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Expression and clinical correlation of NGAL and VEGF in endometrial carcinoma

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Abstract. – OBJECTIVE: To determine the expression of neutrophil gelatinase-associated lipocalin (NGAL) and vascular endothelial growth factor (VEGF) in endometrial carcinoma.

PATIENTS AND METHODS: The clinical features (FIGO staging, pathological type, differentiation level, myometrial invasion depth and tumor size) and immunostaining analysis of endometrial carcinoma (30 cases), atypical hyperplasia (30 cases) and proliferative endometrial tissues (30 cases) were analyzed. Immunohistochemistry and reverse transcription PCR (RT-PCR) were performed to detect the expression of NGAL and VEGF.

RESULTS: The positive tissue immunostaining and mRNA expression of NGAL and VEGF in endometrial carcinoma were significantly higher than in either atypical hyperplasia or proliferative endometrial tissues ($p<0.05$). The relative expression level of NGAL and VEGF was positively correlated with worse FIGO staging, higher differentiation level and a greater myometrial invasion depth ($p<0.05$); but not with patient age, pathological type or tumor size ($p>0.05$).

CONCLUSIONS: The abnormal high expression of NGAL and VEGF observed in the endometrial carcinoma may be an important biomarker for early tumor diagnosis or as a novel target for therapeutic intervention.

Key Words

Endometrial carcinoma, Neutrophil gelatinase-associated lipocalin, Vascular endothelial growth factor.

Introduction

The incidence of endometrial cancer (EC) is increasing; it is second only to cervical cancer in women in China and first in the United States[1]. Neutrophil gelatinase-associated lipocalin (NGAL), also known as lipocalin-2 or transferrin, is highly expressed in various adenocarcinomas (e.g., gastric cancer, breast cancer, and colorectal cancer)[2,3]. NGAL can promote the enzymatic activation of the matrix metalloproteinase (MMP)-9 by forming a complex with it[4] which allows activation and processing of factors relevant for angiogenesis such as vascular endothelial growth factor (VEGF)[5]; VEGF then can promote local angiogenesis, cell proliferation, differentiation, invasion, and migration[6]. NGAL can also participate in tumorigenesis through NGAL receptor (NGAL-R)-mediated iron-dependent pathways[7]. *In vitro*, the expression of NGAL is associated with the degree of tumor differentiation level in EC cell lines (e.g., Ishikawa, HEC-1-A, and KLE) and has been suggested to be a useful marker of early detection of EC lesions[8]. High expression levels of NGAL may also inhibit apoptosis. Is a study of EC tumor-bearing mice model injected with NGAL overexpressing adenovirus, it was reported that[9] the tumor apoptosis rate was decreased along with the reduction of apoptosis-related genes p63, P450arom and PTEN. The purpose of this study is to compare the expression of NGAL and VEGF in various human endometrial tissue samples of varying degrees of differentiation in order to gain a better understanding of its potential role in the tumor pathogenesis and to potentially identify novel targets for therapeutic intervention.

Patients and Methods

Patients

Thirty patients aged 45-76 years (mean 56.7 ± 12.3 years) diagnosed with EC (21 cases of adenocarcinoma, 6 cases of serous carcinoma, and 3 cases of clear cell carcinoma) from the People's Hospital of Dongying City (Dongying City, Shandong) were selected for the investigation from June, 2015 to December 2016 The research team obtained the approval of the Ethics Committee of our hospital and informed consents of patients. Patients were excluded that had a recent history of

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uterine surgery, radiotherapy, and chemotherapy or taking estrogen and progesterone analogues. According to International Federation of Gynecology and Obstetrics (FIGO) clinical staging, 6 were in stage I, 14 were in stage II, 7 were in stage III, 3 were in stage IV; according to differentiation level, 8 cases were poorly differentiated, 15 were moderately differentiated, and 7 were well differentiated. Tumor characteristic included depth of myometrial invasion (11 cases penetrated less than the superficial half, and 19 cases penetrated deeper than the superficial half of the myometrium) and size (the maximum tumor diameter was 0.8-4.5 cm, average (2.6 ± 1.2) cm). Control groups consisted of 30 cases of atypical hyperplasia (age of 40-69 years, mean age (54.5 ± 13.2)) and 30 cases of proliferative endometrium (age of 38-77 years, mean age (55.9±14.3)); there was no significant difference in age between these two groups ($p>0.05$).

Immunohistochemistry and reverse transcription PCR (RT-PCR) were performed to detect the expression of NGAL and VEGF and to analyze the correlation between the expression of NGAL and VEGF with the clinical features of the carcinoma including FIGO staging, histological type, differentiation level, myometrial invasion depth and tumor size. Tissue sections (5 μm thickness), were dewaxed with xylene, hydrated by gradient ethanol washes, incubated with 3% H_2O_2 solution at 27°C for 20 min and then incubated with normal goat serum at 27°C for 30 min. Sections were then probed with a monoclonal antibody (mouse anti-human NGAL or VEGF antibody (Jiangsu Beyotime Biotechnology Co., Ltd., Jiangsu, China working concentration 1: 2000) or normal mouse IgG (negative control), in a humidified chamber at 4°C overnight. After washing, bound antibody was detected using a biotin-conjugated rabbit anti-mouse antibody (Jiangsu Beyotime Biotechnology Co., Ltd., Jiangsu, China working concentration of 1: 500), in a humidified chamber at 27°C for 20 min. A horseradish enzyme-labeled streptavidin working solution (Jiangsu Beyotime Biotechnology Co., Ltd.) was added and incubated in a humidified chamber for 27°C for 20 min. After washing 3 times with phosphate-buffered saline solution (PBS), sections were stained with 3,3′-Diaminobenzidine (DAB), counterstained in hematoxylin. Sections were differentiated in hydrochloric acid alcohol solution, returned to blue in ammonia, gradient eluted using ethanol, cleared in xylene, mounted with neutral gum, dried at room temperature, and observed under an optical microscope (Olympus, Tokyo, Japan).

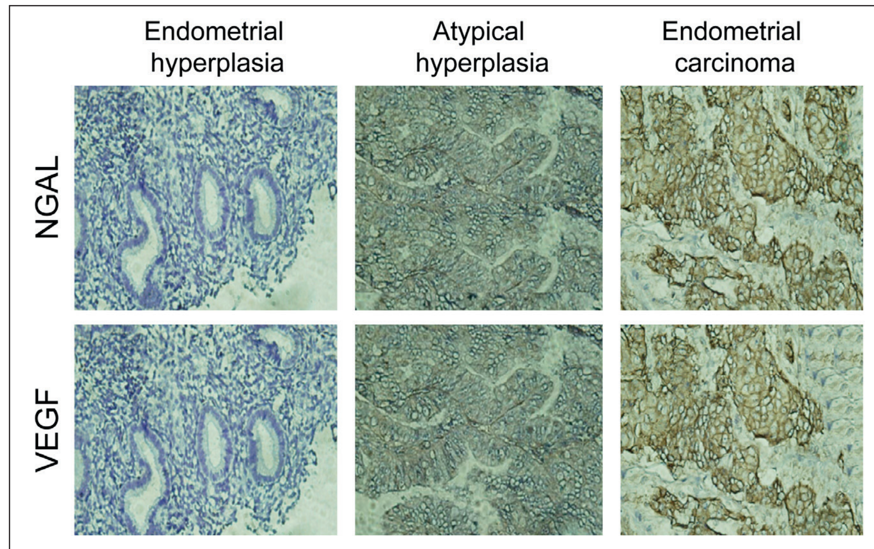
The semi-quantitative scoring method was utilized based on staining intensity and the proportion of positive stained cells (cytoplasm or nucleus stained with yellow to dark brown as positive). Section were allotted up to 3 points for staining intensity (no positive staining was assigned 0 points, weak staining 1 point, medium intensity 2 points, strong staining 3 points) and up to 4 points for the proportion of positive staining cells (if the proportion of positive cells ratio, ≤ 5% was 0 point, 6 to 25% was 1 point, 26 to 50% was 2 points, 51 to 75% was 4 points). Based on the product of the 2 scores, the total score of 0-3 was considered as negative, and a score of 4-12 was considered as positive.

Total cell RNA was extracted by conventional TRIzol reagent (Sigma-Aldrich, St. Louis, MO, USA), concentration and purity were determined by ultraviolet spectrophotometer, cDNA was synthesized by reverse transcription kit (TaKaRa, Tokyo, Japan), Shanghai Biosynthesis Co., Ltd. (Shanghai, China) synthesized primer sequence according to GeneBank sequence, NGAL: (F)5’-GGTTTCATCCAGGATCGAGCAGG-3’,(R)5’-ACAAAGATGGTCACGGTCTGCC-3’,445 bp; VEGF: (F)5’-ACTACTTCTCCCGCCGCTAC-3’,(R)5’-GAAATCAAACAGAGGCCGCATG-3’,332 bp; GAPDH(F): 5’-CGCGAGAAGATGACCCAGAT-3’, R: 5’-GCACTGTGTTGGCGTACAGG-3’, 225bp. The reaction system was: cDNA 2 μl + forward primer 3 μl + reverse primer 3 μl + Taq polymerase 0.5 μl + dNTPs 1 μl + $MgCl_2$ 3 μl + 10×Buffer 5 μl, adjusting the total volume to 50 μl by adding water [PCR reagent (R & D Systems Inc., Minneapolis, MN, USA)], 95°C 30 s, 58°C 30 s, 72°C 60 s for 30 cycles and then 72°C for 10 min. PCR products were resolved on 2% agarose gel electrophoresis and UV spectrophotometry; imaging was performed using a gel imaging analysis system and gray line value analysis performed by digital camera, the results were expressed by $2^{-\Delta\Delta Ct}$ method.

Statistical Analysis

The statistical analysis was performed using SPSS20.0 software (IBM, Armonk, NY, USA). Data were expressed as mean ± standard deviation; single factor ANOVA analysis was used for comparison among groups. LSD *t*-test was used for comparison between two groups. Enumeration data were expressed as a number of cases or (%), χ^2-test or Fisher exact probability method was used for the comparison between groups; $p<0.05$ was considered as statistically significant.

Figure 1. Immunohistochemical staining of NGAL and VEGF in endometrium of endometrial hyperplasia, atypical hyperplasia and endometrial carcinoma (100 ×). The positive expression rate of NGAL and VEGF in EC tissue was significantly higher than that in dysplasia, and was the least in proliferative endometrial tissues ($p<0.05$).



Results

The positive expression of NGAL and VEGF in EC tissue was significantly higher than that in dysplasia and was the least positive in proliferative endometrial tissues ($p<0.05$) (Figure 1 and Table I).

The expression of NGAL and VEGF mRNA in EC was significantly higher than that in atypical hyperplasia and was the least positive in proliferative endometrium ($p<0.05$) (Figure 2). The positive expression of NGAL and VEGF was related to FIGO staging, differentiation grade and myometrial invasion depth ($p<0.05$). The higher the tumor stage and level and the greater the depth of invasion was positively correlated with higher expression of both NGAL and VEGF; this positive correlation was not dependent on patient age, pathological tumor type and tumor diameter ($p>0.05$) (Table II).

Discussion

NGAL is a secretory protein with multiple biological effects that plays an important role in immune and inflammatory responses[10], tubular epithelial morphological transformation[11], and tumorigenesis. VEGF is an important cytokine that regulates the proliferation and directional migration of tumor cells[12]. MMP-9 is an extracellular type IV collagenase that affects cell adhesion and invasion[13]. The metabolic activity of tumor cells requires involvement of numerous iron ions in DNA and protein synthesis, NGAL can improve the liable iron pool (LIP) activity of cellular organism[14]. No-load NGAL without an iron ion and antitumor drugs were combined to form targeted drugs, that specifically bind to the NGALR on the surface of EC cells, which results in increasing the local drug concentration and enhancing the anti-tumor effect[15]; in addition, by increasing the transport of extracellular iron ions into the cell, they inhibit drug resistance due to drug efflux[16]. VEGF activates tyrosine kinase signaling pathways by binding to its receptors Flt4, inducing lymphoid and vascular tissue formation in EC cells[21].

NGAL is expressed in glandular epithelium-derived tumor cells. Yang et al[17] reported that NGAL can promote epithelial-mesenchymal tran-

Table I. Comparison of the positive rate of NGAL and VEGF [Number of cases (%)].

Group	Number of cases	NGAL	VEGF
Endometrial carcinoma	30	12 (40.0%)	15 (50.0%)
Atypical hyperplasia	30	4 (13.3%)	6 (20.0%)
Proliferative endometrium	30	2 (6.7%)	4 (13.3%)
χ^2		11.667	11.409
p		0.003	0.003

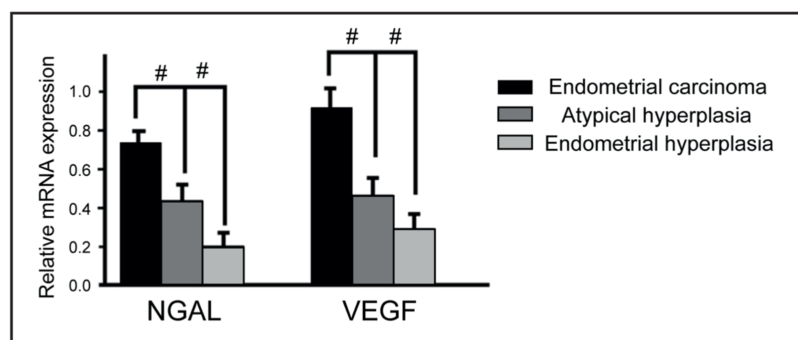


Figure 2. Comparison of NGAL and VEGF mRNA expression (the expression of NGAL and VEGF mRNA in EC was significantly higher than that in atypical hyperplasia, and was the least in proliferative endometrium, # $p<0.05$).

sition (EMT) in human breast cancer cells. Hanai et al[18] showed that NGAL can inhibit the invasion, proliferation, and metastasis of murine breast cancer 4T1-Ras-transformed cells by inducing cell EMT. Lim et al[19] have confirmed that NGAL expression can gradually disappear with change of tumor epithelial cell phenotype and progress of EMT. Results from this study have shown that the expression of NGAL and VEGF in endometrial carcinoma is significantly higher than either atypical hyperplasia or endometrial hyperplasia cases. The higher positive expression of NGAL and VEGF was related to worse FIGO staging, higher differentiation grade and a greater tumor myometrial invasion depth, but not to age, pathological type, and tumor diameter. The positive expression of NGAL in EC decreased with the decrease of differentiation level may secondary to the loss of the glandular cell phenotype and gradual transformation into a stromal cell phenotype. NGAL may play a role in regulation in the epithelial cancer cell differentiation pathway[20]; the specific mechanisms involved needs further analysis.

Conclusions

An abnormal high expression of NGAL and VEGF is closely related to the occurrence and development of endometrial cancer, which may become an important biomarker for early diagnosis. A limitation of this investigation include the limited sample size; therefore further verification for the accuracy of NGAL and VEGF in the diagnosis of EC and whether it can become an important target for novel therapeutic intervention is warranted.

Table II. Relationship between the positive expression of NGAL and VEGF and the clinical features of tumors in EC tissues [Number of cases (%)].

Clinical feature	Number of cases	NGAL positive rate	*p*	VEGF positive rate	*p*
FIGO staging			0.048		0.020
I-II	20	5 (25.0)		7 (35.0)	
III-IV	10	7 (70.0)		8 (80.0)	
Differentiation			0.009		0.011
Poorly	8	2 (25.0)		2 (25.0)	
Moderately	15	5 (33.3)		8 (53.3)	
Well	7	5 (71.4)		5 (71.4)	
Depth of invasion			0.015		0.012
<top 1/2	11	2 (18.2)		3 (27.3)	
≥lower 1/2	19	10 (52.6)		12 (63.2)	
Age (years)			0.254		0.362
<56.7	13	5 (38.5)		6 (46.2)	
≥56.7	17	7 (41.2)		9 (52.9)	
Pathological type			0.241		0.421
Adenocarcinoma	21	8 (38.1)		10 (47.6)	
Non-adenocarcinoma	9	4 (44.4)		5 (55.6)	
Maximum diameter (cm)			0.296		0.312
<2.6	12	6 (50.0)		7 (58.3)	
≥2.6	18	6 (33.3)		8 (44.4)	

Conflict of Interest

The authors declare no conflict of interest.

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