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Original article

Epidermal growth factor-like domain 7 promotes cell invasion and angiogenesis in pancreatic carcinoma

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ABSTRACT

Epidermal growth factor-like domain 7 (EGFL7), also known as vascular endothelial stain, was firstly identified as a modulator of smooth muscle cell migration. Though the expression of EGFL7 was reported to be up-regulated during tumorigenesis, the clinical and biological functions of EGFL7 in pancreatic carcinoma (PC) were still not fully elucidated. In this study, we found that the serum EGFL7 level in PC tissues was statistically higher than that in normal subjects ($p < 0.001$), and its level in non-resectable patients was also higher than that in resectable ones ($p = 0.013$). Among these resectable PC patients, the postoperative EGFL7 expression was significantly down-regulated when tumors were resected ($p = 0.018$). Using the immunohistochemistry method, our results demonstrated that the positive expression of EGFL7 was significantly associated with the TNM stage ($p = 0.024$), lymph node metastasis ($p = 0.003$) and local invasion ($p = 0.022$), and the EGFL7 expression closely correlated to the micro-vessel density (MVD) in PC tissues by *Spearman* analysis ($r = 0.941$, $p = 0.000$). In vitro, EGFL7 was silenced by the small interference RNA in PC cells, and our data indicated that down-regulation of EGFL7 did not influence the cycle progression, proliferation, colony formation and apoptosis of PC cells ($p > 0.05$), whereas inhibition of EGFL7 expression could decrease PaCa-2 cell invasion ($p < 0.05$). More interestingly, by tubular formation, Chick embryo chorioallantoic membrane (CAM) and ELISA assays, our results revealed that silencing EGFL7 expression represented a strong inhibiting effect on tubular formation of micro-vessels through down-regulating the protein levels of VEGF and Ang-2 ($p < 0.05$). Our results raised the possibility of using EGFL7 as a potential prognostic biomarker and therapy target of PC, and down-regulation of EGFL7 might be considered to be a potentially important molecular treatment strategy for patients with PC.

1. Introduction

Pancreatic carcinoma (PC) has been recognized as a leading malignancy with high cancer-related mortality worldwide [1]. The initiation and development of PC is a multi-factorial, multistep and complex process. Though the systemic treatment of PC has been improved, the long-term survival rate of PC still remains below 5% due to the extremely poor early-diagnosis and dismal prognosis [2]. The poor prognosis of PC is related to the high likeliness of invasion, metastasis and resistance to radio- or chemotherapy. Therefore, developing novel and potential predictive factors is essential for PC treatment [3].

Epidermal growth factor-like domain 7 (EGFL7), also known as vascular endothelial stain, is a 41-kDa secreted protein that contains two EGF-like domains [4,5]. EGFL7 was reported to be exclusively expressed in endothelial cells (ECs) and might act in an autocrine fashion [6]. Studies also demonstrated that EGFL7 was essential in the process of angiogenesis during zebra-fish embryogenesis [7]. The expression of EGFL7 was confirmed to

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be down-regulated in almost all mature tissues, but it was over-expressed in many types of cancers, such as hepatocellular carcinoma [8], laryngeal squamous cell carcinoma [9], epithelial ovarian cancer [10] and renal cell carcinoma [11].

In 2014, Zhou et al. found that the EGFL7 expression in tumor tissues was significantly higher than that in non-tumor tissues, and high EGFL7 expression was identified as an independent marker for long-term outcome of PC [12]. Previously, we reported for the first time that EGFL7 could promote the metastasis by inducing the epithelial-mesenchymal transition (EMT) in PC cells [13]. However, the clinical and biological functions of EGFL7 in PC were still not fully elucidated.

In this study, the expressing levels of EGFL7 in PC serum were measured by ELISA, and its clinicopathological values in PC tissues were investigated by the immunohistochemical method (IHC). We evaluated the relationships between EGFL7 expression and clinicpathologic variables in 60 resectable PC patients, and the correlations between EGFL7 expression and MVD were also assessed. Meanwhile, EGFL7 was silenced by the small interference RNA in PC cells, and the influences of EGFL7 on cancer-related growth, apoptosis and invasion were elucidated. Finally, the potential effects of EGFL7 on tumor angiogenesis in PC in vitro were further explored.

2. Materials and methods

2.1. Serum/tissues samples and cell culture

After informed consents had been obtained, serum samples were obtained from 112 PC patients and 32 normal subjects at the Department of General Surgery, the First Affiliated Hospital of Soochow University from 2008 to 2012. 112 PC patients included 60 resectable and 52 non-resectable ones. Serum from 32 normal subjects was used as controls. 10 ml whole blood was collected from each participant. Excluding hemolytic blood, the samples were processed for serum isolation by centrifugationt at 12,000 × g for 10 min at 4 °C. The serum samples were stored at −80 °C until further using. Furthermore, 60 tissue samples with the corresponding pair-matched adjacent non-tumor tissues (NATs) were all obtained from the resectable group, and these specimens were collected for further experiments (IHC assay).

293 T and Mia PaCa-2 cells (human PC cells) were obtained from American Type Culture Collection (ATCC, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma, USA) supplemented with 10% fetal bovine serum (Gibco, Invitrogen, USA). Cells were maintained in a humidified incubator at 37 °C in 5% CO_2.

2.2. Enzyme-linked aptamer sorbent assay (ELISA)

According to the manufacturer's instructions, the protein levels in serum were measured with the ELISA kit. Briefly, the antibody was diluted to 10 μg/ml using coating buffer. Then, 0.1 ml diluted antibody was seeded to 96 wells, maintaining at 4 °C overnight. On the second day, the solution in 96-wells was abandoned and washed with 1x PBS. 0.1 ml sample was seeded into the plate and incubated for 1 h at 37 °C. After washing with 1x PBS, 0.1 ml enzyme labeled antibody (goat anti-rabbit IgG-HRP, Santa Cruz, USA) titration was added to each well, and incubated for 1 h at room temperature. Remove antibody solution and rinse wells with 1x PBS five times, and then followed with dH_2O three times. We added 0.1 ml TMB substrate solution to each well at 37 °C for 30 min and then 0.05 ml sulfuric acid (2 M/L) was added to terminate the reaction. Finally, 450 nm OD was used to measure each well on the ELISA detector.

2.3. Immunohistochemistry (IHC) analysis and hematoxylin–eosin (HE) staining

Tissue sections (4 μm) were incubated in a dry oven at 60°C for 1 h, dewaxed in xylene for 3 × 10 min and rehydrated with graded ethanol each time in 100%, 100%, 95%, 90%, 80% and 70% ethanol for 5 min. Then antigen retrieval was performed by pretreatment of the slides in 0.01 M citrate buffer (pH 6.0) by a microwave oven. In order to block endogenous peroxidase, the sections were treated with 3% hydrogen peroxide (H_2O_2) for 10 min. After washed with 1x PBS, the sections were incubated with rabbit anti-EGFL7 or CD34 antibody (dilution 1: 200, CST) overnight at 4 °C. Then the sections were washed with 1x PBS and incubated with biotinylated goat anti-rabbit IgG at 37°C for 2 h. For each sample, the omission of primary antibody was used as a negative control. Finally, 3,3-diaminobenzine (DAB) was used to visualize the immunoreactive products after washing with 1x PBS.

The results were evaluated and the staining index was expressed as the proportion of positive staining cells (+: <30%; +30–70%; ++: >70%). The value of the micro-vessel density (MVD) was counted by the number of CD34-browned cell. The lumen which had a diameter of less than 8 red blood cells was counted, otherwise the one more than 8 red blood cells was abandoned. Also the lumen which had smooth muscle was not counted. Each tissue was counted in five horizons, with the average value as MVD.

Hematoxylin–eosin staining (HE) was performed using a standard peroxidase-based staining method based on the manufacturer's instructions (Beyotime, China).

2.4. Quantitative reverse transcription-PCR (qRT-PCR)

Total RNA was extracted from cell lines by using Trizol reagent (Invitrogen, CA, USA) and dissolved in 10 μl diethypyrocarbonate-treated water. By the Rever-Aid First Strand cDNA Synthesis (Thermo scientific, Mountain View, CA, USA), specific complementary DNA were reversely transcribed from total RNA following the manufacturer's instructions. The specific primer sequences of EGFL7 (5′-TGAATGCAGTGCTAGGAGGG-3′ and 5′-GCACACAGAGTG-TACCGTCT-3′) and β-actin (5′-ACAACTTTGGTATCGTGGAAGG-3′ and 5′-GCCATCACGCCACAGTTTC-3′) were synthesized from Sangon Biotech (Sangon, Shanghai, China). The quantitative measurement of EGFL7 mRNA expression was performed on the ABI PRISM 7500 Real-time PCR system (Applied Biosystems, USA). Each reaction included 1.5 μl cDNA, 10 μl 2 × Taqman® Universial PCR Master Mix, 1ul gene-specific primers/probe (TaqMan® MicroRNA Assays; ABI, USA), and 7.5 μl nuclease-free water. So, a total volume of 20ul per reaction was incubated in 96-well MicroAmp plates (Applied Biosystems, USA), and began with an initial denaturation step at 95 °C for 10 min, 50 cycles of 15 s at 95 °C and 1 min at 60 °C. All samples were carried out in triplicate. The relative expression of EGFL7 mRNA was quantified by the $2^{-D\Delta Ct}$ method.

2.5. EGFL7-siRNA plasmid infected into Mia PaCa-2 cells

Based on the pGCSIL-GFP vector using the EcoR I and Age I restriction sites, small hairpin RNA (shRNA) of human EGFL7 (GenBank, NM_016215) plasmid transfer vector encoding green fluorescent protein (GFP) sequence was constructed and transfected into Mia PaCa-2 cells by Shanghai Genechem Co (Shanghai, China). After successful infection, the transfection efficiency of EGLF7 was detected by flow cytometry analysis (FACS).

2.6. Flow cytometric analysis

Cell cycle analyses were detected by flow cytometric analysis. After 48-h continuous culture, cells were harvested and fixed by