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The normalization of gene expression data in melanoma: Investigating the use of GAPDH and 18S rRNA as internal reference genes for quantitative real-time PCR

Orsolya Giricz, Janelle L. Lauer-Fields, and Gregg B. Fields *

Department of Chemistry & Biochemistry, Florida Atlantic University, 777 Glades Road, Boca Raton FL 33431

Abstract

We examined a panel of 26 melanoma and fibroblast samples (tissues and cultured cells) to evaluate the suitability of two commonly used housekeeping genes, GAPDH and 18S rRNA, for quantitative real-time PCR. Both genes showed significant variations within the individual cell line and tissue groups. While no overall trends were observed in the expression of the 18S rRNA, GAPDH was up-regulated in melanoma tissue and cultured cells compared to the corresponding normal samples. In melanoma and fibroblast cell lines and tissues, absolute quantification appears to be more appropriate than normalizing mRNA expression via GAPDH or 18S rRNA housekeeping genes.

The number of skin cancer cases has been steadily increasing with the risk of developing malignant cutaneous melanoma doubling every 10-15 years [1]. However, in 2007 the new cases of melanoma was estimated at 59,940, nearly double that of previous estimates [2]. If melanoma is detected early (localized), the five year survival rate is ~99% [3]. However, distant stage (metastatic) melanoma has a very poor prognosis (~15% five year survival) [3]. Thus, there is a critical requirement for accurate assessment of melanoma initiation and progression, as well as the characterization of the more malignant later stages.

The most reliable prognostic factor of melanoma is still the tumor thickness [4], thus underscoring the importance of early detection and surgery. Better knowledge of the underlying molecular mechanisms of transformation and melanoma progression would possibly aid in the discovery of novel biomarkers, potential therapeutic targets, or biochemical pathways. Previous studies have shown that gene expression profiling of melanoma mRNA levels may lead to novel molecular classification methods by identifying the subtypes of disease and possibly predicting phenotypic characteristics ([5] and references cited therein). Microarray analysis can be used for the high-throughput analysis of global gene expression levels, studying hundreds or thousands of targets at once. Quantitative real-time PCR (qRT-PCR or qPCR) allows for the sensitivity to analyze a narrow range of targets or to confirm previously obtained microarray data.

Currently there are two quantification methods available for qPCR, absolute and relative. In the case of absolute quantification, genomic DNA or plasmids containing cloned qPCR target

*Corresponding author. Department of Chemistry & Biochemistry, Florida Atlantic University, 777 Glades Road, Boca Raton, FL 33431-0991, USA. Tel: 1-561-297-2093. Fax: 1-561-297-2759. E-mail address: fieldsg@fau.edu.

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sequences are commonly used as standards. Relative quantification in qPCR relates the expression levels of a target gene transcript to a reference gene transcript. Selecting the appropriate internal standard is critical to properly control the variations in RNA quantification. "Internal reference" genes are presumed to be essential for cell viability and thus constitutively expressed to maintain cellular function [6], and are widely employed as quantification controls, correlating target gene levels to that of the reference gene [7]. The expression of even the most commonly used internal controls may vary as a result of neoplastic growth, leading to inaccurate quantification [8]. In the present study, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and 18S rRNA, two of the most commonly used internal reference genes, were investigated as controls for qPCR analysis of melanoma tissue, native tissue, cultured melanoma cells, and fibroblasts. Also examined was the effect of amplicon length on 18S rRNA quantification (presented in the Supplementary Material).

Results and Discussion

Seven fibroblast and 8 melanoma cell lines and 5 normal and 6 melanoma tissue samples were analyzed (Table 1). The normal tissue samples were all native adjacent samples to the melanoma tissue samples. Due to the uniqueness of the in-house derived melanoma cells, as well as aiming to only look at general trends, the sample pool was kept to a limited number. Samples were categorized based on pathology reports as well as the presence or absence of two widely accepted melanoma markers, tyrosinase and melanA.

Expression of 18S rRNA and GAPDH

The difference between the smallest and the largest observed 18S rRNA value across all samples was 70-fold (Figure 1A). The mean copy number/pg total RNA values were 1.60×10^5 in cultured fibroblasts (an outlier data point, 9.62×10^5 was not used in the calculations), 3.15×10^5 in normal tissues, 3.68×10^5 in cultured melanoma cells, and 2.71×10^5 in melanoma tissues. The Coefficients of Variance (CV) values of 22.8% in cultured fibroblasts, 32.6% in normal tissues, 47.5% in cultured melanoma cells, and 65.4% in melanoma tissues shows high variability within each group. Comparing the average 18S rRNA expression across the four groups, there were no statistically significant differences.

The difference between the smallest and the largest observed GAPDH value is 80-fold (Figure 1B). The mean expression values were 165.1 in melanoma tissues, 79.5 in fibroblasts, 42.2 in native tissues (an outlier data point, 328 was omitted), and 213 in cultured melanoma cells. The CV values were 51.8% in melanoma tissues, 53.8% in fibroblasts, 67.5% in normal tissues, and 77.7% in cultured melanoma cells, and thus even higher than those of the ribosomal target. In melanoma tissue samples, GAPDH was up-regulated 4-fold compared to the normal tissue samples, while cultured melanoma cells showed 3 times higher expression than in fibroblast cells.

GAPDH is well known for catalyzing the reversible oxidative phosphorylation of glyceraldehyde-3-phosphate in the presence of inorganic phosphate and nicotinamide adenine dinucleotide (NAD^+). There have been numerous reports regarding GAPDH regulation in cancer, in tumor samples and in cultured cells, as well as GAPDH response to experimental treatments [9;10;11;12]. Thus, the variability of GAPDH expression levels amongst melanoma samples is not surprising.

When comparing tissues that are in a different developmental stage or proliferation rate, the mRNA/rRNA ratio will be different since they will contain varying amounts of rRNA species [7]. Several growth dependant signaling pathways have been implicated in regulating rRNA levels [13], such as the mammalian target of rapamycin (mTOR) and mitogen activated protein kinase (MAPK) pathways, both of which have been described as crucial for melanoma

activation and progression [14;15]. Thus, as with GAPDH, it is understandable as to why 18S rRNA is not a good choice as a control gene for melanoma and normal tissue types.

A previous study found 18S rRNA to be a more reliable standard compared to GAPDH or β -actin in cultured melanoma cells [8]. However, this prior study utilized regular PCR and duplex relative PCR, both of which are based on the visualization and quantification of the PCR products by gel electrophoresis and densitometric analysis. The suitability of GAPDH or 18S rRNA as an internal control for melanoma or comparison between tissue samples and cultured cells had not been evaluated with the aid of highly sensitive nucleic acid detection assays such as qPCR. There have also been several qPCR studies of the suitability of different housekeeping genes for normalizing RNA expression in normal cell lines or tissues undergoing exogenous stimulation, or comparing housekeeping genes in normal versus diseased states ([16] and references cited therein). However, to the best of our knowledge, comparison of housekeeping genes in melanoma and normal tissues and cell lines has not been reported.

Utilizing genomic or plasmid DNA as an external control is more labor-intensive than using an internal reference, as the standards need to be created and accurately quantified. The main limitation is that the efficiency of the reverse transcription step is not accounted for, thus cDNA synthesis of all samples should be done simultaneously to limit variability. The efficiencies of the external calibration curve (the plasmid standard) and a standard curve prepared from serial dilutions of an RNA sample should be comparable. To determine if the efficiencies of the amplification in both cases is indeed comparable, a plot of the log DNA or RNA dilution versus the ΔC_t has to be made. If the absolute value of the slope is less than 0.1, the efficiencies of amplification in both samples are comparable [17;18]. In this case, it also proves that the reverse transcription step has no effect on the amplification efficiency, thus the external curve can be utilized for absolute quantification. When comparing both internal reference genes, we have found that the slope was below 0.1, thus using the standard curve based on the plasmids was appropriate.

Absolute quantification utilizing an external standard curve leads to units expressed as copies per amount of isolated RNA or copy number/cell, as opposed to relative quantification. The double stranded DNA standards can be easily archived as bacterial glycerol stocks or kept solubilized in TE buffer at -80 °C for years [19]. The quality and stability of RNA cannot be assured during prolonged storage in aqueous solution due to the ubiquitous presence of ribonucleases, not even highly purified and under optimal conditions (at temperatures lower than -70 °C and in the presence of ribonuclease inhibitors) [20]. Therefore, DNA standards are more stable and can be stored longer as well.

Summary

Based on this study and others, relative quantification using endogenous control genes such as GAPDH or 18S rRNA seem to be ineffective as a means of quantifying mRNA levels of target genes in melanoma. Currently, absolute quantification is the best means of evaluating target gene expression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Figure 1.

Expression of (A) 18S rRNA or (B) GAPDH in individual samples. The number of copies of 18S rRNA or GAPDH per total RNA was determined for individual samples of normal tissue (NT1-5), melanoma tissue (MT1-6), cultured fibroblasts (f1-7), and cultured melanoma cells (m1-8). Experiments were performed two times in triplicate.

Table 1**Samples used in this study**

Abbreviation	Type	Origin
NAT56WF	f1	CHTN, lung fibroblasts
NAT56UM	f2	CHTN, lung fibroblasts
NAT58WM	f3	CHTN, skeletal muscle fibroblasts
NAT74UM	f4	CHTN, skin fibroblasts
NAT41UM	f5	CHTN, colon/rectum
NAT50WM	f6	CHTN, lung fibroblasts
BJ	f7	ATCC, foreskin fibroblasts
Mel58WM	m1	CHTN
Mel93WF	m2	CHTN
Mel80WM	m3	CHTN
SK-2-MEL	m4	ATCC, derived from skin metastasis
M14 clone#5	m5	*
WM115	m6	ATCC, derived from primary skin tumor
WM-266-4	m7	ATCC, derived from skin metastasis
Hs 895.T	m8	ATCC, derived from lung metastasis
tNAT56WF	NT1	CHTN, lung tissue
tNAT56UM	NT2	CHTN, lung tissue
tNAT58WM	NT3	CHTN, skeletal muscle
tNAT46WM	NT4	CHTN, skeletal muscle
tNT77WM	NT5	CHTN, skeletal muscle
tMel58WM	MT1	CHTN, muscle metastasis
tMel56UM	MT2	CHTN, lung metastasis
tMel93WF	MT3	CHTN, unknown
tMel46WM	MT4	CHTN, unknown
tMel77WM	MT5	CHTN, muscle metastasis
tMel80WM	MT6	CHTN, lymph node metastasis

f = fibroblast, m = melanoma, NT = native tissue, MMT = metastatic melanoma tissue, CHTN = Cooperative Human Tissue Network, ATCC = American Type Tissue Collection.

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