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Original Article

Expression of vascular endothelial growth factor and basic fibroblast growth factor in extramammary Paget disease

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Abstract: Extramammary Paget's disease (EMPD) is a special type of cancers. The etiology of the disease is still unclear. We aimed to study the expression differences of vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) in EMPD tissues and corresponding adjacent normal tissues. The mRNA expression was detected by RT-PCR and the protein expression was explored by immunohistochemistry. Higher immunostaining signal scores of bFGF and VEGF in EMPD tissues had been found ($z = -3.827$, $P < 0.001$, $z = -3.729$, $P < 0.001$, respectively). In addition, the mRNA expression of bFGF and VEGF was higher in EMPD tissues, which had been validated by RT-PCR ($t = 5.771$, $P < 0.001$, $t = 3.304$, $P = 0.004$, respectively). The VEGF and bFGF might be the key signaling proteins in angiogenesis of EMPD. How to block the VEGF and bFGF in EMPD and to destroy the blood supply of the tumor cells becomes the focus of our future research.

Keywords: Extramammary Paget disease, VEGF, bFGF

Introduction

Paget's disease (PD), also known as eczema-like cancer, was first described by James Paget in a patient with breast cancer in 1874. PD had been subdivided into two categories, mammary Paget's disease (mammary Paget's disease, MPD) and extramammary Paget's disease (extramammary Paget's disease, EMPD) [1, 2].

EMPD could also be subdivided into primary and secondary disease. Primary EMPD is defined as the tumor is confined in the epidermis and rarely metastasize [3]. While secondary EMPD is associated with cutaneous adnexal structure adenocarcinomas or non-cutaneous internal malignancies such as rectal adenocarcinoma, transitional cell carcinoma of the bladder and so on [4-7].

EMPD is a distinct form of rare skin malignant neoplasm with similar clinical features to inflammatory reactions. The pathogenesis of EMPD is multifaceted. It is thought that Paget's cells either originated in the apocrine sweat duct openings cells or from the multipotential cells in the epidermis. Tumor cells could also invade the dermis and metastasize by the lymphatic system [8, 9].

As tumor growth and metastasis are dependent on angiogenesis, tumor angiogenesis plays a crucial role in the progress of the tumor [10]. Tumor angiogenesis is a complex process, by which new blood vessels are formed from pre-existing vessels. Tumor cells, vascular endothelial cells and stromal cells, such as macrophages, tumor infiltrating lymphocytes, et al. were involved in angiogenesis. In addition, a lot of angiogenic factors and angiogenesis inhibitory factors, including vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), also regulated tumor angiogenesis [11, 12].

Overexpression of VEGF and bFGF had been found in a variety of human cancers, such as breast cancer, gastric cancer, and pancreatic

Table 1. Clinical background of 18 samples of EMPD and expression of bFGF and VEGF (IHC)

Cases	Age/Sex	Location	bFGF		VEGF	
			N	C	N	C
C01	59/M	Scrotum	1+	3+	1+	3+
C02	66/F	Perineum	2+	2+	2+	2+
C03	75/M	Scrotum	1+	3+	1+	2+
C04	65/M	Scrotum	1+	2+	1+	2+
C05	55/F	Perineum	1+	2+	2+	3+
C06	65/F	Vulva	1+	3+	1+	2+
C07	56/F	Vulva	1+	2+	1+	2+
C08	59/M	Anal	1+	2+	1+	2+
C09	49/F	Vulva	1+	3+	1+	2+
C10	64/M	Scrotum	1+	3+	1+	3+
C11	44/M	Scrotum	1+	3+	1+	3+
C12	46/M	Scrotum	1+	3+	1+	3+
C13	50/M	Scrotum	1+	3+	1+	3+
C14	55/F	Perineum	1+	3+	1+	3+
C15	37/M	Scrotum	1+	3+	2+	3+
C16	42/F	Vulva	1+	3+	1+	3+
C17	57/M	Scrotum	1+	3+	1+	3+
C18	51/F	Vulva	1+	3+	1+	3+

C: EMPD tissues; N: Adjacent normal skin and mucous membrane tissues.

adenocarcinoma [13-15]. VEGF has been identified as a key mediator of tumor angiogenesis. VEGF involved in the induction of tumor angiogenesis by not only stimulating directly endothelial cells division and proliferation, but also increasing the permeability of endothelial cells. As a mitogen, bFGF stimulated the cancer cells directly through autocrine and paracrine. In addition, bFGF also played a significant role in tumor angiogenesis.

Previous study found that the expression of VEGF was higher in EMPD than those of normal skin [16]. However, expression of bFGF had not been investigated in EMPD so far. Therefore, we investigated the expression of bFGF and VEGF and assessed their potential relationships and roles in the progression of EMPD.

Methods

Ethical approval

The study was conducted in accordance with the Declaration of Helsinki and approved by First Affiliated Hospital of Nanjing Medical University Authorities. The study had no influence on the management of patients, and all patients were provided with written informed consent.

Tissue samples

18 cases of EMPD tissue, accompanying with the corresponding adjacent normal skin and mucous membrane tissue, were obtained from Department of Urology in First Affiliated Hospital of Nanjing Medical University and Shanghai Institute of Dermatology (**Table 1**). The mean age of these patients was 55.3 years (range 37 to 75). All patients were diagnosed by clinical and histopathological findings. All patients were primary EMPD.

Real-time analysis of bFGF and VEGF mRNA

Total RNA was isolated from tissue samples using TRIzol reagent (Molecular Research Center, Cincinnati, OH, USA). A 2 μg aliquot of total RNA from each specimen was reverse transcribed into single-strand cDNA using oligo primer and Superscript II (Invitrogen, Carlsbad, CA, USA). Relative gene expression of bFGF and VEGF were performed by ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA, USA) based on the SYBR green method, with β-actin as an internal standard.

The primers pairs used in the present study were as follows: for bFGF, 5'-GCGACCCTCACATCAAGCTA-3' (forward) and 5'-CTTTCTGCCCAGGTCCTGTT-3' (reverse); for VEGF, 5'-TTGCCTTGCTGCTCTACCTC-3' (forward) and 5'-TGCATGGTGATGTTGGACTC-3' (reverse); and for β-actin, 5'-TGACTG-3' (forward) and 5'-agg ggc cgg act cgt cat act-3' (reverse). The PCR reaction mixture (final volume 20 μL) contained 0.1 μM each primer, 1 X SYBR Premix EX Taq premix reagent (Takara), and 50 ng cDNA. The cycling conditions consisted of 95°C for 2 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Expression of bFGF and VEGF in individual samples were calculated relative to expression of β-actin using a modification of the method described by Lehmann and Kreipe [17]. All analyses were performed in a blinded fashion.

Immunohistochemistry

All samples were fixed with 10% buffered formalin. The archival paraffin-embedded tissue blocks were cut into 4-μm thick sections and

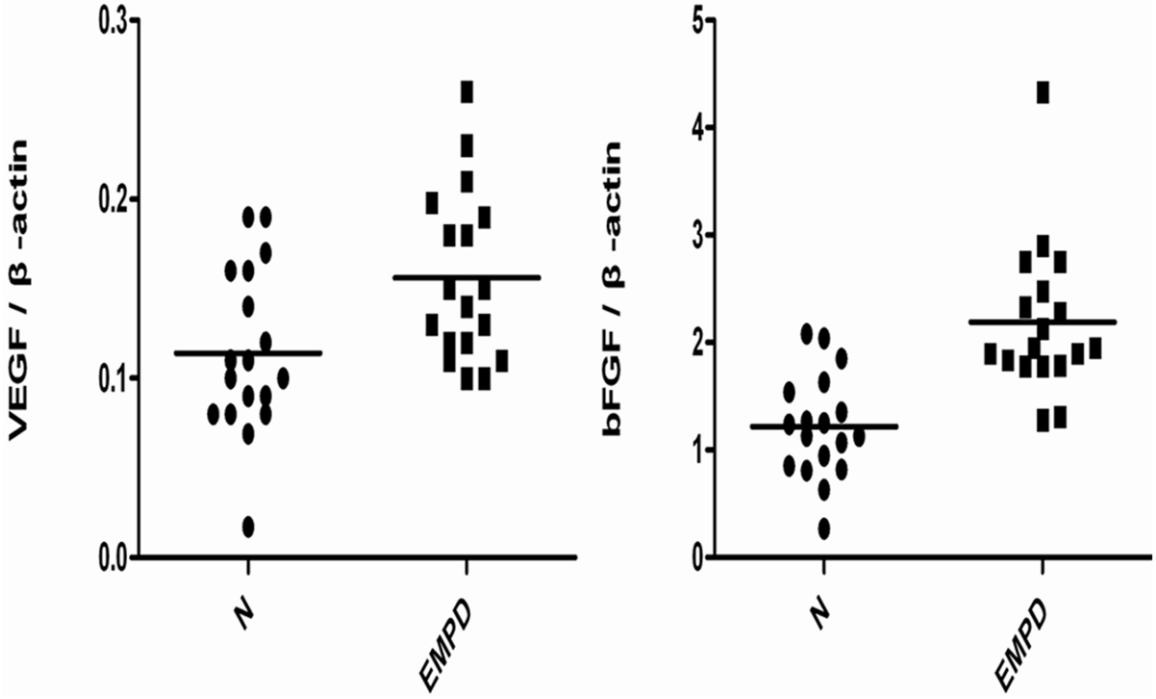

Figure 1. Expression level of bFGF and VEGF mRNA in EMPD tissues and the adjacent normal skin and mucosa. Both bFGF and VEGF mRNA expression level were significantly higher in EMPD tissues.

mounted on slides coated with silane. The sections were deparaffinized with xylene for 10 min and rehydrated through graded ethanol-water solutions, followed by blocking of endogenous peroxidase activity in 0.3% H_2O_2 in methanol for 30 min. The sections set dried at 37°, and then HE staining, immunohistochemical staining and control experiments were performed. Each step was performed according to the manufacturer's protocols. The antibodies were goat anti-human polyclonal antibody and were purchased from Beijing Zhongshan Biotechnology Company.

After immunohistochemical staining for bFGF and VEGF, three high-power fields (HPFs, ×200) were randomly selected in each specimen and 100 tumor cells were counted in each field. And the average percentage positive cells for each staining from the three HPFs were computed for each sample. Expression levels of bFGF and VEGF were assessed semiquantitatively as follows: -, < 5% of Paget cells positive; +, 5-25% of Paget cells positive; 2+, 26-50% of Paget cells positive; 3+, > 50% of Paget cells positive.

Statistical analysis

Paired-samples t-test was used to test the bFGF and VEGF mRNA expression levels in EMPD tissues compared with the controls. Wilcoxon test was used for statistical analysis to compare the expression levels of bFGF and VEGF between EMPD tissues and the controls. Correlations among bFGF and VEGF expression were assessed by Pearson's correlation coefficient analysis. A *P*-value of less 0.05 was considered to be statistically significant. All the statistical analysis was conducted using the SPSS software, version 16.0 (SPSS, Inc.).

Results

bFGF and VEGF mRNA levels in EMPD

As VEGF and bFGF were most extensively studied in pro-angiogenic factors, we aimed to investigate the expression of bFGF/VEGF in EMPD tissues and the adjacent normal skin and mucosa. Therefore, Real-time PCR was performed to detect the mRNA expression of bFGF and VEGF. It was found that bFGF and VEGF mRNA expression levels (normalized against β-actin) in EMPD tissues were significantly higher than those in adjacent normal skin and mucous membrane tissues ($t = 5.771$, $P < 0.001$, $t = 3.304$, $P = 0.004$, respectively, as shown in **Figure 1**).

Table 2. Clinical characteristics of bFGF and VEGF expression in EMPD

	No. of patients	bFGF	Z	*P*	VEGF	Z	*P*
TNM							
T2	4	10+			9+		
T3-4	14	39+	-1.093	0.274	38+	-1.633	0.103
Recurrence							
Yes	2	5+			5+		
No	16	44+	-0.723	0.470	42+	-0.332	0.740
Metastasis							
Yes	0	0			0		
No	18	49+	/	/	47+	/	/

bFGF and VEGF expression in EMPD

Immunostaining signals of bFGF distributed unevenly. bFGF staining was positive in tumor cells, cytoplasm and cell membrane in adjacent normal skin mucosa epithelial cells and interstitial tissue. In addition, the expression of bFGF was significantly higher in tumor cells. Immunostaining signals of VEGF were weaker compared with bFGF. However, VEGF staining were positive in the tumor cells and adjacent normal skin epithelial cell membrane, whereas negative in cytoplasm and interstitial tissue. In addition, the expression of VEGF was slightly higher in tumor cells.

Analysis showed significantly higher immunostaining signal scores of bFGF in EMPD than in adjacent normal skin mucosa ($z = -3.827$, $P < 0.001$). Similarly, significant higher scores of VEGF could also be found in EMPD ($z = -3.729$, $P < 0.001$, as shown in **Figure 2**). Pearson's correlation coefficient analysis showed a significantly positive correlation between the expression levels of bFGF and VEGF in EMPD ($r = 0.523$, $P = 0.026$).

Every patient was well informed of the importance of follow-up. All patients cooperated, and no one was lost to follow-up. All patients were followed up for 1 to 5 years with an average of 3 years. Two patients had local recurrence and none had metastatic disease.

Although the expression of bFGF and VEGF were higher in the T3-4 subgroup, there was no significant difference ($Z = -1.093$, $P = 0.274$, $Z = -1.633$, $P = 0.103$, respectively, **Table 2**). Similarly, no significant results could be found in the subgroup with or without local recurrence (**Table 2**). These negative results may be due to our small samples.

Discussion

EMPD is a special type of cancers. The etiology of the disease is still unclear. It was considered that EMPD originated either from the intraepidermal cells of apocrine gland ducts or from pluripotent keratinocyte stem cells according to current studies. Paget's cells were larger than normal keratinocyte cells and scattered or nested distribution, located at the bottom of the epidermis or oppressed epidermal cells into a network. The mesh is full of eczema-like cancer cells [18].

Lots of studies about cancer research focused on the relationship between angiogenesis and tumor biological behavior. Current basic researches and clinical trial studies demonstrated that angiogenesis played a crucial role in the development and metastasis of tumors. Tumor pro-angiogenic factor increased and angiogenesis inhibitor factor relatively decreased in the process of angiogenesis and metastasis of the tumor [10, 19]. Therefore, we thought that angiogenesis was associated with development and outcome of EMPD. VEGF and bFGF are the most popular pro-angiogenic factors in previous study [20]. Combined detection of their expression in EMPD tissues may contribute to a deeper understanding of the role of pro-angiogenic factors in the pathogenesis of EMPD and their correlations.

VEGF is the strongest family of pro-angiogenic substance. VEGF and receptor Flt-1 and KDR have high affinity and they all have tyrosine kinase activity [21]. Activation of the VEGF pathway triggers a range of signaling processes, stimulating vascular endothelial cell growth, migration, survival, and permeability. In this study, immunohistochemical staining confirmed that the staining signal of VEGF gene protein in EMPD tissues was significantly stronger than those in adjacent normal skin and mucous tissues. bFGF is a class of pro-angiogenic factors. It could bind to the cell membrane specific receptor (FGFR) to play a regulatory role, which was associated with angiogen-

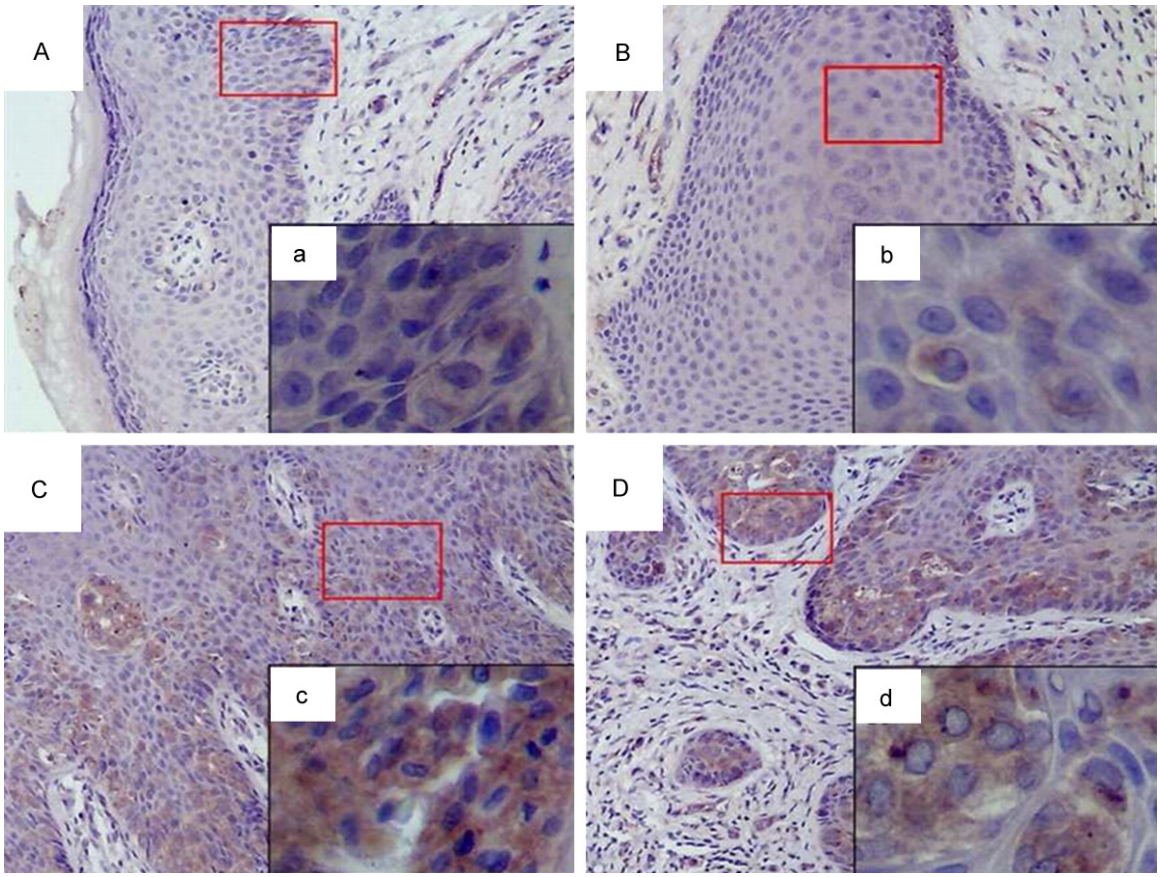

Figure 2. Immunohistochemical staining for bFGF and VEGF (A-D×200, a-d×400). A: a: bFGF immunohistochemical staining in the adjacent normal skin and mucosa; B: b: VEGF immunohistochemical staining in the adjacent normal skin and mucosa; C: c: bFGF immunohistochemical staining in EMPD tissues; D: d: VEGF immunohistochemical staining in EMPD tissues.

esis, chemotaxis, embryonic development, nerve regeneration, tumor growth, and many other physiological and pathological processes [22]. Current studies suggested that bFGF was closely related with development and progression of various cancers. The expression of bFGF in a variety of cancer tissues was significantly higher than those in control tissues and correlated with tumor grade, tumor stage and prognosis. Meanwhile, the expression in the serum and urine of some cancer patients was also significantly higher [23, 24]. In this study, immunohistochemical staining confirmed that the staining signal of bFGF gene protein in EMPD tissues was significantly stronger than those in adjacent normal skin and mucous tissues. Our study indicated that VEGF and bFGF might be related with the development of EMPD. In addition, future researches are warranted.

There were some studies reported that VEGF could stimulate endothelial cells to secrete bFGF. In addition, bFGF could enhance own angiogenic activity in autocrine and paracrine manners. Hence, VEGF produced by endothelial cells could be considered as an important "intermediary" autocrine factor in bFGF-induced angiogenesis [25, 26]. We also found a significant correlation between the expression levels of bFGF and VEGF in EMPD tissues.

In conclusion, the result of this study was consistent with findings of bFGF and VEGF in other tumors, which further demonstrated the reliability of our results. The expression of VEGF and bFGF gene might be the key signaling proteins in angiogenesis of EMPD. In addition, the stimulus signals provided by their interactions might play a crucial role in the development of EMPD. How to block the overexpression of VEGF and bFGF in EMPD tissues and destroy the blood supply of the tumor cells becomes the focus of our future research.

Disclosure of conflict of interest

None.

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