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ORIGINAL ARTICLE

Identification of Suitable Reference Genes for qPCR Analysis of Serum microRNA in Gastric Cancer Patients

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Abstract
Background Circulating microRNA expression profiles may be promising biomarkers for diagnosis and assessment of the prognosis of cancer patients. Quantitative polymerase chain reaction (qPCR) is a sensitive technique for estimating expression levels of circulating microRNAs. However, there is no current consensus on the reference genes for qPCR analysis of circulating microRNAs.
Aims In this study we tried to identify suitable reference genes for qPCR analysis of serum microRNA in gastric cancer patients and healthy individuals.
Methods Six microRNAs (let-7a, miR-16, miR-93, miR-103, miR-192, and miR-451) and RNU6B were chosen as candidate reference genes on the basis of the literature. Expression levels of these candidates were analyzed by qPCR in serum samples from 40 gastric cancer patients and 20 healthy volunteers. The geNorm, Normfinder, bestkeeper, and comparative delta-Ct method algorithms were used to select the most suitable reference gene from the seven candidates. This was then validated by normalizing the expression levels of serum miR-21 across all gastric cancer patients and healthy volunteers.
Results The algorithms revealed miR-16 and miR-93 were the most stably expressed reference genes, with stability values of 1.778 and 2.213, respectively, for serum microRNA analysis across all the patients and healthy controls. The effect of different normalization strategies was compared; when normalized to the serum volume there were no significant differences between patients and controls. However, when the data were normalized to miR-93, miR-16, or miR-93 and miR-16 combined, significant differences were detected.
Conclusions Our results demonstrated that reference gene choice for qPCR data analysis has a great effect on the study outcome, and that it is necessary to choose a suitable reference for reliable expression data. We recommend miR-16 and miR-93 as suitable reference genes for serum miRNA analysis for gastric cancer patients and healthy controls.

Keywords Stomach neoplasms · Circulating microRNA · qPCR · Reference genes · Normalization

Introduction

Gastric cancer, one of the most frequent malignancies in China, is a multistep and multifactorial process resulting from the abnormal expression of related genes, including microRNAs (miRNAs). These are single-stranded RNA molecules approximately 21–23 nucleotides in length that regulate gene expression by binding to the 3′-untranslated region of target mRNA. Dysregulation of miRNAs has been observed in a wide range of diseases, including cardiovascular disease, diabetes, and cancer in particular [1–3]. Research suggests that tissue miRNA expression profiles could be promising biomarkers for diagnosis and assessment of the prognosis of cancer, and for providing a therapeutic target [4, 5].

There have recently been several reports that miRNAs circulate in highly stable, cell-free forms in blood [6, 7]. Because serum and plasma miRNAs are relatively easy to

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access, circulating miRNAs also have great potential to serve as non-invasive biomarkers [8]. It was first reported that high levels of serum miR-21 in patients with diffuse large B-cell lymphoma correlated with improved relapse-free survival [9]. In gastric cancer patients, plasma miR-21 and miR-106b were reported to be potential diagnostic biomarkers for gastric cancer [10], and serum miRNAs (miR-1, miR-20a, miR-27a, miR-34, and miR-423-5p) could serve as fingerprints for gastric cancer diagnosis [11].

Currently, quantitative polymerase chain reaction (qPCR) is the most frequently used approach for evaluation of circulating miRNAs. Normalization is an essential component of a reliable qPCR assay, because this process controls for variations in the extracted yield, reverse-transcription yield, and efficiency of amplification, thus enabling comparisons of miRNA concentrations across different samples. The use of reference genes as an internal control is the most common method for normalization of tissue miRNA data; however, there is no current consensus on reference miRNAs for qPCR analysis of serum miRNAs. The RNA molecules RNU6B, 18S, and miR-16 have previously been used to normalize target serum miRNA expression data [4, 12]. Alternative strategies, for example normalization to the serum volume, RNA quantity, or synthetic miRNA molecules used as spike-in controls, have also been used [11, 13]. The utility of reference genes as a normalization strategy must be experimentally validated for specific tissues or sample types and individual experimental design. This issue has already been discussed for several experimental systems in the context of qPCR for mRNA and miRNA [14–17]. As far as we are aware, however, this issue has not yet been addressed in relation to the relative quantification of circulating miRNAs in gastric cancer patients. To produce reliable relative qPCR data, we have attempted to identify a set of suitable reference genes for serum miRNA expression of gastric cancer patients, to enable further progress in research on this topic.

Table 1 Clinical characteristics of subjects

Clinicopathological variable	Patients, $N = 40$		Healthy controls, $N = 20$	
	n	%	*n*	%
Gender				
Male	27	67.5	12	60
Female	13	32.5	8	40
Age				
Median	59.5		41	
Range	35–88		27–60	
Borrmann's classification				
Ulcerative type	33	82.5		
Diffuse infiltrative	7	17.5		
Tumor thickness				
pT1	8	20		
pT2	6	15		
pT3	19	47.5		
pT4	7	17.5		
Nodal status				
pN0	13	32.5		
pN1	9	22.5		
pN2	7	17.5		
pN3	11	27.5		
Distant metastasis				
M0	37	92.5		
M1	3	7.5		
Tumor differentiation				
Well	5	12.5		
Moderate	22	55		
Poor	13	32.5		
UICC stage				
I	11	27.5		
II	6	15		
III	11	27.5		
IV	12	30		

Materials and Methods

Serum Samples and Candidate Reference Genes

Blood was collected from 40 patients with histologically proven diagnosis of gastric cancer at initial diagnosis and 20 healthy participants during health check-ups at Beijing Friendship Hospital. The patient cohort comprised TNM stage I ($n = 10$), stage II ($n = 10$), stage III ($n = 10$), and stage IV ($n = 10$). The clinical characteristics of study participants are presented in Table 1. Five milliliters of venous blood was collected from each donor and placed in a separate vacuum cube. Each blood sample was immediately centrifuged at 3,000 rpm for 10 min, then the serum supernatant was recovered and stored at −80°C until further analysis. Prior written and informed consent was obtained from each patient and the study was approved by the ethics review board of Beijing Friendship Hospital.

One small nucleolar RNA (RNU6B) and six miRNAs (let-7a, miR-16, miR-93, miR-103, miR-192, and miR-451) were chosen as candidate reference genes for this study, on the basis of literature search and suggestions from Exqion Company (Table 2). As far as we are aware, few detailed profiling studies of circulating miRNA qPCR expression have chosen an internal reference gene as a normalization strategy [4, 12]. miR-16 and RNU6B were used as reference genes for circulating miRNA normalization in these studies. It has also been reported that let-7a is a suitable reference gene for miRNA expression analysis

Table 2 Function of candidate normalization genes

Name	Accession	Function	Ref.	PCR efficiency
Let-7a	MIMAT0000062	Negatively regulates RAS oncogene	[31]	98%
miR-16	MIMAT0000069	Cellular differentiation, anti-apoptotic	[32]	92%
miR-93	MIMAT0000093	Impairs TGFβ-dependent cell-cycle arrest and apoptosis in gastric cancer	[33]	105%
miR-103	MIMAT0000101	Attenuates miRNA biosynthesis by targeting dicer	[34]	116%
miR-192	MIMAT0000222	Regulates dihydrofolate reductase and cellular proliferation	[35]	95%
miR-451	MIMAT0001631	Regulates macrophage migration inhibitory factor production and proliferation of gastrointestinal cancer cells	[36]	106%
RNU6B	NR_002752.1	Guided post-transcriptional modification of cellular RNAs	[37]	Undetected

in human breast cancer [15], thus it was included as a candidate reference gene in our studies. miR-93, miR-103, miR-192, and miR-451 are typically detected at constant levels in serum and plasma samples; they are, therefore, ideal for normalization of expression data [18]. In this study, we systematically evaluated the stability of candidate reference gene expression.

RNA Extraction and Reverse Transcription

RNA was isolated by use of the miRcute miRNA isolation kit (Tiangen Biotech, Beijing, China), in accordance with the manufacturer's procedure for serum/plasma samples, with minor modifications. In brief, 300 µl human serum was mixed with 300 µl MZ lysis buffer, vortex mixed vigorously, and incubated at room temperature for 5 min. After phase separation by addition of chloroform and centrifugation, the aqueous phase was mixed with different volumes of 100% ethanol and applied to miRspin columns and miRelute columns successively in accordance with the manufacturer's procedure. Finally, the microRNA was eluted from the column with 30 µl RNase-free water, of which 18 µl was used for reverse transcription and the rest was stored at −80°C for further analysis. RNA concentration and purity was assessed by use of an Eppendorf Biophotometer. RNA concentrations ranged from 26 to 54 ng/µl, and the purity was verified by use of A260/A280 ratios (range 1.77–1.92). The integrity of the serum RNA could not be assessed by gel electrophoresis because ribosomal RNA, which is used as an integrity marker, is present at extremely low concentrations in serum and plasma.

Reverse transcription was carried out using an all-in-one miRNA first-strand cDNA synthesis kit (Genecopoeia, Rockville, USA). In this step, poly-A tails were added to the 3′ end of miRNAs, which were then reverse-transcribed using a universal Oligo-dT adaptor primer. The reaction mixture consisted of 1 µl 2.5 U/µl Poly-A polymerase, 1 µl RTase Mix, 5 µl 5 × reaction buffer, and 18 µl miRNA template solution, in a total volume of 25 µl. Reverse transcription was performed in a PTC-200 Peltier thermal cycle at 37°C for 60 min and 85°C for 5 min. The resulting reverse transcription reaction product was immediately stored at −20°C for further analysis.

Detection of miRNAs by qPCR

qPCR was performed in 96-well plates by use of an ABI 7500 instrument. qPCR was performed in duplicate for each miRNA, and a non-template control was included on the same plate. To control inter-assay variation, all samples were analyzed on the same plate for a specific miRNA. The qPCR reaction mixture consisted of 10 µl 2× all-in-one qPCR Mix, 2 µl all-in-one miRNA qPCR primer, 2 µl universal adaptor primer, 0.4 µl 50 × ROX reference dye, and 5.6 µl cDNA. All required reagents were supplied in an all-in-one miRNA qPCR kit (Genecopoeia, Rockville, USA). Amplification was performed on an Applied Biosystems 7500 thermal cycler at 50°C for 2 min and 95°C for 10 min, followed by 50 cycles of 95°C for 15 s and 60°C for 1 min. Melting analysis was carried out at the end of the amplification cycle to verify the non-specific amplification.

Data Analysis

Raw fluorescence data were analyzed by use of SDS Relative Quantification Software version 1.4 with automatic baseline (3–15 cycles) and threshold (0.2) settings for quantification cycle (Cq) determination. To reduce inter-plate variance, the Cq values measured for different plates were normalized to an inter-plate calibrator for each miRNA by use of GenEx software. The mean Cq values from duplicate wells were then calculated for further analysis. PCR amplification efficiencies were calculated for each candidate reference miRNA by use of the formula $E = (10^{-1/\text{slope}} - 1) \times 100$, using the slope of the plot of Ct against log input of cDNA. PCR amplification efficiencies for each EC candidate are shown in Table 2.

The software Normfinder [19], geNorm [20], and bestkeeper [21], and the comparative delta-Ct method [22] were used for evaluation of the stability of candidate reference miRNA expression under the experimental conditions used.

By analysis of Cq values, each of the four methods furnishes stability values (M) for each candidate miRNA. Lower M values are indicative of higher expression stability. Because of the different algorithms used by the software, a comprehensive gene-stability value was calculated for a specific candidate as the final stability value (http://www.leonxie.com/referencegene.php?type=reference#). To calculate the expression of the target miR-21 relative to suitable normalizer, the comparative delta-Ct method was used, with relative quantities $Q_{\text{rel}} = E^{-(\text{Cq}_{\text{test sample}} - \text{Cq}_{\text{normalizer}})}$. When normalized to serum volume, the relative expression of miR-21 was calculated by use of the formula: $Q_{\text{rel}} = E^{-(\text{Cq}_{\text{test sample}} - \text{Cq}_{\text{average of all samples}})}$ = PCR amplification efficiency. The relative quantities were corrected for efficiency of amplification.

Statistical analysis was performed by use of SPSS 17.0. One-way ANOVA was used to compare $\log_{10}$-transformed relative quantities of target miR-21 between all groups. Differences in variance between genes were assessed by use of Bartlett's test. P values <0.05 were considered statistically significant for all tests.

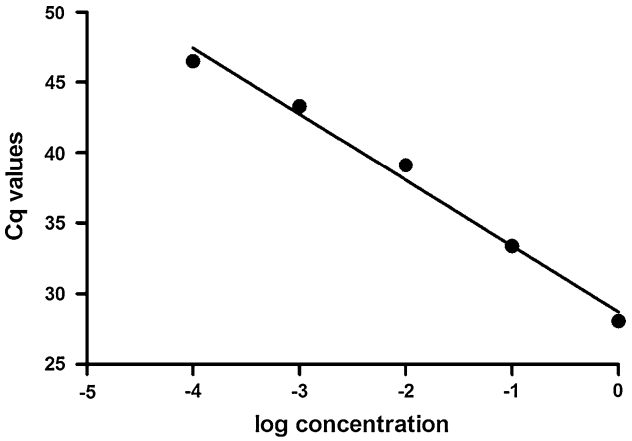

Fig. 1 Linear relationship between Cq values and log concentration of serially diluted serum. The regression curve between Cq values and log concentration for serially diluted serum showed a good linear relationship ($R^2 = 0.9868$) when the Cq values were between 28 and 46. Serial tenfold dilutions of serum were performed and miR-16 expression was measured in these samples. The concentration of primary serum was arbitrarily designated as 1, and thus was 0.1 when the serum was diluted tenfold, etc. Each point represents the mean of duplicate measurements

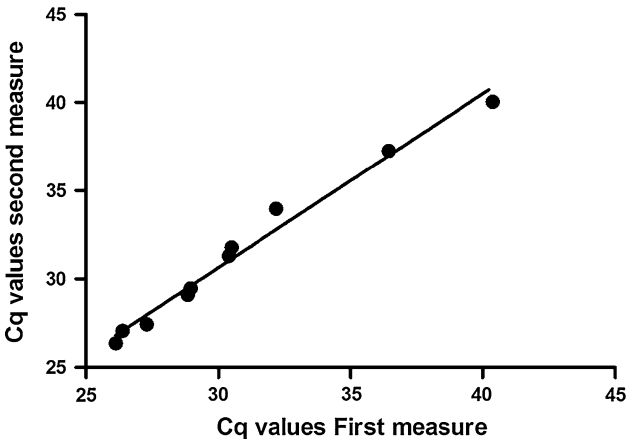

Fig. 2 Comparison of duplicate measurements of individual serum samples. To evaluate the reproducibility of the measurements, miR-16 expression was determined twice in each of 10 serum samples. Each point represents a serum sample. The results showed that all differences of Cq values between the two measurements were <2. Correlation analysis showed correlation between the pairs of measurements was good (Pearson $R = 0.9910$)

Results

Evaluation of Methods for Measuring miRNAs in Serum Samples

To evaluate the range of detectable Cq values, we first examined miR-16 in tenfold serial dilutions of a serum sample and plotted the regression curve as Cq value vs. log concentration. The plot showed that Cq values had a good linear relationship with log concentrations when the Cq values were between 28 and 46 (Fig. 1). We then evaluated the reproducibility of the measurement by assessing miR-16 expression twice in ten serum samples**.** The difference between Cq values from the two measurements was <2, and correlation analysis showed correlation of the pairs of measurements was good (Pearson $R = 0.9910$) (Fig. 2), indicating that reproducibility was good for these levels of miRNAs. These results proved the method of measurement was appropriate for this study.

Expression Levels of Candidate Reference Genes in Serum

Seven candidate reference miRNAs from the serum of gastric cancer patients had a wide expression range, with Cq values between 20.18 and 46.18 (Fig. 3). Expression of Let-7a and miR-93 was relatively low, with median Cq of 38.03 and 40.19 respectively, whereas miR-16, miR-21, and miR-103 were moderately abundant with median Cq of 34.09, 33.30, and 37.22, respectively. miR-451 was more abundant, with a median Cq of 30.06. RNU6B was the lowest expressing candidate reference miRNA with detectable Cqs ranging from 40–48 (data not shown; Cq was not detected in some serum samples). Thus, RNU6B was excluded from further analysis because of its low expression. Using the Cq values of each reference miRNA, there was no evidence for differential expression of any of the candidate reference miRNAs between gastric cancer

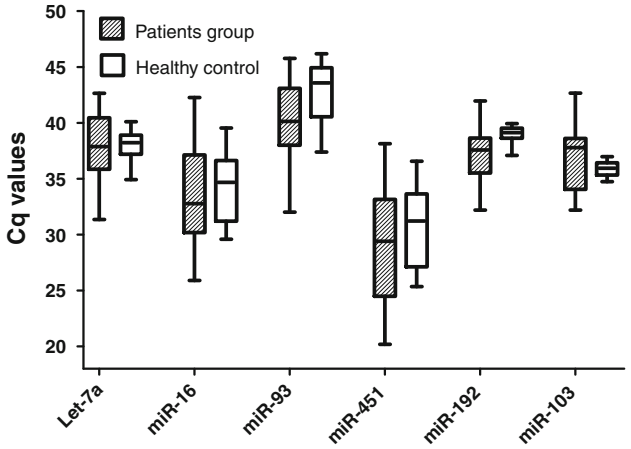

Fig. 3 Cq values of candidate reference miRNAs in all samples. Primary Cq values of candidate reference serum miRNAs in gastric cancer patients and healthy controls. *Boxplots* show interquartile ranges and medians; *whiskers* indicate ranges. No differences were found between miRNAs of the patient group and healthy controls ($P > 0.05$)

patients and healthy controls ($P > 0.05$). This is an important validation step before use of geNorm and NormFinder, because these models assume candidates are not differently expressed between the experimental groups.

miR-93 and miR-16 Are the Most Stable Reference miRNAs

Stability values of candidate reference miRNAs for gastric cancer patients and healthy volunteers are reported in Table 3. miR-451 and miR-103 were found to be the most unstable miRNAs both for gastric cancer patients and for healthy controls, although geNorm ranked miR-451/miR-16 as the most stable miRNA with a stability value of 1.047. More stable miRNAs for gastric cancer patients, for example miR-93 and miR-16, were also found to be highly stable in healthy controls. Although let-7a ranked as the most stable miRNA in healthy controls, its stability was poorer for gastric cancer patients. The similarity of stability profiles between gastric cancer patients and healthy controls provided evidence for further analysis. Next, we attempted to identify reference miRNAs suitable for both patients and healthy volunteers. As shown in Table 4, miR-16 and miR-93 were the top two most stable miRNAs with low stability values. Thus, we inferred these miRNAs may be regarded as universal normalizers within the context of the current dataset, hence we tested the effect of different normalization strategies on serum miR-21 expression in gastric cancer patients.

Table 3 Expression stability of candidate reference miRNAs evaluated in gastric cancer patients and healthy controls

Rank[a]	Gastric cancer group		Healthy controls	
	Gene	Stability	Gene	Stability
1	miR-93	1.86	Let-7a	1.86
2	miR-16	2.11	miR-93	2.63
3	miR-192	2.59	miR-16	2.99
4	Let-7a	3.36	miR-192	3.08
5	miR-451	3.5	miR-451	3.5
6	miR-103	5.05	miR-103	3.83

[a] miRNAs ranked according to comprehensive gene stability value

Effects of Different Normalizers on Relative Quantity of Serum miR-21

To test for the effect of different normalization strategies on the accuracy of the qPCR results, we selected serum miR-21 as the target miRNA, because its expression has been found to be aberrant in the plasma of gastric cancer patients. It has been reported that plasma miR-21 was significantly higher in gastric cancer patients than in healthy volunteers [10]. In our current experiment, depending on the different normalization strategies, serum miR-21 was either significantly different between groups or no difference were detected. First, normalization to serum volume, the most commonly used normalization strategy, was evaluated. In this study, RNA was extracted from 300 μl serum for all samples, thus the Cq values were directly converted to relative quantification by use of the formula already described. There was no significant difference in serum miR-21 between gastric cancer patients and healthy volunteers when the data were normalized to serum volume (Fig. 4a). However, when normalized to miR-16, the most stable candidate ranked by geNorm, and to miR-93, the most stable candidate ranked by Normfinder, serum miR-21 expression was significantly different between stage IV gastric cancer patients and controls (Fig. 4b, c). The effect on serum miR-21 expression of combining miR-93 and miR-16 or of normalization to the mean expression of all candidate miRNAs was assessed. Significant differences in serum miR-21 expression were detected between stage IV gastric cancer patients and controls when either combination approach was used (Fig. 4d). However, none the normalization strategies detected significant differences between stage I/II/III patients and healthy controls, although serum miR-21 tended to higher in stage I/II gastric cancer patients than in healthy controls.

Discussion

In this study we confirmed that normalization with suitable reference genes is important for reliable qPCR results. We tentatively propose that serum miR-16 and miR-93 could be used as normalizers in qPCR assays for detection of gastric cancer patients by serum microRNA expression.

Table 4 Expression stability of candidate reference miRNAs across all patients and healthy volunteers

Rank[a]	Gene	Normfinder	Genorm	Bestkeeper	Delta Ct	Comprehensive
1	miR-16	1.572	1.047	3.301	2.4	**1.778**
2	miR-93	1.387	1.856	2.944	2.43	**2.213**
3	miR-192	1.684	2.409	1.615	2.54	**2.59**
4	Let-7a	1.772	2.205	2.058	2.61	**3.364**
5	miR-451	2.236	1.047	3.723	2.74	**3.5**
6	miR-103	2.617	2.63	2.346	3.07	**5.045**

[a] miRNAs ranked according to comprehensive gene stability value

Bold numbers indicates the geomean of ranking values. They are regarded as the final ranking values, acorrding to which, a recommended comprehensive ranking was obtained

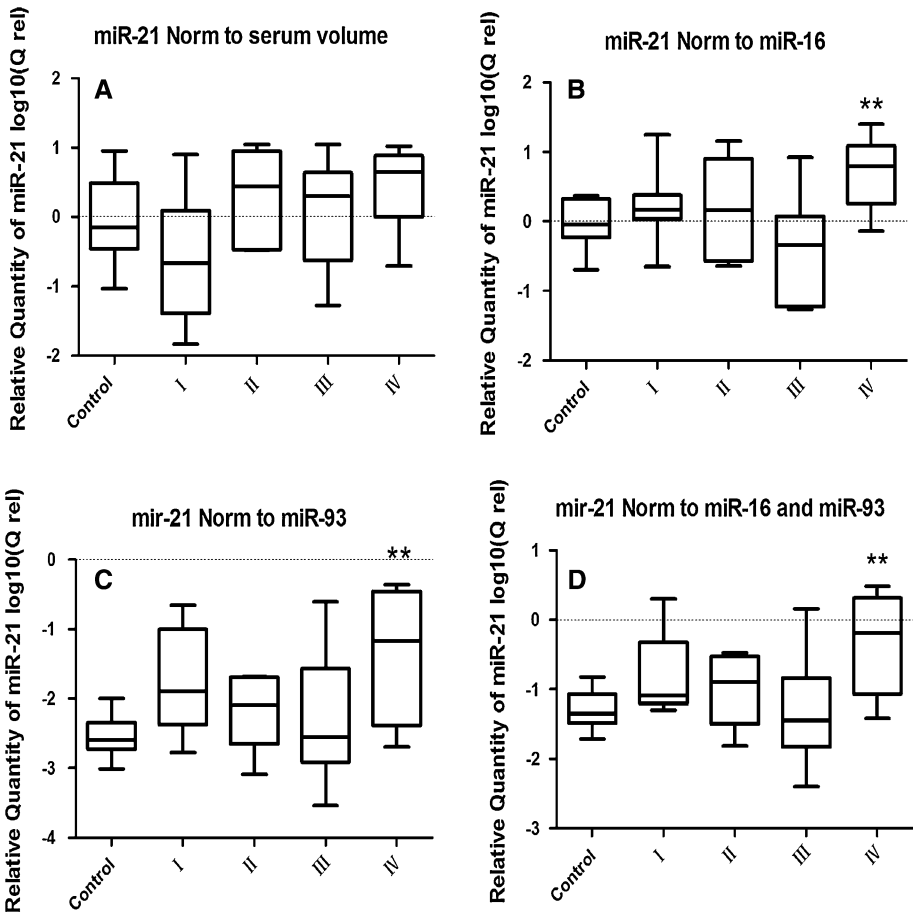

Fig. 4 Normalization strategy strongly affects qPCR analysis. Relative quantities (Q rel $= E^{-\Delta Cq}$) of serum miR-21 in healthy controls and gastric cancer patients using different normalization strategies. *Boxplots* show interquartile ranges and medians; *whiskers* indicate ranges. *Control* represents the healthy controls, and *I*, *II*, *III*, and *IV* indicate different stages of gastric cancer according to the AJCC's staging of gastric cancer (seventh edition, 2010). *Asterisks* indicate significant differences from healthy controls ($P < 0.05$). Expression levels of serum miR-21 were not significantly different when the data were normalized to serum volume (**a**). When the data were normalized to miR-93 (**c**), miR-16 (**b**), and miR-16 and miR-93 combined (**d**), there were significant differences between stage IV and healthy controls ($P < 0.05$)

qPCR is the most frequently used approach for studying miRNA expression. Mature miRNAs were amplified and quantified by use of PCR for the first time in 2005 [23] and recent developments include a 220-plex RQ-PCR enabling analysis of multiple miRNAs from single cells [24]. The high sensitivity of qPCR demands appropriate normalization to correct for non-biological variation, and the use of reference genes as internal controls is the most common method. Although consensus has not yet reached on how best to tackle the normalization strategies in circulating miRNA qPCR studies, numerous RNA species, including rRNA, snRNA, and synthetic miRNA as spike-in controls, have previously been used as internal reference controls. However, there is controversy regarding the abundance and stability of rRNAs and snRNAs in cell-free samples such as serum and plasma.

In our study, RNU6B, which is commonly used to normalize miRNA qPCR data in tissue samples, could not be detected in almost half of the serum samples, whereas in the other half it was detected with Cq values of more than 40. Moreover, RNU6B was found to be less stably expressed than let-7a and miR-16 in normal and cancerous

human solid tissues [25]. Therefore, RNU6B was excluded as a reference candidate because of its low abundance. Non-human (e.g. *C. elegans*) synthetic miRNA is also frequently used as a spike-in control to normalize for technical variability in plasma or serum RNA extraction [26]. However, spike-in controls may not correct for variability arising because of differences in template quality and efficiency of the reverse transcription reaction. The use of specific miRNAs or sets of miRNAs as endogenous controls has been proposed by some investigators. Kroh et al. [26] reported the use of miR-16 and miR-223 as endogenous controls in their laboratory, because both are expressed at high levels in plasma and serum and are relatively invariant across large number of samples. It was also reported that miR-16 and let-7a were suitable normalizers in miRNA expression studies of breast tissue [15].

As far as we are aware, this is the first report detailing the identification and validation of suitable reference genes for qPCR data analysis of circulating miRNAs in gastric cancer patients. In our study, seven candidate reference miRNAs selected from the literature were investigated. RNU6B was not suitable for normalization purposes in this study because of its low expression level. The low abundance of RNU6B may be because it is a large-molecular-weight RNA and is not resistant to RNase in serum or plasma, unlike miRNAs [6]. The other six miRNAs were included for further investigations using the geNorm, Normfinder, bestkeeper, and comparative delta-Ct methods. First, the Cq values of candidate miRNAs were analyzed in the patient and healthy control groups. In the patient group, Normfinder and the comparative delta-Ct method ranked the six candidates identically according to stability, whereas geNorm produced a slightly different result from Normfinder because of their different algorithms. Comprehensive ranking across all four methods identified miR-93, miR-16, and miR-192 as the top three most suitable reference miRNAs. In the healthy control group, the rank order was highly similar to that of the patient group except for let-7a. let-7a was identified as the most stable miRNA by both Normfinder and the comparative delta-Ct method for the healthy group, and the comprehensive ranking recommended let-7a, miR-93, and miR-16 as the top three most stable miRNAs in the healthy group.

Because the patient group and healthy group shared miR-93 and miR-16 in the top three most stable reference miRNAs, we then tried to identify suitable reference miRNAs across both the healthy and patient populations. As expected, miR-16 and miR-93 were also identified as the top-ranked reference miRNAs for normalization across both groups, thereby supporting the validity of the results obtained from different modeling strategies. Therefore, miR-16 and miR-93 could serve as reference miRNAs for qPCR analysis of serum miRNA in gastric cancer patients and healthy controls.

We illustrated the effect of different normalization strategies on the accuracy of qPCR results by analyzing the expression of serum miR-21, for which aberrant expression is observed in both the plasma and gastric cancer tissues of gastric cancer patients [27]. miR-21 has frequently been observed to be aberrantly overexpressed in a variety of tumors [28]. In gastric cancer tissues, miR-21 is reported to be of pivotal importance in pathogenesis and progression by targeting RECK, PDCD4, and PTEN [29, 30]. Tsujiura et al. [10] reported that plasma concentrations of miR-21 were significantly higher in gastric cancer patients than in healthy controls. In our study, the difference between miR-21 expression in the patient and healthy groups varied depending on which single reference miRNA was used in our experiment. When the data were normalized to serum volume, a significant difference between healthy people and patients was not detected. However, when the data were normalized to miR-16, miR-93, or miR-16 and miR-93 combined, a significant difference between stage IV gastric cancer patients and healthy controls was detected. These results indicated that normalization to systematically selected reference miRNAs could detect even smaller differences than normalization to serum volume. Previous studies have reported that use of multiple references for normalization may be better for distinguishing very small differences. When the data were normalized with the mean expression of all candidate miRNAs, differences were also detected between patients and the healthy group (results not shown). These results draw particular attention to the potential effect of reference choice on the outcome of a study, and demonstrate the need for validation of candidate reference genes to produce reliable expression data.

The potential limitations of this study should also be taken into account. First, the number of candidate reference genes studied in our experiment is small; candidate reference genes selected in a genome-wide serum microRNA expression profile would be better. Second, in this study, the identity of miR-16 and miR-93 reference genes was confirmed by normalization to serum miR-21 expression only. The identity of reference genes must be further validated for normalizing the expression of other serum miRNAs. Furthermore, all serum samples used in this study were obtained from gastric cancer patients, and it remains to be determined whether our findings can be applied to other diseases.

In conclusion, our study clearly shows that accurate selection of reliable reference genes is an absolute prerequisite for measurement of serum miRNA expression by use of qPCR. This study has identified two stably-expressed miRNAs for qPCR normalization analysis in human gastric cancer studies. We recommend the combination of miR-16 and miR-93 as normalizers in this context.

Conflict of interest No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

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