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Validation of reference genes for the normalization of RT-qPCR expression studies on human laryngeal cancer and hypopharyngeal cancer

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Abstract. – OBJECTIVE: Selecting stably expressed reference genes is crucial for evaluating real-time quantitative polymerase chain reaction (RT-qPCR) data via the relative quantification method. In the present-day study, our aim was to select optimal reference genes (RGs) for the investigation of target gene (TG) expression profiling in cancerous human laryngeal and hypopharyngeal tissues.

PATIENTS AND METHODS: 12 cancerous laryngeal tissues and 10 cancerous hypopharyngeal tissues were investigated. The expression characteristics of 11 reference genes (18S rRNA, GAPDH, B2M, ACTB, TBP, ALAS1, RPL29, HMBS, HPRT1, GUSB, and PUM1), which were commonly used in RT-qPCR for the analysis of gene expression, were investigated using the geNorm, NormFinder, and BestKeeper algorithm programs.

RESULTS: HMBS, ALAS1, and B2M were suggested as optimal RGs for studying human laryngeal and hypopharyngeal cancerous tissues together, laryngeal cancerous tissue by itself, and hypopharyngeal cancerous tissue by itself, respectively. If 2 or more reference genes are needed to achieve better standardization, 3 reference genes can optimally be used in combination to improve the accuracy of relative quantitation normalization. The recommended combinations for studying human laryngeal and hypopharyngeal cancerous tissues together, laryngeal cancerous tissue by itself, and hypopharyngeal cancerous tissue by itself were HMBS + HPRT1 + GUSB, ALAS1 + GUSB + HMBS, and B2M + HPRT1 + TBP, respectively.

CONCLUSIONS: The recommended reference genes could be used to improve the accuracy of gene expression studies on the molecular mechanisms of cancerous human laryngeal and hypopharyngeal tissues. The selected combination of reference genes can effectively improve the accuracy of the relative quantitative diagnosis of gene expression levels, such as messenger RNA, circular RNA, and long-noncoding RNA.

Key Words:
Reverse transcription quantitative polymerase chain reaction, Reference gene, Human laryngeal cancer, Human hypopharyngeal cancer, Expression stability, Normalization.

Introduction

Relative quantification analysis is a common, accurate, and easy-to-operate method that is widely used in many gene expression investigations of molecular biology. A stably expressed internal control gene is used as a standard to measure and compare the relative expression levels of TGs in the same biological sample. Therefore, evaluating and identifying appropriate reference genes in relative quantification analysis are very important. Only reference genes that are expressed stably under various experimental conditions can be considered ideal[1-3]. However, increasing studies have demonstrated that the expression levels of most RGs commonly used such as β-actin (ACTB), ribosomal RNA (18S rRNA), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), are variable across cell types or distinct tissues[4-7], even between treatments of the same tissue[8-10]. Thus, identifying and choosing optimal reference genes before performing the study among various kinds of cell types and tissues at the gene expression level through relative quantification analysis are very important[11].

Laryngeal cancer, also referred to as LC, is a common squamous cell carcinoma capable of developing in any part of the larynx, and the cure rate of laryngeal cancer is affected by the location

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of the tumor. Therefore, for tumor staging, the larynx is divided into three anatomical regions: the glottis (true vocal cords, anterior and posterior commissures); the supraglottis (epiglottis, arytenoids, aryepiglottic folds, and false cords); and the subglottis[12]. The hypopharynx can also be divided into three anatomic zones: the pyriform fossa, the back of the cricoid cartilage region, and the posterior wall of the hypopharynx region. Hypopharyngeal cancer occurs most often in the piriform fossa, followed by the posterior wall of the hypopharynx region, and minimally, in the back of the cricoid cartilage region. Most (approximately 95%) primary malignant tumors in the hypopharynx are squamous cell carcinomas, and the combination of comprehensive therapy, surgery, and radiation therapy is widely accepted as the most effective treatment. Location and TNM staging are the most important factors affecting the prognosis and treatment of hypopharyngeal cancer[13]. With great advances in cancer research using functional genomics and proteomics, individualized medicine has been made possible. However, the personalized treatment of disease, especially cancer, depends on identifying and validating drivers of the disease. Real-time quantitative polymerase chain reaction (RT-qPCR) is a technique that's frequently utilized to investigate the gene expression differences[14-16]; therefore, establishing normalization standards for quantitative gene expression studies of human laryngeal cancer (HLC) is important [17]. To our best knowledge at present, no study has been systematically performed on selecting the suitable reference genes for profiling human laryngeal cancer and hypopharyngeal cancer target genes between parenchymal and paracancerous tissues.

Some genes including 18S rRNA, GAPDH, ACTB, beta-2-microglobulin (B2M), TATA-box binding protein (TBP), 5'-aminolevulinate synthase 1 (ALAS1), ribosomal protein L29 (RPL29), hydroxymethylbilane synthase (HMBS), hypoxanthine phosphoribosyltransferase 1 (HPRT1), glucuronidase beta (GUSB), and pumilio RNA binding family member 1 (PUM1) have been identified as optimal RGs in other cancers[17-19]. We validated these 11 candidate genes in order to provide useful information on selecting suitable reference genes in the future gene expression studies of HLC and hypopharyngeal cancer by RT-qPCR.

Patients and Methods

Cancerous Laryngeal and Hypopharyngeal Tissue Samples

12 cancerous laryngeal tissues and 10 cancerous hypopharyngeal tissues, of both parenchymal and paracancerous origin, were collected from Department of Otorhinolaryngology, Head and Neck Surgery at the First Clinical Hospital of Jilin University (Changchun, Jilin, China). Patient clinicopathological characteristics and general information are summarized in Table I.

Table I. Clinicopathological characteristics of patients with laryngeal cancer and hypopharyngeal cancer.

Clinicopathological characteristic	Value	
	Laryngeal cancer	Hypopharyngeal cancer
Age (mean ± standard deviation; years)	61.10 ± 7.61	57.36 ± 7.06
Gender		
Male	9	9
Female	3	1
Histopathological type		
Squamous cell carcinomas	12	10
Adenocarcinoma	0	0
TNM stage*		
Stage 0	0	0
Stage I	0	0
Stage II	3	0
Stage III	6	3
Stage IVa	3	6
Stage IVb	0	1
Stage IVc	0	0

According to the Union for International Cancer Control.

Ethics Committee Approval

The Ethics Committee of the First Clinical Hospital of Jilin University fully understood and approved the present study, while written consent was obtained from each patient.

RNA Extraction and Complementary DNA (cDNA) Synthesis

TRIzol reagent (Invitrogen Life Technologies, Waltham, MA, USA) was used to extract total RNA from 10-100 mg of the tissue samples. A NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) was used to measure the concentration and purity of the isolated RNA. DNase I was used to eliminate residual genomic DNA, and 1 μg of total RNA was used for reverse transcription by the M-MuLV First-Strand cDNA Synthesis kit (Sangon Company, Shanghai, China). All operations were carried out according to the manufacturer's instructions.

RT-qPCR (Reverse-Transcrip Quantitative PCR

The primer sequences of 11 candidate reference genes (18S rRNA, GAPDH, B2M, ACTB, TBP, ALAS1, RPL29, HMBS, HPRT1, GUSB, and PUM1) and a target gene (TLR2), all based on the previous studies[17,20,21], are listed in Table II. The RT-qPCR was performed on a Roche LightCycler 480 instrument (Roche, Basel, Switzerland) using 2× SG Fast qPCR Master Mix (Sangon, Shanghai, China). All experiments were repeated twice, and the *Cp*-values were pre-converted into relative quantities (Q) using the equation $Q = 2^{-\Delta Cp}$ for subsequent statistical analysis[22]. This study was done with reference to MIQE recommendation[23].

Statistical Analysis

We divided the samples into 3 groups: total (both laryngeal and hypopharyngeal cancerous tissue), laryngeal cancer and hypopharyngeal cancer. Three algorithm programs, geNorm[24], NormFinder[25], and BestKeeper[26], were utilized to assess the stability of the reference genes.

Table II. Summary of reference genes used in the present study.

Symbol	Official full name	Accession No.	Primer sequence	Product size (bp)
18S rRNA	18S ribosomal RNA	NM_10098.1	F: CGGCTACCACATCCAAGGAA R: GCTGGAATTACCGCGGCT	186
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	NM_002046.5	F: GACAGTCAGCCGCATCTTCT R: TTAAAAGCAGCCCTGGTGAC	127
B2M	Beta-2-microglobulin	NM_004048.2	F: AGCGTACTCCAAAGATTCAGGTT R: ATGATGCTGCTTACATGTCTCGAT	306
ACTB	Actin, beta	NM_001101.3	F: AGAAAATCTGGCACCACACC R: TAGCACAGCCTGGATAGCAA	173
ALAS1	5'-aminolevulinate synthase 1	NM_000688.5	F: GGCAGCACAGATGAATCAGA R: CCTCCATCGGTTTTCACACT	150
GUSB	Glucuronidase, beta	NM_000181.3	F: AGCCAGTTCCTCATCAATGG R: GGTAGTGGCTGGTACGGAAA	160
HPRT1	Hypoxanthine phosphoribosyl transferase 1	NM_000194.2	F: GACCAGTCAACAGGGGACAT R: CCTGACCAAGGAAAGCAAAG	132
HMBS	Hydroxymethylbilane synthase	NM_000190.3	F: AGTGTGGTGGGAACCAGC R: CAGGATGATGGCACTGAACTC	144
PUM1	Pumilio RNA-binding family member 1	NM_001020658.1	F: CAGGCTGCCTACCAACTCAT R: GTTCCCGAACCATCTCATTC	217
RPL29	Ribosomal protein L29	NM_000992.2	F: GGCGTTGTTGACCCTATTTC R: GTGTGTGGTGTGGTTCTTGG	120
TBP	TATA box binding protein	NM_003194.4	F: TGCACAGGAGCCAAGAGTGAA R: CACATCACAGCTCCCCACCA	132
TLR2	Toll like receptor 2	NC_000004.12	F: GGTTCAAGCCCCTTTCTTCT R: TTCCCACTCTCAGGATTTGC	117

F: forward; R: reverse.

Validation of Reference Genes

TLR2 acted as the target gene (TG) invalidating the control genes for the normalization of relative quantities in parenchymal and paracancerous tissues. We evaluated the expression patterns of TLR2 using each of the 11 reference genes as internal controls. In earlier studies, the transcription level of TLR2 was significantly higher in cancerous parenchymal tissue than in paracancerous tissue (ACTB served as the reference gene)[27]. The relative quantity of each sample was normalized to that of each of the 11 control genes using the $2^{-\Delta\Delta Ct}$ method[22].

Results

Candidate Reference Gene Expression Levels

The expression profiles of the candidate RGs were reflected by their *Cp*-values, with higher *Cp*-value indicating lower levels of expression. The *Cp*-values of all the samples ranged between 8.23 (18S rRNA, minimal *Cp*-value among all 3 groups) and 37.48 (HPRT1, maximum *Cp*-value among all 3 groups) (Figure 1). All *Cp*-values are shown in Table III.

Expression Stability of the Candidat Reference Genes GeNor

HMBS and GUSB had the lowest M-values in the total and laryngeal groups as revealed by the geNorm algorithm, indicating the fact that they are the most stably expressed candidate genes in studies of cancerous human laryngeal and hypopharyngeal tissues together and laryngeal cancerous tissue by itself; TBP and HPRT1 had the lowest M-values in the hypopharyngeal cancer group, indicating the fact that they are the most stably expressed candidate genes in human hypopharyngeal cancerous tissues (Figure 2A). A combination of 10 reference genes was optimal for the total and laryngeal groups, with V10/11 values of 0.142 for both; a combination of 9 RGs was optimal for the hypopharyngeal group, with a V9/10 value of 0.150 (Figure 2B).

NormFinder

As shown in Figure 3, NormFinder showed that the combination of HMBS and HPRT1 had the lowest stability value (0.138), indicating that the HMBS + HPRT1 combination was optimal for the total group. When used as a single reference gene, HMBS was considered as the most stable candidate gene in total, followed by HPRT1. In the laryngeal group, ALAS1 was considered the most stable candidate gene, followed by GAPDH. In the hypopharyngeal group, B2M was considered the most stable candidate gene, followed by HPRT1.

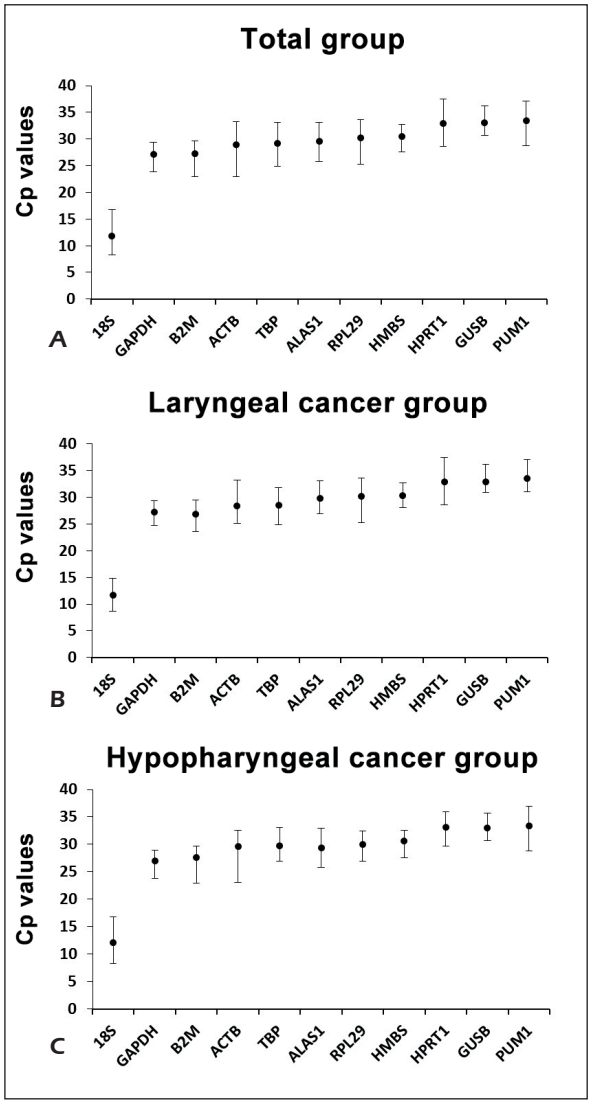

Figure 1. Cp-values of the candidate reference genes in the total group (n=22) (**A**), the laryngeal cancer group (n=12) (**B**), and the hypopharyngeal cancer group (n=10) (**C**). The dots represent the average Cp-values of each candidate reference gene, while the bars represent the minimum and maximum Cp-values.

BestKeeper

The BestKeeper program can evaluate only 10 candidate genes at a time. Even though the most unstable candidate reference genes ranked by geNorm and NormFinder in the laryngeal and hypopharyngeal cancer groups were the same, they were ranked differently in the total group.

Table III. Ct values of candidate control genes in various groups ($\bar{x} \pm s$).

Group	Total group (n=44)	Laryngeal cancer group (n=24)	Hypopharyngeal Cancer group (n=20)
18S rRNA	11.83 ± 2.13	11.64 ± 1.93	12.07 ± 2.37
GAPDH	27.13 ± 1.22	27.27 ± 1.29	26.98 ± 1.31
B2M	27.21 ± 1.70	26.90 ± 1.69	27.59 ± 1.68
ACTB	28.95 ± 2.50	28.38 ± 2.45	29.63 ± 2.43
TBP	29.13 ± 1.69	28.59 ± 1.59	29.77 ± 1.61
ALAS1	29.62 ± 1.54	29.84 ± 1.52	29.35 ± 1.56
RPL29	30.16 ± 1.72	30.25 ± 1.92	30.05 ± 1.48
HMBS	30.45 ± 1.27	30.35 ± 1.35	30.58 ± 1.18
HPRT1	32.95 ± 1.99	32.85 ± 2.28	33.07 ± 1.63
GUSB	32.99 ± 1.38	32.95 ± 1.44	33.04 ± 1.34

To avoid standard confusion, we considered only the geNorm result and removed the most unstable candidate genes from each group before analysis. BestKeeper showed the most stable candidate reference gene in both the overall and laryngeal groups to be HPRT1, followed by 18S rRNA and ALAS1, while in the hypopharyngeal group, the most stable internal reference gene was ACTB, followed by B2M and HPRT1 (Figure 4).

Final Candidate Reference Gene Rankings

The M-values obtained from geNorm, the stability values obtained from NormFinder, the R-values obtained from BestKeeper, and the candidate reference gene rankings obtained from all 3 programs are listed in Table IV. Because the candidate gene rankings varied slightly, the geometric means of the rankings from the 3 programs were calculated to provide an overall ranking regarding the best candidate reference genes[11]. Smaller geometric means are correlated with more stable candidate gene expression[28]. The final rankings of the candidate RGs are also provided in Table IV. According to this ranking, HMBS was considered the optimal reference gene for studying human laryngeal and hypopharyngeal cancerous tissues together, followed by HPRT1; ALAS1 was considered the optimal reference gene for studying human laryngeal cancerous tissue by itself, followed by GUSB; and

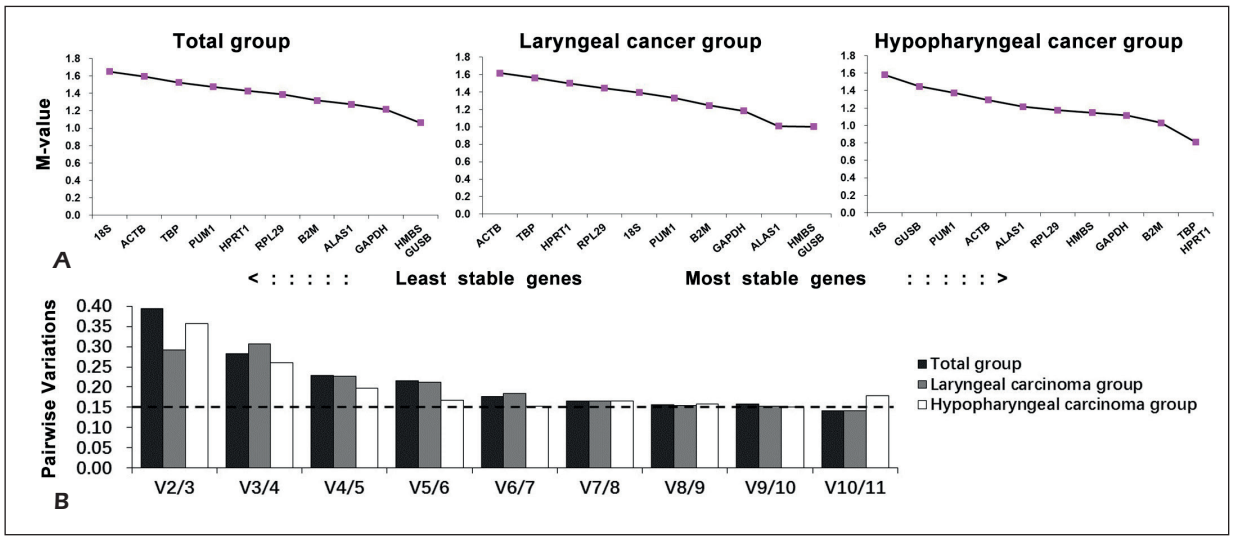

Figure 2. GeNorm analysis of expression stability. **A**, The x-axis indicates the gene rankings according to their expression stability, and the y-axis shows the M-values (total group n=22, laryngeal cancer group n=12, hypopharyngeal cancer group n=10). **B**, The x-axis indicates the number of genes that should be used in combination to achieve satisfactory accuracy in relative quantification analysis, and the y-axis shows the pairwise variation values (n=22).

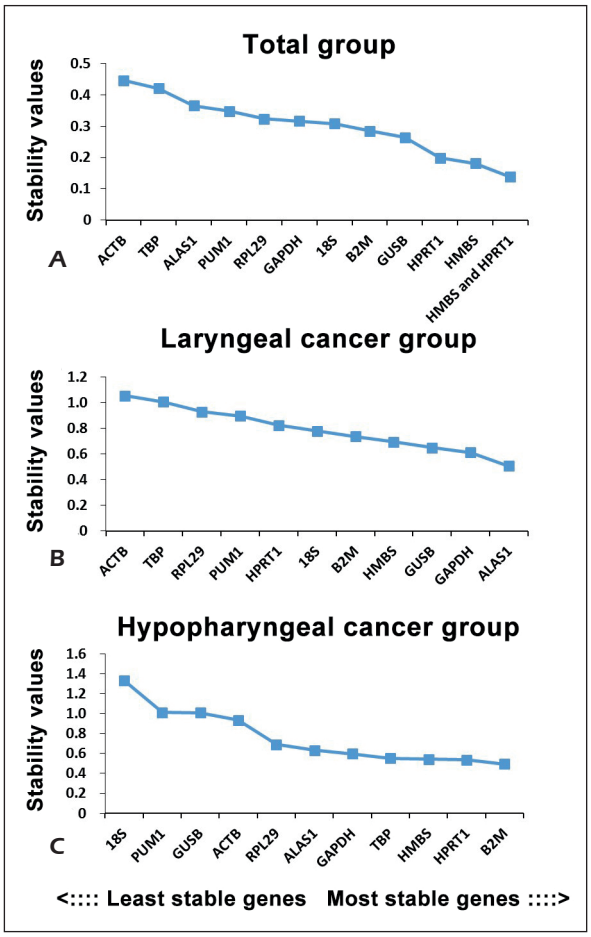

Figure 3. NormFinder analysis of expression stability. The x-axis indicates the gene rankings according to their expression stability, and the y-axis shows the stability values (total group n=22, laryngeal cancer group n=12, hypopharyngeal cancer group n=10).

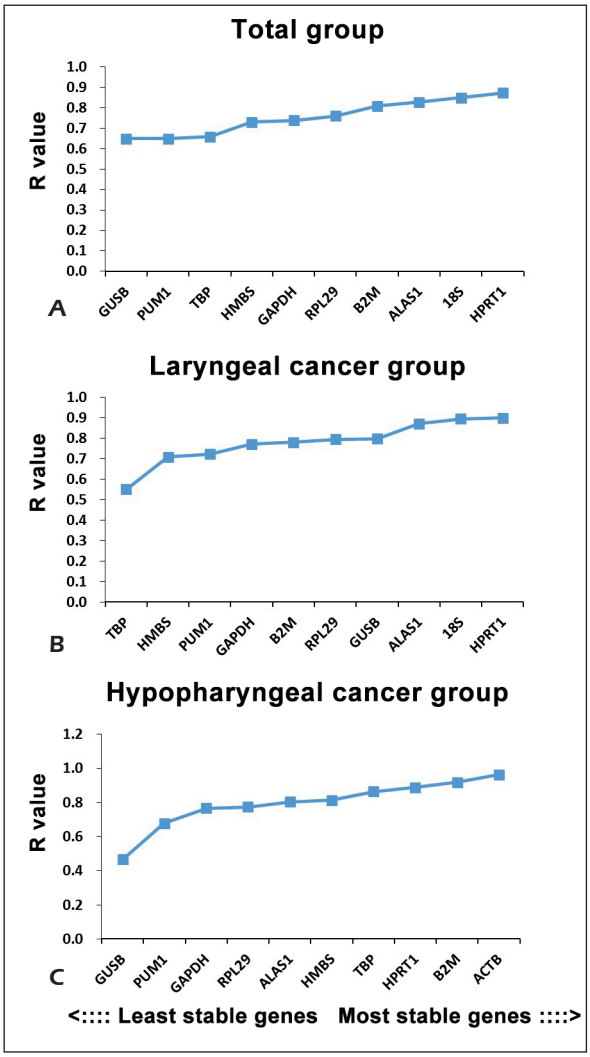

Figure 4. BestKeeper analysis of expression stability. The x-axis indicates the gene rankings according to their expression stability, and the y-axis shows the R values (total group n=22, laryngeal cancer group n=12, hypopharyngeal cancer group n=10).

B2M was considered the optimal reference gene for studying human hypopharyngeal cancerous tissue by itself, followed by HPRT1.

Validation of Reference Genes

In the total group, TLR2 transcript levels were significantly higher in cancerous parenchymal tissue than in paracancerous one when normalizing the expression patterns of TLR2 using GAPDH, ACTB, TBP, B2M, HMBS, ALAS1, GUSB, and PUM1 as reference genes. In both the laryngeal and hypopharyngeal cancer groups, the TLR2 transcript levels were significantly higher in cancerous parenchymal tissue than in paracancerous one when using GAPDH, ACTB, B2M, HMBS, ALAS1, GUSB, and PUM1 as reference genes (Figure 5). Curiously, normalization in each group based on 18S rRNA yielded the opposite results.

Discussion

In this study, the stability and applicability of reference genes were systematically evaluated to identify optimal reference genes for performing more accurate relative quantification analysis of target gene expression in human laryngeal and hypopharyngeal cancers. HMBS, ALAS1, and B2M were suggested to be the optimal RGs for studying human laryngeal and hypopharyngeal cancerous tissues together, laryngeal cancerous tissue by itself, and hypopharyngeal cancerous tissue by itself, respectively. If 2 or more reference genes were needed to achieve better standardiza-

Table IV. Overall comparison of candidate reference genes' stability.

Rank (weight)	Program geNorm		NormFinder		BestKeeper		Final ranking	
	Gene	M-value	Gene	Stability value	Gene	R-value	Gene	Geo Mean
Total group								
1	HMBS	1.062	HMBS	0.182	HPRT1	0.872	HMBS	1.913
2	GUSB	1.062	HPRT1	0.199	18S rRNA	0.849	HPRT1	2.410
3	GAPDH	1.217	GUSB	0.264	ALAS1	0.826	GUSB	3.107
4	ALAS1	1.274	B2M	0.285	B2M	0.808	B2M	4.309
5	B2M	1.318	18S rRNA	0.308	RPL29	0.759	GAPDH	4.762
6	RPL29	1.388	GAPDH	0.316	GAPDH	0.737	ALAS1	4.762
7	HPRT1	1.427	RPL29	0.323	HMBS	0.729	18S rRNA	4.791
8	PUM1	1.476	PUM1	0.348	TBP	0.657	RPL29	5.944
9	TBP	1.526	ALAS1	0.365	GUSB	0.649	PUM1	8.320
10	ACTB	1.594	TBP	0.421	PUM1	0.649	TBP	8.963
11	18S rRNA	1.649	ACTB	0.446	ACTB	–	ACTB	10.488
Laryngeal cancer group								
1	HMBS	1.003	ALAS1	0.507	HPRT1	0.900	ALAS1	2.080
2	GUSB	1.003	GAPDH	0.612	18S rRNA	0.894	GUSB	2.289
3	ALAS1	1.007	GUSB	0.649	ALAS1	0.871	HMBS	3.302
4	GAPDH	1.183	HMBS	0.694	GUSB	0.798	GAPDH	3.826
5	B2M	1.246	B2M	0.736	RPL29	0.794	HPRT1	3.979
6	PUM1	1.331	18S rRNA	0.778	B2M	0.780	18S rRNA	4.380
7	18S rRNA	1.393	HPRT1	0.824	GAPDH	0.771	B2M	5.313
8	RPL29	1.444	PUM1	0.895	PUM1	0.723	RPL29	7.114
9	HPRT1	1.499	RPL29	0.927	HMBS	0.709	PUM1	7.268
10	TBP	1.562	TBP	1.006	TBP	0.550	TBP	10.000
11	ACTB	1.617	ACTB	1.053	ACTB	–	ACTB	11.000
Hypopharyngeal cancer group								
1	TBP	0.810	B2M	0.496	ACTB	0.964	B2M	1.817
2	HPRT1	0.810	HPRT1	0.535	B2M	0.918	HPRT1	1.817
3	B2M	1.032	HMBS	0.542	HPRT1	0.889	TBP	2.520
4	GAPDH	1.115	TBP	0.551	TBP	0.865	ACTB	4.000
5	HMBS	1.148	GAPDH	0.597	HMBS	0.813	HMBS	4.217
6	RPL29	1.176	ALAS1	0.632	ALAS1	0.804	GAPDH	5.429
7	ALAS1	1.214	RPL29	0.689	RPL29	0.775	ALAS1	6.316
8	ACTB	1.292	ACTB	0.936	GAPDH	0.766	RPL29	6.649
9	PUM1	1.375	GUSB	1.010	PUM1	0.679	PUM1	9.322
10	GUSB	1.448	PUM1	1.011	GUSB	0.466	GUSB	9.655
11	18S rRNA	1.581	18S rRNA	1.334	18S rRNA	–	18S rRNA	11.000

tion effects, the HMBS + HPRT1 + GUSB combination was considered optimal for the total group, the ALAS1 + GUSB + HMBS combination was considered optimal for laryngeal cancerous tissue, and the B2M + HPRT1 + TBP combination was considered optimal for the hypopharyngeal cancerous tissue.

In the present study, both parenchymal and paracancerous human laryngeal and hypopharyngeal cancerous tissues were investigated. Due to limitations in indications for surgery, biopsy specimens were not selected by grades or stages. According to previous researches[18,29], the expression levels of the selected RGs are not directly

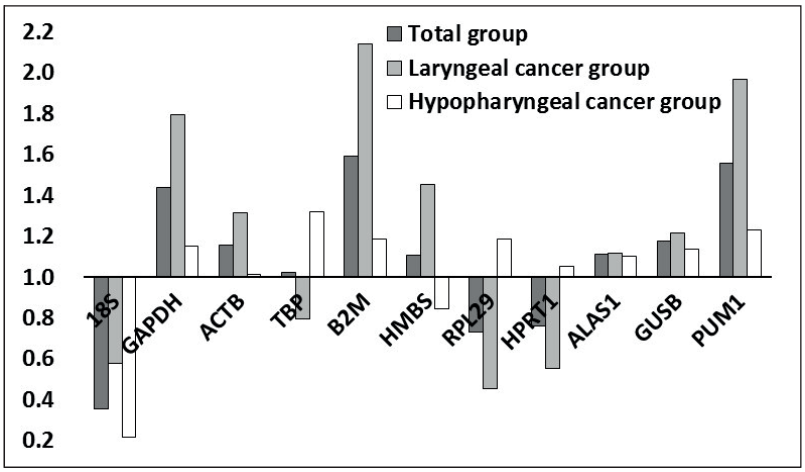

Figure 5. Relative quantities of the TLR2 target gene in parenchymal and paracancerous human laryngeal and hypopharyngeal cancerous tissues using different normalization approaches (n=22).

associated with the grade or stage of malignant tumors. The specimens were confirmed by the Hospital's Pathology Department as malignant, and the samples used here were the most common pathological types of squamous cell carcinoma.

Eleven candidate genes in the present study (18S rRNA, GAPDH, B2M, ACTB, TBP, ALAS1, RPL29, HMBS, HPRT1, GUSB, and PUM1) were commonly used for relative quantification analysis of human tissues or cell lines, and the primer sequences were derived from the previous studies[5-7,11,14]. The expression levels of the 11 genes determined by RT-qPCR are presented as *Cp*-values. In this study, the *Cp*-values of all the samples ranged between 8.23 (18S rRNA) and 37.48 (HPRT1). These values were within an acceptable range, and these genes could thus be used as candidate reference genes, as shown in the previous studies[11,30,31]. To more accurately assess reference gene expression patterns, three specialized programs (geNorm, NormFinder, and BestKeeper) were employed for data analysis. The 3 programs provided slightly different candidate gene expression stability rankings, possibly because of their different calculation algorithms[32,33]. For instance, in the total group, geNorm ranked the top four genes in the order of HMBS, GUSB, GAPDH, and ALAS1, while NormFinder ranked them as HMBS, HPRT1, GUSB, and B2M. Furthermore, BestKeeper included an alternative gene and ranked the top four genes as HPRT1, 18S rRNA, ALAS1, and B2M. In the total group, HMBS was ranked as first by both geNorm and NormFinder but as seventh by BestKeeper. However, regardless of which program was applied, the last two genes were almost the same in each group. In recent studies, RPL family genes showed high stability in both human breast cancer cell lines and formalin-fixed, paraffin-embedded (FFPE) biopsies, but GAPDH performed poorly[34]. GAPDH was also found to be unsuitable for the study of bladder cancer cells, in which B2M was considered the most reliable reference gene[35]. In the study of endometrial carcinoma, PUM1 and PPIA showed high stability, but RPL family genes performed poorly[36]. Taken together, these findings reinforce the notion that the expression of reference genes is context dependent and varies largely across different cell types or between treatments in the same cell type. Thus, validating and evaluating the expression stabilities of reference genes and selecting the most stable reference gene is key to experimental accuracy before subsequent quantitative studies are performed in different samples or experimental conditions[11]. Since the expression stabilities of candidate genes were ranked slightly differently, the geometric means of the ranking numbers from the 3 programs were calculated to provide a ranking of the overall best candidate reference genes. Smaller geometric means were correlated with more stable candidate gene expression[11,28]. The final candidate reference gene rankings suggested HMBS, ALAS1, and B2M as the optimal RGs for studies of human laryngeal and hypopharyngeal cancerous tissues together, laryngeal cancerous tissue by itself, and hypopharyngeal cancerous tissue by itself, respectively. In addition, Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines states that normalization can

be further improved by using multiple reference genes[22]. When multiple reference genes were utilized to improve the accuracy of relative quantification analysis, 9-10 reference genes in combination were suggested to be optimal for each group when considering the V value results that were provided by the geNorm program. However, according to the standardized principle of relative quantification analysis, this result only provides guidance for deciding how many reference genes should be used in combination to further improve normalization[24] instead of providing a stringent standard consideration. Previous studies recommend that using 3 internal control genes in combination is accurate enough to perform a relative quantitative investigation[11,37]. Comprehensively considering the results of all 3 programs, the recommended combinations are listed as follow: for the total group combination, it was HMBS + HPRT1 + GUSB; for the reference gene combination regarding the laryngeal cancerous tissue group, it was ALAS1 + GUSB + HMBS; and for the reference gene combination regarding the hypopharyngeal cancerous tissue group, it was B2M + HPRT1 + TBP. To evaluate the functional significance of the reference gene results, we analyzed the relative expression of the TLR2 gene, whose pattern has already been described for parenchymal and paracancerous human laryngeal and hypopharyngeal cancerous tissues[38]. TLR2 is dramatically more up-regulated in cancerous laryngeal and hypopharyngeal parenchymal tissues than in paracancerous ones. In fact, when expression was normalized using the most stable RGs suggested by the final ranking as the endogenous control, expression of the TLR2 transcript was found to be significantly increased in cancer parenchymal tissue compared with paracancerous one (Figure 5), which was consistent with the pattern of TLR2 expression in laryngeal and hypopharyngeal cancerous tissues. Curiously, when the expression was normalized using 18S rRNA in each group, the opposite results were obtained, which was consistent with our experimental results in that 18S rRNA ranked lower on the stability list.

Conclusions

Because mRNA expression varies largely in tissues between individuals, validating the expression stabilities of reference genes and selecting the most stable reference gene before further quantitative studies are performed in tissue samples are very important. The present study's aim is to analyze and evaluate the expression stability of reference genes in cancerous human laryngeal and hypopharyngeal tissues. To simultaneously investigate human laryngeal and hypopharyngeal cancerous tissues, the HMBS gene or the HMBS + HPRT1 + GUSB genes in combination were considered the most suitable reference genes. For the study on laryngeal cancerous tissues, the ALAS1 gene or the ALAS1 + GUSB + HMBS genes in combination were considered the most suitable reference genes. For the study on hypopharyngeal cancerous tissues, the B2M gene or the B2M + HPRT1 + TBP genes in combination were considered the most suitable reference genes. This information is significant for other researchers that need to evaluate mRNA expression in these tissues and experimental conditions. Selecting the most stable reference gene before performing additional quantitative studies in diverse samples or experimental conditions is recommended. Our recommended RGs might increase the accuracy of quantitating target gene expression during investigations of the molecular mechanisms of human laryngeal and hypopharyngeal cancers. The selected combination of reference genes can effectively improve the accuracy of the relative quantitative diagnosis of gene expression levels, such as messenger RNA, circular RNA, and long-noncoding RNA.

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Authors' statement

The manuscript has been read and approved by all the authors, that the requirements for authorship as stated earlier in this document have been met, and that each author believes that the manuscript represents honest work.

Conflict of Interests

The authors declare that they have no conflicts of interest.

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