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mRNA Expression of the Angiogenesis Markers VEGF and CD105 (Endoglin) in Human Breast Cancer

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Abstract. *Background: Both VEGF and CD105 (endoglin) have been identified as markers of tumor angiogenesis and prognosis in breast cancer. They have always been studied in this kind of tumor by means of immunological methods. Objective: To study, by means of reverse-transcription polymerase chain reaction (RT-PCR), the expression of VEGF and CD105 (endoglin) at the mRNA level in a series of breast cancers and to correlate the results obtained with all available clinical and biological features of the tumors. Materials and Methods: Fresh tumor tissue from 103 previously untreated breast cancer patients was studied for VEGF and CD105 (endoglin) expression. In addition, the following parameters were determined in all tumors: DNA ploidy by means of flow cytometry; hormone receptor (ER & PR), Ki67, c-erb-B2 and p53 expression by means of immunohistochemistry; and h-MAM (mammaglobin) expression by means of RT-PCR. Classical prognostic parameters of the tumors, such as histological and nuclear grade or axillary lymph node invasion, were also included into the statistical analysis. Results: VEGF mRNA expression levels above the 25th percentile were significantly ($p<0.05$) associated with high proliferation (Ki67>10%) and aneuploidy of the tumors and inversely with estrogen receptor expression ($p<0.01$). CD105 (endoglin) mRNA expression levels above the 25th percentile only correlated significantly with nuclear grade 3 ($p<0.05$). The expression of both genes did not correlate with each other. Conclusion: VEGF mRNA expression levels seem to be directly associated with VEGF functions at the protein level, whereas this seems not to be the case for CD105 (endoglin) mRNA levels.*

Angiogenesis is one of the fundamental steps of the oncogenic cascade. It is indispensable for tumor growth and also for the establishment and growth of metastasis. It is most probably triggered by hypoxia and a number of known factors are involved in its regulation. Among these, one of the best studied is VEGF (vascular endothelial growth factor).

VEGF itself is induced by hypoxia and, in its turn, selectively acts on endothelial cells, which are the only ones to possess its specific receptor, VEGFR2. Tumor cells, among them breast cancer cells, but also breast stromal cells, are known to overexpress VEGF. The steroid hormones, most notably the estrogens, regulate VEGF expression, although the exact mechanism of this hormonal regulation is still controversial (1-3).

Different isoforms of VEGF have been described, termed VEGF A to D, although it seems that VEGF-A is the one implicated in tumor angiogenesis, whereas the other forms act upon the lymphatic endothelium. Thus, VEGF-A is commonly meant when dealing with VEGF in the context of tumor biology (3). VEGF expression has been studied in breast cancer both by means of ELISA and immunohistochemistry (microvessel density) and has been shown to correlate with poor prognosis and mutant p53 expression (4,5).

CD105 (endoglin), on the other hand, is also induced by hypoxia in activated endothelial cells, where it acts as a receptor for transforming growth factor (TGF) beta1 and beta3 (6). Endoglin expression has also been studied in breast cancer in an analogous manner to VEGF and has been identified as an independent prognostic marker (7,8). Moreover, CD105 circulating levels in the plasma of breast cancer patients have also been studied and shown to correlate with the presence of metastasis (9). However, despite its proven prognostic potential, the precise role of endoglin in breast cancer is less well defined than that of VEGF at the present time.

Studies of VEGF and CD105 at the mRNA level in breast cancer are lacking, most probably due to the excellent clinical experience with monoclonal antibodies against both markers. However, from a conceptual point of view, such studies are potentially interesting. Transcription is the first step towards protein synthesis and many valuable tumor markers are known to be subject to posttranscriptional regulation, whereas in other cases, mRNA expression dierctly correlates with the level of

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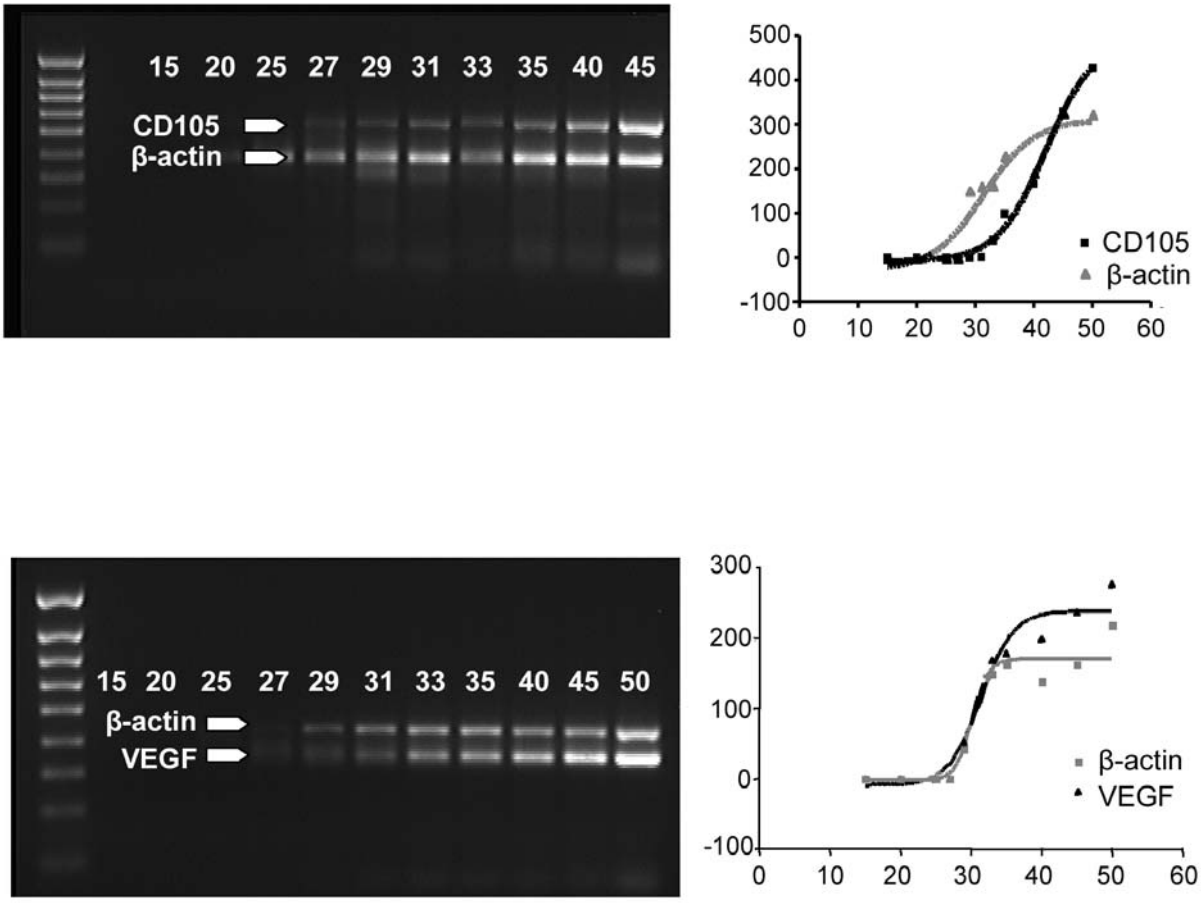

Figure 1. *Saturation curves. Determination of the exponential range of amplification for CD105 and VEGF.*

translation and therefore the ultimate level of protein expression. In these cases, techniques such as RT-PCR or quantitative PCR allow the measurement of the latter with a great level of precision and quantification of the expression, avoiding the sources of error (mainly cross-reactivity between epitopes) and subjectivity inherent in many immunological procedures, such as immunohistochemistry. With this in mind, we devised the present investigation. In it we studied, by means of RT-PCR, the expression of VEGF- and CD105-mRNA in a series of breast cancers and have correlated them with all available clinical and biological features of the studied tumors.

Materials and Methods

Fresh tumor tissue from 103 previously untreated breast cancer patients operated upon at Fundacion Tejerina, Madrid, Spain, was obtained at the time of surgery and immediately snap-frozen or immersed in RNA*later*™ (AMBION Inc., Austin, Texas, USA).

The histologies of the tumors, which were all invasive carcinomas, were as follows: ductal infiltrating, 84; lobular infiltrating, 16; tubular, 3.

In addition to measuring VEGF and CD105 expression, the following parameters were also determined in all tumors: DNA-ploidy by means of flow cytometry; hormone receptor (ER & PR), Ki67, c-erb-B2 and p53 expression by means of immunohistochemistry; and h-MAM (mammaglobin) expression, shown by us recently (10) to be a marker for a less aggressive phenotype in breast cancer, by means of differential RT-PCR. All the procedures employed were exactly the same as described in other papers by our same group, and the reader is referred to them for details (11-13). Finally, conventional features of the tumors, such as histological and nuclear grade or axillary lymph node invasion, were also included into the statistical analysis.

RT-PCR. As stated above, the RNA was extracted from the tumor specimens using the Rneasy™ commercial kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions. The total RNA content was immediately quantified in a spectrophotometer (SmartSpec 3000™ spectrophotometer, BioRad, Hercules, CA, USA) after 1:50 dilution in RNAase-free water and the RNA was then frozen at -80˚C until further use. The mRNA concentration corresponding to each sample was diluted to 10 ng/ml and 1 ml was used for each reaction. The RT-PCR reaction was carried out in an iCycler™ thermal cycler, also from BioRad, using a commercial one-step RT-PCR kit

Table I. *Reaction components for one-step RT-PCR.*

VEGF	
Component	Final concentration
5x QIAGEN OneStep RT-PCR Buffer	1x
dNTP Mix	400 μM of each dNTP
Primer VEGFa	0.6 μM
Primer VEGFb	0.6 μM
Primer ACTIN1	0.22 μM
Primer ACTIN2	0.22 μM
QIAGEN OneStep RT-PCR Enzyme mix	2 μl
Template RNA	10 ng

CD105	
Component	Final concentration
5x QIAGEN OneStep RT-PCR Buffer	1x
dNTP Mix	400 μM of each dNTP
Primer CD105A	0.6 μM
Primer CD105B	0.6 μM
Primer ACTIN1	0.21 μM
Primer ACTIN2	0.21 μM
QIAGEN OneStep RT-PCR Enzyme mix	2 μl
Template RNA	10 ng

Table II. *Primers used in this study.*

Primer	Sequence	Product (bp)
VEGF		
VEGFa	5'-CTCCACCATGCCAAGTGGTC-3'	346
VEGFb	5'-TTGGTCTGCATTCACATTTG-3'	346
CD105		
CD105A	5'-CTTGGCCTACAATTCCAGCC-3'	542
CD105B	5'-CTTGAGGTGTGTCTGGGAGC-3'	542
β-actin		
ACTIN1	5'-CGGATGTCCACGTCACACT-3'	427
ACTIN2	5'-CCACTGGCATCGTGATGGA-3'	427

(QIAGEN). The number of extension cycles of the PCR reaction was adjusted, so that it was within the ascending slope of the saturation curve, which was calculated both for tumor and normal tissue (Figure 1). The primer concentration of the target and the internal control gene (β-actin in this case) was accordingly adjusted, so that the final band densities were similar and both reactions were within the ascending slope of the respective saturation curves using the same number of extension cycles. This allowed for the reactions to be carried out in a single tube. A second tube with the same components, save for the reverse transcriptase, was run in parallel with each reaction, in order to exclude an amplification of a pseudogene from contaminating DNA. The concentrations of the different components are summarized in Table I. The primers used and the conditions of the RT-PCR are shown in Tables II and III, respectively.

Table III. *Thermal cycler conditions.*

Step	Temperature	Time (min)
Reverse transcription	50˚C	30
Initial PCR activation step	95˚C	15
PCR amplification		
Denaturation	94˚C	0.5
Annealing	50˚C	0.5
Extension	72˚C	1
Number of cycles	35 (VEGF) 32 (CD105)	
Final extension	72˚C	10

Table IV. *Correlation of VEGF and CD105 (endoglin) mRNA expression above the 25th percentile with other clinical and biological parameters of breast cancer in the series of tumors with complete available data. n=103.*

	VEGF		CD105 (endoglin)	
	r	*p*	r	*p*
Ki67 > 10%	0.21	<0.05	n.s.	n.s.
Aneuploidy	0.19	<0.05	n.s.	n.s.
Histological grade 3	n.s.	n.s.	n.s.	n.s.
Nuclear grade 3	n.s.	n.s.	0.22	<0.05
Estrogen receptors	-0.29	<0.01	n.s.	n.s.
Progesterone receptors	-0.18	0.07	n.s.	n.s.
c-erb-B2	n.s.	n.s.	n.s.	n.s.
P53	n.s.	n.s.	n.s.	n.s.
h-MAM	n.s.	n.s.	n.s.	n.s.
Axillary metastasis	n.s.	n.s.	n.s.	n.s.
Histological variety	n.s.	n.s.	n.s.	n.s.

Quantification. In order to quantify the expression levels of the target genes, 1.5% agarose gels were run, loading each lane with 10 μl of RT-PCR product, for 2 h at 100 V. They were then analyzed in a UV-transilluminator mini darkroom™ (UVP, Upland, CA, USA), by means of the Labworks analysis software™ (also from UVP). The band density corresponding to the target gene, divided by the one of the internal control genes, gives a figure in arbitrary units which reflects the relative level of expression of the former. The sequence verification of the RT-PCR products was carried out on an automated sequencer ABI PrismTM 377 and 3730 DNA Analyzer (Applied Biosystems).

Statistics. Correlations between continuous variables were calculated using Pearson's test and, in case of dichotomous variables, by means of the Spearman rank correlation test. All tests were carried out using the GraphPad Prism biomedical statistical package (GraphPad Software, Inc., San Diego, CA, USA). Values were considered significant when p was < 0.05.

Results

Reverse transcription and amplification was successful for all cases of VEGF and failed in one for CD105 (endoglin).

VEGF mRNA expression levels above the 25th percentile of the distribution of values for the whole set were significantly associated in a direct way with high proliferation (Ki67-labeling index > 10%) and aneuploidy of the tumors and, inversely, with estrogen receptor expression by them. According to the manufacturer's information (Novocastra, Newcastle, UK), the monoclonal antibody employed for the immunohistochemical detection of estrogen receptor expression in the studied tumors (clone 6-F11) selectively recognizes the estrogen alpha receptor, so that the inverse correlation detected pertains to this isoform of the receptor. An inverse correlation between VEGF mRNA expression and progesterone receptor expression was also found, but did not attain statistical significance ($p=0.07$).

CD105 (endoglin) mRNA expression levels above the 25th percentile, on the other hand, only correlated significantly with nuclear grade 3. All these results are summarized in Table IV. Surprisingly, VEGF and endoglin mRNA expression levels did not correlate with each other, although both encode for proteins related to angiogenesis, according to all previous publications.

Discussion

As was mentioned in the introduction, both VEGF and CD105 (endoglin) expressions have been studied previously in breast cancer, mainly by means of immunological methods (immunohistochemistry and ELISA) (4-8). According to these previous reports, VEGF overexpression not only correlated with an overall worse prognosis of the patients, but also with other biological features defining aspects of this worse prognosis, such as tumor size or p53 status. Our results regarding VEGF mRNA expression agree, in general, with these previous ones obtained at the protein level. In fact, we found that VEGF mRNA expression above the 25th percentile of the distribution significantly correlated with a higher proliferation and aneuploidy of the tumors. All these factors (tumor size, proliferation, ploidy and, to a large extent, also p53) are partial aspects of one common event, namely tumor growth, of which neoangiogenesis is an essential part. Although we did not find a significant correlation between tumor size or p53 status and VEGF mRNA expression, there was a trend towards significance in the statistical analysis, which would have made itself evident with a larger series of cases or if the same techniques had been employed throughout the study. Moreover, we found the strongest (inverse) correlation for VEGF with estrogen receptor-alpha levels of the tumors, something absolutely concordant with findings from previous studies on breast cancer. Indeed, Wen *et al.* (14) also found an inverse correlation between ER-alpha and VEGF, whereas the relationship with the ER-beta isoform was direct and also highly significant. Previously, Bermont *et al.* (15) had reported that, in experimental breast cancer cells, estrogens inhibited VEGF expression. In endometrial cancer xenografts, a tumor subject to hormonal modulation, like breast cancer, Ali *et al.* (1) found that transfection of the ER-alpha down-regulated VEGF expression. We also found significantly higher VEGF mRNA levels in postmenopausal patients, something already reported by Nishimura *et al.* (16), who measured plasma VEGF levels. So considered, VEGF mRNA expression seems to be directly associated to events that take place further on at the translational level and, probably, fluctuations in transcription have an immediate repercussion on the protein function. The picture seems not to be the same at all for CD105 (endoglin). In fact, the only significant correlation we found between endoglin and all other features tested was with high nuclear grade, something hardly specific (although defining a worse prognosis). Worse still, VEGF and CD105 mRNA levels did not correlate with each other, which is hard to explain. Since the retrotranscribed mRNA corresponds to CD105 (corroborated repeatedly by sequencing of the amplified product), other possible causes for this unexpected result must be sought. The most obvious explanation would be that endoglin expression is subject to important posttranscriptional regulation and, thus, mRNA levels do not reflect protein synthesis. Other, highly speculative reasons for the discordant results might be that a still undescribed splicing variant of the CD105 gene, not containing the mRNA fragment studied by us, might be the one encoding the protein normally recognized by monoclonal antibodies. Or, finally, that the monoclonal antibodies do not recognize unbound endoglin, but rather endoglin coupled to one of its substrates, TGF-beta-2 or -3 (for which endoglin acts as a receptor). This could induce a conformational change of the epitope, making it more accessible to the immunological reaction.

In conclusion, VEGF mRNA expression levels correlated with features of the tumors which are in agreement with previous reports on the biological role of VEGF. CD105 (endoglin) mRNA levels, on the other hand, did not reflect the biological features of this angiogenesis marker elicited by means of studies at the protein level. VEGF and CD105 (endoglin) levels, finally, did not correlate with each other, something without a currently plausible explanation and thus warranting further studies.

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