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Embryonic develop-associated gene 1 is overexpressed and acts as a tumor promoter in thyroid carcinoma

Jinkai Xu, Zongyu Li, Qinghua Su, Jun Zhao, Jiancang Ma*

Department of General Surgery, The Second Affiliated Hospital of Xi'an Jiaotong University, Shaanxi, PR China

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ABSTRACT

Embryonic develop-associated gene 1 (EDAG-1), a hematopoietic tissue-specific protein, is usually highly expressed in the placenta, fetal liver, bone marrow and leukemia cells, but the expression status in normal or solid tumor tissues is rarely reported. In this study, we found that EDAG-1 was up-regulated in thyroid carcinoma tissues and cells. Knockdown of EDAG-1 suppressed proliferation and enhanced cisplatin-induced apoptosis of thyroid carcinoma cells. We also demonstrated that knockdown of EDAG-1 inactivated the phosphatidylinositol-3 kinase (PI3K)/Akt signaling pathway *in vitro* and *in vivo*. Moreover, knockdown of EDAG-1 suppressed tumorigenesis of thyroid carcinoma *in vivo*. Taken together, these results suggest that EDAG-1 regulates the proliferation and apoptosis of thyroid carcinoma *via* the PI3K/Akt signaling pathway.

1. Introduction

Thyroid carcinoma (TC) is the most common endocrine malignant tumor, and its incidence has been explosively rising worldwide over the past few decades [1,2]. According to tumor pathology classification, TC including four classification, that are follicular epithelial cell-derived thyroid tumors (FTC), papillary thyroid cancer (PTC), anaplastic thyroid cancer (ATC) and poorly differentiated thyroid cancers (PDTCs) [3–6]. Similar to other cancers, thyroid carcinogenesis involves epigenetic alterations and accumulation of various genetic, leading to gain-of-function in oncogenes and loss of-function in tumor suppressor genes [7]. So far, the factors such as age, sex, tumor size, tumor-node-metastasis (TMN) stage and extra thyroidal invasion have been proposed as poor prognostic factors [8]. Although surgery and chemoradiotherapy are generally effective for thyroid cancer, many of these patients are currently incurable, and novel treatments are needed for them. In order to give a more objective preview of tumor behavior, many attempts to identify more diagnostic and prognostic factors in thyroid carcinoma has been induced, at the time of first surgery.

Cisplatin (diamminedichloroplatinum, DDP) is widely used as the preferred chemotherapeutic agent for different cancers, such as human non-small cell lung cancer treatment and colon cancer [9]. Embryonic develop-associated gene 1 (EDAG-1) is located in chromosome 9, encoding a 484 amino acid protein [10]. EDAG-1 is usually highly expressed in the placenta, fetal liver, bone marrow [10] and leukemia cells [11], but the expression status in normal or solid tumor tissues is rarely reported. Specific expression of EDAG-1 in hematopoietic tissue is related to leukemia and involved in the regulation of proliferation, differentiation and apoptosis of hematopoietic cells [12]. Many signaling pathways have been illustrated their important role in tumorigenesis of thyroid cancer in recent years [13]. Previous studies revealed that EDAG promoted the proliferation and differentiation of hematopoietic cells and resist cell apoptosis through the activation of nuclear factor-kappa B (NF-κB) [14,15]. The PI3K/Akt signaling pathway plays a fundamental role in the regulation of cell growth, proliferation, and in human tumorigenesis in thyroid carcinoma [16,17]. It has been proved that EDAG-1 kept silent in many tumor tissues. While in the present study, EDAG-1 showed a high expression in thyroid carcinoma and important biological functions. Nevertheless, the mechanisms by which EDAG-1 exerts biological functions in thyroid carcinoma are still unknown.

In the present study, we evaluated 118 cases of thyroid carcinoma with known histological properties, including extra thyroidal invasion and TMN stage, to determine the expression level of EDAG-1. The aim of this study was to examine biological functions of EDAG-1, and to investigate the underlying molecular mechanisms in thyroid carcinoma.

* Corresponding author at: Department of General Surgery, The Second Affiliated Hospital of Xi'an Jiaotong University, No.157 Xiwu Road, Xi'an 710004, PR China.
E-mail address: jcangma@163.com (J. Ma).

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2. Materials and methods

2.1. Clinical patients

One hundred and eighteen patients diagnosed with TC were recruited in our hospital during 2012–2014. Their clinical information (Table 1) obtained by reviewing the pathology reports included age, sex, TMN stage, tumor size and extrathyroid invasion. Informed consent was signed by all participants and the study protocol was approved by the Second Affiliated Hospital of Xi'an Jiaotong University.

2.2. Cell culture and transfection

Human thyroid follicular epithelial Nthy-ori 3-1 cells and human thyroid cancer cell Sw579 were maintained in DMEM (Gibco, Invitrogen, CA, USA) with 10% fetal bovine serum (Gibco BRL, Carlsbad, CA). Human thyroid cancer cells TPC-1, BCPAP, FTC-133 and WRO were cultured in RPMI 1640 medium (Invitrogen, Grand Island, NY, USA) with 10% fetal bovine serum. Cells were cultured in an incubator at 37 °C with a humidified atmosphere of 5% CO_2 in the presence of 100 U/ml penicillin, and 100 μg/ml streptomycin. All the cells in the study were purchased from Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China).

A short hairpin RNA (shRNA) specific for EDAG-1 (pGenesil-2-EDAG-1) and a nonspecific control shRNA (pGenesil-2-control) were chemically synthesized by Sangon (Shanghai Sangon Biotech Co., Shanghai, China). The stable EDAG-1 silencing cell line was obtained by transfection pGenesil-2-EDAG-1 cloning vector into TPC-1 cells using DharmaFECT 4 Transfection Reagent (Dharmacon, Lafayette, CO, USA). The knockdown procedure was as described previously [18].

2.3. Quantitative real-time polymerase chain reaction

Total RNA was extracted from different tissues and cells including Nthy-ori 3-1, Sw579, TPC-1, BCPAP, FTC-133 and WRO, using Trizol reagent (Gibco Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. A total of 1 μg total RNA was reverse-transcribed using reverse the transcription kit (Qiagen, Hilden, Germany) and quantitative polymerase chain reaction (qPCR) was performed using the miScript SYBR Green PCR Kit (Qiagen) according to the manufacturer's protocol. GAPDH (F: 5′-ATG GGG CAT GAG GTC ACC AC-3′, R: 5′-TGA AGG TCG GAG TCA ACG GAT TTG GT-3′, Qiagen) was used as internal control. The threshold cycle (Ct) was defined as the number of PCR cycles required for the fluorescence to pass the fixed threshold. Then ΔCt values were calculated according to the formula ($\Delta Ct = Ct_{EDAG\text{-}1} - Ct_{GAPDH}$). ΔΔCt value was determined by the formula ($\Delta\Delta Ct = \Delta C_{experimentalgroup} - \Delta Ct_{controlgroup}$). Last, $2^{-\triangle\triangle Ct}$ was used for analysis of relative expression quantity differences [19]. All reactions were performed in triplicate.

Table 1
Clinicopathologic characteristics of the patients.

Characteristics	*n*	EDAG-1-positive	%	$\chi 2$	P value
Age (y)				0.372	0.542
<45	62	41	66.1		
≥45	56	34	60.7		
Sex				0.173	0.678
Male	33	20	60.6		
Female	85	55	64.7		
TMN stage				15.974	0.001
I	22	8	36.4		
II	41	23	56.1		
III	24	17	70.8		
IV	31	27	87.1		
Tumor size				0.255	0.613
<2 cm	53	35	66		
≥2 cm	65	40	61.5		
Extrathyroid invasion				13.82	0.000
with	42	36	85.7		
without	76	39	51.3		

2.4. Western blot assay

After treatment, cells were collected and washed with phosphate buffered saline (PBS). Briefly proteins were extracted, centrifuged and supernatant collected according to the instruction (Sangon). Total proteins were quantified using the BCA Protein Assay Reagent Kit (Sangon), separated by 8% SDS/PAGE, and transferred onto PVDF membrane using standard procedure. After blocked with 5% BSA for 1 h at room temperature, the membranes were probed with primary antibodies overnight at 4 °C. Primary antibodies (Sigma, St. Louis, MO, USA) against EDAG-1, Ki67, Bax, Caspase-3, Bcl-2, PI3K, p-PI3K (Tyr 199), Akt and p-Akt (Ser 473) were selected for this study. Anti-GAPDH (Cell Signaling Technology, Beverly, MA, USA) was used as a control. The membranes were washed three times with TBST and then, incubated with HRP-labeled Goat Anti-Mouse IgG (H + L) secondary antibodies for 1 h at room temperature. Visualization of the proteins was done using ChemiDoc-It 2 Imaging System (UVP, Sopachem).

2.5. MTT assay

To record the cell viability, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used for assessment. After transfection, the TPC-1 and FTC-133 cell lines were plated in 96-well plates at a density of 10^4 cells per well and incubated for 24 h, 48 h, 72 h and 96 h. Then, added 20 μl 5 mg/ml MTT to each well incubated sequentially at 37 °C for 4 h. Before measurement, the supernatant was discarded, and 200 μl of DMSO (Sigma, St. Louis, MO, USA) was added to each well to dissolve the formazan crystals. The absorbance at 490 nm (A490) of each well was read on a Multiscan Spectrum (Thermo Fisher Scientific, Waltham, MA, USA). Three independent experiments were performed in quadruplicate.

2.6. Assessment of apoptosis

Apoptosis assay was performed with Annexin V-FITC Apoptosis Detection Kit (KeyGEN, Nanjing, China), according to the manufacturer's instructions. An amount of 3×10^5 cells were cultured in 6 well plates and were treated with 50 μM cisplatin for 48 h. The cells were then harvested in complete1640 medium and centrifuged at 1000*g* for 5 min. Each of the cell lines was washed twice with cold PBS, resuspended in 400 μl annexin V-fluorescein isothiocyanate (FITC) binding buffer. The cell suspension was incubated with 5 μl of Annexin V-FITC and 10 μl propidium iodide (PI) at room temperature for 10 min. Finally, the treated cells were immediately analyzed on a FACSCalibur flow cytometer (Becton Dickinson Medical Devices, Shanghai, China). This experiment was conducted for three times independently.

2.7. Xenograft models

BALB/c nude mice aged 4–5 weeks old (SLAC Animal, Shanghai, China) were randomized to control and treated groups (6 mice per group). The two groups were subcutaneously injected in the left