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Quantitative real-time reverse transcription polymerase chain reaction: normalization to rRNA or single housekeeping genes is inappropriate for human tissue biopsies☆

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Abstract

Careful normalization is essential when using quantitative reverse transcription polymerase chain reaction assays to compare mRNA levels between biopsies from different individuals or cells undergoing different treatment. Generally this involves the use of internal controls, such as mRNA specified by a housekeeping gene, ribosomal RNA (rRNA), or accurately quantitated total RNA. The aim of this study was to compare these methods and determine which one can provide the most accurate and biologically relevant quantitative results. Our results show significant variation in the expression levels of 10 commonly used housekeeping genes and 18S rRNA, both between individuals and between biopsies taken from the same patient. Furthermore, in 23 breast cancers samples mRNA and protein levels of a regulated gene, vascular endothelial growth factor (VEGF), correlated only when normalized to total RNA, as did microvessel density. Finally, mRNA levels of VEGF and the most popular housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), were significantly correlated in the colon. Our results suggest that the use of internal standards comprising single housekeeping genes or rRNA is inappropriate for studies involving tissue biopsies.

The reverse transcription polymerase chain reaction (RT-PCR)[1] remains the most sensitive method for characterizing or confirming gene expression patterns and comparing mRNA levels in different sample populations [1]. A recent development, real-time fluorescence-based RT-PCR, integrates the amplification and analysis steps of the PCR, and its sensitivity, specificity, and wide dynamic range make it the method of choice for quantitating steady-state mRNA levels [2]. However, variability in protocols used for samples acquisition and RNA template isolation can introduce errors into the analysis process. These are exacerbated by additional, protocol-dependent inconsistencies characteristic of the RT step as well as variable PCR amplification efficiencies. Third, the identification of a valid reference for data normalization to achieve accurate, reproducible, and biologically relevant mRNA quantification remains a serious problem [3]. This is especially pronounced when the aim is to compare gene expression profiles using in vivo biopsies from different individuals. Accurate quantification and suitable comparison between samples requires equivalent amounts of starting material. Without appropriate normalization, the expression profile of a target gene may be misinterpreted.

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[1] *Abbreviations used:* RT-PCR, reverse transcription polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; VEGF, vascular endothelial growth factor; IPC, internal positive control; MVD, microvessel density; HIF-1, hypoxia-inducible factor 1.

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Ideally sample-to-sample variation could be corrected by measuring simultaneously the levels of a single universal cellular RNA that is present at constant levels during all stages of development, regardless of tissue or experimental treatment [4]. Preferably, its steady-state expression levels should be similar to those of the target gene [5]. Historically, constitutively expressed housekeeping genes such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a key enzyme in glycolysis, have been used widely as internal RNA references for Northern blotting, RNAse protection, and qualitative RT-PCR analyses. Their expression has been assumed to be constant at different times and after many forms of experimental manipulation. Several other housekeeping genes, as well as rRNA, have been proposed as possible alternative internal controls [6,7], but no systematic analysis and comparison of their usefulness has been carried out on in vivo tissue biopsies. While it might have been acceptable to use these internal controls for qualitative assays, this appears not to be so for quantitative RT-PCR. Hence there is an urgent need to identify a more reliable way of normalizing between samples.

In the present study we have quantitated the mRNA levels of 10 housekeeping genes, widely used as convenient reference genes, and of 18S rRNA, in an unselected group of 23 paired normal breast tissue and tumor samples. We normalized the mRNA levels of a regulated gene, vascular endothelial growth factor (VEGF) to total RNA as well as to the 11 internal controls and compared normalized mRNA to VEGF protein levels and to microvessel density as the biological end point of angiogenesis. Finally, we observed a significant correlation between GAPDH and VEGF mRNA levels in normal colon samples as well as colorectal cancer biopsies.

Materials and methods

Tissue samples and RNA extraction

Ten- to 20-mg biopsies of normal and malignant tissue taken from unselected patients undergoing surgery for breast ($n = 23$) or colorectal ($n = 41$) carcinoma, respectively, were snap-frozen in liquid nitrogen. Total RNA from these samples and from the human breast cancer cell line MCF-7 transfected with a coding sequence for $VEGF_{121}$ was extracted using RNeasy reagents (Qiagen, Milan, Italy, or Crawley, UK), according to the manufacturer's recommendations and resuspended in 50 µl of DEPC-treated water. RNA from breast biopsies was quantitated in triplicate after 1:50 dilution in water using a GeneQuant spectrophotometer (Pharmacia, Milan, Italy). The intraassay variability of the 260 nm measurement was 3.0% and of the 260/280 nm ratio was 1.3%. Sufficient RNA from the MCF-7 cell line was prepared to provide enough template for all experiments described in this paper. RNA from colonic biopsies was quantitated using Ribogreen quantification kits (Molecular Probes, Leiden, The Netherlands) after DNase (Qiagen) treatment and quality assessed using a RiboChip on an Agilent 2100 Bioanalyser.

Experimental design

Breast biopsies

We used two lots of 16 Human Endogenous Control Plates (Applied Biosystem (ABI), Foster City, CA) to quantitate mRNA levels in the breast samples. This plate includes an internal positive control (IPC) which consists of a synthetic amplicon, primer, and probe designed to detect the presence of PCR inhibitors in the test samples. If inhibitors are present, the IPC signal will diminish or disappear. In addition, it contains 11 specific primer and dual-labeled probe sets that permit a simultaneous evaluation of the expression of 10 housekeeping genes and 18S rRNA (Table 1). Each plate allows the measurement of 8 replicates of the test samples.

All RT-PCR reagents were purchased from ABI. Total RNA 650 ng from MCF-7 and the 23 breast biopsy samples was reverse-transcribed in duplicate in a final volume of 100 µl in a final concentration of 1× TaqMan RT buffer, 2.5 µM random hexamers, 500 µM each dNTP, 5.5 mM $MgCl_2$, 0.4 U/µl RNase inhibitor, and 1.25 U/µl Multiscribe RT for 10 min at 25 °C, 30 min at 48 °C, and 5 min at 95 °C. Subsequently 225 µl RNase-free water was added to each duplicate of MCF-7 and breast sample cDNA, and amplification was performed using 325 µl of TaqMan PCR Universal Master Mix. Aliquots 50 µl in duplicate (each corresponding to 50 ng RNA of initial sample) were dispensed into each well of the Taqman Human Endogenous Control Plate. PCR was performed on an ABI PRISM 7700 as follows: 2 min 50 °C, 10 min 95 °C followed by 40 cycles of 15 s at 95 °C, 1 min at 60 °C. Inclusion of the MCF-7 RNA on every plate provided the baseline for each internal con-

Table 1
Endogenous genes used in this study

Endogenous control gene	Abbreviation
18S rRNA	18S
Acidic ribosomal protein	PO
β-actin	βa
Cyclophilin	CYC
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH
Phosphoglycerokinase	PGK
β_2-Microglobulin	β2m
β-Glucuronidase	GUS
Hypoxanthine ribosyl transferase	HPRT
TATA-binding protein	TBP
Transferrin receptor	TfR

trol marker for comparison with the breast biopsy samples that were run at different times on different plates.

Colon biopsies

RT-PCR were carried out using 100 ng of total RNA with the one enzyme/one tube EZ assay (ABI) using conditions as previously described [8] on an ABI PRISM 7700.

Primers and probes

The sequences of all primers and probes used in addition to the ones provided with the control plate, together with amplicon lengths, are listed in Table 2. They were designed using Primer Express software version 1.0 (ABI) and synthesized by ABI with FAM at the 5′-end and TAMRA at the 3′-end. The VEGF primers used with RNA prepared from breast biopsies and MCF-7 cells amplify a region common to all five isoforms [9]; those used with colorectal biopsies are specific for $VEGF_{165}$ or $VEGF_{189}$ splice variants. The GAPDH primers and probe were used with the colonic biopsies only, since the endogenous control plate used with the breast samples already contains a GAPDH set.

Copy number calculations

Copy numbers were calculated from external standard curves that were obtained as follows: HPLC-pure sense-strand oligonucleotides specifying an amplicon, e.g., $VEGF_{165}$ or $VEGF_{189}$ were synthesized (ABI or MWG-Biotech, Ebersberg, Germany). The manufacturer's information was used to calculate the copy number of oligonucleotides present in 1 µl TE buffer (molecules/ng $= (1 \times 10^{-9}\,\mathrm{g/MW}) \times N_0$ molecules/mole, where MW is the molecular weight of the oligonucleotides as per product sheet and $N_0 =$ Avogadro's number). Most stock solutions of oligonucleotides were calculated to contain between 1 and 3×10^{12} molecules/µl, depending on the amplicon. Serial dilutions of this stock were carried out in duplicate and dilutions in the range of 10^9–100 were used in triplicate PCR to generate standard curves. We have previously shown that such short sense-strand amplicons, average length 70 bases, reliably mimic the RT step and generate standard curves that are identical to those obtained using T7-transcribed RNA [2]. If use of the higher dilutions did not result in linear standard curves, the dilutions were repeated until a reliable set of standards was obtained. Higher dilutions were stable at −20 °C for no more than 5 days.

Immunohistochemistry for VEGF in breast cancer tissues

Staining for VEGF protein was performed using the LabVision Kit (Neomarkers, Fremont, CA). Briefly, the section were dewaxed in xylene, taken through ethanol, and then incubated with 3% hydrogen peroxide in methanol for 15 min. The sections were washed in phosphate-buffered saline and incubated in 10% normal goat serum for 15 min to reduce nonspecific antibody binding. Polyclonal antibody to human VEGF (Santa Cruz Biotechnology Inc., Santa Cruz, CA) was used as the primary antibody. The slides were incubated in phosphate-buffered saline containing diaminobenzidine and 1% hydrogen peroxidase for 10 min and counterstained with hematoxylin. The staining scores were attributed in a blind fashion and indicated as 0, 1+, 2+, and 3+, corresponding to no staining, weak or focal staining (cytoplasmic staining in less than 30% of cells), moderate staining (staining in 30–60% of the cells), and strong staining (greater than 60%), respectively [10]. We used smooth muscle as a positive control.

Microvessel staining and counting

Microvessel density (MVD) was determined with a monoclonal antibody to CD31 antigen (clone JC/70A; Dako, Carpinteria, CA). In all samples, the mean value of microvessel number was calculated in three high neovascularized areas in an examination area of 0.41 mm^2 with a final magnification of ×250. Results were expressed as the number of microvessel per mm^2 [11].

Statistics

The data were analyzed by nonparametric tests and all statistical calculations were carried out using Winstat for Excel (R. Fitch Software, Staufen, Germany).

Table 2
Primer/probe sequences and sizes of amplicons generated by real-time RT-PCR assay in addition to the ABI control plate

Target	Forward primer (5′–3′)	Reverse primer (5′–3′)	Probe (5′–3′)	Amplicon
$VEGF_{165}$	TGTGAATGCAGACCAAAGAAAGA	GCTTTCTCCGCTCTGAGCAA	AGAGCAAGACAAGAAAATCCCTGTGGGC	73 bp
$VEGF_{189}$	TGTGAATGCAGACCAAAGAAAGA	CGTTTTTGCCCCCTTTCC	AGAGCAAGACAAGAAAATCCCTGTGGGC	77 bp
VEGF	TACCTCCACCATGCCAAGTG	ATGATTCTGCCCTCCTCCTTC	TCCCAGGCTGCACCCATGGC	63 bp
GAPDH	GAAGGTGAAGGTCGGAGT	GAAGATGGTGATGGGATTTC	CAAGCTTCCCGTTCTCAGCC	226 bp

Results and discussion

Fluorescence-based real-time RT-PCR assays permit accurate quantification of steady-state mRNA levels and have become widely used for the expression profiling of regulated genes. However, accurate quantification is not the same as biologically relevant interpretation; this requires appropriate normalization between individual samples. Differential expression patterns are most commonly identified by comparing target mRNA levels to those of an internal reference. This can be a control mRNA whose expression is more or less universal and assumed to be constitutive, i.e., not regulated or rRNA, which is transcribed by a separate RNA polymerase. However, doubts have emerged recently about the reliability and appropriateness of this approach. It has become increasingly obvious that there is no one mRNA with a constant expression level and that housekeeping gene mRNA levels can vary quantitatively in response to numerous factors such as developmental stage, during the cell cycle, and with experimental conditions [2]. Therefore, normalization to accurately quantitated total RNA has been proposed as an alternative strategy.

We have used in vivo breast biopsies to quantitate the mRNA levels of commonly used internal control genes to investigate their possible use as internal standards in clinical diagnostic assays. Results were compared to those obtained with GAPDH in colonic biopsies. VEGF was chosen as the target gene, since its expression is increased at both the mRNA and the protein levels in breast [12] and colorectal cancers [13]. We analyzed two quantitative parameters: interindividual variability of mRNA levels and intraindividual differences between paired normal and cancer tissue.

In addition to acting as a control for the presence of RT-PCR inhibitors in samples, the IPC provides a useful indicator for the reliability of the operator carrying out the experiments. Using the control MCF-7 RNA, the C_ts of the IPC varied very little between the 16 plates, with an average C_t of 23.23 (95% confidence interval (CI) ± 0.76). Since these PCR assays were carried out over the course of several weeks, the results confirm the reproducibility of the operator's technique. When the control RNA was substituted with RNA preparations from in vivo tissue samples, a virtually identical average C_t of 23.11 (CI ± 0.40) was obtained. This suggested that there were no inhibitors of the RT-PCR in any of the RNA or cDNA preparations. The reproducibility of the housekeeping gene plates was tested by amplifying MCF-7 cDNA on all 16 plates and determining the C_ts of the 11 internal controls. There was very little interplate variability for 18S RNA and four of the reference housekeeping genes; six of the housekeeping genes revealed some differences between the plates, particularly when comparing early with late plates. Since plates 1–9 and 10–16 represented two separate lots, we compared the average C_t for each marker between the two lots of plates (Fig. 1). This showed that, except for βA and CYC, there was little variation within the lots. Even then their respective 95% CI were only 2.34 and 2.03, which translate into an 8-fold difference in calculated copy number. This is insignificant compared with the variability observed subsequently with the in vivo biopsies.

The interindividual variability of control gene RNAs in the breast biopsies was enormous, ranging over more than four orders of magnitude (Fig. 2). Similar wide ranges of mRNA levels have been reported previously for regulated genes [14–16]. Intraindividual RNA levels between paired normal and cancer biopsies also varied considerably, although only one pair differed by more than four orders of magnitude, with most differing by one to two. Importantly, there was no consistent pattern: for example, GAPDH mRNA levels were higher in some cancers and lower or no different in others when compared with the paired normal tissue (Fig. 3). This large and unpredictable variability was observed for all other reference genes in the breast biopsies and for GAPDH in colorectal biopsies (data not shown). This variability between individuals is not just confined to breast and colon, as we have obtained similar results in pituitary [17] as well as blood and cervical (unpublished observation) biopsies.

We have previously advocated normalization to total RNA as an appropriate method of comparing steady-state mRNA levels between different in vivo tissue biopsies [2]. We now confirm the validity of this approach by quantitating "absolute" VEGF mRNA levels in breast cancer samples relative to an external standard curve and normalized to total RNA and correlating the results with VEGF protein levels as well as with a biological end point of VEGF activity, microvessel density. VEGF mRNA copy numbers, normalized to total

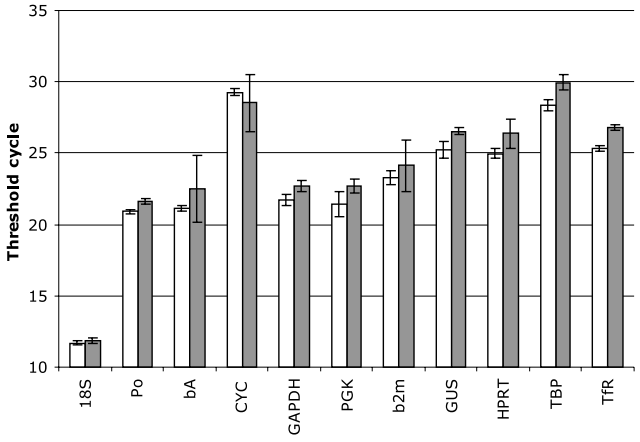

Fig. 1. Comparison of threshold cycles (C_t) for the 11 internal control genes using MHC-7 RNA in plates 1–9 (□) and plates 10–16 (■). The graph shows the average $C_t \pm 95\%$ confidence intervals (CI).

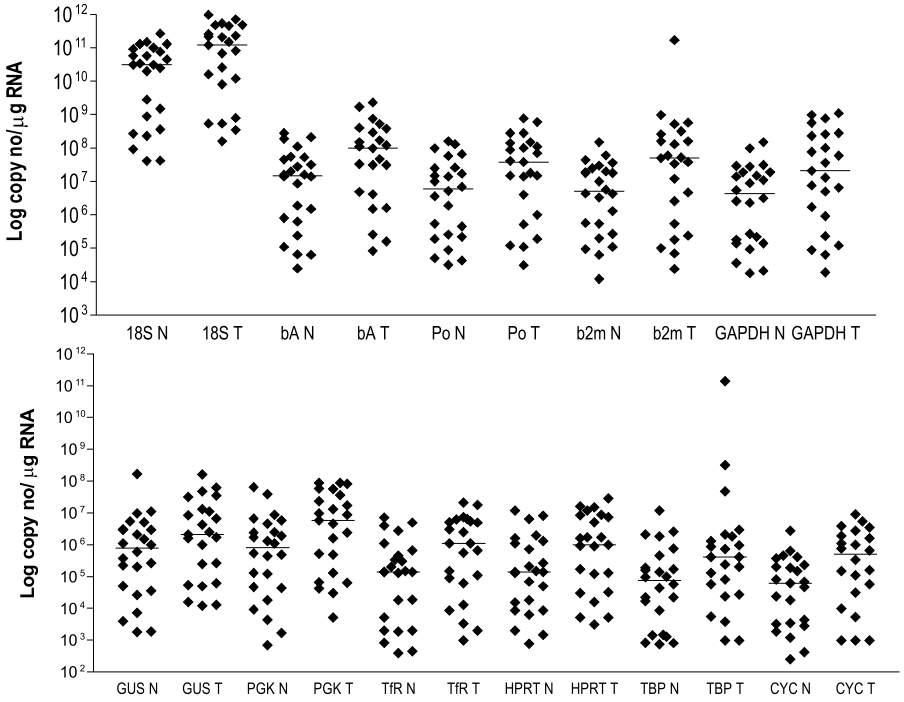

Fig. 2. "Absolute" mRNA levels of housekeeping genes in normal breast (N) and breast cancer (T) samples ($n = 23$) expressed as copy numbers/μg total RNA. Replicates have been averaged. The horizontal bar indicates the median mRNA levels.

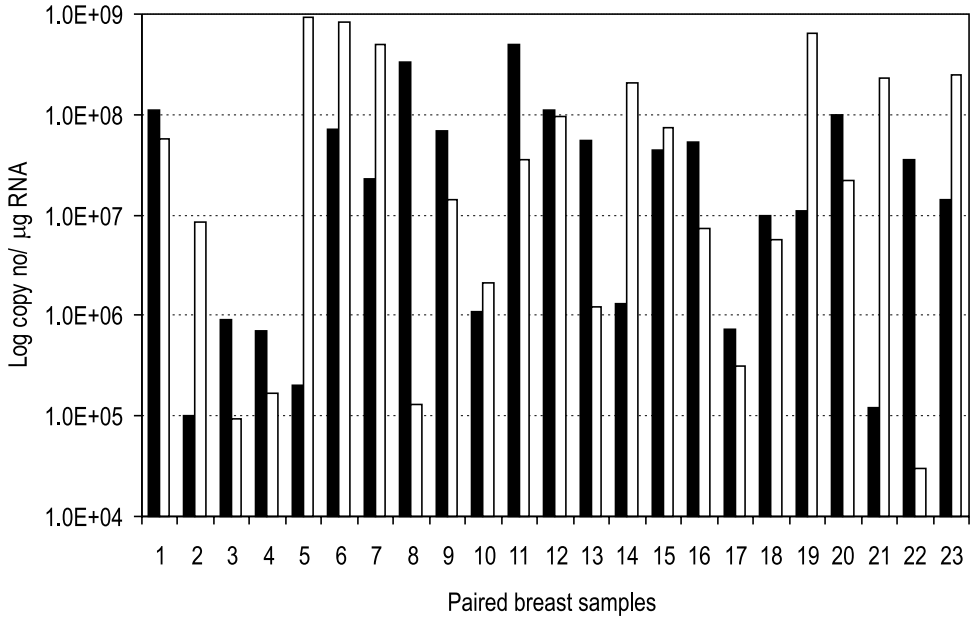

Fig. 3. "Absolute" GAPDH mRNA levels in 23 paired normal (□) and breast cancer samples (■). Copy numbers were normalized to total RNA and quantitated using a GAPDH-specific standard curve.

RNA, were significantly higher in breast cancers than in paired normal tissues (Fig. 4A), in contrast to normalization against GAPDH which showed no difference (Fig. 4B). Normalization to total RNA resulted in higher VEGF mRNA levels in 17/23 paired samples (7/23 with GAPDH), lower levels in 1/23 (4/23 with GAPDH), with no difference in the remainder. This correlated well with the results obtained using immunohistochemistry, which showed that VEGF protein is expressed at higher levels in the tumor compared with the paired normal samples (Fig. 4B). In addition, MVD staining was significantly higher in breast cancer samples

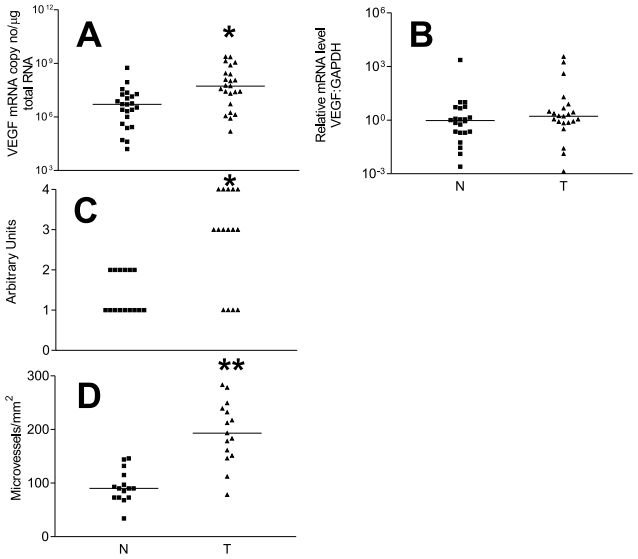

Fig. 4. Correlation between VEGF mRNA and protein expression and MVD in normal and breast cancer biopsies. (A) The scatter plot compares "absolute" VEGF mRNA copy numbers ($P < 0.05$, Mann–Whitney) in paired normal and cancer tissue, with the horizontal line indicating the median mRNA copy number. (B) The same samples expressed relative to GAPDH, with the horizontal line indicating the median mRNA copy number. There is no significant difference between the normal and tumor samples. (C) Immunohistochemical detection of VEGF was recorded as 1 (weak) through 4 (strong) and plotted. The graph shows the enhanced staining for VEGF in the tumor samples ($P < 0.05$, Mann–Whitney). (D) Microvessel density, expressed as the number of microvessel per mm^2, is significantly higher in the breast cancer samples ($P < 0.0001$, Mann–Whitney). The horizontal bars indicate the median microvessel numbers.

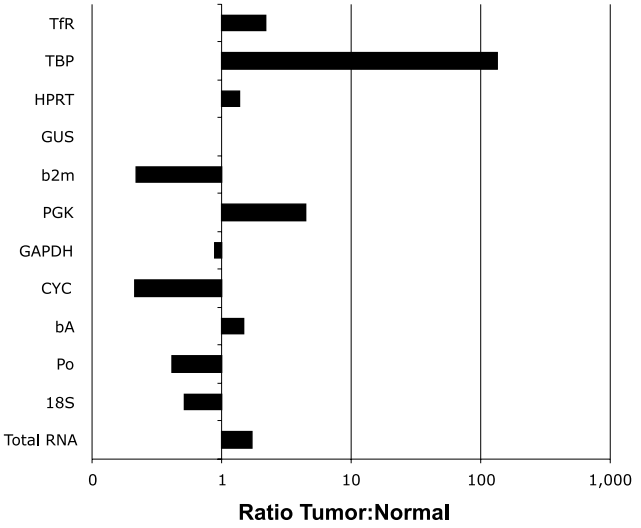

Fig. 5. Comparison of normalization to total RNA or to 11 endogenous control genes in one patient. The Tumor:Normal ration was calculated as (Tumor VEGF copy number, normalized to total RNA or individual endogenous control RNA)/(Paired normal VEGF copy number, normalized to total RNA or individual endogenous control RNA).

compared with the normal tissue biopsies (Fig. 4C). This suggests that under the conditions specified, normalization to accurately quantitated total RNA can generate accurate and biologically relevant data.

In contrast, the normalization of VEGF mRNA to each of the 11 reference RNAs generated confusing and misleading results (Fig. 5) that were contradicted by the protein quantification and activity data. VEGF mRNA levels could be made to appear increased, decreased, or unchanged between paired normal and cancer samples, depending only on the normalizer chosen. Clearly such divergence is unacceptable and results in a meaningless quantification.

If a single housekeeping gene should not be used, would normalization against several such genes be more accurate? The recent demonstration of the usefulness of normalization against a panel of internal reference genes is interesting [18]. However, this method requires extensive practical validation to identify a combination of reference genes appropriate for every individual experiment, something that is not at all trivial. In addition, as the choice of housekeeping gene panel is tissue or cell dependent, this is not a universal method. Finally, it seems reasonable to assume that all genes are regulated and our data suggest significant unpredictable differences in that regulation between and even within the same individual.

Despite this conclusion, it must be stated that the use of total RNA concentration as an internal standard has several drawbacks. First, it is not always possible to quantify total RNA, especially when dealing with very limited amounts of clinical samples, or when mRNA has been extracted. Second, the quality of the RNA preparation must be taken into account. However, the development of the Agilent 2100 Bioanalyser and RNAChip provides a convenient way of combining quality and quantity assessment of most RNA preparations. Most importantly, normalization to total RNA concentration is not ideal for quantifying gene expression between tissues whose transcriptional activities may vary. Transcriptionally active tissues will contain disproportionately fewer target transcripts, whereas transcriptionally quiescent tissues will appear to contain more target mRNAs. This will be particularly relevant to any comparison between normal cells and matched carcinomas. Nevertheless, the validity of using total RNA to normalize quantitative RT-PCR data is suggested by the fact that an analysis of the mRNA levels of the c-myc oncogene in 75 normal colon epithelium and matched tumor samples showed overexpression of c-myc in 69% of tumors [15], which agrees with other published data obtained using alternative techniques [19–21]. Therefore, until a more appropriate standard can be identified, normalization to total RNA should certainly be considered as an appropriate normalization procedure for in vivo biopsies.

Table 3
Correlation of VEGF and GAPDH mRNAs in normal colon and colorectal cancer biopsies. Correlations were calculated using the nonparametric Spearman rank correlation

	$VEGF_{165}$		$VEGF_{189}$	
	r^2	P	r^2	P
GAPDH N	0.56	< 0.00001	0.37	< 0.00001
GAPDH T	0.51	< 0.00001	0.51	< 0.00001

We observed a significant correlation between GAPDH and $VEGF_{165/189}$ mRNA levels in 41 paired colorectal normal and cancer biopsies (Table 3) which suggests that it is not appropriate to normalize the expression of VEGF mRNA to that of GAPDH, something that is still being reported [22]. The correlation might be explained by the observation that hypoxia is known to stimulate the expression of GAPDH [23,24] as well as of VEGF [25]. Hypoxia-inducible facor 1 (HIF-1) is the main transcriptional activator of VEGF and, interestingly, HIF-1 consensus sequences have been identified in the GAPDH promoter [26]. Hypoxia is common in tumors, hence the necrotic core at the center of many solid cancers. Little is known about hypoxic effects on normal human colon epithelium, but problems with peristalsis may result in the induction of local tissue hypoxia. Such conditions would enhance angiogenesis, hence could account for the coordinated regulation of the two genes. Importantly, this finding provides additional authentication of the validity of normalization to total RNA.

In conclusion, our data suggest single housekeeping gene RNA levels should not be used to normalize RNA levels when comparing in vivo tissue biopsies obtained from different individuals. Ideally, cell numbers should be counted to allow the determination of mRNA copy numbers per cell. Since this is often not possible, our results suggest that normalization to total RNA concentration is an acceptable alternative.

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