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VEGF, FGF2, TGFB1 and TGFBR1 mRNA expression levels correlate with the malignant transformation of the uterine cervix

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Abstract

Angiogenesis is a complex procedure induced by the secretion of numerous growth factors from endothelial cells. Vascular endothelial growth factor (VEGF), basic fibroblastic growth factor (FGF2), transforming growth factor-β1, 2, 3 (TGFB1, 2, 3), and transforming growth factor-β receptors (TGFBR1, 2, 3) mRNA expression pattern was evaluated in tissue samples with cervical intraepithelial neoplasia (CIN) and cervical cancer, compared to that of normal cervical tissues, and correlated to the clinical stage of the disease. Transcript levels of the above genes were assessed by RT-PCR analysis in a total of 44 cervical specimens. VEGF, TGFB1, TGFBR1, and FGF2 transcript levels were significantly different in the normal, CIN and cancer specimen groups ($P=0.015$, 0.001, 0.008, and 0.029, respectively). Higher TGFBR1 mRNA levels were observed in parallel with increased severity of the lesion, whereas FGF2 exhibited lower transcript levels. A highly significant increase of VEGF mRNA expression was found upon cervical neoplastic transformation ($P<0.0001$). High-grade squamous intraepithelial lesions exhibited higher VEGF mRNA levels than low-grade lesions ($P=0.039$). TGFBR1 and TGFBR3 receptors demonstrated significant co-expressions with TGFB2 ($P<0.0001$), and TGFB1 ($P=0.005$ and 0.002, respectively) in normal cervical specimens. However, a disruption of co-expression patterns was observed in the groups of CIN and cancer cases, compared to normal tissues. Our findings show that VEGF, FGF2, TGFB1 and TGFBR1 mRNA expression levels correlate with the malignant transformation of the uterine cervix. The involvement of the examined markers in cervical carcinogenesis is furthermore supported by the observed disruption of their mRNA co-expression patterns.

Keywords: Angiogenesis; mRNA Expression; RT-PCR; Cervical cancer; Cervical intraepithelial neoplasia

1. Introduction

Angiogenesis is an important event during the neoplastic process. This complex procedure, also known as neovascularization is essential for tissue development, wound healing and reproduction [1] and

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is an indispensable requirement for tumor progression, invasiveness and metastasis [2]. Tumor cells as well as certain stromal cells such as macrophages, mast cells and fibroblasts are known to secrete a large number of growth factors that activate neovascularization. However, the balance between angiogenic enhancers and endogenous inhibitors adjusts the angiogenic switch [3–5].

Tumor angiogenesis has been described in almost all human cancer types comprising malignancies of the female genital tract [6–13]. Numerous growth factors and cytokines are involved in the angiogenic process that accompanies cervical carcinogenesis. Among these factors VEGF has a predominant role acting as an endothelial cell specific mitogen [3–5], stimulating cell proliferation and increasing vascular permeability. Elevated VEGF expression at advanced stages of the disease has been reported in various cancer types including breast, endometrial, ovarian, bladder, and lung cancer [14–21] and has also been associated with high-grade intraepithelial lesions and cervical cancer [22–27]. VEGF protein level has been found to be correlated with local tumor progression, metastasis and poor prognosis in the uterine cervix, based on immunohistochemical or enzyme immunoassay studies [23–26]. However, other reports provided evidence suggesting that VEGF does not have a prognostic value [27].

Basic Fibroblast Growth factor (FGF2) takes part in various steps of the neovascularization process by promoting angioblast differentiation, cell growth and invasion. Secretion of FGF2 has been described in both tumor and infiltrating inflammatory cells (macrophages). When secreted from tumor cells, FGF2 is responsible for basement membrane dissolution, migration and metastasis of malignant endothelial cells [3–5]. Its role has been described in highly metastatic prostate cells, uterine endometrial cancer, pancreatic and hepatocellular carcinoma [28–31]. Advanced primary cervical cancers have been demonstrated to express high FGF2 mRNA levels [32].

Transforming growth factor beta (TGFB) is a growth modulator involved in angiogenesis, cell proliferation, differentiation, adhesion and migration [33–37]. TGFB has been proved to substantially inhibit cell growth in normal epithelial cells and human keratinocytes in vivo and in vitro [38,39], inducing in parallel its own mRNA expression (autocrine cell growth regulation). TGFB's growth inhibitory effects are attributed to its ability to arrest cells in the G1 phase of the cell cycle [37,39]. It has been shown to inhibit T and B cell function as well as secretion of immunostimulatory cytokines, leading to immune response deficiency and tumor growth. TGFB promotes, via a paracrine action, tumor stroma formation and decreases tumor infiltration providing tumor cells an alternative escape mechanism from the immune response [40]. Five isoforms of TGFB have been identified so far but only three (TGFB1, B2, B3) are expressed in mammalian cells. TGFB1 is the most well characterized isoform to date and along with TGFB3 exhibits stronger inhibitory effects than TGFB2. Reduced expression of TGFB or loss of response to its inhibitory effects has been linked to cell hyperproliferation and tumor progression. Cell function regulation by TGFB arises from his interaction with cell surface receptors (TGFBR1, 2, 3) [33–36]. Immunohistochemical studies report either decreased or increased TGFB1 levels during the neoplastic transformation of cervical epithelium, [41–43], while RT-PCR analyses indicate variations in TGFB1 transcript levels among CIN and normal cervical specimens [44–46]. Elevated levels of TGFB ligands and receptors have been demonstrated in cervical adenocarcinomas [47].

Induction of the angiogenic switch in several cancer types has been mainly associated with VEGF and FGF2 upregulation, while TGFB has been recognized as a growth inhibitor [4,5]. However, the mRNA expression pattern of a panel/group of angiogenic growth factors and receptors has not been investigated in cervical intraepithelial neoplasia and cervical cancer, and their role regarding the neovascularization process in cervical carcinogenesis is poorly understood. In order to investigate the significance of angiogenic markers in malignant transformation of the uterine cervix, we evaluated the combined mRNA expression of VEGF, FGF2, TGFB1, TGFB2, TGFB3 and TGFBR1, TGFBR2, TGFBR3 in tissue samples with cervical intraepithelial neoplasia and cervical cancer. Furthermore, we examined whether the mRNA expression profile of these genes is correlated with the clinical stage of the disease.

2. Materials and methods

2.1. Patients and controls

A total of 44 individuals who underwent surgical treatment due to cervical disease or non-proliferate diseases of the female reproductive system at the Department of Obstetrics and Gynecology of University Hospital of Heraklion, Crete, from 2002 to 2003 were included in this study. Tissue specimens were obtained at the time of the surgical procedure. 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.

Histological cell types of the tumors and intraepithelial lesions were assigned according to the WHO classification: nine patients had cervical carcinoma (eight squamous cell carcinoma, one adenocarcinoma), and 14 patients had CIN. Twenty-one individuals who underwent surgery (transabdominal or transvaginal hysterectomy) for a variety of therapeutic reasons had histologically normal cervix and consisted our control group. Thirteen women were in the reproductive period, 16 were perimenopausal and 15 were in menopause. Staging was reviewed based on International Federation of Obstetrics and Gynaecology (FIGO) staging system; amongst patients with squamous cell carcinoma two were 1A, one IB, four IIB, and two were of stage III. In addition five specimens were CIN I, three CIN II, and six CIN III. Table 1 summarizes the patients' clinical characteristics. Age distribution of the patients was similar in the groups of normal, CIN and cancer cases. The mean age at the time of treatment was 52 years and the median age was 50 years (range, 32–82 years). 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 radical hysterectomy and tissue biopsies for the present study. Patients with CIN had not received any previous treatment, neither surgical nor laser vaporization. Patients of the normal cervical tissue group had not undergone any surgical treatment of the cervix. Ethics Committee of the University of Crete approved the present study, and all participating patients gave written informed consent.

2.2. RNA extraction

Total RNA was extracted from each specimen using the Trizol reagent (Invitrogen Ltd, UK) according to the manufacturer's instructions. Briefly, 1 ml of reagent was added to each tissue specimen (50–100 mg of tissue), which was then homogenized using a power homogenizer and transferred to a 1.5 ml Eppendorf tube. Chloroform (200 μl) was added, and the tube was vortexed and centrifuged at 14,000 rpm for 15 min. RNA was precipitated with an equal volume of isopropanol, washed with 75% ethanol, and resuspended in DEPC treated water. RNA concentration and purity was determined on a UV spectrophotometer (Hitachi Instruments Inc., USA) by absorbance measurements. RNA integrity was examined by denaturing polyacrylamide gel electrophoresis.

2.3. RT-PCR

Reverse transcription reactions for the preparation of first strand cDNA were conducted using the Thermoscript RT-PCR Kit (Invitrogen Ltd., UK) according to the manufacturer's protocol.

Transcribed products were subjected to PCR for the target of interest in a PTC-200 programmable thermal controller (MJ Research Inc., USA). 1 μl of cDNA was amplified in a total volume of 10 μl containing, 1× PCR reaction buffer, 2 mM $MgCl_2$, 0.8 mM dNTPs, and 0.65 U Platinum *Taq* DNA polymerase (Invitrogen Ltd., UK). The sequences of all primer pairs used are listed in Table 2. All primer pairs were designed to span at least one intron in order to avoid amplification of

Table 1
Clinical characteristics of the patients

	Controls	CIN			Cancer	
		I	II	III	Sqamous cell carcinoma	Adenocarcinoma
Patients number	21	5	3	6	IA 2 IB 1 IIB 4 III 1	IB 1

Table 2
Oligonucleotide primer sequences, primer quantities and PCR cycling conditions of each primer pair with Beta 2-microglobulin as an internal control in each reaction

Primer set	Oligonucleotide sequences (5′–3′)	Primer quantities (pmol) [B2M]	Primer annealing temperature (°C)	Amplification cycles	Product size (bp)
VEGF	(F): GCAGAAGGAGGAGGGCA-GAATC	9	62	33	197
	(R): ACACTCCAGGCCCTCGTCATT	[1]			
FGF2	(F): GAAGAGCGACCCTCACAT-CAAG,	12	58	35	236
	(R): CTGCCCAGTTCGTTTCAGTG	[1]			
TGFB1	(F): ACCAACTATTGCTTCAGCTC	12	55	35	198
	(R): TTATGCTGGTTGTACAGG	[1,5]			
TGFB2	(F): CTGTCCCTGCTGCACTTTTGT	12	58	35	227
	(R): TCTTCCGCCGGTTGGTCTGTT	[1,5]			
TGFB3	(F): CCTTTCAGCCCAATGGAGAT	30	57	35	241
	(R): ACACAGCAGTTCTCCTCCAA	[1,5]			
TGFBR1	(F): TCGTCTGCATCTCACTCAT	20	54	35	344
	(R): GATAAATCTCTGCCTCACG	[1,5]			
TGFBR2	(F): GCGGGAGCACCCCTGTGTC	12	62	35	216
	(R): CCCGAGAGCCTGTCCA-GATGC	[1]			
TGFBR3	(F): AATCTGGGCCATGATGCAG	10	57	35	287
	(R): ACTGCTGTTTTCCGAGGCT	[10][a]			
β-actin	(F): AGCCTCGCCTTTGCCGA				175
	(R): CTGGTGCCTGGGGCG				
β2 microglobulin (B2M)	(F): AGCGTACTCCAAAGATT-CAGGTT				297
	(R): TACATGTCTCGATCCCACT-TAACTAT				

[a] Amplification of β-actin RNA was used as an internal control in this case.

contaminating genomic DNA along with cDNA. In the amplification reactions for each specific RNA primer set, RNA primers for β2-microglobulin (B2M) were included as an internal control in all PCR reactions. The corresponding quantities of each specific primer set in the PCR reactions are summarized in Table 2.

PCR products were analysed on 8.5% polyacrylamide gels (acrylamide/bis-acrylamide 29:1 ratio) and silver stained. Gels were scanned on an Agfa SnapScan 1212u (Agfa-Gevaert N.V., Belgium). Integrated density of the bands was used as quantitative parameter and was calculated by digital image analysis (Scion image). The intensity of β2-microglobulin amplification was used as an internal standard. The ratio of the integrated density of each gene tested to that of β2-microglobulin was used to quantify the results. Present analyses conducted on pathological samples may be a manifestation of RNA profiles of endothelial and stromal components.

2.4. Statistical analysis

VEGF, FGF2, TGFB1, 2, 3 and TGFBR1, 2, 3 mRNA expression was compared between the groups of different clinical stages. Three different specimen groupings were applicable: one concerning general condition of the cervix (normal, CIN, cancer), another regarding the grade of cervical intraepithelial lesion [normal, low-grade squamous intraepithelial lesions (LG-SIL, corresponding to CIN I), high grade SIL (HG-SIL, corresponding to CIN II, III) and cancer], and finally one taking into consideration the gradual increased severity of neoplasias (normal tissue, CIN I, II, III, CA). Non-parametric procedures (Kruskal–Wallis and Mann–Whitney test) were applied to the set of data for the evaluation of significant statistical

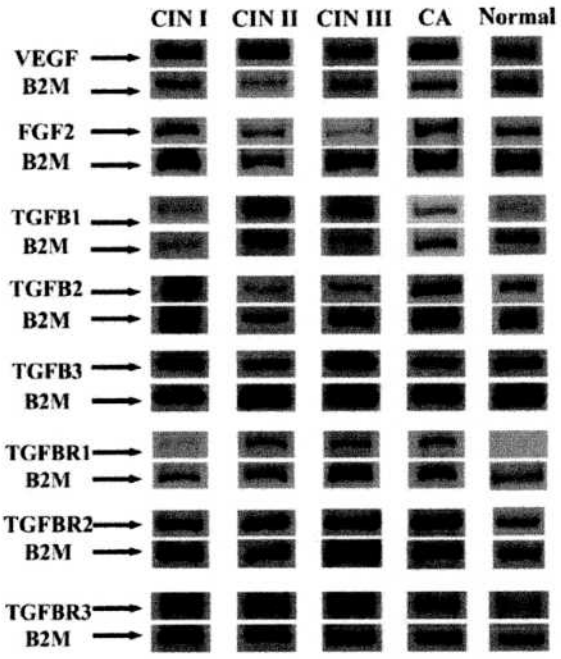

Fig. 1. Representative examples of VEGF, FGF2, TGFB1, 2, 3 and TGFBR1, 2, 3 expression in cervical tissue. Ratio: integrated density of the band of each gene divided by the integrated density of the internal standard band (β2-microglobulin or β-actin).

differences. Age distribution was assessed similarly. Data is presented as the mean and standard error of the mean value (mean ± SEM). The Spearman's rank correlation was used to evaluate the significance of the mRNA of the growth factor co-expression pair wise, in the groups of normal, CIN lesion and cancer tissues. Probability values less than 0.05 were considered statistically significant. Statistical calculations were performed using the SPSS software, version 11.

3. Results

We evaluated the mRNA expression profile of VEGF, FGF2, TGFB1, 2, 3 and TGFBR1, 2, 3 (Fig. 1) in a total of 44 cervical tissue specimens. Twenty-one specimens were normal cervical tissues from hysterectomies performed for non-malignant conditions (48%), 14 were CIN lesions (32%), eight were squamous cell carcinomas and one was an adenocarcinoma (20%). CIN lesions were of grade I (five cases), II (three cases), and III (six cases).

3.1. Transcript levels of growth factors and receptors

Our findings indicate that VEGF, FGF2, TGFB1 and TGFBR1 mRNA expression is significantly related to the condition of the cervix (normal, CIN, cancer) ($P=0.015$, 0.001, 0.008 and 0.029, respectively, Kruskal–Wallis test) (Fig. 2A). Higher TGFBR1 mRNA levels were observed in parallel with increased severity of the lesion, whereas FGF2 exhibited lower transcript levels. Specifically VEGF and TGFBR1 mRNA expression in cancer specimens was significantly elevated compared to normal tissues ($P=0.002$, 0.02, respectively, Mann–Whitney test), whereas FGF2 expression was considerably decreased ($P<0.001$). Significant difference in mRNA expression between CIN and normal specimens was observed only for FGF2 and TGFB1 ($P=0.012$ and 0.008, respectively). No correlation was established between TGFB2, TGFBR2, TGFB3 or TGFBR3 mRNA expression with the grade of the intraepithelial lesion or cervix condition (normal, CIN, cancer), whereas we observed relatively lower expression levels of these genes compared to VEGF.

Transcript levels of VEGF, FGF2, and TGFB1 were significantly different in the groups of normal, LG-SIL, HG-SIL and cancer specimens. ($P=0.001$, 0.002, and 0.020, respectively, Kruskal–Wallis test) (Table 3). TGFBR1 transcript levels exhibited a marginal difference in the above groups ($P=0.06$). Pair wise analysis revealed higher mRNA expression levels of VEGF in HG-SIL than LG-SIL ($P=0.001$ 2-tailed Mann–Whitney test). FGF2 transcript levels in high-grade lesions (HG-SIL) and in cancer specimens were significantly lower than in normal tissues ($P=0.028$ and $P<0.0001$, respectively). The highest mRNA expression levels of TGFB1 and TGFBR1 were observed in high-grade intraepithelial lesions and cancer tissues respectively.

Grouping of cervical specimens in respect of increasing severity of neoplasias (normal tissue, CIN I, II, III, CA) revealed considerable differences in VEGF, FGF2, TGFB1 and TGFBR1 transcript levels in the above groups ($P<0.0001$, $P=0.001$, 0.030 and 0.025, respectively Kruskal–Wallis test) (Fig. 2B). The highest transcript levels of VEGF were observed in CIN III lesions (10.78 ± 5.63) and they were significantly elevated compared to CIN I and II specimens group ($P=0.001$ Mann–Whitney test), but

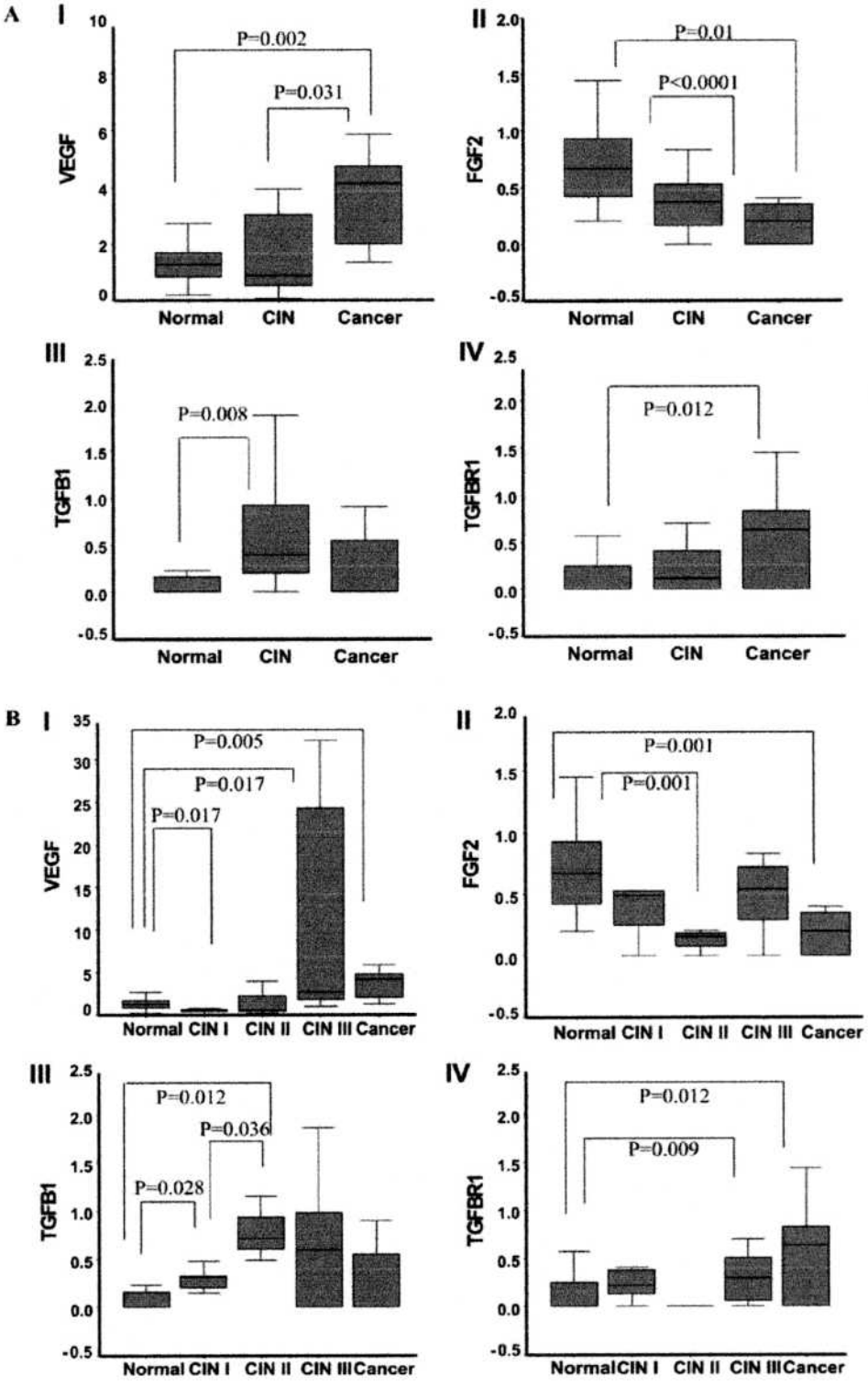
A
I
VEGF
P=0.002
P=0.031
Normal
CIN
Cancer
II
FGF2
P=0.01
P<0.0001
Normal
CIN
Cancer
III
TGFB1
P=0.008
Normal
CIN
Cancer
IV
TGFBR1
P=0.012
Normal
CIN
Cancer
B
I
VEGF
P=0.005
P=0.017
P=0.017
Normal CIN I CIN II CIN III Cancer
II
FGF2
P=0.001
P=0.001
Normal CIN I CIN II CIN III Cancer
III
TGFB1
P=0.012
P=0.036
P=0.028
Normal CIN I CIN II CIN III Cancer
IV
TGFBR1
P=0.012
P=0.009
Normal CIN I CIN II CIN III Cancer

Table 3
mRNA expression in normal, low-grade SIL (CIN I), high-grade SIL(CIN II < III) and cervical cancer specimens

	Controls	Low-grade SIL	High-grade SIL	Cancer	*P* value[a]
VEGF/B2M	1.51±0.24	0.50±0.12	7.41±3.55	3.54±0.60	0.001
FGF2/ B2M	0.71±0.08	0.36±0.10	0.36±0.09	0.19±0.05	0.002
TGFB1/B2M	0.21±0.11	0.29±0.06	0.73±0.18	0.25±0.13	0.020
TGFB2/B2M	0.20±0.06	0.19±0.11	0.45±0.09	0.35±0.12	NS
TGFB3/B2M	0.37±0.10	0.30±0.10	0.47±0.12	0.44±0.11	NS
TGFBR1/B2M	0.13±0.04	0.23±0.08	0.25±0.09	0.58±1.17	0.06
TGFBR2/B2M	1.17±0.34	0.89±0.18	1.07±0.13	1.49±0.48	NS
TGFBR3/βactin	0.93±0.58	0.73±0.22	0.56±0.14	1.33±1.04	NS

Data are presented as Mean±SEM (standard error of the mean).
[a] Kruskal–Wallis test.

similar to those of cancer specimens. FGF2 mRNA expression decreased gradually in CIN I and CIN II lesions compared to normal tissues, whereas CIN III and controls demonstrated similar FGF2 transcript levels. Substantially lower FGF2 mRNA expression levels however, were observed in cervical cancer cases compared to controls ($P=0.012$, Mann–Whitney). On the contrary a significant gradual increase of TGFB1 mRNA expression was demonstrated in CIN I and CIN II lesions, while TGFB1 transcript levels did not differ in the groups of CIN III, cancer cases or controls. Similar TGFBR1 mRNA levels were also found in normal, CIN I and CIN II specimens, although cancer tissues expressed considerably elevated TGFBR1 mRNA levels compared to controls ($P=0.012$).

Our evaluation did not establish any correlation between the mRNA expression of the growth factors and receptors included in the study and patients' age or menopausal status.

3.2. mRNA co-expression analysis pair wise

Spearman correlations for evaluation of VEGF, FGF2, TGFB1, 2, 3 and TGFBR1, 2, 3 co-expression patterns in the groups of normal, CIN and cervical cancer tissue are demonstrated in Tables 4A–4C, respectively). In normal cervical specimens, we observed strong mRNA co-expression between TGFB1 and the receptors TGFBR1 and TGFBR3 ($P<0.0001$ and $P=0.037$, respectively). TGFB2 mRNA expression was positively correlated with TGFB1 and TGFBR3 ($P=0.044$ and $P<0.0001$). Significant positive correlation ($P=0.037$) was also established between the mRNA of TGFBR1 and TGFBR3. Moreover our findings indicated a strong negative correlation in transcript levels of FGF2 and TGFB1 or TGFB2 ($P=0.001$ and 0.026), as well as the TGFBR1 receptor ($P<0.0001$).

In the group of Cervical Intraepithelial lesions, the mRNA angiogenic profile was different since TGFB1 mRNA expression was no longer correlated with any receptor, but was strongly co-expressed with TGFB3 ($P=0.006$). TGFB2 exhibited mRNA co-expression with TGFBR1, FGF2 and VEGF ($P=0.006$, 0.001 and 0.039, respectively). TGFBR1 and FGF2 transcript levels were also co-expressed ($P=0.001$).

In the group of cancer specimens the co-expression pattern of the angiogenic factors included in our evaluation was altered comparing both to the normal and the CIN specimen groups. FGF2 mRNA was significantly co-expressed with TGFB1 ($P=0.042$), and TGFBR1 ($P=0.005$). A negative correlation was

Fig. 2. (A) mRNA levels of VEGF (I), FGF2 (II), TGFB1 (III) and TGFBR1 (IV) in normal, CIN and cancer specimens. Relative values of growth factors expression versus β2-microglobulin exhibited significant differences with respect to the severity of the cervical lesion (normal, CIN and cancer specimens) ($P=0.015$, 0.001, 0.008 and 0.029 Kruskal–Wallis test). Significant differences assessed by Mann–Whitney subgroup analysis pair wise, are shown. (B) mRNA levels of VEGF (I), FGF2 (II), TGFB1 (III) and TGFBR1 (IV) upon malignant transformation of the uterine cervix (normal, CIN I, II, III, cancer). Relative values of growth factors expression versus β2-microglobulin exhibited significant differences with respect to the extent of the lesion towards malignancy ($P<0.0001$, 0.001, 0.030 and 0.025 Kruskal–Wallis test). Significant differences assessed by Mann–Whitney subgroup analysis pair wise, are shown.

Table 4A
Spearman correlation *rho* and *P* values in the normal group of patients

		VEGF	FGF2	TGFB1	TGFB2	TGFB3	TGFBR1	TGFBR2	TGFBR3
VEGF	Spearman's rho	1.000							
	Sig. (2-tailed)								
FGF2	Spearman's rho	−0.121	1.000						
	Sig. (2-tailed)	0.602							
TGFB1	Spearman's rho	0.003	−0.687	1.000					
	Sig. (2-tailed)	0.990	0.001[a]						
TGFB2	Spearman's rho	−0.066	−0.485	0.443	1.000				
	Sig. (2-tailed)	0.777	0.026[b]	0.044[b]					
TGFB3	Spearman's rho	0.127	−0.022	0.108	0.113	1.000			
	Sig. (2-tailed)	0.585	0.925	0.642	0.625				
TGFBR1	Spearman's rho	−0.070	−0.745	0.845	0.362	0.190	1.000		
	Sig. (2-tailed)	0.764	0.000[a]	0.000[a]	0.107	0.408			
TGFBR2	Spearman's rho	0.001	0.034	−0.225	0.121	−0.247	−0.399	1.000	
	Sig. (2-tailed)	0.998	0.882	0.327	0.602	0.280	0.073		
TGFBR3	Spearman's rho	−0.210	−0.383	0.458	0.748	0.066	0.514	0.000	1.000
	Sig. (2-tailed)	0.361	0.087	0.037[a]	0.000[a]	0.776	0.017[b]	0.999	

[a] Correlation is significant at the 0.01 level (2-tailed).
[b] Correlation is significant at the 0.05 level (2-tailed).

observed between VEGF and TGFBR3 mRNA expression ($P=0.019$).

4. Discussion

The induction of the angiogenic process in cytological material derived from female patients with gynaecologic epithelial neoplasia or cancer has been demonstrated. Among the growth factors implicated in neovascularization, VEGF and FGF have been recognized as the main inducers of the angiogenetic switch in human cancers, while other molecules such as members of the TGFB family have been associated with tumor inhibition. Limited information is available on the combined mRNA

Table 4B
Spearman correlation *rho* and *P* values in the CIN lesions group of patients

		VEGF	FGF2	TGFB1	TGFB2	TGFB3	TGFBR1	TGFBR2	TGFBR3
VEGF	Spearman's rho	1.000							
	Sig. (2-tailed)								
FGF2	Spearman's rho	0.427	1.000						
	Sig. (2-tailed)	0.128							
TGFB1	Spearman's rho	0.185	−0.022	1.000					
	Sig. (2-tailed)	0.527	0.940						
TGFB2	Spearman's rho	0.555	0.775	0.186	1.000				
	Sig. (2-tailed)	0.039[a]	0.001[b]	0.524					
TGFB3	Spearman's rho	0.298	0.102	0.696	0.300	1.000			
	Sig. (2-tailed)	0.301	0.728	0.006[b]	0.297				
TGFBR1	Spearman's rho	0.420	0.796	0.072	0.690	0.002	1.000		
	Sig. (2-tailed)	0.135	0.001[b]	0.807	0.006[b]	0.994			
TGFBR2	Spearman's rho	0.004	−0.314	−0.458	−0.051	−0.469	−0.329	1.000	
	Sig. (2-tailed)	0.988	0.274	0.099	0.863	0.091	0.251		
TGFBR3	Spearman's rho	0.209	−0.035	0.253	0.212	−0.113	0.308	0.033	1.000
	Sig. (2-tailed)	0.474	0.904	0.383	0.466	0.702	0.284	0.911	

[a] Correlation is significant at the 0.05 level (2-tailed).
[b] Correlation is significant at the 0.01 level (2-tailed).

Table 4C
Spearman correlation *rho* and *P* values in the cancer group of patients

		VEGF	BFGF	TGFB1	TGFB2	TGFB3	TGFBR1	TGFBR2	TGFBR3
VEGF	Spearman's rho	1.000							
	Sig. (2-tailed)								
FGF2	Spearman's rho	−0.468	1.000						
	Sig. (2-tailed)	0.204							
TGFB1	Spearman's rho	−0.174	0.685	1.000					
	Sig. (2-tailed)	0.654	0.042[a]						
TGFB2	Spearman's rho	−0.520	0.540	0.579	1.000				
	Sig. (2-tailed)	0.151	0.133	0.102					
TGFB3	Spearman's rho	−0.519	−0.342	−0.275	0.018	1.000			
	Sig. (2-tailed)	0.152	0.368	0.475	0.964				
TGFBR1	Spearman's rho	−0.406	0.831	0.248	0.462	−0.309	1.000		
	Sig. (2-tailed)	0.278	0.005[b]	0.520	0.210	0.418			
TGFBR2	Spearman's rho	0.441	−0.383	−0.169	0.114	−0.084	−0.248	1.000	
	Sig. (2-tailed)	0.235	0.309	0.664	0.771	0.829	0.520		
TGFBR3	Spearman's rho	−0.752	0.128	0.109	0.420	0.574	−0.051	0.076	1.000
	Sig. (2-tailed)	0.019[a]	0.743	0.779	0.261	0.106	0.896	0.847	

[a] Correlation is significant at the 0.05 level (2-tailed).
[b] Correlation is significant at the 0.01 level (2-tailed).

expression levels of a variety of angiogenic growth factors in cervical cancer. Moreover their mRNA co-expression profile in CIN lesions has not yet been reported [48].

We evaluated the combined mRNA expression of VEGF, FGF2, TGFB1, 2, 3 and TGFBR1, 2, 3 that are known to be secreted by premalignant and malignant epithelial cells, in tissue specimens with CIN and cervical cancer, as well as in normal cervical tissues. The mRNA expression levels obtained were associated with the clinical stage of the disease followed by assessment of the growth factor mRNA co-expression patterns pair wise.

4.1. Vascular endothelial growth factor

Increased VEGF mRNA levels have been reported in both intraepithelial neoplasia and invasive carcinoma of the uterine cervix and have been associated with increased microvessel density, suggesting that VEGF is an important mediator of angiogenesis in CIN lesions and cervical cancer [22,49]. Our results demonstrate an increase of VEGF mRNA expression levels corresponding to the severity of the cervical lesion (CIN or cancer), while expression remained significantly lower in normal cervix. Interestingly, VEGF transcript levels in cancer specimens were significantly higher compared to CIN lesions. Moreover, substantially higher expression levels of VEGF mRNA were observed in HG-SIL in comparison to LG-SIL. Our findings are consistent with previous immunohistochemical and in situ hybridization studies [22–27] and suggest that VEGF mRNA expression could possibly comprise an indicator of cervical malignant transformation degree. The highest VEGF mRNA levels were encountered in CIN III lesions (10.78 ± 5.63), being significantly elevated compared to CIN I and II specimens, and similar to those of cancer specimens. Our data, in accordance with previous reports [49], support the hypothesis that CIN III may be the actual clinically relevant precursors of invasive cervical cancer, whereas CIN I and II that express significantly less VEGF transcript levels may represent non-aggressive pathologic conditions that do not necessarily progress to cancer.

4.2. Basic fibroblast growth factor (FGF2)

Limited information is available on the significance of FGF2 in cervical disease progression. There are only two reports on FGF2 mRNA levels in cervical cancer and normal cervical tissue by Fujimoto et al. and Van Trappen et al. [32,48], while there are no reports of FGF2 expression in

Cervical Intraepithelial lesions. Both studies found similar levels of FGF2 mRNA in normal cervical tissue and early-stage cervical cancers. Higher FGF2 transcript levels were found in advanced stage cervical cancers compared to normal cervices but the difference was statistically significant only in Fugimoto's evaluation [32]. Limited number of samples was used in both studies, while no immunohistochemical evaluations of FGF2 expression in cervical tissues have been established so far. We evaluated the FGF2 transcript levels in cervical cancer and in CIN lesions. Our results are indicative of lower FGF2 mRNA levels in CIN and cancer tissues compared to normal cervices, suggesting that FGF2's role in cervical carcinogenesis is unclear. We can only speculate that FGF2 transcriptional activation may not be a requirement for the first steps of cervical neoplasia development, or even that other pathways may be responsible for the downregulation of its mRNA expression in these systems. This is not surprising since reports on other cancers such as breast cancer also show conflicting results regarding FGF2 levels, indicating increased FGF2 amounts in tumors compared to normal tissues [50,51], in contrast to others that found no difference [52] or lower levels [53–55], failing in this way to implicate its involvement in disease progression. More studies need to be conducted in larger sets of specimens in order to elucidate the exact mechanism by which FGF2 is involved in cervical malignant transformation.

4.3. Transforming growth factor beta

TGFB is involved in many aspects of cellular function by influencing angiogenesis as well as growth inhibition, cell differentiation, migration, and local immune response. According to our data, TGFB1 is kept at basal levels under normal conditions. However, in primary steps of neoplasia transformation (CIN I, II), a gradual intense TGFB1 mRNA expression may occur as indicated by our results. The observed increase was expected by the activation of the growth inhibitory mechanism of TGFB1, as a consequence of abnormal cellular differentiation [3–5]. In advanced stages of neoplasia (CIN III) or cancer, TGFB1 mRNA expression seems to approach normal levels, possibly explained by the need of inhibition of the immunosuppressive action of TGFB1 overexpression, leading to an effective immune response [56]. Nevertheless most immunohistochemical studies report decreased TGFB1 levels during the neoplastic transformation of cervical epithelium indicating that post-transcriptional mechanisms might regulate TGFB1 protein levels in these systems. Our investigation also designates that TGFBR1 mRNA expression remains constant at the onset of cervical neoplasia (CIN I, II), while in advanced intraepithelial lesions (CIN III) and cancer it is substantially increased. This fact cannot be interpreted without taking into consideration its participation in a complex signalling pathway that includes interactions with other receptors and ligands [3–5].

Regarding TGFB2 and 3 and their receptor mRNA expression levels, our evaluation did not provide evidence on their possible correlation with clinical stage. Thus our estimations support that among the TGFB family members, only TGFB1 and TGFBR1 seem to be transcriptionally dysregulated in the multistep process of cervical tumorigenesis.

The exact mechanism by which TGFB is implicated in cell growth and differentiation in cervical carcinogenesis is the result of many different biochemical pathways that require further investigation.

4.4. mRNA co-expression analysis pair wise

Signaling transduction is activated by the binding and bringing together of the TGFBR1 and TGFBR2 by one of the TGFB ligands (B1, B2, B3). TGFBR1 and TGFBR2 are transmembrane kinases that form a heterotetrameric complex when brought together by a TGFB ligand. It has been demonstrated that TGFBR2 can bind to a TGFB ligand in the absence of TGFBR1 (but not the opposite) but signalling is inhibited without TGFBR1 binding to the complex [35,57,58]. TGFBR3 receptor is a membrane-anchored proteoglycan lacking a kinase activity thus cannot mediate signal transduction. TGFBR3 binds to all three TGFB ligands (B1, B2, B3) and facilitates access to the signalling receptors. In case that TGFBR2 expression or its binding affinity is either reduced or defective in a system, TGFBR3 forms a TGFBR3/TGFB/TGFBR2 complex and expedites access to the signalling receptors. Loss or reduction of expression

of the signalling receptors is associated with reduced responsiveness to the TGFB tumor inhibitory effects [59–61].

mRNA co-expression analysis of all angiogenic factors and receptors included in this study in the groups of normal, CIN and cancer tissues, revealed considerable differences. More correlations between angiogenic factors were found in the group of normal tissues, and they were considerably stronger than those observed in the other two groups. Correlations became even fewer in the group of cancer tissues in comparison with the CIN lesion group of tissues.

In the normal specimen group we observed positive correlations between TGFB1, TGFB2, TGFBR1 and TGFBR3 mRNA expression. TGFBR1 and TGFBR3 were also significantly co-expressed. This is consistent with our knowledge of the mechanism, which initiates TGFB signal transduction through the surface receptors. Most interesting is the substantial role of TGFBR3 in signalling activation. Its significant positive correlation with TGFBR1 mRNA expression suggests that it is an essential requirement for the bringing together of the two signalling receptors in these systems by mediating TGFB ligands' access and binding to TGFBR2. TGFB1 and TGFB2 mRNA was found to be considerably co-expressed, which leads to the assumption that they might be the main TGFB ligands that take part in the heterotetrameric complex with the signalling receptors rather than TGFB3. In addition our results demonstrate a strong negative correlation of FGF2 mRNA expression with that of TGFB1, TGFB2 and TGFBR1 in the normal group of tissues.

In the group of CIN lesions the mRNA co-expression profile is substantially altered. Novel mRNA co-expressions take place such as that of TGFB2 with FGF2 and VEGF, while others are abolished such as that of TGFB1 and the TGFBR1 and TGFBR3 receptors. Furthermore TGFB3 mRNA (instead of TGFB2) is significantly co-expressed with TGFB1. On the other hand TGFB2 transcript levels continue to be positively correlated with TGFBR1, as in the normal tissues group. Interestingly FGF2 mRNA expression, which exhibited negative correlations in the normal group of tissues, is positively correlated to TGFBR1. These findings suggest a disruption of the mRNA co-expression profile of the angiogenic factors that we studied in this group of CIN lesions.

In the group of cancer specimens, FGF2 mRNA is significantly co-expressed with TGFB1 and TGFBR1, similarly to the CIN specimens group, while VEGF mRNA expression is negatively correlated with that of TGFBR3. All other correlations observed in normal or CIN lesion tissues are absent in this case.

TGFB is involved in many aspects of cellular function. Given the growth inhibitory action of TGFB it is obvious that its role in the tumorigenic process is essential. Loss of TGFB function has been linked with tumor growth and cancer progression. Our results suggest that loss or reduced expression of TGFB is not the only probable cause of a malignant phenotype. TGFB may be expressed in CIN or cervical cancer tissue and it may even be overexpressed as indicated by our results in primary steps of neoplasia (CIN I, II) in order to suppress the initiation or expanding of a tumor but other factors may be responsible for loss of TGFB signalling. The disrupted co-expression pattern of TGFB and cognate receptors evidenced from the present data in CIN lesions and cervical cancer tissues compared to normal cervices could possibly explain the premalignant and malignant phenotype respectively of these tissues by accounting for loss of TGFB signaling and eventually loss of its growth inhibitory action.

Interestingly, the observed negative correlation of FGF2 and TGFBR1 in normal cervical tissues becomes positive in CIN lesions and cancer specimens as well. The type I receptor of the TGFB Receptor family seems to be the most important, since TGFB signalling is inhibited without its presence. Specifically it has been demonstrated that TGFBR2 can bind to a TGFB ligand in the absence of TGFBR1 (but not the opposite) but signalling is inhibited without TGFBR1 binding to the complex. Therefore it is not surprising that FGF2 is positively co-expressed with TGFBR1 in CIN lesions and cervical cancer. FGF2 is known to stimulate cell proliferation and differentiation. Overexpression of FGF2 in premalignant and malignant cervical cells is accompanied by TGFBR1 overexpression so that FGF2's stimulating effects on proliferation will be counteracted by TGFB's growth inhibitory effects. When FGF2 is underexpressed on the other hand,

there is no need for growth inhibition and TGFBR1 which seems to control TGFB signalling is also underexpressed.

In conclusion, our findings indicate that cervical malignant transformation is accompanied by many alterations in the co-expression profile of the referred genes. We can only speculate that corresponding alterations might take place in the mechanism and efficiency of TGFB signal transduction during cervical carcinogenesis.

Summarizing, our results give indirect evidence that the dysregulation of VEGF, FGF2, TGFB1 and TGFBR1 mRNA expression may be involved in the malignant transformation process of the uterine cervix. Additionally, disruption of co-expression patterns of the factors included in this study, in the CIN and cancer specimen groups compared to controls, suggests a transcriptional dysregulation during cervical cancer development. Further studies are needed to elucidate the potential use of mRNA expression profiles of angiogenetic factors as progression indicators in cervical carcinogenesis.

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