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Development of a multiplexed PCR-coupled liquid bead array assay for vascular endothelial growth factor (VEGF) splice variants

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ABSTRACT

Objectives: To develop a multiplex PCR-coupled liquid bead array assay for the expression of *VEGF* splice variants.
Design and methods: The assay was based on the combination of multiplex PCR with liquid bead array technology, and optimized and evaluated in terms of analytical sensitivity, specificity, and reproducibility using the MCF-7 cell line. Clinical performance was evaluated in 16 pairs of fresh frozen cancerous and corresponding non-cancerous adjacent tissues from NSCLC patients.
Results: The assay is highly sensitive, reproducible and can detect specifically VEGF splice variants in clinical samples. When applied in 32 clinical samples it gave comparable results to RT-qPCR (concordance of 81%, 75%, 88% and 81% for *PBGD*, $VEGF_{121}$, $VEGF_{165}$, and $VEGF_{189}$ respectively).
Conclusions: The developed assay can simultaneously detect three VEGF splice variants with high specificity and sensitivity and can be further used to evaluate the role of each individual VEGF splice variant in molecular therapies targeted against VEGF.

Introduction

Angiogenesis is the process of formation of new blood vessels from preexisting vasculature so that cells are able to obtain oxygen beyond its diffusion limit [1]. Angiogenesis is not only very crucial in physiological but also in pathological conditions such as tumor progression and metastasis [2].Vascular endothelial growth factor (VEGF) is an endothelial specific mitogen [3] and a key regulator of the "angiogenic switch" which determines whether an endothelial cell will be in a quiescent or an angiogenic state [4]. VEGF has also a key role in physiological processes involving angiogenesis such as embryogenesis [5], bone formation [6], formation of the female reproductive system [7] and in many pathological conditions as well such as diabetic retinopathy [8] and rheumatoid arthritis [9].

Multiple isoforms of VEGF are created through alternative splicing. To date nine VEGF splice variants have been reported, ranging in length from 111 to 206 amino acids (subscripts denote the number of amino acids after signal sequence cleavage): $VEGF_{206}$, $VEGF_{189}$, $VEGF_{183}$, $VEGF_{165}$, $VEGF_{162}$, $VEGF_{148}$, $VEGF_{145}$, $VEGF_{121}$ and $VEGF_{111}$ [10–12], while most VEGF-producing cells express $VEGF_{121}$, $VEGF_{165}$ and $VEGF_{189}$ [13]. An important biological property that differentiates these VEGF isoforms is the sulfate-binding capacity of heparin and heparan as a result of the presence or not of exons 6 and 7. $VEGF_{121}$ 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, $VEGF_{189}$ is almost completely sequestered in the extracellular matrix because of its high binding affinity for heparin [3]. Based on these characteristics, each of these VEGF isoforms is responsible for the formation of vessels with different properties. In tumors expressing $VEGF_{121}$ only, vascularization is limited to the periphery of the tumor mass in contrast with those expressing $VEGF_{189}$, where the vessels are formed throughout the tumor mass. Tumors over-expressing $VEGF_{165}$ are described by vessels located mainly in the tumor periphery [14–16]. More VEGF isoforms, named $VEGF_{xxx}b$ (subscript xxx denotes the number of amino acids), are created through splicing at a C-terminal distal site in exon 8: $VEGF_{189}b$, $VEGF_{165}b$ and $VEGF_{121}b$ are already assigned with an anti-angiogenic action [17–20].

The antiangiogenic treatment era has begun in 1971 thanks to Judah Folkman's hypothesis [21]. The only FDA approved antiangiogenic treatment so far is the monoclonal antibody bevacizumab (Avastin®) while several monoclonal antibodies and tyrosine kinases inhibitors (TKIs) are currently tested [22,23]. A new approach in antiangiogenic treatment is based on the alternative C-terminal distal splice site of *VEGF* exon 8 mentioned above. Intervention at the signaling of transcriptional factors and especially of splicing factors of *VEGF*, leads in specific changes to its splicing. In particular it is possible to favor the splicing toward the $VEGF_{xxx}b$ isoforms resulting in reduced vascularization [12,24].

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Our group has previously developed a sensitive and specific RT-qPCR for the quantification of VEGF splice variants [25] which was also applied in samples originating from patients with diabetes [26] and NSCLC [27]. In addition, we have recently developed a highly specific and sensitive liquid bead array assay for the detection of gene expression in circulating tumor cells [28]. The aim of this study was to develop and validate a highly specific and sensitive liquid bead array assay based on multiplex RT-PCR for the simultaneous detection of $VEGF_{121}$, $VEGF_{165}$ and $VEGF_{189}$ splice variants in a variety of clinical samples.

Materials and methods

Cell line and clinical samples

The human breast cancer cell line MCF-7 was used for the development of the assay, and the preparation of *$VEGF_{121}$*, *$VEGF_{165}$* and *$VEGF_{189}$* splice variants and *PBGD* external standards as previously described [25–27]. Cells were counted in a hemocytometer and their viability was assessed by trypan blue dye exclusion. Serial dilutions of a known number of MCF-7 cells corresponding to 1–1000 MCF-7 cells, for which total RNA isolation and cDNA synthesis was performed, were prepared. These cDNAs were kept in aliquots at −20 °C and used for the validation of the assay, prior to the analysis of patients' samples.

Sixteen paired clinical specimens (lung carcinoma and adjacent noncancerous tissues) that were the same used in our previous study [27] were used for the evaluation of the developed assay.

RNA extraction and cDNA synthesis

Total cellular RNA was isolated using the Qiagen RNeasy Mini Reagent Set (Qiagen, Germany) according to the manufacturer's recommendations. RNA concentration and purity were determined by using the Nanodrop-1000 spectrophotometer and the isolated RNA was stored at −70 °C until analyzed. Reverse transcription of RNA was performed with the High Capacity RNA-to-cDNA Kit (Applied Biosystems, USA) according to the manufacturer's instructions, using 2 μg of total RNA as template.

Multiplex PCR

Based on our previous study [27] we developed a multiplex PCR, for the three most common VEGF splice variants, using exactly the same sequences of primers as before. In this way we didn't need to perform any additional specificity experiments for the PCR step. A common forward primer for all splice variants located on exon 3 was used, whereas specific amplification of each variant was achieved by using highly specific reverse primers spanning variant-specific exon boundaries. Concerning PBGD specific forward and reverse primers were used for its amplification. (Supplementary Fig. 1).

Multiplex PCR was carried out with 2 μL cDNA in a final volume of 25 μL while a PCR negative control containing no target was included in each assay run. The reaction consisted of 12.5 μL Master Mix, 2.5 μL Q-Solution (Multiplex PCR Kit, Qiagen, Germany) and 0.08 μmol/L of each primer (forward:5′-GGTGGGTGTGCTGCACGAT-3′ and reverse:5′-ATCTTCATGCTGGGCAGGGA-3′ primer for *PBGD* and 1 common forward and 3 reverse primers specific for *VEGF* splice variants as previously described [27]). PCR was performed in an Eppendorf Mastercycler. After experimental condition optimization for all primer pairs, the final PCR conditions were as follows: 95 °C for 15 min and 35 cycles of 95 °C for 30 s, 60 °C for 1 min and 72 °C for 30 s. Samples were then held at 72 °C for 10 min and kept at 4 °C until use.

Treatment with ExoSAP-IT® reagent

The multiplex PCR products were then treated with ExoSAP-IT® PCR product cleanup reagent (Affymetrix — USB, USA) as follows: a mixture of 5 μL of PCR product and 2 μL of ExoSAP-IT® reagent was incubated in DNA Engine thermal cycler (MJ Research Inc., USA) first at 37 °C for 20 min in order to degrade the excess of primers and dephosphorylate the unused dNTPs and then at 80 °C for 15 min resulting in the deactivation of the participating enzymes.

TSPE (Target Specific Primer Extension) and biotinylation of multiplex PCR products

ExoSAP-IT treated PCR products (5 μL) were then placed in a 15-μL reaction volume containing 1 μL of a solution containing 500 nmol/L of each sequence-tagged target specific primer (Table 1), 1 μL of a solution containing 100 μmol/L of each deoxynucleoside triphosphate except for dCTP, 0.25 μL of a solution containing 400 μmol/L of biotinylated-dCTP (Invitrogen, USA), 0.5 μL of 50 mmol/L $MgCl_2$, 0.15 μL of 5 U/μL Platinum Tsp Taq DNA Polymerase and 2 μL of 10xPCR buffer included with this enzyme (Invitrogen, USA) and 10.1 μL of diethylpyrocarbonate (DEPC)-treated water. Samples were cycled in an Eppendorf Mastercycler under the following conditions: denaturation at 96 °C for 2 min, 20 cycles of denaturation at 95 °C for 30 s, annealing at 50 °C for 30 s and extension at 74 °C for 30 s. Samples were kept at 4 °C until use.

Bead array hybridization

A bead mix consisting of 2500 beads from each of four different bead sets with specific spectral addresses was prepared. After microcentrifugation at 13,400 rpm for 4 min, the supernatant was discarded and the beads were re-suspended in 1× Tm buffer [0.2 mol/L sodium chloride, 0.1 mol/L Tris (pH 8.0) and 0.08% Triton® X-100] to a final volume of 48 μL. We then added 7 μL of the capture sequence-tagged TSPE products to the bead mix, denatured the products and bead mix at 95 °C for 5 min and allowed the suspension to hybridize at 37 °C for 30 min. The coupled microspheres were transferred to a filter plate, the supernatant was removed by vacuum filtration and the microspheres were re-suspended in 75 μL of reporter solution [10 μg/mL streptavidin-conjugated phycoerythrin in hybridization buffer (1× Tm)] and incubated at room temperature for 10 min.

Bead analysis

Resuspended microspheres were placed in 96-well microtiter plates and analyzed with a Luminex® 100 instrument (Luminex Corporation, USA). The sample volume was set at 50 μL, and the flow rate was 60 μL/min. A minimum of 100 events were recorded

Table 1
Sequences of tagged target specific primers and corresponding anti-TAG microspheres used in this study.

Target	FlexMAP bead set #	Sequence, 5′–3′
$VEGF_{121}$	LUA 30	Tagged primer: TTACCTTTATACCTTTCTTTTTAC–TCGGCTTGTCACATTTTTCTTG Anti-TAG: GTAAAAAGAAAGGTATAAAGGTAA
$VEGF_{165}$	LUA 49	Tagged primer: TCATCAATCTTTCAATTTACTTAC–CAAGGCCCACAGGGATTTTC Anti-TAG: GTAAGTAAATTGAAAGATTGATGA
$VEGF_{189}$	LUA 28	Tagged primer: CTACAAACAAACAAACATTATCAA–CACAGGGAACGCTCCAGGAC Anti-TAG: TTGATAATGTTTGTTTGTTTGTTGTAG
PBGD	LUA 12	Tagged primer: TACACTTTCTTTCTTTCTTTCTTT–GGTGGGTGTGCTGCACGAT Anti-TAG: AAAGAAAGAAAGAAAGAAAGTGTA

for each bead set, mean fluorescence intensities (MFIs) were computed and analysis was completed in less than 60 s for each sample.

Results

The developed assay is based on the simultaneous amplification of three *VEGF* splice variants and one reference gene (*PBGD*) via multiplex PCR using highly specific primers. Multiplex PCR products were biotinylated and PCR amplicons were hybridized against a pool of four sets of optically addressed fluorescent microspheres. Each set of these microspheres was carrying immobilized capture probes complementary to a TAG-sequence specific for each target gene. After hybridization, these microspheres were further incubated with streptavidin–phycoerythrin. Fluorescently labeled captured amplicons were then quantified, and the beads were decoded by liquid bead array on the Luminex platform. All these steps were optimized in different experiments. The whole experimental flowchart is outlined in Fig. 1.

Assay optimization

To optimize the assay, we used total RNA from the breast cancer cell line MCF-7. In all cases, optimized conditions were selected according to the best signal-to-noise ratio. The conditions for multiplex PCR were established in a series of preliminary experiments that evaluated the number of PCR and TSPE cycles (Fig. 2A). The hybridization protocol was optimized according to the number of fluorescent microspheres used for each target (Fig. 2B) and the volume of biotinylated PCR products used (Fig. 2C). All experiments were performed in triplicate.

Comparison between multiplex and singleplex PCR for each VEGF splice variant and PBGD

We have tested the efficiency of the 4 primer pairs in this multiplex assay, compared to singleplex reactions. For this reason we have used the same cDNA (positive control) sample and amplified

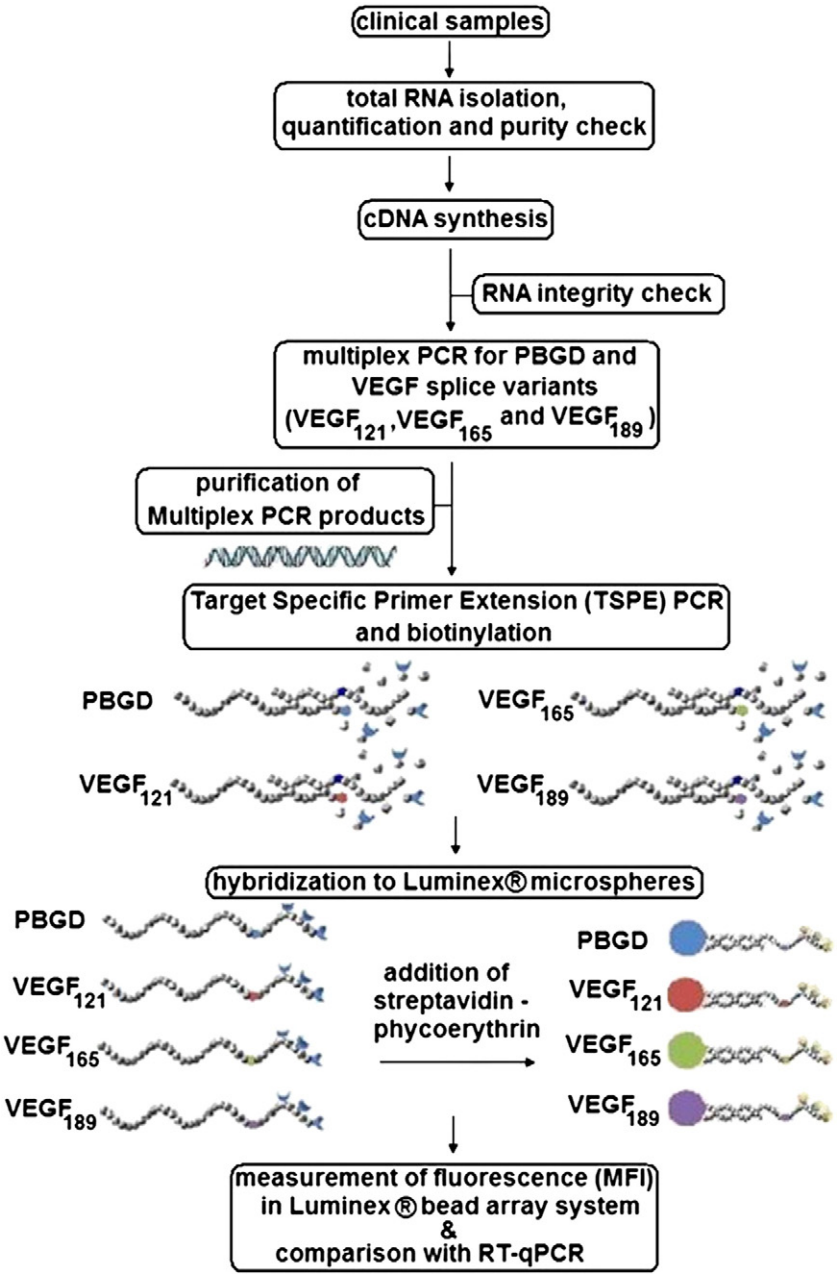

Fig. 1. Experimental flowchart of the VEGF multiplex liquid bead array assay.

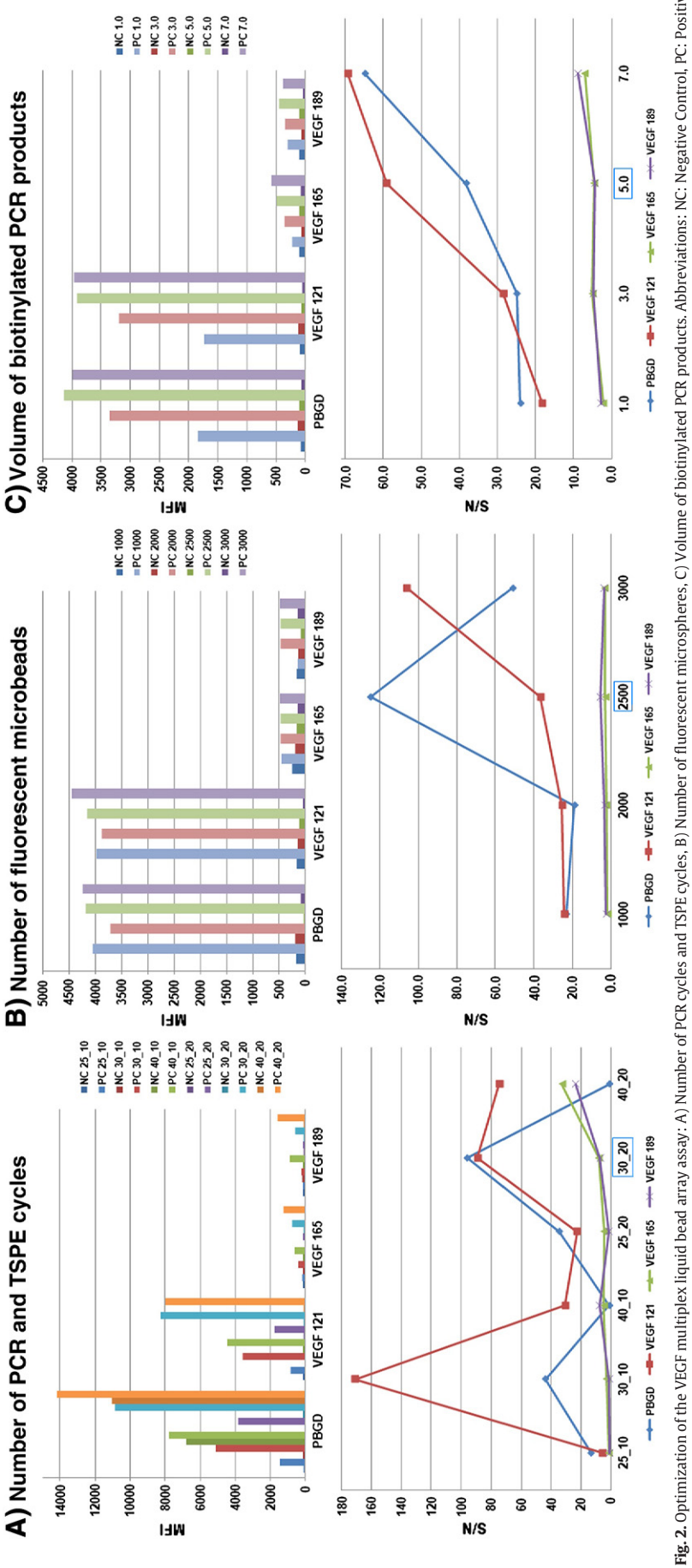

Fig. 2. Optimization of the VEGF multiplex liquid bead array assay: A) Number of PCR cycles and TSPE cycles, B) Number of fluorescent microspheres, C) Volume of biotinylated PCR products. Abbreviations: NC: Negative Control, PC: Positive Control, S/N: Signal/Noise, PC a_ b: Positive Control number of multiplex PCR cycles _ number of TSPE PCR cycles.

first each VEGF splice variant and PBGD separately in singleplex reactions and then altogether in a multiplex format. In all cases the fluorescent signals were higher when multiplex PCR was used (Supplementary Fig. 2). All reactions were performed in triplicate.

Assay specificity

We checked the analytical specificity of the primers used for multiplex PCR, as well as those for the sequence-tagged target specific primers both in the presence and in the absence of each target.

First, we assessed analytical specificity when only one VEGF splice variant was used as a template. For this experiment, multiplex PCR was performed in the presence of only one VEGF splice variant and all primer pairs, then multiplex PCR products were hybridized in triplicate in the presence of all four microspheres sets. The assay was highly specific as it could discriminate the expression of each individual VEGF splice variant, since we did not observe any of the twelve nonspecific interactions that theoretically could have occurred between the PCR products and the specific oligonucleotides attached on the microspheres (Fig. 3A).

Secondly, we assessed analytical specificity in the absence of each individual splice variant. For this experiment, biotinylated PCR products of multiplex PCR reactions that had been performed in the presence of two VEGF splice variants and in the absence of one VEGF splice variant at each time and in the presence of all primer pairs were hybridized in the presence of all four microspheres sets. The assay was highly specific in this case too, since only the splice variants that were present in the sample were detected (Fig. 3B).

Assay sensitivity

For the evaluation of the analytical sensitivity of the developed bead array assay we decided to prepare four different PCR amplicons corresponding to the three VEGF splice variants studied ($VEGF_{121}$, $VEGF_{165}$, and $VEGF_{189}$), and PBGD as previously described [27]. For this purpose, cDNA was synthesized from total RNA extracted from MCF-7 cells and served as a template for the amplification of the targets of interest by real-time PCR using the above described specific primers. When PCR products were run on a 2% agarose gel, no other non-specific products were observed but the band of proper size (data not shown). These splice variant specific bands were excised and the amplicons were purified by passing through a column of the QIA Quick Gel Extraction Kit (Qiagen, USA). These four different amplicons were quantified spectrophotometrically in the Nanodrop and the corresponding concentration of the purified amplicons expressed as ng/mL, was converted to copies per μL by using the Avogadro constant and their molecular weight [27]. Serial dilutions of these stock amplicon solutions ranging from 10^6 to 10 copies/μL were used in triplicate to evaluate the analytical sensitivity of the assay. In this way the assay sensitivity is independent of the cell line used. We are presenting these data in terms of Median Fluorescence Intensity (MFI) (Fig. 3C) and in terms of S/N (Fig. 3D). As can be seen in these figures the assay could reliably

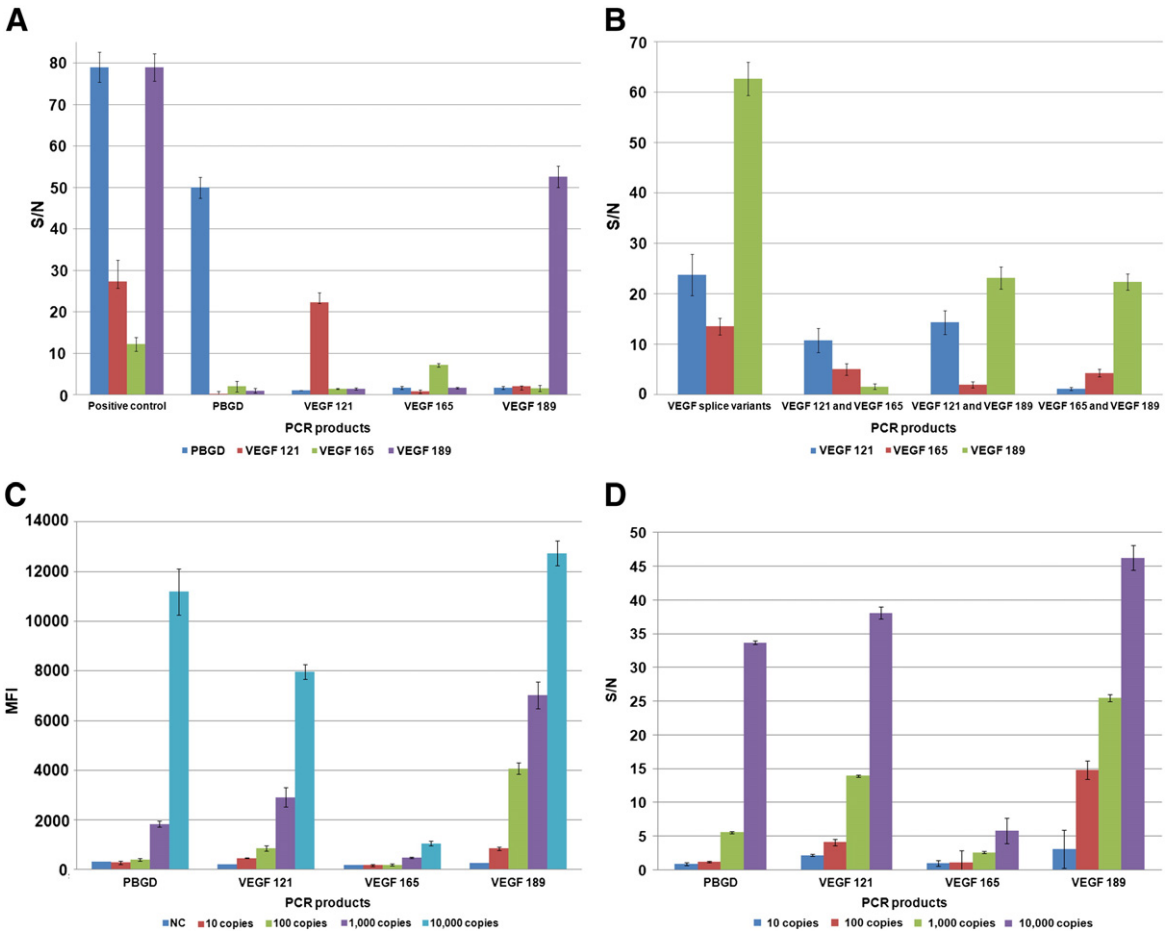

Fig. 3. Specificity and sensitivity of the VEGF multiplex liquid bead array assay. All experiments were performed in triplicate. A) Four different set of microspheres hybridized with only one individual biotinylated PCR product. B) Four different set of microspheres hybridized with two out of three biotinylated PCR products. C) Sensitivity of the assay: MFI units of serial dilutions corresponding to 10, 10^2, 10^3 and 10^4 copies/μL for each splice variant subjected to the entire analytical procedure, D) Sensitivity of the assay: S/N of serial dilutions corresponding to 10, 10^2, 10^3 and 10^4 copies/μL for each splice variant subjected to the entire analytical procedure.

Table 2
Precision of the VEGF multiplex liquid bead array assay.

Target	Concentration, copies/μL	Intra-assay precision, MFI, mean ± SD (n = 3), (CV %)	Inter-assay precision, MFI, mean ± SD (n = 3), (CV %)
PBGD	10^3	1840 ± 132 (7.2)	1893 ± 68 (3.6)
	10^4	11,190 ± 926 (8.3)	11,358 ± 462 (4.1)
	Positive control[a]	11,797 ± 561 (4.8)	9257 ± 351 (3.8)
$VEGF_{121}$	10^3	2913 ± 387 (13.3)	2883 ± 296 (10.3)
	10^4	7960 ± 288 (3.6)	7917 ± 261 (3.3)
	Positive control[a]	4747 ± 117 (2.5)	7610 ± 1002 (13.2)
$VEGF_{165}$	10^3	468 ± 28 (5.9)	477 ± 32 (6.7)
	10^4	1053 ± 101 (9.6)	1050 ± 85 (8.1)
	Positive control[a]	971 ± 48 (5.0)	1613 ± 297 (18.4)
$VEGF_{189}$	10^3	7020 ± 527 (7.5)	7027 ± 500 (7.1)
	10^4	12,737 ± 505 (4.0)	12,663 ± 500 (3.9)
	Positive control[a]	8340 ± 640 (7.7)	8227 ± 685 (8.3)

[a] cDNA synthesized from total RNA isolated from MCF-7 cells (total RNA: 2 μg).

detect VEGF121 and VEGF 189 in the presence of as low as 10^2 copies/μL, while VEGF165 and PBGD in the presence of as low as 10^3 copies/μL.

Precision

We evaluated intra-assay precision by analyzing in triplicate three different samples at three different concentration levels: two samples that contained 10^3 and 10^4 copies/μL for each target and a sample of cDNA synthesized from total RNA isolated from MCF-7 cells (total RNA: 2 μg). For this reason, we analyzed these samples in the same run in 3 parallel determinations and followed the entire analytical procedure as described in Fig. 1. We evaluated inter-assay precision by analyzing the same samples as previously in 3 separate assays in 3 different days. Intra-assay CVs ranged from 2.5% to 13.3%, and inter-assay CVs ranged from 3.3% to 18.4% (Table 2).

Application of the developed liquid bead array assay in paired NSCLC clinical samples — comparison with RT-qPCR

The developed assay was used to study the expression of VEGF splice variants in fresh frozen cancerous and corresponding noncancerous adjacent tissue samples originating from patients with NSCLC. RNA quality of all samples was checked by PBGD expression, used as a reference gene. In this way only paired samples that were found to be of adequate quality were included in this study. Sixteen paired samples (11 pairs of adenocarcinomas and 5 pairs of squamous cell carcinomas) were found to be of good RNA quality among 20 pairs checked in the beginning. We observed a high qualitative and quantitative heterogeneity in the gene expression profile in the VEGF splice variant expression between the cancerous and corresponding noncancerous adjacent tissue samples as can be seen in Fig. 4.

In order to evaluate the concordance of the new developed liquid bead array assay and RT-qPCR we analyzed the same clinical samples in parallel by a highly sensitive and accurate real time RT-qPCR assay for the quantification of *VEGF* splice variants that we have previously described [27]. We estimated the concordance between the two methodologies, by comparing the MFI values of the liquid bead array to the corresponding Cq values obtained by RT-qPCR. In the bead array assay we defined as positive a sample when its MFI signal was statistically different than the corresponding adjacent tumor sample signal. In this way, a high MFI value for the cancerous sample compared to that of the corresponding noncancerous adjacent tissue sample should correspond to a lower C_q value obtained by RT-qPCR. Based on this approach, our analysis revealed a satisfactory agreement (total of positive and negative samples by both methods/total number of samples analyzed) between the RT-qPCR assay and the liquid bead array assay; for *PBGD*: 13/16 (81%) pairs, for $VEGF_{121}$: 12/16 (75%) pairs, for $VEGF_{165}$: for 14/16 (88%) pairs and finally for $VEGF_{189}$: 13/16 (81%) pairs (Table 3).

Discussion

The VEGF pathway controls angiogenesis, a necessary step in tumor growth, metastasis, and malignancy. Indeed, tumor vascularization is a prognostic indicator of disease progression in various cancers, including NSCLC [29], and renal cancer [30]. Up-regulation of VEGF has been observed in NSCLC tumor samples and is correlated with tumor angiogenesis, shorter postoperative recurrence time, and shorter survival time [29].

The presence of various isoforms deriving from alternative splicing of a single VEGF gene has been reported, and some studies have

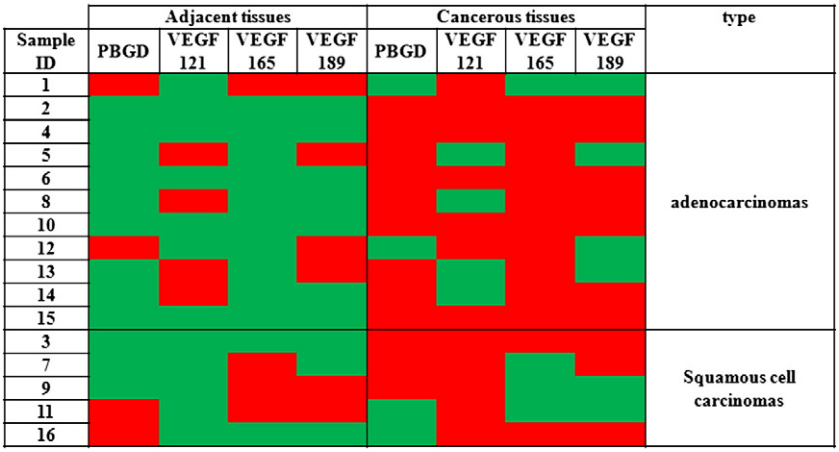

Fig. 4. Expression levels of VEGF splice variants in NSCLC as evaluated by the multiplex liquid bead array assay. Comparison between 16 NSCLC tissues and corresponding non cancerous adjacent tissues (N: normal, T: Tumor). (N: normal, T: tumor, red: overexpression, green: underexpression).

Table 3
VEGF multiplex liquid bead array assay in comparison to RT-qPCR.

VEGF-R121	Liquid bead array assay		
	VEGF-R121 (+)	VEGF-R121 (−)	Total
RT-qPCR			
VEGF-R121 (+)	11	3	14
VEGF-R121 (−)	1	1	2
Total	12	4	16
Agreement	12/16, concordance:75%		
VEGF-R165	Liquid bead array assay		
	VEGF-R165 (+)	VEGF-R165 (−)	Total
RT-qPCR			
VEGF-R165 (+)	11	1	12
VEGF-R165 (−)	1	3	4
Total	12	4	16
Agreement	14/16, concordance: 88%		
VEGF-R189	Liquid bead array assay		
	VEGF-R189 (+)	VEGF-R189 (−)	Total
RT-qPCR			
VEGF-R189 (+)	8	1	9
VEGF-R189 (−)	2	5	7
Total	10	6	16
Agreement	13/16, concordance: 81%		
PBGD	Liquid bead array assay		
	PBGD (+)	PBGD (−)	Total
RT-qPCR			
PBGD (+)	12	3	15
PBGD (−)	0	1	1
Total	12	4	16
Agreement	13/16, concordance: 81%		

shown that the expression of certain splice variants is related to tumor progression [17,18,31]. 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 [32].

The importance of VEGF splice variants could only be evaluated after the development of highly specific and sensitive methodologies, suitable for clinical specimens. Wellmann et al. were the first to use real-time RT-PCR assay for the detection of different VEGF mRNAs in seven healthy tissues (each pooled from healthy donors) and seven correlated malignant tissues. According to this methodology results were normalized to beta(2)-microglobulin mRNA and amplification of VEGF splice variants was performed with variant-specific reverse primers, whereas forward primer and fluorescent probe were common to obtain similar RT-PCR kinetics [33]. Our previous study on the splice variant profile of VEGF in NSCLC that revealed differences in VEGF splice variant expression profile between NSCLC and their corresponding non malignant paired tissues has also been based on RT-qPCR [27]. Stimpfl et al. have used RT-PCR and automated laser fluorescence fragment analysis to study VEGF isoform expression in breast and ovarian cancer cell lines, as well as in breast carcinomas and ovarian tumors. They correlated the expression pattern with the in vitro invasiveness of the breast carcinoma cell lines and the clinicopathological characteristics of the tumors [34]. Pio et al. have very recently described the development of a novel splice array platform and this methodology was then applied to the analysis of differential splice forms in lung cancer samples compared to matched normal lung tissue [35].

The developed multiplex liquid bead array assay for VEGF splice variant expression combines the advantages of multiplex RT-PCR and the liquid bead microarray technology. Its main advantage over our previously reported RT-qPCR method is that it enables the reliable gene expression analysis for three splice variants of VEGF in parallel using a very limited amount of sample. The assay is specific for each splice variant in complex multiplexed formats thereby saving precious sample and reducing the costs and time of analysis. The assay produces results comparable to those of RT-qPCR for each individual splice variant and has the potential to be scaled up to all known VEGF splice variants while the use of an internal control such as a DNA competitor could further enable quantification [36]. Our previously developed quantitative RT-PCR for 6 VEGF splice variants required up to 6 μL of the cDNA while in the present method only 2 μL of sample was required regardless the number of splice variants that would be analyzed. In this context this assay can be further developed to an angiogenesis panel of genes, testing not only all known VEGF splice variants but VEGF receptor family members as well.

Since VEGF represents the main proangiogenic stimulator [2], it is currently in focus for therapeutic interventions. Angiogenesis inhibitors based on the VEGF pathway for the treatment of cancer have been approved by the Food and Drug Administration in the United States, and in 28 other countries and clinical application of this new class of drugs is informed by certain principles from angiogenesis research [1]. The efficacy and tolerability of these therapies in the first-line treatment of patients with colorectal [37] and metastatic breast cancer [38] have been recently reviewed. We strongly believe that this assay could be a useful tool for clinical researchers that design clinical studies to evaluate the response to VEGF-based therapy in a larger number of patients.

Conclusions

The developed liquid bead array assay can be used for the simultaneous detection of the three VEGF splice variants in clinical samples with high specificity and sensitivity. This methodology can be further used to evaluate the role of each individual VEGF splice variant in molecular therapies targeted against VEGF.

Supplementary materials related to this article can be found online at doi:10.1016/j.clinbiochem.2012.01.025.

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