Specific Reverse Transcription-PCR Quantification of Vascular Endothelial Growth Factor (VEGF) Splice Variants by LightCycler Technology


**Background:** Overexpression of vascular endothelial growth factor (VEGF) is associated with increased angiogenesis, growth and invasion in solid tumors, and hematologic malignancies. The expression of isoforms of VEGF, which mediate different effects, can be discriminated by splice-variant-specific quantitative reverse transcription-PCR (RT-PCR), but current methods have only modest sensitivity and precision and suffer from heteroduplex formation.

**Methods:** We used a real-time RT-PCR assay on the LightCycler system. Applicability for detection of different VEGF mRNAs and total VEGF message was tested on seven healthy tissues (each pooled from healthy donors) and seven correlated malignant tissues. Results were normalized to $\beta_2$-microglobulin mRNA. Amplification of VEGF splice variants was performed exclusively with variant-specific reverse primers, whereas forward primer and fluorescent probe were common to obtain similar RT-PCR kinetics.

**Results:** Highly specific detection of VEGF splice variants was achieved with minor intra- and interassay variation (<0.22 threshold cycle). Total VEGF expression was higher in malignant tissues. In healthy tissues, the mRNA encoding diffusible variants VEGF$_{121}$ and VEGF$_{165}$ constituted on average 78% (SD = 9.3%) of the total VEGF message, and the cell-adherent variant VEGF$_{189}$ constituted on average 22% (SD = 5.4%). In contrast, in malignant tissues VEGF$_{121}$ and VEGF$_{165}$ accounted for 94% (SD = 7.6%) and VEGF$_{189}$ only 6% (SD = 3.7%).

**Conclusions:** Because of the ability for quantification of VEGF splice variants with high specificity, sensitivity, and reproducibility, this new LightCycler assay is superior to conventional semiquantitative competitive RT-PCR and immunological assays and may contribute to better understanding of VEGF-mediated angiogenesis.

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Vascular endothelial growth factor (VEGF), also known as vascular permeability factor, is an endothelial cell-specific mitogen and a significant mediator of angiogenesis during a variety of nonpathological and pathological processes (1–3). VEGF is expressed in response to hypoxia and other stimuli by healthy as well as neoplastic cells (4–7). The concentrations of VEGF mRNA and the expression of VEGF protein in human solid tumors correlate positively with malignant progression (8, 9). Recent evidence indicates that increased angiogenesis mediated by VEGF also plays a pivotal role in hematopoietic tumors (10–14).

VEGF is a 34- to 42-kDa heparin-binding, dimeric, disulfide-bound glycoprotein existing in at least five homodimeric isoforms. The monomers consist of 121, 145, 165, 189 and 206 amino acids (VEGF$_{121}$, VEGF$_{145}$, VEGF$_{165}$, VEGF$_{189}$, and VEGF$_{206}$), respectively (15, 16). The precise functional differences among the isoforms are not yet known. Differences in solubility, receptor affinity, and mitogenic potency have been described [reviewed in Neufeld et al. (17)]. The primary VEGF transcript derives from a single VEGF gene coding for eight exons. Variable
alternative mRNA splicing involves exons 6 and 7, whereas the amino acids encoded by exons 1–5 and 8 are conserved in all isoforms. Exons 6 and 7 encode two distinct heparin-binding domains. The presence or absence of these domains influences solubility and receptor binding. The heparin-binding domain encoded by exon 6 involves the domains encoded by exons 6 and 7, is highly diffusible.

At present, little is known on the relative abundance of the different VEGF splice variants in healthy cells of different origin and in the malignant counterparts. Whereas the major splice variants VEGF121, VEGF165, and VEGF189 are expressed in nearly all investigated tissues in different ratios, the variants VEGF145 and VEGF206, as well as the recently described variants VEGF148 (21) and VEGF185 (22) are restricted to only a very few cell types and seem to be of minor importance.

In recent investigations based on competitive reverse transcription (RT)-PCR assays, the increased expression of VEGF165 and VEGF189 in osteosarcoma (23), renal cell carcinoma (24), non-small-cell lung cancer (25), and colon cancer (26) was correlated with neovascularization, tumor progression, and poor prognosis. Cheung et al. (27) pointed out that during malignant progression, an angio-

In this study, a novel real-time RT-PCR was developed for specific quantification of VEGF splice variants to investigate the expression of VEGF splice variants and their ratio in healthy and malignant tissues, using the new LightCycler technology.

**Materials and Methods**

**PREPARATION OF CALIBRATORS**

Total cellular RNA was isolated from Jurkat cells by the Qiagen RNeasy Mini reagent set (Qiagen) according to the manufacturer’s recommendations. Reverse transcription was carried out as described elsewhere (30). The transcripts that encode for VEGF splice variants VEGF121, VEGF165, and VEGF189 as well as total VEGF (VEGFtotal) were amplified by RT-PCR using specific primers (sequences for all primers see below). The housekeeping gene β2-microglobulin (B2M) served as internal control. PCR products were separated on a 3% agarose gel, and bands were excised, purified, cloned, and sequenced. For cloning, the TOPO TA Cloning reagent set pCRII (Invitrogen Corporation) was used according to the manufacturer’s recommendations. Plasmids were purified using the Qiagen Miniprep reagent set (Qiagen) and sequenced (Big Dye Terminator Cycle on ABI Prism 377 Automated Sequencer; both from PE Applied Biosystems).

**QUANTITATIVE REAL-TIME RT-PCR**

**Primer and probe design.** Quantification of the mRNA message coding for VEGF121, VEGF165, VEGF189, and VEGFtotal was performed using LightCycler technology (Roche Diagnostics). To reach high conformity of the PCR kinetics for the VEGF splice variants and VEGFtotal, a common forward primer, ex3fo (5’-CCCTGATGAGTCGAGTACATT-3’), and a common fluorescent hydrolyzation probe, VEGF-probe (5’-ATCCTGTGTCGCCCT-TATACCG-3’), both located in exon 3 (part of the conserved region of all VEGF splice variants) were designed (Fig. 1). Specific amplification of each splice variant was performed exclusively with specific reverse primers spanning the variant specific exon boundaries: ex5/8re (5’-CCCTGCGTTTCGATTTTT-3’, 254-bp amplicon) for VEGF121, spanning the boundaries of exons 5 and 8; ex5/7re (5’-AGCAAGCCACAGGGATT-3’, 254-bp amplicon) for VEGF165, spanning the boundaries of exons 5 and 7; and ex6re (5’-ACCCTCAGAAGATT-3’, 310-bp amplicon) for VEGF189, located in exon 6. For quantification of the complete VEGF message (VEGFtotal), the common forward primer, the common fluorescent probe, and a common reverse primer located in exon 8 (ex8re: 5’-ACCCTCAGAAGATT-3’) were used for simultaneous amplification of all variants by external PCR. For normalization, the published B2M primer/probe set (31) was adapted to our conditions: B2M forward primer (5’-ATCCTGTGTCGCCCT-TATACCG-3’) and B2M reverse primer (5’-AACCTCAGAAGATT-3’), which produce a 114-bp amplicon; and B2M hydrolyzation probe (5’-CTCAGAAGATT-3’). Hydrolyzation probes were labeled with a reporter dye (6-carboxy-fluorescein phospho-
phoramidite) at the 5’ end and a quencher dye (5-carboxytetramethylrhodamine) at the 3’ end. The similar calculated melting temperatures for all primers enabled the use of the same cycling program for all samples. The melting temperatures for primers and probes were calculated using OLIGO 5.0 (MedProbe) or the JAVA OLIGO program (TIB Molbiol).

**PCR conditions.** Quantitative PCR was performed in a total reaction volume of 20 μL per capillary for the LightCycler format. For conventional PCR, the amounts of reagents were scaled up to 30 μL. Bovine serum albumin (Sigma) was added only to the LightCycler reaction mixture to avoid nonspecific binding of reagents to the LightCycler glass capillary. The 20-μL reaction mixture contained 0.75 U of a temperature-release Taq DNA polymerase (Platinum DNA Polymerase; Gibco BRL, Life Technologies), 2 μL of the supplied 10× PCR buffer, 7.5 mM MgCl₂, 0.2 mM dNTPs (Gibco BRL, Life Technologies), 3 μg of bovine serum albumin (Sigma), 0.25 μM each primer, 0.1 μM fluorescent probe (primers and probes; TIB Molbiol), and ± 0.4 ng of cDNA. The amplification conditions for LightCycler consisted of an initial 1.5-min denaturation step at 95 °C, followed by 40 cycles of denaturation at 95 °C for 8 s, annealing at 68 °C for 12 s, and extension at 72 °C for 20 s. Conventional PCR cycling conditions were as follows: 3-min denaturation step at 95 °C, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 68 °C for 15 s, and extension at 72 °C for 25 s.

**Samples.** Fourteen cDNA samples from seven healthy and seven malignant tissues (Clontech Laboratories) were investigated by quantitative real-time PCR to demonstrate the applicability of the new method. Healthy tissues were pooled from several healthy Caucasians: lung, pancreas, kidney, liver, heart, skeletal muscle, and placenta. Malignant tissues were obtained from well-characterized tissues were obtained from seven malignant tumors (Clontech Laboratories) were investigated by quantitative real-time PCR to demonstrate the applicability of the new method. Healthy tissues were pooled from several healthy Caucasians: lung, pancreas, kidney, liver, heart, skeletal muscle, and placenta. Malignant tissues were obtained from well-characterized tumors that had been explanted and propagated as xenografts in athymic nude mice, and used as model systems for cancer research: tumor cell lines LuCa1, a lung carcinoma explanted from a metastasis; LuCa2, a lung carcinoma explanted from a tumor; PaCa, a pancreatic adenocarcinoma; BrCa, a breast carcinoma; OvCa, an ovarian carcinoma; PrCa, a prostatic adenocarcinoma; and CoCa, a colon adenocarcinoma. Each provided cDNA preparation (± 0.2 ng/μL) was diluted 1:1 in water, and 4 μL of this dilutions was used per 20-μL real-time PCR reaction volume.

**Normalization and quantification.** To determine the absolute copy number of the target transcripts, the cloned plasmid cDNAs for B2M and for the VEGF mRNA splice variants VEGF₁₂₁, VEGF₁₆₅, and VEGF₁₈₉ were used to generate a calibration curve. Purified plasmid cDNA templates were measured in a photometer, and copy numbers were calculated from the absorbance at 260 nm (A₂₆₀). Plasmid cDNA was serially diluted in log steps from 10⁸ copies down to 10 copies in a 1-μL volume. A calibration curve was created by plotting the threshold cycle (Ct) vs the known copy number for each plasmid template in the dilutions. The copy numbers for all unknown samples were determined by LightCycler software 3.1, according to the calibration curve. To correct for differences in both RNA quality and quantity between samples, data were normalized by dividing the copy number of the target cDNA by the copy number of B2M. Quantitative results are presented as copies of target gene per 1000 copies of B2M. For each tissue, B2M and the target genes VEGF₁₂₁, VEGF₁₆₅, VEGF₁₈₉, and VEGF₅₇ were quantified simultaneously in triplicate in one LightCycler run, together with dilutions of calibrators spanning four orders of magnitude from 4 × 10⁶ to 4 × 10² copies per reaction mixture in duplicates and the appropriate non-template controls for each PCR. The range from 4 × 10⁶ to 4 × 10² copies (corresponding to cycles 20–35) was chosen because the Cts for all measured target copies were within ± 2 cycles of this range.

To have comparable conditions for analyzing VEGF expression in all 14 tissues, we set the mean Ct value of the first dilution of the calibrators (4 × 10⁶) to cycle 20 by adjusting the baseline. Statistics were performed using Excel 97 computer software (Microsoft Inc.).

**Results**

After extensive optimization of primer, MgCl₂, and bovine serum albumin concentrations as well as reaction temperatures and times, we obtained a highly specific, sensitive, and reproducible quantitative real-time RT-PCR assay for specific detection of the three most abundant VEGF splice variants as well as the complete VEGF message (VEGF_57).

**SPECIFICITY**

Specific detection of different VEGF variants was achieved with the following primer pairs: for VEGF₁₂₁, primer pair ex3fo-ex5/8re; for VEGF₁₆₅, primer pair ex3fo-ex5/7re; and for VEGF₁₈₉, primer pair ex3fo-ex6re. For the variants VEGF₁₂₁, VEGF₁₆₅, and VEGF₁₈₉, 10⁶ plasmid copies were detectable with the appropriate primer sets with a mean Ct of 24. Real-time RT-PCR with the “non-specific” primer set for the same copy number did not generate any reporter fluorescence signal even after 40 PCR cycles, and did not show any PCR product in 3% agarose gel electrophoresis after ethidium bromide staining (Fig. 2).

**DETERMINATION OF PCR SENSITIVITY AND EFFICIENCY**

The sensitivity of the PCR method using different primer/probe sets was determined from the Ct values obtained with known quantities of plasmid cDNA. All calibration curves for the plasmid cDNAs VEGF₁₂₁, VEGF₁₆₅, VEGF₁₈₉, and for B2M cDNA (each diluted 10-fold from 4 × 10⁶ to 4 × 10² copies of plasmid cDNA per reaction mixture) showed linearity over the entire
quantification range with correlation coefficients >0.99, indicating a precise log-linear relationship. Because the amplicons of the three VEGF splice variants and B2M have different sizes (range, 114–310 bp), PCR efficiency may vary. However, for all dilutions of the four different plasmid calibrators, the mean SD of the Ct values for each dilution step was 0.21 cycles. This deviation was not significantly different from the SD for the dilution series for the individual plasmid calibrators (SD = 0.18 cycles). The slopes for the dilution series for all four plasmid were 3.34–3.48, indicating comparable PCR amplification efficiencies. Thus, for the different targets, all experiments were performed using one calibration curve for all targets, including B2M (Fig. 3).

REPRODUCIBILITY

The intrarun variability, calculated from triplicate samples for all of the targets, showed an average SD for the Ct of 0.16 cycles. The difference in absolute Ct values for each set of triplicates was never >0.53 cycles. The interrun variability was assessed by comparing the results of 14 different runs (corresponding to 14 different tissues) using duplicates of the calibrator dilutions. When we compared the means of two replicate measurements at each dilution, the difference in the Ct was always <1.1 cycle,
with a mean SD for all runs of 0.21 cycles. The slopes of the calibration curves of all 14 runs were 3.32–3.52.

**VEGF Splice Variants and VEGFtotal Expression in Healthy and Malignant Tissues.**

The measured copy numbers for VEGFtotal cDNA were, on average, 90% (SD = 5.6%) of the sum of the different VEGF splice variant measurements in the 14 cDNA samples investigated. The LightCycler PCR products of all 14 cDNA samples were collected, and gel electrophoresis was performed. The copy numbers for the VEGF splice variants in all 14 tissues are presented in Table 1.

### Discussion

Increasing evidence indicates that the ratio of the VEGF isoforms rather than total VEGF is responsible for VEGF-mediated angiogenesis in physiological and pathological conditions (23–27). We present here for the first time a specific, sensitive, and reproducible real-time RT-PCR approach for absolute quantification of the most abundant VEGF splice variants (VEGF₁₂₁, VEGF₁₆₅, and VEGF₁₈₉) as well as of the total amount of VEGF (VEGFtotal), using LightCycler technology to obtain better insight into the regulation and distribution of the VEGF isoform ratio in healthy and malignant tissues.

Common immunological assays allow quantification of VEGF but fail to discriminate among isoforms because of a lack of VEGF isoform-specific monoclonal antibodies. Therefore, at present, investigation of the impact of differential expression of the various VEGF isoforms is possible only on the mRNA level. Several publications have indicated that expression of VEGF is controlled at the mRNA level by regulation of transcription rate and/or by changing mRNA stability (32,33). To date, competitive RT-PCR has been the only method for semi-quantitative detection of variant mRNA transcripts. This approach has two major disadvantages: (a) PCR endpoint analysis permits only semiquantitative analysis at a low sensitivity; and (b) formation of heteroduplexes between the different variants (34,35), especially for the extensively spliced VEGF variants (36), further impairs quantification. In contrast, real-time quantitative RT-PCR, where quantification is performed during the exponential phase of amplification, permits accurate quantification over a wide dynamic range without any post-PCR processing. Heteroduplex formation does not play any role in real-time RT-PCR amplification. In the assay described here, each splice variant was separately and specifically amplified.

As shown in Fig. 4, expression of the different VEGF splice variants is detectable when the external primer pair for VEGFtotal is used, but absolute quantification of each variant as well as VEGFtotal from the gel is not exact. Therefore, statements concerning the absolute quantities of different splice variants obtained by competitive PCR should be judged critically.

The presented standardized VEGF real-time RT-PCR assay using variant-specific reverse primers in combination with a common hydrolyzation fluorescent probe and a common forward primer ensures highly specific quantification with very good sensitivity and enables comparison of results generated by different investigators.

In this study, the mRNA message of VEGF splice variants and VEGFtotal in 14 (7 healthy and 7 malignant) tissues of different origin was investigated. The total VEGF message was generally up-regulated in malignant tissues (20.5–728 copies/10⁵ copies of B2M) compared with healthy tissues (4.2–81 copies/10⁵ copies of B2M). Most remarkably, the copy number for VEGFtotal and the sum of the different VEGF splice variant copies showed excellent correlation (on average, 90%; SD = 5.6%). In all 14 tissues, the splice variants VEGF₁₆₅ and VEGF₁₂₁ showed higher expression than VEGF₁₈₉. In healthy tissues, the mRNA encoding diffusible variants VEGF₁₂₁

| Table 1. Expression of VEGF splice variants in seven healthy and seven malignant tissue samples. |
|--------------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Healthy tissues                       | Malignant tissues |                 |
|                                      | LuCa1           | LuCa2           | PaCa            | BrCa            | OvCa            | PrCa            | CoCa            |
| VEGF₁₈₉                               |                 |                 |                 |                 |                 |                 |                 |                 |
| Lu                                    | 2.8             | 3.6             | 6.9             | 4               | 7.1             | 1.1             |                 |                 |
| Pa                                    | (0.1)           | (0.2)           | (0.3)           | (1.4)           | (0.2)           | (1.9)           | (0.3)           |                 |
| Ki                                    | 22%             | 22%             | 23%             | 23%             | 32%             | 22%             |                 |                 |
| Li                                    | 3                | 1               | 8               | (2.3)           | (2.3)           | (2.3)           |                 |                 |
| He                                    | 31.5            | 31.5            | 50.8            | 50.8            | 52              | 37              |                 |                 |
| SM                                    | 1.1             | 1.1             | 1.8             | 1.8             |                 |                 |                 |                 |
| Pl                                    |                 |                 |                 |                 |                 |                 |                 |                 |
| VEGF₁₆₅                               |                 |                 |                 |                 |                 |                 |                 |                 |
| Lu                                    | 4.1             | 12.5            | 14.4            | 6.2             | 6.9             | 50.8            | 1.8             |                 |
| Pa                                    | (0.1)           | (1.0)           | (1.1)           | (0.2)           | (0.2)           | (2.3)           | (0.2)           |                 |
| Ki                                    | 32%             | 45%             | 35%             | 35%             | 53%             | 53%             | 37%             |                 |
| Li                                    | 22%             | 22%             | 23%             | 23%             | 32%             | 22%             |                 |                 |
| He                                    | 7.1             | 1.1             | 8              | (2.3)           | (2.3)           | (37)            | (2.3)           |                 |
| SM                                    | 3.1             | 15.1            | 15.1            | 2               |                 |                 |                 |                 |
| Pl                                    |                 |                 |                 |                 |                 |                 |                 |                 |
| VEGF₁₂₁                               |                 |                 |                 |                 |                 |                 |                 |                 |
| Lu                                    | 6               | 15              | 10.4            | 7.5             | 3.1             | 15.1            | 2               |                 |
| Pa                                    | (0.8)           | (0.5)           | (1.2)           | (0.5)           | (1.4)           | (1.9)           |                 |                 |
| Ki                                    | 46%             | 46%             | 42%             | 42%             | 16%             | 41%             |                 |                 |
| Li                                    | 3.1             | 15.1            | 15.1            | 2               |                 |                 |                 |                 |
| He                                    | 81              | 4.2             |                 |                 |                 |                 |                 |                 |
| SM                                    | 0.7             |                 |                 |                 |                 |                 |                 |                 |
| Pl                                    |                 |                 |                 |                 |                 |                 |                 |                 |
| VEGFtotal                             |                 |                 |                 |                 |                 |                 |                 |                 |
| Lu                                    | 12.7            | 27.5            | 28              | 15               | 11.4            | 81              | 4.2             |                 |
| Pa                                    | (0.6)           | (2.1)           | (1.7)           | (0.9)           | (0.1)           | (8.1)           | (0.7)           |                 |
| Ki                                    | 47              | 20.5            | 114             | 728             | 30.6            | 33.2            | 24.5            |                 |
| Li                                    | (1.5)           | (1.2)           | (0.3)           | (3.5)           | (43.8)          | (0.5)           | (2.9)           | (1.2)           |

*All data represent mean of triplicates, presented as copy numbers of target gene per 1000 copy numbers of B2M. Standard deviation of copy numbers is given in parentheses. The relative amounts of the individual splice variants related to the total sum are given as percentages.

*b Healthy tissues: Lu, lung; Pa, pancreas; Ki, kidney; Li, liver; He, heart; SM, skeletal muscle; Pl, placenta.

*c Malignant tissues: LuCa1, lung carcinoma, explant from metastasis; LuCa2, lung carcinoma, explant from tumor; PaCa, pancreatic adenocarcinoma; BrCa, breast carcinoma; OvCa, ovarian carcinoma; PrCa, prostatic carcinoma; CoCa, colon adenocarcinoma.
and VEGF<sub>165</sub> constituted on average 78% (SD = 9.3%) of the total VEGF message, and the cell-adherent variant VEGF<sub>189</sub> constituted on average 22% (SD = 5.4%). In contrast, in malignant tissues, VEGF<sub>121</sub> and VEGF<sub>165</sub> constituted 94% (SD = 7.6%) of the total VEGF message, whereas VEGF<sub>189</sub> was 6% (SD = 3.7%). When we compared the absolute copy numbers for VEGF<sub>189</sub> in all healthy and malignant tissues, no difference was found (Table 1), whereas VEGF<sub>165</sub> and VEGF<sub>121</sub> were up-regulated in malignant tissues. Our data, indicating that overexpression of VEGF in tumors is caused by the more diffusible variants, support the observations that during malignant progression an angiogenic switch favoring the shorter diffusible isoforms (VEGF<sub>121</sub> and VEGF<sub>165</sub>) occurs (27).

Because of the small number of correlated tissues investigated, differences in VEGF expression mark only a trend and allow no final conclusions. To verify the findings and to confirm the utility of the presented method, fresh malignant and healthy tissues from the same individuals should be measured. Furthermore, the prognostic significance of this assay could be demonstrated by examining malignant tissues from individuals with carcinomas in situ and by comparing with individuals with metastatic cancers.

In conclusion, the presented quantitative real-time RT-PCR assay clearly demonstrates many advantages over other existing methods for (semi)quantitative analysis of VEGF splice variants, including specificity, sensitivity, reduced carryover contamination, and rapid, accurate, and simultaneous quantification of multiple samples. Thus, we believe that this assay may be a promising tool for further investigations in the field of tumor angiogenesis and prognostic impact of VEGF in cancer.

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