Vascular Endothelial Growth Factor Splice Variants and Their Prognostic Value in Breast and Ovarian Cancer

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ABSTRACT

Purpose: **Vascular endothelial growth factor (VEGF) is a promotor for tumor angiogenesis, and is known to be elevated in breast and ovarian cancers. Through alternative splicing six VEGF isoforms were identified. We studied VEGF isoform expression in breast and ovarian cancer cell lines, as well as in breast carcinomas and ovarian tumors, and correlated the expression pattern with the *in vitro* invasiveness of the breast carcinoma cell lines and the clinicopathologic characteristics of the tumors.**

Experimental Design: **Reverse transcription-PCR and automated laser fluorescence fragment analysis were used to determine the expression of each splice variant. This method allowed the detection of all of the splice variants simultaneously, especially VEGF145 for the first time in tumor tissue.**

Results: **VEGF121 and VEGF165 were the most dominantly expressed variants in all of the tumor samples and cell lines investigated. VEGF145 was very weakly or not expressed in breast and ovarian cancers. Statistical analysis showed no correlation between VEGF splice variant expression in the tumors and histological type, differentiation grade, tumor size, Fédération Internationale des Gynaecologistes et Obstetristes, and nodal status from cancer patients. There was also no correlation between the invasive capacity of breast cell lines and VEGF isoform expression.**

Conclusions: **Even though expression levels of VEGF have been shown to be important for tumor invasion and progression, the present data indicate no relation of VEGF isoform pattern with invasion and progression.**

INTRODUCTION

VEGF[2] is a highly specific mitogen for vascular endothelial cells. The expression of VEGF is potentiated in response to hypoxia, by activated oncogenes, and by a variety of cytokines. VEGF induces endothelial cell proliferation, promotes cell migration, and inhibits apoptosis. *In vivo* VEGF induces angiogenesis as well as permeabilization of blood vessels, and plays a central role in the regulation of vasculogenesis (1). Deregulated VEGF expression contributes to the development of solid tumors by promoting tumor angiogenesis (2, 3). Angiogenesis is a key factor in solid tumor growth. Tumors stimulate angiogenesis by secreting angiogenic substances that activate nearby endothelial cells to express a cell autonomous pattern of behavior that culminates in the formation of new vessels. VEGF as a potent angiogenic cytokine stimulates endothelial cells, thereby contributing to the angiogenesis of solid tumors, like breast or ovarian carcinomas (4).

VEGF is a M_r 40,000–46,000 disulfide-linked dimeric glycoprotein that dissociates on reduction into two M_r 20,000–23,000 subunits (5, 6). Six VEGF isoforms are generated as a result of alternative splicing from a single VEGF gene, consisting of 121, 145, 165, 183, 189, or 206 amino acids (7–11). The domain encoded by exons 1–5 contains information required for the recognition of the known VEGFRs KDR/flk-1 and flt-1 (12), and is present in all of the VEGF isoforms. The amino acids encoded by exon 8 are also present in all of the VEGF splice variants. The VEGF isoforms are distinguished by the presence or the absence of the peptides encoded by exons 6a, 6b, and 7 of the VEGF gene (Fig. 1). VEGF121 lacks these exons. VEGF165 contains the exon 7-encoded peptide, whereas VEGF189 contains both exon 6a and exon 7-encoded peptides (13–15). VEGF183 develops because of the usation of a conserved alternate splicing donor site within exon 6a; so a 18-bp long part from exon 6a is missing (11). VEGF145 contains exon 6a but lacks exon 7 (10). VEGF206 is the full length form containing additionally to exon 6a and exon 7 the 51-bp long part from intron 3, exon 6b, neighboring exon 6a (9).

The different VEGF isoforms differ in their heparin binding ability as well as in their binding capacity to tyrosine-kinase receptors VEGFR-1 (flt-1) and VEGFR-2 (KDR/flk-1), and to neuropilin-1 and neuropilin-2 (1, 9, 10, 16–19).

Most cell types produce several VEGF variants simultaneously. Usually the 121 and 165 isoforms are the predominant forms. VEGF121 was estimated to be more angiogenic and tumorigenic than the other isoforms (20). VEGF145 is one of

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[2] The abbreviations used are: VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; RT-PCR, reverse transcription-PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ALF, automated laser fluorescence; ATCC, American Type Culture Collection.

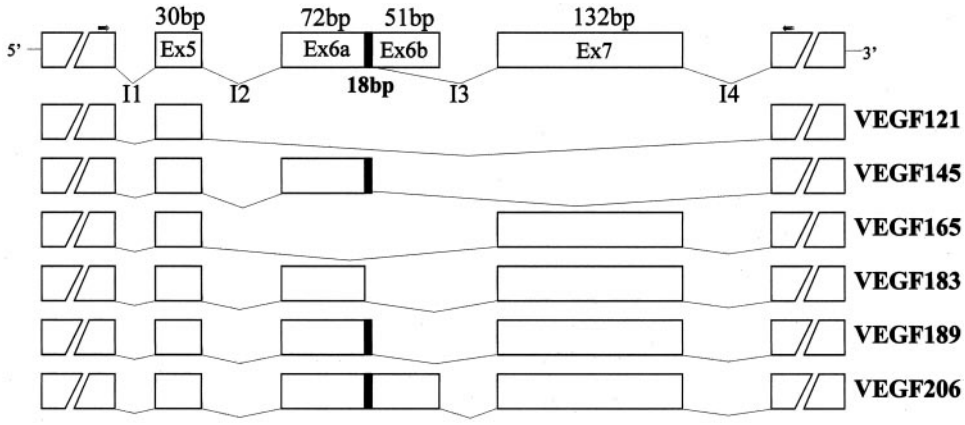

Fig. 1 Model for alternative splicing of VEGF mRNA generating six VEGF variants. Exons are represented by □, introns by *solid lines*. *Arrows* indicate the sense and antisense primers. Representation is done according to GenBank sequence and to the paper from Houck *et al.* (9).

the main VEGF isoforms expressed by several cell lines derived from carcinomas of the female reproductive system, even at levels comparable with the expression levels of VEGF165 (10). Additionally, it is expressed by 100% of human blastocysts (21). VEGF189 plays important roles in the establishment of human colon and esophageal cancer xenografts, suggesting that VEGF189 contributes to the successful xenotransplantability of various human solid tumors via augmentation of stromal vascularization (22). Recently, VEGF189 isoform expression was demonstrated to correlate with tumor angiogenesis, patient survival, and postoperative relapse in non-small cell lung cancer (23). VEGF206 is found mainly in human fetal liver library (9).

In normal ovary, cystadenoma, and carcinoma of the ovary the predominant isoforms are VEGF121, 165, and 189 in that order (24). Also in another study examining malignant ovarian cells and solid tumors, the VEGF121 and 165 isoforms were the principal products (25). Correlations with the tumor characteristics showed that VEGF165 is elevated in all of the stages of ovarian carcinoma, regardless of histopathologic type (26). In summary, only elevated levels of VEGF expression are associated with poorer survival. Other possible prognostic variables had minimal impact on survival, including age, stage, grade, cytology, and tumor size (26–28). In all of the studies elucidating ovarian carcinomas, splice variant VEGF145 was not investigated, although this isoform seems to be specific for the female reproductive system. Also, breast carcinoma is an angiogenesis-dependent tumor. Eight of nine published retrospective studies reported that VEGF is significantly associated with relapse-free survival, overall survival, or both (29). However, no correlation between the VEGF isoforms and patient history was reported.

There is still no clearness about the role of all of the splice variants. Therefore, investigations of many tumor samples investigating all of the VEGF isoforms may give more insight in the function of them. Our method of fragment analysis of fluorescently labeled PCR products allows the simultaneous detection of VEGF isoform expression. Consequently, we were able to investigate for the first time VEGF expression, including all of the known splice variants, in tumor samples from breast and ovarian cancer patients, and from breast and ovarian cancer cell lines. Specifically, it is the first report describing VEGF145 splice variant expression in tumor samples and not only in cultured cell lines derived from carcinomas of the female reproductive system.

MATERIALS AND METHODS

Cell Lines. Ovarian cancer cell lines ES-2, PA-1, NIH: OVCAR-3, and SK-OV-3 were purchased from the ATCC (Rockville, MD), A2780 and A2780 ADR were purchased from the European Collection of Cell Cultures (Salisbury, United Kingdom). Breast cancer cell lines BT-549, Hs 578T, BT-474, MDA-MB-231, MDA-MB-157, MDA-MB-436, BT-20, MDA-MB-435s, MDA-MB-361, MDA-MB-175-VII, UACC-893, SK-BR-3, ZR-75–30, BT-483, CAMA-1, T-47D, DU4475, ZR-75-1, UACC-812, MCF-7, MDA-MB-453, MDA-MB-134-VI, and MDA-MB-468 were purchased from the ATCC. The breast epithelial cell line HBL100 derived from the milk of a nursing mother, MCF-12F, and MCF-12A were established from mammary tissue from a patient with fibrocystic breast disease. They were purchased from the ATCC. All of the cell lines were cultured according to the ATCC and European Collection of Cell Cultures instructions.

Patients. Ninety-one samples from patients with ovarian tumors (67 ovarian carcinomas and 24 cystadenomas) and 165 tumor specimens from patients with breast carcinoma were investigated.

RT-PCR Analysis. Total RNA was extracted by isopyknic centrifugation as described previously (30). For reverse transcription, 0.5 μg of RNA was incubated at 75°C in a volume of 6.5 μl for 10 min before adding the RT master mix: 20 units of placental RNase inhibitor, 10 μl random-primed reverse transcriptase-mix (ViennaLab, Vienna, Austria), and 200 units of Moloney murine leukemia virus reverse transcriptase (ViennaLab) in a volume of 25 μl. The mixture was incubated for 60 min at 37°C and terminated by 95°C for 10 min. cDNA (1 μl) was used as a template for PCR in a total volume of 25 μl. The PCR reaction mixture included 2.5 μl 10× amplification buffer [10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.01% w/v gelatin, 1.5 mM $MgCl_2$, and 0.1% Triton X-100; ViennaLab], 5 pmol sense and 5 pmol antisense primer, 125 μM deoxynucleotide triphosphates (ViennaLab), and 0.8 unit super Taq polymerase (HT Biotechnology Ltd., Cambridge, United Kingdom).

Table 1 VEGF isoform expression in breast cell lines[a]

Cell line	VEGF121	VEGF145	VEGF165	VEGF183	VEGF189
BT-20	48.1%	0	45.0%	0.9%	6.0%
BT-474	47.1%	0.8%	35.0%	2.7%	14.4%
BT-483	70.6%	0	29.4%	0	0
BT-549	51.7%	0	39.7%	1%	7.6%
CAMA-1	47.9%	0.3%	43.2%	1.5%	7.1%
DU4475	53.1%	0	46.9%	0	0
HS 578T	54.7%	0.8%	28.4%	1.8%	14.3%
MCF-7	49.2%	1.8%	40.7%	1.3%	7.0%
MDA-MB-134-VI	47.7%	0	52.3%	0	0
MDA-MB-157	53.5%	0	40.2%	0.6%	5.6%
MDA-MB-175-VII	45.3%	0	48.8%	0	5.9%
MDA-MB-231	45.7%	0	44.9%	1.2%	8.2%
MDA-MB-361	48.6%	0	43.7%	1.1%	6.6%
MDA-MB-435s	81.1%	0	18.9%	0	0
MDA-MB-436	54.5%	0	39.5%	0	6.0%
MDA-MB-453	64.5%	0.9%	34.6%	0	0
MDA-MB-468	75.5%	0	24.5%	0	0
SK-BR-3	49.4%	0	42.8%	0.9%	6.9%
T-47D	54.9%	0.4%	40.2%	0	4.5%
UACC-812	67.7%	0	24.8%	0	7.5%
UACC-893	49.4%	0	40.6%	1.0%	9.0%
ZR-75-1	80.5%	0	0	0	19.5%
ZR-75-30	69.2%	0	25.6%	0	5.2%
HBL-100	58.1%	0	26.6%	1.8%	13.5%
MCF-12F	82.3%	0	17.7%	0	0
MCF-12A	72.1%	0	27.9%	0	0

[a] Percentage of overall expression within one cell line determined by ALF analysis.

The antisense primer was fluorescently labeled at the 5′end with Cy5. The PCR was performed on a Perkin-Elmer GeneAmp PCR system 9600 with 40 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 90 s. All of the reactions were preceded by a primary denaturation step at 94°C for 1 min and terminated by 72°C for 7 min. PCR products were resolved on a 6% polyacrylamide gel using an automated laser fluorescent sequencer (ALF express DNA Sequencer; Pharmacia, Uppsala, Sweden). Sizes of the fragments were calculated using an external standard and the Fragment Manager TM Software (Pharmacia).

Primers. VEGF primers were designed according to the literature (31): sense 5′-ATGAACTTTCTGCTGTCTTGGGT-3′ and antisense 5′-TCACCGCCTCGGCTTGTCAC-3′. GAPDH primers were: sense 5′-GAAGGTGAAGGTCGGAGTC-3′ and antisense 5′-ATGAGTCCTTCCACGATAC-3′, resulting in a 516-bp product.

Statistic. Pearson's χ^2 test was performed to calculate correlations. For the analysis of the correlation between nonparametric data, the Mann-Whitney *t* test was used. The ratios VEGF121:165, VEGF121:189, and VEGF165:189 were calculated by comparing relative peak areas from ALF analysis that are equivalent to the used amounts of PCR products.

RESULTS

Primers span all of the exons, so that all of the possible splice variants could be analyzed by RT-PCR reaction and ALF analysis, resulting in 444, 516, 576, 630, 648, and 699 bp fragments corresponding to VEGF121, 145, 165, 183, 189, and 206, respectively. All of the splice variants except VEGF206 were found to be expressed in the samples analyzed. Among the subtypes of VEGF, VEGF121 and VEGF165 were dominant in all of the specimens investigated, followed by VEGF189, VEGF183, and VEGF145. VEGF206 was not detected.

Breast Carcinomas and Cell Lines. Among 26 breast cell lines 23 showed higher VEGF121 expression compared with VEGF165. One cell line only expressed VEGF121 and not VEGF165 (ZR-75-1). Additionally, a correlation was seen between the expression of VEGF183 and VEGF189 ($P = 0.002$; $\chi^2 = 9.90$), *i.e.*, in 46% both splice variants were simultaneously expressed, and in 31% both variants were not expressed. VEGF189 was expressed in 18 cell lines, VEGF183 in 12, and VEGF145 in 6 cell lines (Table 1). The expression level was highest for VEGF121, followed by VEGF165, VEGF189, VEGF183, and VEGF145. There was no correlation between VEGF isoform expression and the invasive capacity (32) of the cell lines (data not shown).

In 165 tumor samples from breast cancer patients VEGF121 and VEGF165 were dominantly expressed. VEGF121 was more strongly expressed than VEGF165 in 113 samples. Eighteen samples expressed VEGF165 stronger than VEGF121. VEGF189 was next intensively expressed, followed by VEGF145 and VEGF183. VEGF145 was expressed in 12 samples, VEGF183 in 3 samples, and VEGF189 in 52 samples. Eleven samples showed no VEGF expression, although GAPDH control was positive. Considering all of the splice variants a significant correlation was seen between VEGF121 and VEGF165 ($P = <1 \times 10^{-6}$; $\chi^2 = 38.45$), *i.e.*, in 79% both splice variants were expressed and both variants were absent in 7%; VEGF121 and VEGF189 ($P = 0.02$; $\chi^2 = 5.44$), *i.e.*, in 31% both variants were expressed and in 9% not; VEGF145 and VEGF189 ($P = 0.02$; $\chi^2 = 5.60$), *i.e.*, in 5% both variants were expressed and in 65% not, and VEGF165 and VEGF189 ($P =$

Table 2 VEGF isoform expression and clinicopathologic characteristics in 165 breast tumors

		VEGF121		VEGF145		VEGF165		VEGF183		VEGF189	
	n^a	Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg
Histological type[b]											
IDK	122	109	13	9	113	99	23	3	119	40	82
ILK	24	24	1	2	22	22	2	0	24	10	14
Differentiation grade											
G1	5	5	0	0	5	5	0	0	5	0	5
G2	88	80	8	5	83	75	13	3	85	31	57
G3	52	49	3	6	46	41	11	0	52	20	32
Tumor size											
pT1	59	52	7	4	55	45	14	2	57	21	38
pT2	71	68	3	7	64	63	8	1	70	25	46
pT3	7	6	1	1	6	5	2	0	7	3	4
pT4	10	8	2	0	10	9	1	0	10	2	8
Nodal status											
pN0	77	69	8	6	71	65	12	3	74	26	51
pN1	62	57	5	3	59	51	11	0	62	23	39

[a] *n*, number of samples; IDK, ILK, infiltrating ductal, infiltrating lobular.
[b] Histological type, differentiation grade, tumor size, and nodal status were unknown in 19, 20, 18, and 26 cases, respectively.

Table 3 VEGF isoform expression in ovarian carcinoma cell lines[a]

Cell line	VEGF121	VEGF145	VEGF165	VEGF183	VEGF189
ES-2	57.1%	0.4%	37.6%	0.7%	4.2%
PA-1	43.8%	0	56.2%	0	0
NIH:OVCAR-3	47.0%	0	42.1%	4.8%	6.1%
SK-OV-3	70.5%	0	24.5%	0	5.0%
A2780	67.4%	0	32.6%	0	0
A2780 ADR	46.7%	0	53.3%	0	0

[a] Various VEGF isoforms as percentage of overall expression within one cell line determined by ALF analysis.

0.001; $\chi^2 = 10.90$), *i.e.*, a coexpression in 31% and an absence of both in 17%. Statistical analysis considering the histopathologic type or clinical stage (grading, pT, and pN) of 165 patients and VEGF splice variant expression showed no correlation (Table 2).

Ovarian Tumors and Cell Lines. In ovarian cancer cell lines as well as in ovarian tumor specimens VEGF121 and VEGF165 were dominantly expressed. Among all of the ovarian cell lines VEGF121 and VEGF165 were always simultaneously expressed. Beside the coexpression of VEGF121 and VEGF165, no correlation was seen between the other splice variants (Table 3).

Examining 67 ovarian tumor specimens, 2 samples showed no VEGF expression, although GAPDH control was positive. VEGF121 was stronger expressed than VEGF165 in 57 cases. VEGF189 was next intensively expressed, followed by VEGF183 and VEGF145. There was a significant correlation between the expression of VEGF121 and VEGF165 ($P = 0.002$; $\chi^2 = 9.45$). *i.e.*, they were coexpressed in 82% of all cases and absent in 3%. There was a significant expression pattern between VEGF145 and VEGF183 ($P = <10^{-5}$; $\chi^2 = 24.56$), *i.e.*, a coexpression in 18% and an absence of both in 63% of all of the cases. Additionally, a significant expression pattern was seen for VEGF165 and VEGF189 ($P = 1 \times 10^{-5}$; $\chi^2 = 19.83$), *i.e.*, a coexpression in 63% and an absence of both in 16%. Furthermore, the expression of VEGF189 and VEGF183 was significantly correlated ($P = 0.002$; $\chi^2 = 9.84$); *i.e.*, a coexpression in 33% and no expression of both variants in 31%. Statistical analysis considering the histopathologic type or clinical parameters (grading and Fédération Internationale des Gynaecologistes et Obstetristes) for 67 patients and the VEGF splice variant expression showed no correlation (Table 4).

Cystadenomas. Twenty-four benign cystadenomas showed a similar expression pattern than the malignant tumors. VEGF121 and VEGF165 were dominantly expressed. VEGF121 was more strongly expressed in 18 cases, VEGF165 in only 2 cases, and 4 samples showed no VEGF expression at all. VEGF145 was never expressed. A correlation was seen between the expression of VEGF121 and VEGF165 ($P = 0.0006$; $\chi^2 = 11.66$); *i.e.*, a coexpression in 71% and no expression of both variants in 17%, and between VEGF121 and VEGF189 ($P = 0.044$; $\chi^2 = 4.06$), *i.e.*, a coexpression was detectable in 46% of all cases and no expression in 17%. There was no correlation between each of the VEGF splice variants to histological type (mucinous and serous). There was no significant difference between the benign and the malignant samples considering the splice variant expression patterns.

DISCUSSION

In this study we examined the expression of all known VEGF splice variants (VEGF121, 145, 165, 183, 189, and 206) in breast and ovarian cell lines, and malignant and benign tumor samples of the patients. We describe the detection of the PCR

Table 4 VEGF isoform expression and clinicopathologic characteristics in 67 ovarian tumours

		VEGF121		VEGF145		VEGF165		VEGF183		VEGF189	
	n^a	Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg	Pos	Neg
Histologic type[b]											
Serous	49	48	1	11	38	42	7	22	27	33	16
Mucinous	2	2	0	0	2	2	0	0	2	0	2
Endometrioid	10	10	0	0	10	8	2	2	8	8	2
Clear-cell	2	2	0	0	2	1	1	0	2	0	2
Differentiation grade											
G1	7	7	0	1	6	7	0	3	5	4	3
G2	13	13	0	3	10	12	1	4	9	8	5
G3	40	40	0	7	33	34	6	15	25	29	11
FIGO											
I	8	7	1	1	7	5	3	3	5	4	4
II	2	2	0	0	2	1	1	0	2	1	1
III	47	47	0	6	41	42	5	16	31	33	14
IV	4	4	0	3	1	4	0	4	0	4	0

[a] n, number of samples; IDK, ILK, infiltrating ductal, infiltrating lobular; FIGO, Fédération Internationale des Gynaecologistes et Obstetritstes.
[b] Histologic type, differentiation grade, and FIGO were unknown in 4, 7, and 6 cases, respectively.

products from the splice variants after analysis of the polyacrylamide gel with an ALF detector. Current results are nonquantitative with regard to intertumor levels of various VEGF isoforms, but the method allows the sensitive simultaneous detection of VEGF isoform expression (Fig. 2). We could demonstrate that all of the splice variants except splice variant VEGF206 were expressed in the examined tissue specimens and cell lines with variants 121 and 165 being the most dominant. VEGF145, although described to be expressed by several cell lines derived from carcinomas of the female reproductive system (10), was only rarely detected in breast and in ovarian carcinomas, as well as in breast and ovarian cell lines. If detectable the expression level was 10–100-fold lower compared with VEGF121 or VEGF165.

Interestingly, VEGF206 is lacking in breast and ovarian tumor specimens, cystadenomas, as well as cell lines, although the presence of isoform 206 in cystadenomas and carcinomas of the ovary was described recently, detected with RT-PCR (24). A comparison between breast and ovarian samples showed no significant difference in the expression pattern of the splice variants. Breast tumor cell lines showed more frequently VEGF145 expression than ovarian tumor cell lines. Differences were seen in the correlations between each of the splice variant expressions of tumor material from patients: a correlation was found between VEGF145 and VEGF189 expression in breast carcinomas. In contrast, in ovarian tumors a correlation between VEGF145 and VEGF183, and VEGF183 and VEGF189 expression was noted.

To look for differences between malignant and benign ovarian tumors, 24 cystadenomas were also included in this study. Other studies did not examine VEGF145 expression in primary tumors. Interestingly, all of the cystadenomas did not express VEGF145. However, malignant tumors also showed a lack of VEGF145 expression frequently. Furthermore, cystadenomas showed a significant correlation between the expression of VEGF121 and VEGF165, and VEGF121 and VEGF189. Carcinomas of the ovary showed a correlation between VEGF121 and VEGF165, VEGF121 and VEGF189, VEGF145 and VEGF183, VEGF165 and VEGF189, and VEGF183 and VEGF189.

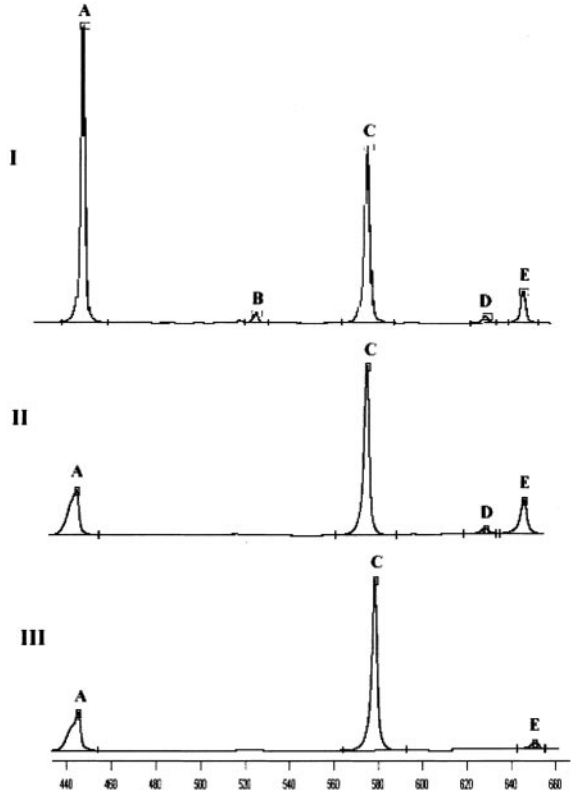

Fig. 2 ALF analysis. Computer representations of the analysis of the amplification products from the VEGF isoforms by automated PAGE from three ovarian tumor specimens (*I-III*). *A*, splice variant VEGF121 (444 bp); *B*, VEGF145 (516 bp); *C*, VEGF165 (516 bp); *D*, VEGF183 (630 bp); and *E*, VEGF189 (648 bp).

To consider the fact that the expression level of individual isoforms may be different, the ratios VEGF121:VEGF165,

VEGF121:189, and VEGF165:189 were calculated, and correlated with clinicopathologic characteristics of patient samples and invasion data from breast cell lines. No correlation was found. Also, the actual percentage of distribution of VEGF isoforms in cell lines and tumor material showed no correlation with invasive data or clinicopathologic characteristics.

Because the physiological significance of the differential splicing is still unknown, we examined for the first time all of the known splice variants simultaneously in tumor material from breast and ovarian cancers, and looked for possible clinical correlations. In breast carcinomas, no correlations could be found. Only the fact that the isoform 121 is more strongly expressed in human breast carcinomas could be confirmed (33) indicating the conclusion that this isoform has a stronger induction of tumorigenesis than the others (20). Also in ovarian carcinomas no correlation could be found between clinicopathologic characteristics and VEGF isoform expression, as already described for isoforms 121, 165, and 189 (24), and isoforms 165 and 121 (26). In conclusion, the splice variant expression shows no association with either organ type (breast or ovary) nor with clinicopathologic characteristics. Additionally, the VEGF145 isoform, which was investigated for the first time in tumor material, seems not to be specific for ovarian or breast tumor cancer.

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