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Original Contribution

Progranulin promotes lymphangiogenesis through VEGF-C and is an independent risk factor in human esophageal cancers☆,☆☆

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Summary Lymph node metastasis is one of the most important predictors of the prognosis for esophageal cancer (EC) patients. However, the mechanism underlying the lymph node metastasis is largely unknown. Progranulin (PGRN) is shown to be highly expressed in various types of cancers and could promote the angiogenesis and epithelial-mesenchymal transition of cancer cells in previous studies. However, the expression status of PGRN and its effects on the lymphangiogenesis in EC are largely unclear. In this study, we show for the first time that PGRN is expressed in EC tissue samples and cell lines and could promote the expression of VEGF-C in vitro, a well-known lymphangiogenesis inducer, through the putative signaling transducers p-ERK and p-AKT. Besides, increased levels of PGRN are correlated with lymph node metastasis, high levels of lymph microvessel density, and lymph vessel space invasion in tissue samples of EC patients. In addition, Cox proportional risk model shows that patients with high levels of PGRN would have 2-fold increases in 5-year mortality compared with patients with low levels of PGRN. Finally, we establish a clinically useful nomogram to predict the possibility of mortality for individual EC patients. In conclusion, PGRN may play an important role in the lymphangiogenesis through activation of VEGF-C in the EC patients.

Abbreviations: PGRN, Progranulin; EC, esophageal cancer; IHC, immunohistochemistry; LMVD, lymph microvessel density; LVSI, lymph vessel space invasion; PFS, progression-free survival; OS, overall survival.

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1. Introduction

EC is one of the most common malignancies in the world [1]. Although we have achieved some advances in the diagnosis and treatment of EC, the overall prognosis of these patients is still very dismal [2]. Lymph node metastasis has been proved to be an independent prognostic factor for EC patients [3-7]. However, the mechanism of lymphangiogenesis in EC is largely unclear.

PGRN (also known as PGRN-epithelin precursor, proepithelin, or PC cell–derived growth factor) has been shown to be highly expressed in various cancer types including colorectal and epithelial ovarian cancers [8,9]. Besides, studies have also proved that PGRN could promote tumor growth, apoptosis resistance, and epithelial-mesenchymal transition in these cancers [8,9]. However, expression status of PGRN in EC and effects of PGRN on EC biological behavior, especially lymphangiogenesis, are still unknown.

VEGF-C, a member of VEGF families, could promote the formation and growth of lymph tube through activation of VEGFR-3 [10-12]. The expression of VEGF-C has been shown to be positively correlated with LMVD and lymph node metastasis in a variety of cancers [13-17]. Besides, in vitro studies have shown that overexpression of VEGF-C could induce the lymphangiogenesis and promote the cancer cells to invade the lymph tube, whereas blockage of this pathway could prevent the lymphangiogenesis in several types of cancers [18-20]. However, the mechanism underlying the regulation of VEGF-C in EC is still unknown.

We therefore try to determine the expression levels of PGRN in EC cell lines and tissue samples and explore the effects of PGRN on the expression of VEGF-C. Besides, we sought to ascertain the association between the PGRN levels and several clinicopathological predictors, including LMVD, LVSI, lymph node metastasis, and others, in EC tissue samples. In addition, we also try to elucidate the effects of PGRN expression on the OS and PFS of EC patients. Finally, we try to establish a clinically useful nomogram to predict the specific mortality for individual EC patients.

2. Materials and methods

2.1. Cell lines and antibodies

Human esophageal squamous cancer cell lines TE-1, Eca109, k410, and k450 were purchased from the Institutes of Biochemistry and Cell Biology (Shanghai, China), which were originated from American Type Culture Collection (Manassas, VA). TE-1 cells were cultured in RPMI 1640 medium (Invitrogen, Shanghai, China), Eca109 in McCoy's 5A medium (Invitrogen, Shanghai, China), and k410 and k450 cells in Dulbecco modified Eagle medium (Gibco Life Technologies, Grand Island, NY). All culture media were supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA). All cells were maintained in a 37°C humidified incubator containing 5% CO_2. The antibodies used are as follows: PGRN (Enzo Life Science, Farmingdale, NY), VEGF-C (CST Biological Reagents Co, Ltd, Danvers, MA), p-ERK (Abcam, Cambridge, UK), ERK (Abcam), p-AKT (Abcam), AKT (Abcam), p-JNK (Abcam), JNK (Abcam), and GAPDH (Proteintech Group, Inc, Wuhan, China).

2.2. PGRN overexpression and knockdown

EC cell line TE-1 was transfected using HP Xtreme GENE HP Reagents (Roche, Basel, Switzerland) by p-GPU6/GFP/Neo/PGRN or shRNA control (Shanghai GenePharma Co, Ltd, Shanghai, China) to interfere PGRN expression, whereas pEZ-M61/GFP/PGRN or vector control (Gene Copoeia, Guangzhou, China) was used to up-regulate PGRN expression in TE-1 cell line. Transfection procedures were performed according to the manufacturer's instructions. Total messenger RNA and protein were extracted for further study. To determine whether AKT and ERK signal pathways were required for regulation of VEGF-C expression, TE-1 cells transfected with vector were preplated in 6-well plates at 2×10^5 cells per well for 24 hours, then the medium was replaced by Dulbecco modified Eagle medium without fetal bovine serum containing different concentrations of ERK inhibitor U0126 (4 ng/mL, diluted with dimethyl sulfoxide) and AKT inhibitor LY294002 (4 ng/mL, diluted with dimethyl sulfoxide). Forty-eight hours later, Western blot assay was performed, and PGRN, VEGF-C, p-ERK, ERK, p-AKT, AKT, p-JNK, and JNK expression was calculated.

2.3. Reverse-transcription polymerase chain reaction analysis

Total RNA from cells was extracted by TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Complementary DNA was synthesized using PrimeScript RT-PCR Kit (TakaRa, Dalian, China) as described in the protocol. Then reverse-transcription polymerase chain reaction (RT-PCR) was performed as described previously [9]. All experiments were performed in a minimum of 3 replicates.

2.4. Protein extraction and Western blotting

Cells were harvested in RIPA lysis buffer. Protein from cellular lysates was resolved with polyacrylamide gel electrophoresis, transferred to polyvinylidene fluoride membrane (Millipore Corp, Billerica, MA), and then blocked in Tris Buffered Saline with Tween 20 (TBST) containing 5% nonfat milk followed by incubation with primary antibodies. After being washed in TBST, the membrane was