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Technical Advance

Molecular Profiling of Angiogenesis Markers

Shu-Ching Shih,* Gregory S. Robinson,* Carole A. Perruzzi,† Alfonso Calvo,‡ Kartiki Desai,‡ Jeffery E. Green,‡ Iqbal U. Ali,§ Lois E. H. Smith,* and Donald R. Senger†

From the Department of Ophthalmology, Children's Hospital, Harvard Medical School, Boston, Massachusetts; the Division of Cancer Prevention,‡ National Cancer Institute, National Institutes of Health, Bethesda, Maryland; the Center for Cancer Research,§ National Cancer Institute, National Institutes of Health, Bethesda, Maryland; and the Department of Pathology,† Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts*

The goal of this study was to develop a sensitive, simple, and widely applicable assay to measure copy numbers of specific mRNAs using real-time quantitative reverse transcriptase-polymerase chain reaction (RT-PCR), and identify a profile of gene expression closely associated with angiogenesis. We measured a panel of nine potential angiogenesis markers from a mouse transgenic model of prostate adenocarcinoma (TRAMP) and a mouse skin model of vascular endothelial growth factor (VEGF)-driven angiogenesis. In both models, expression of VEGF correlated with expression of mRNAs encoding other angiogenic cytokines (angiopoietin-1 and angiopoietin-2), endothelial cell receptor tyrosine kinases (Flt-1, KDR, Tie-1), and endothelial cell adhesion molecules (VE-cadherin, PECAM-1). Relative to control, in dermis highly stimulated by VEGF, the Ang-2 mRNA transcript numbers increased 35-fold, PECAM-1 and VE-cadherin increased 10-fold, Tie-1 increased 8-fold, KDR and Flt-1 each increased 4-fold, and Ang-1 increased 2-fold. All transcript numbers were correspondingly reduced in skin with less VEGF expression, indicating a relationship of each of these seven markers with VEGF. Thus, this study identifies a highly efficient method for precise quantification of a panel of seven specific mRNAs that correlate with VEGF expression and VEGF-induced neovascularization, and it provides evidence that real-time quantitative RT-PCR offers a highly sensitive strategy for monitoring angiogenesis. ***(Am J Pathol 2002, 161:35–41)***

Angiogenesis, the growth of new blood vessels, is widely regarded as an attractive target for controlling cancer and other important pathologies including retinopathy. Angiogenesis occurs early in tumor progression.[1,2] Moreover, angiogenesis correlates not only with the onset of tumor development but also with growth and invasion of established tumors.[1,3] Consequently, tools for quantifying angiogenesis are valuable for molecular profiling of tumors, monitoring the status of tumor progression and estimating the malignant potential of established tumors. In addition, molecular quantification of angiogenesis would be useful for the study of a variety of experimental models of pathological angiogenesis including retinopathy and arthritis.

The most widely used technique to assess angiogenesis in clinical settings relies on immunohistochemical staining of blood vessels in fixed biopsies of specimens. Even with the use of sophisticated computerized imaging systems, this technique is labor-intensive and cannot be used as a quick and quantitative screening procedure. Individual vascular markers have been studied by quantitative and semi-quantitative techniques usually in a single tissue, but there has been little work on a comprehensive assay suitable for a varying degrees of angiogenesis. Thus, there is a need to develop a sensitive molecular assay, that allows molecular profiling of new vessel growth and that can be used to monitor angiogenesis and the efficacy of chemopreventive and other drug-based therapies toward suppressing angiogenesis.

Real-time quantitative (kinetic) PCR technology offers high sensitivity and high throughput capacity.[4–6] This technique uses SYBR Green I dye to detect and verify product by melting curve analysis, and is applicable for validation of samples containing cDNA fragments prepared with gene-specific primers.[4–6] The PCR amplification and detection in real-time quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) occur

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The present address for Gregory S. Robinson is Pharmacia Corporation, St. Louis, MO 63198.

Address reprint requests to Lois E. H. Smith. Department of Ophthalmology, Children's Hospital, 300 Longwood Avenue, Boston, MA 02115. E-mail: lois.smith@tch.harvard.edu.

simultaneously, thereby avoiding the need for post-PCR analysis and minimizing the risk of carryover contamination. Furthermore, this technique is highly reproducible over a wide dynamic range, and permits simultaneous quantitative analysis of a large number of samples with varying input concentrations allowing for statistically significant data. Using real-time quantitative PCR technology and precisely quantified external cDNA templates as standards, the TaqMan system (Applied Biosystem) is capable of detecting copy numbers of nucleic acid targets with sensitivity as low as 10 copies with optimal detection from 100 to 500 copies. Recent studies have applied the technique to measure copy numbers of target genes relative to cell number[7] or total RNA.[8] However, a method for precise standardization is required to accurately and reproducibly measure mRNA copy number from different sources.

Therefore, in this study we used real-time quantitative RT-PCR in combination with specific cDNA standards to determine absolute mRNA copy numbers. Since it is likely that no single mRNA marker is predictive of the degree of angiogenesis in all tumor types and all tissues, we evaluated a panel of potential markers. Such markers include those directly correlated with endothelial cell number and/or mRNAs encoding proteins directly associated with the process of neovascularization. VE-cadherin, an adhesion receptor specific to endothelium, is one candidate for which the expression level would be expected to correlate with endothelial cell number.[9,10] Other candidates, expressed in relative abundance by endothelial cells in comparison with other cell types, include those encoding the adhesive protein PECAM-1[11,12] and the receptor tyrosine kinases Flt-1, KDR, Tie-1, and Tie-2.[13–18] Finally, VEGF[14,19,20] and two other cytokines associated with angiogenesis, angiopoietin-1 (Ang-1) and angiopoietin-2 (Ang-2),[21–24] were included in our analyses.

Two independent animal models served as sources of tissue for testing the correlation of the marker panel with angiogenesis. One of them is a mouse transgenic model of prostate adenocarcinoma (TRAMP), in which spontaneous prostate intraepithelial neoplasia (PIN) develop at about 6 to 12 weeks of age and progress to invasive carcinoma and neoplasia between 18 and 24 weeks of age. Tumor progression and histological features of carcinomas in this animal model highly resemble human prostate carcinoma. In the TRAMP model, the probasin promoter drives the expression of SV40 Tag in the prostate causing a functional inactivation of P53 and Rb tumor suppressor genes.[25] Tumors are highly vascularized[26] and metastatic[27] and therefore, TRAMP mice constitute an excellent model to study angiogenic factors. The other is a mouse Matrigel model of skin angiogenesis in which neovascularization is provoked by subcutaneous implantation of Matrigel containing human VEGF-transfected human melanoma cells. Real time RT-PCR data from both models identified the same panel of mRNAs which corresponded to both VEGF expression and VEGF-induced neovascularization. Overall, this study indicates that real-time RT-PCR offers a sensitive new approach for molecular profiling of a panel of mRNA markers that is closely associated with tumor neovascularization.

Materials and Methods

Skin Angiogenesis Model

Athymic NCr nude mice (7 to 8 weeks old, females) were injected subdermally midway on the right and left back sides with 0.25 ml Matrigel (BD Biosciences, San Diego, CA) at a final concentration of 10 mg/ml protein, together with 1.5×10^6 SK-MEL-2 cells transfected with pcDNA3.1 (Invitrogen) containing a human $VEGF_{165}$ insert under the direction of the CMV promoter. To provoke different intensities of neovascularization, two clonal transfectants expressing different levels of VEGF were isolated and characterized (see Results). Soon after injection, the Matrigel implant solidified, and persisted without apparent deterioration throughout the 6-day assay interval. After 6 days, the animals were euthanized, and specimens harvested by dissection.

TRAMP Mice

The C57BL/6 male TRAMP mice were obtained from Jackson Laboratories and were bred and tested positive by slot blot. Animals were observed weekly for tumor appearance and prostate tumors were removed at age of 8 to 9 months. Normal prostates were obtained from C57BL/6 non-transgenic male mice at the same age. Part of each tumor was frozen in liquid nitrogen before RNA extraction and the rest was immersed in a 4% paraformaldehyde solution for histological analysis. Fixed samples were dehydrated through ethanol and xylene, embedded in paraffin, sectioned (4-μm in thickness) and stained with hematoxylin and eosin.

RNA Isolation and cDNA Preparation

Total RNA was extracted by using RNeasy RNA extraction kit (Qiagen, Chatsworth, CA). Briefly, tissues were lysed in guanadinium isothiocyanate buffer, and RNA was purified following manufacturer's instructions. The purified RNA was suspended in diethyl pyrocarbonate (DEPC)-treated H_2O. To generate cDNA, 1 μg total RNA was treated with DNase I (Ambion, Austin, TX) to remove any contaminating genomic DNA. The DNase-treated RNA (100 ng) was then converted into cDNA by using murine leukemia virus reverse transcriptase (Gibco BRL Life Technologies, Bethesda, MD). All cDNA samples were aliquoted and stored at −80°C.

Northern Analyses

Northern blotting was performed as described previously.[28] 10 μg of total RNA was used from each sample and hybridization was carried out overnight at 65°C with $\alpha[^{32}P]$dCTP-labeled human VEGF165 (823-bp fragment encompassing the coding region and 330-bp of 3′UTR)

Table 1. TaqMan Primer Sequences

Gene	Forward	Reverse
Ang-1	329–CATTCTTCGCTGCCATTCTG–348	431–GCACATTGCCCATGTTGAATC–411
Ang-2	1478–TTAGCACAAAGGATTCGGACAAT–1500	1598–TTTTGTGGGTAGTACTGTCCATTCA–1574
Cyclophinin	5–CAGACGCCACTGTCGCTTT–23	137–TGTCTTTGGAACTTTGTCTGCAA–115
Flt-1	2421–GAGGAGGATGAGGGTGTCTATAGGT–2445	2536–GTGATCAGCTCCAGGTTTGACTT–2514
KDR	4570–GCCCTGCTGTGGTCTCACTAC–4590	4632–CAAAGCATTGCCCATTCGAT–4666
PECAM	1652–GAGCCCAATCACGTTTCAGTTT–1673	1769–TCCTTCCTGCTTCTTGCTAGCT–1748
Tie-1	742–CAAGGTCACACACACGGTGAA–762	863–GCCAGTCTAGGGTATTGAAGTAGGA–839
Tie-2	1804–ATGTGGAAGTCGAGAGGCGAT–1824	2081–CGAATAGCCATCCACTATTGTCC–2059
VE-cadherin	1853–TCCTCTGCATCCTCACTATCACA–1875	1974–GTAAGTGACCAACTGCTCGTGAAT–1951
VEGF	805–GGAGATCCTTCGAGGAGCACTT–826	933–GGCGATTTAGCAGCAGATATAAGAA–909

and control β-actin cDNA. Probes were prepared by the random-primed synthesis method using the Multiprime kit (Amersham Corp., Arlington Heights, IL). Blots were washed at high stringency (1% SDS and 1X SSC at 55°C) and exposed to Kodak MR film.

TaqMan Quantitative Real Time RT-PCR

Real-time quantitative RT-PCR primers targeting murine VEGF, Flt-1, KDR, Tie-1, Tie-2, Ang-1, Ang-2, PECAM, VE-cadherin, and cyclophilin were designed by using Primer Express software (Applied BioSystems, Foster City, CA) and sequences are listed in Table 1 (5′ to 3′). Specificity of each primer to the sequence of choice was checked by National Center for Biotechnology Information (NCBI) Blast module and was synthesized by Genemed Synthesis (South San Francisco, CA). To assure the specificity of each primer set, amplicons generated from PCR reactions were analyzed for specific melting point temperatures by using the first derivative primer melting curve software supplied by Applied BioSystems. The SYBR Green I assay and the ABI Prism 7700 sequence detection system (Applied Biosystems) were used for detecting real-time quantitative PCR products from 0.25–2.5 ng reverse-transcribed cDNA samples and performed as described previously.[29] SYBR Green I dye intercalation into the minor groove of double-stranded DNA reaches an emission maximum at 530 nm. PCR reactions for each sample were done in duplicate for both target gene and cyclophilin control. The level of target gene expression was calculated following normalization of the cyclophilin level in each sample and presented as relative units.

cDNA Template Construction for Absolute Quantitation of Specific mRNAs

For each of nine genes studied, we cloned cDNA that covers the region of real-time quantitative RT-PCR primers and inserted the cDNA into PCRScript vector (Strategene, La Jolla, CA). To use as an external standard for real-time quantitative PCR detection, each cDNA construct was purified, precisely quantified, and 10-fold serially diluted to 10 copies per microliter. SYBR Green I provided reproducible detection of the cDNA template that ranged from less than 10 copies per reaction to more than 10^7 copies per reaction. In each real-time quantitative PCR assay, a 10-fold serially diluted cDNA template series was added to measure standard curve for copy number. Each sample was analyzed in triplicate and copy numbers determined from each corresponding standard curve. Each gene was then normalized to 10^6 copies of cyclophilin control and data presented as copy numbers/10^6 cyclophilin copies.

Results

To measure quantitatively the level of gene expression in cDNA samples, specific TaqMan PCR primers were designed for each potential angiogenesis marker listed in Table 1. The specificity of each primer set was validated by analyzing the melting point temperature of each amplicon in the ABI Prism 7700 Sequence Detection System. Cyclophilin, a protein known as a primary cytosolic receptor for cyclosporine A, is abundant (0.05% to 0.4% of total protein) in the cytosol and is ubiquitous in cells and tissues of eukaryotic organisms.[30] The expression level of cyclophilin was constant in both TRAMP and skin samples in comparison with 18S rRNA (data not shown). Since the level of 18S rRNA transcript is almost 2^{12} to 2^{30} times higher than most mRNAs in real-time quantitative real-time quantitative RT-PCR detection, it is difficult to use 18S rRNA as a housekeeping gene and accurately calculate the target gene expression. Therefore, cyclophilin mRNA was used as the internal control gene in our real-time quantitative RT-PCR studies.

First, to identify potential tumor angiogenesis markers, total RNA was isolated from two normal and two TRAMP C57 mice prostates and was reverse-transcribed into cDNA. Tumor TRAMP1 was isolated at 8 months of age and was 1 cm^3 in size, whereas tumor TRAMP2 was isolated at 9 months of age and was nearly double the size of TRAMP1. The histological analysis for both tumors revealed the presence of advanced prostate adenocarcinomas with the characteristics described previously. Neoplastic cells were relatively small and showed hyperchromatic nuclei and small amount of cytoplasm. Both tumors also showed high vascularization and areas of necrosis. Relative to normal prostate, both TRAMP1 and TRAMP2 tumors expressed higher levels of all eight angiogenesis associated mRNAs listed in Table 1 (Figure 1). Interestingly, the TRAMP2 tumor, whose VEGF expression was 3.7-fold higher than TRAMP1 tumor, also expressed higher levels of Ang-1, Ang-2, Flt-1, KDR,

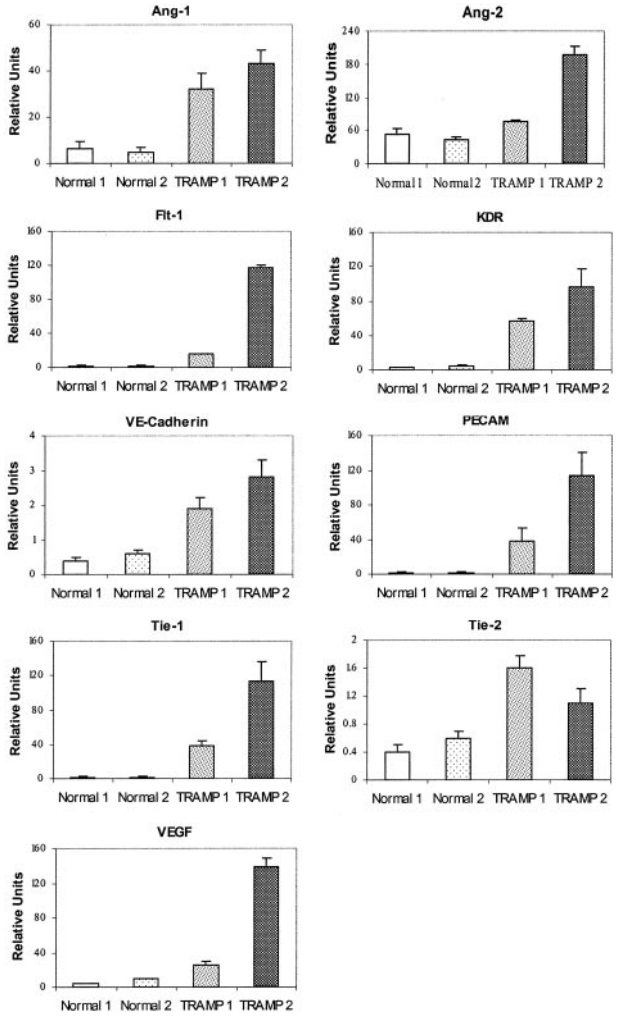

Figure 1. Real-time quantitative RT-PCR quantitation of gene expression in mouse TRAMP tumors. Total RNA and cDNA was prepared from two normal prostate (Normal 1 and Normal 2) and two TRAMP tumors (TRAMP 1 and TRAMP 2). The level of gene expression in the cDNA was measured in the presence of its respective primer and SYBR Green I Dye in real-time quantitative PCR. PCR reactions for each sample were performed in duplicate for both target gene and cyclophilin normalizer. The level of gene expression was calculated after normalizing against the cyclophilin level in each sample and presented as relative units in the graph.

PECAM, Tie-1, and VE-cadherin mRNAs. Tie-2 expression did not follow the same trend, although Tie-2 expression was higher in both TRAMP tumors as compared with normal prostate controls.

To precisely measure the expression of the candidate marker mRNAs identified above in relation to VEGF expression, we transfected human SK-MEL-2 melanoma cells with a human $VEGF_{165}$ cDNA construct and isolated two clones expressing different levels of human VEGF mRNA (Figure 2). To determine absolute mRNA numbers, we cloned and isolated individual cDNA templates that cover the sequences bracketed by the real-time quantitative PCR primers (Table 1) and used precisely quantified template preparations to establish standard curves of amplification (see Methods). Quantitation of cyclophilin mRNA provided an internal standard, and data are presented as specific mRNA copy numbers/10^6 copies of cyclophilin. As shown in Figure 2A, Northern blot analyses clearly demonstrated higher expression of VEGF mRNA in hVEGF-High SK-MEL-2 melanoma cells in comparison with hVEGF-Low SK-MEL-2 melanoma cells. Densitometric analyses suggested that the difference was approximately ninefold. Absolute quantitation of VEGF copy numbers with real-time quantitative RT-PCR showed that hVEGF-High transfectants expressed 5.2-fold more VEGF mRNA than the hVEGF-Low transfectants (1.5×10^5 copies of VEGF/10^6 copies of cyclophilin, *vs.* 2.9×10^4 copies of VEGF/10^6 copies of cyclophilin) (Figure 2B). When implanted subdermally with Matrigel in nude mice, the hVEGF-High transfectants clearly induced a significantly greater degree of skin angiogenesis than the hVEGF-Low transfectants, whereas Matrigel alone did not provoke any detectable angiogenesis (Figure 2C). Consequently, this Matrigel hVEGF-transfectant model served as a well-controlled source of neovascularized tissue for identification of mRNAs that correlate with VEGF expression and angiogenesis.

To quantify the candidate angiogenesis marker mRNAs expressed in the area of hVEGF-induced skin neovascularization, the implanted Matrigel was removed and cDNAs were prepared from skin specimens associated with control Matrigel, hVEGF-Low transfectants, and hVEGF-High transfectants. As shown in Figure 3, cDNAs prepared from skin specimens associated with both hVEGF-Low and hVEGF-High transfectants clearly demonstrated higher copy numbers of Flt-1, KDR, VE-cadherin, PECAM, Ang-1, Ang-2, and Tie-1 in comparison with skin specimens associated with Matrigel alone. Furthermore, the levels of each of these markers were highest in samples associated with the hVEGF-High transfectants. In skin specimens associated with control Matrigel, KDR had the highest mRNA copy number (7.5×10^4 copies of KDR/10^6 copies of cyclophilin), whereas PECAM, and Tie-2 had approximately $1.5–1.9 \times 10^4$ copies/10^6 copies of cyclophilin. Ang-1, Ang-2, Flt-1, Tie-1, and VE-cadherin had the lowest copy numbers (less than 10^4 copies/10^6 copies of cyclophilin). In skin specimens associated with hVEGF-High transfectants, for every 10^6 copies of cyclophilin mRNA we found approximately 3.1×10^5 copies of KDR, 1.8×10^5 copies of PECAM, 8.8×10^4 copies of VE-cadherin, 4.3×10^4 copies of Ang-2, 2.9×10^4 copies of Tie-1, 2×10^4 copies of Tie-2, 1.8×10^4 copies of Flt-1, and 1.3×10^4 copies of Ang-1. This indicates a ~35-fold induction of Ang-2, 10-fold induction of PECAM and VE-cadherin, 8-fold induction of Tie-1, and a 4-fold induction of Flt-1 and KDR in skin specimens associated with VEGF-High transfectants in comparison with control Matrigel (Table 2). Interestingly, as observed with the TRAMP tumors (Figure 1), Tie-2 expression did not correlate with VEGF expression or the other candidate markers. All three types of skin samples (control, hVEGF-High, and hVEGF-Low) showed similar levels of endogeneous VEGF expression (approximately 1.9×10^4 copies VEGF/10^6 copies of cyclophilin); thus the skin angiogenesis induced in human VEGF transfectants was attributable to exogenous human VEGF released from Matrigel.

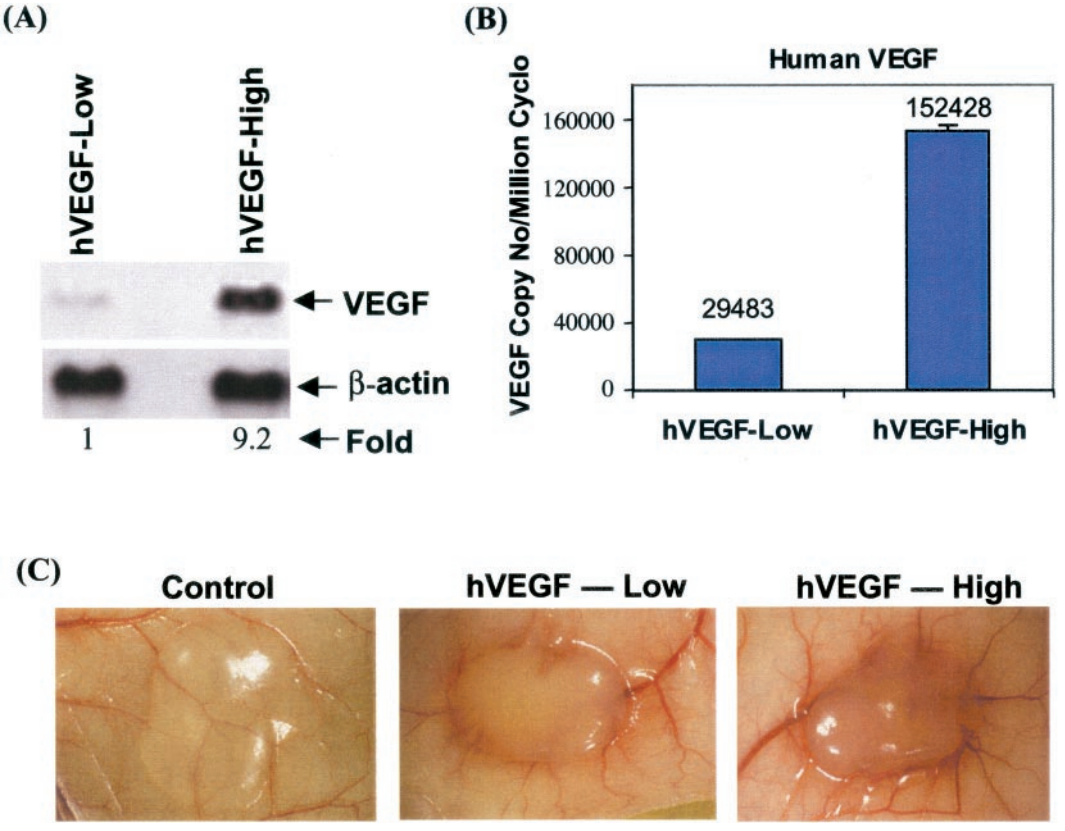

Figure 2. Induction of neovascularization in nude mouse skin by SK-MEL-2 human melanoma VEGF-transfectants. Human SK-MEL-2 melanoma cells were transfected with human $VEGF_{165}$ under the direction of a CMV promoter and clones (hVEGF-Low and hVEGF-High) were isolated. **A:** Northern blot detection of VEGF expression in hVEGF-Low and hVEGF-High clones. The fold increase of hVEGF-High over hVEGF-Low was calculated after normalization to the β-actin. **B:** Real-time quantitative RT-PCR detection of absolute VEGF copy numbers in VEGF-Low and VEGF-High clones. **C:** Angiogenesis in nude mouse skin was induced by subdermal injection of 0.25cc Matrigel containing 1.5×10^6 transfected cells as indicated or no cells (control). Tissue was harvested and photographed at day 6.

Discussion

An important early event during the progression of many cancers is the acquisition and maintenance of new blood vessels, which is now recognized both as a promising prognostic indicator and a target for therapy.[1,20,31] In this study, we used real time RT-PCR to analyze relative changes and absolute mRNA copy numbers of a panel of potential angiogenesis markers in mouse transgenic model of prostate adenocarcinoma (TRAMP) and hVEGF-driven skin angiogenesis, respectively. Results showed an excellent quantitative correlation between VEGF and Ang-1, Ang-2, Flt-1, KDR, VE-cadherin, PECAM, and Tie-1 in these two experimental models of angiogenesis.

The strength of a real-time quantitative RT-PCR assay lies in its potential to rapidly and precisely measure a

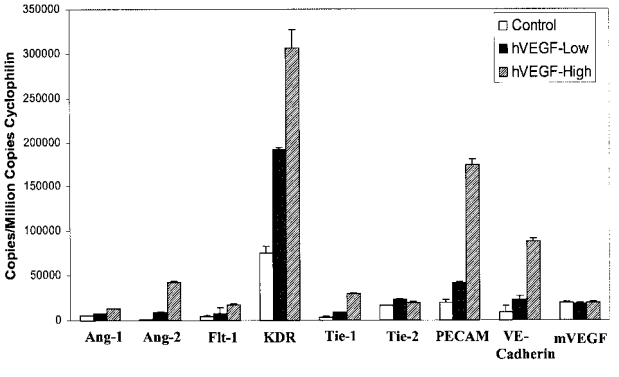

Figure 3. Real-time quantitative RT-PCR quantitation of mRNA copy number in skin specimens from nude mice injected with Matrigel +/− hVEGF-transfectants. On dissection, Matrigel was removed and total RNA and cDNA was prepared from the overlying skin associated with control Matrigel, Matrigel + hVEGF-Low transfectants, and Matrigel + hVEGF-High transfectants. The precisely measured and 10-fold serially diluted cDNAs templates (Ang-1, Ang-2, Flt-1, KDR, PECAM, VE-cadherin, Tie-1, and Tie-2) were used as external standard curve. The copy numbers of each mRNA was calculated after normalizing to million copies of cyclophilin in each sample and data are presented as copy number/million cyclophilin mRNA molecules.

Table 2. Fold Induction of Gene Expression by hVEGF-High and hVEGF-Low Transfectant

Gene	Control	hVEGF-Low	hVEGF-High
Ang-1	1	1.29	2.28
Ang-2	1	7.49	35.06
Flt-1	1	1.46	3.64
KDR	1	2.56	4.10
Tie-1	1	2.44	8.61
Tie-2	1	1.42	1.25
PECAM	1	2.18	9.14
VE-cadherin	1	2.76	10.44
mVEGF	1	0.98	1.03

Fold induction of gene expression over control was calculated from data presented in Figure 3.

large number of transcripts with limited material.[4–6] By contrast, quantification with Northern blot analysis requires approximately 5000 times more RNA. Furthermore, with a precisely quantified cDNA template standard that covers the region of real-time quantitative RT-PCR primer, real-time quantitative RT-PCR is also able to measure absolute transcript copy numbers.[7,8,32] Thus, with its superior sensitivity and accuracy, wide linear dynamic range, good intra-assay and inter-assay reproducibility, real-time quantitative RT-PCR offers potential utility in clinical diagnosis and offers an alternative route to current immunohistochemical methods for assessing angiogenesis.

Studies have shown a direct relationship between microvessel density and the level of VEGF expression during tumor progression.[32–34] As demonstrated here, increasing the number of VEGF mRNA transcripts from 2.9×10^4 copies to 1.5×10^5 copies for every 10^6 copies of cyclophilin in human SK-MEL-2 melanoma transfectants induced significantly greater degree of neovascularization in mouse skin. In particular, the panel of angiogenesis markers identified here clearly distinguished skin neovascularization induced by hVEGF-High transfectants from that induced by hVEGF-Low transfectants. This implies that proteins necessary to induce and retain blood vessels are coordinately regulated along with VEGF during tumor growth. Thus, detection of mRNA copy numbers with real-time quantitative RT-PCR is able to produce precise transcript profiles, and these profiles may serve to monitor different phases of tumor progression and efficacy of therapy.

Ang-1 is a secreted growth factor that binds to and activates the Tie-2 receptor tyrosine kinase, whereas Ang-2 binds the same receptor but fails to activate it, thus acting as a natural inhibitor of Ang-1.[14,21,22] Recent studies showed that Ang-2 destabilizes capillary integrity and facilitates vessel sprouting when VEGF levels are high, but causes vessel regression when VEGF levels are low.[21,22] Our real-time quantitative RT-PCR showed that hVEGF-High transfectant contained $\sim 4.3 \times 10^4$ copies of Ang-2 for every million copies of cyclophilin while control contained only 1200 copies, resulting in a 35-fold induction of Ang-2 expression. Thus, in agreement with others' findings, our studies suggest that higher rates of skin neovascularization are associated with higher levels of VEGF and Ang-2 expression. Interestingly, induction of Tie-2 was not correspondingly increased with VEGF in either the TRAMP or skin angiogenesis models. As determined by measurement of absolute mRNA copy number, both control and VEGF-stimulated skin samples expressed moderate yet equal levels of Tie-2 mRNA (for every million copies of cyclophilin, control skin contained 1.5×10^4 copies of Tie-2 and vascularized skin associated with hVEGF-High transfectants expressed 2.2×10^4 copies). Thus the ratio of Tie-2 and its ligand Ang-2 increased from 1.0/0.08 in control skin to 1.0/2.8 in hVEGF-High transfectant skin. This suggests that although Tie-2 mRNA level did not increase significantly during skin neovascularization, the pre-existing levels of Tie-2 are able to accommodate Ang-2 ligand-induced signaling. This particular observation underscores the significance of rigorously quantifying mRNA copy number and illustrates that such quantification may provide important insights.

In summary, this study demonstrates a method for rigorous quantitative profiling of specific mRNAs closely correlated with VEGF expression and angiogenesis. We have defined a strategy that precisely quantifies a complex panel of marker transcripts, and our findings provide evidence that detection of absolute mRNA transcript numbers with real-time quantitative RT-PCR can serve to sensitively monitor angiogenesis. Because there may exist significant differences in gene expression among different tissues and during various phases of tumor progression, the panel of markers we have identified more accurately predicts the level of angiogenesis than singular markers and may provide for tumor and stage specific fingerprinting of neovascularization.

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