

Expression of Efp, VEGF and bFGF in normal, hyperplastic and malignant endometrial tissue

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Abstract. Vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) are two important angiogenic and prognostic factors for endometrial cancer. Estrogen is considered to be associated with increasing risk of endometrial adenocarcinoma. We investigated the expression of estrogen-responsive ring finger protein (Efp), VEGF and bFGF in endometrial cancer and correlated the results with clinicopathological features. We measured the expression of Efp, VEGF and bFGF in normal, hyperplastic and malignant endometrial tissues by quantitative RT-PCR, ELISA or Western blot analysis. The expression of Efp mRNA was significantly decreased in the groups of endometrial cancer (EC) in comparison with the groups of normal endometrium (NE) ($P<0.05$). Expression of VEGF mRNA in the groups of EC and atypical hyperplasia (AH) was significantly increased in comparison with the groups of NE ($P<0.05$). The expression of bFGF mRNA in groups of EC and AH was higher than that of NE groups ($P<0.01$ and $P<0.05$). There was positive relevance of bFGF expression and histologic grade and negative relevance of Efp expression and histologic grade. The expression of Efp, VEGF and bFGF protein was in agreement with the mRNA. The high expression of VEGF and bFGF indicate that both contribute in the angiogenesis of endometrial cancer. The regulation of Efp and bFGF expression during endometrial carcinogenesis suggests their potential utility as a prognostic biomarker.

Introduction

Endometrial carcinoma is one of the most common gynecological malignancies and its incidence has recently increased. Endometrial adenocarcinoma accounts for ~80%

of endometrial tumors. A causal link between endometrial adenocarcinoma and estrogen is widely accepted (1). Estrogen acts by binding to estrogen receptor (ER) α or β , the ER α predominate in breast and uterus, whereas the ER β predominate in bone and blood vessels. Estrogen-responsive ring finger protein (Efp) is one of downstream genes of ER α , it is predominantly expressed in estrogen target tissues including mammary glands, uteri and osteoblasts (2,3). Solid tumors beyond 2 mm in diameter require angiogenesis for growth and nutrition (4). Angiogenic factors have been identified as basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF) and platelet derived endothelial cell growth factor (PD-ECGF) (5-7). VEGF and bFGF have been identified as important regulators of tumor angiogenesis in the endometrium (8). VEGF is a vasoactive factor that alter the growth behavior of various tissues and has a role in angiogenesis (9,10), it might be involved in the promotion of angiogenesis in endometrial cancer and might contribute to the aggressive potential of high grade tumors or certain histological subtypes with unfavorable prognosis through the induction of angiogenesis (11). Dai *et al* demonstrate that estradiol may regulate the growth of endometrial carcinoma cells by stimulating VEGF production through Efp (12).

The aim of the present study was to characterize the expression of Efp, VEGF and bFGF in normal, hyperplastic and malignant endometrial tissues. Furthermore, we sought to determine any correlation between the expression of Efp, VEGF and bFGF and clinicopathological features of endometrial adnecarcinoma.

Materials and methods

Tissue samples. The study was approved by the Ethics Committee and written informed consent for participation in the study was obtained from each subject. One hundred and fifteen fresh endometrial tissue specimens were obtained from the Department of Gynecology, the Affiliated Hospital of Qingdao University Medical School, between January 2005 and December 2006. The patient age range was 30-70 years (median 54 years). Specimens were collected from patients who were scheduled to undergo fractional curettage and hysterectomy for benign gynecological conditions or endometrial carcinoma. No initial hormonal therapy or radiotherapy was performed prior to endometrium excision. All tissue samples were snap-frozen in liquid nitrogen within 30 min of

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surgical removal and stored at -70°C until use. The histopathological diagnoses of the selected endometrium were as follows: normal endometrium (NE, $n=30$ including proliferative endometrium, $n=15$ and secretory endometrium, $n=15$); simple hyperplasia (SH, $n=20$); complex hyperplasia (CH, $n=15$); atypical hyperplasia (AH, $n=15$); endometrial cancer (EC, $n=35$). Carcinomas were staged according to International Federation of Gynecology and Obstetrics criteria and histologically classified according to World Health Organization criteria. Twenty-three cases represented stage I, 7 stage II and 5 stage III. Fifteen of the malignant tumors were well differentiated (G1), 13 moderately differentiated (G2) and 7 poorly differentiated (G3).

Reagents. Antibodies against Efp and secondary antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). VEGF and bFGF ELISA kit were obtained from R&D system.

RNA extraction and cDNA synthesis. Total RNA was isolated from tissue using TRIzol reagent (Invitrogen) according to the manufacturer's instructions and stored at -70°C until use. Total RNA was reverse transcribed into cDNA using the reverse transcription reagents (Takara, Dalian, China) in a final volume of $10\ \mu\text{l}$ containing PrimeScript™ Buffer 2 μl , PrimeScript™ RT Enzyme Mix I 0.5 μl , 25 pmol of Oligo dT Primer, 50 pmol of Random 6 mers and 500 ng of total RNA. The samples were incubated at 37°C for 15 min and reverse transcriptase was inactivated by heating at 85°C for 5 sec. Samples were preserved at -20°C until use.

QRT-PCR. Primers and probes for Efp, VEGF, bFGF and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were designed with Primer Express 1.5 software (Applied Biosystems), and synthesized by Takara. The sequences for all primers and probes were as follows: Efp, forward, 5'-CGA GGTGGAAGTGAACCACAA-3'; reverse, 5'-TTCACAGGG CGTGTGGATTT-3'; probe, 5'-FAM-AAACGAGCTGAAG CAGTGCATCGGG-TAMRA-3'; VEGF, forward, 5'-AAGA TCCGCAGACGTGTAAATGTT-3'; reverse, 5'-CGGCTTG TCACATCTGCAAGTA-3'; probe, 5'-FAM-TTGCAAGGC GAGGCAGCTTGAGTTA-TAMRA-3'; bFGF, forward, 5'-GGC TTCTTCCTCCTGCGCATCCA-3'; reverse, 5'-GCTCTTAG CAGACATTGGAAGA-3'; probe, 5'-FAM-CTGCCAGGC GAGGGAGTTGAGCCTA-TAMRA-3'; GAPDH, forward, 5'-GAAGGTGAAGGTCCGAGTCA-3'; reverse, 5'-GAAG ATGGTGATGGGATTTC-3'; probe, 5'-FAM-CAAGCTTC CCGTTCTCAGCC-TAMRA-3'. All probes were labeled with FAM as reporter and with TAMRA as quencher. The PCR kit (Takara) was used for the PCR reaction. In a total volume of $20\ \mu\text{l}$, $2\ \mu\text{l}$ of the cDNA, 160 nM probe and 200 nM primer were used. PCRs were performed with the ABI Prism 7500 Sequence Detection System (Applied Biosystems). The PCR conditions were 95°C for 10 sec, followed by 40 cycles of 95°C for 5 sec and 60°C for 34 sec. Human GAPDH was used as an internal control, and each total RNA sample was normalized to the content of GAPDH mRNA using the comparative $\Delta\Delta\text{CT}$ method.

Western blot analysis. Snap-frozen tissues were prepared by homogenization in a protein extraction buffer with proteinase

inhibitors, analyzed for protein content by the method of Lowry. Protein samples ($30\ \mu\text{g}/\text{lane}$) was separated by 10% SDS-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride membrane. Membranes were subsequently blocked for 1 h at room temperature in 5% non-fat milk in PBS, incubated with anti-Efp antibody at a dilution of 1:400 overnight at 4°C , then rinsed and incubated for 1 h with horseradish peroxidase-conjugated secondary antibody at a dilution of 1:2000. Enhanced chemiluminescence was carried out using enhanced chemiluminescence kit (Amersham, Buckinghamshire, UK). Efp was readily detectable at $\sim 68\ \text{kDa}$. The reactions were analyzed by FluorChem IS-9900 Imaging System and band intensities were quantified using Alpha EaseFC Imaging software 4.0 (Alpha Innotech Corp., San Leandro, CA). An internal reference sample (same on each blot) was included as the standard for quantification. The standard was set to 100%. The signal from each band was correlated to the standard and this relative number was used for statistical analysis.

VEGF and bFGF measurement. All steps were carried out at 4°C . Tissues (wet weight 10-20 mg) were homogenized in a protein extraction buffer with proteinase inhibitors, analyzed for protein content by the method of Lowry. VEGF and bFGF levels were measured using an ELISA kit (R&D system) according to the manufacturer's instructions.

Statistical analysis. The statistical significance of data was evaluated by using one-way ANOVA and Student's t-test. A P-value ≤ 0.05 was considered to be significant. All analysis was performed using SPSS version 10.0 and Microsoft Excel 2003. Each experiment was repeated 3 times to verify the reproducibility of the results. All experiments are shown as mean of triplicate \pm standard error (SE).

Results

Expression of Efp mRNA and protein in normal, hyperplastic and malignant endometrial tissue. The expression of Efp mRNA and protein was significantly decreased in the groups of endometrial cancer in comparison with the groups of normal endometrium (Fig. 1).

Expression of VEGF mRNA and protein in normal, hyperplastic and malignant endometrial tissue. Expression of VEGF mRNA in the groups of EC and atypical hyperplasia (AH) was significantly increased in comparison with the groups of NE (Fig. 2A) and the protein expression of VEGF in the groups of EC was significantly increased in comparison with the groups of NE (Fig. 2B).

Expression of bFGF mRNA and protein in normal, hyperplastic and malignant endometrial tissue. The expression of bFGF mRNA and protein in groups of EC and AH was higher than that of NE groups (Fig. 3).

Correlation between Efp, VEGF and bFGF mRNA expression and clinicopathological features. To further illustrate whether or not the expression of Efp, VEGF and bFGF in EC correlates with clinicopathologic factors, several parameters

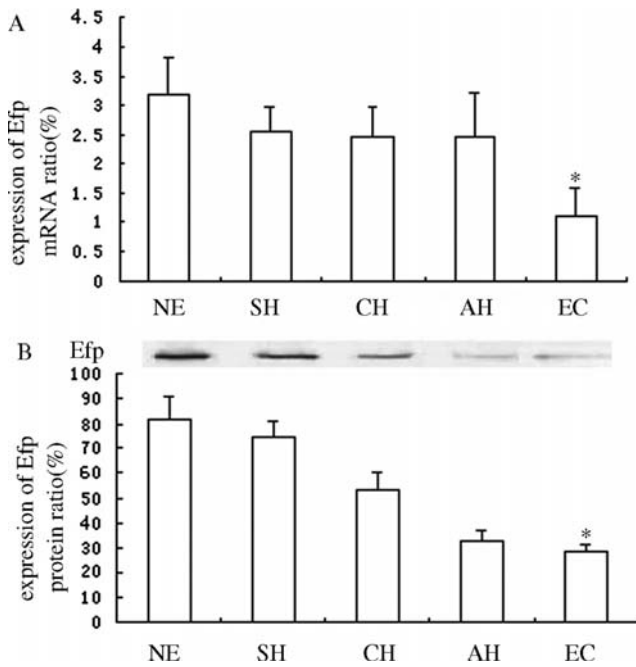


Figure 1. Expression of Efp mRNA and protein in normal, hyperplastic and malignant endometrial tissue. (A) Expression of Efp mRNA. (B) Expression of Efp protein. Results were obtained from three separate experiments and expressed as mean \pm SE. The asterisk indicates significant difference compared with the control (* P <0.05).

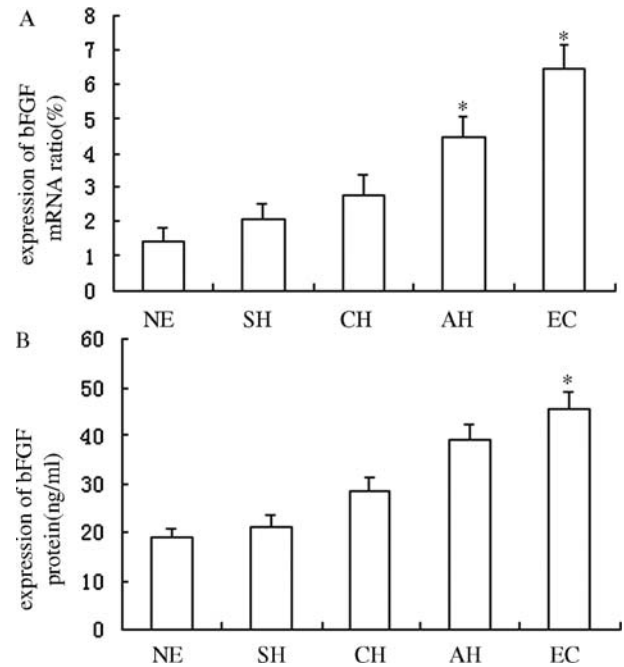


Figure 3. Expression of bFGF mRNA and protein in normal, hyperplastic, and malignant endometrial tissue. (A) Expression of bFGF mRNA. (B) Expression of bFGF protein. Results were obtained from three separate experiments and expressed as mean \pm SE. The asterisk indicates significant difference compared with the control (* P <0.05, ** P <0.01).

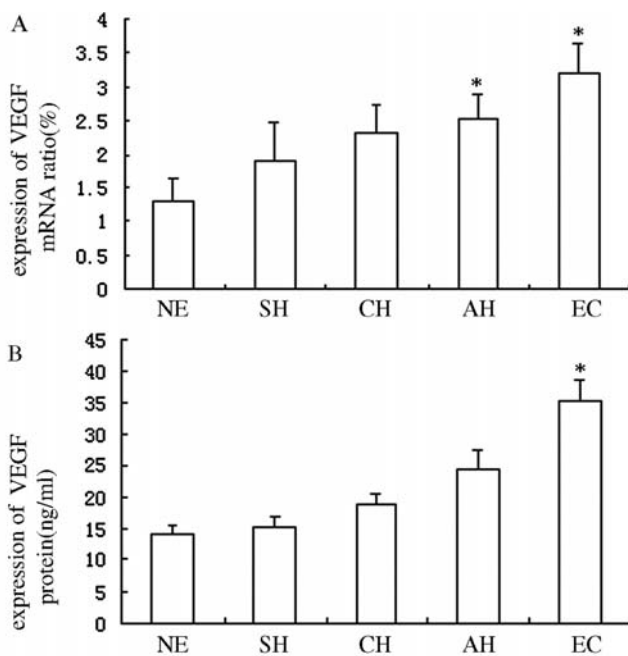


Figure 2. Expression of VEGF mRNA and protein in normal, hyperplastic, and malignant endometrial tissue. (A) Expression of VEGF mRNA. (B) Expression of VEGF protein. Results were obtained from three separate experiments and expressed as mean \pm SE. The asterisk indicates significant difference compared with the control (* P <0.05).

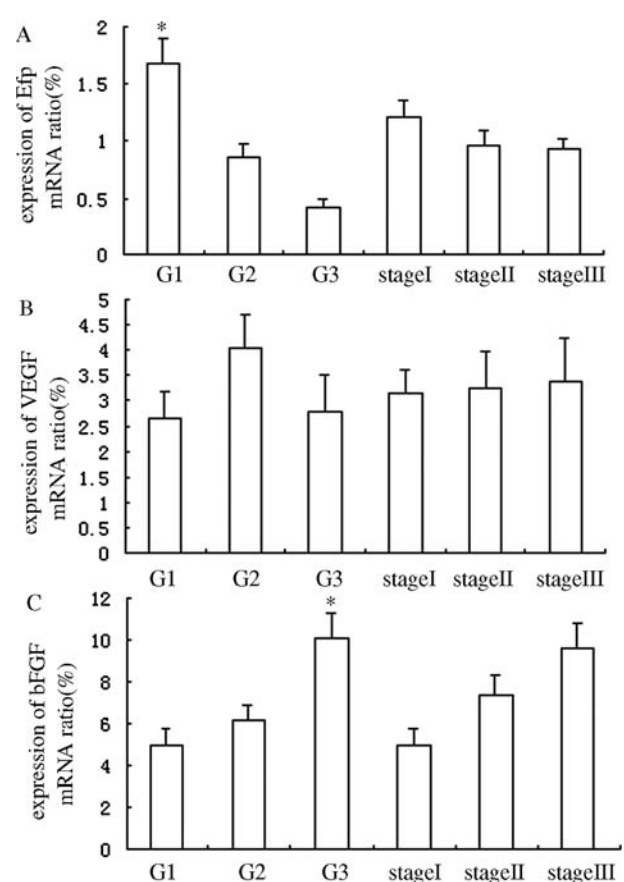


Figure 4. Correlation between Efp, VEGF and bFGF mRNA expression and clinicopathological features. (A) Expression of Efp mRNA. (B) Expression of VEGF mRNA. (C) Expression bFGF mRNA. Results were obtained from three separate experiments and expressed as mean \pm SE. The asterisk indicates significant difference compared with the control (* P <0.05).

were assessed using QRT-PCR for all specimens. As showed in Fig. 4, the expression of Efp mRNA was significantly increased in the groups of G1 endometrial cancer in comparison with the groups of G3. The difference of VEGF

mRNA expression did not reach statistical significance. The expression of bFGF mRNA was significantly increased in the groups of G3 endometrial cancer in comparison with the groups of G1. Although there appeared to be a trend for increasing bFGF with increasing surgical pathologic stage of the tumors, the differences were not statistically significant.

Discussion

Endometrial cancer is an important hormone-dependent tumor that develop in women. The major risk factor is associated with lifetime exposure to estrogen (13). Estrogen is known as a major cause of tumorigenesis in the endometrium. The stimulatory effect of estrogen on cell proliferation may be regulated by up-regulation of CyclinD₁ and CyclinA (14), or by stimulating VEGF production through Efp (12).

Estrogen acts by binding to estrogen receptor (ER) α or β , Efp is one of downstream genes of ER α (2). Efp was first isolated from HeLa cells and found to be inducible when cells were treated with estrogen (15). Efp belongs to family of nuclear proteins, that contains a Ring finger motif, which has been implicated in transcriptional regulation, DNA repair and site-specific recombination. Efp expression in cancer has been studied predominantly in breast cancer. Efp was significantly correlated with poor prognosis of breast cancer patients and it is considered to be a new potential biomarker for breast cancer (16). Efp mRNA was detected in Ishikawa human endometrial carcinoma cells and was induced by estrogen treatment, suggesting that Efp can mediate estrogen actions such as cell growth (12). There are few previous studies on Efp in endometrial cancer. In the current study, we found that expression of Efp decreased gradually from normal endometrium to SH and CH. Significantly down-regulated expression was observed in EC. Our findings were consistent with those of Nakayama *et al* (17). We also found that expression of Efp decreased significantly from G1 to G3, though a trend for decreasing Efp levels with the increasing in stage was found, it was not statistically different. These results suggest that the expression of Efp was positively correlated with histological subtypes, in endometrial adenocarcinoma of high-grade histological subtypes (G3), Efp expression was weak, and in low-grade histological subtypes (G1), Efp expressed more strongly. With the increasing of the histological subtype, the expression of the Efp was decreasing, we speculated that the down-regulation is related to loss of estrogen dependency on uterine endometrial cancers during their progression.

VEGF is one of the most potent angiogenic factors favoring the development of new blood vessels and, therefore, contribute to the progression of tumors that depend on neovascularization (18). In Ishikawa endometrial carcinoma cells, VEGF expression was transiently increased by estradiol through Efp (12). The transient up-regulation of VEGF is considered to be logical, as the upstream of VEGF gene conserves estrogen-responsive elements (19). Therefore, it is natural that VEGF expression should be down-regulated with advancement and dedifferentiation in endometrial cancers. The expression of VEGF in endometrial cancer, actually increased compared to normal endometrium, a lack of correlation was found between VEGF and stage, grade of tumor. We speculate that

it is related to loss of estrogen dependency on uterine endometrial cancers during their progression. Our study is not consistent with Fujimoto *et al* (5). Yoshiji *et al* explained that more advanced breast cancers may not require VEGF for continued growth of large tumors. Their study revealed that VEGF was necessary during the initial stages of tumor growth but was not essential for continued growth once the tumor reached a critical size. Other growth factors (bFGF, TGF) may assume its role in tumor progression in large tumors following suppression of VEGF (20). Therefore, it can be hypothesized that VEGF may play the same role in the progression of endometrial cancers.

Basic FGF is expressed in cancer and stromal cells and studies on basic angiogenesis. The expression of bFGF is significantly higher in uterine endometrial cancers than in normal uterine endometrium and increased with advancement of dedifferentiation, this is consistent with Fujimoto *et al* (6). Soufla *et al* also showed the up-regulation of FGF2 mRNA in malignant endometrium (21). So we speculate that bFGF might be an excellent indicator for advancement of endometrial cancers.

In conclusion, this study suggest that VEGF and bFGF may contribute in the angiogenesis of endometrial cancer. The regulation of Efp and bFGF expression during endometrial carcinogenesis suggests their potential utility as a prognostic biomarker and VEGF was necessary during the initial stages of tumor growth and bFGF may assume a more direct and important role in tumor progression.

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