Available online at www.sciencedirect.com
SCIENCE DIRECT®
Cancer Letters 235 (2006) 100–113
CANCER Letters
www.elsevier.com/locate/canlet

Transcriptional deregulation of VEGF, FGF2, TGF-β1, 2, 3 and cognate receptors in breast tumorigenesis

Giannoula Soufla[a], Filippos Porichis[a], George Sourvinos[a], Stamatis Vassilaros[b], Demetrios A. Spandidos[a,*]

[a]*Department of Virology, Medical School, University of Crete, P.O. Box 1527, Heraklion 710 03, Crete, Greece*
[b]*Prolepsis Breast Cancer Diagnosis and Research Center, Athens, Greece*

Received 12 November 2004; received in revised form 8 April 2005; accepted 11 April 2005

Abstract

Angiogenesis is an important event during the neoplastic process and is induced by the secretion of numerous growth factors from endothelial cells. Vascular endothelial growth factor (VEGF), basic fibroblastic growth factor (FGF2), and transforming growth factor-β1, β2, β3 (TGF-β1, 2, 3) and cognate receptors (TGF-βRI, II, III) mRNA expression pattern was evaluated by RT-PCR in 25 breast cancer tissue samples and adjacent normal tissues, and correlated to clinicopathological features. Western blot analysis was performed to evaluate VEGF and TGF-β1 protein levels. TGF-β1 and TGF-β3 mRNA levels were significantly different in breast cancer specimens of differing histology (ductal, lobular, other) ($P=0.020$ and $P=0.043$). No statistically significant difference was observed at the mRNA level of VEGF between normal and tumor tissues while elevated VEGF protein levels in tumors were associated with patients' menopausal status. A strong hormonal influence of ER and PR on TGF-β mRNA expression was established. FGF2 transcript levels were substantially decreased in cancer compared to adjacent normal specimens ($P=0.031$). A disruption of mRNA co-expression patterns was observed in malignant breast tissues compared to controls. Western blot analysis revealed differences between VEGF and TGFβ1 mRNA and their corresponding protein levels. A substantial negative correlation of TGF-β1 protein and TGF-β1 mRNA levels ($P=0.016$) was demonstrated by breast tissue-pair analysis. Summarizing, our findings suggest that transcript levels of the examined markers in breast cancer are associated with menopausal and hormonal status, while their co-expression pattern is altered in malignant tissues compared to controls. In addition the difference between VEGF and TGF-β1 mRNA and protein levels observed, indicates that post-transcriptional mechanisms may regulate expression of these molecules in breast cancer.

Keywords: Angiogenesis; VEGF; TGF-β1; FGF2; Breast cancer

1. Introduction

Angiogenesis, the formation of new blood vessels from the pre-existing vascular network is a physiological process involved in tissue development, wound healing and reproduction [1]. However it is

* Corresponding author. Tel.: +30 2810 394631; fax +30 2810 394759.
E-mail address: spandidos@spandidos.gr (D.A. Spandidos).

0304-3835/$ - see front matter © 2005 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.canlet.2005.04.022

also essential in tumor progression, invasiveness and metastasis [2]. Tumor cells as well as non-malignant cells (macrophages, mast cells, fibroblasts) secrete a large number of growth factors that either stimulate or suppress neovascularization controlling endothelial cell growth and survival [3,4]. Among these, VEGF is considered to be the most important.

Vascular endothelial growth factor (VEGF) is a multifunctional glycoprotein that acts as an endothelial cell specific mitogen [4,5]. In addition to stimulating cell proliferation, VEGF has been proved to increase vascular permeability and induce capillary tube formation. Elevated VEGF expression at advanced stages of the disease has been reported in a variety of human malignancies including breast, endometrial, ovarian, bladder, and lung tumors [6–11]. Its prognostic value has been extensively studied mostly by enzyme-linked immunosorbent assays and immunohistochemical methods in early breast cancer patients with controversial results [12,13]. Attempts to establish a relationship between VEGF protein as well as mRNA expression and clinicopathological characteristics demonstrated only an association with estrogen receptor status in some reports while others indicated otherwise [12,14–17]. Most studies however, reported that VEGF expression is positively associated with relapse-free survival or overall survival in breast cancer patients [4].

Basic Fibroblast Growth factor mediates the neovascularization process, by promoting angioblast differentiation, cell growth and invasion. FGF-2 acts in a paracrine and autocrine manner and is produced either by tumor cells, or infiltrating inflammatory cells (macrophages). When secreted from tumor cells, FGF2 is responsible for basement membrane dissolution, migration and metastasis of endothelial cells [3–5]. It can upregulate other important angiogenic factors like VEGF and inhibits endothelial cell apoptosis. Its role has been described in highly metastatic prostate cells, uterine endometrial cancer, pancreatic and hepatocellular carcinoma [18–21] but most studies failed to implicate FGF2 among the major players in the development of breast cancer, although they are suggestive of some involvement [22].

Transforming growth factor beta (TGF-β) has a highly complex role acting as a growth modulator involved in angiogenesis, cell proliferation, differentiation, adhesion and migration [23–28]. TGF-β has been proved to substantially inhibit cell growth in normal epithelial cells in vivo and in vitro [29,30], inducing in parallel its own mRNA expression. TGF-beta's growth inhibitory effects are attributed to its ability to arrest cells in the G1 phase of the cell cycle [27,30]. Immunosuppression however, is another major effect of this multifunctional molecule. It has been shown to inhibit T and B cell proper function, leading to immune response deficiency and tumor growth. Moreover TGF-β via a paracrine action promotes tumor stroma formation and decreases tumor infiltrate providing tumor cells an alternative escape mechanism from the immune response [31]. Five isoforms of TGF-β have been identified so far but only three (TGF-β1, 2, 3) are expressed in mammalian cells. TGF-β1 is the most characterized isoform todate and along with TGF-β3 exhibits stronger inhibitory effects than TGF-β2. Cell function regulation by TGF-β arises from his interaction with cell surface receptors I, II, III (TGF-βRI, II, III) [23–26]. It has been suggested that TGF-β may play a role as autocrine/paracrine regulator of breast tumor progression. Furthermore deficiency in TGF-βRII expression has been associated with malignant progression of breast cancer since tumor cells become insensitive to TGF- β mediated growth inhibition.

A variety of angiogenic growth factors have been reported to be overexpressed during breast cancer development. These genes may represent potential targets for prognosis and therapy of breast cancer. However angiogenesis is recognized as a process that involves the coordinated action of a group of genes that either induce or suppress neovascularization. The mRNA expression profile of a panel of growth factors in breast cancer tissues has not been investigated, and their role as an ensemble in the underlying mechanism of breast malignant transformation is poorly understood. In order to provide a distinct molecular portrait of each tumor, we evaluated the combined expression of VEGF, FGF2, TGF-β1, 2, 3 and their receptors TGF-βRI, II, III in breast cancer tissues and compared it with adjacent normal specimens. Most importantly we investigated the co-expression pattern of these factors in breast cancer and compared it to that of the adjacent normal specimens, with the scope of identifying alterations that may favor or account for the loss of the balance between enhancers and inhibitors of angiogenesis supporting the tumorigenic

process. Furthermore, we examined whether the expression profile of these genes is correlated with clinicopathological features.

2. Materials and methods

2.1. Patients and controls

A total of 25 individuals who were admitted to Prolepsis Breast Diagnosis and Research Center, Athens, Greece and underwent surgical treatment from 2003 to 2004 due to breast cancer disease, without having received any radiotherapeutic or chemotherapeutic treatment prior to surgery 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 while the rest was fixed in 10% formaldehyde solution for histopathological examination. Only histologically confirmed normal or malignant breast tissues were processed to RNA extraction.

Histological cell types of the tumors were as follows: ductal infiltrating carcinoma ($n=15$) (60%), lobular infiltrating carcinoma ($n=1$) (4%), multifocal infiltrating carcinoma ($n=1$) (4%), in situ intraductal/microductal and nodular carcinoma ($n=2$) (8%), adenoma ($n=1$) (4%), mucinous carcinoma ($n=1$) (4%) and of mixed histological differentiation ($n=4$) (16%). Histologically normal tissue specimens adjacent to the tumor of each patient consisted our control group.

Age at the time of surgery ranged from 35 to 72, mean (55.5 ± 2.1). Thirteen women were in the reproductive period and 12 were in menopause. One (5%) of the 21 patients with invasive cancer had well (grade I), 6 (29%) moderately (grade II), and 14 (67%) poorly (grade III) differentiated cancer.

Patients were classified according to hormonal status (positive, negative, unknown). The ER, PR and HER-2 status was determined in the laboratory of Prolepsis research and Diagnosis Center by immunohistochemistry. In all cases the results were assessed as follows: a) no staining or weak staining (<10%) was considered negative, whereas b) moderate (10–50%) or strong (>50%) staining was considered positive. Table 1 summarizes patients' clinical characteristics.

Ethics Committee of the University of Crete approved the present study and all participating patients gave written informed consent.

2.2. RNA and protein extraction

Total RNA and protein were extracted from each specimen using the Trizol reagent (Invitrogen Ltd, UK) according to the manufacturer's instructions. 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. Protein concentration was determined by Bradford assay.

2.3. RT-PCR

Reverse transcription reactions for the preparation of first strand cDNA were conducted using the

Table 1
Clinicopathological characteristics of the patients

Characteristics	No. of patients	(%)
Age (years)		
≤50	10	40
>50	15	60
Menopausal status		
Premenopausal	13	52
Postmenopausal	12	48
Histological type		
Ductal invasive	15	60
Lobular invasive	1	4
Other	9	36
Tumor grade		
I	1	4
II	6	24
III	14	56
No grade	4	16
Receptor status		
ER+	15	60
ER−	9	36
Unknown	1	4
PR+	13	52
PR−	11	44
Unknown	1	4
HER2		
Positive	12	48
Negative	12	48
unknown	1	4

Thermoscript RT-PCR Kit (Invitrogen Ltd, UK) according to the manufacturer's protocol.

Transcribed products were subjected to PCR for the growth factors 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, 1x PCR reaction buffer, 2 mM $MgCl_2$, 0.8 mM dNTPs, and 0.65 U Platinum *Taq* DNA polymerase (Invitrogen Ltd., UK). All primer pairs were designed to span at least one intron in order to avoid amplification of contaminating genomic DNA along with cDNA. Primer pair sequences and amplification conditions were previously described [32]. Cycling conditions for performing the analysis in the exponential phase of each PCR reaction were determined experimentally (data not shown). In the amplification reactions for each specific RNA primer set, RNA primers for β2-microglobulin were included as an internal control in all PCR reactions.

PCR products were analysed on 8.5% polyacrylamide gels 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. All RT PCR reactions were performed in triplicates. Present analysis conducted on normal and pathological samples may be a manifestation of RNA profiles of endothelial and stromal components.

2.4. Western blot analysis

Twenty-five micrograms of protein were electrophoresed through a 10% polyacrylamide gel and the separated proteins were transferred onto PVDF membranes by overnight blocking at 4 °C. After blocking with 3% milk powder in PBS-T for 1 h at room temperature, the membranes were incubated with a commercially available mouse anti-TGF-β1 (1:250 dilution) or anti-VEGF (1:500 dilution) (R and D systems) or anti-beta-actin (Sigma) antibody for 1 h. The blots were then incubated, after the standard washes, for 1 h with an anti-mouse IgG conjugated to horseradish peroxidase and after further washing with PBS-T, bands were visualised using the ECL method (Chemicon Int. CA), as described in the manufacturer's protocol.

2.5. Statistical analysis

One-sample Kolmogorov–Smirnov-test was applied to our results for all factors studied and revealed that the distribution of expression values in our study was not normal. Therefore, non-parametric procedures (Kruskal Wallis and Mann–Whitney-test) were applied to the set of data for the evaluation of significant statistical differences in mRNA expression of VEGF, FGF2, TGF-β1, 2, 3 and TGF-βRI, II, III between the groups of different clinicopathological characteristics. Probability values less than 0.05 were considered statistically significant exhibiting significant differences between groups. Age distribution was assessed similarly. Data are 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 growth factors' mRNA co-expression pair wise, in the groups of normal, and cancer tissues. Spearman's rho correlation coefficient values range from −1 to 1. The sign of the correlation coefficient indicates the direction of the relationship (positive or negative). Probability values less than 0.05 were considered statistically significant showing linearly related variables. Statistical calculations were performed using the SPSS software, version 11.

3. Results

We evaluated the mRNA expression profile of VEGF, FGF2, TGF-β1, 2, 3 and TGF-βRI, II, III (Fig. 1) in a total of 25 breast cancer and adjacent normal tissue specimens. An infiltrating ductal carcinoma was reported in the majority of patients. The most frequent histological grade was G3.

3.1. Transcript levels of growth factors and receptors

Our findings indicate that FGF2 and TGF-β3 mRNA expression is significantly different between tumor and corresponding adjacent normal breast tissues ($P=0.031$ and $P=0.043$, respectively, Mann–Whitney-test) (Table 2). Specifically, TGF-β3

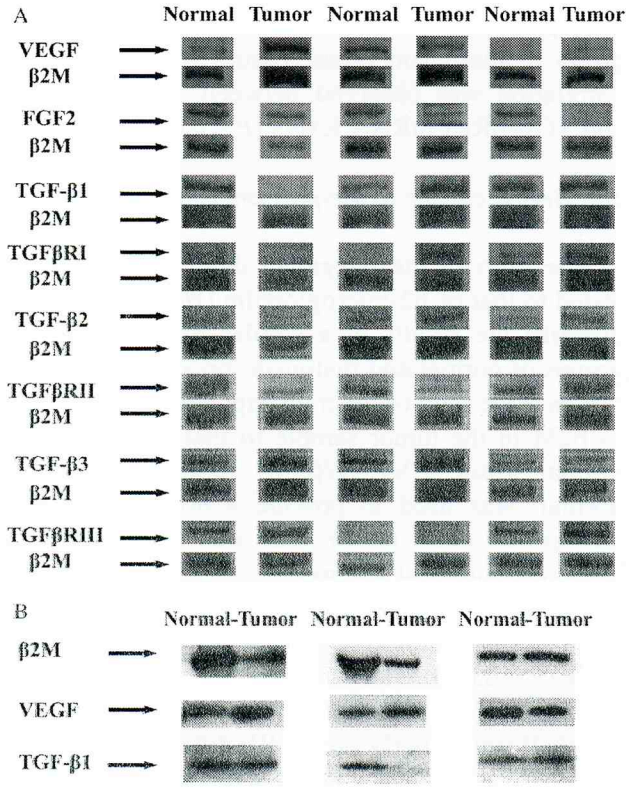

Fig. 1. (A) Representative examples of VEGF, FGF2, TGF-β1, 2, 3 and TGFβRI, II, III mRNA expression in cancer and adjacent normal breast tissues. Ratio: integrated density of the band of each gene divided by the integrated density of the internal standard band (β2-microglobulin or β-actin). (B) Representative examples of VEGF and TGF-β1 protein expression assessed by western blot analysis in cancer and adjacent normal breast tissues. Ratio: integrated density of the band of each gene divided by the integrated density of the internal standard band (β2-microglobulin or β-actin).

transcript levels in cancer specimens were significantly elevated compared to normal tissues, whereas FGF2 expression was considerably decreased. VEGF mRNA expression was higher in tumor (mean = 0.34 ± 0.03) compared to normal tissues (0.30 ± 0.04) but the difference was not statistically significant. Similarly, TGF-β1 and TGF-β2 exhibited higher mRNA levels in cancer specimens compared to controls, whereas all TGF-β Receptors (TGF-βRI, RII, RIII) had lower transcript levels in tumors than normal tissues (Table 2); the differences however were not statistically significant.

Breast cancer tissues exhibited significantly different TGF-β1 and TGF-β3 mRNA levels in respect of differing histology (ductal, lobular, other type of carcinoma) ($P=0.020$ and $P=0.036$, respectively Kruskal Wallis test). Lower levels were observed in ductal carcinoma compared to other types of cancer, while the lowest TGF-β1 mRNA levels were found in lobular carcinoma.

Significantly higher TGF-β2 and TGF-β3 mRNA levels were found in ER positive breast cancer tissues compared to ER negative cancer specimens ($P=0.002$ and $P=0.014$, respectively). TGF-β2 and TGF-βRII transcript levels in the normal specimen group were also significantly higher in ER positive tissues ($P=0.002$ and $P=0.014$, respectively). Progesterone Receptor (PR) positive breast cancer tissues express significantly higher TGF-β2, TGF-β3 and TGF-βRIII mRNA than PR negative tumors ($P=0.004$, $P=0.006$ and $P=0.004$, respectively), and this was observed in PR positive normal breast tissues concerning TGF-β2 mRNA ($P=0.031$) as well (Table 3). Tumors as well as adjacent normal breast tissues expressing the cerbB2 (HER2) were found to express significantly lower FGF mRNA levels than HER2 negative tissues ($P=0.018$ and $P=0.043$, respectively). No correlation was established between transcript levels of any the growth factors studied in normal or malignant breast tissues and grade of the tumors, patients' age or menopausal status.

3.2. mRNA co-expression analysis pair wise

Spearman correlations for evaluation of VEGF, FGF2, TGF-β1, 2, 3 and TGF-βRI, II, III co-expression patterns in the groups of cancer and adjacent normal breast tissue are demonstrated in Table 4 (A, B, respectively). In normal specimens, VEGF mRNA was co-expressed with that of FGF2 and the ligands TGF-β1 and TGF-β2 ($P=0.018$, $P=0.029$ and $P=0.048$, respectively). TGF-β1 mRNA expression was positively correlated with TGF-β2, TGF-β3 and TGF-βRII ($P=0.032$, $P=0.037$ and $P=0.007$). Significant positive correlations were also established between TGF-β2 and TGF-β3 ($P=0.033$), as well as the receptor TGF-βRII mRNA ($P<0.001$). TGF-βRII and TGF-βRIII transcript levels were considerably co-expressed ($P=0.026$).

In the group of breast cancer specimens the mRNA angiogenic profile appeared to be different since VEGF mRNA expression was only correlated with TGF-β2 ($P=0.017$). TGF-β1 exhibited a marginal

Table 2
mRNA expression in breast cancer and adjacent normal tissues

Growth factor	Tumor	Normal	*P*-value
VEGF/β2M	0.34±0.03	0.30±0.04	NS
FGF2/β2M	0.45±0.02	0.62±0.09	0.031
TGF-β1/β2M	0.28±0.02	0.25±0.02	NS
TGF-β2/β2M	0.20±0.05	0.12±0.02	NS
TGF-β3/β2M	0.63±0.22	0.32±0.12	0.043
TGFβRI/β2M	0.16±0.03	0.30±0.14	NS
TGFβRII/β2M	0.37±0.05	0.46±0.08	NS
TGFβRIII/β-actin	1.47±0.69	2.94±0.84	NS

Data are presented as Mean±SEM (standard error of the Mean). Mann–Whitney-test. $P<0.05$ is statistically significant; NS, not significant

mRNA co-expression with TGF-βRI ($P=0.052$), while all previous correlations were abolished. TGF-β2 transcript levels were correlated with those of TGF-β3 and TGF-βRIII ($P=0.028$ and $P=0.011$, respectively). A strong positive correlation was observed between TGF-β3 and TGF-βRIII mRNA expression ($P<0.0001$).

3.3. Protein levels of VEGF and TGFB1

VEGF and TGF-β1 protein levels in the tumors and adjacent normal breast tissues were determined by western blot analysis (Fig. 1). Our findings did not indicate any association of VEGF and TGF-β1 protein levels with clinicopathological characteristics such as patients' age, histological type of the tumor, differentiation grade, ER, PR, or HER-2 status, with the exception of VEGF protein levels in tumor specimens that were found to be significantly elevated in postmenopausal women (Fig. 2).

Co-expression pattern analysis in the group of normal breast tissues demonstrated that TGF-β1 protein levels were significantly correlated with TGF-βRII mRNA levels ($P=0.048$), while in the group of malignant breast tissues a strong positive correlation was observed between TGF-β1 protein and TGF-βRIII mRNA levels ($P<0.0001$).

3.4. Breast cancer tissue-pair analysis

The ratio of the integrated density of each gene tested to that of β2-microglobulin (β2M) was used to quantify the results in a similar manner as in the groups of normal and malignant breast tissues, but in this case the ratio of the transcript levels of each gene to β2M in the tumor sample to that of the adjacent normal tissue [(VEGF/β2M) tumor/(VEGF/β2M) normal] was used to provide a distinct molecular portrait of each tumor that was compared with clinicopathological features.

Significantly different TGF-β1 mRNA levels were observed in breast tissue pairs in respect to tumor stage ($P=0.046$). Pair-wise analysis revealed marginally higher TGF-β1 expression levels in moderately (grade II) than poorly (grade III) differentiated cancer ($P=0.052$). VEGF transcript levels were found to be substantially elevated in postmenopausal compared to premenopausal women ($P=0.035$) (Fig. 2). ER positive tissue-pairs expressed significantly higher TGF-β2 mRNA than ER negative specimens ($P=0.009$). TGF-β3 transcript levels were elevated in EGFR positive compared to EGFR (HER-2) tissue-pairs ($P=0.049$).

Spearman correlations for evaluation of VEGF, FGF2, TGF-β1, 2, 3 and TGF-βRI, II, III co-expression patterns in breast tissue-pairs revealed a significant positive correlation between VEGF and TGF-β2 mRNA expression ($P=0.017$) as well as a marginally non significant co-expression of TGF-β2 and TGF-βRII mRNA ($P=0.059$).

Table 3
Statistically significant difference of mRNA expression of tumor and adjacent normal tissues in respect to ER and PR status

Growth factor	ER		*P* value	PR		*P* value
	Positive	Negative		Positive	Negative	
(TGFβ2/β2M)tumor	0.30±0.07	0.19±0.05	0.002	0.32±0.07	0.06±0.03	0.005
(TGFβ3/β2M) tumor	0.81±0.33	0.65±0.22	0.014	0.90±0.39	0.38±0.19	0.052
(TGFβRIII/β-actin) tumor	2.01±0.99	1.46±0.69	NS	2.62±1.24	0.31±0.11	0.026
(TGFβ2/β2M) normal	0.17±0.03	0.12±0.02	0.002	0.19±0.02	0.04±0.02	0.009
(TGFβRII/β2M) normal	0.59±0.09	0.44±0.07	0.014	0.56±0.11	0.31±0.10	NS

Data are presented as Mean±SEM (standard error of the Mean). Mann–Whitney test. $P<0.05$ is statistically significant; NS, not significant.

Table 4

		VEGF	FGF	TGFβ1	TGFβ2	TGFβ3	TGFβRI	TGFβRII	TGFβRIII	VEGF protein	TGF-β1 protein
A Spearman correlation *rho* and *P* values in breast cancer tissue specimens											
VEGF	Spearman rho	1.000									
	Sig. (2-tailed)										
FGF	Spearman rho	0.068	1.000								
	Sig. (2-tailed)	0.747									
TGFβ1	Spearman rho	0.361	0.006	1.000							
	Sig. (2-tailed)	0.076	0.977								
TGFβ2	Spearman rho	0.481	0.371	0.131	1.000						
	Sig. (2-tailed)	**0.017**[a]	0.074	0.542							
TGFβ3	Spearman rho	0.289	−0.090	0.363	0.478	1.000					
	Sig. (2-tailed)	0.193	0.689	0.097	**0.028**[a]						
TGFβRI	Spearman rho	0.172	0.004	0.402	0.210	0.112	1.000				
	Sig. (2-tailed)	0.422	0.987	0.052	0.337	0.629					
TGFβRII	Spearman rho	0.006	0.277	0.274	0.259	0.411	0.105	1.000			
	Sig. (2-tailed)	0.980	0.213	0.218	0.258	0.080	0.651				
TGFβRIII	Spearman rho	0.158	0.133	0.109	0.702	0.606	0.225	0.529	1.000		
	Sig. (2-tailed)	0.623	0.681	0.737	**0.011**[a]	**0.037**[a]	0.482	0.077			
VEGF	Spearman rho	0.058	−0.363	0.434	0.431	0.396	0.479	0.419	0.571	1.000	
Protein	Sig. (2-tailed)	0.851	0.223	0.138	0.162	0.203	0.098	0.228	0.180		
TGF-β1	Spearman rho	−0.110	0.082	0.138	0.372	0.370	0.272	0.067	0.964	0.190	1.000
Protein	Sig. (2-tailed)	0.720	0.789	0.654	0.234	0.213	0.392	0.854	**0.000**[b]	0.651	
B Spearman correlation *rho* and *P* values in the adjacent normal breast specimens											
VEGF	Spearman rho	1.000									
	Sig. (2-tailed)										
FGF	Spearman rho	0.437	1.000								
	Sig. (2-tailed)	**0.029**[a]									
TGFβ1	Spearman rho	0.469	0.073	1.000							
	Sig. (2-tailed)	**0.018**[a]	0.728								
TGFβ2	Spearman rho	0.399	0.317	0.430	1.000						
	Sig. (2-tailed)	**0.048**[a]	0.122	**0.032**[a]							
TGFβ3	Spearman rho	0.296	−0.212	0.438	0.446	1.000					
	Sig. (2-tailed)	0.170	0.332	**0.037**[a]	**0.033**[a]						
TGFβRI	Spearman rho	0.147	0.162	−0.168	0.089	0.130	1.000				
	Sig. (2-tailed)	0.484	0.440	0.423	0.672	0.555					
TGFβRII	Spearman rho	0.341	0.298	0.546	0.747	0.327	0.027	1.000			
	Sig. (2-tailed)	0.111	0.167	**0.007**[b]	**0.000**[b]	0.148	0.901				
TGFβRIII	Spearman rho	−0.014	0.280	0.011	0.236	−0.084	−0.231	0.498	1.000		
	Sig. (2-tailed)	0.964	0.355	0.972	0.438	0.785	0.448	0.099			

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

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

VEGF	Spearman rho	0.074	0.126	−0.187	0.284	0.322	0.466	0.456	0.000	1.000	
Protein	Sig. (2-tailed)	0.809	0.681	0.541	0.347	0.283	0.109	0.159	1.000		
TGF-β1	Spearman rho	−0.061	−0.099	0.252	0.346	0.492	0.382	0.606	0.076	0.310	1.000
Protein	Sig. (2-tailed)	0.844	0.748	0.406	0.247	0.087	0.198	**0.048**[a]	0.847	0.456	
C Spearman correlation *rho* and *P* values in breast tissue-pairs											
VEGF	Spearman rho	1.000									
	Sig. (2-tailed)										
FGF	Spearman rho	0.280	1.000								
	Sig. (2-tailed)	0.175									
TGFβ1	Spearman rho	−0.025	−0.158	1.000							
	Sig. (2-tailed)	0.907	0.451								
TGFβ2	Spearman rho	0.482	−0.119	−0.123	1.000						
	Sig. (2-tailed)	**0.017**[a]	0.579	0.567							
TGFβ3	Spearman rho	0.241	−0.038	−0.017	0.216	1.000					
	Sig. (2-tailed)	0.280	0.865	0.940	0.346						
TGFβRI	Spearman rho	−0.013	−0.161	−0.145	0.124	0.031	1.000				
	Sig. (2-tailed)	0.951	0.453	0.499	0.572	0.894					
TGFβRII	Spearman rho	0.062	−0.173	0.198	0.430	−0.110	−0.013	1.000			
	Sig. (2-tailed)	0.789	0.454	0.390	0.059	0.665	0.958				
TGFβRIII	Spearman rho	−0.539	−0.358	0.115	−0.293	−0.248	0.232	0.486	1.000		
	Sig. (2-tailed)	0.108	0.310	0.751	0.444	0.489	0.519	0.154			
VEGF	Spearman rho	0.160	−0.162	−0.154	0.264	0.251	−0.521	0.323	−0.469	1.000	
Protein	Sig. (2-tailed)	0.444	0.440	0.464	0.213	0.260	**0.009**[b]	0.153	0.172		
TGF-β1	Spearman rho	−0.126	−0.016	−0.652	−0.322	0.047	−0.557	0.024	−0.036	0.465	1.000
Protein	Sig. (2-tailed)	0.681	0.957	**0.016**[a]	0.307	0.879	0.060	0.947	0.939	0.109	

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

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

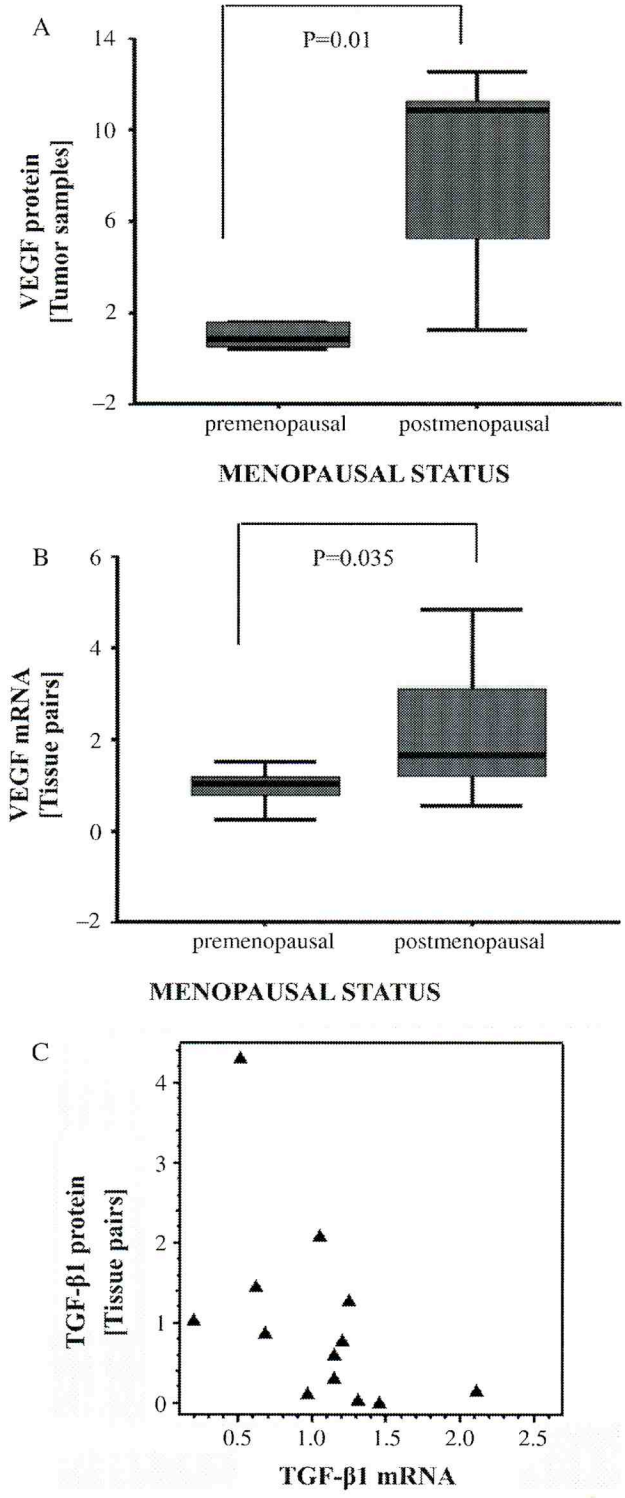

Fig. 2. (A) VEGF protein levels of breast cancer *tissue specimens* derived from premenopausal and postmenopausal women. Relative values of protein expression versus β2-microglobulin exhibited significant differences with respect to menopausal status ($P=0.010$, Mann–Whitney test). (B) mRNA levels of VEGF in breast cancer *tissue pairs* derived from premenopausal and postmenopausal women. Relative values of VEGF mRNA expression versus β2-microglobulin in each tissue-pair exhibited significant differences with respect to menopausal status ($P=0.035$, Mann–Whitney test). (C) Scatterplot of TGF-β1 protein expression versus TGF-β1 mRNA expression in breast cancer tissue pairs ($r=-0.652$, $P=0.016$).

An association between VEGF and TGF-β1 protein levels of breast tissue pairs and clinicopathological characteristics of the specimens was not established by our findings. However TGF-β1 protein levels were found to be considerably increased in tissue pairs in which TGF-β1 mRNA was under-expressed compared to tissue specimens with normal TGF-β1 mRNA expression ($P=0.049$). Furthermore, co-expression pattern analysis demonstrated a substantial negative correlation of TGF-β1 protein and TGF-β1 mRNA levels ($P=0.016$). A significant negative correlation was also observed between VEGF protein and TGF-βRI mRNA levels ($P=0.009$), while a marginally non-significant negative correlation was indicated by our findings between TGF-β1 protein and TGF-βRI transcript levels ($P=0.060$).

4. Discussion

Angiogenesis has been shown to play a major role in breast cancer development. 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 TGF-β family have been associated with tumor inhibition.

In the present study we evaluated the combined mRNA expression of VEGF, FGF2, TGF-β1, 2, 3 and TGF-βRI, II, III that are known to be secreted by premalignant and malignant epithelial cells, in cancer and adjacent normal breast tissues. The mRNA expression levels obtained were associated with clinicopathological features followed by co-expression analysis.

4.1. Vascular endothelial growth factor

Increased VEGF mRNA levels have been reported in breast tumors compared to normal tissues [33,34]. VEGF protein levels assessed by ELISA have been directly associated with increased microvessel density during tumor progression, suggesting that VEGF is an important mediator of angiogenesis in breast carcinogenesis [15,35]. According to our results the mean value of VEGF mRNA expression levels in breast cancer specimens was higher than that observed in normal breast tissues, but the difference was not statistically significant. Interestingly, VEGF protein levels in breast tumors were found to be significantly elevated in postmenopausal women, while VEGF mRNA did not exhibit a similar association with menopausal status. Furthermore, breast tissue pair analysis revealed that the actual difference of VEGF mRNA in each tissue pair (ratio of VEGF mRNA of each tumor to its adjacent normal tissue specimen) was significantly higher in postmenopausal patients; however a similar association was not established for VEGF protein. Our findings suggest that VEGF expression is possibly associated to menopausal status but the responsible mechanism as well as other pathways probably involved in its post-transcriptional regulation remain obscure.

4.2. Basic Fibroblast Growth factor (FGF2)

The majority of studies evaluating the role of FGF2 in breast carcinogenesis indicated lower FGF2 mRNA levels in tumors compared to normal breast tissues [36–39]. This finding is consistent with our results that demonstrated a significant decrease in FGF2 mRNA expression in breast cancer specimens as opposed to adjacent normal tissues. Immunohistochemical evaluations have reported FGF2 expression to be mainly associated with normal tissues of different origin and particularly with stromal components, while little or no expression has been attributed to cancer cells [37, 40]. Furthermore breast cancer cell lines have not been found to express FGF2, in contrast to cell lines derived from normal breast cells. Diminished FGF2 mRNA expression seems to be a feature of cervical cancer as well, since lower FGF2 transcript levels were also found by our group in cervical cancer tissues compared to normal cervical specimens [32].

Other reports however presented conflicting results regarding FGF2 mRNA levels indicating increased FGF2 amounts in tumors compared to normal tissues, while others found no difference failing in this way to implicate its involvement in disease progression [39–41].

4.3. Transforming growth factor beta

TGF-β 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, TGF-β1 protein is at basal levels in normal breast specimens. However in breast tumor tissues at early stages of malignant transformation (Stage I) TGF-β1 protein displays high values, which gradually decrease in advanced stages of cancer (II, III) and finally reaches basal levels (stage III) as indicated by our results. Our observations were likely expected by the activation of the growth inhibitory mechanism of TGF-β1, as a consequence of abnormal cellular differentiation [3–5]. In advanced stages of cancer however, TGF-β1 protein expression seems to approach normal levels, possibly explained by the need of inhibition of the immunosuppressive action of TGF-β1, leading to an effective immune response [42]. Our data support the hypothesis that TGF-β1 acts as a tumor suppressor in early epithelial carcinogenesis and switches to pro-oncogenic agent during tumorigenic progression [43–45].

It has been previously suggested that loss of TGF-β growth-inhibitory effects are not attributed to either loss of TGF-βRI and TGF-βRII expression [46] or mutational inactivation of these two receptors which is a very rare event in breast cancer [47–49]. Our findings, consistent with previous reports based on immunohistochemical evaluations [46], indicate reduced TGF-βRII mRNA expression with increasing tumor grade. Moreover, TGF-βRI transcript levels are not significantly different among breast cancer tissues of differing grade, in agreement with TGF-βRI protein evaluating reports [46]. Thus, diminished responsiveness to TGF-β is not likely to be a consequence of defective TGF-βRI or TGF-βRII expression according to our evaluation which suggests that signal transduction pathways

downstream of the TGF-β receptors may be responsible for defective TGF-β signalling.

Our evaluation provides evidence of a strong hormonal influence of TGF-β mRNA expression by both Estrogen and Progesterone receptors (ER, PR). Specifically TGF-β2 and TGF-β3 mRNA levels were significantly elevated in ER positive breast tumor tissues whereas TGF-βRII displayed a similar increase in normal tissues (Table 4). These data along with the fact that transcript levels of TGF-β2, TGF-β3 and TGF-βRIII were positively correlated with PR status, reinforces previous findings indicating the existence a cross-talk between TGF-β signalling and steroid hormone receptors that seems to control growth and differentiation processes in breast carcinogenesis [50]. Since loss of ER has been associated with loss of TGF-β inhibitory effects, elucidation of the exact mechanism that mediates ER and TGF-β interaction as well as TGF-β signal transduction could influence the choice of an appropriate therapeutic approach.

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

4.4. mRNA co-expression analysis pair wise

TGF-beta signalling is activated by the binding and bringing together of the two receptors TGF-βRI and TGF-βRII by one of the TGF-β ligands (β1, β2, β3). TGF-βRI and TGF-βRII are transmembrane kinases that form a heterotetrameric complex when brought together by a TGF-β ligand. TGF-βRIII receptor is a membrane-anchored proteoglycan lacking a kinase activity thus cannot mediate signal transduction. TGF-βRIII binds to all three TGF-β ligands (β1, β2, β3) and facilitates access to the signalling receptors. In case that TGF-βRII expression or its binding affinity is either reduced or defective in a system, TGF-βRIII forms a TGF-βRIII/TGF-β/TGF-βRII complex and expedites access to the signalling receptors. Loss or reduction of expression of the signalling receptors is associated with reduced responsiveness to the TGF-β tumor inhibitory effects [51–53].

In the normal specimen group we observed a positive correlation between VEGF and FGF2 mRNA expression, as expected since FGF2 is known to be an inducer of VEGF. Moreover, VEGF was co-expressed with TGF-β1 and TGF-β2 as a consequence of the need for counteraction of VEGF angiogenic activity by TGF-β growth inhibitory effects. All three TGF-β isoforms were positively correlated suggesting that it is possible that all ligands (each at a time) actively take part in the heterotetrameric complex with the signalling receptors in these settings. Furthermore the significant co-expression of TGF-β1 and TGF-β2 with TGF-βRII is consistent with our knowledge of the mechanism that initiates TGF-β signal transduction through the surface receptors. However in the group of tumor tissues the co-expression profile was substantially altered. Surprisingly transcript levels of TGF-βRII were no longer correlated with any TGF-β isoforms. Moreover, the significant TGF-βRIII mRNA co-expression with TGF-β2 and TGF-β3 observed, reveals that TGF-βRIII could comprise an essential requirement for the bringing together of the two signalling receptors in these systems by mediating TGF-β ligands' access and binding to TGF-βRII. The latter is reinforced by our finding that TGF-β1 protein levels exhibited a positive correlation with TGF-betaRIII mRNA in tumors instead of TGF-βRII as demonstrated by our results in adjacent normal tissues. The substantial role of TGF-βRIII in signalling activation in malignant tissues has also been observed by our group in cervical carcinogenesis [32]. These findings suggest a disruption of the mRNA co-expression profile of the angiogenic factors that we have studied, in the group of tumor specimens.

4.5. Breast cancer tissue-pair analysis

Breast cancer tissue-pair analysis demonstrated decreasing TGF-β1 mRNA expression in respect to increasing tumor grade but these findings were not confirmed by the corresponding results at the protein level. TGF-β1 protein levels were inversely correlated to TGF-β1 mRNA expression values suggesting that post-transcriptional regulation of TGF-β1 may occur in agreement with previous in vitro studies [45,54,55].

The significant positive correlation between VEGF and TGF-β2 mRNA levels revealed by co-expression analysis, suggests that TGF-β mRNA increases in breast malignant transformation, however it is unclear if this happens as a need for counteracting the growth

promoting action of VEGF, or TGF-β has switched its role to tumor promoter in each specific case. Clearly TGF-beta's role in breast carcinogenesis is highly sophisticated and the mechanism that underlies the opposing roles of TGF-β depends on disease stage as well as context and relative amounts of ligands and receptors that mediate signal transduction.

Our results give indirect evidence that the dysregulation of growth factor mRNA expression may be involved in the breast tumorigenic process. Additionally, disruption of co-expression patterns of the factors included in this study, in cancer specimen groups compared to controls, suggests a transcriptional deregulation during breast cancer development. Further studies are needed to elucidate the potential use of mRNA expression profiles of angiogenic or other factors as progression indicators in breast carcinogenesis.

Acknowledgements

Dr Giannoula Soufla was funded by the 'Propontis Foundation'.

References

[1] J. Folkman, Y. Shing, Angiogenesis, J. Biol. Chem. 267 (1992) 10931–10934.
[2] J. Folkman, What is the evidence that tumors are angiogenesis dependent, J. Natl Cancer Inst. 82 (1990) 4–6.
[3] P. Carmeliet, R.K. Jain, Angiogenesis in cancer and other diseases, Nature 407 (2000) 249–257.
[4] G. Gasparini, Clinical significance of determination of surrogate markers of angiogenesis in breast cancer, Crit. Rev. Oncol. Hematol. 37 (2001) 97–114.
[5] G. Bergers, L.E. Benjamin, Tumorigenesis and the angiogenic switch, Nat. Rev. Cancer 3 (2003) 401–410.
[6] L.F. Brown, B. Berse, R.W. Jackman, K. Tognazzi, A.J. Guidi, H.F. Dvorak, et al., Expression of vascular permeability factor (vascular endothelial growth factor) and its receptors in breast cancer, Hum. Pathol. 26 (1995) 86–91.
[7] C.A. Boocock, D.S. Charnock-Jones, A.M. Sharkey, J. McLaren, P.J. Barker, K.A. Wright, et al., Expression of vascular endothelial growth factor and its receptors flt and KDR in ovarian carcinoma, J. Natl Cancer Inst. 87 (1995) 506–516.
[8] G.M. Abu-Jawdeh, J.D. Faix, J. Niloff, K. Tognazzi, E. Manseau, H.F. Dvorak, et al., Strong expression of vascular permeability factor (vascular endothelial growth factor) and its receptors in ovarian borderline and malignant neoplasms, Lab. Invest. 74 (1996) 1105–1115.
[9] A.J. Guidi, G. Abu-Jawdeh, K. Tognazzi, H.F. Dvorak, L.F. Brown, Expression of vascular permeability factor (vascular endothelial growth factor) and its receptors in endometrial carcinoma, Cancer 78 (1996) 454–460.
[10] L.F. Brown, B. Berse, R.W. Jackman, K. Tognazzi, E.J. Manseau, H.F. Dvorak, et al., Increased expression of vascular permeability factor (vascular endothelial growth factor) and its receptors in kidney and bladder carcinomas, Am. J. Pathol. 143 (1993) 1255–1262.
[11] P. Macchiarini, G. Fontanini, M.J. Hardin, F. Squartini, C.A. Angeletti, Relation of neovascularisation to metastasis of non-small-cell lung cancer, Lancet 340 (1992) 145–146.
[12] V. Ludovini, A. Sidoni, L. Pistola, G. Bellezza, V. De Angelis, S. Gori, et al., Evaluation of the prognostic role of vascular endothelial growth factor and microvessel density in stages I and II breast cancer patients, Breast Cancer Res. Treat. 81 (2003) 159–168.
[13] E.M. Howard, S.K. Lau, R.H. Lyles, G.G. Birdsong, T.S. Tadros, J.N. Umbreit, et al., Correlation and expression of p53, HER-2, vascular endothelial growth factor (VEGF), and e-cadherin in a high-risk breast-cancer population, Int. J. Clin. Oncol. 9 (2004) 154–160.
[14] D.H. Zhang, M. Salto-Tellez, L.L. Chiu, L. Shen, E.S. Koay, Tissue microarray study for classification of breast tumors, Life Sci. 73 (2003) 3189–3199.
[15] L. Ryden, B. Linderholm, N.H. Nielsen, S. Emdin, P.E. Jonsson, G. Landberg, Tumor specific VEGF-A and VEGFR2/KDR protein are co-expressed in breast cancer, Breast Cancer Res. Treat. 82 (2003) 147–154.
[16] F. Gomez-Esquer, D. Agudo, F. Martinez-Arribas, M.J. Nunez-Villar, J. Schneider, mRNA expression of the angiogenesis markers VEGF and CD105 (endoglin) in human breast cancer, Anticancer Res. 24 (2004) 1581–1585.
[17] M. Stimpfl, D. Tong, B. Fasching, E. Schuster, A. Obermair, S. Leodolter, et al., Vascular endothelial growth factor splice variants and their prognostic value in breast and ovarian cancer, Clin. Cancer Res. 8 (2002) 2253–2259.
[18] T. Nakamoto, C.S. Chang, A.K. Li, G.W. Chodak, Basic fibroblast growth factor in human prostate cancer cells, Cancer Res. 52 (1992) 571–577.
[19] L.I. Gold, B. Saxena, K.R. Mittal, M. Marmor, S. Goswami, L. Nactigal, et al., Increased expression of transforming growth factor beta isoforms and basic fibroblast growth factor in complex hyperplasia and adenocarcinoma of the endometrium: evidence for paracrine and autocrine action, Cancer Res. 54 (1994) 2347–2358.
[20] Y. Yamanaka, H. Friess, M. Buchler, H.G. Beger, E. Uchida, M. Onda, et al., Overexpression of acidic and basic fibroblast growth factors in human pancreatic cancer correlates with advanced tumor stage, Cancer Res. 53 (1993) 5289–5296.
[21] Y. Motoo, N. Sawabu, Y. Nakanuma, Expression of epidermal growth factor and fibroblast growth factor in human hepatocellular carcinoma: an immunohistochemical study, Liver 11 (1991) 272–277.

[22] C. Dickson, B. Spencer-Dene, C. Dillon, V. Fantl, Tyrosine kinase signalling in breast cancer: fibroblast growth factors and their receptors, Breast Cancer Res. 2 (2000) 191–196.
[23] J. Massague, S. Cheifetz, M. Laiho, D.A. Ralph, F.M. Weis, A. Zentella, Transforming growth factor-beta, Cancer Surv. 12 (1992) 81–103.
[24] J. Massague, L. Attisano, J.L. Wrana, The TGF-beta family and its composite receptors, Trends Cell Biol. 4 (1994) 172–178.
[25] J. Massague, TGF-beta signal transduction, Annu. Rev. Biochem. 67 (1998) 753–791.
[26] A. Hata, Y. Shi, J. Massague, TGF-beta signaling and cancer: structural and functional consequences of mutations in Smads, Mol. Med. Today 4 (1998) 257–262.
[27] L.H. Hartwell, M.B. Kastan, Cell cycle control and cancer, Science 266 (1994) 1821–1828.
[28] E. Christeli, V. Zoumpourlis, H. Kiaris, M. Ergazaki, S. Vassilaros, D.A. Spandidos, TGF-β1 overexpression in breast cancer: correlation with clinicopathological data, Oncol. Rep. 3 (1996) 1115–1118.
[29] G.D. Shipley, M.R. Pittelkow, J.J. Wille, R.E. Scott, H.L. Moses, Reversible inhibition of normal human prokeratinocyte proliferation by type beta transforming growth factor-growth inhibitor in serum-free medium, Cancer Res. 46 (1986) 2068–2071.
[30] C.C. Bascom, J.R. Wolfshohl, R.J. Coffey, L. Madisen, N.R. Webb, A.R. Purchio, et al., Complex regulation of transforming growth factor beta 1, beta 2, and beta 3 mRNA expression in mouse fibroblasts and keratinocytes by transforming growth factors beta 1 and beta 2, Mol. Cell. Biol. 9 (1989) 5508–5515.
[31] S. Hazelbag, A. Gorter, G.G. Kenter, L. van den Broek, G. Fleuren, Transforming growth factor-beta1 induces tumor stroma and reduces tumor infiltrate in cervical cancer, Hum. Pathol. 33 (2002) 1193–1199.
[32] G. Soufla, S. Sifakis, S. Baritaki, A. Zafiropoulos, E. Koumantakis, D.A. Spandidos, VEGF, FGF2, TGF-BETA1 and TGF-BETAR1 mRNA expression levels correlate with the malignant transformation of the uterine cervix, Cancer Lett 221 (2005) 105–118.
[33] P.A. Scott, K. Smith, R. Poulsom, A. De Benedetti, R. Bicknell, A.L. Harris, Differential expression of vascular endothelial growth factor mRNA vs protein isoform expression in human breast cancer and relationship to eIF-4E, Br. J. Cancer 77 (1998) 2120–2128.
[34] S.W. Kim, S. Park, S. Ahn, K.W. Chung, W.K. Moon, J.G. Im, et al., Identification of angiogenesis in primary breast carcinoma according to the image analysis, Breast Cancer Res. Treat. 74 (2002) 121–129.
[35] M. Toi, K. Inada, H. Suzuki, T. Tominaga, Tumor angiogenesis in breast cancer: its importance as a prognostic indicator and the association with vascular endothelial growth factor expression, Breast Cancer Res. Treat. 36 (1995) 193–204.
[36] S.Y. Anandappa, J.H. Winstanley, S. Leinster, B. Green, P.S. Rudland, R. Barraclough, Comparative expression of fibroblast growth factor mRNAs in benign and malignant breast disease, Br. J. Cancer 69 (1994) 772–776.
[37] Y.A. Luqmani, M. Graham, R.C. Coombes, Expression of basic fibroblast growth factor, FGFR1 and FGFR2 in normal and malignant human breast, and comparison with other normal tissues, Br. J. Cancer 66 (1992) 273–280.
[38] C. Yiangou, J.J. Gomm, R.C. Coope, M. Law, Y.A. Luqmani, S. Shousha, et al., Fibroblast growth factor 2 in breast cancer: occurrence and prognostic significance, Br. J. Cancer 75 (1997) 28–33.
[39] R. Colomer, J. Aparicio, S. Montero, C. Guzman, L. Larrodera, H. Cortes-Funes, Low levels of basic fibroblast growth factor (bFGF) are associated with a poor prognosis in human breast carcinoma, Br. J. Cancer 76 (1997) 1215–1220.
[40] K. Smith, S.B. Fox, R. Whitehouse, M. Taylor, M. Greenall, J. Clarke, et al., Upregulation of basic fibroblast growth factor in breast carcinoma and its relationship to vascular density, oestrogen receptor, epidermal growth factor receptor and survival, Ann. Oncol. 10 (1999) 707–713.
[41] M. Relf, S. LeJeune, P.A. Scott, S. Fox, K. Smith, R. Leek, et al., Expression of the angiogenic factors vascular endothelial cell growth factor, acidic and basic fibroblast growth factor, tumor growth factor beta-1, platelet-derived endothelial cell growth factor, placenta growth factor, and pleiotrophin in human primary breast cancer and its relation to angiogenesis, Cancer Res. 57 (1997) 963–969.
[42] K.E. De Visser, W.M. Kast, Effects of TGF-beta on the immune system: implications for cancer immunotherapy, Leukemia 13 (1999) 1188–1199.
[43] B. Tang, M. Vu, T. Booker, S.J. Santner, F.R. Miller, M.R. Anver, et al., TGF-beta switches from tumor suppressor to prometastatic factor in a model of breast cancer progression, J. Clin. Invest. 112 (2003) 1116–1124.
[44] L.M. Wakefield, Y.A. Yang, O. Dukhanina, Transforming growth factor-beta and breast cancer: lessons learned from genetically altered mouse models, Breast Cancer Res. 2 (2000) 100–106.
[45] J.R. Benson, Role of transforming growth factor beta in breast carcinogenesis, Lancet Oncol. 5 (2004) 229–239.
[46] M.B. Buck, P. Fritz, J. Dippon, G. Zugmaier, C. Knabbe, Prognostic significance of transforming growth factor beta receptor II in estrogen receptor-negative breast cancer patients, Clin. Cancer Res. 15 (2004) 491–498.
[47] J. Barlow, D. Yandell, D. Weaver, T. Casey, K. Plaut, Higher stromal expression of transforming growth factor-beta type II receptors is associated with poorer prognosis breast tumors, Breast Cancer Res. Treat. 79 (2003) 149–159.
[48] R. Anbazhagan, H. Fujii, E. Gabrielson, Microsatellite instability is uncommon in breast cancer, Clin. Cancer Res. 5 (1999) 839–844.
[49] C.D. Lucke, A. Philpott, J.C. Metcalfe, A.M. Thompson, L. Hughes-Davies, P.R. Kemp, et al., Inhibiting mutations in the transforming growth factor beta type 2 receptor in recurrent human breast cancer, Cancer Res. 15 (2001) 482–485.
[50] T. Matsuda, T. Yamamoto, A. Muraguchi, F. Saatcioglu, Cross-talk between transforming growth factor-beta and estrogen receptor signaling through Smad3, J. Biol. Chem. 16 (2001) 42908–42914.

[51] M. Laiho, M.B. Weis, J. Massague, Concomitant loss of transforming growth factor (TGF)-beta receptor types I and II in TGF-beta-resistant cell mutants implicates both receptor types in signal transduction, J. Biol. Chem. 265 (1990) 18518–18524.

[52] T.Y. Chu, J.S. Lai, C.Y. Shen, H.S. Liu, C.F. Chao, Frequent aberration of the transforming growth factor-beta receptor II gene in cell lines but no apparent mutation in pre-invasive and invasive carcinomas of the uterine cervix, Int. J. Cancer 80 (1999) 506–510.

[53] J.F. DeCoteau, P.I. Knaus, H. Yankelev, M.D. Reis, R. Lowsky, H.F. Lodish, et al., Loss of functional cell surface transforming growth factor beta (TGF-beta) type 1 receptor correlates with insensitivity to TGF-beta in chronic lymphocytic leukemia, Proc. Natl Acad. Sci. USA 94 (1997) 5877–5881.

[54] J.R. Benson, A.A. Colletta, Transforming growth factor β-prospect for cancer prevention and treatment, Clin. Immunother. 4 (1995) 249–258.

[55] C. Knabbe, M.E. Lippman, L.M. Wakefield, K.C. Flanders, A. Kasid, R. Derynck, et al., Evidence that transforming growth factor-beta is a hormonally regulated negative growth factor in human breast cancer cells, Cell 13 (1987) 417–428.