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Selection of valid reference genes for expression studies of hepatic cell lines under IFN-α treatment

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ABSTRACT

The proper selection of reference genes to normalize the quantitative real-time PCR (RT-qPCR) results under particular experimental conditions is crucial for validation of the gene quantification data. Herein, using SYBR green RT-qPCR, five reference genes (GAPDH, ACTB, HMBS, HPRT-1 and TBP) were evaluated to determine the most stable reference genes in hepatic cell lines (Huh-7 and $HepG_2$) under IFN-α treatment conditions. Analyses by geNorm program ranked GAPDH and HPRT-1 in Huh-7 and that of ACTB and HMBS in $HepG_2$ cells as the most stable reference genes under IFN-α treatment. While, same reference gene pairs were ranked by NormFinder program in Huh-7 cells, GAPDH was assessed as the most stable gene in $HepG_2$ group by this program, implying the importance of the employed algorithm in comparative interpretation of the data. Finally, cumulative analyses by one-way ANOVA, geNorm and NormFinder programs indicated that use of two reference genes (HMBS and GAPDH) in Huh-7 and three (HMBS, ACTB and GAPDH) in $HepG_2$ cells would greatly improve the normalization of the RT-qPCR data under IFN-α. Data presented in this paper will aid the selection of the most stable reference genes in RT-qPCR studies on evaluation of hepatic viral proteins and IFN pathway.

1. Introduction

Real-time quantitative polymerase chain reaction (RT-qPCR) is a specific, sensitive and broadly used technique in gene expression studies. However, non-specific variations in the quality of starting materials and RNA extraction or transcription efficiencies as well as presence of inhibitors or operator errors may lead to severe quantification inaccuracies and misinterpretation of the results [1]. Application of appropriately selected endogenous reference genes is the common approach to overcome these shortcomings for normalization of data in RT-qPCR analyses [1]. An ideal reference gene is one of which the expression is assumed to be unaffected under normal and various experimental conditions [2]. Although a number of such constitutively expressed reference genes such as glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or hypoxanthine guanine phosphoribosyl transferase-1 (HPRT-1) have been employed in different RT-qPCR studies, recent reports have indicated that the expression levels of commonly used reference genes may depend on the tissue [3] or cell type [4]. In addition, variations in reference genes have been implied during cellular processes, cancer progression [5] and different experimental [6,7] or environmental [8] conditions. Thus, in different experimental conditions, a "suitable reference gene" for RT-qPCR application has been determined empirically [1].

Hepatocellular carcinoma (HCC, the third most common cause of cancer mortality) and viral hepatitis (liver inflammation) are among research areas where the qPCR method is widely being employed to study the molecular mechanisms of the pathogenesis [9]. In this context, previous studies demonstrated that hydroxyl methyl-bilane synthase (HMBS) and GAPDH were the most stable reference genes for RT-qPCR gene expression analyses in HCC and hepatic cell lines including $HepG_2$ and Huh-7 [10]. However, deregulation of these reference genes in HCC arising from hepatitis C virus (HCV) [11] and Hepatitis B virus (HBV) [5] infections was recently reported. In fact, TATA box binding protein (TBP) and HPRT-1 were identified as the most stable reference genes for normalization of RT-qPCR data in HBV-related HCC specimens [5,11,12]. HCV and HBV usually develop persistent and chronic infection which may result to progressive liver diseases such as severe fibrosis, cirrhosis and HCC [9]. Although Interferon-alpha (IFN-α) is one of the most significant therapies approved for treatment of HBV and HCV infections [9,13], it is not effective in a large proportion of the chronic patients [9]. This implies the possible immune evasion mechanisms employed by HBV/HCV through interaction with the IFN-signaling pathways, which has been suggested by a few previous *in vitro* studies [9,14]. In this context, extensive research in HBV/HCV infections involving the effect of

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IFN-α treatment has been emerged to address the altered gene expression in either virus infected or transfected (by viral proteins) hepatic cell lines. Since IFNs have multiple biological effects through induction of a large subset of genes [15], common reference genes might not be stable in the IFN-α treated condition. Thus, selection of the most stable reference genes in IFN-α treated-hepatic cell lines is an important RT-qPCR analysis step in such studies.

In this study, five reference genes (GAPDH, ACTB (β-actin), HMBS, HPRT-1 and TBP) were evaluated to determine the most stable genes for normalization of gene expression results in IFN-α stimulated hepatic cell lines including $HepG_2$ and Huh-7. The geNorm [16] and NormFinder [17] softwares were used for data analysis.

2. Materials and methods

2.1. Cell cultures and IFN-α treatments

Human hepatic cell lines; Huh-7 (Invitrogen, USA) and $HepG_2$ (NCBI, IR) were cultured in DMEM (Biosera, UK) supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Invitrogen, USA) and antibiotics (0.1 mg/mL streptomycin and 100 U/mL penicillin) (Biosera, UK) at 37 °C in a humidified incubator supplied with 5% CO_2 for 24 h. For IFN-α treatments, cells were incubated with media containing different concentrations (125, 250, 500 IU/ml) of recombinant human IFN-α (Peprotech, UK) for 6, 12 and 24 h prior to RNA extraction (Supplementary Table 1).

2.2. Preparation of total cellular RNA and cDNA synthesis

Total RNA was isolated by RNeasy plus Mini Kit (Qiagen, Germany) according to the manufacturer's instructions and was used for first-strand cDNA synthesis (all reagents from Fermentas, France). RNA concentration and the A260/280 absorbance ratios were measured by a picodrop spectrophotometer (Picodrop Limited, UK). The integrity of RNA samples was evaluated by 28S and 18S ribosomal RNA bands on ethidium bromide-stained 1.0% agarose gels. Briefly, a mixture of 2 μg of total RNA and 0.2 μg of Random Hexamer Primers was incubated for 5 min at 65 °C and rapidly cooled on ice. Subsequently, the template–primer mixture was mixed with the reaction buffer (50 mM Tris/HCl, 50 mM KCl, 4 mM $MgCl_2$, 10 mM DTT, pH 8.3), ribolock RNase Inhibitor (20 U), dNTPs mix (1 mM final concentration), and Revert Aid M-MulV Reverse Transcriptase (200 U) in a 20 μL final volume. The reaction was incubated at 25 °C for 10 min, followed by 60 min at 42 °C. Finally, Transcriptase was heat inactivated at 70 °C for 10 min. The synthesized cDNA was stored at −20 °C for further analyses.

2.3. Selection of candidate reference genes and primer design

Five reference genes including ACTB and GAPDH (which are the most frequently used reference genes for normalization of gene expression results) as well as HMBS, HPRT-1 and TBP (recently shown to be suitable reference genes for RT-qPCR normalization in HCC specimens or hepatoma cell lines [10] were considered in the present study (Table 1). The DNA sequences of the selected reference genes were obtained from GenBank database and the corresponding Primer sequences were designed by "Primer 3 software" (Primer 3 software, USA) so that each primer spanned at least one intron to minimize inaccuracies due to genomic DNA contamination in RNA samples [18]. Primers (Table 1) were synthesized at the highest quality and purified by HPLC (eurofins MWG operon, Germany). The specificity of the primers was confirmed by melt curve analysis and evaluation of the RT-qPCR products via electrophoresis in 1.5% agarose gel.

2.4. RT-qPCR with SYBR green

RT-qPCR was performed using Rotor-Gene 6000 (Corbett Robotics, USA) and SYBR® Premix Ex Taq™ II kit (TaKaRa Biotechnology, Japan). Reactions were prepared in a total volume of 10 μl containing: 0.5 μl of template cDNA (corresponding to 50 ng of RNA), 0.5 μl of each primer (5 pmole/μl), 5 μl of SYBR Green master mix and 3.5 μl of RNase/DNase-free sterile water. The PCR was performed by an initial single heating cycle (95 °C for 30 s) followed by 35 amplification cycles (95 °C for 5 s, 64 °C for 26 s). All samples were analyzed in a single analytical run (RT-qPCR reaction) to exclude any potential alteration of the results due to inter-run variations. Finally, for each primer pair, a dissociation protocol with a heat gradient ranging from 50 °C to 99 °C was used to confirm product specificity and absence of the primer dimer formation. To control the DNA contamination in the reagents, No Template Control (NTC) reactions were included in each experiment. Each reaction was performed four times (two technical replicate in duplicate) for each gene and the mean value was used for statistical analyses.

2.5. Assessment of the RT-qPCR efficiencies

Efficiency of RT-qPCR for each separate reaction (each primer pair) was derived from the slopes of the standard curves obtained by four-fold dilution series covering a 4 log dynamic range starting from cDNA samples of untreated (IFN-α) cells. Assuming a value of 2.0 for 100% efficiency, the obtained correlation coefficients (R^2) ranged between 0.990 and 0.998 with the PCR efficiencies from 1.93 to 2.15, respectively (Supplementary Figs. 1 and 2).

Table 1
Description of the selected reference genes and their corresponding designed primers used in RT-qPCR assays.

Symbol	Gene name		GenBank ID	Primer sequences	Amplicon length (bp)
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Glycolytic enzyme	NM 002046	FP: 5′-CTCTGCTCCTCCTGTTCGAC-3′ RP: 5′-TTAAAAGCAGCCCTGGTGAC-3′	144
ACTB	β-Actin	Cytoskeletal structural protein	NM 001101	FP: 5′-GCACAGAGCCTCGCCTT-3′ RP: 5′-CCTTGCACATGCCGGAG-3′	112
HPRT-1	Hypoxanthine phosphoribosyltransferase	Purine synthesis in salvage pathway	NM 000194	FP: 5′-TCCTCCTCCTGAGCAGTCA-3' RP: 5'-ACCCTTTCCAAATCCTCAGC-3'	147
TBP	TATA box binding protein	General RNA polymerase II transcription factor	NM 003194	FP: 5-′TTCGGAGAGTTCTGGGATTGTA-3′ RP: 5′-TGGACTGTTCTTCACTCTTGGC-3′	227
HMBS	Hydroxymethyl-bilane synthase	Heme synthesis, porphyrin metabolism	NM 000190.3	FP: 5′-AGCCTACTTTCCAAGCGGAG-3′ RP: 5′-GTACCCACGCGAATCACTCT-3′	96

2.6. Statistical analyses

Results of each experiment were normalized to the average level of Ct value of the respective control group using the relationship "$E^{-\Delta Ct}$" (Supplementary Tables 2 and 3). One-way ANOVA (two pair-wise comparisons analysis) using Graph-Pad Prism 4 software (Graph-Pad software, USA) was used to determine the significant difference in the mean level for each transcript ($P < 0.05$). The data presented as mean ± SD of multiple measurements.

2.7. Analyses of expression stabilities using geNorm and NormFinder

The expression stabilities were analyzed by both geNorm version 3.5 and NormFinder programs. GeNorm calculates the gene stability measure (M) of each reference genes to provide the average pairwise variation of that gene compared with all other examined genes. Besides, geNorm was used to calculate the Pair-wise variation ($Vn/Vn+1$) between the 2 sequential normalization factors (NFn and NF$n+1$) [16] to evaluate the optimum number of reference genes. The NormFinder algorithm estimates both the overall expression variation of the reference genes and the variation among subgroups of the sample set [17]. All the analyses were performed separately for three sample groups: Huh-7, $HepG_2$ and total samples (sum of the Huh-7 and $HepG_2$ samples together).

3. Results

3.1. Analyses of the RNA quality and primer specificity

The mean value of A260/280 of the isolated RNA samples was 2.01 ± 0.045 indicating the required purity. Evaluation of the 28S and 18S ribosomal RNA bands by agarose gel electrophoresis indicated ratios greater than 1:5 (28S/18S) pointing to proper RNA integrities (data not shown). Appearance of single peaks (with no indication of primer dimers) in melt curves of the RT-qPCR products (Supplementary Fig. 3) and single bands of expected size in agarose gel suggested the specificity of the designed primers.

3.2. Expression profiles of candidate reference genes

The distribution of the Ct values of each reference gene under different experimental conditions was displayed by Box and Whiskers plots (Fig. 1A and B). Selected reference genes displayed a variety of expression levels with Ct values ranging from 12.68 to 20.83 for Huh-7 groups (Fig. 1A) and from 13.45 to 21.50 for $HepG_2$ groups (Fig. 1B). TBP with a mean (±SD) Ct value of 20.83 ± 0.92 in Huh-7 group and 21.50 ± 1.25 in $HepG_2$ group was the least abundant gene in all investigated samples. In contrast, GAPDH was the most abundant gene with mean (±SD) Ct values of 12.68 ± 0.49 and 13.45 ± 0.84 in Huh-7 and $HepG_2$ cells, respectively (Fig. 1A and B). The expression levels of reference genes under different experimental conditions relative to the control group (untreated cells) are demonstrated in Supplementary TableS 2 and 3. As shown in Table 2, while average expression values for IFN-α treated $HepG_2$ cells were significantly different for all evaluated reference genes ($P < 0.05$), in Huh-7 group, the expression levels were significantly different only for ACTB, TBP and HPRT-1 ($P < 0.05$). In contrast, both GAPDH and HMBS demonstrated statistically equal expression levels ($P > 0.05$) under different IFN-α treatments in Huh-7 group (Table 2).

3.3. Expression stability of reference genes

In the geNorm program the average gene stability measurements (M-values) are calculated by stepwise exclusion of the least stable gene and lower M values indicate higher stabilities. Using this program, all five reference genes were ranked according to their M-values in different sample groups (Fig. 2). As shown in Fig. 2A the most reliable reference genes for normalizing the RT-qPCR data in total samples (in both the Huh-7 and $HepG_2$ groups) were the combination of GAPDH and HPRT-1. Similarly, M-values of all five reference genes were also analyzed separately in Huh-7 and $HepG_2$ groups. In Huh-7 group (similar to total sample group) GAPDH and HPRT-1 were ranked as the most stable and ACTB as the least stable reference genes (Fig. 2B). In contrast, ACTB and HMBS were found as the most and TBP as the least stable genes in $HepG_2$ group (Fig. 2C). In addition, NormFinder program was applied to the same data sets to calculate the stability values. Similar to geNorm program, lower values in NormFinder indicate higher expression stability of the genes. The results of NormFinder for Huh-7 and both cell line groups were exactly the same as the geNorm results in the ranking order of the reference genes (Table 3). In NormFinder, TBP was identified as the least stable gene in $HepG_2$ group. However, in contrast to geNorm results, GAPDH was assessed as the most stable gene in $HepG_2$ group by NormFinder (Table 3).

3.4. The optimal number of reference genes for normalization of the data

The optimal number of reference genes required for a valid normalization of the RT-qPCR data was also evaluated by geNorm. A large variation in the normalized results (by including a second reference gene) means that the included gene has a significant effect and should preferably be included for a more reliable

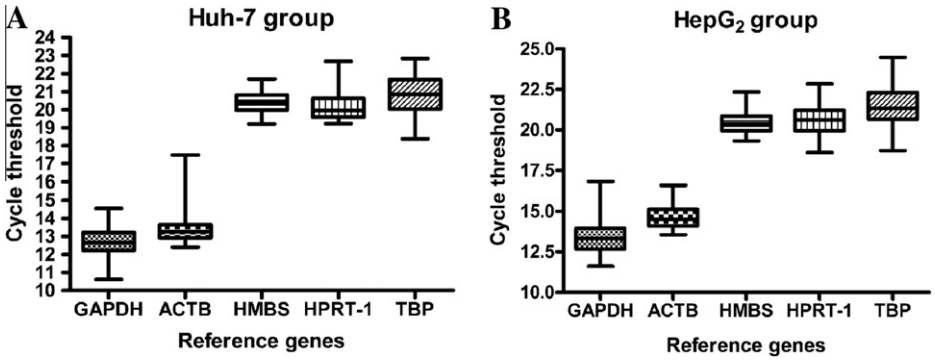

Fig. 1. RT-qPCR cycle threshold values of the selected five reference genes in different conditions. Expression data displayed as cycle threshold (Ct) values in (A) Huh-7 and (B) $HepG_2$ cells grown on different IFN-α concentration (125, 250, 500, 1000 IU/ml) and time incubation (6, 12, 24 h). Expression levels are shown as median (lines), 25th percentile to the 75th percentile (boxes), and ranges (whiskers) for 10 different culture conditions ($n = 40$).

Table 2
P values for the five reference genes following statistical analysis in HepG2 and Huh-7 cell lines.

Reference gene	*P* value	
	$HepG_2$	Huh-7
GAPDH	0.0048	0.2488
ACTB	0.0041	<0.0001
HMBS	0.0307	0.0532
HPRT-1	0.0008	0.0182
TBP	<0.0001	0.0002

Pair-wise comparisons across the different IFN-α concentrations/incubation periods treatments were done in Huh-7 and HepG2 cells by one-way ANOVA test.

normalization factor (NF) calculation [16]. According to a previous study [19], we considered the suggested value of 0.20 for geNorm program as the threshold below in which the inclusion of additional reference genes was not required for further validation of the RT-qPCR data. As shown in Fig. 2D, in both Huh-7 and total sample groups, the pairwise variation (V2/3) between the NF2 (including the two most stable reference genes) and the NF3 (including the three most stable reference genes) were already below the threshold. Although the inclusion of the third reference gene significantly improved the pairwise variation considering all sample groups, application of two reference genes (GAPDH and HPRT-1) would be adequate for normalization of the data in this group. On the contrary, in $HepG_2$ group, application of three reference genes as internal controls could greatly reduce the variability and improve the normalization of the RT-qPCR data (Fig. 2D). Since application of more than three optimal reference genes would diminished the accuracy, therefore employing the three best ranked reference genes (ACTB, HMBS and GAPDH) in this group would be sufficient for comparative gene expression studies in $HepG_2$ cell lines under IFN-α treatment (Fig. 2D).

4. Discussion

Validation of expression stabilities of employed reference genes for normalization of RT-qPCR results under particular experimental condition is essential to obtain accurate data [1]. Prior studies reported stability of a few selected reference genes in relation to HCV or HBV induced HCC in hepatic cell lines [5,10–12]. The present study is the first to assess the stability of reference genes under IFN-α treatment in hepatic cell lines. We have used a range of IFN-α concentrations similar to previously published *in vitro* studies addressing the effect of IFN-α treatment on gene expression in cells [20–22]. The stabilities of five selected reference genes (ACTB, GAPDH, HMBS, HPRT-1 and TBP) were compared using both geNorm and NormFinder programs (Fig. 2A–C) (Table 3). The results in both Huh-7 and in all the groups demonstrate consistent reference gene ranking in both programs. In contrast to Huh-7 and the total sample groups, the results calculated by geNorm and NormFinder for $HepG_2$ groups were not consistent with each other (Fig. 2A–C) (Table 3). Such discrepancies have been reported in few recent studies [8,19] and perhaps are the result of different mathematical models employed by these two programs (geNorm and NormFinder). In fact, geNorm selects the gene pairs with similar expression patterns while NormFinder ranks reference genes according to their inter- and intra-group variations [16,17]. This finding is important since it implies the significance of the use of algorithm for comparative analyses of gene expression stabilities.

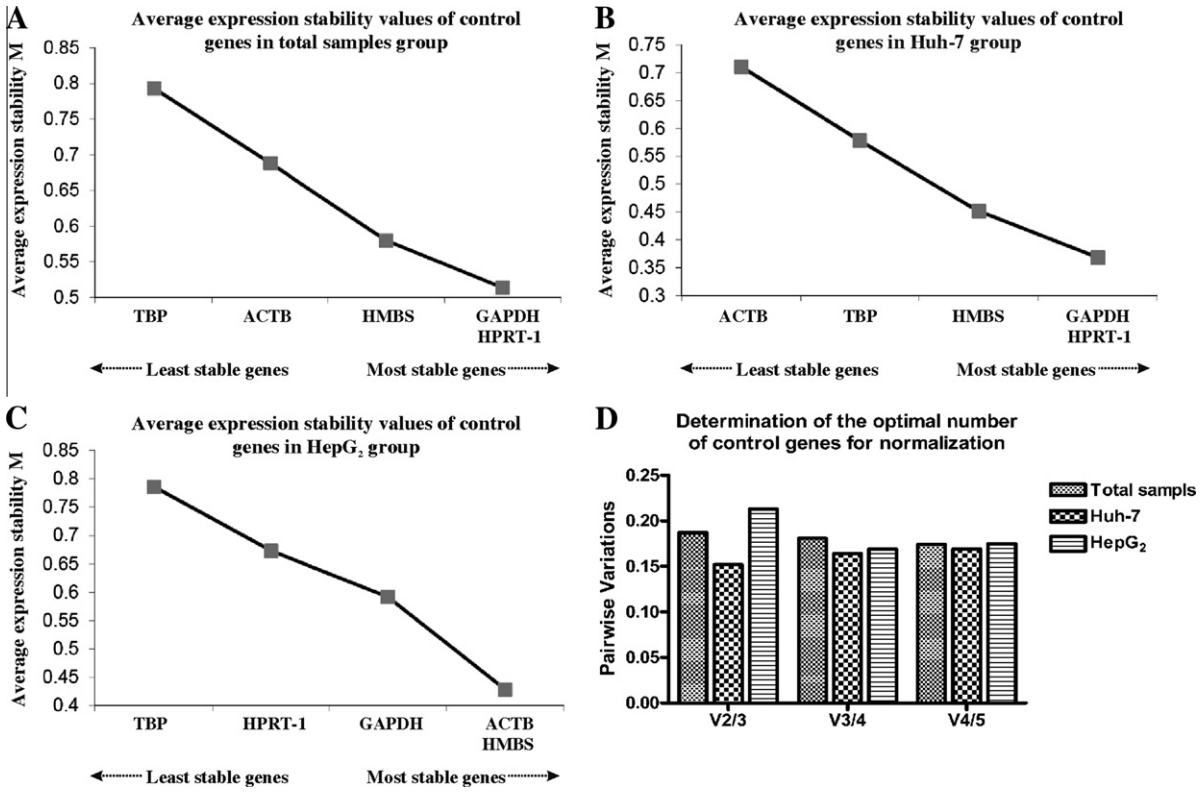

Fig. 2. Expression stability values (*M*) and pair-wise variation (*V*) analyses of the candidate reference genes calculated by geNorm. Expression stability was plotted in (A) Total samples, (B) Huh-7 and (C) $HepG_2$ groups. The most stable reference genes were measured during stepwise exclusion of the least stable reference genes. A lower M value indicates more stable expression. (D) To determine the optimal number of reference genes, pairwise variation was estimated by comparing $Vn/n+1$ in total samples, Huh-7 and $HepG_2$ groups. 0.20 was set as the cut-off value. There is no need to include more than two reference genes for Huh-7 cells while inclusion of a third reference gene is required for $HepG_2$ cells.

Table 3
Ranking of the candidate reference genes based on the evaluation of their expression stability by NormFinder in Huh-7, HepG2 and total samples groups.

Gene name	[a]Gene stability values		
	Total samples	Huh-7 group	HepG2 group
HPRT-1	0.225 (1)	0.156 (1)	0.295 (2)
GAPDH	0.236 (2)	0.220 (2)	0.283 (1)
HMBS	0.268 (3)	0.221 (3)	0.341 (3)
ACTB	0.541 (4)	0.566 (5)	0.412 (4)
TBP	0.578 (5)	0.466 (4)	0.584 (5)

[a] Genes with lower stability values have higher expression stability. Numbers in brackets show the ranking of the stability in the three groups.

Therefore, to prevent misleading conclusions in comparative studies, only data analyzed by the same program in different reports should be compared.

As shown in Table 2, GAPDH, HMBS, HPRT-1 in Huh-7 study group and HMBS, ACTB and GAPDH in $HepG_2$ study group showed the highest stability. TBP was previously suggested as one of the most stable reference genes in HBV or HCV induced HCC or hepatic cell lines [5,10,12]. Contrary to the previous reports, in our study, TBP was ranked as one of the least stable genes in all three examined groups (Huh-7, $HepG_2$ and total samples groups) (Fig. 2A–C) (Tables 2 and 3). The main reason for the different TBP stability in hepatic cell lines could be the IFN-α treatment of hepatic cell lines. This observation further indicated the importance of the selection of the most stable reference genes for validation of RT-qPCR results in different experimental conditions (even for the same employed cell lines). HPRT-1 is another reference gene recommended in gene profiling studies of cancers like HBV related HCC [5]. We have found that HPRT-1 is one of the least affected genes in IFN-α treated Huh-7 cells (Fig. 2B) (Table 2 and Table 3). HPRT-1 has been reported as a stable reference gene in cells under BDNF (Brain-derived neurotrophic factor) treatment in hippocampal neurons [23] and curcumin or diacerein treatments in C-28/I2 chondrocytes [24]. Our results are in agreement with these reports indicating HPRT-1 as a reliable reference gene. GAPDH is one of the most widely used reference genes for gene expression studies in various human tissues [3]. Furthermore, GAPDH is shown to be a stable reference gene in treatment of C-28/I2 chondrocytes with IL-1ß or glucosamine [24,25] and in liver cancer cell lines including Huh-7 and $HepG_2$ [10]. Although lack of GAPDH stability in some experimental conditions has been recently reported [3] our data, in accordance with most of previous reports, indicate GAPDH as a stable reference gene in Huh-7 and $HepG_2$ cells under IFN-α treatment (Tables 2 and 3).

In summary, to our best of knowledge, present study is the first report on evaluation of reference genes in IFN-α treated hepatic cell lines. Analyses by geNorm and NormFinder programs indicate that the use of two reference genes (GAPDH and HMBS) in Huh7 and three (ATCB, HMBS and GAPDH) in $HepG_2$ cells will validate RT-qPCR data of comparative gene expression studies of IFN-α treated hepatic cell lines. TBP was found the least stable reference gene in all IFN-α treated groups. Data accumulated in this article will provide further knowledge for selection of the most stable reference genes to normalize RT-qPCR data evaluating gene profiling of IFN-α treated hepatic cell lines as well as the molecular mechanisms of hepatic viral proteins expression and interactions with IFN pathway.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.09.009.

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