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Original Research

Validation of housekeeping genes for the normalization of RT-qPCR expression studies in oral squamous cell carcinoma cell line treated by 5 kinds of chemotherapy drugs

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Abstract: Reverse transcription quantitative polymerase chain reaction (RT-qPCR) has become a frequently used strategy in gene expression studies. The relative quantification method is an important and commonly used method for the evaluation of RT-qPCR data. The key of this method is to identify an applicable internal control gene because the usage of different internal control genes may lead to distinct conclusions. Herein, we report the validation of 12 common housekeeping genes for RT-qPCR for gene expression analysis in the Oral squamous cell carcinoma (OSCC) cell line (KB and Tca-8113) treated by 5 kinds of Chemotherapy Drugs. The gene expression stability and applicability of the 12 housekeeping gene candidates were determined using the geNorm, NormFinder, and BestKeeper software programs. Comprehensive analyzing the results of the three software, ALAS1/GAPDH, ALAS1 and GUSB were suggested to be the most stable candidate genes for the study of both KB and Tca-8113 cell line together, KB cell line, and Tca-8113 cell line, respectively. This study provides useful information to normalize gene expression accurately for the investigation of target gene profiling in cell lines of OSCC. Further clarification of tumor molecular expression markers with our recommended housekeeping genes may improve the accuracy of diagnosis and estimation of prognostic factors as well as provide novel personalized treatments for OSCC patients.

Key words: Reverse transcription quantitative polymerase chain reaction, Housekeeping gene, Oral squamous cell carcinoma, Chemotherapy treatment, Expression Stability.

Introduction

Real-time quantitative polymerase chain reaction (RT-qPCR) is a common strategy for investigating gene expression in biomedical studies (1). RT-qPCR is accurate, sensitive, and rapid method for the determination of gene expression. It is considered as the gold standard for gene expression studies. Among all analysis strategies, relative quantification is a relatively simple and common technique to evaluate the expression levels of target genes (2). A stably expressed endogenous control gene is used as a standard to determine the expression levels of target genes in the same biological sample. Therefore, the accurate determination of gene expression levels depends on the selection of a reliable internal control gene for normalization (3). The identification of appropriate internal control genes is a crucial in the relative quantification analysis. An ideal internal control gene should be universally stable under various experimental conditions (4-6). In general, housekeeping genes, such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-actin (ACTB), and ribosomal RNA (18S rRNA), are chosen as internal control genes for the relative quantification analysis between clinical samples. However, amounting studies have demonstrated variability of these commonly used housekeeping genes in different tissues or between treatments in the same tissue (7-10), as well as across cell types (11-13). Thus, with the advance of precise medicine, it is of high importance to identify and validate housekeeping genes for the study of target gene expression profiles for different cell types and tissues.

Oral squamous cell carcinoma (OSCC) represents more than 80% of all forms of head and neck cancer, and during the past decade its incidence has increased by 50% (14). Due to the poor response to therapy and high recurrence rates (15), researchers are still working on identifying new targets point and therapy for OSCC. KB and Tca-8113 are two commonly used cell line for studying OSCC. Human oral epidermoid carcinoma cell line KB first established in 1954, it is squamous cell carcinoma cell line which is primary in the Mouth Floor (16). The cells are positive for keratin by immunoperoxidase staining. KB cells have been reported to contain human papillomavirus 18 (HPV-18) sequences. Squamous cell carcinoma of tongue cell line Tca-8113 was established from in situ biopsy tissue of a I grade squamous cell carcinoma (T2N1Amo, II stage) tongue carcinoma case in 1987 by dry adherent method. Among the treatment strategies of OSCC, Cyclophosphamide (CTX), cis-Dichlorodiamineplatinum (II) (DDP), Paclitaxel (PTX), Vincristine (VCR) and 5-Fluorouracil (5-FU) are the most widely used chemotherapy drugs (17-20). With the great advance of functional genomic and proteomics studies in cancer research, personalized medicine has become possible. However, personalized treatment of a disease, especially cancer, relies on the identification and validation of the drivers for the di-

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sease. RT-qPCR is a frequently used technique to investigate gene expression differences; thus, it is important to establish the normalization standards used in the quantitative gene expression studies of human laryngeal cancer. To the best of our knowledge, no systematic study has been performed on the selection of suitable housekeeping genes for investigating target gene profiling of OSCC cell lines which are treated by 5 kinds of Chemotherapy Drugs.

A number of genes including 18S rRNA, GAPDH, ACTB, HPRT1, RPL29, HMBS, PPIA, ALAS1, TBP, PUM1, GUSB, and B2M have been identified as optimal housekeeping genes in certain other cancers (21, 22). In order to provide useful information for the selection of suitable housekeeping genes in further gene expression studies of OSCC by RT-qPCR, we validated these 12 candidate genes for gene expression studies in KB (human oral epidermoid carcinoma cell line) and Tca-8113 (squamous cell carcinoma of tongue cell line).

Materials and Methods

Cell culture and treatment

KB and Tca-8113 were provided by the Research Center of Second Hospital, Jilin University (Changchun, China) and cultivated, according to the recommendation of the supplier, in IMDM containing 10% FBS with 100 units of Penicillin-Streptomycin for KB and RPMI 1640 containing 10% FBS with 100 units of Penicillin-Streptomycin for Tca-8113, maintained at 37 °C in 5% CO_2 humidified atmosphere.

KB and Tca-8113 cell lines were treated at a confluency of 80–90% with CTX (final concentration was 2 mg/ml for KB and Tca-8113), DDP (final concentration was 6 μg/ml for KB and Tca-8113), PTX (final concentration was 9 μg/ml for KB and 6 μg/ml for Tca-8113), VCR (final concentration was 2.75 μg/ml for KB and 0.1 μg/ml for Tca-8113) and 5-FU (final concentration was 50μg /ml for KB and Tca-8113) respectively. Cells that were treated with medium were used as the control. The cells were collected 24 h after the treatment.

RNA extraction and complementary DNA (cDNA) synthesis

Total RNA was extracted from each group using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) following the manufacturer's protocol. Remove growth media from culture dish, then add 1 ml TRIzol reagent directly to the cells in the 9.6 cm^2 culture dishes. Lyse the cells directly in the culture dish by pipetting the cells up and down several times, then incubate for 5 min at room temperature. Add 0.2 mL of chloroform, shake tube vigorously by hand for 15 seconds, and incubate for 2 minutes at room temperature. Centrifuge the sample at 12,000 g for 15 minutes at 4°C. Remove the aqueous phase into a new tube, add 0.5 ml isopropanol, and then incubate at room temperature for 10 minutes. Centrifuge at 12,000 g for 10 minutes at 4°C. Remove the supernatant from the tube, add 1 ml of 75% ethanol. Vortex the sample briefly, then centrifuge the tube at 12,000 g for 5 minutes at 4°C. Discard the wash and air dry the RNA pellet for 5 minutes. Resuspend the RNA pellet in 1 μl of RNase-free water. 1 μg of total RNA was used for cDNA synthesis reaction which was performed using an M-MuLV First Strand cDNA Synthesis kit (Sangon Company, China), according to the manufacturer's instructions.

RT-qPCR

The primers of 12 putative housekeeping genes were selected based on previous studies (23, 24) and synthesized by Sangon Company (Shanghai, China) (Table 1). RT-qPCR analysis using 2×SG Fast qPCR Master Mix (Sangon Company, China) was performed on Roche LightCycler 480 detection system (Roche Diagnostics, Germany) as previously described (8, 9). The RT-qPCR was repeated three times for each sample. The cycle threshold value (Cp value) data were analyzed using the equation of relative quantities (Q): $Q = 2^{-\Delta Cp}$ (25).

Statistical analysis

All the samples were divided into 3 groups: total group, KB group and Tca-8113 group. Three frequently used software programs, geNorm (26) (http://medgen.ugent.be/~jvdesomp/genorm/), NormFinder(27) (http://moma.dk/normfinder-software), and BestKeeper(28) (http://www.gene-quantification.de/bestkeeper.html), were utilized to better evaluate the stability of the reference genes. GeNorm was designed to sort reference genes by the stability of expression through analyzing and determinng the M-value. M is the mean pair-wise variation for a given gene compared to other tested genes with stepwise exclusion of the gene with the highest M value, and it is recalculated in order to select the two most stable genes. Lower M value indicates more stable of the gene, while higher M value indicates less stable of the gene. The gene is not suitable for use as a reliable reference gene if its M is higher than 1.5. The other function of GeNorm software is to analyze the pair-wise variation value of the normalization factor (V) to determine whether adding a new reference gene will affect the normalization factor. If the value of V(n/n+1) is higher than 0.15, it is necessary to increase the number of reference genes as internal controls in order to obtain accurate results, until the value is lower than 0.15. NormFinder has a similar operation principle to geNorm and ranks the set of candidate normalization genes according to their expression stability. The other function of NormFinder is to compare the inter- and intra-group stabilities of candidate genes and propose an optimal combination of two genes. BestKeeper evaluates candidate reference gene stability based on the correlation coefficient (R-value). Higher R value indicates more stable and reliable the gene.

Results

The Cp value was employed to assess the expression levels of the candidate reference genes. A higher Cp value indicates a lower amount of expression. As shown in Table 2, the Cp values of all the samples ranged between 8.19 (18S rRNA) and 29.94 (PUM1).

The expression stability of the candidate housekeeping genes

To better evaluate the expression stability of the housekeeping genes, geNorm, NormFinder, and BestKeeper software were utilized. According to the results of

Table 1. Summary of housekeeping genes used in the present study.

Symbol	Official full name	Accession Number	Primer sequence	Product size (bp)
18S	18S ribosomal RNA	NM_10098.1	F: CGGCTACCACATCCAAGGAA	186
			R: GCTGGAATTACCGCGGCT	
GAPDH	glyceraldehyde-3-phosphate dehydrogenase	NM_002046.5	F: GACAGTCAGCCGCATCTTCT	127
			R: TTAAAAGCAGCCCTGGTGAC	
B2M	beta-2-microglobulin	NM_004048.2	F: AGCGTACTCCAAAGATTCAGGTT	306
			R:ATGATGCTGCTTACATGTCTCGAT	
ACTB	actin, beta	NM_001101.3	F: AGAAAATCTGGCACCACACC	173
			R: TAGCACAGCCTGGATAGCAA	
ALAS1	5'-aminolevulinate synthase 1	NM_000688.5	F: GGCAGCACAGATGAATCAGA	150
			R: CCTCCATCGGTTTTCACACT	
GUSB	glucuronidase, beta	NM_000181.3	F: AGCCAGTTCCTCATCAATGG	160
			R: GGTAGTGGCTGGTACGGAAA	
HPRT1	hypoxanthine phosphoribosyl transferase 1	NM_000194.2	F: GACCAGTCAACAGGGGACAT	132
			R: CCTGACCAAGGAAAGCAAAG	
HMBS	hydroxymethylbilane synthase	NM_000190.3	F: AGTGTGGTGGGAACCAGC	144
			R: CAGGATGATGGCACTGAACTC	
PPIA	peptidylprolyl isomerase A	NM_021130.4	F: AGACAAGGTCCCAAAGAC	118
			R: ACCACCCTGACACATAAA	
PUM1	pumilio RNA-binding family member 1	NM_001020658.1	F: CAGGCTGCCTACCAACTCAT	217
			R: GTTCCCGAACCATCTCATTC	
RPL29	ribosomal protein L29	NM_000992.2	F: GGCGTTGTTGACCCTATTTC	120
			R: GTGTGTGGTGTGGTTCTTGG	
TBP	TATA box binding protein	NM_003194.4	F: TGCACAGGAGCCAAGAGTGAA	132
			R: CACATCACAGCTCCCCACCA	

Table 2. Cp values of candidate housekeeping genes in various groups ($\bar{x} \pm s$).

Group	Total group	KB group	Tca-8113 group
18S	8.70 ± 0.29	8.77 ± 0.23	8.63 ± 0.35
GAPDH	16.99 ± 0.90	17.32 ± 1.09	16.67 ± 0.57
ACTB	17.39 ± 1.07	17.78 ± 1.15	16.99 ± 0.92
PPIA	18.57 ± 1.11	19.30 ± 1.12	17.85 ± 0.44
TBP	18.73 ± 1.05	18.85 ± 0.93	18.61 ± 1.24
B2M	22.17 ± 1.00	22.08 ± 1.36	22.26 ± 0.59
HMBS	23.41 ± 1.03	23.92 ± 0.86	22.90 ± 0.99
RPL29	24.32 ± 0.94	24.36 ± 1.29	24.29 ± 0.51
ALAS1	24.60 ± 1.29	25.00 ± 1.58	24.21 ± 0.89
HPRT1	24.71 ± 0.82	24.97 ± 1.05	24.45 ± 0.48
GUSB	24.84 ± 1.13	25.60 ± 0.91	24.09 ± 0.80
PUM1	25.15 ± 1.96	24.69 ± 1.75	25.61 ± 2.21

geNorm, in the total group, ALAS1 and GAPDH had the lowest M-values, suggesting that they are the most stable candidate genes for both the study of KB and Tca-8113 in the condition of being treated by 5 kinds of Chemotherapy Drugs; in KB group, ALAS1 and GUSB had the lowest M-values; in Tca-8113 group, B2M and RPL29 had the lowest M-values (Table 3). A combination of 3 housekeeping genes was optimal for the total group with V3/4 of 0.131; a combination of 2 housekeeping genes was optimal for the KB group and Tca-8113 group, with V2/3 of 0.143 and 0.093 (Figure 1).

According to the results of NormFinder program, the combination of GAPDH and ALAS1 (Stability value 0.115) was the most stable in the total group, while ALAS1 was the most stably expressed gene in this group, followed by GAPDH. In the KB group, ALAS1 was the most stably expressed gene, followed by PPIA. In the Tca-8113 group, GAPDH was the most stably expressed gene, followed by RPL29 (Table 3).

The limitation of BestKeeper program is that only 10 reference genes can be analyzed for one time. Therefore we should remove the two most unstable internal reference genes as indicated by the geNorm program from each group before analyzing. In terms of the R-value, the most stable internal housekeeping gene in total group was GAPDH, followed by ALAS1; while in the

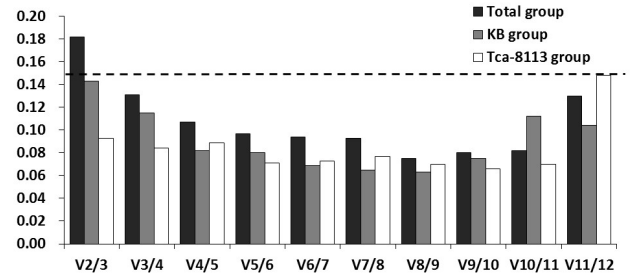

Figure 1. Determination of the optimal number of housekeeping genes for normalization by the result of geNorm analysis. The x-axis represents the number of genes used for the combined analysis, and the y-axis represents the pair-wise variation value. In V(n/n+1), n is incremented by 1; when the first occurrence of the V(n/n+1) value is less than 0.15, n represents the optimal number of housekeeping genes that should be combined to achieve a satisfactory accuracy in the analysis.

KB group, the most stable internal housekeeping gene was B2M, followed by ALAS1; in the Tca-8113 group, the most stable internal housekeeping gene was GUSB, followed by HMBS (Table 3).

Final ranking of the candidate housekeeping genes

Housekeeping gene rankings obtained with all 3 algorithms (GeNorm, NormFinder and BestKeeper) were compared. While housekeeping gene rankings vary slightly by algorithm, a method previously described (29) was used to give an overall ranking of the best candidate housekeeping genes. The geometric means of the 3 ranking numbers were calculated, and the gene with a smaller geometric mean is the most stable housekeeping gene. The recommended comprehensive rankings were also given in Table 3. Using the results from all 3 algorithms, an overall ranking of candidate

Table 3. Twelve candidate housekeeping genes ranked by different software.

Rank (weight)	Program								
	geNorm		NormFinder		BestKeeper			Final ranking	
	Gene	M-value	Gene	Stability value	Gene	R	SD	Gene	Geo Mean
Total group									
1	ALAS1	0.429	ALAS1	0.133	GAPDH	0.930	0.611	ALAS1	1.260
2	GAPDH	0.429	GAPDH	0.152	ALAS1	0.913	0.556	GAPDH	1.260
3	B2M	0.533	ACTB	0.261	ACTB	0.899	0.745	ACTB	3.557
4	GUSB	0.569	PPIA	0.266	B2M	0.894	0.750	B2M	4.932
5	ACTB	0.596	TBP	0.315	GUSB	0.883	0.852	HPRT1	6.000
6	HPRT1	0.628	HPRT1	0.329	HPRT1	0.862	0.772	GUSB	6.037
7	TBP	0.665	HMBS	0.339	PPIA	0.850	0.781	PPIA	6.316
8	RPL29	0.708	18S rRNA	0.379	TBP	0.824	0.644	TBP	6.542
9	PPIA	0.734	RPL29	0.382	RPL29	0.773	0.717	RPL29	8.653
10	18S rRNA	0.789	B2M	0.436	18S rRNA	0.243	0.240	HMBS	8.775
11	HMBS	0.836	GUSB	0.439	HMBS	-	-	18S rRNA	9.283
12	PUM1	0.972	PUM1	0.631	PUM1	-	-	PUM1	12.000
KB group									
1	ALAS1	0.272	ALAS1	0.140	B2M	0.977	0.733	ALAS1	1.260
2	GUSB	0.272	PPIA	0.219	ALAS1	0.975	0.764	B2M	2.289
3	B2M	0.387	GAPDH	0.224	TBP	0.971	0.872	GUSB	3.271
4	GAPDH	0.447	B2M	0.246	RPL29	0.970	0.902	GAPDH	3.915
5	PPIA	0.465	GUSB	0.282	GAPDH	0.947	0.708	PPIA	4.309
6	RPL29	0.494	RPL29	0.304	ACTB	0.921	0.845	RPL29	5.241
7	TBP	0.515	HPRT1	0.328	GUSB	0.915	0.696	TBP	5.518
8	ACTB	0.537	TBP	0.329	PPIA	0.914	0.661	ACTB	7.560
9	HPRT1	0.567	ACTB	0.369	HPRT1	0.879	0.632	HPRT1	8.277
10	PUM1	0.618	PUM1	0.575	PUM1	-	1.188	PUM1	10.000
11	18S rRNA	0.738	18S rRNA	0.826	18S rRNA	-	-	18S	11.000
12	HMBS	0.842	HMBS	0.860	HMBS	-	-	HMBS	12.000
Tca-8113 group									
1	B2M	0.229	GAPDH	0.145	GUSB	0.924	0.638	GUSB	2.410
2	RPL29	0.229	GUSB	0.175	HMBS	0.904	0.629	GAPDH	2.621
3	18S rRNA	0.276	HMBS	0.247	GAPDH	0.885	0.409	B2M	3.107
4	TBP	0.324	ACTB	0.295	HPRT1	0.848	0.786	HMBS	3.634
5	ALAS1	0.388	B2M	0.308	ACTB	0.842	0.616	RPL29	4.642
6	GAPDH	0.421	ALAS1	0.316	B2M	0.831	0.343	18S rRNA	5.518
7	GUSB	0.464	HPRT1	0.343	18S rRNA	0.772	0.286	ACTB	5.646
8	HMBS	0.521	18S rRNA	0.378	ALAS1	0.745	0.381	ALAS1	6.214
9	ACTB	0.563	TBP	0.393	TBP	0.720	0.428	HPRT1	6.542
10	HPRT1	0.599	RPL29	0.411	RPL29	0.702	0.443	TBP	6.868
11	PPIA	0.648	PPIA	0.497	PPIA	-	-	PPIA	11.000
12	PUM1	0.842	PUM1	1.224	PUM1	-	-	PUM1	12.000

housekeeping genes was obtained. ALAS1 and GAPDH were suggested to be the most stable candidate genes for the study of KB and Tca-8113 cell lines treated by chemotherapy drug; ALAS1 was suggested to be the most stable candidate genes for the study of KB with chemotherapy drug treatment, follow by B2M; GUSB was suggested to be the most stable candidate genes for the study of Tca-8113 with chemotherapy drug treatment, follow by GAPDH.

Discussion

In relative quantitative analysis, the expression stability of reference genes is crucial to the accuracy of the results. Many studies have shown that the expression level of common reference genes vary significantly among different samples, for example, under various experimental conditions, in different cell types and tissues, from different individuals, and during different cell proliferation or organ development stages (8-10). With the rapid requirement for studies investigating laryngeal cancer gene expression profiles, it is necessary to confirm stable and reliable internal control genes for RT-qPCR. To the best of our knowledge, the present study is the first to compare the stability of commonly used housekeeping genes in OSCC cell lines with common chemotherapy drug treatments.

To obtain accurate experimental data and reliable conclusions, the present study used an experimental process with a number of characteristics. A total of 12 common housekeeping genes (18S rRNA, GAPDH, ACTB, HPRT1, RPL29, HMBS, PPIA, ALAS1, TBP, PUM1, GUSB, and B2M) were investigated in terms of their expression stability. The data were analyzed by the geNorm, NormFinder, and BestKeeper software programs, which are designed for investigating the stability of reference genes. Since the ranking of the candidate gene stability was slightly different, which was possibly caused by different calculation algorithms (30, 31), The geometric means of the 3 ranking numbers were calculated to give an overall ranking of the best candidate housekeeping genes, the gene with a smaller geometric mean is the most stable housekeeping gene. The final ranking of the candidate housekeeping genes shows that ALAS1/GAPDH, ALAS1 and GUSB were suggested to be the most stable candidate genes for the study of both KB and Tca-8113 cell line together, KB cell line, and Tca-8113 cell line, respectively. In addition, by calculating the value of V, the optimal number of housekeeping genes used in combination was 2-3 in each group. Based on the results of the 3 software programs, the recommended combination for the total group was GAPDH + ALAS1; for the KB cell line, it was ALAS1 + B2M; for the Tca-8113 cell line, it was GUSB + GAPDH.

In summary, the expression of mRNA has a large variation in different cell types or between treatments in the same cell types, it is important to validate the expression stabilities of housekeeping genes and select the most stable housekeeping gene before further quantitative studies in tissue samples. The present study identified the most suitable housekeeping genes and housekeeping gene combinations for gene expression profile analysis of OSCC cell line treated by 5 kinds of chemotherapy drugs. The relevant clarification of tumor molecular expression markers with our recommended reference genes may improve the accuracy of gene expression studies in OSCC. However, in the specific implementation process, it is suggested to further test the internal reference genes recommended according to the specific experimental conditions, as many factors can influence reference gene stability.

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