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FGF2 transcript levels are positively correlated with EGF and IGF-1 in the malignant endometrium

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Abstract

Background. Epidermal and insulin-like growth factors (EGF, IGFs) act as mitogens promoting endothelial cell proliferation and differentiation. Accumulating evidence for interactions between EGF and IGF signaling pathways has been reported. Fibroblast Growth Factor-2 (FGF2) is also mitogenic for various cell types and is associated with regulation of tumor angiogenesis and metastasis. However EGF, IGF-1 and FGF2 transcript levels have been scarcely investigated in endometrial carcinoma.

Methods. In the present study, we evaluated the mRNA expression pattern of EGF, IGF-1 and FGF2 by using Comparative Quantitative real-time RT-PCR assay in 30 endometrial cancer specimens and an equal number of adjacent normal tissues.

Results. Both overexpression and down-regulation of EGF, IGF-1 and FGF2 was demonstrated in endometrial cancer compared to the adjacent normal specimens; however the main features of cancer were IGF-1 and EGF down-regulation and FGF2 up-regulation. Different co-expression patterns of all three factors were displayed in normal and malignant endometrium. Interestingly, FGF2 mRNA was positively correlated with EGF and IGF-1 transcript levels in endometrial cancer ($P = 0.024$ and $P = 0.006$, respectively), while no co-expression was observed in the adjacent normal specimens. Furthermore, endometrial tissue-pair analysis revealed a significant positive correlation between EGF and IGF-1 ($P = 0.010$), supporting the hypothesis of a cross-talk between IGF- and EGF-mediated signaling pathways in endometrial cancer. EGF transcript levels were marginally higher in endometrioid than non-endometrioid tumors ($P = 0.050$), and in grade I compared to grade II tumors ($P = 0.053$).

Conclusions. Up-regulation as well as down-regulation of EGF, IGF-1 and FGF2 transcript levels is observed in endometrial cancer; however IGF-1 and EGF down-regulation and FGF2 up-regulation seem to comprise the main features of endometrial carcinogenesis. The disruption of their mRNA co-expression pattern observed supports the hypothesis of a cross-talk between IGF- and EGF-mediated signaling pathways in promoting endothelial cell proliferation and differentiation in endometrial cancer.

Keywords: EGF; IGF-1; FGF2; Real-time PCR; Endometrial cancer

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1. Introduction

Endometrial cancer is one of the most common gynaecological malignancies [1]. Tumor development and progression in the endometrium, as in most human carcinomas, is mediated by angiogenesis. This complex procedure is an indispensable requirement for tumor growth, as well as invasiveness and metastasis [2]. The uterine endometrium however, is a dynamic organ that undergoes periodic growth, remodeling and breakdown under hormonal influence during the menstrual cycle. Nevertheless induction of abnormal angiogenesis during endometrial cancer development has been well documented [1]. Tumor and stromal vascularization has been shown to provide prognostic information for patients with endometrial cancer [3,4].

Numerous growth factors and cytokines are involved in the angiogenic process accompanying endometrial carcinogenesis. Among them, Epidermal and Insulin growth factors (EGF, IGFs) act as mitogens promoting endothelial cell proliferation and differentiation. Fibroblast Growth Factor-2 (FGF2) is also mitogenic for various cell types and is associated with regulation of tumor angiogenesis and metastasis.

EGF exerts its proliferative function through binding to its receptor EGFR, a 170-kDa transmembrane protein with tyrosine kinase action. Extracellular ligand binding induces dimerization and consequently activation of the intracellular tyrosine kinase. Limited information is available regarding EGF mRNA levels in the premalignant and malignant endometrium [5,6]. Previous studies on the field are suggestive of either higher or lower EGF transcript levels in endometrial cancer compared to normal endometrium, therefore they are not adequate to elucidate EGFs exact role in the malignant transformation of the endometrium.

The IGF signaling pathway on the other hand is more complex. IGF-1 is a hormone-dependent growth factor with a molecular weight of 7650 kDa, which binds to its receptor IGFR-1 under the regulation of IGF binding proteins [7]. Estrogen has been shown to stimulate IGF-1 gene expression in the endometrium [7,8] and IGF-1 is assumed to mediate estrogen action through autocrine as well as paracrine mechanisms. Originally it was considered that these agents manifest their mitogenic functions through separate pathways, but a growing body of evidence supports the hypothesis of interactions between estrogen and IGF signaling pathways. Increased levels of plasma IGF-1 in women have been associated with increased risk of developing cervical, ovarian or endometrial cancer [9,10]. Giudice et al. provided evidence of IGF-I involvement in endometrial growth regulation [11] but the underlying mechanism of its participation in endometrial carcinogenesis is not yet clarified.

FGF2 can affect tumor vasculature not only through paracrine but also through autocrine or possibly intracellular signaling [12]. FGF2 exists as five distinct isoforms with distinct subcellular localizations. FGF2 is produced as a cytoplasmic 18-kDa isoform that can be released and as four high-molecular weight (HMW) isoforms that are nuclear. The 18-kDa FGF2 is the predominant isoform released from cells, and enters the nucleus via a receptor-dependent pathway. FGF2 mediates tumor metastasis through the remodeling of the extracellular matrix. FGF2 has been proposed to be a key regulator of angiogenesis in prostate cancers [13,14], skin melanoma [15] and pancreatic cancer [16]. It has also been implicated in cervical cancer development by our research group [17], but its role in endometrial carcinogenesis has scarcely been examined.

In the present study, we evaluated the mRNA expression pattern of the most important mitogens that regulate angiogenesis in endometrial cancer: EGF, IGF-1 and FGF2. Our goal was to examine their significance as possible markers of malignant transformation of the endometrium, as well as to investigate the underlying mechanism mediating their expression during endometrial cancer development and progression. Therefore, EGF, IGF-1 and FGF2 mRNA levels were assessed by quantitative real-time PCR analysis in endometrial cancer and adjacent normal tissue specimens. A few cases of atypical endometrial hyperplasia were additionally investigated. The expression profile of the above genes was correlated with clinical parameters. Moreover, the ratio of the transcript levels of each gene in the tumor specimen to that of the adjacent normal tissue was used to provide a distinct molecular portrait of each tumor that was subsequently compared with clinicopathological features.

This is the first assessment according to our knowledge of the mRNA co-expression pattern of EGF, IGF-1 and FGF2 (pair-wise) in normal and malignant endometrium, with the scope of elucidating possible interactions between these mitogenic growth factors during the process of endometrial carcinogenesis.

2. Materials and methods

2.1. Tissue specimens

Specimens were surgically obtained from a total of 34 patients who underwent hysterectomy as a therapy at the Department of Obstetrics and Gynecology of PAGNH University Hospital Crete, from 2003 to 2004. Tissue specimens were obtained at the time of the surgery and each specimen was bisected. Half of the sample was snap frozen and stored at −80 °C until required for RNA extraction. The other half was fixed in 10% formaldehyde solution for histopathological examination. Age at the time of surgery ranged from 31 to 88, mean (64.8 ± 14.1). Only one woman was in the reproductive period and the rest were in menopause. Staging was reviewed based on the International Federation of Obstetrics and Gynecology (FIGO) staging system: 14 cancer specimens were of stage I, 12 of stage II and 4 of stage III. Endometrial cancer patients were stratified according to tumor histological cell type and differentiation grade. In addition, four patients suffering from atypical endometrial hyperplasia (AEH) were also examined. Table 1 summarizes the clinical characteristics of the patients' with endometrial cancer. Tissue biopsies had been previously received from all the patients with cancer to establish the diagnosis, but none of them had undergone any radiotherapeutic or chemotherapeutic treatment prior to hysterectomy and tissue biopsies for the present study. The Ethics Committee of the University of Crete approved the present study, and all participating patients gave written informed consent.

Table 1
Clinical and histological characteristics of patients with endometrial carcinoma

Characteristic	No. of patients
Total no. of patients	34
AEH	4
Endometrial cancer	30
Age	
Mean ± SEM	64.8 ± 14.1
Range	31–88
Menopausal status	
Pre	1
Post	29
Histological cell type	
Endometrioid	23
Non-endometrioid	7
Serous papillary	2
Clear cell	1
Mixed	4
Histological grade	
G1	6
G2	17
G1–G2	2
G3	5
FIGO stage	
I	14
II	12
III	4
Myometrial invasion	
<50%	16
>50%	14
Cervical involvement	
Positive	10
Negative	20
Extra-uterine disease	
Positive	6
Negative	24
LVS involvement	
Positive	0
Negative	30

2.2. RNA extraction and reverse transcription (RT-PCR)

Total RNA was isolated from fresh tissues using the Trizol reagent (Life Technologies Ltd., UK) according to the manufacturer's instructions. Tissue specimens were homogenized in the TRIzol® reagent (Invitrogen, Carlsbad, CA) using a power homogenizer and incubated at room temperature, followed by chloroform addition and centrifugation. Total RNA was precipitated from the supernatant with isopropanol, washed with 75% ethanol and resuspended in 50 μl DEPC-treated water. RNA concentration was calculated after measuring on a UV spectrophotometer (Hitachi Instruments Inc., USA) its 260-nm absorbance and 260/280-nm absorbance ratio. One percent agarose gel electrophoresis and ethidium bromide staining were used to examine RNA integrity.

Reverse transcription reactions for the preparation of first-strand cDNA from 2 μg of total RNA, were performed using the "Reverse transcription kit", according to the manufacturer's protocol (Promega USA). Random hexamers were used as amplification primers. In detail, 2 μg of total RNA, 50 ng of random hexamers and 1 mM dNTPs were heated at 70 °C for 10 min, in order to remove RNA secondary structures, and placed on ice until the addition of cDNA synthesis mix, which contained 1× cDNA synthesis buffer (50 mM Tris–acetate, pH 8.4, 75 mM potassium acetate, 8 mM magnesium acetate), 5 mM dithiothreitol (DTT), 40 U RNasin™ (Promega) and 15 U reverse transcriptase (Promega). The final mix (volume 20 μl) was incubated for 10 min at 25 °C for primer extension, and cDNA synthesis was conducted at 42 °C for 60 min. The reaction was terminated by heating at 95 °C for 5 min. In order to remove the RNA template, cDNA was incubated at 37 °C for 20 min with 2 U of *Escherichia coli* RNaseH, and stored at −20 °C until use.

2.3. Real-time PCR

Real-time PCRs were carried out in the Mx3000P Real-Time PCR system (Stratagene, USA) by using the SYBR® Green I Master Mix (Stratagene, USA) according to the manufacturer's instructions. Data were collected and analysed with the use of the Mx3000P Real-Time PCR software version 2.00, Build 215 Schema 60 (Stratagene, La Jolla, CA). Specifically all growth factors' mRNA expression was measured using a real-time RT-PCR assay with SYBR® Green I. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as internal control, in order to normalize EGF, IGF-1 and FGF2 mRNA expression levels. The mRNA-specific primers used are listed in Table 2. After initial experiments, in order to optimize primers' concentration and annealing temperature, 1 μl cDNA from normal, hyperplasia or malignant endometrial samples was amplified in a PCR containing 2× Brilliant SYBR® Green QPCR Master Mix (containing 2.5 mM $MgCl_2$), 300 nM of each primer and 30 μM Rox passive reference dye, in a final volume of 20 μl. To ensure the accuracy of quantification measurements, a representative pool of all samples was diluted in a series of seven 2× dilutions, and was run in the same plate, in order to construct a standard curve for the quantification process. After initial denaturation at 95 °C for 10 min, samples were subjected to 45 cycles of amplification, comprised of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s and elongation at 72 °C for 30 s, followed by a melt curve analysis, in which the temperature was increased from 55 to 95 °C at a linear rate of 0.2 °C/s. Data collection was performed both during annealing and extension, with two measurements at each step, and at all times during melt curve analysis. To verify the results of the melt curve analysis, PCR products were analyzed by electrophoresis in 2% agarose gels, stained with ethidium bromide and photographed on a UV light transilluminator. The PCR product length of all growth factors analyzed is presented in Table 2. In each PCR two negative controls were included, one with no cDNA template and one with no reverse transcription treatment. Peptide growth factor transcription levels were calculated using the following formula: $\text{NormalizedSample/Control} = (1+E_{GF})^{-\Delta C_t}\text{GF}/(1+E_{GAPDH})^{-\Delta C_t}\text{GAPDH}$. Twofold increased or decreased expression was considered significant. Representative examples of real-time quantitative PCR amplification plots and dissociation curves corresponding to the target genes or internal control (GAPDH) are presented in Fig. 1. Procedures were repeated with cDNA template synthesized 3 times from the same RNA. Each sample's mRNA levels for every growth factor tested represent the mean value of data acquired from three independent RT-PCR experiments.

Table 2
Primer sequences used for quantitative real-time RT-PCR

Growth factor	Primer pair sequence (5′–3′)	Product size
EGF	CTTGTCATGCTGCTCCTCCTG TGCGACTCCTCACATCTCTGC	118
IGF-1	CCTCCTCGCATCTCTTCTACCTG CTGCTGGAGCCATACCCTGTG	166
FGF2	CTGGCTATGAAGGAAGATGGA TGCCCAGTTCGTTTCAGTG	149
GAPDH	GGAAGGTGAAGGTCGGAGTCA GTCATTGATGGCAACAATATCCACT	101

2.4. Statistical analysis

One sample Kolmogorov–Smirnov test was employed to assess the normal distribution of the mRNA expression values of all genes studied. The mRNA expression of EGF, IGF-1 and FGF2 was compared between the groups of normal and pathological samples, as well as between the groups of different clinicopathological features (histological cell type, differentiation grade, myometrial invasion, etc.) by the use of non-parametric procedures (Kruskal Wallis and Mann–Whitney test). The Spearman rank correlation (non-parametric test) was employed to examine the growth factor mRNA correlation pair-wise. Finally the Chi-Square (χ^2) test was used to assess differences in EGF, IGF-1 and FGF2 mRNA expression status (overexpression or down-regulation) in the groups of endometrial cancer and endometrial atypical hyperplasia cases. Probability values less than 0.05 were considered statistically significant. Statistical calculations were performed using the SPSS software, version 11.

3. Results

In the present study, we evaluated the mRNA expression profile of EGF, IGF-1 and FGF2 using a quantitative real-time RT-PCR method. FGF2 and GAPDH amplification plots and dissociation curves are representative examples of the analysis and are presented in Fig. 1. Our study group consisted of 30 endometrial cancer tissues, and adjacent normal tissue specimens of all subjects. We additionally examined four atypical endometrial hyperplasia (AEH) and adjacent normal specimens. Transcript levels of the housekeeping gene GAPDH were also evaluated in all samples by real-time PCR analysis, in order to be used as an internal control. The ratio of each growth factor's (EGF or IGF-1 or FGF2) transcript levels, versus GAPDH mRNA levels of the same specimen served as its normalized mRNA levels.

3.1. Transcript levels of EGF, IGF-1 and FGF2

Transcript levels of all three mitogenic growth factors studied (normalized to GAPDH) were found to be similar in normal and malignant endometrial speci-