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Expression profiling and significance of VEGF-A, VEGFR2, VEGFR3 and related proteins in endometrial carcinoma

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ABSTRACT

Background: Angiogenesis plays a key role in the progression of various tumors, including endometrial carcinomas. Several cytokines and their associated receptors are shown to be involved, particularly VEGF-A with VEGFR1, -2 and -3.
Methods: The expressions of VEGF-A, VEGFR2 and VEGFR3 were studied in by immunohistochemistry in 76 endometrial carcinoma specimens. VEGFR2 and VEGFR3 receptor expression were also studied by qRT-PCR in 17 tumors in comparison to normal endometrium. The expression profiles were correlated with tumor type, grade, stage, lymphovascular invasion, disease free survival, and the expressions of other cytokine receptors (EGFR, CXCR1 and CXCR2).
Results: Immunohistochemically, 63% of endometrial cancers expressed VEGF-A, 55% VEGFR2 and 26% VEGFR3. VEGFR3 was significantly correlated with tumor stage ($p = 0.02$), with a trend towards poorer disease free survival ($p = 0.09$). VEGF-A was significantly correlated with microvessel density ($p < 0.01$). Using qRT-PCR, increased expression of VEGFR2 (17.2-fold) and VEGFR3 (21.9-fold) was seen in endometrial carcinomas compared with normal endometrium, with significant correlations among the expression levels of VEGFR2, VEGFR3, EGFR, CXCR1 and CXCR2.
Conclusion: Our study suggests that evaluation of VEGFR3 expression in tumors may provide prognostic data, and help identify patients who would best benefit from anti-angiogenic therapeutic agents. This is the first report showing correlations between the expressions levels of the different receptors.

1. Introduction

Angiogenesis is an important but poorly understood process which allows tumors to grow and to metastasize. Several angiogenic growth factors have been found which promote and regulate angiogenesis in physiological conditions, as well as in tumor progression [1,2]. Of these, the most studied is the vascular endothelial growth factor (VEGF) family, comprising VEGF-A to -D and their receptors VEGFR1 (flt-1), VEGFR2 (KDR/flk-1) and VEGFR3 (flt-4). Although VEGF-A and -B act primarily via VEGFR1 and -R2, while VEGF-C and -D act via VEGFR3 (and appear most important in lymphangiogenesis), cross-signalling between the different ligands and receptors occurs. Physiologically, VEGFs are secreted by fibroblasts and inflammatory cells, and bind to VEGFRs on endothelial cells to promote angiogenesis. However, in tumor angiogenesis, expression of these ligands and receptors are also seen in tumor cells, resulting in both autocrine-induced tumor growth and angiogenesis.

However, the VEGF/VEGFR pathway is not the sole pathway for angiogenesis. Other signalling pathways, such as fibroblast growth factor 2 (FGF2) and its receptors FGFR1 and FGFR2, epidermal

Abbreviations: CXCR, CXC chemokine receptor; DFS, disease-free survival; (c)DNA, (complementary) deoxyribonucleic acid; EGF(R), epidermal growth factor (receptor); FGF(R), fibroblast growth factor (receptor); IL-8, interleukin-8; (m)RNA, (messenger) ribonucleic acid; qRT-PCR, Quantitative Real-Time Polymerase Chain Reaction; VEGF(R), vascular endothelial growth factor (receptor).

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growth factor (EGF) and its receptor EGFR [3] are also involved. In addition, interleukin-8 (IL-8), a pro-inflammatory cytokine, which binds to its receptors CXC chemokine receptor 1 (CXCR1) and CXCR2, also initiates many different signalling pathway resulting in angiogenesis, mitogenesis and motogenesis [4]. These other pathways may explain the resistance of tumors to VEGF inhibition therapy [2].

The expression of these various angiogenic factors and their receptors have therefore been studied and found to be expressed in a wide variety of tumors, including breast, pancreatic and colorectal cancers [5–7]. Their expressions in endometrial cancers have also been studied in the past [8–11]. However, some of these studies have shown conflicting data, with some showing increased expression compared with normal endometrium, while others do not show this. Similarly, some studies have shown the expression of VEGFs, or their receptors to be correlated with prognostic factors, while others have not shown this correlation.

In this paper, we studied the expression of VEGF-A, VEGFR2 and VEGFR3 in endometrial tumors. We correlated the expressions of these receptors with EGFR, CXCR1 and CXCR2 expressions, as well as other pathological prognostic factors and clinical outcome.

2. Materials and methods

2.1. Case and sample selection

A cohort of patients diagnosed with endometrial carcinomas was identified from the database of Hammersmith Hospital, London, United Kingdom. All patients had undergone a total hysterectomy with bilateral salpingo-oopherectomy at Hammersmith Hospital. Ethical approval for the use of human tissue was obtained from the Hammersmith and Queen Charlotte's and Chelsea Hospitals Research Ethics Committee (Reference number: 2000/5875).

The clinical history and histopathological reports and slides were reviewed. A representative tissue block containing adenocarcinoma was selected for each case for immunohistochemistry staining. To analyse the mRNA expression levels, 17 patients who had fresh tissue snap frozen in liquid nitrogen and stored at −80 °C were selected. The frozen blocks for adenocarcinoma and matched normal endometrium tissue were obtained from the Imperial College Healthcare Tissue Bank.

Clinico-pathological data including patient age, histological tumor type, tumor grade, tumor stage and the presence of lymphovascular invasion were collected. For a number of patients, clinical outcome data was available as disease free survival (DFS).

2.2. Immunohistochemistry

The expressions of EGFR, VEGF-A, VEGFR2, VEGFR3 and CD31 were evaluated by immunohistochemistry using the avidin-biotin immunodetection complex method.

Two micron thick sections from formalin-fixed, paraffin embedded tissue were prepared, deparaffinised and rehydrated. Endogenous peroxidase was blocked by incubation in hydrogen peroxide. Antigen retrieval was performed by microwaving in citrate or ER1 (Leica, citrate equivalent) buffer. Sections were incubated with normal goat serum for 10 min, then with the primary antibody for 60 min at room temperature. The primary antibodies used were mouse anti-EGFR antibody (NCL-EGFR, Leica, 1:50 dilution), mouse anti-VEGF-A (sc-7269, Santa Cruz, 1:500 dilution), rabbit anti-VEGFR2 (LVRB1526R7, Neomarkers, 1:200 dilution), mouse anti-VEGFR3 (NCL-L-VEGFR-3, Leica, 1:50 dilution), mouse anti-CD31 (NCL-CD31-1A10, Leica, 1:50 dilution).

The sections were washed, and then incubated with goat anti-mouse or anti-rabbit biotinylated immunoglobulin for 30 min, followed by streptavidin peroxidase for 30 min. The slides were developed in DAB, followed by a haematoxylin counterstain. Sections from normal colon and placenta were used as positive controls and for each case a section from which the primary antibody was replaced by phosphate buffered saline was used as a negative control.

2.3. Assessment of expression

All sections were examined by light microscopy for the presence of expression and cellular distribution of the proteins (between the cell membrane, cytoplasm and nucleus) in the endometrial adenocarcinomas. Cell staining intensity was scored as negative (0), weak (1+), moderate (2+) and strong (3+). Tumors showing antigen expression showed intratumoral heterogeneity in the intensity of staining. For each case, the percentage of cells with the predominant staining intensity was estimated. For statistical purposes, cases which were moderately (2+) or strongly (3+) positive in more than 10% of cells were designated overall positive, while cases in which expression was weak (1+) or seen in less than 10% of cells were considered overall negative. Slides were scored independently by three investigators (JW, IB and MEB), and disagreements were resolved by review and a consensus reached.

Assessment of microvessel density (MVD) was performed as previously described [12]. Essentially, microvessel density was assessed but scanning the area of highest vessel density at low power magnification. Individual microvessels were then counted on a 200× field (0.80 mm^2 per field). Any endothelial cell cluster, which was positive for CD31 and contained a visible lumen was considered a single countable microvessel. For each section, three fields were counted, and the mean value calculated. This value was converted to the MVD count, expressed as vessels/mm^2.

2.4. Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total ribonucleic acid (RNA) isolated from 10-μm sections of frozen tissue using the RNeasy Mini kit (Qiagen, Crawley, UK), was reverse transcribed using the Superscript III reverse transcriptase and random primers (Invitrogen). qRT-PCR was performed with ABI PRISM 7700 Sequence Detection System using SYBR Green Mastermix (Applied Biosystems). L19, non-regulated ribosomal housekeeping gene was used as an internal control to normalize input complementary deoxyribonucleic acid (cDNA).The transcript levels were quantified using the standard curve method. All experiments were performed in duplicates.

The following gene-specific primer pairs were designed using the ABI Primer Express software (Applied Biosystems, Brackley, UK):

CXCR1-sense: 5′-ATCTGTCCCTGCCCTTCTT-3′
CXCR1-antisense: 5′-AGTGTACGCAGGGTGAATCC-3′
CXCR2-sense: 5′-ACATGGGCAACAATACAGCA-3′
CXCR2-antisense: 5′-TGAGGACGACAGCAAAGATG-3′
EGFR-sense: 5′-GGCTGGTTATGTCCTCATTGC-3′
EGFR-antisense: 5′-TCTGCAGGTTTTCCAAAGGAA-3′
VEGFR2-sense: 5′-CAGCGATGGCCTCTTCTGTAA-3′
VEGFR2-antisense: 5′-TCCAGTGTCATTTCCGATCACT-3′
VEGFR3-sense: 5′-CCAGGCCCTACTGCAAGGT-3′
VEGFR3-antisense: 5′-GCCTGTGTCGTTGGCATGT-3′
L19-sense 5′-GCGGAAGGGTACAGCCAAT-3′
L19-antisense 5′-GCAGCCGGCGCAAA-3′.

2.5. Statistical analysis

The presence of significant differences in immunohistochemical expression of VEGF-A, VEGFR2, VEGFR3 and EGFR, between