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Quantitative Real-Time Reverse Transcription–PCR Study of the Expression of Vascular Endothelial Growth Factor (VEGF) Splice Variants and VEGF Receptors (VEGFR-1 and VEGFR-2) in Non–Small Cell Lung Cancer

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Background: **Vascular endothelial growth factor (VEGF) is a major regulator of angiogenesis and its expression is increased in non–small cell lung cancer (NSCLC). We aimed to determine the expression pattern of VEGF splice variants in NSCLC and its correlation with the clinicopathological characteristics of tumors.**

Methods: **We used real-time reverse transcription PCR to quantify the mRNA expression of total VEGF, 4 VEGF splice variants ($VEGF_{121}$, $VEGF_{165}$, $VEGF_{183}$, and $VEGF_{189}$), and 2 VEGF receptors (VEGFR-1 and VEGFR-2) in 27 pairs of cancerous and adjacent noncancerous tissues originating from patients with NSCLC.**

Results: **Total VEGF, $VEGF_{121}$, and $VEGF_{165}$ were expressed in all specimens, whereas $VEGF_{183}$ and $VEGF_{189}$ were present in small amounts in certain samples. Total VEGF, $VEGF_{121}$, and $VEGF_{165}$ mRNA was upregulated in cancerous compared with healthy tissues, whereas $VEGF_{183}$ and $VEGF_{189}$ expression tended to be higher in healthy tissues. The expression of VEGFRs was similar between matched specimens. No correlation was found between the expression of total VEGF or VEGF splice variants and the clinicopathological characteristics of tumors. The expression patterns of VEGF splice variants differed between tissue pairs. $VEGF_{121}$ was the major variant expressed in all samples; however, its relative expression was higher in cancerous tissues. The relative expression of $VEGF_{183}$ and $VEGF_{189}$ was upregulated in healthy lung tissues, whereas the ratio of $VEGF_{165}$ to total VEGF was similar between matched specimens.**

Conclusions: **The expression pattern of certain VEGF splice variants is altered during tumorigenesis. Our data support the hypothesis that during malignant progression an angiogenic switch favoring the shorter diffusible isoforms occurs.**

Angiogenesis is the complex process by which new blood vessels arise from preexisting vasculature. The development of a vascular supply is essential not only for organ development and differentiation during embryogenesis but also for wound healing and reproductive functions in the adult. Dysregulation of angiogenesis, however, contributes to several pathological conditions, such as diabetic retinopathy, rheumatoid arthritis, and solid tumor development *(1)*. Although recent evidence indicates that new vessel growth and maturation are highly complex and coordinated processes, vascular endothelial growth factor (VEGF)[4] signaling is generally agreed to be a crucial step for the regulation of angiogenesis *(2)*.

VEGF is a highly specific mitogen for endothelial cells, inducing angiogenesis as well as the permeabilization of blood vessels and playing a central role in the regulation of vasculogenesis *(2)*. Through alternative splicing of a single VEGF gene, various isoforms are produced. To date 7 VEGF splice variants have been reported, ranging in length from 121 to 206 amino acids *(3–7)*. All splice variants contain exons 1–5 and 8 and differ only by

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[4] Nonstandard abbreviations: VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor; NSCLC, non–small cell lung cancer; RT-PCR, reverse transcription PCR.

various combinations of either no additional exon [$VEGF_{121}$ *(3)*] or addition of exon 6 [$VEGF_{145}$ *(4)*], exon 7 [$VEGF_{165}$ *(3)*], exons 6 and 7 [$VEGF_{189}$ *(3)*], or exons 6, 6′, and 7 [$VEGF_{206}$ *(5)*]. $VEGF_{148}$ has the same amino acid sequence as $VEGF_{165}$, apart from a 35-bp-long deletion at the end of exon 7 *(6)*, whereas in $VEGF_{183}$ the final 18 bp of exon 6a are missing *(7)*. An important biological property that differentiates the various VEGF isoforms is the sulfate-binding capacity of heparin and heparan. $VEGF_{121}$ does not bind to heparin and is a freely soluble protein; $VEGF_{165}$ is also secreted, although a significant fraction remains bound to the cell surface and the extracellular matrix. In contrast, $VEGF_{189}$ and $VEGF_{206}$ are almost completely sequestered in the extracellular matrix because of their high binding affinity for heparin *(1, 2, 8)*. VEGF splice variants bind to 2 tyrosine-kinase receptors, VEGF receptor 1 (VEGFR-1; flt-1) and VEGFR-2 (KDR/flk-1), and 2 isoform-specific receptors, neurophilin-1 and neurophilin-2, which bind to $VEGF_{165}$ but not $VEGF_{121}$ *(9)*. One soluble receptor, sVEGFR-1, created by alternative splicing of VEGFR-1, was reported to act as a competitive antagonist to VEGF *(10)*. Most cell types produce several VEGF variants simultaneously. Usually $VEGF_{121}$ and $VEGF_{165}$ are the predominant forms, followed by $VEGF_{189}$ *(3)*. $VEGF_{183}$ also has wide tissue distribution and may have gone undetected because of confusion with $VEGF_{189}$ *(7)*. In contrast, $VEGF_{145}$, $VEGF_{148}$, and $VEGF_{206}$ are comparatively rare and detected in trace amounts *(4–6, 11)*.

The expression of VEGF splice variants has been investigated in various neoplasms, but current data are contradictory *(12–18)*. The increased expression of $VEGF_{165}$ and $VEGF_{189}$ in osteosarcoma *(12)*, renal cell carcinoma *(13)*, non–small cell lung cancer (NSCLC) *(14–16)*, and colon cancer *(17)* was correlated with neovascularization, tumor progression, and poor prognosis. On the other hand, another study reported no relation of VEGF isoform patterns with invasion and progression in breast and ovarian cancer *(18)*. In most studies, semiquantitative techniques such as conventional reverse transcription PCR (RT-PCR) were used to determine the expression of VEGF splice variants, and only malignant tissues and not pairs of cancerous and healthy tissues were investigated. We recently developed a real-time RT-PCR method for the quantification of mRNA of individual VEGF splice variants *(11)*. In the present study, we used this method to quantify the expression of total VEGF and 4 VEGF splice-variant mRNA ($VEGF_{121}$, $VEGF_{165}$, $VEGF_{183}$, and $VEGF_{189}$) in pairs of cancerous and adjacent noncancerous tissues originating from patients with NSCLC. In addition, we developed a similar real-time RT-PCR approach to quantify the expression of VEGFRs, VEGFR-1 and VEGFR-2, in the same tissue pairs. The purpose of our study was to apply this highly sensitive and accurate technique to evaluate possible correlations between the expression pattern of VEGF splice variants and the clinicopathological characteristics of tumors and possible differences in VEGF splice-variant expression patterns in malignant vs healthy tissues.

Materials and Methods

SELECTION OF PATIENTS

Lung carcinoma and adjacent nonneoplastic tissues were obtained sequentially from 27 male NSCLC patients. All patients and donors gave their informed consent, and the study was approved by the ethics and scientific committees of the participating institutions. The tumor types and stages were determined according to the WHO classification. Tissue samples were immediately flash frozen in liquid nitrogen and stored at −80 °C until use. All samples were analyzed histologically to assess the amount of tumor component (at least 70% of tumor cells) and the quality of material (i.e., absence of necrosis). The clinicopathological characteristics of the tumors investigated are summarized in Table 1.

RNA ISOLATION AND cDNA SYNTHESIS

We isolated total cellular RNA with the Qiagen RNeasy Mini Reagent Set (Qiagen) according to the manufacturer's instructions. All preparation and handling of RNA took place in a laminar flow hood, under RNase-free conditions, and the isolated RNA was stored at −70 °C until used. RNA concentration was determined with a NanoDrop ND-100 spectrophotometer (NanoDrop Technologies). Reverse transcription of RNA was carried out with the ImProm II Reverse Transcription System (Promega) according to the manufacturer's instructions, using 1 μg of total RNA as template. RNA integrity of the cDNA preparations was tested by real-time PCR amplification of the porphobilinogen deaminase housekeeping gene.

REAL-TIME RT-PCR

Real-time RT-PCR for the quantification of total VEGF and 4 VEGF splice variants ($VEGF_{121}$, $VEGF_{165}$, $VEGF_{183}$, and $VEGF_{189}$) was performed as previously described *(11)*. For the quantification of VEGFRs, specific primers and probes were designed with the Primer Premier software. To prevent amplification of genomic DNA, the oligonucleotides designed were intron spanning. VEGFR-1 primers are located in an area present in both membrane-associated and soluble receptors and can

Table 1. Clinicopathological features of patients included in the study.

Variable	
Mean age (range), years	62.1 (48–78)
Tumor size [mean (SD)], cm	4.0 (2.2)
Lymph-node positive	17/27
Lymph-node negative	10/27
Stage I/II	21/27
Stage III/IV	6/27
Adenocarcinoma	15/27
Squamous cell carcinoma	12/27

therefore amplify both forms of VEGFR-1. The sequences of the primers and probes for VEGFRs are presented in Table 2. For the quantification of both VEGFRs the cycling protocol consisted of an initial 5-min denaturation step at 95 °C, followed by 45 cycles of denaturation at 95 °C for 10 s and annealing/extension at 60 °C for 1 min.

STATISTICS

Data for each continuous variable were examined with the Shapiro–Wilk *W*-test to determine whether assumptions of gaussian distribution were valid. Because the data did not show gaussian distribution, nonparametric tests were used. We used the Wilcoxon rank-sum test for statistical analysis of total VEGF, VEGF splice variants, and VEGFR expression in pairs of malignant and healthy tissue and Spearman rank R to test for unadjusted associations between total VEGF, VEGF splice variants, and VEGFR values and independent variables. Descriptive data for continuous variables are reported as medians and ranges (25th and 75th percentiles). Two-sided *P* values <0.05 were considered statistically significant. Data were analyzed with the Statistica software (version 7.0, StatSoft).

Results

We investigated the expression of total VEGF, VEGF splice variants, and VEGFRs in 27 paired samples of tumor specimens and adjacent healthy tissue. Our results were normalized to the amount of total RNA present in each sample and are expressed as copy number of the gene of interest per micrograms of total RNA. All specimens investigated (healthy and malignant) expressed total VEGF. The splice variants $VEGF_{121}$ and $VEGF_{165}$ were most abundantly expressed in all samples, followed by $VEGF_{189}$, which was detected in 7 (25.9%) of 27 malignant tissues and 14 (51.9%) of 27 healthy tissues. $VEGF_{183}$ was detected in small amounts and was present in 9 (33.3%) of 27 malignant and 15 (55.6%) of 27 healthy tissues. Thirteen (48.1%) of the 27 healthy tissues expressed all 4 splice variants; in 12 (44.4%) of 27 cases only the 2 secretory forms ($VEGF_{121}$ and $VEGF_{165}$) were concomitantly detected, whereas in the remaining 2 cases (7.4%), the variants $VEGF_{121}$, $VEGF_{165}$, and $VEGF_{183}$ were present simultaneously. On the other hand, the majority of cancerous tissues (18 of 27 samples; 66.7%) expressed only the shorter variants $VEGF_{121}$ and $VEGF_{165}$. All 4 variants were present in 7 (25.9%) of 27 cases, whereas in the remaining 2 cases (7.4%), the 3 variants $VEGF_{121}$, $VEGF_{165}$, and $VEGF_{183}$ were simultaneously expressed. VEGFR-1 was detected in 22 (81.5%) of the 27 of the noncancerous lung tissues investigated, and VEGFR-2 was present in 22 (81.5%) of 27 healthy tissues. For malignant specimens, these percentages were 23 (85.2%) of 27 for VEGFR-1 and 24 (88.9%) of 27 for VEGFR-2.

The expression of all genes investigated is presented in Table 3. Total VEGF mRNA expression was found to be significantly higher in tumor samples than in their corresponding adjacent healthy tissue ($P<0.01$; Table 3; Fig. 1), and the same applied for the splice variants $VEGF_{121}$ and $VEGF_{165}$ ($P<0.01$ and $P = 0.01$, respectively; Table 3; Fig. 2, A and B). On the other hand, $VEGF_{183}$ and $VEGF_{189}$ displayed higher expression in healthy tissues than in their corresponding malignant tissues (Table 3; Fig. 2, C and D), but this trend did not reach statistical significance ($P = 0.08$ and 0.11, respectively). The expression of both VEGFRs, VEGFR-1 and VEGFR-2, was similar between paired specimens (Table 3; Fig. 2, E and F). We observed significant correlations between the expression of total VEGF and all 4 VEGF splice variants as well as between all 4 VEGF splice variants in both healthy and malignant tissues ($P<0.01$ in all cases). We also observed strong linear relationships between total VEGF or VEGF splice variants and VEGFR-1 in normal lung tissues ($P<0.01$ in all cases), whereas in malignant samples a linear relationship existed only between $VEGF_{189}$ and VEGFR-1 expression ($P<0.01$). The expression of total VEGF, $VEGF_{121}$, and $VEGF_{165}$ correlated significantly with the expression of VEGFR-2 in normal lung tissue ($P<0.01$ in all cases); in cancerous tissues, however, a linear relationship existed between total VEGF or $VEGF_{121}$ and VEGFR-2 ($P = 0.04$ in both cases).

We also examined the relative expression (%) of VEGF splice variants, i.e., the ratio of a specific variant to the sum of all VEGF splice variants (copy number:micrograms total RNA) in healthy and malignant tissues. We have found different expression patterns of the VEGF splice variants between different normal and tumor tissues (Fig. 3). We observed that whereas in noncancerous

Table 2. Sequences of VEGFRs primers and probes used in this study.

Oligonucleotide		Oligonucleotide sequence (5′–3′)
VEGFR-1[a]	Forward primer	ATC ATT CCG AAG CAA GGT GTG
	Reverse primer	AAA CCC ATT TGG CAC ATC TGT
	TaqMan probe	AAG GAA AGA ATA AGA TGG CTA GCA CCT TGG TT
VEGFR-2[b]	Forward primer	AGG CAG CTC ACA GTC CTA GAG C
	Reverse primer	GTC TTT TCC TGG GCA CCT TCT A
	TaqMan probe	GAG ACC CTT GTA GAA GAC TCA GGC ATT GTA TTG

[a] GenBank accession no.: NM_002019.
[b] GenBank accession no.: NM_002253.

Table 3. Expression of total VEGF, VEGF splice variants, and VEGFRs in malignant and adjacent noncancerous tissues (n = 27).

	NSCLC tissues	Adjacent noncancerous tissues	P[a]
Total VEGF	6.4×10^3 (2.7×10^3, 1.5×10^4)[b]	2.1×10^3 (1.0×10^3, 5.8×10^3)	<0.01
$VEGF_{121}$	4.4×10^3 (1.8×10^3, 1.1×10^4)	1.2×10^3 (7.6×10^2, 3.1×10^3)	<0.01
%	73.3 (67.5, 78.8)[c]	60.8 (50.3, 68.4)	<0.01
$VEGF_{165}$	1.5×10^3 (6.2×10^2, 3.0×10^3)	5.6×10^2 (3.7×10^2, 1.1×10^3)	<0.01
%	24.4 (18.8, 31.3)	23.6 (16.6, 31.6)	NS
$VEGF_{183}$	0 (0, 9.2×10)	4.3×10 (0, 1.6×10^2)	NS
%	0 (0, 0.4)	1.0 (0, 3.2)	<0.01
$VEGF_{189}$	0 (0, 4.1×10^2)	0 (0, 2.0×10^3)	NS
%	0 (0, 1.7)	0 (0, 27.6)	<0.01
VEGFR-1	3.9×10^3 (1.7×10^3, 1.5×10^4)	2.5×10^3 (4.4×10^2, 7.8×10^3)	NS
VEGFR-2	5.4×10^3 (2.3×10^3, 1.5×10^4)	4.3×10^3 (9.4×10^2, 1.7×10^4)	NS

[a] Wilcoxon rank-sum test.

[b] Values for total VEGF, VEGF splice variants, and VEGFRs are given as copies of target per microgram RNA. All values are given as medians and ranges (25th and 75th percentiles). NS, Not significant.

[c] Percentage of each splice variant in with respect to total VEGF expression.

tissues, mean (SD) $VEGF_{121}$ expression constituted 59.7% (11.9%) of the total VEGF transcript, in NSCLC tissues the percentage was 73.1% (9.8%; $P < 0.01$). The expression ratio of $VEGF_{165}$ was similar between the 2 tissue types [23.8% (7.7%) in NSCLC and 24.8% (9.4%) in adjacent noncancerous tissues]. The percentage of $VEGF_{189}$ and $VEGF_{183}$ as part of total VEGF expression was higher in normal than malignant samples [13.7% (15.7%) vs 2.9% (6.9%) and 1.8% (2.2%) vs 0.2% (0.3%), respectively; $P < 0.01$ in both cases, Fig. 3)].

We did not find any correlation between total VEGF, VEGF splice variants, or VEGFRs expression and age or tumor size. Also, the expression of total VEGF and VEGF splice variants and relative expression of the variants did not differ between adenocarcinoma specimens and squamous cell carcinoma specimens; the same applied for VEGFR-1 and VEGFR-2. We observed, however, that the expression of total VEGF tended to be higher in tumor specimens originating from lymph-node–positive than lymph-node–negative patients, although the difference did not reach statistical significance. The expression of the investigated genes did not differ depending on tumor stage (I/II vs III/IV).

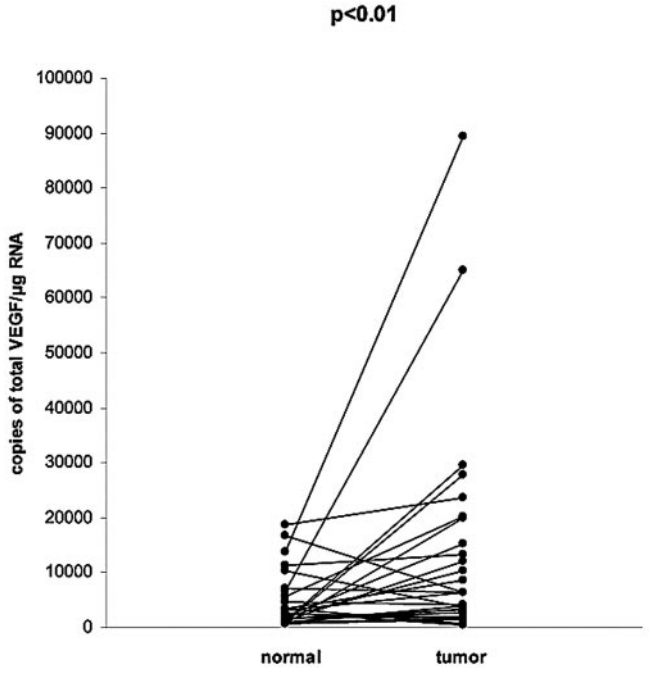

Fig. 1. Total VEGF mRNA in paired NSCLC and adjacent noncancerous tissues (n = 27).

Discussion

VEGF is a potent angiogenic factor, and its expression has been studied in both mRNA and protein in many different tumors. VEGF upregulation is reported in a wide variety of cancer cells and was associated with a poor prognosis in patients with breast, colon, renal, and lung carcinomas *(13, 16–23)*. The presence of various isoforms deriving from alternative splicing of a single VEGF gene has been reported, and some studies have shown that the expression of certain splice variants is related to tumor progression *(12, 14, 15)*. Current data are contradictory, however, and further studies are required to elucidate the biological role of VEGF splice variants. Most studies have investigated VEGF protein expression in lung carcinomas using immunohistochemical staining; only a few studies have examined VEGF expression at the transcriptional level. Usually RT-PCR, a semiquantitative technique, is used, and only the variants $VEGF_{121}$, $VEGF_{165}$, and $VEGF_{189}$ are investigated. In the present study we used real-time RT-PCR, which is a more sensitive and accurate technique, for the quantification in pairs of malignant and adjacent normal lung tissues of mRNA for total VEGF, 4 VEGF splice variants ($VEGF_{121}$, $VEGF_{165}$, $VEGF_{183}$, and $VEGF_{189}$), and 2 VEGFRs (VEGFR-1 and VEGFR-2). Although $VEGF_{183}$ has wide tissue distribution and was

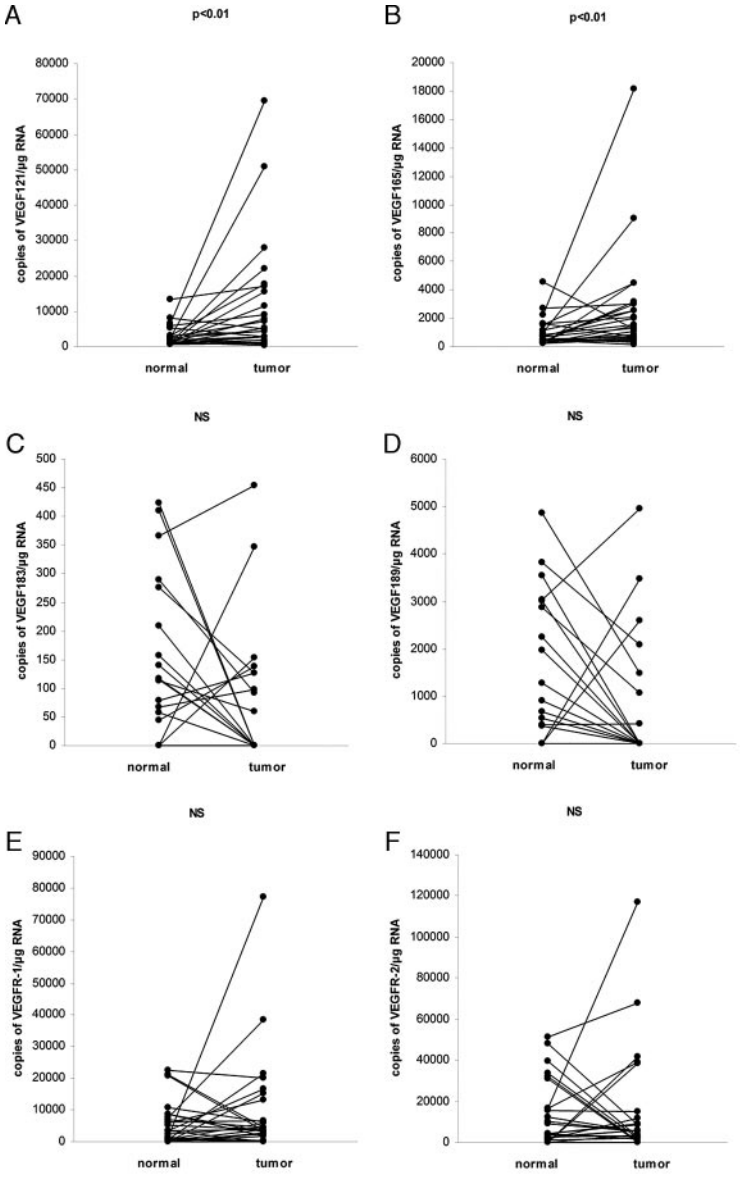

Fig. 2. (*A*), $VEGF_{121}$; (*B*), $VEGF_{165}$; (*C*), $VEGF_{183}$; (*D*), $VEGF_{189}$; (*E*), VEGFR-1; (*F*), VEGFR-2 mRNA in paired NSCLC and adjacent noncancerous tissues (n = 27). *NS*, not significant.

detected in cancer and ovarian cell lines, to our knowledge this study is the first to detect $VEGF_{183}$ in lung tissue. We did not include the variants $VEGF_{145}$, $VEGF_{148}$, or $VEGF_{206}$ in our study, because they are expressed in trace amounts and seem to be of minor importance.

The primary VEGF transcript derives from a single gene, and various isoforms are produced through alternative splicing. Although the precise biological role of VEGF isoforms remains unclear, it is believed that individual VEGF isoforms mediate different aspects of vascular growth. This concept is supported by the fact that different VEGF isoforms exhibit different binding affinities toward heparin sulfate and thus differ in extracellular localization and accessibility *(1, 2)*. VEGF isoforms are not functionally equivalent; previous studies have shown that the presence of all isoforms is required for normal vascular development *(14)*. Moreover, examination of mRNA expression of VEGF splice variants 121, 165, and 189 revealed that the relative amounts of VEGF splice variants varied among different organs and that their expression patterns changed during organ development *(24, 25)*. In agreement with these findings, we report that the expression profile of VEGF splice variants varies between malignant and healthy lung tissues. In both cases $VEGF_{121}$ is the major variant expressed, but its percentage as part of the total VEGF message is significantly enhanced in tumor specimens. $VEGF_{121}$ is the predominant splice variant expressed in various carcinomas and is considered to be more strongly tumorigenic than the other splice variants in vivo *(26)*. Although the ratio of $VEGF_{165}$ to total VEGF was similar between matched specimens, the ratios of $VEGF_{183}$ and $VEGF_{189}$ were downregulated in

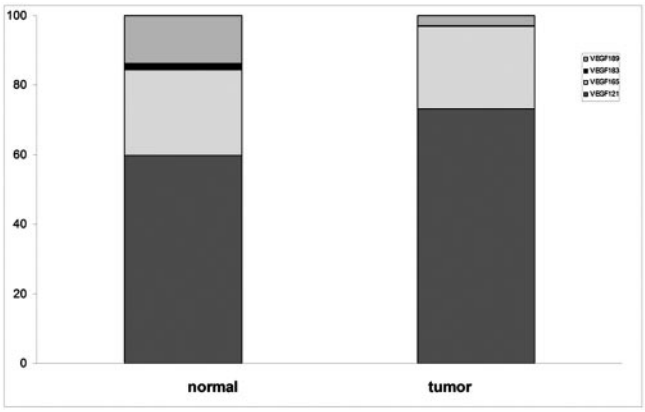

Fig. 3. Mean percentage (%) of expression of individual VEGF splice variants in respect to total VEGF in paired NSCLC and adjacent noncancerous tissues (n = 27).

lung carcinomas. Total VEGF mRNA showed significantly higher expression in cancer tissues than in paired adjacent normal lung tissues. The analysis of mRNA expression in VEGF splice variants revealed overexpression of $VEGF_{121}$ and $VEGF_{165}$ and lower expression of $VEGF_{183}$ and $VEGF_{189}$ in all NSCLC tumor samples compared with their corresponding noncancerous specimens. These results are in keeping with the findings of a recent study *(27)*. Our data indicate that overexpression of VEGF in lung tumors is caused by the most diffusible variants and support the observation that during malignant progression an angiogenic switch occurs and favors the shorter diffusible isoforms *(28)*.

We did not find any correlation between the expression of total VEGF or VEGF splice variants and clinicopathological characteristics of the tumors investigated. We observed that lymph-node–positive tumors overexpressed total VEGF, although this tendency did not reach statistical significance, but found no differences in the expression profiles of VEGF splice variants. Previous studies have reported that VEGF expression is associated with nodal involvement in NSCLC *(16, 20)*. Biological behavior differs between the 2 main histological types of NSCLC: adenocarcinoma has a higher metastatic potential than squamous cell carcinomas *(16)*. Some studies report that VEGF expression is higher in adenocarcinomas than squamous cell carcinomas *(16, 21)*, but data also suggest that there is no difference in VEGF levels between those 2 subtypes *(29, 30)*. We observed that the 2 histological types of NSCLC not only displayed similar expression of VEGF and VEGF splice variants, but also showed similar relative expression of VEGF splice variants, and therefore we conclude that their different metastatic potential cannot be associated with VEGF.

Discrepancies between our results and the results of previous studies can be attributed to the use of different methods for the quantification of VEGF and, in cases when RT-PCR is used, to the housekeeping gene selected for the normalization of results. A number of different genes are used as internal controls for qualitative RT-PCR analyses since their expression is assumed to be constant at different times and after many forms of experimental manipulations. However, a recent study reported a high interindividual variability of control gene RNAs in breast and colon biopsies and significant differences in intraindividual RNA levels between paired normal and cancer specimens *(31)*. For this reason, we did not use a housekeeping gene, but our results were normalized to the amount of total RNA present in each sample as previously described *(11)*. The validity of this approach has been confirmed, since normalization of VEGF mRNA expression to total RNA in breast cancer biopsies correlated well with protein levels, and VEGF mRNA expression levels were found augmented in comparison with paired normal tissues *(31)*. On the other hand, normalization of VEGF mRNA to various housekeeping genes generated confusing and misleading results that were contradicted by protein quantification. VEGF mRNA levels could be made to appear increased, decreased, or unchanged between matched samples depending on the normalizer chosen *(31)*.

In conclusion, our study shows for the first time the splice variant profile of VEGF in a quantitative aspect in NSCLC. Our study has revealed a different VEGF splicing variant expression profile in NSCLC in respect to nonmalignant paired tissues. More specifically, our results show that absolute expression, as well as the expression pattern of certain VEGF splice variants, is altered during tumorigenesis and support the hypothesis that during malignant progression an angiogenic switch favoring the shorter diffusible isoforms occurs. The low number of specimens studied constitutes a limitation for the detection of significant associations between isoform or receptor expression and clinical prognostic variables. It would be of interest to extend this study in a larger number of clinical samples and different cancers, since most studies so far are based on the expression of total VEGF. Taking into account the great importance of VEGF in cancer, as a specific molecular target of very recently approved angiogenesis-based novel cancer therapeutics (AVASTIN, Roche), we strongly believe that our results will be of importance for clinical researchers who will design clinical studies to evaluate the response to VEGF-based therapy in a larger number of patients.

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