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.

INTRODUCTION

Gastric cancer (GC) is the fourth most common cancer and the second leading cause of cancer-related death[10]. 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 biological subtypes of adenocarcinoma, and other variables.

Among these factors, ethnicity can determine different biological subtypes of adenocarcinoma, and other variables. 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[14]. However, to obtain reliable data, the gene expression levels must be normalized using two or more reference genes[9]. Ideally, reference genes should be stable, unregulated, and invariant under the conditions of the experiment[10-13]. Therefore, a validation experiment for the evaluation of reference gene expression stability for each target tissue and disease is recommended[12,15]. 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 Dr. Song Wei, China. All cell lines were obtained from the American Type Culture Collection (ATCC) and maintained in plastic culture dishes (Nunc, Nerman). The cultures were passaged weekly and maintained at 37℃ in RPMI media 1640 (Invitrogen, Grand Island, NY) supplemented with 10% fetal bovine serum (Invitrogen, Grand Island, NY), 0.02 mg/mL kanamycin (Invitrogen, Grand Island, NY), and 2 mM L-glutamine (Invitrogen, 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 Dr. Song Wei, China. All the gastric samples were obtained from the Chinese Academy of Sciences, Institute of Biomedical Sciences, National Cancer Institute. All the gastric samples were obtained from the Chinese Academy of Sciences, Institute of Biomedical Sciences, National Cancer Institute.

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
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/publications/normfinder.htm)\[17\], geNorm™ (http://medgen.ugent.be/~jvdesomp/genorm/index.html)\[7\], and DataAsist™ (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.genequantification.info) was used to determine the optimal number of reference genes by calculating the Accumulated Standard Deviation (Acc.SD).

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-

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**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.
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-
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 reference 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), $ACTB + B2M$ ($P = 0.61$, TaqMan® probes were purchased as Assays-on-Demand Products for Gene Expression (Life Technologies, Foster City, CA).
were the best single and combination of reference genes, respectively, for all the cell lines. Our results showed that $\text{ACTB} + \text{B2M}$ 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 $\text{RPL29}$ and $\text{RPL29} + \text{B2M}$ and $\text{B2M}$ and $\text{GAPDH} + \text{B2M}$ as the best single and combination of reference genes, respectively. Interestingly, the genes suggested by Rho et al$^{[14]}$, $\text{RPL29}$ and $\text{GAPDH}$, 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$^\text{®}$ 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 $\text{ACTB}$ and $\text{GAPDH} + \text{B2M}$ or $\text{ACTB} + \text{B2M}$ as the best single and combination of reference genes for all gastric tissues, with $\text{ACTB}$ and $\text{ACTB} + \text{B2M}$ 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 $\text{ACTB} + \text{B2M}$ 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

| 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 |
|--------------------|---------------------------------|----------------|-------------------------------------------------|-------------------|------------|-----------------------------|
| $\text{ACTB} + \text{B2M}$ | $\text{GAPDH} + \text{B2M}$ | $\text{GAPDH} + \text{ACTB}$ | $\text{ACTB} + \text{B2M}$ | $\text{ACTB} + \text{B2M}$ | $\text{ACTB} + \text{B2M}$ | $\text{ACTB} + \text{B2M}$ |
| $\text{GAPDH} + \text{B2M}$ | $\text{GAPDH} + \text{B2M}$ | $\text{GAPDH} + \text{ACTB}$ | $\text{GAPDH} + \text{B2M}$ | $\text{GAPDH} + \text{B2M}$ | $\text{ACTB} + \text{B2M}$ | $\text{ACTB} + \text{B2M}$ |
| $\text{GAPDH} + \text{B2M}$ | $\text{ACTB} + \text{B2M}$ | $\text{GAPDH} + \text{B2M}$ | $\text{GAPDH} + \text{B2M}$ | $\text{GAPDH} + \text{B2M}$ | $\text{ACTB} + \text{B2M}$ | $\text{ACTB} + \text{B2M}$ |
| $\text{ACTB} + \text{B2M}$ | $\text{GAPDH} + \text{B2M}$ | $\text{GAPDH} + \text{B2M}$ | $\text{GAPDH} + \text{B2M}$ | $\text{GAPDH} + \text{B2M}$ | $\text{ACTB} + \text{B2M}$ | $\text{ACTB} + \text{B2M}$ |
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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