Original Article

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

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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 many tissues [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 (A260). Purity of total RNA was determined by A260/A280 ratio. RNA samples with A260/A280 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 (HPRT1, 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>
<thead>
<tr>
<th>Gene</th>
<th>Accession number</th>
<th>Name</th>
<th>Function</th>
<th>Localization</th>
</tr>
</thead>
<tbody>
<tr>
<td>B2M</td>
<td>NM_004048</td>
<td>Beta-2-microglobulin</td>
<td>Beta-chain of major histocompatibility complex class I molecules</td>
<td>15q21–q22</td>
</tr>
<tr>
<td>ACTB</td>
<td>NM_001101</td>
<td>Beta-actin</td>
<td>Cytoskeletal structural protein</td>
<td>7p15–p12</td>
</tr>
<tr>
<td>GAPDH</td>
<td>NM_002046</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
<td>Oxidoreductase in glycolysis and gluconeogenesis</td>
<td>12p13</td>
</tr>
<tr>
<td>HMBS</td>
<td>NM_000190</td>
<td>Hydroxymethyl-bilane synthase</td>
<td>Heme synthesis, porphyrin metabolism</td>
<td>11q23</td>
</tr>
<tr>
<td>HPRT1</td>
<td>NM_000194</td>
<td>Hypoxanthine phosphoribosyl-transferase 1</td>
<td>Purine synthesis in salvage pathway</td>
<td>Xq26</td>
</tr>
<tr>
<td>TBP</td>
<td>NM_003194</td>
<td>TATA box binding protein</td>
<td>General RNA polymerase II transcription factor</td>
<td>6q27</td>
</tr>
<tr>
<td>UBC</td>
<td>M26880</td>
<td>Ubiquitin C</td>
<td>Protein degradation</td>
<td>12q24</td>
</tr>
<tr>
<td>YWHAZ</td>
<td>NM_003406</td>
<td>Tyrosine 3-monoxygenase/tryptophan 5-monoxygenase activation protein, zeta polypeptide</td>
<td>Signal transduction by binding to phosphorylated serine residues on a variety of signaling molecules</td>
<td>2p25</td>
</tr>
</tbody>
</table>

Reference gene for qRT-PCR

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 60°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 on 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.

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>
<thead>
<tr>
<th>Ranking order</th>
<th>Gene</th>
<th>Stability value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>TBP</td>
<td>0.075</td>
</tr>
<tr>
<td>2</td>
<td>HPRT1</td>
<td>0.082</td>
</tr>
<tr>
<td>3</td>
<td>YWHAZ</td>
<td>0.093</td>
</tr>
<tr>
<td>4</td>
<td>HMBS</td>
<td>0.115</td>
</tr>
<tr>
<td>5</td>
<td>B2M</td>
<td>0.124</td>
</tr>
<tr>
<td>6</td>
<td>GAPDH</td>
<td>0.125</td>
</tr>
<tr>
<td>7</td>
<td>ACTB</td>
<td>0.186</td>
</tr>
<tr>
<td>8</td>
<td>UBC</td>
<td>0.240</td>
</tr>
</tbody>
</table>

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.
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

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

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.
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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**References**


