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Original Article

Identification of genes for normalization of quantitative real-time PCR data in ovarian tissues

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Increased attention has been paid to the determination of the potential biomarker and therapeutic target for ovarian cancer in recent years. However, the normalization of quantitative real-time PCR is important to obtain accurate gene expression data. We investigated the stability of 20 reference genes in ovarian tissues under different conditions to determine the most adequate for this application. The study characterized the expression of 20 possible reference genes among 52 ovarian tissue samples involving the normal, non-malignant, and primary ovarian carcinomas. One-way analysis of variance (ANOVA) method was used to compare the candidate gene changes brought about by the disease progression. The stability and suitability of the genes with no statistic difference were further validated employing geNorm and NormFinder softwares. Results showed that the expression levels of the 20 reference genes varied, while the *RPL4*, *RPLP0*, *HSPCB*, *TPT1*, *RPL13A*, *18S rRNA*, *PPIA*, *TBP*, and *GUSB* kept statistic stability despite different ovarian tissue conditions. *RPL4*, *RPLP0*, and *HSPCB* were demonstrated as the most stable reference genes and the combination of the *RPLP0* and *RPL4* should be recommended as a much more reliable normalization strategy.

Keywords ovarian tissues; quantitative real-time PCR; reference genes; GeNorm; NormFinder

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Introduction

Ovarian carcinoma is a significant cause of mortality worldwide, largely due to the high proportion of cases that present at a late stage, when survival is extremely poor. Early detection of epithelial ovarian cancer is therefore a promising strategy for saving lives. Consequently, candidate biomarkers and therapeutic targets associated with ovarian cancer have been addressed in recent years [1–3].

Quantitative real-time PCR (qPCR) is a technique that is frequently used to measure the expression of the target molecules. However, the accurate normalization of relative quantities of marker gene expression relies on accurate comparison of the expression of reference genes. An ideal reference gene should be expressed at the same level in all samples, for example, samples from different tissues, during all developmental stages, and before or after experimental manipulation [4]. There is emerging evidence that common reference genes can significantly vary in expression over different conditions. Therefore, not only new reference genes were used besides traditional genes such as *GAPDH* and *ACTB*, but also the combination of multiple reference genes were considered [5–7].

In recent years, many papers have been published which were on gene expression analysis in human ovarian tissues using qPCR, aiming to seek a candidate biomarker or therapeutic target. In these publications, multiple ovarian tissue types were involved and traditional reference genes such as *ACTB*, *GAPDH*, *B2M*, or *18S rRNA* were just commonly chosen as housekeeping without validation. Therefore, it is essential to identify optimal genes for qPCR normalization in ovarian tissue-related studies.

Up to now, rare data involved ovarian tissues was reported except one recent publication, in which the recruit of only normal and primary serious ovarian cancer subtypes led to the greatest limitation [8]. More appropriate systematic evaluation of reference gene stability should be performed in those who stably expressed across samples with different status (or in different conditions). In order to identify the optimal genes, more ovarian tissues should be included, for example, normal tissues, benign tumors, primary ovarian cancers, and other well-known ovarian diseases such as ovarian chocolate cyst.

In this study, more reference genes and much more ovarian tissue types were covered to determine the optimal reference gene, which could be more universal in ovarian tissue-related experiments and diagnosis. This study focused on ovarian tissues under three different conditions: normal ovarian tissues, non-malignant ovarian tissues including benign tumors (serious cystadenoma and mucinous tumors), and ovarian endometrioma; and primary serous ovarian cancer with malignancy Grade I–III. Systematic evaluations of 20 endogenously expressed genes were performed with the aim of identifying the most useful genes for normalization adapted for ovarian tissues in different stages. Results revealed that many of the 'housekeeping' genes are indeed differentially expressed across the ovarian tissues of different types, and a detailed comparison of the stability of 20 reference genes at different stages was made.

Materials and Methods

Patients and sample collection

A total of 52 ovarian tissues from patients at the Affiliated Hospital of Academy of Military Medical Sciences (Beijing, China) were recruited (mean age of 52 years, range from 21 to 69 years; no chemotherapy before surgery). The use of the tissue material for research was approved by the ethics commission of the Affiliated Hospital of Academy of Military Medical Sciences. Samples were divided into three groups, based on the clinicopathological characteristics and clinical staging of tissues: Group A, normal ovarian tissues ($n = 12$); Group B, 19 cases of non-malignant ovarian tissues including benign tumors (serous cystadenoma, $n = 6$; mucinous tumors, $n = 5$) and ovarian endometrioma ($n = 8$); Group C, 21 cases of primary serous ovarian cancer with malignancy Grade I–III (Stage I, $n = 5$; Stage II, $n = 8$; Stage III, $n = 8$).

Ovarian samples were kept in E.Z.N.A.^{TM} RNA safe stabilizer reagent (Omega Biotek Inc., USA) immediately after surgical removal and then frozen in liquid nitrogen for storage at -80°C until further processing.

RNA extraction and cDNA synthesis

Strict RNA quality control and precise RNA concentration determination were performed. Tissues were homogenized in liquid nitrogen and RNA was extracted by TRI Reagent (Molecular Research Center, Inc., Cincinnati, USA) according to the manufacturer's instructions. The integrity of RNA obtained from frozen tissues was also detected by gel electrophoresis. The concentration of purified RNA was measured and genome DNA contamination was removed by RQ1 RNase-free DNase (Promega, Madison, USA). The RNA quality was assessed with absorbance measurements using an ND-1000 UV–Vis spectrophotometer (NanoDropTechnologies, Wilmington, USA). Only RNA of high quality was used in the subsequent PCR reactions. The acceptance criteria were that A_{260}/A_{280} should range from 1.9 to 2.1 and the 28S/18S ratio be above 1.7 on 1% agarose gels.

Total RNAs (1–3 μg) were reverse-transcribed into cDNA using a RevertAid first strand cDNA synthesis Kit (Fermentas Life Sciences, Burlington, Canada) according to the manufacturer's instructions. The cDNA samples were measured for DNA content and stored at -20°C until use.

Quantitative real-time PCR

To obtain similar and optimal qPCR reaction efficiencies, gene-specific primers were selected. Primers for all of the investigated genes were generated using Primer Premier 5 software or according to published references. Most primers were designed to span introns in order to detect any genomic DNA contamination, and the lengths of products were designed to range from 60 to 250 bp. All primers were located near the 3′-end of cDNA to assure accurate quantification. For each candidate reference gene, at least two pairs of primers were investigated to determine the optimal primers (**Table 1**).

A standard curve of serially diluted (320, 160, 80, 40, and 20 ng) standard sample cDNA (bulked cDNA of analyzed samples) was obtained for each candidate gene [9,10]. Amplification efficiencies were determined based on the slope the of standard curve using the following equation: $E\% = [10^{(-1/\text{slope})} - 1]\%$, and the correlation coefficient (R^2) [10] was calculated by Origin Software version 7.5. The standard sample was included in every PCR run to control intra-assay variability. Maxima SYBR Green qPCR Master Mix (2×) (Fermentas Life Sciences, Burlington, Canada) was used for all reactions for the genes shown in **Table 1**. Briefly, 25 μl of qPCR reaction mixture was used containing 1× Master Mix, 1 μl of cDNA (equal to 300 ng per reaction) or dilution series, and RNase/DNase-free water. A no-template control was used in every assay. PCR reactions were carried out at 50°C for 2 min, then with an initial 10 min hot start activation at 95°C, followed by 40 cycles of 30-s denaturation at 95°C, 30-s annealing at 50–60°C as appropriate for each primer, and a 30-s extension at 72°C. Fluorescence data were acquired after each cycle. The specificity of products was verified by melting curve analysis (65–95°C) and agarose gel electrophoresis. All qPCR reactions were performed on the Opticon2 Chromo4 system (Bio-Rad, CA, USA) and were measured in triplicate to ensure methodological reproducibility.

Data acquisition and statistical analysis

Expression levels were determined as quantification cycles (C_q). For stability comparisons of candidate reference

Table 1 Summary of candidate reference genes

Symbol (accession number)	Gene name	Primer sequences (5′ → 3′)
ACTB[a] (NM_001101.3)	Beta actin	F: ATGTGGCCGAGGACTTTGATT
		R: AGTGGGGTGGCTTTTAGGATG
ATP5B[a] (NM_001686.3)	ATP synthase, H+ transporting, mitochondrial F1 complex, beta-polypeptide	F: CACCCAGGCTGGTTCAGA
		R: AGTGGCCAGGGTAGGCTGAT
B2M (NM_004048.2)	Beta-2-microglobulin	F: TGACTTTGTCACAGCCCAAGATA
		R: CGGCATCTTCAAACCTCCA
GAPDH[a] (NM_002046.3)	Glyceraldehyde-3-phosphate dehydrogenase	F: TCTCCTCTGACTTCAACAGCGAC
		R: CCCTGTTGCTGTAGCCAAATTC
HMBS (NM_000190.3)	Hydroxymethylbilane synthase	F: GGCAATGCGGCTGCAA
		R: GGGTACCCACGCGAATCAC
HPRT1[a] (NM_000194.2)	Hypoxanthine phosphoribosyltransferase 1	F: TGACACTGGCAAAACAATGCA
		R: GGTCCTTTTCACCAGCAAGCT
RPL13A[a] (NM_012423.2)	Ribosomal protein L13a	F: CCTGGAGGAGAAGAGGAAAGAGA
		R: TTGAGGACCTCTGTGTATTTGTCAA
SDHA (NM_004168.2)	Succinate dehydrogenase complex, subunit A	F: TGGGAACAAGAGGGCATCTG
		R: CCACCACTGCATCAAATTCATG
YWHAZ[b] (NM_003406.3)	Tyrosine 3-monoox-ygenase/tryptophan 5-monoox-ygenase activation protein, zeta polypeptide	F: ACTTTTGGTACATTGTGGCTTCAA
		R: CCGCCAGGACAAACCAGTAT
UBC[a] (NM_021009,4)	Ubiquitin C	F: ATTTGGGTCGCAGTTCTTG
		R: TGCCTTGACATTCTCGATGGT
RPLP0[a] (NM_001002.3)	Large ribosomal protein P0	F: TTAAACCCTGCGTGGCAATCC
		R: CCACATTCCCCCGGATATGA
HSPCB[a] (NM_007355.2)	Heat shock 90-kDa protein 1, _beta	F: AAGAGAGCAAGGCAAAGTTTGAG
		R: TGGTCACAATGCAGCAAGGT
PPIA[a] (NM_021130.3)	Peptidylprolyl isomerase A (cyclophilin A)	F: AGGGTTCCTGCTTTC
		R: ATGCCTTCTTTCACTTT
TPT1[a] (NC_000013.10)	Tumor protein, translationally controlled 1	F: GATCGCGGACGGGTTGT
		R: TTCAGCGGAGGCATTTCC
TEGT (NM_003217.2)	Testis enhanced gene transcript (BAX inhibitor 1)	F: TGCTGGATTTGCATTCCTTACA
		R: ACGGCGCCTGGCATAGA
PGK1 (NM_000291.3)	phosphoglycerate kinase 1 [11]	F: ATTAGCCGAGCCAGCCAAAATAG
		R: TCATCAAAAACCCACCAGCCTTCT
18S rRNA (X03205.1)	18S Ribosomal RNA[12]	F: GGCGCCCCCTCGATGCTCTTAG
		R: GCTCGGGCCTGCTTTGAACACTCT
RPL4 (NM_000968.2)	Ribosomal protein L4[13]	F: GCTCTGGCCAGGGTGCTTTTG
		R: ATGGCGTATCGTTTTTGGGTTGT
GUSB (NM_000181.3)	Glucuronidase, beta	F: TGGTGCTGAGGATTGG
		R: TGTTGATGGCGATAGTGA
TBP[a] (NM_001172085.1)	TATA box binding protein (TBP), transcript variant 2	F: GCACAGGAGCCAAGAG
		R: GTGGGTGAGCACAAGG

The primers were designed by Primer Premier 5 software in our study except the *PGK1*, *18S rRNA* and *RPL4* were used according to the reference. [a]Presence of the pseudogene. [b]The primers were designed spanning the intron except the *YWHAZ*, primers of which were within the same exon.

genes, the software geNorm, vers. 3.4 [12] (http://medgen.ugent.be/~jvdesomp/genorm/Download.php) and Norm Finder (http://www.mdl.dk/publicationsnormfinder.htm) were used. Two parameters were chosen to quantify the reference gene stability: *M* (average expression stability) and *V* (pairwise variation). GeNorm generates an *M* value for each gene and a pairwise stability measure to determine the benefit of adding extra reference genes for the normalization process (with a lower value indicating greater stability of the normalization factor). Similarly, NormFinder

generates a stability measure in which a lower value indicates increased stability in gene expression and group samples. These allow the direct estimation of expression variation and the ranking of genes according to their expression similarity. Data were analyzed by one-way analysis of variance (ANOVA) followed by the Tukey method for pairwise multiple comparisons using SPSS 13.0 program (SPSS Software, Chicago, USA). $P < 0.1$ was considered statistically significant.

Results

Expression levels of candidate reference genes

qPCR was used to measure the expression levels of 20 common reference genes in 52 ovarian tissue samples. The annealing temperature (T_a), melting curve temperature (T_m), product length, PCR amplification efficiencies, and R^2 of each primer were documented (**Table 2**). The melting curve analysis of these 20 genes was shown in **Supplementary Fig. S1**. Minimum information for the publication of qPCR experiments (MIQE Guidelines) according to the reference [13] was filled out (**Supplementary Table S1**).

These 20 reference genes displayed a wide expression range, with C_q values from 9 to 34. Highly expressed genes with C_q values below 20 cycles were *18S rRNA* and *RPL13A*. Intermediately expressed genes with C_q values from 20 to 25 cycles included *B2M*, *ACTB*, *GAPDH*, *RPLP0*, *PPIA*, *RPL4*, *HSPCB*, and *TPT1*. Genes with low expression and C_q values above 25 cycles were *ATP5B*, *HMBS*, *HPRT1*, *UBC*, *SDHA*, *YWHAZ*, *PGK1*, *GUSB*, *TBP*, and *TEGT* (**Fig. 1**).

Correlation in the expression levels of candidate genes in ovarian tissues at different stages

The correlation was investigated using one-way ANOVA to compare the reference gene changes brought about by disease progression (**Fig. 2**). For these 20 reference genes, no statistical differences were detected between Groups A and B. With regard to gene expression, significant differences between Groups A and C were observed in 11 genes, between Groups B and C in 5 genes. To avoid type 2 errors, we set the $P < 0.1$ as the statistical difference. The expression levels of *RPL4*, *RPLP0*, *HSPCB*, *TPT1*, *RPL13A*, *18S rRNA*, *PPIA*, *TBP*, and *GUSB* in ovarian tissues at different stages showed no statistically significant differences ($P > 0.1$).

Determination of optimal reference genes for all tested ovarian tissues

In our search for the most stable reference genes across the ovarian tissues with difference status, the expression stabilities of tested genes were validated with two software programs, geNorm and NormFinder. Reference genes with no statistically significant differences were calculated further by these two software. The expression stabilities of tested genes varied dramatically, with *M*

Table 2 Annealing temperature, melting temperature, product length, R^2, and PCR efficiency for all SYBR green I real-time PCR assays

Primer Name	*Ta* (°C)	*Tm* (°C)	R^2	Efficiency (%)	Product length (bp)
ACTB	60	77.3 ± 0.2	0.99	100	107
ATP5B	60	80.8 ± 0.2	0.99	88	80
B2M	60	79.4 ± 0.2	0.98	92	75
GAPDH	60	81.6 ± 0.2	0.96	87	126
HSPCB	60	76.8 ± 0.2	0.97	98	120
HMBS	60	79.2 ± 0.2	0.98	95	64
HPRT1	60	78.2 ± 0.2	0.99	85	94
RPL13A	60	78.8 ± 0.2	0.99	89	126
SDHA	60	75.8 ± 0.2	0.98	85	86
YWHAZ	60	77.4 ± 0.2	0.98	87	94
UBC	60	80.6 ± 0.2	0.99	88	91
RPLP0	60	84.2 ± 0.2	0.99	88	288
TPT1	60	80.8 ± 0.2	0.98	91	100
PPIA	50	81.2 ± 0.2	0.97	85	262
TEGT	60	82.8 ± 0.2	0.97	85	151
18S rRNA	60	81.8 ± 0.2	0.99	100	89
RPL4	60	82.1 ± 0.2	0.99	98	154
GUSB	54	85.2 ± 0.2	0.97	90	165
TBP	54	79.2 ± 0.2	0.98	92	170
PGK1	60	81.0 ± 0.2	0.97	96	152

SYBR green I real-time PCR assays.

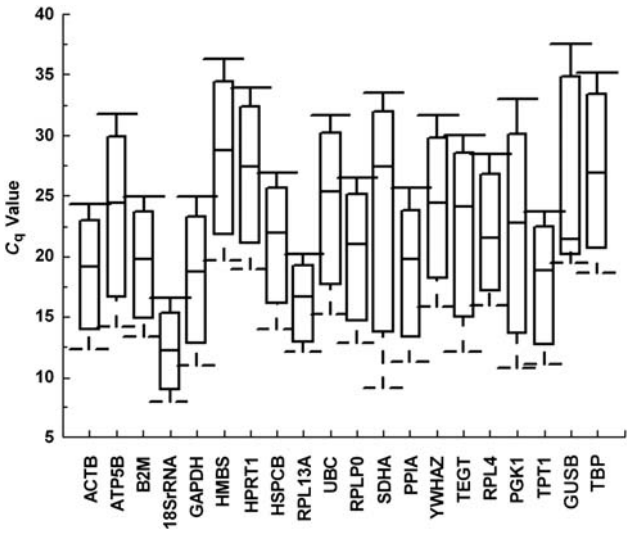

Figure 1 Expression levels of candidate reference genes Expression levels of 20 candidate reference genes in ovarian tissues with three different statuses were shown as C_q. Boxes represent the lower (lower limit) and upper (upper limit) quartiles with medians; bars indicate the ranges of the data. All assays were carried out at least three times, and values are expressed as mean ± SD.

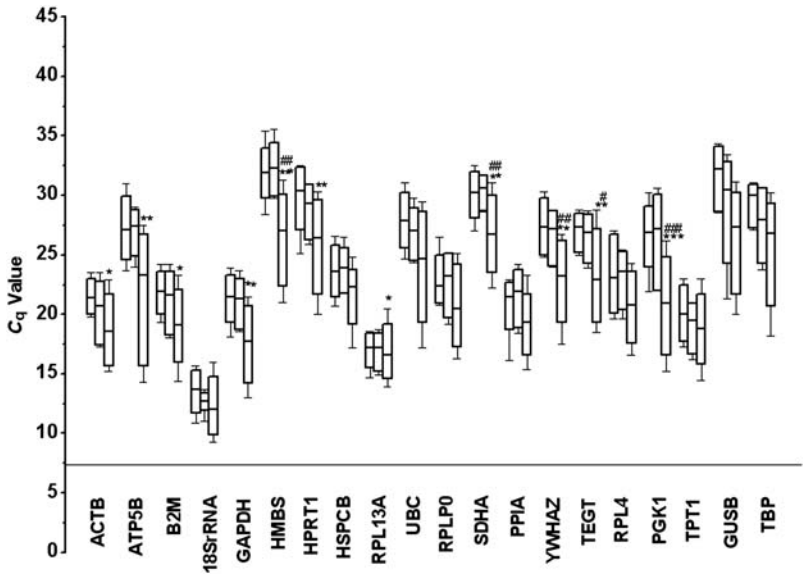

Figure 2 Box plot of the absolute C_q values of 20 reference genes investigated in three groups The correlation between the expression levels of 20 candidate genes and ovarian tissues in different stages was investigated using one-way ANOVA, to compare the reference gene changes brought about by disease progression. Three boxes from the left to the right represented the expression levels of three groups for each candidate genes. Group A, normal ovarian tissues; Group B, non-malignant ovarian tissues including benign tumors (serious cystadenoma and mucinous tumors) and ovarian endometriosis; Group C, primary serous ovarian cancer with malignancy Grade I–III. $^{*}P < 0.1$ vs A, $^{**}P < 0.05$ vs A, $^{***}P < 0.001$ vs A; $^{\#}P < 0.1$ vs B, $^{\#\#}P < 0.05$ vs B, $^{\#\#\#}P < 0.001$ vs B.

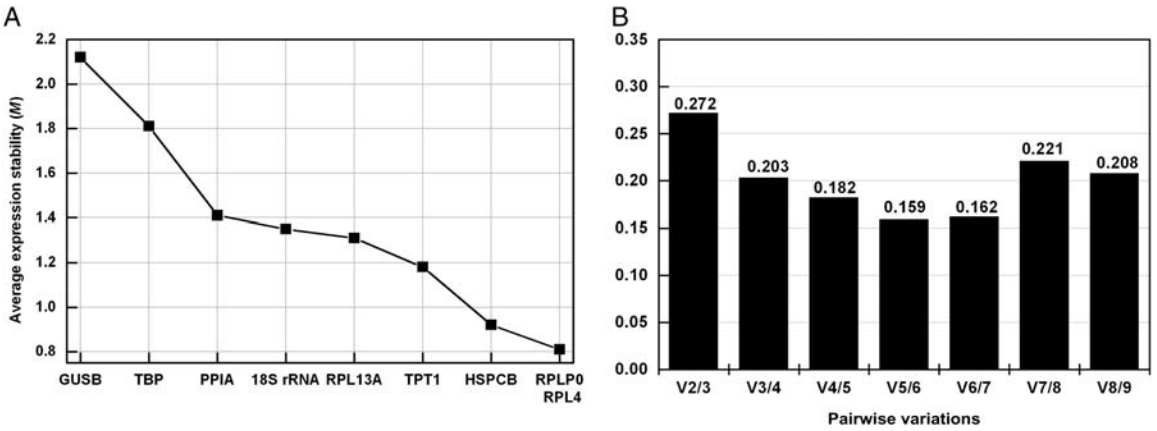

Figure 3 Selection of the most suitable reference genes for normalization in ovarian tissues using geNorm analysis The expression stability of candidate genes across the 52 ovarian tissues in three stages was determined. (A) Stepwise exclusion of the least stable genes by calculating the average expression stability measure *M*. The value of *M* was calculated for each gene, and the least stable gene with the highest *M* value was automatically excluded from the next calculation round. The *X*-axis from left to right indicates the ranking of the genes according to their expression stability. (B) Determination of the optimal number of reference genes for normalization; V_n/V_{n+1} is the pairwise variation between normalization factors of n and $n + 1$ genes.

values ranging from 2.12 to 0.81. The *GUSB* was the least stable reference gene with an *M* value of 2.12, whereas *RPLP0* and *RPL4* were identified as the most stable genes, with *M* value of 0.81 [**Fig. 3(A)**, **Table 3**]. In addition, the optimal number of candidate genes for normalization was determined. This was calculated from two or more genes, with variable *V* value as the pairwise variation (V_n/V_{n+1}) between two sequential normalization factors (NF_n and NF_{n+1}). *RPL4*, *RPLP0*, and *HSPCB* were demonstrated as the most stable reference genes and the combination of two or all the three genes should be recommended as a much more reliable normalization strategy to improve the accuracy of normalized data across all tested ovarian tissues [**(Fig. 3(B)**].

NormFinder program analysis showed that stability values for these 20 genes ranged from 0.92 (*GUSB*) to 0.34 (*HSPCB*). *GUSB* was determined to be the least stable gene and the combination of *RPL4* and *RPLP0* was identified as the most stable gene pair with *M* value of 0.276, which was the same with the geNorm result. The rank of reference genes by both geNorm and NormFinder programs were shown in **Table 3**.

Table 3 Expression stability measures (*M*) calculated by geNorm and Normfinder for all candidate reference genes; the lower *M* values indicate higher expression stability

GeNorm[a]		NormFinder[b]	
Gene	*M* value	Gene	*M* value
RPL4	0.81	*HSPCB*	0.34
RPLP0	0.81	*RPL4*	0.39
HSPCB	0.92	*RPLP0*	0.39
TPT1	1.18	*TPT1*	0.48
RPL13A	1.31	*PPIA*	0.50
18S rRNA	1.35	*RPL13A*	0.53
PPIA	1.41	*18SrRNA*	0.57
TBP	1.81	*TBP*	0.63
GUSB	2.12	*GUSB*	0.92

[a]Best combination for *RPLP0* + *RPL4* is 0.81. [b]Best combination for *RPLP0* + *RPL4* is 0.276.

Discussion

Strict quality controls were performed in the present study to obtain reliable data, including the RNA integrity, RNA concentration determination, and primer selection. SYBR green qPCR was adopted instead of the Taqman probe method, which indicated that more attention must be paid to primer design and selection. Another two reference genes that were optimal in other studies, *PMM1* [8,10] and *SFRS4* [7], did not give good amplification and were not incorporated into our study. In addition, we determined the optimal reference genes combining the results from the two programs (geNorm and NormFinder) and the statistical analysis, which could lead to better reliability than using geNorm alone [4].

Our study showed that commonly used traditional reference genes, *ACTB*, *GAPDH*, *B2M*, and *18S rRNA*, were unsuitable for normalization in some ovarian tissue-related studies. *GAPDH* expression was significantly increased in malignant samples (Group C) compared with normal and non-malignant samples. Similarly, the expression levels of *ACTB*, *B2M*, and *18S rRNA* showed low expression stability among the three groups. Up to now, there has been only one paper reporting the reference genes study about the ovarian cancers. The combination of *GUSB*, *PPIA*, and *TBP* was demonstrated to be useful as reference genes for normalization in gene profiling studies of serous ovarian cancer [8]. Although the *GUSB*, *PPIA*, and *TBP* indeed showed no statistical difference among the three groups in our study, which supported the previous results, the other reference genes, such as *RPL4*, *RPLP0*, *HSPCB*, *TPT1*, and *RPL13A* showed a more stability expression profile than the three genes. Our current study demonstrated that the combination of three most stable genes *RPL4*, *RPLP0*, and *HSPCB* could be used as reference genes for normalization in all the ovarian-related researches.

Nevertheless, the samples used in the current study were limited and additional similar samples are needed to support and extend our observations. This small sample size might be a limitation of the study design when performing statistical tests. In addition, all ovarian samples in this study were obtained from Chinese patients. It remains to be determined whether similar results would be obtained with other populations.

The greatest contribution of our study was that we performed a systematic stability comparison of the multiple traditional reference genes for different research purposes. This study, unlike the published ovarian cancer studies [8] that had compared normal tissues versus primary carcinomas, examined three different conditions of ovarian tissues. On the basis of the ANOVA analysis and stability analysis results concluded from two programs, the combination of *RPL4* and *RPLP0* were recommended as the best combination of two genes for the normalization despite which condition the ovarian tissues were in.

Supplementary Data

Supplementary Material is available at *ABBS* online.

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