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ORIGINAL ARTICLE

Prognostic value of tgfb1 protein in endometrioid adenocarcinoma

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ABSTRACT

Background Angiogenesis is a prerequisite for tumour development, progression and metastasis; however, its underlying molecular mechanisms in endometrial carcinoma are poorly understood.

Design In this study, the mRNA and protein expression profiles of two key regulators of angiogenesis, vascular endothelial growth factor (VEGF) and transforming growth factor beta-1 (TGFB1), were evaluated by real-time PCR and western blot analysis in 23 endometrial cancer tissue-paired specimens (malignant vs. adjacent normal tissues). We aimed to investigate whether VEGF and TGFB1 serve as markers of the malignant transformation of the endometrium and whether VEGF or TGFB1 expression can constitute a useful prognostic marker of survival in patients with endometrial carcinoma.

Results Tissue-pair analysis revealed VEGF transcriptional up-regulation and TGFB1 mRNA down-regulation as the most frequent transcriptional features. VEGF and TGFB1 mRNA were positively correlated ($P < 0{\cdot}001$). VEGF protein levels were higher in endometrioid-type tissue pairs ($P = 0{\cdot}047$). TGFB1 protein and mRNA levels were negatively correlated ($P = 0{\cdot}042$). TGFB1 protein expression was related to survival only in patients with endometrioid adenocarcinoma ($P = 0{\cdot}045$).

Conclusions Tissue-pair mRNA and protein analysis reveals VEGF transcriptional up-regulation and TGFB1 down-regulation that are correlated with the malignant transformation of the endometrium, while post-transcriptional mechanisms control VEGF and TGFB1 protein. TGFB1 protein demonstrated a prognostic value only in endometrioid adenocarcinoma.

Keywords Endometrial cancer, mRNA expression, protein, RT-PCR, TGFB1, vascular endothelial growth factor.

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Introduction

The molecular mechanisms that control endometrial carcinogenesis are poorly understood, and the molecular indicators of disease progression are currently being investigated. The induction of the angiogenic process during endometrial cancer development has been well documented [1]. Tumour and stromal vascularization, assessed by microvessel density counting, has been shown to provide prognostic information for patients with endometrial cancer [2,3].

Vascular endothelial growth factor (VEGF) plays a significant role in angiogenesis acting as an endothelial cell-specific mitogen, stimulating cell proliferation and increasing vascular permeability. Elevated VEGF expression at advanced stages of the disease has been reported in various types of cancer, including breast, ovarian and bladder cancer [4–6], and has also been associated with increased angiogenesis in the endometrium [7]. However, whether VEGF levels could be used as a molecular prognostic indicator of disease progression is still a matter of controversy. Studies attempting to correlate VEGF expression with either survival or metastasis have had conflicting results, and the utility of VEGF as a prognostic indicator remains to be determined. Immunohistochemical or enzyme immunoassay studies have found VEGF protein levels to be correlated with local tumour progression, metastasis and poor prognosis in the

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endometrium [8–11]. However, other reports have provided evidence, suggesting that VEGF does not have a prognostic value [12,13].

Transforming growth factor beta-1 (TGFB1) is involved in cell proliferation, adhesion, differentiation and migration. TGFB1 interacts with cell surface receptors (TGFBR1, TGFBR2 and TGFBR3) to regulate cell function [14]. Its role in angiogenesis and cancer development is highly complex, involving aspects of tumour suppression at the initial stages of oncogenesis and, as tumours evolve, pro-oncogenic activities [15]. The growth-inhibitory effects of TGFB are attributed to its ability to arrest cells in the G1 phase of the cell cycle [16]. It promotes tumour stroma formation [17] and inhibits T- and B-cell function, as well as the secretion of immunostimulatory cytokines, leading to immune response deficiency and tumour growth. Previous studies elucidated the loss of TGFB1 signalling in endometrial cancer [18,19]. Nevertheless, the exact mechanism underlying the transition of TGFB1 from tumour suppressor to pro-metastatic factor in endometrial carcinogenesis remains to be defined.

The present study evaluated the mRNA and protein expression patterns of the two most important regulators of angiogenesis VEGF and TGFB1 in endometrial cancer and adjacent normal tissue samples and correlated their expression profile with clinical parameters. We aimed to investigate whether VEGF and TGFB1 serve as markers of the malignant transformation of the endometrium and whether VEGF or TGFB1 expression can constitute a useful prognostic marker of survival in patients with endometrial carcinoma.

Materials and methods

Patients and controls

Samples were surgically obtained from 23 female patients who underwent therapeutic hysterectomy at the Department of Obstetrics and Gynaecology, PAGNH University Hospital of Heraklion, Crete, between 2002 and 2006. Tissue samples were obtained at the time of the surgery. Half of each sample was snap frozen and stored at −80 °C until RNA extraction, while the other half was fixed in 10% formaldehyde solution for histopathological examination. Tissue samples comprised > 80% tumour cells, minimum or no infiltrate and ∼20% stroma cells. The mean age (± SEM) of the patients at the time of surgery was 67·9 (± 1·9), with a range of 52–88 years. Staging was reviewed based on the International Federation of Obstetrics and Gynecology (FIGO) staging system. Table 1 summarizes the clinical and histological characteristics of the patients. None of the patients had undergone any radiotherapeutic or chemotherapeutic treatment prior to hysterectomy. The study was approved by the Ethics Committee of the University of Crete, and all participants gave their written informed consent.

Table 1 Clinical and histological characteristics of 23 patients with endometrial carcinoma

Characteristic	No. of patients
Age (years)	
Mean ± SEM	67·9 ± 1·9
Range	52–88
Menopausal status	
Pre	1
Post	22
Histological cell type	
Endometrioid	15
Nonendometrioid	8
Serous papillary	2
Clear cell	1
Mixed	5
Histological grade	
G1	6
G2	12
G1–G2	2
G3	3
FIGO stage	
I	13
II	7
III	3
Myometrial invasion	
< 50%	12
> 50%	11
Cervical involvement	
Positive	9
Negative	14
Extra-uterine disease	
Positive	3
Negative	20

RNA and protein extraction

Total RNA and protein were extracted from each tissue sample using TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) with the aid of a power homogenizer according to the

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manufacturer's instructions. RNA concentration and purity were evaluated by a spectrophotometer. Protein concentration was determined using the Bradford assay. Aliquots of RNA and protein were stored at −80 and −20 °C, respectively, until use.

Reverse transcription and real-time PCR

Reverse transcription for the preparation of first-strand cDNA from 2 μg of total RNA with random hexamers was performed using the 'reverse transcription kit' according to the manufacturer's protocol (Promega, Madison, WI, USA).

Real-time PCR was carried out using the MX3000P Real-Time PCR system (Stratagene, La Jolla, CA, USA) with SYBR® Green I Master Mix (Stratagene) according to the manufacturer's instructions. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control to normalize VEGF and TGFB1 mRNA expression levels. The sequences of the primer pairs used are as follows: VEGF, forward: 5′-ATGACGAGGGCCTGGAGTGTG-3′ and reverse: 5′-CCTATGTGCTGGCCTTGGTGAG-3′ (91-bp PCR product); TGFB1, forward: 5′- AAGGACCTCGGCTGGAAGTG-3′ and reverse: 5′-CCCGGGTTATGCTGGTTGTA-3′ (137-bp PCR product); and GAPDH, forward: 5′-GGAAGGTGAAGGTCGGAGTCA-3′ and reverse: 5′-GTCATTGATGGCAACAATATCCACT-3′ (101-bp PCR product). Annealing was set at 60 °C. To avoid the amplification of the contaminating genomic DNA, the primer pairs were designed to span at least one intron. A representative pool of the samples was diluted in a series of seven 2× dilutions and used to construct a standard curve for the quantification process. Melting curves for each amplicon were generated to evaluate the specificity of the products. Data were collected and analysed using MX3000P Real-Time PCR software version 2.00, Build 215, Schema 60 (Stratagene). Reproducibility of the real-time PCR results (mean value of data acquired from three independent RT-PCR experiments) for the same samples was 99%. Peptide growth factor transcription levels (normalized to GAPDH) were calculated using the formula: normalized sample or control = $(1 + E_{GF})^{-\Delta Ct}$GF/$(1 + E_{GAPDH})^{-\Delta Ct}$GAPDH. A twofold increased or decreased expression was considered significant over-expression or down-regulation, respectively.

Western blot analysis

Protein extracts (30 μg) were electrophoresed through a 10% polyacrylamide gel, transferred onto nitrocellulose membranes and incubated with a mouse anti-TGFB1 antibody (Cat. Number: MAB240; R&D Systems, Minneapolis, MN, USA), an anti-VEGF antibody (Cat. Number: MAB293; R&D Systems) and an anti-beta-actin antibody (Cat. Number: MAB3128; Chemicon Int., Temecula, CA, USA). Antibody binding was revealed by a peroxidase-labelled secondary antibody. Bands were visualized using the ECL reagent (Chemicon Int.), as described in the manufacturer's protocol. The film was photographed with the Alpha Imager © system (Alpha Innotec Corp., Santa Clara, CA, USA). Western blot analysis was performed twice for each sample (normal or tumour). VEGF and TGFB1 protein levels in normal and pathological endometrial tissue samples were quantified using ALPHA INNOTEC image analysis software. The beta-actin protein levels of each sample were used as an internal control. VEGF and TGFB1 protein expression status (over-expression, normal expression or under-expression) was determined as the ratio of expression of each tumour specimen vs. the expression of its adjacent normal specimen. A twofold increased or decreased expression was considered over-expression or down-regulation, respectively.

Statistical analysis

The one-sample Kolmogorov–Smirnov test was employed to assess the normal distribution of the mRNA expression values of the genes studied. The mRNA and protein expression of VEGF and TGFB1 in the normal and pathological sample groups, as well as in groups of different clinicopathological features were compared using nonparametric procedures. Spearman's rank correlation was employed to examine the growth factor mRNA and protein correlation pairwise; therefore, the correlations observed are distinct for each individual patient. The chi-square (χ^2) test was used to assess differences in VEGF and TGFB1 mRNA and protein expression status (over-expression or down-regulation) within or between different sample groups. Survival analysis was performed according to the Kaplan–Meier method and was analysed using the log-rank test. Survival was assessed as corrected survival from the date of surgery to the date of death, 5 years (60 months) survival time or overall survival. Multivariate analysis was performed using the Cox proportional hazards model with hazard ratios (HRs) and 95% confidence intervals (CIs) to evaluate independent prognostic factors. Probability values or differences < 0·05 were considered statistically significant. Statistical calculations were performed using SPSS software version 11 (SPSS Inc., Chicago, IL, USA).

Results

Using a quantitative real-time RT-PCR method and western blot analysis, the present study evaluated the mRNA and protein expression profiles of VEGF and TGFB1, respectively, in 23 endometrial cancer and adjacent normal tissue samples. The ratio of the transcript levels of each growth factor (VEGF or TGFB1) to the GAPDH mRNA levels (internal control) of the same specimen served to normalize for cDNA input.

VEGF and TGFB1 transcript levels

Vascular endothelial growth factor mRNA expression levels were significantly elevated in endometrial tumours [mean ± SEM = (4·8 ± 3·4), median = 0·6] compared with adjacent normal tissues (0·5 ± 0·2, median = 0·03) (P = 0·020, Mann–Whitney test) (Fig. 1a). Substantially, elevated VEGF mRNA levels were observed in tissues derived from endometrial tumours invading the cervix (P = 0·015, Mann–Whitney test) (Fig. 1b). The mean TGFB1 mRNA levels showed a tendency to be higher in the tumour compared with the nontumour tissues; however, the difference was not statistically significant (Fig. 1c).

The mean VEGF (4·8 ± 0·2) and TGFB1 (1·7 ± 0·2) transcript levels were found to be higher and lower, respectively, in the endometrial cancer tissue of FIGO stage II compared with stage I tumours [(0·9 ± 0·3) and (3·9 ± 3·1)], although the difference was not statistically significant.

mRNA co-expression analysis pairwise

The same co-expression pattern was displayed in normal and malignant endometrium, because in both specimen groups VEGF mRNA levels were found to be positively correlated with TGFB1 transcript levels (P < 0·001, P = 0·003 Spearman's correlation) Fig. 1D,E, respectively. Specifically, Fig. 1D shows the

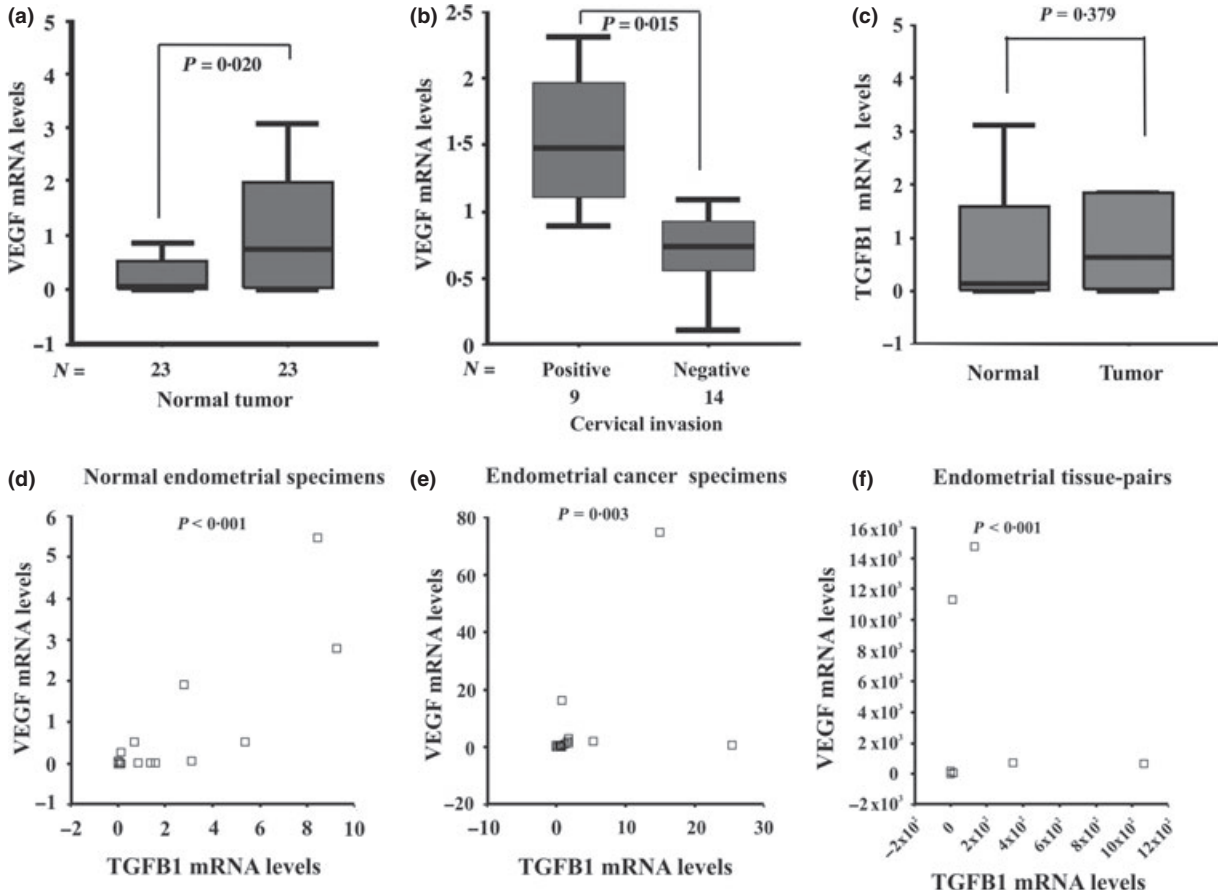

Figure 1 (a) VEGF mRNA levels (mean ± SEM = (4·8 ± 3·4), median = 0·6) were significantly elevated in endometrial tumours compared with adjacent normal tissues. (0·5 ± 0·2, median = 0·03) (P = 0·020, Mann–Whitney test). (b) Substantially elevated VEGF mRNA levels were observed in tissues derived from endometrial tumours invading the cervix (P = 0·015, Mann–Whitney test). (c) The mean TGFB1 mRNA levels showed a tendency to be higher in the tumour compared with the nontumour tissues; however, the difference was not statistically significant. (d) VEGF mRNA levels were positively correlated with TGFB1 transcript levels in the normal endometrial tissues (P < 0·001, P = 0·003 Spearman's correlation). (e) A significant positive correlation between VEGF and TGFB1 mRNA levels was observed in the endometrial cancer specimens (P = 0·003, P = 0·003 Spearman's correlation). (f) VEGF mRNA levels were positively correlated with TGFB1 transcript levels in endometrial tissue pairs (P < 0·001, P = 0·003 Spearman's correlation). Boxplots represent the median expression values, quartiles and extreme values in each category.

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positive pairwise correlation of VEGF mRNA expression levels with the TGFB1 mRNA expression levels in the normal endometrial tissues ($P < 0{\cdot}001$). Figure 1E shows the positive pair-wise correlation of VEGF transcript levels with the TGFB1 transcript levels in the endometrial cancer specimens ($P = 0{\cdot}003$).

Endometrial tissue-pair analysis

In the present study, adjacent normal endometrial tissue samples were available for all pathological specimens. It was therefore considered more appropriate to compare the mRNA levels of each pathological sample with those of its adjacent normal specimen. Consequently, the ratio of the transcript levels of each gene to GAPDH in the tumour sample was calculated to that of the adjacent normal tissue, that is, [(VEGF/GAPDH)tumour/(VEGF/GAPDH) normal]. This ratio was used to provide a distinct molecular portrait of each tumour, subsequently compared with clinicopathological features. A twofold increased or decreased expression ratio was considered to reflect over-expression or down-regulation of the gene of interest. Table 2 shows the VEGF and TGFB1 mRNA and protein expression ratios (T/N) in the 23 endometrial tissue pairs included in the study, as well as their correlation with clinicopathological data corresponding to each case, including patients' age, menopausal status, tumour histological cell type and grade, FIGO stage, myometrial and cervical invasion and the presence or absence of extra-uterine disease.

Notably, VEGF mRNA up-regulation was found to occur significantly more often (60% of the endometrial tissue pairs) than no significant change in expression (15%) or down-regulation (25%) in endometrial cancer ($P < 10^{-7}$, OR = 4·50, CI = 2·36–8·64; and $P < 10^{-7}$, OR = 8·50, CI = 4·11–17·82). TGFB1 mRNA down-regulation (53%) was the most frequent event in endometrial cancer compared with up-regulation (37%) ($P = 0{\cdot}029$, OR = 0·52, CI = 0·28–0·95). Table 3a shows the percentage of the endometrial tissue pairs examined that exhibited VEGF and TGFB1 mRNA over-expression, down-regulation or no significant change in mRNA expression.

A significant positive correlation was shown between VEGF and TGFB1 mRNA by endometrial tissue-pair analysis ($P < 10^{-4}$, Spearman's correlation) (Fig. 1F).

The expression ratio of the tissue pairs for each growth factor included in the study was correlated with the following clinicopathological parameters: FIGO stage, histological grade, histological cell type, myometrial invasion, cervical involvement and presence of extra-uterine disease. The TGFB1 mRNA ratio was marginally higher in FIGO stage I compared with stage II endometrial tissue pairs ($P = 0{\cdot}053$, Mann–Whitney test). TGFB1 mRNA expression status was inversely correlated with cervical invasion ($P = 0{\cdot}006$, Spearman's correlation). In cases with cervical invasion, TGFB1 mRNA was down-regulated (100% of the cases), whereas in the absence of cervical invasion, TGFB1 mRNA was mainly over-expressed (57% of the cases). Furthermore, TGFB1 mRNA expression status exhibited an inverse correlation with tumour grade ($P = 0{\cdot}025$ Spearman correlation's). Well-differentiated tumours (grade I) exhibited TGFB1 mRNA over-expression (72% of cases), whereas low- or moderately differentiated tumours (grade II and III) exhibited TGFB1 mRNA down-regulation (64% of the cases).

VEGF and TGFB1 protein levels

Vascular endothelial growth factor and TGFB1 protein levels in the tumour and adjacent normal endometrial tissues were determined by western blot analysis (Fig. 2).

Protein tissue-pair analysis. Endometrial tissue-pair analysis was performed for the protein levels of each tumour sample. Specifically, the ratio of VEGF and TGFB1 protein levels to beta-actin protein levels in each tumour sample to that of the adjacent normal tissue was calculated as follows: (VEGF/beta-actin) tumour/(VEGF/beta-actin) adjacent normal. This ratio was used to provide a distinct protein portrait of each tumour; subsequently compared with clinicopathological features.

Vascular endothelial growth factor protein over-expression (35%) was significantly more frequent in endometrial cancer tissue pairs than down-regulation (12% of the cases) ($P < 10^{-4}$, OR = 3·95, CI = 1·81–8·77, χ^2 test). However, normal (35%) or no protein expression (18%) was also observed. Tissue pairs periodically exhibited TGFB1 protein over-expression (26%), down-regulation (16%), normal (32%) or no protein expression (26%). Table 3b shows the percentage of the endometrial tissue pairs examined that exhibited VEGF and TGFB1 protein over-expression, down-regulation, no significant change or absence of mRNA expression.

Vascular endothelial growth factor protein levels did not correlate with mRNA levels. VEGF protein levels were higher in nonendometrioid-type adenocarcinoma tissue pairs ($P = 0{\cdot}047$, Mann–Whitney test) (Fig. 3a). TGFB1 protein was lower in tumours with extended (> 50%) myometrial invasion ($P = 0{\cdot}016$) (Fig. 3b). Concerning tumour/normal ratios in endometrial tissue pairs, TGFB1 protein was negatively correlated with TGFB1 tumour/normal transcript level ratios ($P = 0{\cdot}002$, CC: −0·778, Spearman's correlation).

Prognostic value of VEGF and TGFB1 mRNA and protein expression

Vascular endothelial growth factor and TGFB1 mRNA or protein expression did not correlate with tumour stage, depth of myometrial invasion or grade of differentiation, except for TGFB1 mRNA, which exhibited an inverse correlation with the differentiation grade. Grade 2 and 3

Table 2 VEGF and TGFB1 mRNA and protein expression ratios (T/N) in the 23 endometrial tissue pairs included in the study, as well as their correlation with clinicopathological data corresponding to each case, including patients' age, menopausal status, tumour histological cell type and grade, FIGO stage, myometrial and cervical invasion and the presence or absence of extra-uterine disease

Patient no.	VEGF mRNA (T/N)	TGFB1 mRNA (T/N)	VEGF Protein (T/N)	TGFB1 protein (T/N)	Age	Menopausal status (pre/post)	Histological cell type	Histol. grade	FIGO Stage	Myometrial invasion (%)	Cervical invasion	Extra-uterine disease
1	NA	NA	0	0	58	Post	Endometrioid	G2	II	< 50	Yes	No
2	1·32	NA	0	0	59	Post	Nonendometrioid	G1	II	< 50	Yes	No
3	0·2604	NA	4·7	1·73	52	Post	Endometrioid	G1	I	< 50	No	No
4	NA	0·5445	0·06	0	60	Post	Endometrioid	G1	I	< 50	No	No
5	11290·07	12·9333	0	0	62	Post	Endometrioid	G1	I	< 50	No	No
6	666·2743	1066·413	0	0	73	Post	Endometrioid	G1	I	> 50	No	Yes
7	1·5082	11·3046	1·95	0	72	Post	Nonendometrioid	G1	II	> 50	No	No
8	0·0429	0·0276	0	0	75	Post	Endometrioid	G2	I		No	No
9	0·1138	0·0622	0	0·54	NA	Post	Endometrioid	G2	I	< 50	No	No
10	11·457	0·0181	1·66	5·37	55	Pre	Endometrioid	G2	I	< 50	No	No
11	3·7715	2·2404	0	0	60	Post	Endometrioid	G2	I	< 50	No	No
12	1·0463	1·0135	0	0	74	Post	Endometrioid	G2	I	> 50	No	No
13	12·4027	0·0863	0	0	67	Post	Nonendometrioid	G3	II	> 50	Yes	No
14	221·816	0·2552	0	0	70	Post	Endometrioid	G2	II	> 50	Yes	No
15	1425147	NA	5·31	0	88	Post	Nonendometrioid	G2	II	> 50	Yes	No
16	0·0032	0·0019	10·33	25	63	Post	Nonendometrioid	G2	III	< 50	No	Yes
17	348·4826	NA	0	0	82	Post	Nonendometrioid	G2	III	> 50	Yes	Yes
18	14753·28	133·8098	0	0	70	Post	Endometrioid	G1	I	> 50	No	No
19	0·0861	0·0056	1·58	1	61	Post	Endometrioid	G3	I	< 50	No	No
20	710·7832	342·6083	0	0	70	Post	Endometrioid	G3	I	< 50	No	No
21	0·5811	0·4669	0·93	0	68	Post	Endometrioid	G3	II	> 50	Yes	No
22	54·3111	15·8561	4·23	0	78	Post	Nonendometrioid	G2-G3	I	< 50	No	No
23	4·0452	0·4674	4·56	0	78	Post	Nonendometrioid	G2-G3	II	> 50	Yes	No

tumours were correlated with worse patient outcome compared with grade 1 tumours (P = 0·023). Kaplan–Meier analysis revealed that endometrial cancer patients without cervical invasion had a significantly favourable prognosis (P = 0·019) (Fig. 4). FIGO stage I patients had a significantly favourable prognosis compared with stage II patients (P = 0·032).

The expression status (expression or not, over-expression or down-regulation) of VEGF and TGFB1 mRNA or protein was not a significant prognostic indicator of 5-year disease-free or overall survival (Kaplan–Meier analysis). However, following the stratification for tumour histology, endometrioid adenocarcinoma patients with TGFB1 protein expression had a significantly favourable prognosis compared with those that did not express the protein (P = 0·045, Kaplan–Meier analysis), and this is of clinical importance.

Results of the Cox multivariate analysis showed that only cervical invasion (P = 0·023) was a significant prognostic indicator of 5-year disease-free and overall survival in endometrial cancer samples. Stepwise linear regression analysis for the group of endometrioid tumours showed cervical invasion (P = 0·037) and TGFB1 protein expression status (expression or no expression) (P = 0·001) to be significant prognostic indicators of survival.

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Table 3 (a) Percentage of the endometrial tissue pairs examined that exhibited VEGF and TGFB1 mRNA over-expression, down-regulation or no significant change in mRNA expression. VEGF mRNA up-regulation was a significantly more frequent event than no significant change in expression ($P < 10^{-7}$, χ^2 test) (df = 2). TGFB1 mRNA down-regulation (53%) was the most frequent event in endometrial cancer compared with up-regulation (37%) ($P = 0{\cdot}029$, χ^2 test) (df = 2). (b) Percentage of the endometrial tissue pairs examined that exhibited VEGF and TGFB1 protein over-expression, down-regulation, no significant change or absence of mRNA expression. VEGF protein over-expression (35%) was significantly more frequent in endometrial cancer tissue pairs than down-regulation ($P < 10^{-4}$, χ^2 test) (df = 2)

	VEGF			TGFB1		
Growth factor	↑[†]	=	↓	↑	=	↓
(a)						
% of endometrial tissue pairs	60	15	25	37	10	53
P-value*		$< 10^{-7}$			0·029	

	VEGF				TGFB1			
Growth factor	↑[‡]	=	↓	▬	↑	=	↓	▬
(b)								
% of endometrial tissue pairs	35	35	12	18	26	32	16	26
P-value**		$< 10^{-4}$						

[†]↑: over-expression.
[‡]↑: over-expression.
↓: reduced expression.
=: no significant change in expression.
*$P < 0{\cdot}050$ is considered statistically significant; χ^2 test (df = 2).
▬: no expression.
**χ^2 test (df = 2).

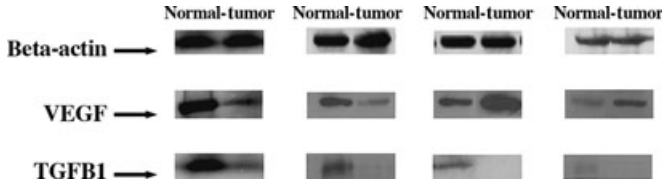

Figure 2 Representative examples of western blot analysis of VEGF, TGFB1 and beta-actin protein expression in endometrial tissue pairs.

Discussion

In the present study, the mRNA and protein expression and co-expression profiles of VEGF and TGFB1 were evaluated in malignant and adjacent normal endometrial tissues. To the best of our knowledge, this is the first time that expression and co-expression patterns have been evaluated in paired endometrial pathological and normal tissue samples from the same patients, reflecting the molecular portrait of each tumour (endometrial tissue-pair analysis).

VEGF transcript levels

Our results show VEGF mRNA expression in all the malignant and adjacent normal specimens, confirming previously published reports [7,12,20,21]. Moreover, a significant increase in VEGF mRNA levels in tumour compared with normal endometrial tissue samples was observed providing evidence that VEGF transcriptional up-regulation is one of the main characteristics of endometrial carcinogenesis. This observation is in accordance with O'Toole *et al.* (2005), who detected higher VEGF-A mRNA levels in malignant tissues [22].

Of note is that VEGF transcript levels were found to be higher in endometrial cancer tissues of FIGO stage II vs. stage I tumours, although this difference did not reach statistical significance. If verified in a larger set of samples, our findings would suggest that VEGF mRNA levels correlate with the malignant transformation of the endometrium. Stage II tumours comprise extended malignancy beyond the endometrium and specifically to the cervix. The elevated VEGF mRNA in tissues derived from endometrial tumours invading the cervix suggests that VEGF transcript levels could serve as a marker of disease spreading to the cervix. Consequently, our results indicate the induction of VEGF mRNA in endometrial cancer and therefore support the involvement of VEGF in endometrial tumorigenesis.

VEGF mRNA by endometrial tissue-pair analysis

The VEGF mRNA ratios (T/N) in the 23 endometrial tissue pairs included in the study are demonstrated in Table 2. Of note, there are cases such as that of patient 15 where the fold induction of VEGF mRNA is a number in the tens of thousands range. Specifically, this is true for patients 5, 15 and 18. Therefore, we examined the mRNA levels obtained by the malignant as well as the normal endometrial specimens of these patients separately. We observed minimal VEGF mRNA expression in the normal specimens; however, in all three cases, the VEGF mRNA levels of the malignant tissues were substantially higher (at least 0·3-fold or more) than the mean ± SEM (0·5 ± 0·2) values of the normal endometrial specimens. Consequently, even though the fold induction of VEGF mRNA obtained in the paired samples is extremely high (and this does not have a biological significance), it truly represents VEGF over-expression and not a modest VEGF induction.

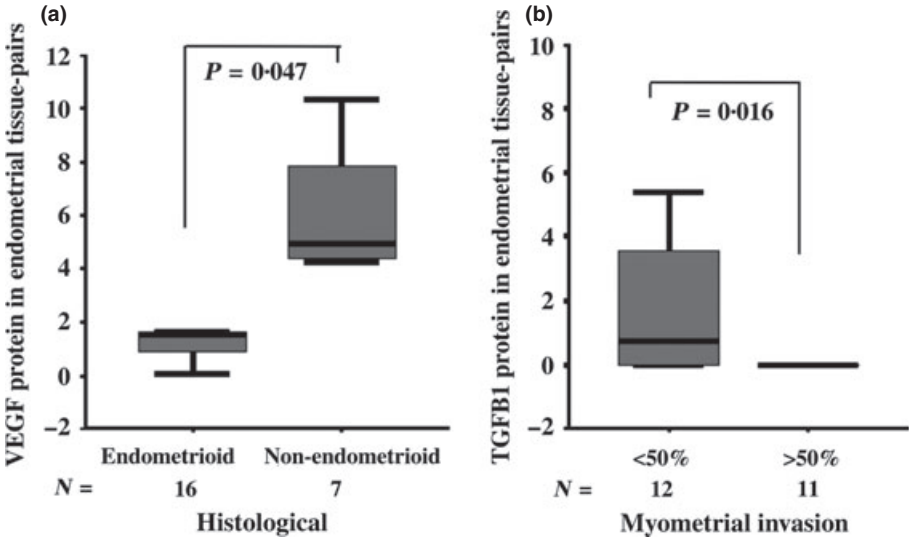

Figure 3 (a) VEGF protein levels were higher in nonendometrioid-type adenocarcinoma tissue pairs (P = 0·047, Mann–Whitney test). (b) TGFB1 protein was lower in tumours with extended (> 50%) myometrial invasion (P = 0·016 Mann–Whitney test).

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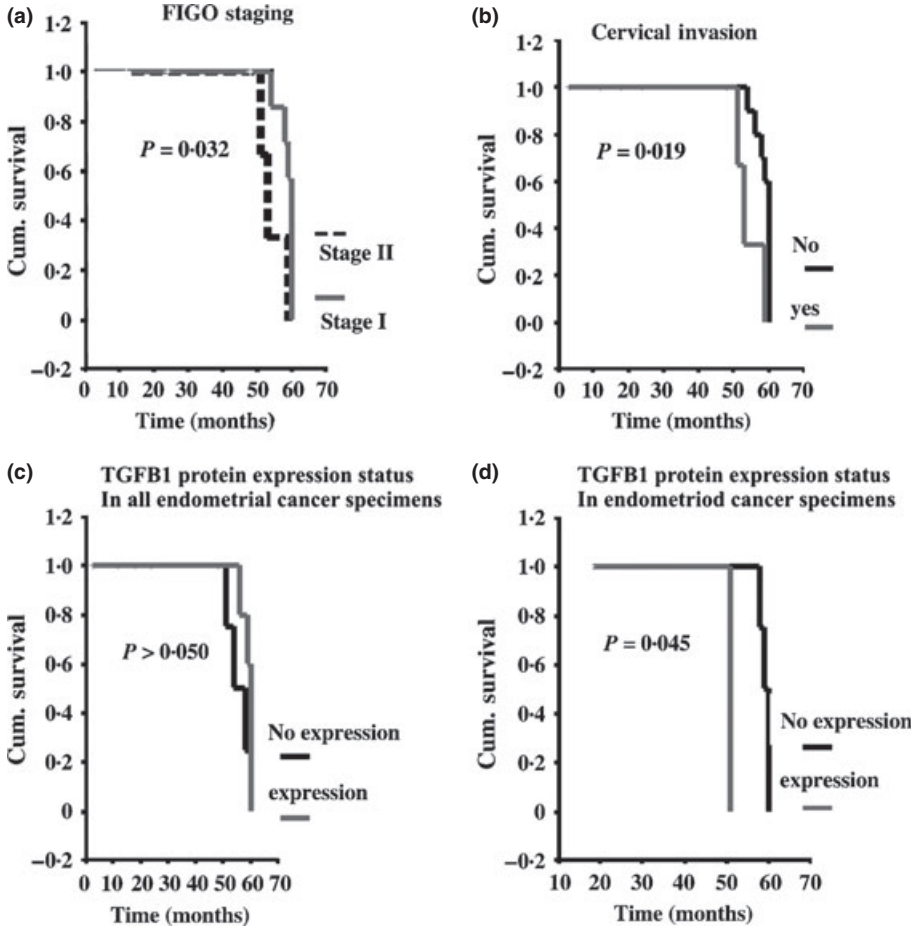

Figure 4 Survival plots of Kaplan–Meier analysis from all patients with endometrial cancer included in the study demonstrating the effect of: (a) FIGO stage I, II of the endometrial malignancies examined (P = 0·032), (b) cervical invasion of the endometrial malignancies examined (P = 0·019), (c) TGFB1 protein expression status (expression or no expression) in the endometrial tumour tissues examined (P = 0·054) at the end-point, defined as the time period in months of disease-free survival after five years of patient follow-up. (d) Survival plots of Kaplan–Meier analysis from N = 10 patients with endometrioid endometrial cancer included in the study demonstrating the effect of TGFB1 protein expression status (expression or no expression) in the endometrial tumour tissues examined at the end-point, defined as the time period in months of disease-free survival after five years of patient follow-up (P = 0·045).

VEGF protein by endometrial tissue-pair analysis

In the present study, VEGF protein assessed by western blot assay was found to be expressed in 82% of endometrial cancer cases. This finding is similar to previous reports [21,23,24], but contrary to others [8,13] that have reported VEGF expression in 66% and 39% of endometrial cancer specimens, respectively. These differences could be attributed to the different clinical characteristics of the specimens (histological type, FIGO stage and grade) included in each study. Interestingly, within the samples that expressed VEGF protein, we observed an equal incidence of normal protein expression or over-expression (35%) and a small percentage of down-regulation. Based on the fact that VEGF mRNA was mainly down-regulated but mRNA levels did not correlate with VEGF protein levels, we can only speculate that post-transcriptional mechanisms control protein expression in these settings.

A mounting body of evidence suggests that high VEGF expression is associated with aggressive features in uterine carcinoma. Specifically, VEGF expression has been shown to be stronger in endometrial cancer types that exhibit aggressive behaviour, such as papillary serous adenocarcinoma and carcinosarcoma [3,12,24]. Nonendometrioid histological subtypes in general account for 10% of endometrial cancers and carry an increased risk of recurrence and distant metastasis [25,26]. Notably, in our study, nonendometrioid tissue pairs exhibited significantly higher VEGF protein levels than tumours of the endometrioid type. It is therefore fair to presume that high VEGF protein levels could serve as a marker of the malignant potential of the neoplasm or even to speculate that the elevated VEGF protein levels observed contribute to the aggressiveness of these tumours. However, this hypothesis requires further investigation.

Immunohistochemical studies have shown a positive correlation between VEGF protein expression and grade [27], myometrial invasion and shorter disease-free survival [8,24], while others have failed to provide significant correlations with clinicopathological features or prognosis [12,23]. In our analysis, both VEGF transcript and protein levels failed to provide prognostic information about patient outcome or disease-free and overall survival. VEGF protein levels were higher in disease

involving cervical invasion, but – despite the fact that cervical invasion was correlated with significantly poorer patient prognosis according to the Kaplan–Meier analysis – our results provide evidence suggesting that VEGF does not have a prognostic value, which is in accordance with previous reports [12,13].

TGFB1 transcript levels

TGFB constitutes a potent endogenous inhibitor of epithelial cell growth and has been shown to induce growth arrest in normal proliferative endometrial cells [18]. On the other hand, endometrial cancer epithelial cells have been shown to be refractory to the tumour-suppressive action of TGFB. In fact, release from the tumour-suppressive actions of TGFB has been suggested to be an early event in endometrial carcinogenesis [18,19].

Limited information is available regarding TGFB1 mRNA and protein levels in tissue samples derived from normal compared with malignant endometrium. As in the case of Strick *et al.* [28], our findings demonstrated similar normalized TGFB mRNA levels in tumours vs. control specimens. On the other hand, using northern blotting, Perlino *et al.* [29] found a substantial decrease in TGFB1 mRNA expression in the endometrial carcinoma cases compared with proliferative endometrium. The difference was greater compared with normal atrophic tissues. What is important here is to point out that the epithelial and stromal compartments are not equivalent in normal atrophic endometrium and in endometrial carcinomas. Of note, in all above-mentioned studies (including ours), the normal and tumour samples were processed as an entity without separating epithelial from stromal cells. This fact could have an impact on the results obtained; therefore, careful interpretation and comparison of the data between studies is required, especially taking into consideration that the exact percentage of epithelial/stromal cells included in the tissue specimens of each study cannot be estimated.

Our observation of lower (although not significant) mean TGFB mRNA levels in FIGO stage II endometrial tumours compared with stage I carcinomas, if verified in a larger set of specimens, could lead to the speculation that TGFB mRNA is down-regulated as tumours evolve, thus contributing to the loss of growth inhibition and to disease progression. Significant down-regulation of TGFB1 mRNA was observed in all the endometrial malignancies with cervical invasion examined, while endometrial carcinomas without cervical involvement exhibited mainly over-expression, indicating that perhaps TGFB1 transcriptional down-regulation could be used as a molecular indicator of disease spreading beyond the endometrium. Low- or moderately differentiated endometrial tumours (grades III and II) are associated with worse patient outcome compared with well-differentiated carcinomas. It is therefore not surprising that grade III and II endometrial tissue pairs exhibited TGFB1 mRNA down-regulation, because the loss of TGFB1 growth inhibition could explain the poorer prognosis observed. Indeed, TGFB1 mRNA over-expression observed in well-differentiated tumours possibly acts in a protective manner, sustaining growth inhibition and leading to improved patient outcome. The findings of this study suggest that TGFB1 transcriptional down-regulation correlates with the malignant transformation of the endometrium.

TGFB1 mRNA and protein by endometrial tissue-pair analysis

Our endometrial tissue-pair analysis demonstrates that TGFB1 mRNA down-regulation is the most frequent transcriptional event observed. TGFB1 transcriptional down-regulation has also been detected in cervical cancer by our investigators [30], as well as in other malignancies, such as prostate and breast cancer [4,31]. Of note is that our results periodically exhibit over-expression, or down-regulation or even absence of expression of TGFB1 protein in endometrial tissue pairs, suggesting that the role and signalling action of TGFB is context dependent and varies in endometrial tissues, possibly depending on the tumour microenvironment or the clinicopathological characteristics of the specimens. The latter is reinforced by our finding that TGFB1 protein inversely correlates with TGFB1 transcript levels in endometrial tissue pairs, demonstrating a post-transcriptional regulation of TGFB1 in these systems. However, the differences in the epithelial and stromal compartments of the normal atrophic endometrium and the endometrial carcinomas may have influenced our results. Perlino *et al.* [29] demonstrated higher TGFB1 protein in the epithelial compared with stromal cells in endometrial cancer, whereas the opposite was observed in atrophic endothelium. To elucidate this issue, future studies should be conducted to evaluate TGFB1 expression separately in the different cell types in both normal and pathological endometrial tissues. An understanding of how and when endometrial cancer cells escape the growth-inhibitory effects of TGFB1, thereby augmenting their oncogenic potential due to the invasive and metastatic properties of TGFB, would greatly contribute to the clarification of the mechanisms of endometrial carcinogenesis.

TGFB1 protein is known to participate in tumour promotion. Muinelo-Romay *et al.* [32] have recently demonstrated that TGFB1 has a principal role in the initial steps of endometrial carcinoma invasion through the promotion of epithelial-to-mesenchymal transition that leads to the individualization of cells and the acquisition of an invasive phenotype. The same researchers have proposed that once TGFB1 has initiated tumour infiltration, its contribution must be counteracted for further persistent invasion. Our finding that TGFB1 protein was lower in tumours with extended myometrial invasion is in accordance with the above

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observations because TGFB1 is proposed to be an inducer of endometrial cancer dissemination and metastasis and a limiting step in deep tumour invasion.

Correlation of VEGF and TGFB1 transcript levels

Our finding that TGFB1 transcript levels are positively correlated with VEGF mRNA levels in both pathological and disease-free specimen groups, as well as in endometrial tissue pairs, which supports previous findings regarding the angiogenic role of TGFBs. We can only assume that TGFB1 regulates VEGF production by endometrial cells, as previously demonstrated in breast cancer cell models by Donovan and colleagues [33]. In addition, as the angiogenic process physiologically takes place in the normal tissue, we can speculate that VEGF may act as an inducer of TGFB1 along with other growth factors and cytokines exclusively in the normal settings.

Prognostic significance of VEGF and TGFB1 mRNA or protein levels

Similar to VEGF, neither TGFB1 transcript nor protein levels were found to be significant prognostic indicators of patient disease-free or overall survival. However, what is clinically important is our finding that TGFB1 protein expression as opposed to absence of expression was significantly correlated with a favourable prognosis in endometrioid cancer patients. This is expected taking into consideration the growth-inhibitory effect of TGFB1. Most recently, Mhawech-Fauceglia *et al.* [34] demonstrated that the TGFB1 and Smad4 mRNA levels were associated with disease-free survival in human endometrial cancer. Moreover, increased TGFB1 mRNA levels were shown to be an independent factor of poor prognosis in the same study, probably as a consequence of tumour cells escaping the growth-inhibitory response of TGFB1. Our findings, in addition to the above, provide evidence of the prognostic significance of the multiple components of the TGFB–Smad signalling pathway in endometrial cancer.

Conclusion

In conclusion, our results provide evidence of VEGF and TGFB1 involvement in endometrial carcinogenesis through transcription activation and down-regulation, respectively, and suggest their potential use as molecular indicators of disease progression. Moreover, our results reinforce previous findings regarding the dual role of TGFB in cancer development and progression and delineate its prognostic value in endometrioid adenocarcinomas. Finally, our findings provide new insight into the molecular mechanisms mediating the malignant transformation of the endometrium as, according to the results, post-transcriptional mechanisms appear to control VEGF and TGFB1 expression in endometrial cancer.

Conflict of interest

None declared.

Contributions

Giannoula Soufla designed and performed the research/study and collected and analysed the data. Stavros Sifakis collected the data, received funding and critically reviewed the manuscript. Filippos Porichis performed the research/study and collected and analysed the data. Demetrios A. Spandidos received funding, critically reviewed the manuscript and supervised the research work. All authors involved in the writing of the manuscript.

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