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BRIEF ARTICLE

Reference genes for quantitative RT-PCR data in gastric tissues and cell lines

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Abstract

AIM: To evaluate the suitability of reference genes in gastric tissue samples and cell lines.

METHODS: The suitability of genes *ACTB*, *B2M*, *GAPDH*, *RPL29*, and *18S rRNA* was assessed in 21 matched pairs of neoplastic and adjacent non-neoplastic gastric tissues from patients with gastric adenocarcinoma, 27 normal gastric tissues from patients without cancer, and 4 cell lines using reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR). The ranking of the best single and combination of reference genes was determined by NormFinder, geNorm™, BestKeeper, and DataAssist™. In addition, GenEx software was used to determine the optimal number of reference genes. To validate the results, the mRNA expression of a target gene, *DNMT1*, was quantified using the different reference gene combinations suggested by the various software packages for normalization.

RESULTS: *ACTB* was the best reference gene for all gastric tissues, cell lines and all gastric tissues plus cell lines. *GAPDH* + *B2M* or *ACTB* + *B2M* was the best combination of reference genes for all the gastric tissues. On the other hand, *ACTB* + *B2M* was the best combination for all the cell lines tested and was also the best combination for analyses involving all the gastric tissues plus cell lines. According to the GenEx software, 2 or 3 genes were the optimal number of references genes for all the gastric tissues. The relative quantification of *DNMT1* showed similar patterns when normalized by each combination of reference genes. The level of expression of *DNMT1* in neoplastic,

adjacent non-neoplastic and normal gastric tissues did not differ when these samples were normalized using *GAPDH* + *B2M* (P = 0.32), *ACTB* + *B2M* (P = 0.61), or *GAPDH* + *B2M* + *ACTB* (P = 0.44).

CONCLUSION: *GAPDH* + *B2M* or *ACTB* + *B2M* is the best combination of reference gene for all the gastric tissues, and *ACTB* + *B2M* is the best combination for the cell lines tested.

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Key words: Gastric cancer; Reference gene; Normalization; Gene expression; Quantitative real-time polymerase chain reaction

Core tip: Gene expression studies have revealed much about the molecular basis of gastric cancer. However, the normalization of expression data using reference genes without validation may undermine the results. In the present study, we evaluated the suitability of possible reference genes in gastric tissues and cell lines. To our knowledge, our study is the first to determine and validate reference genes for gastric samples in a Western population. In addition, the inclusion of normal gastric tissues from patients without cancer in determining the best reference genes is original in the literature.

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INTRODUCTION

Gastric cancer (GC) is the fourth most common cancer and the second leading cause of cancer-related death[1]. Although approximately 90% of gastric tumors are adenocarcinomas, the etiology and disease evolution may vary among populations, primary tumor location, histological subtypes of adenocarcinoma, and other variables. Among these factors, ethnicity can determine different levels of susceptibility and aggressiveness of gastric tumors[2,3]. An understanding of GC biology is important to identify cancer biomarkers, which may help in early diagnosis and in the development of new targets therapies and, therefore, contribute to reduce mortality or morbidity rates.

Although gene expression studies have revealed much about the molecular basis of GC, the detailed mechanisms remain unclear. Reverse transcription quantitative polymerase chain reaction (RT-qPCR) is currently considered the gold standard for accurate, sensitive, and rapid measurements of gene expression[4-6]. However, to obtain reliable data, the gene expression levels must be normalized using two or more reference genes[7-9]. Ideally, reference genes should be stable, unregulated, and invariable under the conditions of the experiment[10,11]; therefore, a validation experiment for the evaluation of reference gene expression stability for each target tissue and disease is recommended[12,13]. To our knowledge, only one previous study has aimed to assess the best single and combination of reference genes for gastric adenocarcinoma and non-neoplastic samples in an East Asian population[14]. In contrast, there is no information about the stability of candidate reference genes in gastric samples from other populations.

In this study, we assessed the suitability of 5 possible reference genes in 21 matched pairs of neoplastic and non-neoplastic gastric tissues from patients with gastric adenocarcinoma and 27 normal gastric tissues from patients without cancer. We also included 4 cell lines in the analysis. The stability analysis was performed using 4 freely available software packages.

MATERIALS AND METHODS

Cell lines

The ACP02 and ACP03 cell lines were established by our research group from primary gastric adenocarcinomas classified as diffuse and intestinal types, respectively[15]. The PG100 and MRC-5 cell lines were obtained from Rio de Janeiro Cell Bank, Brazil, and were established from a primary gastric adenocarcinoma and from normal human fibroblasts, respectively. All the cell lines were cultured at 37 ℃ in RPMI media 1640 (GIBCO®, Grand Island, NY) supplemented with 10% fetal bovine serum (GIBCO®, Grand Island, NY), and 0.02 mg/mL kanamycin (GIBCO®, Grand Island, NY).

Patients

Twenty-one matched pairs of neoplastic and adjacent non-neoplastic gastric tissues were obtained from patients with gastric adenocarcinoma who were subjected to gastric resection. Twenty-seven normal gastric tissues were obtained from patients subjected to routine endoscopic examination. Table 1 shows the clinicopathological features of the studied patients. All the gastric tissue samples were obtained from João de Barros Barreto University Hospital (HUJBB) in Pará State, Brazil, and were snap-frozen in liquid nitrogen and stored frozen until use. All patients had negative histories of exposure to either chemotherapy or radiotherapy before surgery, and there was no other co-occurrence of diagnosed cancers. Written informed consent with approval of the ethics committee of HUJBB was obtained from all patients prior to sample collection.

RNA extraction and cDNA synthesis

Total RNA was extracted from the cell lines and tissue samples using the AllPrep DNA/RNA/Protein Kit (Qiagen, Hilden, Germany) according to the manufacturer's

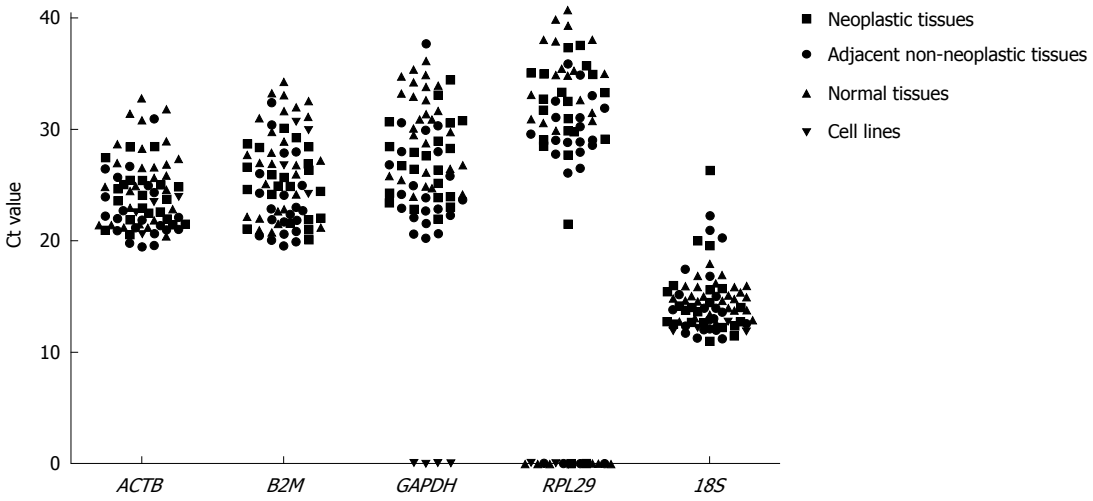

Figure 1 Expression level of five candidate reference genes detected by quantitative real-time polymerase chain reaction. A lower cycle threshold (Ct) value indicates higher gene expression.

Table 1 Clinicopathological features of the studied patients *n* (%)

Clinicopathological feature	Patients with gastric adenocarcinoma	Patients without gastric adenocarcinoma
Age (yr, mean ± SD)	57 ± 15.6	49 ± 14.5
Gender		
Male	15 (71)	12 (44)
Female	6 (29)	15 (56)
Location		
Cardia	2 (9)	0 (0)
Non-cardia	19 (91)	27 (100)
Histopathological type[1]		
Intestinal	16 (76)	NA
Diffuse	5 (24)	NA
Stage[2]		
Early	4 (19)	NA
Advanced	17 (81)	NA
Tumor Invasion		
T1/T2	9 (43)	NA
T3/T4	12 (57)	NA
Lymph node metastasis		
Absent	3 (14)	NA
Present	18 (86)	NA
Distant metastasis		
Unknown/absent	18 (86)	NA
Present	3 (14)	NA

[1]According to the Lauren classification[29]; [2]according to AJCC[30]. NA: Not applicable.

instructions. The concentration and quality of the extracted RNA were measured using a Nanodrop ND-1000 (Thermo Scientific, Wilmington, DE), and the integrity was determined by gel electrophoresis. The complementary DNA was synthesized using High-Capacity® cDNA Reverse Transcription (Life Technologies, Foster City, CA) following the manufacturer's protocol.

RT-qPCR

The reaction to detect the expression range of the 5 candidate reference genes was performed in triplicate using TaqMan® inventoried Assays-on-Demand probes (Life Technologies, Foster City, CA) and the Applied Biosystems 7500 fast real-time PCR system. We also quantified the mRNA expression of a target gene, *DNMT1*, using the possible candidate genes for normalization. For this analysis, we evaluated 18 matched pairs of adjacent non-neoplastic and neoplastic gastric tissues from patients with gastric adenocarcinoma and 19 normal gastric tissues from patients without cancer. The analyzed genes, their respective TaqMan® assay identification and efficiencies are provided in Table 2. The relative quantification (RQ) of *DNMT1* expression was calculated according to the Livak method[16]. A sample from a patient without cancer was designated as a calibrator.

Analysis of reference gene stability

We categorized the gastric tissues and cell lines into the following groups: (1) neoplastic tissues; (2) adjacent non-neoplastic tissues; (3) matched pairs of adjacent non-neoplastic and neoplastic gastric tissues; (4) normal tissues; (5) all gastric tissues; (6) cell lines; and (7) all gastric tissues plus cell lines. For the stability comparisons of the candidate reference genes, we used the software NormFinder version 20 (http://www.mdl.dk/publicationsnormfinder.htm)[17], geNorm™ (http://medgen.ugent.be/~jvdesomp/genorm/http://medgen.ugent.be/~jvdesomp/genorm/)[7], BestKeeper1 (http://www.gene-quantification.de/bestkeeper.html)[18], and DataAssist™ (http://www.lifetechnologies.com/us/en/home/technical-resources/software-downloads/dataassist-software.html) according to the recommendations of the authors. The software GenEx (http://genex.gene-quantification.info/) was used to determine the optimal number of reference genes by calculating the Accumulated Standard Deviation (Acc.S.D).

In the analysis using geNorm, the reference genes were ranked according to the expression stability value M (average pair-wise variation of a gene with all other tested candidate reference genes). Using NormFinder, the set of candidate reference genes was ranked accord-

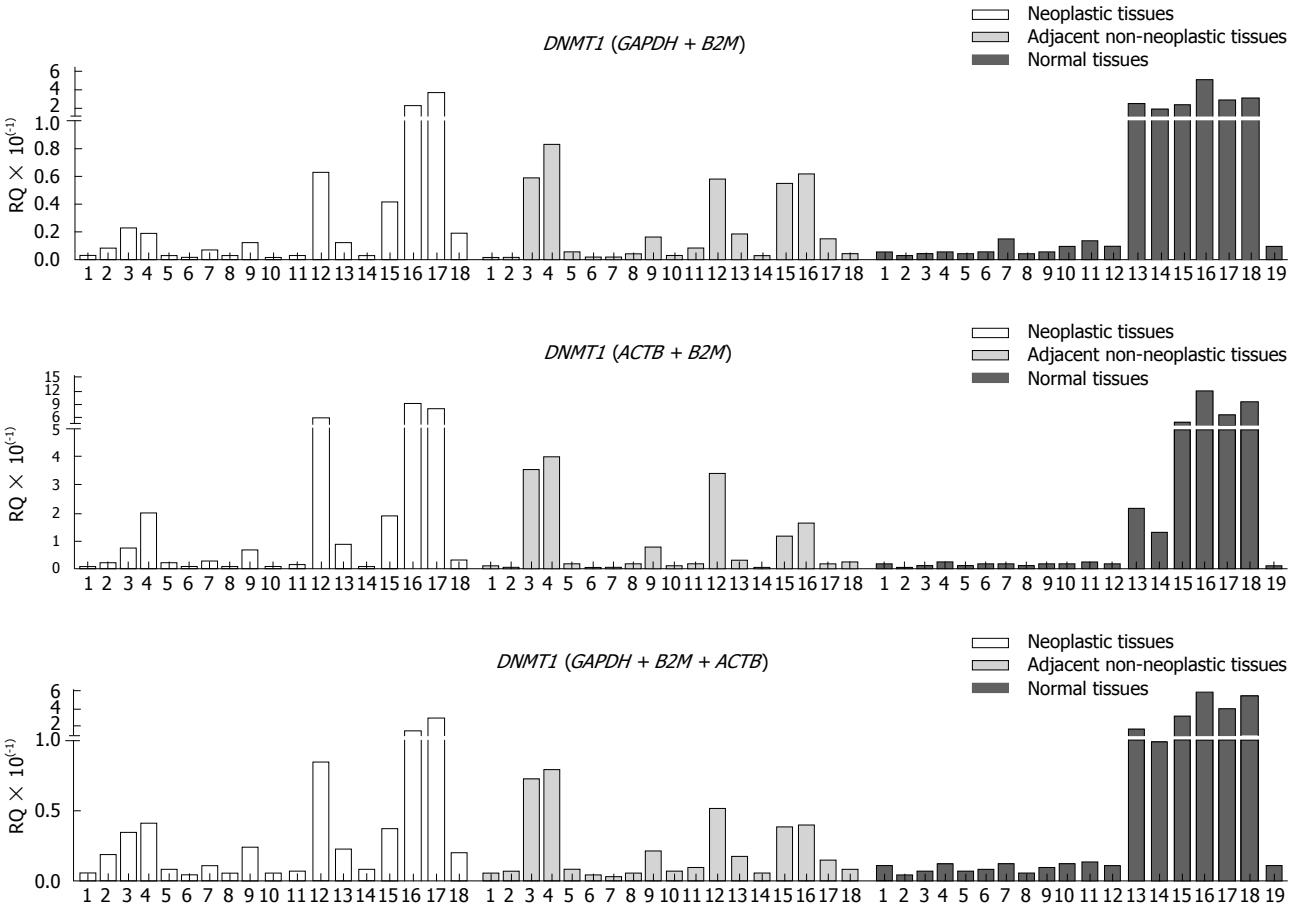

Figure 2 Relative quantification of *DNMT1*, as normalized by *GAPDH + B2M*, *ACTB + B2M*, and *GAPDH + B2M + ACTB* in gastric tissues.

ing to their expression stability (combination of the intra- and intergroup variation). The ranking of the 5 reference genes by Bestkeeper was based on the standard deviation (SD) and coefficient of variance (CV) expressed as a percentage of the cycle threshold (Ct) level. Lastly, DataAssist provides a metric to measure reference gene stability based on the geNorm algorithm. Unlike all the other programs, DataAssist uses RQ to calculate the stability value of individual candidate reference genes. The two genes that showed the highest stability were considered the best combination of reference genes.

RESULTS

Expression level of candidate reference genes

The expression levels of 5 candidate reference genes as the Ct value are shown in Figure 1. These genes displayed a wide range of expression levels. *18S rRNA* showed the highest expression level in the gastric tissues and cell lines. In contrast, *RPL29* showed the lowest expression level and did not amplify in 3 samples of neoplastic tissue, 2 samples of adjacent non-neoplastic tissue, and 9 samples of normal tissue. Similarly, *RPL29* and *GAPDH* did not amplify in any of the 4 cell lines studied. Therefore, *RPL29* was excluded from the ensuing analysis, and *GAPDH* was excluded from the set of candidate reference genes in the cell line analysis.

Expression stability of candidate reference genes

Table 3 demonstrates the stability value ranking of the single candidate reference genes calculated using the 4 different software packages. Although the various software packages suggested different reference genes, *ACTB* was the gene most cited as the best reference gene in the different gastric tissue categories, followed by *GAPDH* and *B2M*. *ACTB* was also the best reference gene in the cell line and all gastric tissues plus cell line categories.

Table 4 shows the best combination of reference genes suggested by the 4 software packages. Overall, for the different gastric tissue categories, *GAPDH + B2M* were the genes more cited as the best combination of reference gene, followed by *ACTB + B2M* and *GAPDH + ACTB*. *ACTB + B2M* was also the best combination of reference genes suggested for the cell lines and all gastric tissues plus cell line categories.

Although the software indicated up to 2 genes as the best combination of reference genes, we also used GenEx software to determine the optimal number of reference genes. This software revealed that an Acc. S.D. of 0.03 was the lowest when 2 or 3 reference genes were used for both the matched pairs of adjacent non-

Table 2 Summary of five reference genes and a target gene

Symbol	Gene name (Assay ID[1])	Location	Description	Efficiency
ACTB	β-Actin (Hs03023943_g1)	7p22	Cytoskeletal structural protein	109%
B2M	β-2-Microglobulin (Hs00984230_m1)	15q21	Beta-chain of major histocompatibility complex class I molecules	104%
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase (Hs99999905_m1)	12p13	Oxidoreductase in glycolysis and gluconeogenesis	102%
RPL29	Ribosomal protein L29 (Hs00426490_g1)	3p21	Structural constituent of the ribosome	100%
18S rRNA	18S ribosomal RNA (Hs99999901_s1)	22p12	Ribosome subunit	110%
DNMT1	DNA (cytosine-5-)-methyltransferase 1 (Hs00945875_m1)	19p13	Regulation of tissue-specific patterns of methylated cytosine residues	98%

[1]TaqMan® probes were purchased as Assays-on-Demand Products for Gene Expression (Life Technologies, Foster City, CA).

Table 3 Ranking of the candidate single reference genes by each software package used

	NormFinder		geNorm™		BestKeeper		DataAssist™	
	Stability value[1]	Ranking	M value[1]	Ranking	Coefficient of variance[1]	Ranking	Score[1]	Ranking
Neoplastic tissues								
	0.24	*ACTB*	0.07	*GAPDH*	9.78	*ACTB*	1.58	*ACTB*
	0.73	*B2M*	0.07	*B2M*	11.77	*B2M*	1.70	*B2M*
	0.99	*GAPDH*	0.08	*ACTB*	13.39	*GAPDH*	1.88	*GAPDH*
	1.41	*18S rRNA*	0.12	*18S rRNA*	17.14	*18S rRNA*	2.25	*18S rRNA*
Adjacent non-neoplastic tissues								
	0.61	*B2M*	0.05	*GAPDH*	7.80	*ACTB*	1.65	*B2M*
	0.65	*GAPDH*	0.05	*B2M*	9.61	*B2M*	1.77	*ACTB*
	0.65	*ACTB*	0.07	*ACTB*	10.52	*GAPDH*	1.81	*GAPDH*
	1.53	*18S rRNA*	0.13	*18S rRNA*	16.57	*18S rRNA*	2.46	*18S rRNA*
Matched pairs of adjacent non-neoplastic and neoplastic gastric tissues								
	0.14	*ACTB*	0.06	*GAPDH*	9.24	*ACTB*	1.83	*GAPDH*
	0.29	*B2M*	0.06	*B2M*	11.31	*B2M*	1.83	*ACTB*
	0.41	*GAPDH*	0.07	*ACTB*	12.52	*GAPDH*	2.00	*B2M*
	0.54	*18S rRNA*	0.13	*18S rRNA*	16.86	*18S rRNA*	2.60	*18S rRNA*
Normal gastric tissues								
	0.38	*GAPDH*	0.06	*GAPDH*	7.38	*18S rRNA*	1.82	*GAPDH*
	0.41	*ACTB*	0.06	*ACTB*	11.08	*GAPDH*	2.24	*ACTB*
	1.07	*B2M*	0.07	*B2M*	11.46	*ACTB*	2.40	*B2M*
	2.24	*18S rRNA*	0.11	*18S rRNA*	13.34	*B2M*	3.91	*18S rRNA*
All gastric tissues								
	0.14	*ACTB*	0.07	*GAPDH*	10.89	*ACTB*	2.04	*ACTB*
	0.36	*B2M*	0.07	*B2M*	13.12	*B2M*	2.09	*B2M*
	0.69	*GAPDH*	0.08	*ACTB*	13.24	*18S rRNA*	2.12	*GAPDH*
	0.96	*18S rRNA*	0.13	*18S rRNA*	13.43	*GAPDH*	3.34	*18S rRNA*
Cell lines								
	0.53	*ACTB*	0.06	*ACTB*	2.36	*18S rRNA*	1.71	*ACTB*
	1.55	*B2M*	0.06	*B2M*	4.81	*ACTB*	2.45	*B2M*
	1.73	*18S rRNA*	0.13	*18S rRNA*	8.60	*B2M*	2.63	*18S rRNA*
All gastric tissues + cell lines								
	0.45	*ACTB*	0.09	*ACTB*	10.67	*ACTB*	2.73	*B2M*
	1.11	*B2M*	0.09	*B2M*	13.02	*B2M*	3.32	*ACTB*
	1.19	*18S rRNA*	0.16	*18S rRNA*	13.41	*18S rRNA*	3.38	*18S rRNA*

[1]A lower value indicates increased stability in gene expression.

neoplastic and neoplastic gastric tissues and all gastric tissues categories.

Target gene normalization using different combined reference genes

Because *GAPDH* + *B2M* and *ACTB* + *B2M* or *GAPDH* + *B2M* + *ACTB* were identified as the best combinations of references genes for gastric tissues, we evaluated the expression of *DNMT1*, as normalized by these combinations of reference genes. The RQ of *DNMT1* normalized by each combination of reference genes showed similar patterns (Figure 2). The level of expression of *DNMT1* in neoplastic, adjacent non-neoplastic and normal gastric tissues did not differ when these samples were normalized using *GAPDH* + *B2M* (P = 0.32, Kruskal-Wallis test), *ACTB* + *B2M* (P = 0.61,

Table 4 Best reference gene combinations according to each software

Neoplastic tissues	Adjacent non-neoplastic tissues	Normal tissues	Matched pairs of adjacent nonneoplastic and neoplastic gastric tissues	All gastric tissues	Cell lines	All gastric tissues[+] cell lines
ACTB + B2M	*GAPDH + B2M*	*GAPDH + ACTB*	*ACTB + B2M*	*ACTB + B2M*	*ACTB + B2M*	*ACTB + 18S rRNA*
GAPDH + B2M	*GAPDH + B2M*	*GAPDH + ACTB*	*GAPDH + B2M*	*GAPDH + B2M*	*ACTB + B2M*	*ACTB + B2M*
GAPDH + B2M	*GAPDH + B2M*	*GAPDH + B2M*	*GAPDH + B2M*	*GAPDH + B2M*	*ACTB + B2M*	*ACTB + B2M*
ACTB + B2M	*GAPDH + B2M*	*GAPDH + B2M*	*GAPDH + B2M*	*GAPDH + B2M*	*ACTB + B2M*	*ACTB + B2M*

Kruskal-Wallis test), or *GAPDH + B2M + ACTB* (P = 0.44, Kruskal-Wallis test).

DISCUSSION

Reference genes have been described for RT-qPCR studies in several diseases and tissues[19-23]. However, with regard to gastric adenocarcinoma samples, there is only one previous study that evaluated the best single and combination of reference genes in an East Asian Population[14]. Because ethnicity can determine different levels of gene expression, it is important to determine the suitability of reference genes considering the population in addition to the disease type and target tissue. To our knowledge, our study is the first to determine and validate reference genes for gastric samples in a Western Population. The population in Pará State, Brazil, is composed of interethnic crosses between three main groups: European (mainly represented by Portuguese), Africans, and Amerindians[24]. In addition, to our knowledge, the study of normal gastric tissue from patients without gastric cancer and its inclusion in determining the best single and combination of reference genes is original in the literature.

The software packages NormFinder, geNorm™, BestKeeper, and DataAssist™ are statistical tools that aid in the selection of appropriate reference genes. Although these software packages differed in the suggestion of the best single and combination of reference genes, at least two programs agreed with the results for each group evaluated (Tables 3 and 4), emphasizing the importance of using more than one software to assess the best reference genes among a set of candidate genes. When considering all gastric tissues, our results showed that *ACTB* and *GAPDH + B2M* or *ACTB + B2M* were the best single and combination of reference genes, respectively. Despite the software packages indicating up to 2 genes as the best combination, the GenEx software revealed that 2 or 3 reference genes were necessary for gene normalization in all gastric tissue. When the expression of the target gene *DNMT1* was evaluated using the 3 different combinations of reference genes for normalization (*GAPDH + B2M, ACTB + B2M*, and *GAPDH + B2M + ACTB*), no differences in *DNMT1* expression were detected among the neoplastic, adjacent non-neoplastic, and normal tissues. These results validated the combination of reference genes suggested by the software, proving that combinations of 2 genes can be used and that it is not necessary to use 3 or more reference genes for all gastric tissues. *ACTB* and *ACTB + B2M* were the best single and combination of reference genes, respectively, for all the cell lines. Our results showed that *ACTB + B2M* was the best option under circumstances that require the use of the same combination of reference genes for all gastric tissues and cell lines. Although the measure of stability for *18S rRNA* was within the range of acceptance when using Bestkeeper, it has repeatedly been documented that this is not a good reference gene because the regulation of its synthesis is not representative of mRNA levels[25-28].

Rho *et al*[14] proposed different reference genes for the study of gene expression in gastric tissues and cell lines, suggesting *RPL29* and *RPL29 + B2M* and *B2M* and *GAPDH + B2M* as the best single and combination of reference genes, respectively. Interestingly, the genes suggested by Rho *et al*[14], *RPL29* and *GAPDH,* did not amplify in our cell lines and in some tissue samples. The different methodologies applied can explain the different results. In the present study, we evaluated gene expression using commercially available TaqMan® assays, whereas Rho *et al*[14] evaluated gene expression using SYBR green and primers previously reported in the literature that can detect non-specific reaction products with variable sensitivity. In addition, it should be considered that samples obtained from different ethnicities could contribute to the different results of our group and Rho *et al*[14].

In conclusion**,** our suitability analysis suggested *ACTB* and *GAPDH + B2M* or *ACTB + B2M* as the best single and combination of reference genes for all gastric tissues, with *ACTB* and *ACTB + B2M* as the best single and combination of reference genes for all cell lines tested. When circumstances require the use of the same combination of reference genes for all gastric tissues and cell lines, our results showed that *ACTB + B2M* was the best option. The use of these genes for RT-qPCR data normalization may enhance the robustness of transcription level determination in gastric samples.

COMMENTS

Background

Gastric cancer is the fourth most common cancer worldwide, with high rates of mortality and morbidity. Reverse transcription quantitative polymerase chain reaction is currently considered the gold standard for the accurate, sensitive, and rapid measurement of gene expression. To obtain reliable data, a validation experiment to evaluate the best reference genes for the normalization of gene expression data is recommended for each target tissue and disease.

Research frontiers

The etiology and disease evolution of gastric adenocarcinomas vary among patients due to several factors. Among them, ethnicity can determine different levels of gastric tumor susceptibility and aggressiveness. The understanding

of gastric cancer biology is important to identify cancer biomarkers, which may help in the early diagnosis and development of new targets therapies and, therefore, contribute to reduce mortality and morbidity rates.

Innovations and breakthroughs

Only one previous study aimed to evaluate the best reference genes for gastric adenocarcinoma in an East Asian population. To their knowledge, the present study is the first to determine and validate reference genes for gastric samples in a Western population. In addition, the analysis of normal gastric tissue from patients without gastric cancer and its inclusion in determining the best reference genes is original in the literature.

Applications

The use of the combination of reference genes determined and validated in our study for reverse transcriptional quantitative polymerase chain reaction data normalization may enhance the robustness of transcription level determination in gastric samples.

Terminology

Reference genes are internal controls used in reverse transcription quantitative polymerase chain reaction analysis to avoid the sample biases related to variability in the total RNA content, RNA stability, and enzymatic efficiency. Ideal reference genes should be stable, unregulated, and invariable under the conditions of the experiment.

Peer review

The authors evaluated the suitability of five possible reference genes in matched pairs of non-neoplastic and neoplastic gastric tissues from patients with gastric adenocarcinoma and normal gastric tissues from patients without cancer. Four cell lines were also included in this analysis. The stability analysis was performed using four freely available software packages. This study validated *GAPDH* + *B2M* or *ACTB* + *B2M* as the best combination of reference genes for all gastric tissues. In addition, *ACTB* + *B2M* were suggested as the best combination of reference genes for cell lines. When circumstances require the use of the same combination of reference genes for all gastric tissues and cell lines, the *ACTB* + *B2M* combination was found to be the best option.

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