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. 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). NGAL can promote the enzymatic activation of the matrix metalloproteinase (MMP)-9 by forming a complex with it which allows activation and processing of factors relevant for angiogenesis such as vascular endothelial growth factor (VEGF). VEGF then can promote local angiogenesis, cell proliferation, differentiation, invasion, and migration. NGAL can also participate in tumorigenesis through NGAL receptor (NGAL-R)-mediated iron-dependent pathways. 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. 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 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 analagues. 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 differenti-ated, 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 myo-metrium) 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 and 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% H2O2 solution at 27°C for 20 min and then incubated with normal goat serum at room temperature, and observed under an optical microscope (Olympus, Tokyo, Japan).

NGAL and VEGF antibody (Jiangsu Beyotime Biotechnology Co., Ltd. (Shanghai, China) synthesized primer sequence according to GeneBank sequence, NGAL: (F)5'-GGTTTCTATCCAGATCGAGCAGG-3', (R)5'-ACAAAGATGGTCACGGTGCTT-GCC-3', 445 bp; VEGF: (F)5'-ACTACTTCTCCGCCGCTAC-3', (R)5'-GAAATCAAA-CAGAGGCACCAGAT-3', 332 bp; GAPDH(F): 5'-CGCGAGAAGATGACCCAGAT-3', R: 5'-GCACTGTGTGGCGTGACAGG-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, 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 (%), $\chi^2$ test or Fisher exact probability method was used for the comparison between groups; $p<0.05$ was considered as statistically significant.
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-
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Hanai et al. 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. 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; 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.

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

<table>
<thead>
<tr>
<th>Clinical feature</th>
<th>Number of cases</th>
<th>NGAL positive rate</th>
<th>( \rho )</th>
<th>VEGF positive rate</th>
<th>( \rho )</th>
</tr>
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<tbody>
<tr>
<td>FIGO staging</td>
<td></td>
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<tr>
<td>I-II</td>
<td>20</td>
<td>5 (25.0)</td>
<td>0.048</td>
<td>7 (35.0)</td>
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<td>III-IV</td>
<td>10</td>
<td>7 (70.0)</td>
<td></td>
<td>8 (80.0)</td>
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<tr>
<td>Differentiation</td>
<td></td>
<td></td>
<td>0.009</td>
<td></td>
<td>0.111</td>
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<tr>
<td>Poorly</td>
<td>8</td>
<td>2 (25.0)</td>
<td></td>
<td>2 (25.0)</td>
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<tr>
<td>Moderately</td>
<td>15</td>
<td>5 (33.3)</td>
<td></td>
<td>8 (53.3)</td>
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<tr>
<td>Well</td>
<td>7</td>
<td>5 (71.4)</td>
<td></td>
<td>5 (71.4)</td>
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<tr>
<td>Depth of invasion</td>
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<td></td>
<td>0.015</td>
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<td>0.012</td>
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<td>&lt;top 1/2</td>
<td>11</td>
<td>2 (18.2)</td>
<td></td>
<td>3 (27.3)</td>
<td></td>
</tr>
<tr>
<td>≥lower 1/2</td>
<td>19</td>
<td>10 (52.6)</td>
<td></td>
<td>12 (63.2)</td>
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<tr>
<td>Age (years)</td>
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<td>0.254</td>
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<td>&lt;56.7</td>
<td>13</td>
<td>5 (38.5)</td>
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<td>6 (46.2)</td>
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<td>≥56.7</td>
<td>17</td>
<td>7 (41.2)</td>
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<td>9 (52.9)</td>
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<td>Adenocarcinoma</td>
<td>21</td>
<td>8 (38.1)</td>
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<td>Non-adenocarcinoma</td>
<td>9</td>
<td>4 (44.4)</td>
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<tr>
<td>Maximum diameter (cm)</td>
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<td>7 (58.3)</td>
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<td>≥2.6</td>
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<td>8 (44.4)</td>
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Conflict of Interest

The authors declare no conflict of interest.

References


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