

Quantitative RT-PCR assay for VEGF mRNA in human tumors of the kidney

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ABSTRACT: *Angiogenesis is the formation of new capillaries from pre-existing vessels, and recent evidence has demonstrated that tumor growth is controlled mainly by angiogenesis. Vascular endothelial growth factor (VEGF) is an endothelium-specific growth factor which is strongly angiogenic in vitro and in vivo. We have developed a quantitative RT-PCR assay for the measurement of VEGF mRNA expression using a real-time procedure based on the use of fluorogenic probes and the ABI PRISM 7700 Sequence Detector System. The assay performance of this method in terms of practicability and reliability is reported with results that seem promising for its widespread use in the clinical laboratory. The method has been applied to the measurement of mRNA of VEGF in human renal cell carcinomas (RCC). Preliminary results show a significantly higher VEGF mRNA expression (ratio values between 181 and 2222) in tumor specimens compared to non-adjacent, non-tumoral tissue of the same subjects. (Int J Biol Markers, 1999; 14: 247-50)*

KEY WORDS: *TaqMan, Quantitative RT-PCR, VEGF, Renal tumors*

INTRODUCTION

Several factors have been shown to display angiogenic activity. Among these factors vascular endothelial growth factor (VEGF) appears to play a key role in tumor angiogenesis (1). Increased VEGF mRNA levels have been observed in various kinds of tumors including kidney tumors (2-4). In particular upregulation of VEGF expression has been reported in tumors compared with non-tumoral, non-adjacent tissue of the same subjects.

We have developed a quantitative RT-PCR assay for the measurement of VEGF mRNA expression using a real-time procedure based on the use of fluorogenic probes and the ABI PRISM 7700 Sequence Detector System (5). With this method we have investigated the levels of VEGF mRNA expression in six renal tumor specimens and in the corresponding non-adjacent, non-neoplastic tissue from the same kidneys.

MATERIALS AND METHODS

Patients and specimens

Six RCC tumors have been characterized by histotype, tumor size, stage (TNM) and grade (Fuhrman) (6). Clinical data are summarized in Table I. RNA was extracted from renal tissues by conventional phenol-chloroform extraction.

Real-time RT-PCR assay

The TaqMan™ principle has recently been described in detail. Briefly, the method is based on the use of fluorogenic probes labeled both with a fluorescence emitter and a fluorescence quencher. The special fluorogenic TaqMan probe has been designed to hybridize within the target sequence and to generate a signal that accumulates during PCR cycling in a manner proportional to the con-

VEGF mRNA in renal tumors

centration of amplification products. The fluorescence emission is monitored in real time (5). The ΔRn values reflect the quantity of degraded fluorescent probe and fit an exponential function generating an amplification plot for each sample (Fig. 1). At the same time the algorithm determines an experimental threshold. The number of the cycle at which the fluorescence of each sample reaches this threshold (Ct) is proportional to the number of target copies present in the initial sample. The Ct of each sample is utilized to quantitate the specific mRNA, using a standard curve generated with an RNA standard (Fig. 2). Finally, the VEGF mRNA concentration in samples is expressed as molecules/ μ g total RNA.

RESULTS

VEGF mRNA assay performance

As mentioned in the introduction, by using real-time quantitative RT-PCR the specific RNA in the sample is reversely transcribed, amplified and quantitated without any further downstream processing, thus decreasing the risk of contamination. In fact, the use of fluorogenic probes allows the development of a homogeneous assay with excellent characteristics in terms of analytical quality and practicability. In our experiments the standard curve points, control sam-

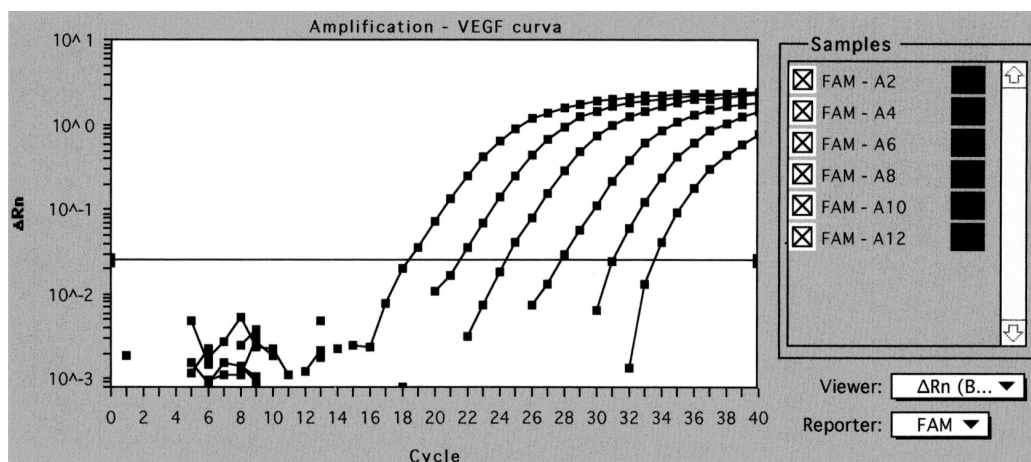


Fig. 1 - Amplification plots in Taq-Man technology: ΔRn (emission intensity of the reporter dye) vs cycle number for VEGF gene expression performed at six points with increasing amounts of standard.

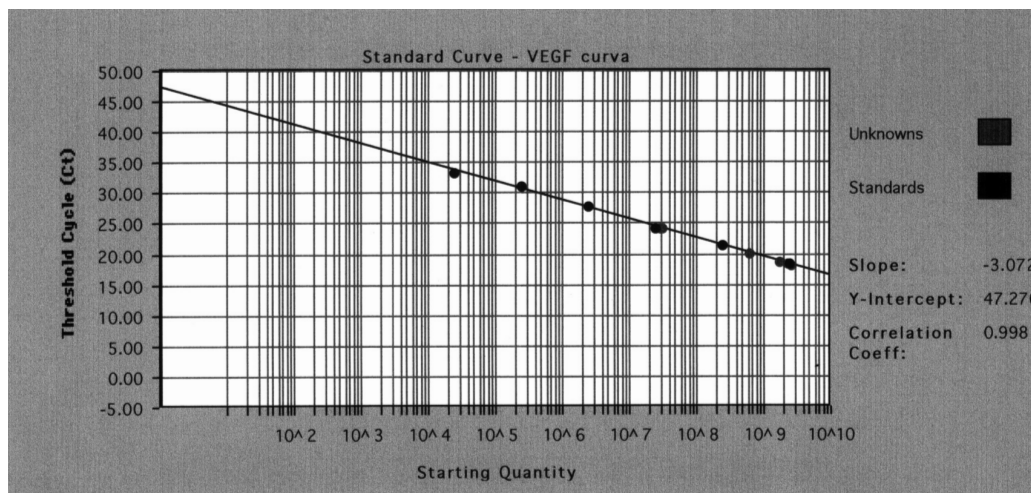


Fig. 2 - Standard curve for VEGF gene expression reported as starting quantity (in molecule number) vs threshold cycle (Ct). Points refer to the experiment and amplification plot described in Figure 1.

ples and unknown samples were elaborated in triplicate after retrotranscription.

As an example of the performance of the VEGF mRNA RT-PCR assay we report the reproducibility data of the calibration curve (see Fig. 3). The practicability of the proposed method (assay in microtiter plate format and in homogeneous phase can perform amplification steps and analysis of the results in 4-6 hours) is also satisfactory and both these aspects indicate that this type of technique should be widely adapted in the clinical laboratory in the near future.

VEGF mRNA expression levels in tumors of the kidney

The proposed method has been applied to VEGF mRNA measurement in six renal tumors. A significant increase in VEGF has been observed in tumors (mean value 9.2×10^7 VEGF mRNA molecules/ μg total RNA) compared to non-adjacent non-neoplastic tissue (mean value 1.6×10^5 VEGF mRNA molecules/ μg total RNA, $p < 0.05$) (Tab. II) with a ratio ranging from 181 to 2222.

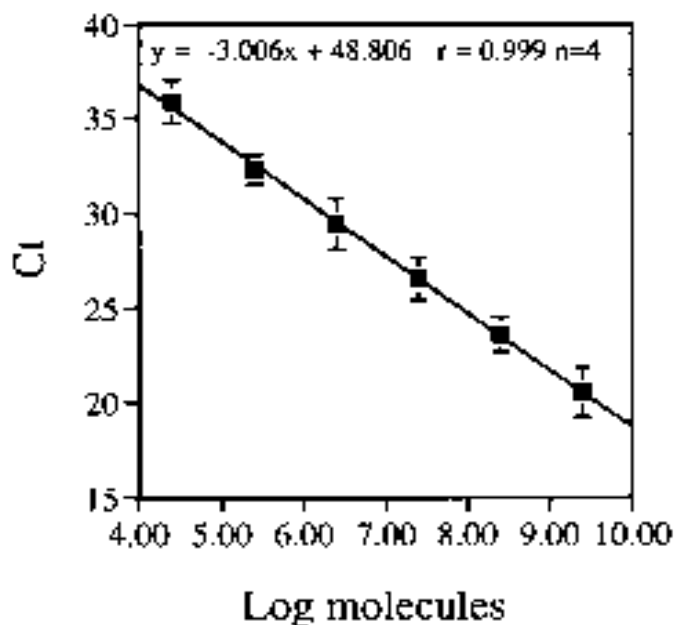


Fig. 3 - Calibration curve reproducibility obtained as mean \pm SD of four different assays.

TABLE I - CLINICAL DATA AND HISTOLOGICAL TYPE OF SIX PATIENTS AFFECTED BY RENAL CARCINOMA

Sample	Age yrs/sex	Stage (TNM)	Grading (Fuhrman)	Histological type	Tumor diameter (cm)
B.B.	73/F	pT2	G2	Clear cell	3.3x3.8x3
P.L.	60/M	pT2	G2	Clear cell	15x14.5x11
C.B.	49/M	pT1	G2	Clear cell	3.6x3.2x3
S.R.	51/M	pT2	G3	Clear cell	9x8x8
T.A.	66/F	pT3a	G4	Sarcomatoid	-
T.G.	84/M	pT1	G2	Papillary	2.5x2x1.8

TABLE II - VEGF LEVELS AND RATIO IN TISSUE SPECIMENS OF KIDNEY TUMORS AND IN THE CORRESPONDING NON-NEOPLASTIC TISSUE

Patient	VEGF in normal kidney tissue molecules/ μg RNA tot	VEGF in kidney tumor molecules/ μg RNA tot	VEGF ratio in kidney tumor/normal tissue
B.B.	4.3×10^5	3.7×10^8	860.47
P.L.	1×10^5	3.1×10^7	310
C.B.	4.3×10^4	7.8×10^6	181.40
S.R.	7.1×10^4	3.8×10^7	535.21
T.A.	3.5×10^5	9.8×10^7	280
T.G.	2.7×10^3	6×10^6	2222

DISCUSSION

We have developed an assay suitable for the measurement of VEGF mRNA expression. Other authors have already reported quantitative RT-PCR assays for VEGF but with different analytical approaches. Fujimoto et al (7) employed primers suitable for monitoring the expression of all the VEGF isoforms and expressed the results as relative units with respect to the GAPDH reference gene. These authors observed a markedly higher VEGF expression in uterine cervical cancer but they did not find any significant difference in the expression pattern of VEGF121 and VEGF165 in comparison to normal cervical tissue. Ikeda et al (8) and Wong et al (9) quantified the expression pattern of the VEGF isoforms and expressed the results as relative units to the β -actin reference gene. Ikeda et al (8) reported a relative increase in VEGF mRNA expression in 67.5% of pancreatic cancers. Wong et al (9) observed a mean increase of total VEGF mRNA expression both in colorectal adenomas and carcinomas in comparison to normal colon tissue. Finally Thelen et al (10) and Boldrini et al (11) developed a quantitative RT-PCR assay for VEGF isoforms based on the use of competitors. In this case the assay results are expressed as attomoles of VEGF/ μ g of total cellular RNA. Thenel et al (10) reported an increase in VEGF expression in renal cell carcinoma in comparison to normal tissue. Boldrini et al (11) obtained similar results in non-small cell lung carcinomas. In spite of a consistently observed mean increase in VEGF expression in various tumors, the dif-

ferent analytical approaches used by the various authors do not allow direct comparison of the results.

In conclusion, the method proposed here includes several novel aspects with respect to previously reported methods, in particular the use of fluorogenic probes and the adequate performance and practicability. This approach seems therefore appropriate for extensive study of the role of VEGF expression in tumors, and the preliminary results observed in a limited number of tumors of the kidney are suggestive of an important role of the measurement of this growth factor in tumor specimens. However, correlation studies between these data and other clinical or diagnostic parameters are required in larger patient series before quantification of VEGF expression can be considered as a suitable index of tumor growth and metastasis.

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