Real-Time Reverse Transcription-PCR Quantification of Vascular Endothelial Growth Factor Splice Variants, Eleni Zygalaki,1 Aliki Statidopoulou,1 Christos Kroupis,2 Loukas Kaklamanis,2 Zenon Kyriakides,3 Dimitrios Kremastinos,2 and Evi S. Lianidou1*

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Vascular endothelial growth factor (VEGF) is an endothelial cell–specific mitogen and a key regulator of angiogenesis in a variety of physiologic and pathologic processes (1). The human gene for VEGF resides on chromosome 6p21.3 and is organized into 8 exons (2, 3). Alternative exon splicing of the VEGF gene produces various splice variants (subscripts denote the number of amino acids after signal sequence cleavage): VEGF121, VEGF165, VEGF183, VEGF148, VEGF145, and VEGF206 (4–8). Exons 1–5 and 8 are preserved in all variants, whereas the presence or absence of exons 6a, 6b, and 7 distinguish the VEGF isoforms (Table 1). Most VEGF-producing cells appear to preferentially express VEGF121, VEGF165, and VEGF183 (4). VEGF183 has a wide tissue distribution and may have not been detected earlier because of confusion with VEGF189 (6). VEGF145 is one of the main variants produced by several cell lines derived from carcinomas of the female reproductive system (7), and it has also been detected in ovarian and breast cancer tissue samples (10). VEGF148 was identified in human glomeruli (5), but there are no data concerning its expression in other tissues. VEGF206 is a very rare isoform found in a human fetal liver library (8).

We developed a real-time reverse transcription-PCR (RT-PCR) method for the quantification of 6 VEGF splice variants (VEGF121, VEGF145, VEGF148, VEGF145, VEGF183, and VEGF189) and applied it to 2 human breast cancer cell lines, MCF-7 and MDA-MB-231, and a human colorectal cancer cell line, COLO-205.

The sequences of the primers and probes used are presented in Table 1, and the principle of the proposed assay is shown in Fig. 1A. A common forward primer (VEGFex3F) and a common pair of hybridization probes (VEGF-FL and VEGF-LC) were used (TIB MOLBIOL). The VEGFex3F primer and the VEGF-FL probe are located on exon 3, whereas the VEGF-LC probe spans the exon 3/4 boundaries to avoid genomic DNA detection. For the quantification of total VEGF mRNA, we used the VEGFex8R reverse primer located on exon 8 (common for all variants), which generated amplicons of various sizes (233, 305, 330, 365, 419, and 437 bp for VEGF121, VEGF145, VEGF145, VEGF183, VEGF165, VEGF183, and VEGF189, respectively). Amplification of each variant was performed with reverse primers spanning variant-specific exon boundaries: 5′/8 for VEGF121 (VEGFR121; 223 bp), 6a/8 for VEGF145 (VEGFR145; 295 bp), 7a/8 for VEGF148 (VEGFR148; 320 bp), 5′/7 for VEGF165 (VEGFR165; 224 bp), 6a/7 for VEGF183 (VEGFR183; 274 bp), and 6a/7 for VEGF189 (VEGFR189; 289 bp). The VEGFR165 reverse primer is not specific for VEGF165 because VEGF148 can also be amplified because the region exon 5/exon 7 is common in both variants.

Real-time PCR was performed in the LightCycler Instrument (Roche Applied Science) in a total volume of 10 µL per glass capillary. For each reaction, 1 µL of cDNA was placed in a 9-µL reaction mixture containing 0.1 µL of water, 0.15 mM MgCl2, 0.2 µL of deoxynucleotide triphosphates (10 mM; Platinum DNA Polymerase; Invitrogen), 1 µL of the supplied 10× PCR buffer, 0.7 µL of bovine serum albumin [10 µg/µL; Sigma (11)], 0.5 µL of the primers and probes (3 µM), and diethylpyrocarbonate-treated H2O. The cycling protocol was identical for all splice variants and consisted of an initial 5-min denaturation step at 95 °C for activation of the DNA polymerase, followed by 50 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 15 s, and extension at 72 °C for 20 s.

For the development and analytical evaluation of the assay, we generated and used as calibrators PCR amplimers corresponding to the 6 VEGF splice variants studied. For this purpose, total RNA was extracted from MCF-7 cells; cDNA was synthesized and served as a template for the amplification of the targets of interest by real-time PCR. PCR products were separated on a 2% agarose gel.

### Table 1. Sequences of primers and hybridization probes used in this study.

<table>
<thead>
<tr>
<th>Name</th>
<th>Oligonucleotide sequence, 5′–3′</th>
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<tbody>
<tr>
<td>Forward primer</td>
<td>VEGFex3F ATCTTCACGCGCATCCTGTGTCGC</td>
</tr>
<tr>
<td>Reverse primers</td>
<td>VEGFR121 TGCCCTGTACATTTTCTTG</td>
</tr>
<tr>
<td></td>
<td>VEGFR145 TGCCCTGTACATCAGCCCTCC</td>
</tr>
<tr>
<td></td>
<td>VEGFR148 TGCCCTGTACATCAGCCCAAC</td>
</tr>
<tr>
<td></td>
<td>VEGFR183 GCCCACAGGGGATTT</td>
</tr>
<tr>
<td></td>
<td>VEGFR189 CGACAGGGACGGAGGAGGASTT</td>
</tr>
<tr>
<td>Hybridization probes</td>
<td>VEGF-FL AGTGCTGTCACAGCTAGGAGGAGTCC FLa</td>
</tr>
<tr>
<td></td>
<td>VEGF-LC LC Red 640/ACATCAGGCAAGGCAGGAGTC PHb</td>
</tr>
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a 3′-End labeled with fluorescein (FL).

b 5′-End labeled with LC Red 640; phosphorylated (Ph) on the 3′ end to avoid extension of the probe.
The bands were excised and purified by use of the QIAQuick Gel Extraction Kit (Qiagen), and the amplicons were quantified with the PicoGreen DNA Quantification Kit (Molecular Probes). Concentrations were converted to copies/µL by use of the Avogadro constant and the molecular weight of each amplicon [number of bases of the PCR product multiplied by the mean molecular weight of a pair of nucleic acids which is 660 (12)].

To establish a specific, sensitive, and reproducible real-time RT-PCR assay, we performed extensive optimization of primers, probes, and MgCl₂ concentrations as well as reaction temperatures and times. The analytical evaluation of the assay was performed with the prepared VEGF calibrators. For each splice variant, a calibration curve was generated from serial dilutions ranging from 10⁶ to 10 copies/µL of the target of interest. All calibration curves showed linearity over the entire quantification range with correlation coefficients >0.99. The between-day imprecision of the calibration curves ranged from 0.5% to 3.9% (Table 1 of the Data Supplement that accompanies the online version of this Technical Brief at http://www.clinchem.org/content/vol51/issue8/). The analytical detection limit of the method, defined as 3.3 times the SD of the crossing point for the less concentrated calibrator divided by the mean slope of the calibration curve, corresponded to 1 copy/µL for each target, whereas the limit of quantification, defined as 3 times the detection limit, was 3 copies/µL.

Because the amplicons generated by PCR have different sizes, the reaction efficiency could vary. However, the mean (SD) slopes of the 6 calibration curves ranged from −3.63 (0.29) to −3.95 (0.08), indicating comparable PCR efficiencies (1.79–1.89), and the mean (SD) intercepts of the calibration curves ranged from 37.3 (0.9) to 38.3 (0.6) copies/µL from day to day. When we used all of the data generated from the 6 different calibration curves to plot a single calibration curve, the correlation coefficient was 0.99, indicating that this grand mean calibration curve could be used for the quantification of all 6 splice variants as well as total VEGF. The assay was highly specific for each splice variant. When cDNA from 10⁵ MCF-7 cells was used as a template for real-time PCR, agarose gel electrophoresis of the PCR products after 50 cycles revealed only one specific product of the desired length for each splice variant (Fig. 1B).

We investigated the expression of VEGF splice variants in 3 cancer cell lines (MCF-7, MDA-MB-231, and COLO-205). VEGF₁₂₁ and VEGF₁₆₅ were the major variants expressed, followed by VEGF₁₈₉, whereas VEGF₁₄₅, VEGF₁₄₈, and VEGF₁₈₃ were present only in small amounts (Fig. 1C). VEGF₁₄₈ was detected for the first time in cancer cell lines.

In conclusion, we have developed a sensitive, specific real-time RT-PCR assay for the quantification of VEGF splice variants and total VEGF. A similar approach has already been described, but only the major VEGF variants VEGF₁₂₁, VEGF₁₆₅, and VEGF₁₈₉ were investigated (11). In addition to these splice variants, we also included in our work the less-studied variants VEGF₁₄₅, VEGF₁₄₈, and VEGF₁₈₃. The analytical performance of the assay was validated through a series of experiments based on VEGF calibrators, which were specifically designed, synthesized, and quantified in a novel way in our laboratory (13). The use of PCR amplicon–based calibrators to generate calibration curves has also been described by others (14). This approach is an alternative to the use of plasmid vectors and is useful for laboratories that do not have the facilities for transformation of bacterial cultures and subsequent plasmid DNA isolation.

The biological significance of the alternative splicing of the VEGF gene is still uncertain. We believe that the developed real-time RT-PCR assay, which provides rapid,
accurate quantification of the expression of 6 VEGF variants, can be a promising tool for the elucidation of their role in promoting physiologic and pathologic angiogenesis. Our method can be applied to various samples for the separate quantification of a single VEGF splice variant of interest or of all 6 variants together and could serve as a model for the development of real-time RT-PCR assays for the quantification of other alternatively spliced genes.

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References


Comparing Whole-Genome Amplification Methods and Sources of Biological Samples for Single-Nucleotide Polymorphism Genotyping, Ji Wan Park,1,2 Terri H. Beatty,1 Paul Boyce,3 Alan F. Scott,2 and Iain McIntosh1,* (1 Department of Epidemiology, Bloomberg School of Public Health, and 2 McKusick-Nathans Institute of Genetic Medicine, School of Medicine, Johns Hopkins University, Baltimore, MD; * address correspondence to this author at: Johns Hopkins University, McKusick-Nathans Institute of Genetic Medicine, 733 N. Broadway/BRB 407, Baltimore, MD 21205; fax 410-502-5677, e-mail mcintosh@jhmi.edu)

High-throughput genotyping systems promise to be an efficient means of identifying susceptibility genes involved in the etiology of non-Mendelian disorders. Adequate amounts of high-quality DNA are essential, however, for large-scale genotyping studies (1). The supply of genomic DNA is frequently limited, and the quality of DNA obtained from oral buccal swabs or Guthrie cards has not been thoroughly evaluated for high-throughput single-nucleotide polymorphism (SNP) genotyping (2,3). Whole-genome amplification (WGA) technologies offer the opportunity to expand DNA from depleted biological samples. The first generation of WGA strategies (i.e., PCR-based methods) (4,5), however, was limited by substantial amplification bias and incomplete coverage of genetic markers (6,7). Recently, new strategies for WGA, such as multiple displacement amplification (MDA) or OmniPlex® WGA technology (Rubicon Genomics) have been developed. MDA is an isothermal amplification with the bacteriophage φ29 DNA polymerase (6,8), whereas OmniPlex uses in vitro libraries with fragmented DNA (~1.5 kb) to amplify the entire genome by PCR (9).

To apply WGA technology to BeadArray™ genotyping (Illumina), the utility of MDA and/or OmniPlex on DNA samples derived from lymphoblast cells has been evaluated (9,10). In this study, we determined the genotyping success rate and reliability of 2 MDA variants (8,11) and OmniPlex with and without 7-deaza-dGTP, using buccal swabs, whole blood, dried blood spots, and sheared genomic DNA on 1260- and 1228-SNP BeadArray panels. The 7-deaza-dGTP nucleotide analog was included in an attempt to ensure amplification of GC-rich DNA.

After Institutional Review Board approval and informed consent were obtained, DNA samples were collected from participants in a study of oral clefts (12). Genomic DNA samples were prepared from peripheral blood by protein precipitation (13). Buccal cells were obtained by rubbing the inside of cheeks with a brush (Medical Packaging), and DNA was extracted with 600 μL of 50 mmol/L NaOH (2). For blood spot samples dried on Guthrie cards, two to three 2-mm circles punched from the blood spots with a micro-punch (Fitzco) were placed in 5 g/100 mL Chelex® 100 chelating resin (Bio-Rad Laboratories) (14) and treated as described (blood spot A) (15) or stored at −20 °C for ~3 years (blood spot C). As an alternative method, 2 to 3 circles punched from the blood spots were placed in a cell lysis solution (10 μL of 0.2

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