

Reference gene expression in human osteosarcoma cell lines treated by EGB and CTX

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Abstract: The selection of stably expressed reference genes is crucial for quantitative real-time polymerase chain reaction (qRT-PCR) assay when assessing osteosarcoma treated with cyclophosphamide (CTX) and *Ginkgo biloba* L. extract (EGB). We aimed to identify the reference genes that are stably expressed in human osteosarcoma cell lines after the above treatments. We used qRT-PCR assay to determine the expression levels of *18S*, *ACTB*, *ALAS1*, *GAPDH*, *TBP*, *HPRT1*, *RPL29*, *HMBS*, *PPIA*, *PUM1*, *GUSB*, and *B2M* in the cell lines MG-63, Saos-2, and U2OS, which were treated with CTX or EGB or both. We analyzed the expression stability of these genes using geNorm, NormFinder, and BestKeeper. The optimal reference gene combinations were *18S* + *PPIA* + *HPRT1* in the total group, *HMBS* + *ALAS1* + *18S* in the EGB-treated group, *PPIA* + *RPL29* in the EGB + CTX-treated group, *PPIA* + *HPRT1* in the MG-63 group, *18S* + *PPIA* in the Saos-2 group, and *HPRT1* + *ALAS1* + *GAPDH* in the U2OS group. We conclude that no single reference gene was most stably expressed in all three treated cell lines. Our findings provide a suitable approach through which qRT-PCR can be applied to investigate the pharmacological effects and the molecular mechanisms of CTX and EGB on human osteosarcoma.

Key words: Reverse transcription quantitative polymerase chain reaction, reference gene, human osteosarcoma, *Ginkgo biloba* L. extract, cyclophosphamide

1. Introduction

The quantitative real-time polymerase chain reaction (qRT-PCR) assay is frequently used to study gene expression and is currently considered the gold standard. A stably expressed endogenous gene is often used as an internal control and reference to calculate the relative levels of target genes.

It is important to appropriately select reference genes. The ideal reference genes should be stably expressed under nearly all experimental conditions (Radonic et al., 2004; Derveaux et al., 2010). Cellular maintenance genes, such as glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), β -actin (*ACTB*), and ribosomal RNA (*18S* rRNA), are frequently selected as reference genes. However, several studies have established a variation in the expression levels of these commonly used reference genes in different tissues. Moreover, there could be variations in the expression levels of these genes when they are subjected to different treatments in the same tissue (Yang et al., 2014; Ali et al., 2015; Ma et al., 2015; Yu et al., 2015) or across different cell

types (He et al., 2015; Li et al., 2015a, 2015b; Yang et al., 2015). Therefore, it is difficult to identify a reference gene that is universally appropriate.

Osteosarcoma is an aggressive malignant neoplasm that arises from primitive transformed cells of mesenchymal origin. Although different osteosarcoma cell lines share some features of osteoblasts, they differ in many aspects (Grigolo et al., 1998; Pautke et al., 2004). In particular, proliferation kinetics and osteoid production of these osteoblasts varies significantly (Declercq et al., 2004). Cyclophosphamide (CTX) is a chemotherapeutic drug that is frequently used in the treatment of osteosarcoma. *Ginkgo biloba* L. extract (EGB) is a traditional herbal medicine that can remove free radicals. EGB is well known for its antioxidant and antiinflammatory activities (Ren et al., 2013). Previous studies showed that EGB may have antitumor effects that are related to its antioxidant, antiangiogenic, and gene-regulatory actions (Dias et al., 2013; Park et al., 2013; Zhao et al., 2013). Flavonoids and terpenoids are major biologically active components

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of EGB. Ginkgo flavonoids may inhibit the growth and induce the apoptosis of osteosarcoma cells by inhibiting STAT3 and activating caspase-3/9 (Xiong et al., 2016). Furthermore, EGB enhances the chemotherapeutic sensitivity of patients. EGB reverses chemoresistance by suppressing the ERK1/2 pathway in cancer cells. The underlying mechanism may be related to its antioxidative activity (Liu et al., 2015). In clinical practice, the combination of EGB and 5-fluorouracil is superior to 5-fluorouracil alone in treating patients with advanced colorectal cancer (Hauns et al., 2001). EGB enhances the efficacy of CTX, which is used in the treatment of osteosarcoma cells. The mechanism of the combined use of EGB and chemotherapeutic drugs in the treatment of cancer remains unclear. Therefore, we propose that qRT-PCR must be used to investigate the pharmacological effects of CTX and EGB, which are used in combinatorial therapy. However, scientists have not been able to select appropriate reference genes for conducting such studies.

In this study, our aim was to identify the most suitable reference gene or set of genes in human osteosarcoma cell lines. Therefore, we treated the cell lines MG-63, Saos-2, and U2OS with EGB and CTX or both; the resultant gene sequences were analyzed using the geNorm (Ohl et al., 2005; Huan et al., 2012), NormFinder (Andersen et al., 2004), and BestKeeper (Andersen et al., 2004) software packages. We selected the following 12 candidate genes: *ACTB*, *ALAS1*, *GAPDH*, *TBP*, *HPRT1*, *RPL29*, *HMBS*, *PPIA*, *PUM1*, *GUSB*, *B2M*, and *18S rRNA*. These genes are frequently used as endogenous controls in treating human osteosarcoma. A number of these genes were classified as optimal reference genes for other cancer types (Ohl et al., 2005; Huan et al., 2012).

Our findings provide a suitable approach through which qRT-PCR can be applied. Using this approach, we are able to investigate the pharmacological effects and underlying mechanisms through which a combinatorial therapy of CTX and EGB can be applied for human osteosarcoma in future studies.

2. Materials and methods

2.1. Cell culture and treatment

The human osteosarcoma cell lines MG-63, Saos-2, and U2OS were provided by the Research Center of the Second Hospital, Jilin University (Changchun, China). These cells were prepared in McCoy's 5A Medium modified with tricine. The cells were treated with each of the following components: 15% fetal bovine serum (FBS), Minimum Essential Medium with Earle's Balanced Salts (containing 10% FBS and 1% NEAA), and McCoy's 5A Medium modified with tricine (containing 10% FBS). The cell culture was maintained at 37 °C in a humidified atmosphere containing 5% CO₂. These media were supplemented with 100 units of penicillin.

The MG-63, Saos-2, and U2OS cell lines were treated at a confluency of 80%–90% with either EGB (final concentration of EGB was 200 mg/mL) or CTX (final concentration of CTX was 2 mg/mL). Furthermore, the same cell lines were also treated with a combination of EGB (a final concentration of 200 mg/mL) and CTX (a final concentration of 2 mg/mL). Cells that were treated with a vehicle (medium) were used as the control. The cells were collected 48 h after the treatment.

2.2. Isolation of RNA and synthesis of cDNA

Total RNA was extracted from each group using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol. DNase I was used to eliminate genomic DNA, the contaminant. The concentrations of isolated RNA samples were determined using a NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA). cDNA was synthesized using a M-MuLV First Strand cDNA Synthesis Kit (Sangon Company, Shanghai, China) according to the manufacturer's instructions. The total reaction volume was 20 µL, which included 1 µg of total RNA, 10 ng/µL of random primer, and RNase-free distilled water.

2.3. Quantitative real-time PCR

The primers for the 12 genes were designed and are listed in the Table. These primers were synthesized by Sangon Company. qRT-PCR was performed using 2X SG Fast qPCR Master Mix (Sangon Company). The instrument was equipped with a Roche LightCycler 480 detection system (Roche Diagnostics, Germany) according to the manufacturer's instructions. All the samples were run in triplicate, and 2 µL of cDNA was used in each PCR reaction. The program for performing the PCR reaction was set as follows: 55 °C for 5 min, 95 °C for 5 min, 40 cycles of 95 °C for 20 s and 55 °C for 20 s, and 72 °C for 4 min. Thereafter, we ran the program for melting curve analysis. Subsequently, we recorded baseline and cycle threshold values (Cp values). Additionally, the product isolated from qRT-PCR was resolved by performing electrophoresis. To perform electrophoresis, we used 1% agarose gel and verified the specificity of primers. A standard curve was constructed for each primer pair, and the specificity of the resultant product was determined.

The amplification specificity of the primers was confirmed and showed a single bright band with an expected molecular size in each PCR product (Figure 1A) and a single peak in each melting curve (Figure 1B).

The Cp values were used to calculate relative quantities (Q) in terms of the PCR efficiencies of the candidate genes. For this purpose, we used the following equation: $Q = 2^{-\Delta C_p}$ (Livak and Schmittgen, 2001). The expression level of the candidate reference genes was determined using the Cp value, which is inversely proportional to the expression level of the gene. Higher Cp values indicated lower expression levels.

Table. Selected genes and their primers for qRT-PCR.

Symbol	Official full name	Accession number	Primer sequence	Product size (bp)
<i>18S</i>	18S ribosomal RNA	NM_10098.1	F: CGGCTACCACATCCAAGGAA R: GCTGGAATTACCGCGGCT	186
<i>GAPDH</i>	Glyceraldehyde 3-phosphate dehydrogenase	NM_002046.5	F: GACAGTCAGCCGCATCTTCT R: TTAAAAGCAGCCCTGGTGAC	127
<i>B2M</i>	Beta-2-microglobulin	NM_004048.2	F: AGCGTACTCCAAAGATTCAAGTT R: ATGATGCTGCTTACATGTCTCGAT	306
<i>ACTB</i>	Actin, beta	NM_001101.3	F: AGAAAATCTGGCACCACACC R: TAGCACAGCCTGGATAGCAA	173
<i>ALAS1</i>	5'-Aminolevulinate synthase 1	NM_000688.5	F: GGAGCACAGATGAATCAGA R: CCTCCATCGGTTTTCACACT	150
<i>GUSB</i>	Glucuronidase, beta	NM_000181.3	F: AGCCAGTTCCTCATCAATGG R: GGTAGTGGCTGGTACGGA	160
<i>HPRT1</i>	Hypoxanthine phosphoribosyltransferase 1	NM_000194.2	F: GACCAGTCAACAGGGGACAT R: CTGACCAAGGAAAGCAAAG	132
<i>HMBS</i>	Hydroxymethylbilane synthase	NM_000190.3	F: AGTGTGGTGGGAACCAGC R: CAGGATGATGGCACTGAAGTC	144
<i>PPIA</i>	Peptidylprolyl isomerase A	NM_021130.4	F: AGACAAGGTCCCAAAGAC R: ACCACCCTGACACATAAA	118
<i>PUM1</i>	Pumilio RNA-binding family member 1	NM_001020658.1	F: CAGGCTGCCTACCAACTCAT R: GTTCCCGAACCATCTCATTC	217
<i>RPL29</i>	Ribosomal protein L29	NM_000992.2	F: GGCGTTGTTGACCCTATTTC R: GTGTGTGGTGTGGTTCTTGG	120
<i>TBP</i>	TATA box binding protein	NM_003194.4	F: TGCACAGGAGCCAAGAGTGAA R: GTGTGTGGTGTGGTTCTTGG	132

2.4. Calculation of PCR efficiency

cDNA samples were randomly selected and used for preparing 10-fold serial dilutions, which varied from $1 \times$ to $0.001 \times$. The diluted samples were used as templates for performing qRT-PCR. The PCR efficiency (E) was calculated according to the following formula: $E = 10^{-1/\text{slope}}$. In this equation, the slope was extracted from the calibration curve.

2.5. Data analysis

Data from all samples were divided into six groups: 1, the Total group contained data from all three cell lines treated with and without EGB, CTX, or EGB + CTX; 2, the EGB group contained data from three cell lines treated with EGB; 3, the EGB + CTX group contained data from three cell lines treated with EGB and CTX; 4, the MG-63 group contained data from the MG-63 cell line treated with and without EGB, CTX, or EGB + CTX; 5, the Saos-2 group contained data from the Saos-2 cell line treated with and without EGB, CTX, or EGB + CTX; 6, the U2OS

group contained data from the U2OS cell line treated with and without EGB, CTX, or EGB + CTX. To evaluate the expression stability of the selected genes, we used the following three software programs: geNorm (<http://medgen.ugent.be/~jvdesomp/genorm/>), NormFinder (<http://moma.dk/normfinder-software>), and BestKeeper (<http://www.gene-quantification.de/bestkeeper.html>). In the geNorm and NormFinder software programs, the relative quantities (Q) were input ($Q = 2^{-\Delta C_p}$, $\Delta C_p = C_p - \text{lowest } C_p$). In the BestKeeper software program, C_p values were input.

The GeNorm software is designed to identify reference genes for qRT-PCR; in this program, the M-value is used as the defining parameter. Compared to other tested genes, M is the mean pairwise variation for a given gene. We excluded the gene with the highest M value. M was recalculated to select the two most stable genes. The default M value is 1.5. A higher M value denoted that the expressed genes were less stable, and a lower M value

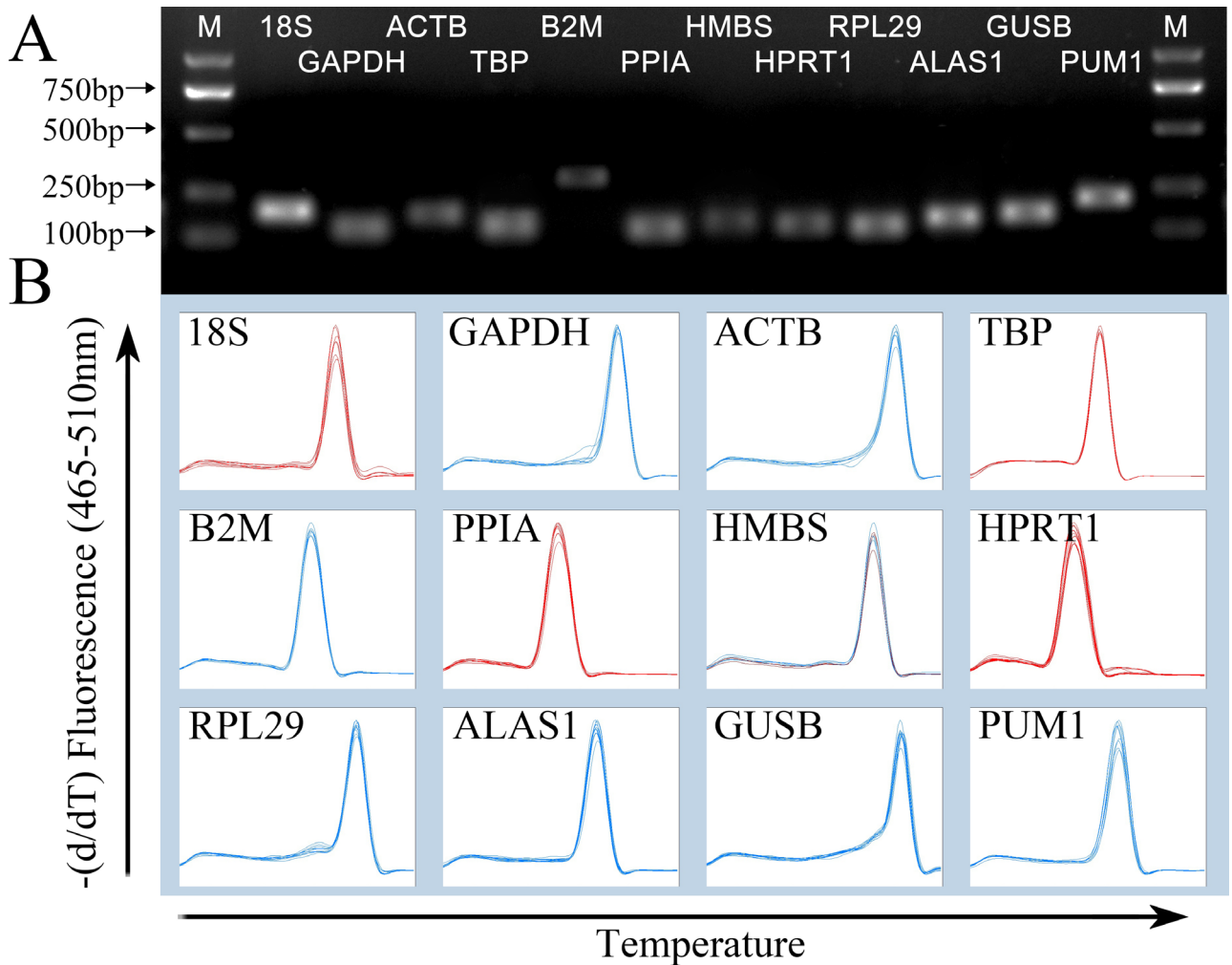


Figure 1. Amplification specificity of primers and amplicon length. qRT-PCR amplification products of 12 reference genes were analyzed using agarose gel electrophoresis (A) and melting curve analysis (B). qRT-PCR products were run using 1% agarose gel. M: DNA standard size markers.

indicated a higher stability of gene expression. If M is >1.5 , the gene is not suitable for use as a reliable reference gene.

GeNorm software can also be used to analyze the pairwise variation value of the normalization factor (V). The default V was 0.15. The value of V_n/V_{n+1} can be used to determine whether adding a new reference gene affects the normalization factor. If the value of V_n/V_{n+1} is >0.15 , it is necessary to use $n + 1$ reference genes as an internal control. If it is <0.15 , then it is not necessary to use new reference genes.

NormFinder software is a tool designed to identify an optimal reference gene among a set of candidates. This program analyzes expression data and ranks the set of candidate genes according to their expression stability. Furthermore, this software considers the gene with the

minimal expression data as the most stable gene. This software was used to compare the stability of inter- and intragroup reference genes, proposing an optimal combination of the two genes.

BestKeeper software evaluates the stability of gene expression based on the standard deviation (SD) and correlation coefficient (R-value). If the SD is >1 , then the gene is unsuitable for use as a stable and reliable reference gene. The remaining genes were ranked according to their R-values. A higher R-value indicates greater stability and reliability of using the gene as the reference. The limitation of the BestKeeper program is that only 10 reference genes can be assessed at one time. Therefore, we removed the two least stably expressed housekeeping genes as indicated by the geNorm program from each group before analysis.

3. Results

3.1. The levels of reference gene expression

Among the six groups, the Cp value of the 12 genes varied from 7.50 to 24.43 (Figure 2). Furthermore, *18S* (RPS18) had the lowest mean Cp values (8.61 ± 0.81), and *PUM1* had the greatest mean Cp values (22.64 ± 0.96).

3.2. Stability and variation analysis of gene expression using GeNorm software

We analyzed the stability of gene expression using GeNorm software. The results are shown in Figure 3A. The candidate genes were sorted according to their expression stabilities in each group. These results indicated that *HPRT1* had the most stable expression for all groups with the exception of the EGB + CTX group.

We analyzed the variation of gene expression using GeNorm software. The results indicated that a combination of five reference genes was optimal in the total group ($V5/6 = 0.116$), whereas a combination of three genes was optimal in the EGB and U2OS groups ($V3/4 = 0.126$, 0.134). In other groups, a combination of two genes was optimal ($V2/3 = 0.142$, 0.138 , 0.089) (Figure 3B).

3.3. Stability analysis of gene expression using NormFinder program

We analyzed the stability of gene expression using the NormFinder program. The results are shown in Figure 4. The candidate genes were sorted according to their expression stabilities in each group. The results indicated that the combinations of *HPRT1* and *ALAS1* in the Total group, *PPIA* and *ALAS1* in the EGB group, *PPIA* and *RPL29* in the EGB + CTX group, *PPIA* and *HPRT1* in the MG-63 group, *18S* and *PPIA* in the Saos-2 group, and *HPRT1* and *ALAS1* in the U2OS group were the most stably expressed combinations.

These results indicated that when *HPRT1* was used in a combinatorial form, the expression was most stable for nearly all groups, with the exception of the EGB + CTX and EGB groups. The *ALAS1* and *PPIA* gene combination had the most stable expression among the groups.

3.4. Stability analysis of gene expression using the BestKeeper program based on standard deviation and R-value

We analyzed the stability of gene expression using the BestKeeper program. We analyzed the SD using this program. A higher SD value represents lower stability. The results are shown in Figure 5. The candidate genes were sorted according to their expression stabilities in each group. These results indicated that *ALAS1* had the most stable expression among all groups. Additionally, *PPIA* had the most stable expression in nearly all groups, with the exception of the EGB + CTX and Saos-2 groups.

We analyzed the stability of gene expression based on the correlation coefficient (R-value). For this purpose, we used the BestKeeper program. A higher R-value indicates greater stability and reliability of using the gene as the reference. The results are shown in Figure 6. The candidate genes were sorted according to their expression stabilities in each group. These results indicated that *18S* and *HPRT1* had the most stable expressions in most groups.

4. Discussion

The reference gene is essential for performing qRT-PCR. This technique was used to determine the relative expression levels of genes. In the current study, we analyzed the expression stability of the 12 most commonly used reference genes: *18S*, *ACTB*, *ALAS1*, *GAPDH*, *TBP*, *HPRT1*, *RPL29*, *HMBS*, *PPIA*, *PUM1*, *GUSB*, and *B2M*.

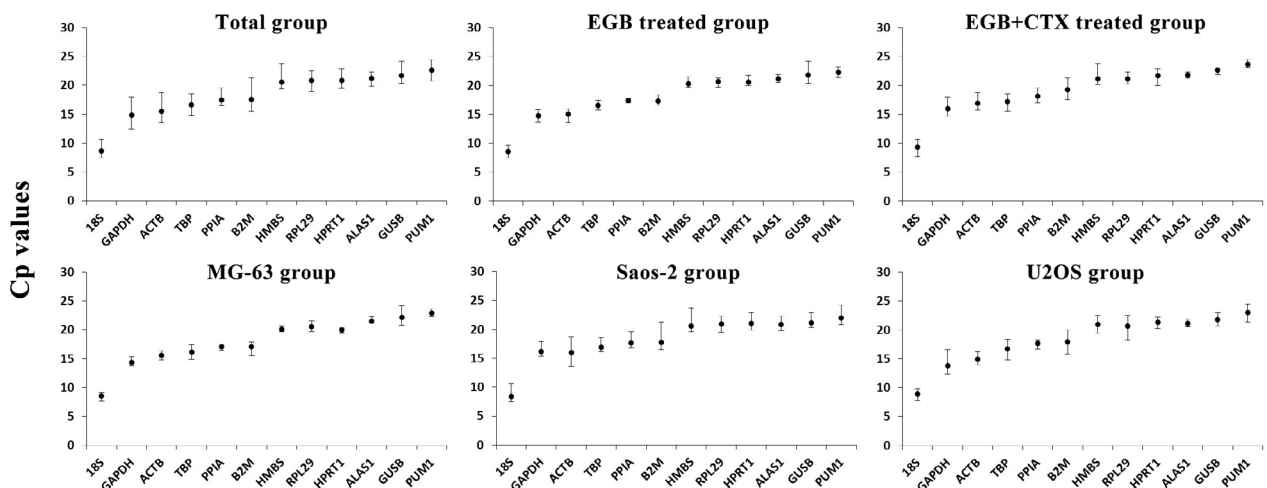


Figure 2. Mean Cp values of reference genes in data groups (bars represent the mean $\pm$ standard deviation, $n = 15$ in the Total group, $n = 3$ in other groups).

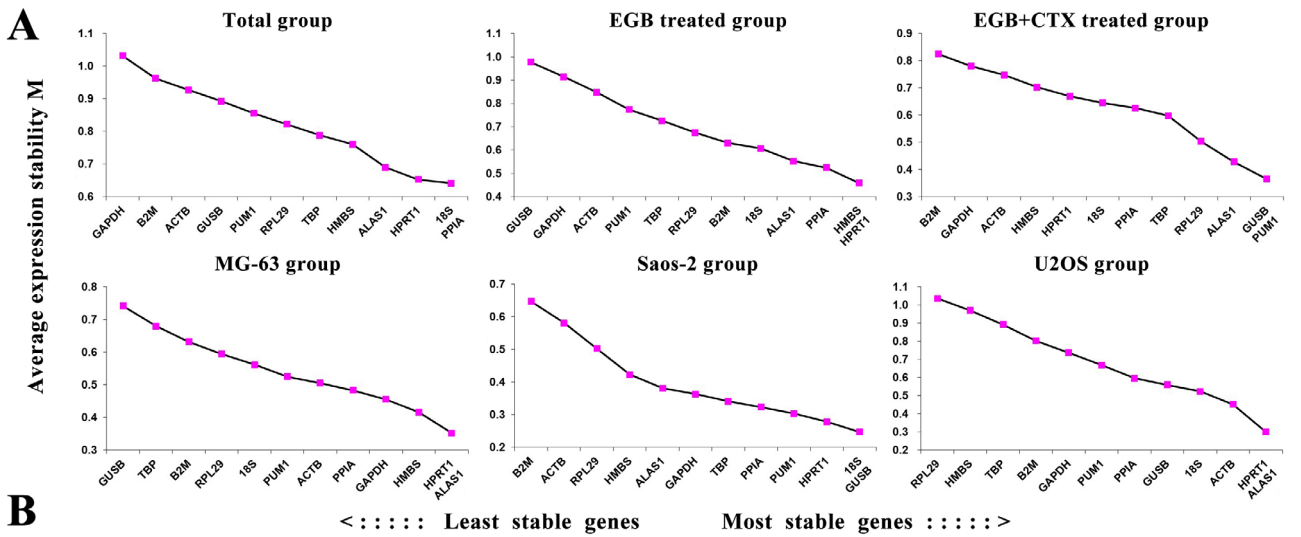


Figure 3. Stability analysis of gene expression using the GeNorm program. (A) Stepwise exclusion of the least stable genes by calculating the M value. The x-axis from the left to right indicates the ranking of reference genes according to their expression stability, and the y-axis indicates M. Low M values imply stable expression. (B) Determination of the optimal number of reference genes for normalization. The V value defines pairwise variation between two sequential normalization factors, which were calculated using geNorm.

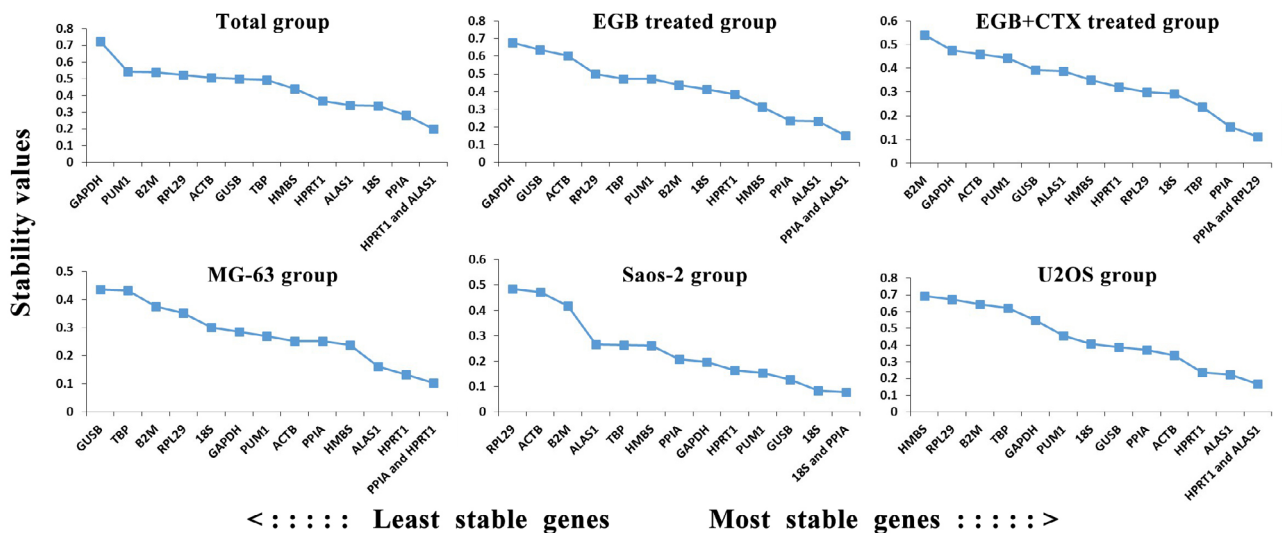


Figure 4. Stability analysis of gene expression using the NormFinder program. The y-axis represents the stability value. Low stability values imply stable expression. The x-axis from the left to right represents the ranking of stability for reference genes.

For this purpose, we used the geNorm, NormFinder, and BestKeeper software programs. We found that no single selected reference gene was most stably expressed

in all three human osteosarcoma cell lines, which were treated with EGB and CTX or both. This is in agreement with previous studies stating that the expression levels of

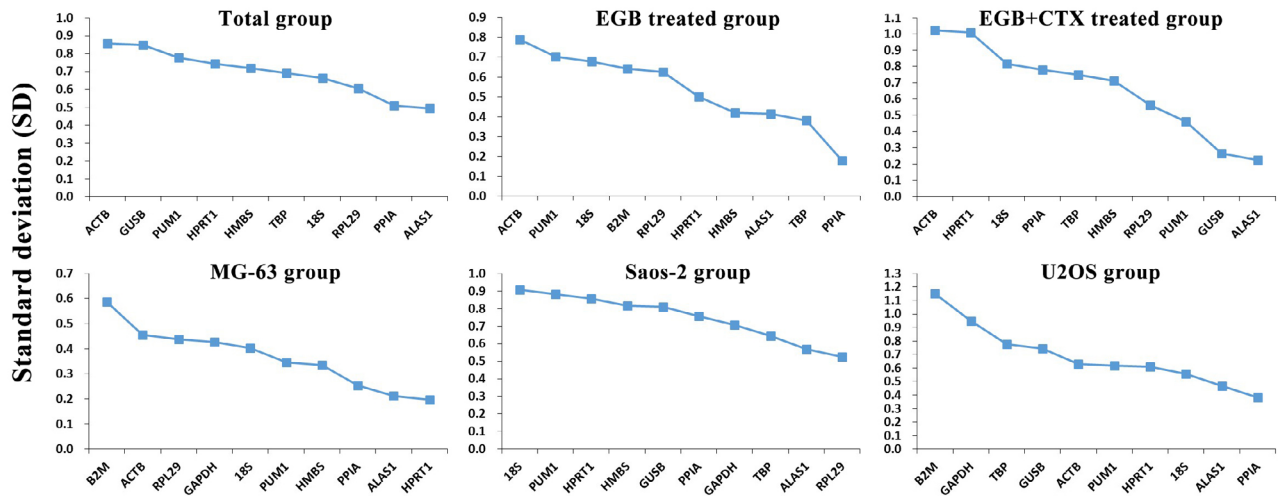


Figure 5. Stability analysis of gene expression, which is based on the standard deviation, using BestKeeper software. The x-axis from left to right represents the ranking of standard deviation values of candidate reference genes.

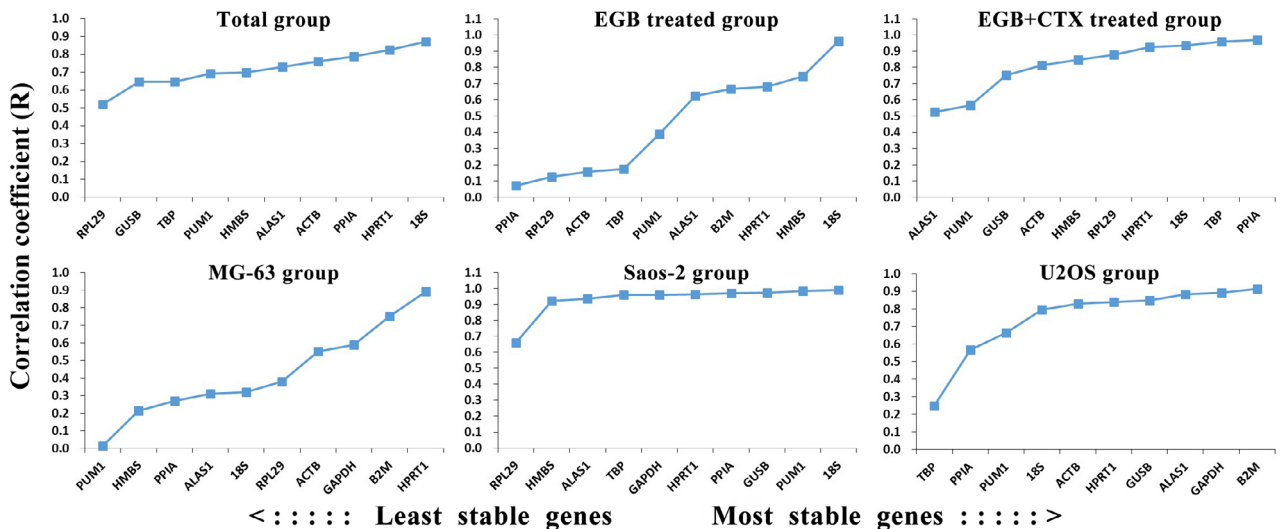


Figure 6. Stability analysis of gene expression, which is based on a correlation coefficient, using the BestKeeper software. The x-axis from left to right represents the ranking of the correlation coefficient values of candidate reference genes. High stability values imply stable expression.

reference genes vary significantly depending on distinct experimental conditions. This includes different cell types and tissues, different individuals, and different stages of cell proliferation, organ development, and in vitro culture (Yang et al., 2014; Ma et al., 2015; Yu et al., 2015). MG-63, Saos-2, and U2OS were the most commonly used osteosarcoma cell lines in previous studies that were carried out in vitro. Although they are all derived from osteosarcoma cells, they differ in many aspects, especially in their proliferation kinetics and osteoid production (Pautke et al., 2004). The rank of the stability of candidate genes was slightly different. This could be attributed to

different calculation algorithms (Brugge et al., 2011; Chang et al., 2012). Therefore, we should use different reference genes under distinct conditions.

To identify the most suitable reference genes of each group, we first used geNorm software and determined the optimal number of combinations of reference genes in each group. During this process, we used pairwise variation values. If the optimal combination number was 2, we used NormFinder software to determine the most suitable reference genes recommended by the combination. If the optimal combination number was 3, we recommended the combination of genes that were

ranked in the forefront of each group as the most suitable reference genes among the three software programs. If the optimal number of combination is more than 3, then it agrees with the standardized principle of qRT-PCR. This finding was supported in previous studies. In these studies, at least three internal control genes were used to perform a relative quantitative investigation (Wisniewski et al., 2013). Moreover, the present study recommended that a combination of three reference genes is sufficient for normalizing the relative expression levels of genes. Among the three software programs, the combination of genes that are ranked in the forefront of each group is recommended as the most suitable reference gene. According to this rule, in the total group, the recommended combination was *18S* + *PPIA* + *HPRT1*. In the EGB-treated group, the recommended combination was *HMBS* + *ALAS1* + *18S*. In the EGB + CTX-treated group, the recommended combination was *PPIA* + *RPL29*. In the MG-63 group, the recommended combination was *PPIA* + *HPRT1*. In the Saos-2 group, the recommended combination was *18S* + *PPIA*. In the U2OS group, the recommended combination was *HPRT1* + *ALAS1* + *GAPDH*.

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In summary, we found that no single reference gene was most stably expressed in all three human osteosarcoma cell lines following treatment with EGB or CTX or both. Instead, we recommend using a combination of three reference genes to normalize the expression of each group. Our findings provide a suitable approach for improving the accuracy in quantitating target gene expression during investigation of the pharmacological effects and underlying mechanisms, through which a combination of CTX and EGB could be used in the treatment of human osteosarcoma.

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