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Selection of reference genes for RT-qPCR analysis in tumor tissues from male hepatocellular carcinoma patients with hepatitis B infection and cirrhosis

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Abstract.

BACKGROUND: Reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) has been widely used to quantify relative gene expression because of the high specificity, sensitivity and accuracy of this technique. However, its reliability is strongly depends on the expression stability of reference gene used for data normalization. Therefore, identification of reliable and condition specific reference genes is critical for the success of RT-qPCR.

OBJECTIVE: Hepatitis B virus (HBV) infection, male gender and the presence of cirrhosis are widely recognized as the leading independent risk factors for the development of hepatocellular carcinoma (HCC). This study aimed to select reliable reference gene for RT-qPCR analysis in HCC patients with all of those risk factors.

METHODS: Six candidate reference genes were analyzed in 33 paired tumor and non-tumor tissues from untreated HCC patients. The genes expression stabilities were assessed by geNorm and NormFinder.

RESULTS: C-terminal binding protein 1(CTBP1) was the most stable gene among the 6 candidate genes evaluated by both geNorm and NormFinder. The expression stability values were 0.08 for CTBP1 and UBC, 0.09 for HPRT1, 0.12 for HMBS, 0.14 for GAPDH and 0.18 for 18S with geNorm analysis. The stability values suggested by NormFinder software were CTBP1: 0.044, UBC: 0.063, HMBS: 0.072, HPRT1: 0.072, GAPDH: 0.098 and 18S rRNA: 0.161.

CONCLUSION: This is the first systematic analysis which suggested CTBP1 as the highest expression-stable gene in human male HBV infection related-HCC with cirrhosis. We recommend CTBP1 as the best candidate reference gene when RT-qPCR was used to determine gene(s) expression in HCC. This may facilitate the relevant HBV related HCC studies in the future.

Keywords: Hepatocellular carcinoma, hepatitis B virus, liver cirrhosis, male gender, C-terminal binding protein 1, reference gene, RT-qPCR

Abbreviation

Reverse transcription quantitative real-time polymerase chain reaction	RT-qPCR
Hepatitis B Virus	HBV
Hepatocellular carcinoma	HCC
C-terminal binding protein 1	CTBP1
Galyceraldehyde-3-phosphate dehydrogenase	GAPDH

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1. Introduction

The technique of quantitative real-time reverse transcription polymerase chain reaction (RT-qPCR) has provided a powerful tool for quantifying gene expression. RT-qPCR is characterized with both higher sensitivity and specificity, as compared with traditional mRNA analysis [1,2]. However, the accuracy of RT-qPCR is influenced by a number of variables, such as RNA stability, quantity, purity, enzymatic efficiency in cDNA synthesis and PCR amplification [3]. Thus, it is extremely important to use highly reliable reference gene to normalize the experiments. The most commonly used measure for normalization is to use one or several housekeeping genes as reference gene in experiment. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) has been widely used as internal control for RT-qPCR analysis in human hepatocellular carcinoma (HCC) [4]. However, several recent lines of evidences implicate GAPDH has also been involved in some physiological functions, such as tRNA transport [5], translational control [6], binding with viral RNAs [7]. Moreover, increased GAPDH expression has been reported in breast cancer, renal cell carcinoma, lung cancer, prostate carcinoma and HCC [8–11]. Separate studies even suggested that GAPDH expression was correlated with chemotherapy-induced DNA damage response in vitro [12–14]. A study even suggested the heavily regulation of GAPDH during HCC carcinogenesis and progression in patients with HBV related HCC [15], as it can affect the proliferative potential of cancer cells in HCC [16]. HBV infection, the presence of cirrhosis and male gender are repeatedly recognized as the independent risk factors for the development of HCC [17–19]. Therefore, it is worthwhile to select an appropriate reference gene for RT-qPCR analysis in HCC patients with those high risk factors. In present study, 6 commonly used reference genes (CTBP1, UBC, HPRT1, HMBS, GAPDH and 18s RNA) were investigated by RT-qPCR in 33 paired tumor and non-tumor tissues from male un-treated HCC patients with evidence of chronic HBV infection and cirrhosis. The expression stability of these genes was assessed by both geNorm and NormFinder softwares.

2. Materials and methods

2.1. Patients information and sample collection

From January to April, 2013, we have collected 33 paired tumor and adjuvant non-tumor tissues from HCC patients in the Henan Cancer Hospital (Zhengzhou, China). The selected HCC patients fulfilled the following entire criterion: seropositive for HBV surface antigen (HBsAg) or HBV DNA positive in tumor tissues, receiving no chemotherapy before surgery, male gender and with liver cirrhosis. Liver cirrhosis and HCC were diagnosed by the experienced pathologists.

The paired tumor and non-tumor tissues were collected when patients underwent hepatectomy. Then specimens were immediately snap-frozen in liquid nitrogen. The study protocol was approved by the Ethic Committee of the Henan Cancer Hospital.

2.2. Analysis of mRNA expression by RT-qPCR

The tissue specimens were ground in liquid nitrogen and homogenized in Trizol (Invitrogen, Carlsbad, CA) by mortar. Total RNA was extracted according to the manufacturer's instruction. Genomic DNA contamination was removed by the on-column digestion with the RNase-free DNase (TaKaRa Biotechnology, Japan). The concentrations of the isolated total RNA were measured at 260 nm to 280 nm with NanoDrop ND-2000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The threshold inclusion values for the RNA samples were > 1.90 for the A260/A280 ratio and 28S/18S ratio $\geqslant 1.7$. The integrity of RNA samples was confirmed by electrophoresis on a 1% agarose gel.

First-strand cDNA was synthesized using Random Primer according to the manufacturer's instructions (Revert Aid First strand cDNA Synthesis Kit, Fermentas, Lithuania). Primers for RT-qPCR assays of CTBP1, HPRT1, HMBS, 18S rRNA, GAPDH and UBC were designed using Primer Premier 5.0 (Table 1). The Roche LightCycler 480 detection system (Roche Diagnostics, Germany) was used for real-time PCR instruments. Reactions were prepared in a total volume of 20 μL containing 10 μL of SYBR Green master mix (Roche Diagnostics, Germany), 0.5 μL of each 10 μM primer (500 nM), 1 μL cDNA, and 8 μL nuclease-free sterile water. All standards and samples were run in triplicate on 96-well reaction plates. The cycle conditions were set as follows: start with 10 min template denaturation at 95°C, 40 cycles of denaturation at 95°C for 30 s, and elongation at 72°C for 30 s. This cycle was followed by a melting curve analysis, baseline and cycle threshold values (Ct values) were automatically determined for all plates using Roche LightCycler 480 Software. Ct values difference be-

Table 1
Six candidate reference genes

Gene symbol	Gene name	Primer sequence(5' → 3')	Accession no.	Amplicon size (bp)
HMBS	hydroxymethylbilane synthase	F1: CCCTGGAGAAGAATGAAGTGGA R1: TTTGGGTGAAAGACAACAGCATC	NM_000190.3	133bp
HPRT1	hypoxanthine phosphoribosyltransferase 1	F1: TTGCTGACCTGCTGGATTACAT R1: CTTGCGACCTTGACCATCTTTG	NM_000194.2	262bp
CTBP1	C-terminal Binding Protein 1	F1: TTCACCGTCAAGCAGATGAGAC R1: CTGGCTAAAGCTGAAGGGTTCC	NM_001012614.1	156bp
18S rRNA	18S ribosomal RNA	F1: GGTGGAGCGATTTGTCTGGTTA R1: CGGACATCTAAGGGCATCACAG	NR_003286.2	167bp
UBC	Ubiquitin C	F1: CCCTTCTCGGCGATTCTG R1: CATTGTCAAGTGACGATCACAGC	NM_021009.4	155bp
GAPDH	glyceraldehyde-3-phosphate dehydrogenase	F:TGTTCCAATATGATTCCACCC R:CTTCTCCATGGTGCGTGAAGA	NM_002046.3	185bp

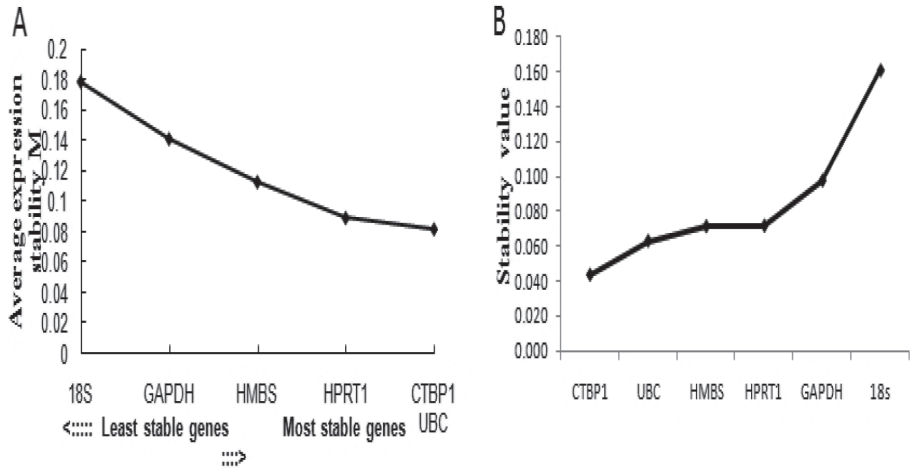

Fig. 1. Relative expression stability of 6 candidate reference genes evaluated by geNorm software (A) and NormFinder program (B). A. Low 'M value' correlates to higher gene expression stability. M values of CTBP1 and UBC, HPRT1, HMBS, GAPDH and 18S rRNA are as follows: The expression stability values were 0.08, 0.09, 0.12, 0.14 and 0.18 geNorm analysis; B. Low 'Stability Value' correlates to higher gene expression stability. Stability values of CTBP1, UBC, HMBS, HPRT1, GAPDH and 18S rRNA was as follows: 0.044, 0.063, 0.072, 0.072, 0.098, 0.161. Both geNorm and Normfinder software showed that CTBP1 was the most stable gene.

tween triplicates no more than 1 was considered acceptable and used for average Ct values calculation. For data set, those genes that had greater than twofold increase ($2^{-\Delta Ct} > 2$) or decrease ($2^{-\Delta Ct} < 0.5$) of expression were considered for differential expression, or will be defined as equal expression ($0.5 \leqslant 2^{-\Delta Ct} \leqslant 2$), as previously reported [20].

2.3. Candidate reference genes selection by geNorm and NormFinder

Data were analyzed by two broadestly used softwares geNorm (version 3.5) [3] and NormFinder [21]. GeNorm calculates an expression stability value (M value) for each candidate gene based on pairwise comparisons of variability. The M value calculated for each gene is used to rank genes from least to most stable. The M-value of 1.5 was set as cut-off value of an endogenous control gene, as the authors suggested [22]. The NormFinder software is available at http://www.mdl.dk. It can calculate the stability values of each candidate reference genes for normalization. A low stability value means a low combined intra- and inter-group variation proves high expression stability [21]. Using these two softwares, the most stable gene is calculated.

2.4. Statistical analyses

Student's t test or wilcoxon rank test was used for statistical analyses with SAS software (version 9.1, SAS Institute, Inc., 2004), $P \leqslant 0.05$ was considered statistically significant. Results were the means ± SEM of 3 separate experiments.

3. Results

The Candidate Gene Expression Profile in Paired Tumor and Non Tumor Tissues Analyzed by geNorm.

The expression stabilities (M value) of overall candidate reference genes ranged from 0.08 to 0.18. High M values indicated the highly variable and less stably expression of the genes. On the other hand, genes with low M values suggested the stable expression and low variability. Based on data obtained, the 18S was the least stable reference gene with M value of 0.18, GAPDH was better than 18S, but CTBP1 and UBC were the two most stable genes with the M value of 0.08 (Fig. 1A).

The Candidate Gene Expression Profile in Paired Tumor and Non Tumor Tissues Analyzed by NormFinder.

Meanwhile, the expression stabilities of overall candidate reference genes were analyzed by NormFinder. Again, CTBP1 was also the most stable gene. GAPDH, UBC, HMBS and the 18S was the subsequently less stable reference gene as the above demonstrated (Fig. 1B).

Therefore, the two software programs together suggest CTBP1 as the most expression-stable gene in HBV-related HCC, either in tumorous or in non-tumorous tissues, or both. In contrast, the commonly used GAPDH is not as stable as CTBP and may not be suitable to be used as the reference gene for RT-qPCR analysis when quantificationally measuring a gene's expression in HCC tissues.

4. Dissusion

About 600 000 people die every year due to the acute or chronic consequences of hepatitis B, including liver failure, cirrhosis and HCC [25]. Presently about 80% of HCC patients have chronic HBV infection in China. Cirrhosis due to chronic hepatitis B is also one of the leading risk factor for HCC. The other upcoming risk factors include male gender, old age, obesity, diabetes and related nonalcoholic fatty liver disease [17–19]. Although the risk factors are clear, the underlying mechanisms remain largely elusive. The change of genes expression profiles in HCC may provide clues for the hunting of key genetic event relevant to HCC development. RT-qPCR has been widely used to quantify the relative gene expression. However, the most challenging part of real-time PCR data analysis is identifying suitable housekeeping genes stably expressed to normalize among all of the samples. GAPDH was most frequently used as a reference gene for RT-qPCR analysis in HCC [4]. However, several recent studies supporting revealed the increased GAPDH expression in both mRNA and protein levels in HCC [15,23,24]. So selection of suitable reference gene to instead of GAPDH for RT-qPCR data analysis in HCC patients is in an urgent need. Cirrhosis due to chronic hepatitis B and male gender are two well known leading risk factors for HCC. Therefore we investigated 6 generally used housekeeping genes as candidate genes in tumor tissues from 33 male HCC patients with evidence of chronic HBV infection and the presence of cirrhosis.

The geNorm and NormFinder are two well-known softwares, each with different strategies to evaluate reference genes [21,22]. To ensure getting reliable result, both softwares were employed in this study. The raw Ct values were analyzed by both software in parallel.

The gene expression stability spectrums were somehow different when evaluated by the two analysis methods. Using geNorm analysis the expression stabilities is evaluated by M value. The M value ranged from 0.08 to 0.18. CTBP1 and UBC ranked as the best gene, and then it was HPRT1, HMBS, GAPDH, and 18S. Interestingly, CTBP1 was the most stable gene evaluated by the NormFinder software as well, UBC, HMBS, HPRT1, GAPDH and the 18S subsequently was the less stable reference gene. Therefore, based on the both software programs CTBP1 exhibited the highest expression stability for RT-qPCR analysis in untreated HCC tumorous and non-tumorous tissues for male patients with HBV infection and cirrhosis. In contrast, GAPDH was only better than 18S, but worse than CTBP1, HPRT1 and UBC. As to our knowledge, it is the first systemic study of reference genes in cohort composed of male HCC patients with underlying HBV infection and cirrhosis.

In conclusion, the current study reveals CTBP1 as the most suitable and reliable reference genes for RT-qPCR data analysis in HCC patients with underlying HBV infection and cirrhosis. This result may facilitate HBV related HCC study in the future.

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