer tissues and adjacent normal controls of the tumor periphery; (B) The association of DPP3 expression with the overall survival of ESCC patients displayed by Kaplan–Meier survival analysis. (Log-rank test, P<0.001).
Figure 2

The effect of DPP3-depletion by lentiviral expression of shRNA targeting DPP3 mRNA in Eca109 and TE-1 esophageal cancer cells. a. Baseline expression of DPP3 in Eca-109, KYSE450, EC9706 and TE-1 esophageal cancer cells detected by qRT-PCR; b. qRT-PCR detection of DPP3-depletion by lentiviral expression of shRNAs, shDPP3-1, shDPP3-2 and shDPP3-3, targeting DPP3 mRNA. c. Assessment of lentiviral infection by microscopic detection of GFP simultaneously expressed with shRNA in Eca-109 and TE-1 cells. d, e. Detection of DPP3 expression by western blotting and qRT-PCR analyses after DPP3-depletion in Eca-109 and TE-1 cells. The data were presented as the mean ± SD (n = 3), *P < 0.05, **P < 0.01, ***P < 0.001

Figure 3

The change in cellular functions after DPP3-depletion in Eca-109 and TE-1 cells. (a) Detection of cell proliferation by Celigo Cell Counting Assay; (b) Flow cytometric detection of cell cycle check points by propidium iodide (PI) staining; (c) Flow cytometric detection of apoptosis by Annexin V-APC staining. The quadrants in the flow cytometry plots are as follows: Upper left, living cells; upper right, early apoptotic cells; lower right, late apoptotic cells; and bottom left, cell necrosis (***P<0.05). The migration was
assessed by the wound healing assay (d) and Transwell assay (e). Data was presented as mean ± SD of 3 independent experiments performed in triplicate (**P<0.01; ***P<0.001).

Figure 4

Analysis of protein expression profile related apoptosis and altered after DPP3-depletion and in vivo analyses of DPP3-depletion esophageal cancer cells by tumor xenograft model in nude mice. (a) A protein expression profile related to apoptosis was analyzed before and after DPP3-depletion using an antibody array recognizing 43 proteins associated with apoptosis signaling; (b) A number of proteins differentially expressed after DPP3-depletion; (c) Nude mice were intracutaneously injected with Eca-109 esophageal cancer cells with and without DPP3-depletion, and by measuring of tumor formation, tumor size, tumor weight; (d) The growth of tumor xenografts in nude mice was visualized by detection of life fluorescence from green fluorescence protein expressed by lentiviral vectors with or without shRNA targeting DPP3 mRNA; (e) The growth of xenograft tumor was confirmed by HE staining and the expression of Ki67 was detected as proliferation biomarker by immunohistochemistry.