OncoTargets and Therapy

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ORIGINAL RESEARCH

Identification and Validation of Reference Genes Selection in Ovarian Cancer Exposed to Hypoxia

This article was published in the following Dove Press journal:
OncoTargets and Therapy

OncoTargets and Therapy downloaded from https://www.dovepress.com/
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Introduction: Hypoxia-mediated tumor metastasis, progression and drug resistance are major clinical challenges in ovarian cancer. Meanwhile, the genetic basis of these traits is still not clear. RT-qPCR, as an efficient and sensitive gene expression technique, has been widely used for gene analyses, providing a basis for in-depth understanding of molecular changes in different microenvironments. However, there is currently a lack of suitable reference genes to normalize the data associated with hypoxia in ovarian cancer cells.

Methods: A systematic method is needed to select the most suitable reference gene. Here, eight candidate reference genes (GAPDH, β-actin, 18S RNA, TUBB, PPIA, TBP, RPL13A and SDHA) from humans were selected to assess their expression levels in SKOV3 cells under hypoxia. The geNorm and NormFinder programs were utilized to evaluate the expression stabilities of these selected candidate reference genes.

Results: Interestingly, 18S RNA was considered to be an ideal reference gene for the normalization of target gene expression under hypoxic conditions. Furthermore, this result was confirmed in another two ovarian cancer cell line, CAOV3 and OVCAR3 cell line. Finally, these results suggest that appropriate reference genes should be selected before performing gene expression analysis during hypoxic environmental exposure.

Conclusion: 18S RNA can be used as an appropriate reference gene for the study of gene expression in ovarian cancer samples under hypoxia by RT-qPCR.

Keywords: reference genes, ovarian cancer, hypoxia, EMT

Introduction

Among all gynecological malignancies, ovarian cancer has the highest mortality rate and most patients are diagnosed with advanced cancer.[1] The metastatic capacity and growth of malignant tumors are highly dependent on the tumor microenvironment. However, although it is recognized that the migration and invasion ability of ovarian tumor cells is a necessary prerequisite for their metastasis, the exact molecular events leading to ovarian tumor cell metastasis have not been well elucidated. These processes involve a change named epithelial–mesenchymal transition (EMT) in the morphology of cuboidal, rigid or cobblestone epithelia into a mesenchymal form.[2]

In order to study the molecular mechanism of EMT, quantitative gene expression analysis is essential. Due to its high sensitivity, simplicity and specificity, quantitative real-time PCR (RT-qPCR) has become a popular biological tool for detecting gene expression levels.[3,4] However, in order to obtain the ideal outcomes for gene expression analysis, minimum requirements of RT-qPCR must be met, which include internal quality control for the mRNA and primers, PCR efficiency

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submit your manuscript | www.dovepress.com
DovePress
http://doi.org/10.2147/OTT.S249733

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determination and appropriate reference gene selection.[5] Furthermore, the obtained gene expression profiles are different when using different internal reference genes.[3] Therefore, the correct selection of the reference gene ensures the accuracy of the RT-qPCR analysis data.[4]

In the process of quantitative gene expression analysis, researchers often choose internal reference genes based on their own laboratory experience, literature recommendation or their experience and habits. For example, GAPDH and β-actin were always selected as potential candidate reference genes, based on previous experience. However, previous studies have shown that the expression levels of these housekeeping genes (HKGs) can vary between cell types[6–8] and in different experimental conditions.[9,10] Therefore, prior to their initial use, the stability of the expression of certain HKGs should be validated first.

The purpose of this research was to select a reliable and stable reference gene for the study of ovarian cancer under hypoxic conditions (1%O_2). Two different specific algorithms, geNorm and NormFinder, were utilized to analyze the stabilities of the selected candidate housekeeping genes (GAPDH, β-actin, 18S RNA, TUBB, PPIA, TBP, RPL13A and SDHA) in a human ovarian cancer cell line under exposure to hypoxia using SYBR green RT-qPCR.

Methods and Materials

Cell Culture

The ovarian cancer cell lines performed in this study were SKOV3, CAOV3 and OVCAR3 (ATCC, Rockville, MD, USA). The SKOV3 cells were cultured as monolayer in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C under a 5% CO_2 atmosphere. CAOV3 and OVCAR3 cells were maintained in RPMI 1640 and supplemented with 10% FBS. Cells at 70% to 80% confluency in monolayers were starved by culturing them in serum-free medium for 24 h. SKOV3, CAOV3 and OVCAR3 cells were incubated for 24 h under either hypoxic (1% O_2) or normoxic (21% O_2) conditions.

RNA Samples and cDNA Synthesis

According to the manufacturer's instructions, 10^6 cells were used to isolate the total cellular RNA using TRIzol reagent (ThermoFisher Scientific) and the RNA was quantified with NanoDrop microspectrophotometer (ThermoFisher Scientific). cDNA was synthetized from 1.0 μg of total RNA by using the SuperScritp IV VILO kit with ezDNase (ThermoFisher Scientific).

Candidate Genes Used for RT-qPCR

Eight candidate human reference genes, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-actin, 18S RNA, β-tubulin (TUBB), ribosomal protein L13 (RPL13A), peptidylprolyl isomerase A (PPIA), succinate dehydrogenase complex, subunit A (SDHA) and TATA box binding protein (TBP), were selected for the evaluation of gene expression stability, and the genes sequences were obtained from the NCBI database linked with the GenBank accession numbers, as shown in Table 1. Specific primers used to amplify the indicated gene were designed by BLAST software in NCBI. Except for those primers for 18S RNA, all primers span an intron to avoid genomic DNA contamination of total RNA. The experimental design was based on the Minimum Information for the Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines.[11]

Real-Time Quantitative PCR with SYBR Green

RT-qPCR was conducted with SYBR Green THUNDERBIRD qPCR Mix (TOYOBO) on a CFX-96 Touch real-time PCR system (Bio-Rad) according to standard protocols. For each reaction, 1 μL of synthetized cDNA was added to 19 μL of reaction mixture containing 8 μL of H_2O, 10 μL of SYBR Green mixture and 0.5 μL each of the forward and reverse primers. The thermocycler program was as follows: 3 min at 95°C followed by 40 cycles of 20 s at 95°C, 20 s at 60°C, and 20 s at 72°C. Each run was concluded with a melting curve analysis to assess the quality of the RT-qPCR products by observation of a single peak melt curve, which represented a single product. All experiments were performed in Four technical repetitions in three independent experiments.

Primer Specificity and Amplification Efficiency of the Reference Genes

In order to calculate out the amplification efficiency (E) and correlation coefficient (R^2), each candidate reference gene was tested by generating a standard curve by RT-qPCR of serially diluted (1/10, 1/100, 1/1000, 1/10,000, and 1/100,000) cDNA samples; the results were obtained with the equation E (%) = $(10^{-1/\text{slope}} - 1) \times 100$.[12] A range of 90–110% for the amplification efficiency and an R^2 value of 0.98 were considered acceptable (Table 2).

Table 1 Primer Sequence Information for RT-qPCR Amplification

Symbol	Gene Name	Accession Number	Forward Primer Sequence [5′-3′]	Position in cDNA	Reverse Primer Sequence [5′-3′]	Position in cDNA	Production Size
GAPDH	Glyceraldehyde	NM_002046.5	TCCAAAATCAAGTGGGGCGA	4th exon	TGATGACCCTTTTGGCTCCC	5th exon	115bp
β-actin	β-actin	NM_001101.3	CTTCCAGCCTTCCTTCCTGG	4th exon	CTGTGTTGGCGTACAGGTCT	5th exon	110bp
18S RNA	18S RNA	NR_003286.4	CAGATCAAAACCAACCCG		GCCCTATCAACTTTCGATGG		152bp
TUBB	β-tubulin	NM_178014.4	CACCTTGTCTCAGCCACCAT	6th exon	AGCTCGATACTGCTGGCTTC	8th exon	171bp
PPIA	Peptidylprolylisomerase A	NM_021130.3	GACTGAGTGGTTGGATGGCA	4th exon	TCGAGTTGTCCACAGTCAGC	5th exon	141bp
RPL13A	Ribosomal protein L13	NM_012423.3	AAAAGCGGATGGTGGTTCCT	6th exon	GCTGTCACTGCCTGGTACTT	7th exon	118bp
TBP	TATA-Box binding protein	NM_003194.4	CAGCTTCGGAGAGTTCTGGG	3th exon	TATATTCGGCGTTTCGGGCA	4th exon	117bp
SDHA	Succinate dehydrogenase complex, subunit A	NM_004168.3	AAACTCGCTCTTGGACCTGG	10th exon	TCTTCCCCAGCGTTTGGTTT	11th exon	111bp

Analysis of the Stability of Candidate Genes Expression

geNorm software was utilized to analyze the measurements of gene expression stability (M), and a normalization factor less than 0.15 indicates that the number of reference genes met the requirements and that no housekeeping gene needed to be added.[4] According to the M value generated by geNorm, the lower the M value, the more stable the expression of the gene. NormFinder[13] aims to find the different genes with the least intragroup and intergroup expression variation and determines the expression stability of each tested candidate reference gene. Finally, according to the ranking of each candidate reference gene in geNorm and NormFinder, a score was determined and used to select the most suitable internal reference gene. The housekeeping gene corresponding to the smallest determination score was the most stable among the eight candidate housekeeping genes.

Confirmation of the Stability of the Selected Reference Gene

Another two ovarian cancer cell lines, CAOV3 and OVCAR3, were used in this experiment to verify whether the selected reference gene was stably expressed in other cell lines under hypoxic conditions. After starvation for 24 h, CAOV3 and OVCAR3 cells were exposed to hypoxia (1% O_2) for 24 h, and then RNA sample collection, cDNA synthesis and the analysis of the stability of reference gene expression were conducted as described previously.

Results

Identification of the Specificities and Amplification Efficiencies of the Primers

The expression stability of eight candidate reference genes normoxia or hypoxia were analyzed by a relative quantification method. For each reference gene, the specificity of the primers was also demonstrated by the observation of a single PCR product, which was indicated by a single peak in the melting curve analysis (Figure 1). According to the method described previously,[12] the amplification efficiencies of the eight reference genes were calculated out ranged between 93.9% and 109.7%. The correlation coefficients of the standard curves for each gene showed R^2 values greater than 0.98 (Table 2).

Table 2 RT-qPCR Analysis for Determination of the Amplification Efficiency

Gene	Slope	E (%)	R^2
GAPDH	−3.531	91.9	0.997
β-actin	−3.110	109.7	0.995
18s RNA	−3.358	98.5	0.996
TUBB	−3.450	94.9	0.999
PPIA	−3.476	93.9	0.996
RPL13A	−3.397	96.9	0.998
TBP	−3.386	97.4	0.999
SDHA	−3.1904	105.8	0.999

Note: E, efficiency; R^2, correlation coefficient.

Study on Expression Levels of Candidate Genes in Cells Under Hypoxia Exposure

In order to study the expression stability of candidate genes in hypoxia environment, we first treated the cells with 1% O_2, and then detected and evaluated the expression level of these genes in this condition by RT-qPCR and Shapiro–Wilk test (Table 3). The results showed that the value of cyclic quantification (Cq), the number of cycles required to detect the real signal of the sample, varied between 16.78 (PPIA) and 26.18 (SDHA) in normoxic culture (Table 3 and Figure 2A), and between 17.37 (PPIA) and 26.45 (SDHA) in hypoxic culture (Table 3 and Figure 2B). In general, the expression levels of PPIA and RPL13A were higher than other evaluated candidate genes before and after hypoxia exposure (Figure 2), while the expression levels of SDHA and TBP were lower than other candidate genes (Figure 2).

Candidate Reference Genes Stability: geNorm

For accurate gene expression measurements, it is essential to normalize results from the quantitative real-time PCR experiments to a stable reference gene, which is not affected by the experimental conditions. In order to assess the stability of the candidate HKGs, an algorithm named geNorm was utilized to generate the M values to indicate the constancy of the candidate genes.[4] The results indicated that the M values for all candidate internal reference genes were less than 1.5 for each sample. Specifically, among the eight candidate HKGs, TUBB and 18S RNA are the most stable expressed genes under the condition of normoxia and hypoxia, respectively (Table 4). Furthermore, the analysis of the samples under all conditions confirmed that 18S RNA indicated as the most stable gene (Table 4).

Using the geNorm program, the optimum number of reference genes required for accurate normalization was calculated from the pairwise variation value ($V_{n/n+1}$) to determine whether the addition of another reference gene (n + 1) was recommended. The cut-off threshold value (0.15) was utilized to generate the optimal number of reference genes required for normalization.[4] In general, the more internal reference genes are used for standardization, the higher the reliability of target gene expression level.[6] However, according to the results, $V_{2/3}$ was less than 0.15, which means only two HKGs were enough for gene expression analysis of the cells under normoxia (Figure 3A and D) or hypoxia (Figure 3B and E) cultural environment. When each sample was analyzed together, the values ($V_{n/n+1}$) ranged between 0.013 and 0.020

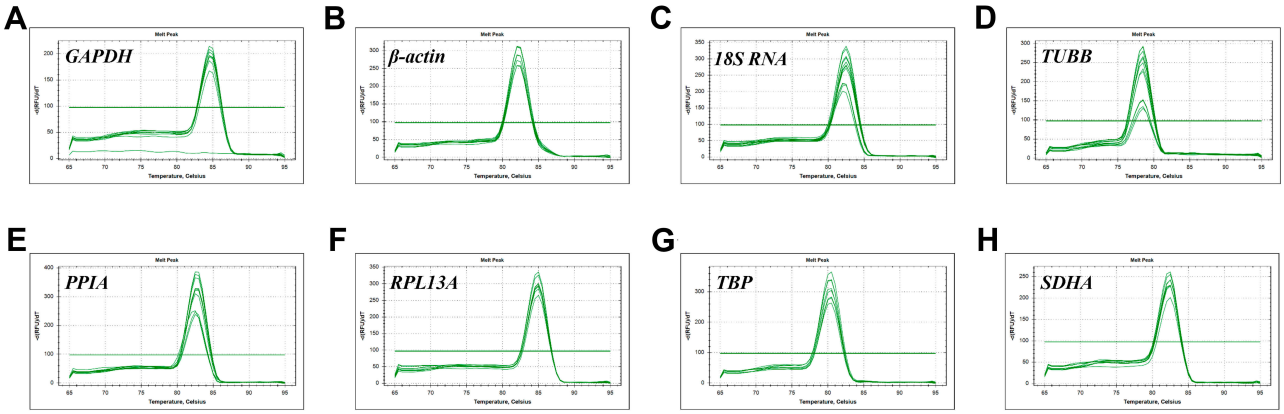


Figure 1 (A-H) Melting curves with single peaks generated from all amplifications. Specificity of RT-qPCR showing the amplification of a single product without dimer formation for each candidate housekeeping gene.

Table 3 Descriptive Statistics and Normality Evaluation of the Reference Genes Ct Values in Normoxic and Hypoxic Environment

	Gene	Mean	SD	CV(%)	Min Ct	Max Ct	SW-Test p
Normoxia	GAPDH	23.57	0.16	0.68	23.37	23.82	0.798
	β-actin	20.98	0.08	0.38	20.82	21.10	0.497
	18S RNA	23.28	0.07	0.30	23.15	23.37	0.507
	TUBB	21.32	0.05	0.23	21.21	21.38	0.350
	PPIA	16.78	0.12	0.72	16.64	17.02	0.166
	RPL13A	18.69	0.15	0.80	18.48	18.93	0.896
	TBP	24.53	0.18	0.73	24.24	24.77	0.895
	SDHA	26.18	0.13	0.50	26.03	26.44	0.300
Hypoxia	GAPDH	23.97	0.07	0.29	23.84	24.06	0.662
	β-actin	21.98	0.09	0.41	21.87	22.13	0.665
	18S RNA	23.95	0.03	0.13	23.91	24.00	0.712
	TUBB	22.22	0.11	0.50	22.08	22.38	0.740
	PPIA	17.37	0.15	0.86	17.18	17.57	0.622
	RPL13A	19.22	0.07	0.36	19.14	19.34	0.519
	TBP	25.36	0.25	0.99	25.08	25.75	0.448
	SDHA	26.45	0.08	0.30	26.36	26.55	0.415

Note: SW-test p, p-value of the Shapiro–Wilk test.
Abbreviations: SD, standard deviation; Min Ct, minimum Ct value; Max Ct, maximum Ct value.

and were all less than 0.15 (Figure 3C and F). Therefore, only two candidate genes are needed to normalize the target gene expression analysis, and then correct the system deviation to get reliable results, especially the small gene expression differences among the subjects under hypoxia.

Candidate Reference Genes Stability: NormFinder

The NormFinder algorithm ranks candidate housekeeping genes according to the intra- and intergroup variation in gene expression.[12] These results indicated that TUBB, GAPDH, β-actin and 18S RNA in the normoxia group (Table 5) and 18S RNA, GAPDH, SDHA and RPL13A in the hypoxia group (Table 5) were the steadiest expression candidate genes. 18S RNA, PPIA, RPL13A and GAPDH were the four most steady expression candidate genes in hypoxic and normoxic conditions (Table 5).

Candidate Reference Gene Stability: Determine Score

According to the combined outputs of geNorm and NormFinder, a determine score was used to determine the overall ranking of the final comprehensive expression stability. As shown in Table 6, TUBB (normoxia) and 18S RNA (hypoxia) were ranked as the most stable HKGs before and after exposure to a hypoxic environment, respectively. In

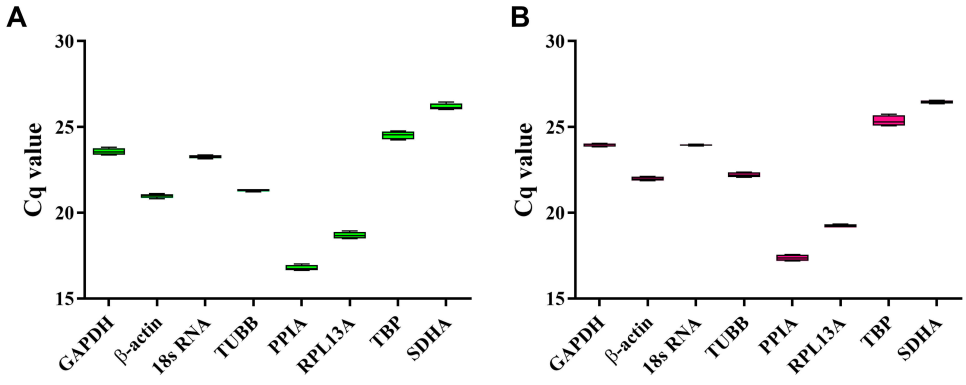


Figure 2 Cq value distributions for the candidate housekeeping genes. Boxplots of the Cq values for four samples of cells in normoxic (**A**) and hypoxic (**B**) culture conditions for each of the eight candidate reference genes.

Table 4 Calculation of Candidate Reference Genes M Value by the geNorm

Ranking Order	Gene	M Value (N+H)	Gene	M Value (N)	Gene	M Value (H)
1	18S RNA	0.013	TUBB	0.009	18S RNA	0.009
2	TBP	0.015	GAPDH	0.010	GAPDH	0.010
3	RPL13A	0.016	TBP	0.010	SDHA	0.010
4	GAPDH	0.016	β-actin	0.010	RPL13A	0.011
5	TUBB	0.017	18S RNA	0.011	β-actin	0.013
6	PPIA	0.018	SDHA	0.011	TUBB	0.015
7	β-actin	0.019	RPL13A	0.014	TBP	0.016
8	SDHA	0.020	PPIA	0.015	PPIA	0.016

both conditions, 18S RNA was indicated to be the most stable candidate gene for the normalization of the target genes expression analysis.

Confirmation of the Selected Reference Gene

Another two ovarian cancer cell lines, CAOV3 and OVCAR3, were used in this experiment to verify whether the selected reference gene was stably expressed in other cell lines under hypoxic conditions. After CAOV3 and OVCAR3 cells were exposed to hypoxia, the stability of the eight HKGs was also analyzed by geNorm and NormFinder. The results still indicated that 18S RNA was the most stably expressed housekeeping gene in CAOV3 and OVCAR3 cells after exposure to hypoxia (Tables 7 and 8, Supplemental Files 1-8).

Discussion

The improvement of the understanding of the mechanisms of hypoxia-induced epithelial–mesenchymal transition is a major focus of ovarian cancer research. Rapid and accurate analysis of gene expression by RT-qPCR is a common strategy to understand the mechanisms of EMT process.[14] Since the expression levels of candidate reference genes can vary by the tissue origin (eg, ovary or uterus) or disease origin (eg, adenoma or health tissue) of the sample and also by the experimental parameters (eg, hypoxia-treated cells or non-treated),[15,16] the appropriate internal reference gene

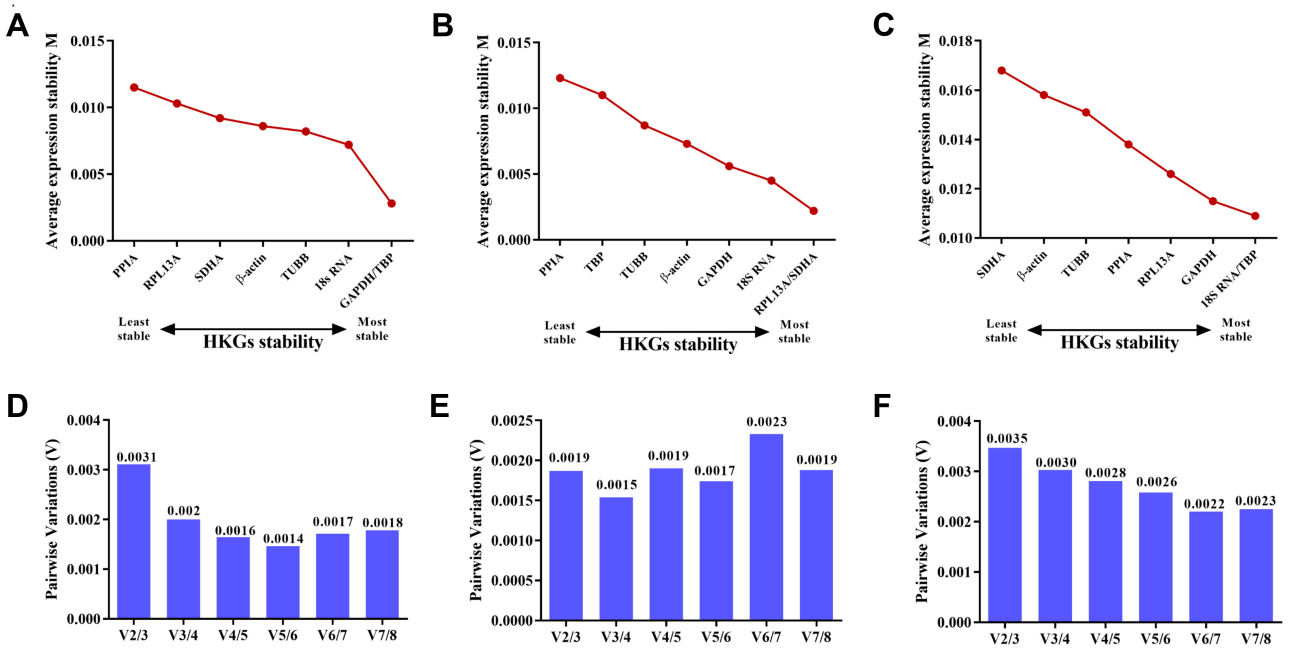


Figure 3 The geNorm selection analysis of +candidate reference genes. The average expression stability value (M) was calculated by geNorm for each gene in cells cultured under normoxia (**A**), hypoxia (**B**) or in both conditions (**C**). Pairwise variation (V) between the normalization factors (V_n and V_{n+1}) was used to determine the optimal number of reference genes for normalization under normoxia (**D**), hypoxia (**E**) or in both conditions (**F**).

Table 5 Calculation of Candidate Reference Genes Expression Stability by the NormFinder

Ranking Order	Gene	Stability Value (N+H)	Gene	Stability Value (N)	Gene	Stability Value (H)
1	18S RNA	0.063	TUBB	0.049	18S RNA	0.041
2	PPIA	0.133	GAPDH	0.094	GAPDH	0.047
3	RPL13A	0.135	β-actin	0.101	SDHA	0.074
4	GAPDH	0.169	18S RNA	0.119	RPL13A	0.092
5	TUBB	0.190	SDHA	0.143	β-actin	0.140
6	TBP	0.205	TBP	0.148	PPIA	0.162
7	β-actin	0.236	RPL13A	0.166	TUBB	0.206
8	SDHA	0.243	PPIA	0.166	TBP	0.296

Abbreviations: N, Normoxia; H, Hypoxia.

Table 6 Stabilities of HKGs Ranked by Determine Score

Rank	geNorm			NormFinder			Gene Name (Determine Score)		
	N	H	N+H	N	H	N+H	N	H	N+H
1	TUBB	18S RNA	18S RNA	TUBB	18S RNA	18S RNA	TUBB (2)	18S RNA (2)	18S RNA (2)
2	GAPDH	GAPDH	TBP	GAPDH	GAPDH	PPIA	GAPDH (4)	GAPDH (4)	RPL13A (6)
3	TBP	SDHA	RPL13A	β-actin	SDHA	RPL13A	β-actin (7)	SDHA (6)	GAPDH (8)
4	β-actin	RPL13A	GAPDH	18S RNA	RPL13A	GAPDH	18S RNA (9)	RPL13A (8)	PPIA (8)
5	18S RNA	β-actin	TUBB	SDHA	β-actin	TUBB	TBP (9)	β-actin (10)	TBP (8)
6	SDHA	TUBB	PPIA	TBP	PPIA	TBP	SDHA (11)	TUBB (13)	TUBB (10)
7	RPL13A	TBP	β-actin	RPL13A	TUBB	β-actin	RPL13A (14)	PPIA (14)	β-actin (14)
8	PPIA	PPIA	SDHA	PPIA	TBP	SDHA	PPIA (16)	TBP (15)	SDHA (16)

Abbreviations: N, normoxia; H, hypoxia

should be selected to correct the systematic error and obtain reliable results when analyzing the gene expression of ovarian cancer cells under hypoxia conditions. In fact, researchers usually choose only one single commonly used reference gene based on research experience and habits, such as GAPDH or β-actin, which was used in most gene expression studies of ovarian cancer under hypoxic conditions,[2,17] without estimation the expression stabilities of the internal reference genes under the specific environment. Therefore, in order to obtain reliable results, it is necessary to estimate the stabilities of candidate genes under hypoxic conditions. However, there have been few studies that have evaluated optimal reference gene(s) in normoxic and hypoxic conditions in ovary cancer.

In this study, eight commonly used housekeeping genes were selected to verify and evaluate their stability in normoxia and hypoxic environments using the NormFinder and geNorm programs. The present study revealed that 18S RNA was

Table 7 Stabilities of HKGs Ranked by Determine Score in CAVO3 Cells

Rank	geNorm	NormFinder	Gene Name (Determine Score)
1	18S RNA	18S RNA	18S RNA (2)
2	SDHA	SDHA	SDHA (4)
3	RPL13A	RPL13A	RPL13A (6)
4	TBP	TBP	TBP (8)
5	GAPDH	GAPDH	GAPDH (10)
6	PPIA	TUBB	PPIA (13)
7	TUBB	PPIA	TUBB (13)
8	β-actin	β-actin	β-actin (16)

Table 8 Stabilities of HKGs Ranked by Determine Score in OVCAR3 Cells

Rank	geNorm	NormFinder	Gene Name (Determine Score)
1	18S RNA	18S RNA	18S RNA (2)
2	RPL13A	SDHA	SDHA (5)
3	SDHA	RPL13A	RPL13A (5)
4	GAPDH	TUBB	TBP (10)
5	TBP	TBP	TUBB (10)
6	TUBB	PPIA	GAPDH (11)
7	β-actin	GAPDH	PPIA (14)
8	PPIA	β-actin	β-actin (15)

stably expressed in the presence of hypoxic condition and can be used as a reliable reference gene for relative gene normalization and quantification in normoxic or hypoxic environments.

Previous studies have reported that the β2-MG level was steady in the presence of different oxygen concentrations.[18] In bladder cancer cells under hypoxic conditions, it was indicated that hypoxanthine phosphoribonucleotransferase-1 (HPRT) and β2-MG were optimal and stable housekeeping genes for the determination of gene expression.[19] Studies of human adipose-derived stem cells revealed that tyrosine 3/tryptophan 5-monooxygenase activation protein (YMHAZ), β-glucuronidase (GUSB) and TBP were the most stable reference genes in all conditions.[20] Therefore, this study indicated that the stress response related genes β2-MG and TBP were not eligible for the normalization of target genes expression in ovarian cancer cells under hypoxia.

Under normoxia, the most stable expression genes were GAPDH/TBP in ovarian cancer cells, whereas under hypoxia, the most stable candidate housekeeping gene were RPL13A/SDHA according to the geNorm algorithm results. When combining samples treated under different conditions, 18S RNA was selected as the most stable candidate housekeeping gene based on the NormFinder and geNorm algorithm. Furthermore, 18S RNA was also stably expressed in another two ovarian cancer cell lines, CAOV3 and OVCAR3, in the same hypoxic environment. These results indicated that 18S RNA could be used as a reference gene when conducting RT-qPCR experiments in ovarian cells subjected to hypoxic stress.

In normoxic and hypoxic conditions, the stability of candidate housekeeping genes should be routinely analyzed for target genes expression analysis in cancer cells prior to performing RT-qPCR. However, previous studies on target gene expression analysis in ovarian cancer cells under hypoxia used GAPDH[21] as the reference gene for normalization. Such conventional reference genes always vary in different culture environments. In the present study, the candidate reference genes expression stabilities were estimated in cells under normoxia and hypoxia stress. According to the present results, 18S RNA, which was estimated as the most stable and suitable gene, should be used as the internal reference for normalizing target gene expression in ovarian cancer cells with different oxygen concentrations in culture environment.

Conclusion

The present study identified TUBB in ovarian cancer cells under normoxia as the most stable reference gene, whereas 18S RNA in ovarian cancer cells under hypoxia was the most stable reference gene. Among the selected stably expressed housekeeping genes in both normoxic and hypoxic conditions, 18S RNA was the most highly recommended, but this depended on the ovarian cancer cells used as well as the normoxic and hypoxic conditions.

Data Sharing Statement

All data supporting the conclusion of this article are contained within the manuscript (Supplement Files 9-12).

Acknowledgments

The authors thank Jiawen Zhang for helpful discussion and review of this manuscript.

Author Contributions

All authors contributed to data analysis, drafting or revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

Disclosure

The authors have no conflicts of interest.

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