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Original Article

Evaluation of potential reference genes for qRT-PCR studies in human hepatoma cell lines treated with TNF-α

Chang Wu, Xiang Wang, Ming Zhong, Hailing Liu, Qiongqiong He, Xiaojing Yang, Jifang Wen, and Deyun Feng*

Department of Pathology, School of Basic Medical Sciences, Central South University, Changsha 410078, China
*Correspondence address. Tel: +86-731-82650400; Fax: +86-731-82650410; E-mail: deyunfeng743@sina.com

In this study, the expression of eight candidate reference genes, *B2M*, *ACTB*, *GAPDH*, *HMBS*, *HPRT1*, *TBP*, *UBC*, and *YWHAZ*, was examined to identify optimal reference genes by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis in two human hepatoma cell lines, BEL-7402 and SMMC-7721, treated with tumor necrosis factor-α (TNF-α) for different time periods. The expression stability of these genes was analyzed by three independent algorithms: geNorm, NormFinder, and BestKeeper. Results showed that *TBP* was the most stably expressed gene in BEL-7402 and SMMC-7721 cell lines under current experimental conditions, and that the optimal set of reference genes required for accurate normalization was *TBP* and *HMBS*, based on the pairwise variation value determined with geNorm. *UBC* and *ACTB* were ranked as the least stable genes by same algorithms. Our findings provide evidence that using *TBP* alone or in combination with *HMBS* as endogenous controls could be a reliable method for normalizing qRT-PCR data in human hepatoma cell lines treated with TNF-α.

Keywords human hepatoma cell lines; qRT-PCR; reference gene; cytokine; tnf-α

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Introduction

In gene expression analysis, quantitative reverse transcription-polymerase chain reaction (qRT-PCR) has become the most common method for quantification of mRNA transcription levels due to its outstanding accuracy, broad dynamic range, and sensitivity [1,2]. It is fast, easy to use, and highly reproducible, requiring a minimal amount of RNA and no post-PCR handling. It is essential to have a reliable normalization control when comparing gene expression levels between different biological samples. Reference genes, which are stably expressed in different samples and unaffected by any experimental treatment [3,4], are often used to normalize qRT-PCR data to eliminate experimental differences, such as RNA quantity and quality, overall transcriptional activity, and cDNA synthesis.

Commonly used reference genes, including glyceraldehyde-3-phosphate dehydrogenase gene (*GAPDH*), beta-actin gene (*ACTB*), and TATA-binding protein gene (*TBP*), are routinely used in gene expression studies [5–9]. However, accumulating evidence has suggested that *ACTB* [10] and *GAPDH* [1,10] vary considerably and are consequently unsuitable endogenous controls for RNA transcription analysis. Glare *et al.* [10] described erroneous readings of target gene expression between experimental groups when *ACTB* was used as an internal control. In fact, it was the *ACTB* rather than the target gene that changed. GAPDH, a key enzyme in glycolysis constitutively expressed in many tissues, is still widely used as an internal control, even though wide variations in its expression levels have been observed in tumor cell lines [11], tissues at different developmental stages [12], and under hypoxic conditions [13]. Many other reference genes also show unacceptable variability in expression, which can lead to increased 'noise' or erroneous results [10,14–16]. Therefore, it is crucial to validate appropriate reference genes in given experimental system.

It has been reported that chronic inflammation is involved in tumor initiation, promotion, and progression in clinical and epidemiological studies [17–22]. Cytokines, which are produced by activated innate immune cells and may stimulate tumor growth and progression, are important components in the linkage between inflammation and cancer, and elucidating the mechanism of cytokine regulation is expected to contribute strongly to identify altered signaling pathways which are suitable targets for therapy.

It has been revealed that the pro-inflammatory cytokine tumor necrosis factor-α (TNF-α) plays a pivotal role in the promotion and progression of cancer [23,24], which is one of the major mediators of cancer-related inflammation and acts as a tumor-promoting factor [25]. In the present study, experiments were performed to identify appropriate reference genes for accurate normalization in human hepatoma cells treated with TNF-α.

Materials and Methods

Cell culture and treatment

Human hepatoma cell lines, BEL-7402 and SMMC-7721, were purchased from Shanghai Cell Bank of the Chinese Academy of Sciences (Shanghai, China). All cells were cultured in Roswell Park Memorial Institute 1640 medium (Gibco, Carlsbad, USA) containing 10% fetal bovine serum, unless stated otherwise, in a humidified incubator at 37°C in 5% carbon dioxide. The cultures were free of mycoplasma species and maintained for no longer than 8 weeks after recovery from frozen stocks. Human recombinant TNF-α was purchased from PEPROTECH (Rocky Hill, USA). To analyze basal levels of reference genes expression, two cell lines were cultured to 70% confluence, washed three times in phosphate buffer saline, and maintained in the absence of serum for 24 h. Subsequently, cells were treated with TNF-α for 0, 4, 8, or 12 h at a final concentration of 10 ng/ml.

Hepatocellular carcinoma sample

Samples were obtained from hepatocellular carcinoma (HCC) patients undergoing surgical resection from October 2012 to January 2013 at the Second Xiangya Hospital of Central South University (Changsha, China). Informed consent was obtained from all patients, and the protocols were approved by the ethics committee of the Second Xiangya Hospital of Central South University. In order to keep warm ischemia time short (usually below 2 min), all samples were handed over for further processing by the surgeon removing the tumor. All tissues obtained were examined for the presence of tumor cells. As a minimum criterion for usefulness for our studies, we only chose tumor tissues in which tumor cells occupied the major portion of tumor biopsy. Immediately after resection, tissue samples were frozen in liquid nitrogen and stored at −80°C until use.

RNA extraction

Total RNA was extracted by using RNAiso Plus kit (TaKaRa, Dalian, China) according to the manufacturer's instructions. RNA was finally quantified using a plastic cuvette (Eppendorf, Germany). Samples were dissolved in RNase-free water and quantified by the average of triplicate spectrophotometric readings at 260 nm (A_{260}). Purity of total RNA was determined by A_{260}/A_{280} ratio. RNA samples with A_{260}/A_{280} ranging from 1.9 to 2.1 were used for experiments. Before cDNA synthesis, the integrity of RNA samples was confirmed by electrophoresis on 1% agarose gels.

First-strand cDNA synthesis

Isolated RNA was reverse transcribed with the PrimeScript® RT kit with gDNA Eraser (TaKaRa) according to the manufacturer's instructions. For all samples, 1 μg of total RNA was used for cDNA synthesis and the protocol included a specific incubation step by using gDNA eraser buffer to eliminate any contamination with genomic DNA that may be contained in samples.

qRT-PCR primers production

Candidate reference genes were selected according to the following criteria [5,6,26–30]: (i) high frequency of use (*ACTB* and *GAPDH*); (ii) used for normalization in other cancer types (*HPTR*1, *TBP*, and *B2M*); (iii) belonging to a different functional class (*HMBS*, *UBC*, and *YWHAZ*). These eight genes described above were selected to evaluate their expression profiles in the present study (**Tables 1** and **2**).

Table 1 Internal control genes evaluated in this study

Gene	Accession number	Name	Function	Localization
B2M	NM_004048	Beta-2-microglobulin	Beta-chain of major histocompatibility complex class I molecules	15q21–q22
ACTB	NM_001101	Beta-actin	Cytoskeletal structural protein	7p15–p12
GAPDH	NM_002046	Glyceraldehyde-3-phosphate dehydrogenase	Oxidoreductase in glycolysis and gluconeogenesis	12p13
HMBS	NM_000190	Hydroxymethyl-bilane synthase	Heme synthesis, porphyrin metabolism	11q23
HPRT1	NM_000194	Hypoxanthine phosphoribosyl-transferase 1	Purine synthesis in salvage pathway	Xq26
TBP	NM_003194	TATA box binding protein	General RNA polymerase II transcription factor	6q27
UBC	M26880	Ubiquitin C	Protein degradation	12q24
YWHAZ	NM_003406	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide	Signal transduction by binding to phosphorylated serine residues on a variety of signaling molecules	2p25

Table 2 Primers, PCR conditions, and qPCR efficiency

Gene	Primers 5′→3′ (forward, reverse)	Amplicon length (bp)	T_a (°C)	PCR efficiency (%)	Correlation with dilution series (R^2)
B2M	CACCCCCACTGAAAAAGATG	167	60	100.37	0.997
	ATATTAAAAAGCAAGCAAGCAGAA				
ACTB	ATGTGGCCGAGGACTTTGATT	107	60	103.22	0.999
	AGTGGGGTGGCTTTTAGGATG				
GAPDH	TGAACGGGAAGCTCACTGG	307	60	99.59	0.996
	TCCACCACCCTGTTGCTGTA				
HMBS	CTGCAAGCGGGAAAACCCT	148	60	108.20	0.998
	CTCCAGATGCGGGAACTTTCT				
HPRT1	CTGACCTGCTGGATTACA	256	60	101.25	0.998
	GCGACCTTGACCATCTTT				
TBP	GCCTCCCCCACCCCCTTCTTT	106	60	101.35	0.998
	GCCACACCCTGCAACTCAACATCC				
UBC	ATTTGGGTCGCGGTTCTTG	133	60	101.78	0.997
	TGCCTTGACATTCTCGATGGT				
YWHAZ	ACTTTTGGTACATTGTGGCTTCA	94	60	102.67	0.994
	CCGCCAGGACAAACCAGTAT				

qRT-PCR analysis and data processing

qRT-PCR was carried out using the SYBR Premix Ex Taq™ II kit (TaKaRa) on 96-well reaction plates. The reaction mixture, in a total volume of 20 μl, contained 2.0 μl of cDNA, 2 μl of each primer (4 μM each), 10 μl of SYBR Premix Ex Taq™ II (2×), 0.4 μl of ROX reference dye II (50×), and 3.6 μl of RNase/DNase-free sterile water. Each reaction was run in triplicate as was the no-template control. All PCRs were performed by using an ABI Prism 7500 real-time PCR system (Applied Biosystems, Foster City, USA). The thermal cycling conditions comprised an initial denaturation step at 95°C for 30 s and 40 cycles at 95°C for 5 s, and 65°C for 34 s, followed by dissociation stage at 95°C for 15 s, 60°C for 1 min, and 95°C for 15 s.

Three different statistical algorithms including geNorm [6], NormFinder [31], and BestKeeper [32] were used to assess gene stability and rank these reference genes.

Genorm. GeNorm is a visual basic application for Microsoft Excel, which determines the most stable reference genes from a set of tested candidate reference genes in a given cDNA sample panel. It calculates the gene expression stability measure *M* to each gene in a pool of candidate reference genes. The *M* value calculated by geNorm is based on pairwise variation. Stepwise exclusion of the reference gene with the highest *M* value allows ranking of the tested genes according to their expression stability.

Normfinder. NormFinder is also a visual basic applet which identifies the optimal normalization gene among a set of candidates. The algorithm is based on a mathematical model of gene expression that enables estimation not only of the overall variation of the candidate normalization genes but also of the variation between sample subgroups of the sample set. NormFinder provides a stability value for each single gene independently and focuses on the inter- and intra-group expression variations.

Bestkeeper. The BestKeeper program index was created using the geometric mean of each candidate gene's Ct values. Gene expression variation was calculated for all the candidate reference genes based on Ct values and displayed as the standard deviation (SD) and coefficient of variance (CV). The program showed an overall stability in gene expression. Genes with SD > 1 were considered inconsistent.

Results

qRT-PCR analysis

Primer dimer formation and unspecific amplification can falsely increase gene expression levels and must be avoided. In the present study, the specific amplification of candidate genes was confirmed by a single peak in real-time melt curve analysis. A standard curve was generated for each gene, using the 5-fold serial dilutions of pooled cDNA. Amplification efficiencies of these eight genes ranged from 99.59% to 108.20%.

Data analysis by geNorm, normFinder, and bestKeeper

Based one average expression stability (*M* value), the ranking of these eight candidate reference genes was

TBP/HMBS < *GAPDH* < *HPRT1* < *B2M* < *YWHAZ* < *ACTB* < *UBC* [**Fig. 1**(**A**)], from the most stable one (lowest *M* value) to the least stable one (highest *M* value). All eight genes reached high expression stability with low *M* values, below the default limit of $M = 1.5$. GeNorm analysis revealed that the pairwise variation value $V_{(n/n+1)}$, generated by using the top three genes from the panel, was below the default cut-off value of 0.15 [6] [**Fig. 1**(**B**)], indicating that the inclusion of the third reference gene was not necessary and *TBP/HMBS* would be the most stable reference gene set.

NormFinder analysis showed that the most stable expressed reference gene was *TBP*, and these eight genes ranked as follows: *TBP* < *HPRT1* < *YWHAZ* < *HMBS* < *B2M* < *GAPDH* < *ACTB* < *UBC* (**Table 3**). In addition, the ranking of the two least stably expressed genes was consistent between geNorm and NormFinder results.

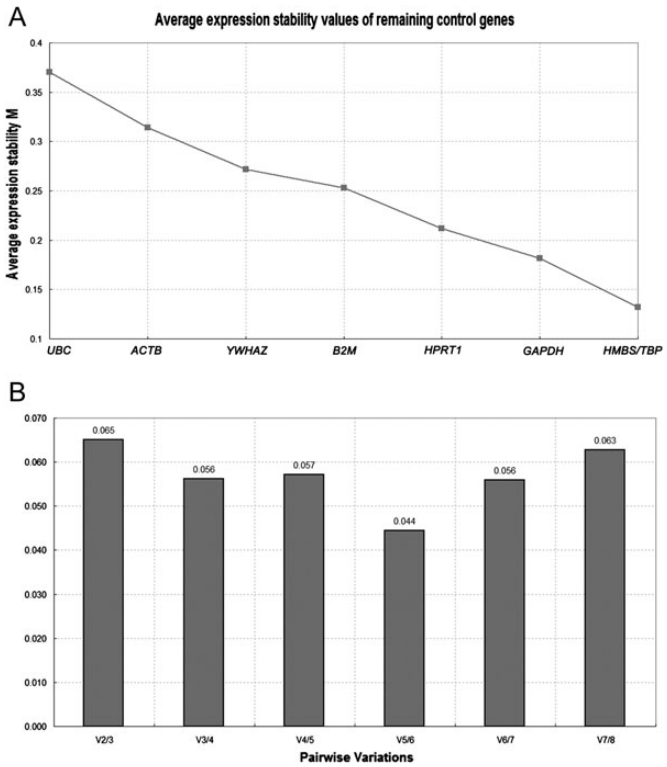

Figure 1 Selection of the most suitable reference genes for samples using geNorm analysis Stepwise exclusion of the least stable genes by calculating the average expression stability (A) was conducted. The value of *M* was calculated for each gene, and the least stable gene with the highest *M* value was automatically excluded for the next round of calculations. The *x*-axis from left to right indicates the ranking of the genes according to their expression stability. Determination of the optimal number of reference genes for normalization (B) was conducted. Pairwise variation analysis between the normalization factors NF_n and NF_{n+1}, to determine the optimal number of reference genes for normalization. The value V2/3 is 0.065, generated using the top three genes from the panel, which is below the default cut-off value of 0.15. This indicates that the inclusion of the third reference gene is not necessary and TBP/HMBS would be the most stable reference gene set.

These eight candidate reference genes showed an SD < 1 in all samples by BestKeeper analysis. Among them, *B2M*, *GAPDH*, and *HMBS* had the lowest Ct value and *ACTB* (SD = 0.42) had the highest Ct value (**Table 4**). However, compared with BestKeeper, geNorm was considered a pairwise variation as the most important factor. Thus, geNorm did not indicate any one gene as the best reference gene but indicated a gene pair. Based on the BestKeeper program, *TBP* was selected as the best reference gene. The ranking of the best stably expressed gene was consistent between NormFinder and BestKeeper.

Paradigm normalization

To illustrate the consequences of choosing an inappropriate reference gene on the calculation of relative mRNA expression level, *HMBS*, one of the two most stable genes in our panel, was normalized to *TBP*, the other most stable gene, or *ACTB*, a gene with irregular unstable expression. Normalization to *TBP* resulted in relatively stable *HMBS* expression in all cell lines and conditions [**Fig. 2**(**A**)]. However, when *HMBS* was normalized to *ACTB*, there was apparently lower expression in BEL-7402 cells treated with TNF-α for 8 and 12 h, and in SMMC-7721 cells treated with TNF-α for 8 h [**Fig. 2**(**B**)].

TBP stability in HCC samples

TBP was considered as the most stable reference gene from a set of tested candidate reference genes by three different statistical algorithms. To further verify its stability, HCC samples were used. Results showed that the expression level of *TBP* was stable in HCC samples (**Fig. 3**).

Discussion

An ideal reference gene should maintain a constant RNA transcription level in different tissues and cell subtypes, and in malignant transformation and tumor progression. It should also be resistant to regulative factors and its

Table 3 Candidate reference genes for normalization listed according to their expression stability calculated by NormFinder

Ranking order	Gene	Stability value
1	*TBP*	0.075
2	*HPRT1*	0.082
3	*YWHAZ*	0.093
4	*HMBS*	0.115
5	*B2M*	0.124
6	*GAPDH*	0.125
7	*ACTB*	0.186
8	*UBC*	0.240

Table 4 Results from Bestkeeper descriptive statistical analysis ($n = 8$)

Gene	Geometric mean (CP)	Arithmetic mean (CP)	min (CP)	max (CP)	SD (± CP)	CV (%CP)
B2M	16.65	16.65	16.67	16.96	0.17	1.01
ACTB	15.97	15.98	15.40	16.71	0.42	2.63
GAPDH	15.76	15.76	15.51	16.01	0.17	1.09
HMBS	24.11	24.11	23.61	24.54	0.26	1.09
HPRT1	20.51	20.51	20.23	21.03	0.22	1.07
TBP	24.33	24.33	23.95	24.80	0.20	0.83
UBC	19.35	19.35	18.75	19.86	0.29	1.47
YWHAZ	19.58	19.58	19.30	19.91	0.17	0.88

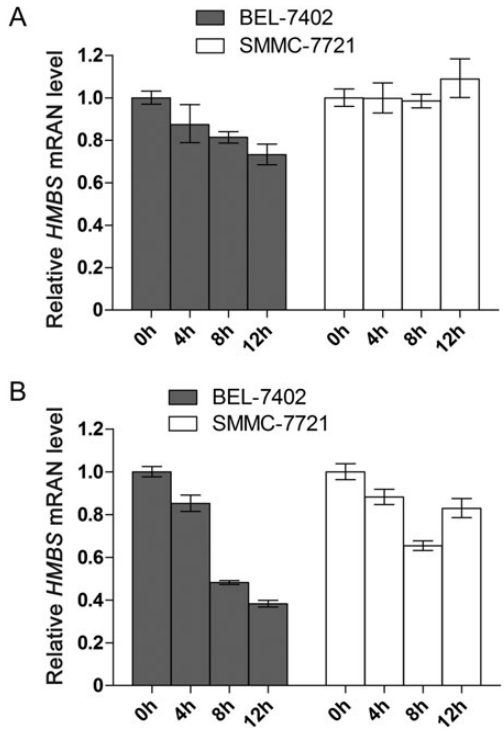

Figure 2 Examples of gene expression calculations Relative mRNA expression calculated by normalization of a stably expressed gene (*HMBS*) to a stable gene (*TBP*, A) and an unstable gene (*ACTB*, B).

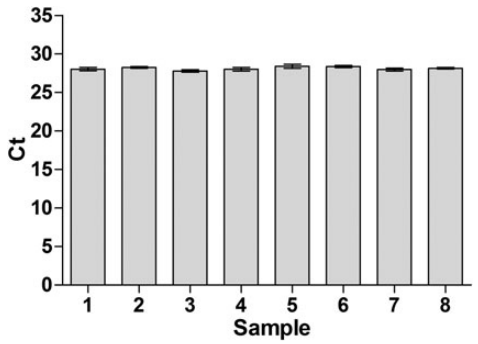

Figure 3 The expression stability of TBP in eight HCC samples Ct values from individual samples were used for evaluating the expression stability of TBP. Data are shown as means ± SD.

expression should not vary by treatment. It has been found that TNF-α plays a tumor-promoting role in cholestatic liver cancer, resulting from chronic liver inflammation in mice lacking drug and phospholipid transporter MDR2 [33]. Treatment with TNF-α-specific-neutralizing antibody during tumor promotion stage results in the apoptosis of transformed hepatocytes and a failure to progress to HCC, suggesting TNF-α plays an important role in the promotion phase of HCC [34].

GAPDH and *ACTB*, two of the most common internal reference genes, are regulated during HCC carcinogenesis and progression, suggesting the involvement in hepatocarcinogenesis [3]. Specifically, *GAPDH* is pathologically implicated in apoptosis and neurodegenerative disease [23], and its mRNA levels are highly heterogeneous even in cellular subpopulations of the same pathological origin [25]. In bladder cancer, it has been reported that *GAPDH*, *G6PD*, and *HMBS* are significantly changed between malignant and non-malignant tissues [29]. Similarly, in adenocarcinomas of colon, the expression of *RPLP0*, *RPS14*, and *GAPDH* varied between primary tumors and corresponding resection margins [35]. Furthermore, in prostate cancer, *ACTB*, *RPL13A*, and *HMBS* showed significant differences between cancer and non-cancerous tissues [7]. Taken together, reference genes whose products have basic functions in cellular metabolisms are possibly differentially expressed between tumor and non-tumor tissues.

To the best of our knowledge, systematic comparison was first performed in the present study, in which some candidate reference genes were analyzed with their potential applications as internal controls in human hepatoma cells treated with TNF-α.

GeNorm analysis demonstrated that two valuable reference genes (*TBP* and *HMBS*) were required for accurate normalization when analyzing gene expression levels in human hepatoma cells treated with TNF-α. Additionally, this study showed that *ACTB*, commonly used as a single internal

control in many studies, was one of the least stably expressed reference genes, suggesting that it was not suitable for normalization in human hepatoma cell system, especially as a single reference gene; otherwise, *ACTB* might result in possible misinterpretation of qRT-PCR data.

When a stable gene, such as *TBP*, was used as a reference gene, the expression of *HMBS* gene was relatively stable in two cell lines [**Fig. 2**(**A**)]. However, when *ACTB*, a reference gene with variable expression, was used, erroneous conclusions appeared in calculation of the relative expression level of target gene [**Fig. 2**(**B**)]. The amount of *HMBS* was markedly lower in both cell lines treated with TNF-α at different time points, actually due to a higher *ACTB* expression level, suggesting that *ACTB* was likely regulated by experimental conditions.

In summary, it was recommended to use a set of *TBP* and *HMBS* for a guarantee of suitable normalization in human hepatoma cells treated with TNF-α. If only one reference gene could be used, *TBP* should be chosen because it was the most stably expressed reference gene in similar types of studies.

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