# A proposed method for the relative quantification of levels of circulating microRNAs in the plasma of gastric cancer patients

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**Abstract.** Gastric cancer (GC) is the fifth most common type of malignancy and the third leading cause of cancer-associated mortality worldwide. It is necessary to identify novel methods aimed at improving the early diagnosis and treatment of GC. MicroRNA expression profiles in the plasma of patients with GC have demonstrated a potential use in the opportune diagnosis of this neoplasm. However, there are currently no standardized targets for use in the normalization of microRNA Cq values for different neoplasms. The present study tested two normalization approaches while analyzing plasma derived from patients with GC and non-atrophic gastritis. The first method utilized a panel of small nucleolar RNAs (snoRNAs) and a small nuclear RNA (snRNA) provided by a commercial array. The second normalization approach involved the use of hsa-miR-18a-5p and hsa-miR-29a-3p, which were identified by a stability analysis of the samples being tested. The results revealed that the snoRNAs and snRNA were not expressed in all samples tested. Only the stable microRNAs allowed a narrow distribution of the data and enabled the identification of specific downregulation of hsa-miR-200c-3p and hsa-miR-26b-5p in patients with GC. hsa-miR-200c-3p and hsa-miR-26b-5p have been previously linked to cancer, and a Kyoto Encyclopedia of Genes and Genomes analysis demonstrated that these microRNAs were associated with cell adhesion, cell cycle and cancer pathways.

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## Introduction

Cancer is a leading cause of mortality worldwide, with 8.2 million cancer-associated mortalities being reported in 2012. Gastric cancer (GC) is the fifth most common type of cancer, with 952,000 new cases per year, and is also the third leading cause of cancer-associated mortality, of which 70% are reported in developing countries, particularly those in East Asia and Latin America (1). The number of cases in these countries is expected to rise in the coming years due to the aging of the population and the lack of public strategies being undertaken to reduce the risk factors.

There is an urgent requirement to develop new methods for the early detection of this neoplasm using minimally invasive procedures with high sensitivity and specificity. Previous research has suggested that circulating levels of microRNAs are specific and sensitive for the detection of GC lesions (2). MicroRNAs are stable due to their packaging in exosomes and apoptotic bodies and their ability to form complexes with lipoproteins and binding proteins, which make them resistant to RNase activity (3). Furthermore, certain microRNAs have been implicated in the development of cancer, and demonstrate specific expression patterns in various types of tumor (4-6), including GC (7-9).

Studies have suggested that microRNAs may be candidate biomarkers for use in the detection of cancer. A recent meta-analysis revealed that circulating microRNAs can be used as targets for the detection of various types of gastrointestinal cancers, and that plasma samples have a higher accuracy in this regard compared with serum samples (10).

Li *et al* (11) identified hsa-miR-223 and hsa-miR-21 to be overexpressed and hsa-miR-218 underexpressed in the plasma of patients with GC, and suggested that these microRNAs are involved in the processes of tumorigenesis and metastasis. Gorur *et al* (12) conducted an analysis of the expression profiles of 740 different microRNAs in patients with GC, and reported that only hsa-miR-195-5p was underexpressed in sera. Song *et al* (13) identified 16 microRNAs that were overexpressed in the sera of patients with GC; however, following additional investigation, the authors suggested that only hsa-miR-221, hsa-miR-744 and hsa-miR-376c were potential biomarkers. A study based on a Chinese population revealed that hsa-miR-148a, hsa-miR-142-3p, hsa-miR-26a and hsa-miR-195 were downregulated in patients with GC; in particular, the plasma levels of hsa-miR-26a were significantly reduced (14). Furthermore, a meta-analysis revealed that 35 microRNAs have been reported as being candidate biomarkers for GC detection, but that hsa-miR-21 is the only microRNA to be consistently reported (15).

This inconsistency of results may be a result of differences in the GC subtypes (9) or populations being studied, differences in microRNA extraction methods or differences in the methods of detection used (16). A critical drawback is the lack of targets to which data from sera or plasma samples may be normalized, similar to the used of β-actin or GAPDH levels to normalize intracellular RNAs. Several studies have proposed to use 5S ribosomal RNA (17), hsa-miR-93, hsa-miR-191, RNU6-2, the small nucleolar RNAs: SNORD48, SNORD61, SNORD68, SNORD72, SNORD95 and SNORD96a (18), and hsa-miR-16-5p (19) for normalization. However, a number of these genes have been dismissed due to their instability in circulation (20,21). The present study presents evidence suggesting that the standardization of microRNA data expression must be established according to the neoplasm and the type of sample. Steady levels of hsa-miR-18a-5p and hsa-miR-29a-3p were identified in the panel of samples used in the present study, and the use of those microRNAs as normalizers of the array data allowed the identification of hsa-miR-200c-3p and hsa-miR-26b-5p underexpression in plasma samples from patients with GC.

## Materials and methods

*Patients and samples*. The present study included 6 adult patients (5 males and 1 female) with distal GC, of which 3 had intestinal-type (IGC) and 3 had diffuse-type (DGC), without any other type of neoplasia (Table I). None of the patients had undergone treatment. The comparison (control) group comprised plasma samples from 2 patients with non-atrophic gastritis (Table I). Plasma samples were stored at -70˚C until use.

The inclusion criteria for the study were individuals who attended the Oncology Hospital and the General Hospital of the XXI Century National Medical Center (Mexican Institute of Social Security, Mexico City, Mexico) due to gastric symptoms and were diagnosed with either GC or non-atrophic gastritis. The exclusion criteria were as follows: Individuals and samples that did not have a diagnosis of GC or non-atrophic gastritis; patients with other types of cancer; patients with recurrent cancer; and patients who had already received cancer therapy.

Samples were not included in the study if they lacked the RNA quantity and/or quality for the microRNA analysis, or if a sufficient quality of complementary DNA (cDNA) was not identified in the GAPDH multiplex reverse transcription (RT)-polymerase chain reaction (PCR).

The present study was approved by the Scientific and Ethics Committees of the Mexican Institute of Social Security, and patients were informed regarding the nature of the study and asked to sign a consent letter. The study was conducted according to the best clinical practices of our institution and the identity of the patients was anonymized for the duration of the study.

*RNA extraction*. RNAs were extracted from 100 µl plasma samples using the miRNeasy Serum/Plasma kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's protocol. The RNA concentration and purity [using the optical density (OD) 260/280 nm ratio] were determined in an Epoch spectrophotometer (BioTek Instruments, Inc., Winooski, VT, USA).

*cDNA synthesis*. cDNA synthesis was performed using a miScript II RT kit (Qiagen GmbH, Hilden, Germany). Mature microRNAs were polyadenylated by poly-A polymerase followed by cDNA synthesis using oligo-dTs. The PCR reaction mixture (20 µl) contained 1X miScript HiSpec buffer, 1X miScript Nucleics mix, 1X miScript reverse transcriptase mix and 250 ng of RNA as template. The mixture was incubated for 60 min at 37˚C for polymerization, followed by 5 min at 95˚C to inactivate the miScript reverse transcriptase.

*GAPDH multiplex PCR*. cDNA quality was determined using a Multiplex PCR kit (Qiagen GmbH). In this reaction, 7 fragments (100-700 bp) of the GAPDH gene were amplified, and the sequences of the primers were described previously (22). The PCR reaction mixture (12.5 µl) contained 1X Master mix, 0.2 µM of each primer and 5 µl of cDNA. Amplification was performed in a MasterCycler GSX1 (Eppendorf, Hamburg, Germany) as follows: 95˚C for 15 min; followed by 40 cycles of 94˚C for 30 sec, 57˚C for 90 sec and 72˚C for 90 sec; and a final extension at 72˚C for 10 min. PCR products were separated by electrophoresis on a 2.0% agarose gel with a 100 bp DNA ladder (Promega Corporation, Madison, WI, USA), stained with 1X GelRed™ (Biotium, Inc., Hayward, CA, USA) and visualized under an ultraviolet light transilluminator and photodocumenter system (Syngene, Frederick, MD, USA).

*RT-quantitative PCR (qPCR) array*. The microRNA expression profile was analyzed using a miScript SYBR® Green PCR kit (Qiagen GmbH) and a Serum and Plasma miScript miRNA PCR Array for the detection of microRNAs in human serum or plasma (Qiagen GmbH; catalog no. MIHS-106ZF). The array comprises microRNAs that have been reported in blood circulation in various types of cancer, including GC; these microRNAs include members of the let-7 and microRNA-200 families. The following RNAs are used as controls in the array: One oligo for the detection of miR-39 *Caenorhabditis elegans*; 5 small nucleolar RNAs (snoRNAs: SNORD61, SNORD68, SNORD72, SNORD95, SNORD96A) and 1 small nuclear RNA (snRNA: RNU6-6P)

Table I. Clinical characteristics of patients with GC or non-atrophic gastritis.

A, GC samples

| Sample ID | Age, years | Gender | Cancer type |
|---|---|---|---|
| 1GC | 72 | Male | Intestinal[b] |
| 2GC | 58 | Male | Diffuse[b] |
| 3GC | 91 | Male | Intestinal[b] |
| 4GC | 76 | Male | Diffuse[b] |
| 5GC | 76 | Male | Diffuse[a,b] |
| 6GC | 52 | Female | Intestinal |

B, Gastritis samples

| Sample ID | Age, years | Gender | Diagnosis |
|---|---|---|---|
| 1C | 64 | Male | Non-atrophic gastritis[b] |
| 2C | 62 | Male | Non-atrophic gastritis[b] |

None of the samples had been subjected to treatment. [a]Without history of hereditary GC; [b]*Helicobacter pylori*-negative. GC, gastric cancer; ID, identification code.

for data normalization; a control for the RT reaction (miRTC) and a positive PCR control (PPC). The PCR reaction mixture (25 µl/well) contained 1X QuantiTect SYBR® Green PCR Master Mix, 1X miScript Universal Primer-T, and 1 µl/well of cDNA. The following thermal cycling was employed: Initial activation at 95˚C for 15 min; followed by 45 denaturation cycles at 94˚C for 15 sec, annealing at 55˚C for 30 sec and extension at 70˚C for 30 sec. The reaction was performed using a Roche® LightCycler® 480 (Roche Diagnostics, Indianapolis, IN, USA).

*Stability analysis*. A stability analysis of the microRNAs was performed using the NormFinder algorithm (23). This software determined the overall expression variation of microRNA targets and controls between samples.

*Analysis of RT-qPCR data*. Data were analyzed using the miScript miRNA PCR Array Data Analysis tool (http://pcrdataanalysis.sabiosciences.com/mirna; free access). The relatively quantified levels of the expression of microRNAs, based on their quantification cycle (Cq) were estimated using the ΔΔCq method (24). Firstly, microRNAs with a Cq>35 were discarded. The remaining microRNAs complied with the values established in the miRTC (ΔCq<7) and PPC (Cq=19±2) controls. Cq values were normalized independently using the snoRNAs and snRNA panel and the microRNAs identified using the stability analysis.

*Pathways associated with microRNAs and GC*. *In silico* analyses were performed to determine the pathways associated with the microRNAs of interest; miRPath DIANA software (version 3.0) (25) was used to perform a Kyoto Encyclopedia of Genes and Genome (KEGG) pathway analysis based on the TarBase database (version 7.0).

*Statistical analysis*. A non-parametric Mann-Whitney U test/Wilcoxon rank-sum test was performed to assess differences between the normalization strategies employed in this study. Analyses were performed using the statistical software package SPSS (version 22.0; IBM SPSS, Armonk, NY, USA) and the R Project (version 3.2.3; https://CRAN.R-project.org/doc/FAQ/R-FAQ.html) to corroborate the results. Following the *in silico* analysis of the targets and pathways associated with microRNAs, a false discovery rate (FDR) correction with a P-value <0.05 was used. P<0.05 was considered to indicate a statistically significant difference.

## Results

*Patients and samples*. In total, 3 IGC, 3 DGC and 2 non-atrophic gastritis samples were analyzed. The pathological characteristics of the samples were determined by two independent pathologists. The clinical characteristics of the patients are shown in Table I.

*RNA extraction*. RNA extraction produced on average 90±3 ng/µl RNA in a final volume of 50 µl. RNA quality was determined by the OD 260/280 nm, which produced a value of 1.9±0.1.

*GAPDH multiplex PCR*. The quality of the cDNA was tested by a GAPDH multiplex PCR, from which the shortest fragment amplified in all samples was of 200 bp, indicating homogeneous cDNA quality between samples.

*RT-qPCR array*. Following the array, the miRTC (ΔCq=3±1) and PPC (Cq=18±1) values obtained complied with the standard of quality. By contrast, the snoRNAs and snRNA control panel included in the array demonstrated inconsistent Cq

Table II. Cq values for 6 quantitative polymerase chain reaction controls, including 5 small nucleolar RNAs (SNORD61, SNORD68, SNORD72, SNORD95 and SNORD96A) and a small nuclear RNA (RNU6-6P) for data normalization.

| Sample ID | SNORD61 | SNORD68 | SNORD72 | SNORD95 | SNORD96A | RNU6-6P |
|---|---|---|---|---|---|---|
| 1C | UD | UD | UD | UD | 31.66 | 32.84 |
| 2C | 33.55 | 28.87 | UD | 31.81 | 32.78 | 34.50 |
| 1GC | 33.70 | 32.98 | UD | UD | 33.99 | UD |
| 2GC | 31.98 | 32.60 | UD | 30.48 | 31.86 | 32.72 |
| 3GC | UD | UD | UD | 33.42 | 34.77 | UD |
| 4GC | UD | 34.43 | UD | UD | UD | UD |
| 5GC | UD | 31.72 | UD | 34.42 | UD | UD |
| 6GC | 31.83 | 32.40 | UD | 32.23 | UD | UD |

Cq, quantification cycle; ID, identification code; C, non-atrophic gastritis sample; GC, gastric cancer sample; UD, undetermined (Cq value reported as >35 cycles).

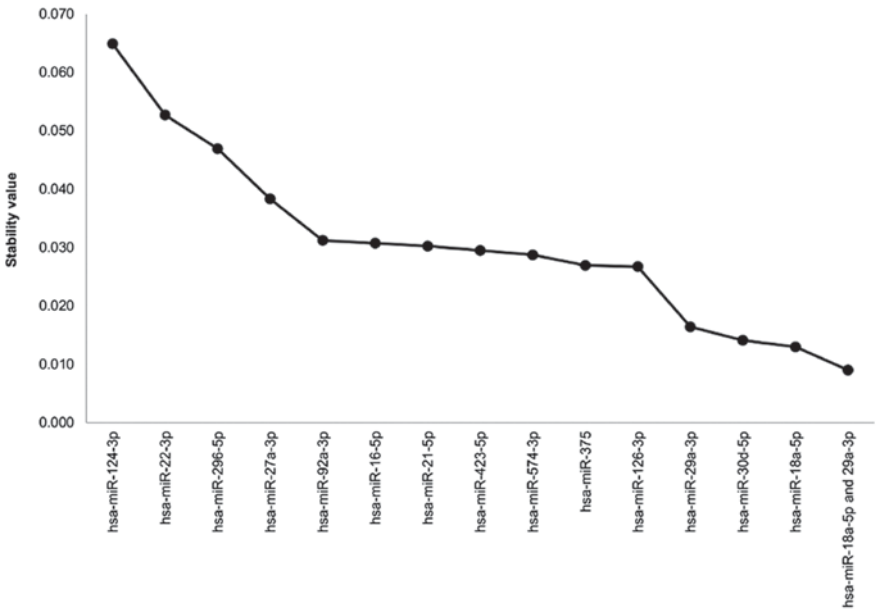


Figure 1. Stability values for 14 microRNAs with constant expression in gastric cancer and non-atrophic gastritis samples. The stability values for the 14 microRNAs were calculated using NormFinder. The values were plotted from the least stable microRNA (hsa-miR-124-3p; stability value, 0.065) to the most stable (hsa-miR-18a-5p; stability value, 0.013). Additionally, the improved stability value using the combination of two microRNAs (hsa-miR-18a-5p and 29a-3p; stability value, 0.009) was included.

values, with some of the samples yielding positive readings only following 35 cycles (Table II); those samples were scored as 'undetermined' for the snoRNAs and snRNA normalizing controls.

*Stability analysis*. Since a single snoRNA and/or snRNA with positive values across all of the samples was not obtained, other targets were investigated for normalization. A total of 14 microRNAs with positive Cq values in all samples were identified, and their stability values were determined using the NormFinder algorithm. Smaller stability values are indicative of fewer variations in expression. A total of 3 microRNAs with a score <0.02 were identified: hsa-miR-18a-5p, hsa-miR-30d-5p, and hsa-miR-29a-3p (Fig. 1). The NormFinder algorithm demonstrated that a combination of hsa-miR-18a-5p and hsa-miR-29a-3p had a stability value of 0.009, and this combination was selected as the best array data normalizer.

*Data normalization to evaluate microRNA expression levels*. The relative expression of 84 microRNAs was determined in GC and non-atrophic gastritis samples. Briefly, the relative expression levels were used to construct a clustergram (http://pcrdataanalysis.sabiosciences.com/mirna) to observe the microRNA expression profiles of the samples. Firstly, the snoRNAs and snRNA normalizer control panel provided by the array was used. Following this normalization strategy, no specific expression profile was observed that would allow the distinction of non-atrophic gastritis from GC, or IGC from DGC (Fig. 2). Subsequently, each microRNA was evaluated manually and normalized against the combination of hsa-miR-18a-5p and hsa-miR-29a-3p. More consistent

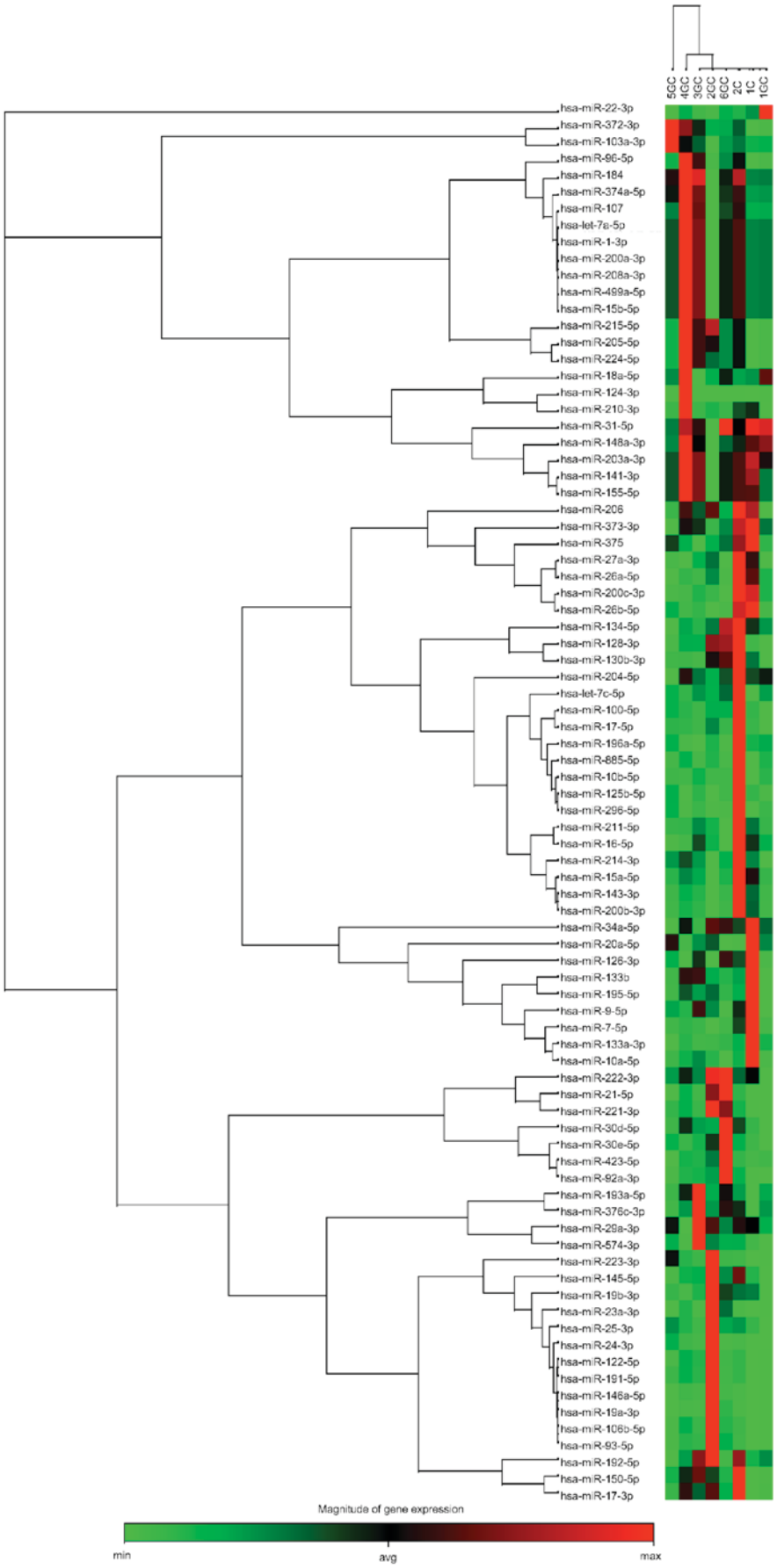


Figure 2. Differential expression levels of 84 microRNAs in plasma samples from patients with GC or non-atrophic gastritis, normalized using snoRNAs/snRNA panel. Of the 84 microRNAs that were evaluated, no specific expression profile was identified that would allow the distinction of non-atrophic gastritis (1C and 2C) from GC samples (1-6GC), or of intestinal-type GC samples (1GC, 3GC and 6GC) from diffuse-type GC samples (2GC, 4GC and 5GC). GC, gastric cancer; min, minimum; avg, average; max, maximum.

Table III. KEGG pathways associated with hsa-miR-200c-3p and/or hsa-miR-26b-5p.

| KEGG pathway | P-value | Number of genes | Associated microRNAs |
|---|---|---|---|
| microRNAs in cancer | $2.18163x10^{-39}$ | 71 | hsa-miR-200c-3p and hsa-miR-26b-5p |
| Hippo signaling pathway | $6.73503x10^{-7}$ | 46 | hsa-miR-200c-3p and hsa-miR-26b-5p |
| p53 signaling pathway | $3.60141x10^{-6}$ | 33 | hsa-miR-200c-3p and hsa-miR-26b-5p |
| Glycosaminoglycan biosynthesis-chondroitin sulfate/dermatan sulfate | $1.96115x10^{-5}$ | 9 | hsa-miR-26b-5p |
| TGF-β signaling pathway | $3.86866x10^{-5}$ | 29 | hsa-miR-200c-3p and hsa-miR-26b-5p |
| Cancer pathways | $5.33533x10^{-5}$ | 116 | hsa-miR-200c-3p and hsa-miR-26b-5p |

miRPath DIANA v3.0 software was used to perform KEGG analysis using TarBase database. Statistical analysis with a false discovery rate correction was performed and P<0.05 was considered to indicate statistical significance. KEGG, Kyoto Encyclopedia of Genes and Genomes; miR, microRNA; p53, tumor protein p53; TGF-β, transforming growth factor-β.

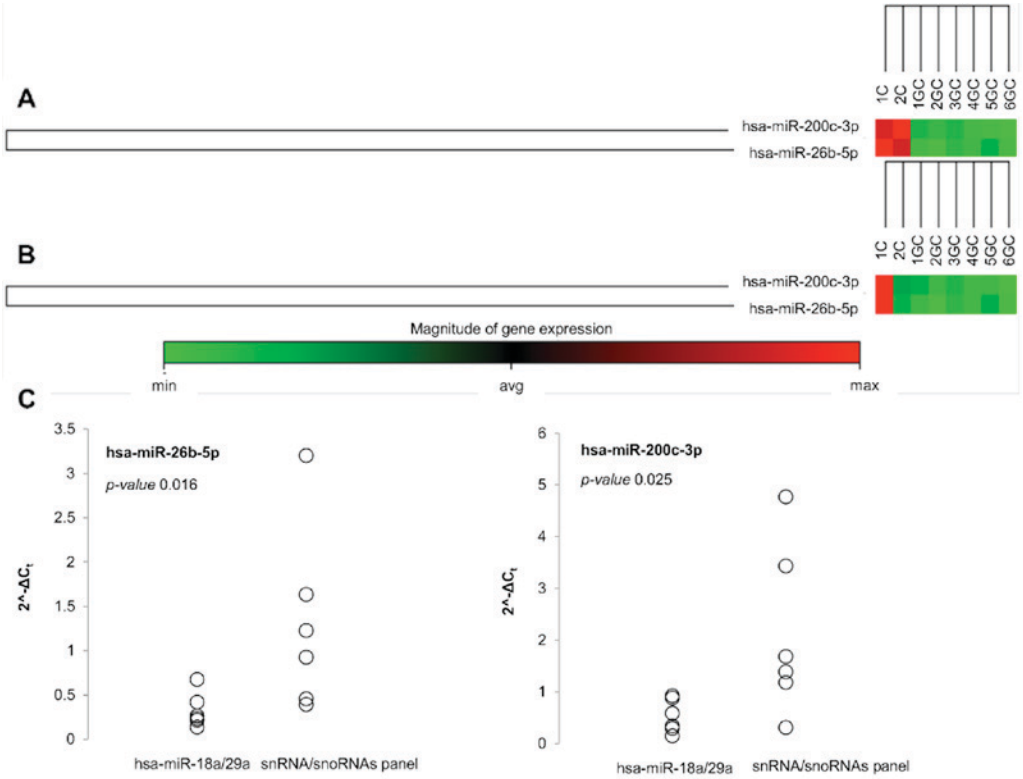


Figure 3. Differential expression levels of hsa-miR-200c-3p and hsa-miR-26b-5p obtained by two different methods of normalization in plasma samples from patients with GC or non-atrophic gastritis. (A and B) Clustergrams showing hsa-miR-200c-3p and hsa-miR-26b-5p expression levels in patients with GC (1-6GC) or non-atrophic gastritis (1C and 2C) normalized using (A) the stable microRNAs hsa-miR-18a-5p and hsa-miR-29a-3p, or (B) the snoRNA/snRNA panel. (C) Distribution of $2^{-\Delta\Delta Cq}$ values of downregulated microRNAs in the plasma samples of patients with GC, obtained by the two different normalization methods. Circles represent the values of $2^{-\Delta\Delta Cq}$ for hsa-miR-200c-3p and hsa-miR-26b-5p normalized using hsa-miR-18a-5p/hsa-miR-29a-3p or the snoRNA/snRNA panel. For the two microRNAs assessed, a smaller amount of variation was observed when using hsa-miR-18a-5p/hsa-miR-29a-3p for normalization compared with the use the snoRNA/snRNA panel for normalization. The P-values obtained for each of the microRNAs indicates a significant difference between the two methods of normalization (P<0.05). GC, gastric cancer; snoRNA, small nucleolar RNA; snRNA, small nuclear RNA; min, minimum; avg, average; max, maximum; Cq, quantification cycle.

results were observed following normalization against these stable microRNAs, which allowed the differentiation between GC and non-atrophic gastritis by the expression profiles of hsa-miR-200c-3p (ΔΔCq and Cq P=0.00293) and hsa-miR-26b-5p (ΔΔCq and Cq P=0.00293), which were significantly decreased in GC samples. A comparison of the clustergrams following normalization with hsa-miR-18a-5p and hsa-miR-29a-3p is presented in Fig. 3. There is a difference between CG and non-atrophic gastritis in data that has been normalized to hsa-miR-18a-5p and hsa-miR-29a-3p (Fig. 3A) compared with those that have been obtained with the snoRNA/snRNA panel (Fig. 3B).

A scatter analysis was also performed in order to observe the distribution of hsa-miR-26b-5p and hsa-miR-200c-3p

expression levels determined by normalization with hsa-miR-18a-5p and hsa-miR-29a-3p, or with the snoRNAs and snRNA panel; this revealed that the distribution of the expression values was narrower when hsa-miR-18a-5p and hsa-miR-29a-3p were used for normalization (Fig. 3C).

*Pathways associated with hsa-miR-200c-3p and hsa-miR-26b-5p expression*. DIANA miRPath reported a total of 841 targets for hsa-miR-200c-3p and 2,904 targets for hsa-miR-26b-5p using the TarBase database. The KEGG analysis identified 22 pathways in which hsa-miR-200c-3p and hsa-miR-26b-5p are most likely to have an important regulatory role, of which 6 were found to have significant P-values (Table III). These 6 identified pathways were mainly involved in processes such as cell adhesion, cell cycle and cancer.

## Discussion

The detection of microRNAs is increasingly being considered as a reliable tool to assess cancer diagnosis and prognosis, and the detection of circulating microRNAs has the additional benefit of overcoming the necessity for invasive procedures (26). However, uncertainty remains regarding the best candidate targets for data normalization, since different types of tissues and cancers secrete variable levels of microRNAs into the circulation. As microRNAs are critical regulators of cellular processes, their own expression is strongly regulated. In GC, varying profiles of expression should be expected from tumors of different histological types or arising from different regions of the stomach, or depending on whether the patient is positive or negative for *H. pylori*. Furthermore, the stability of secreted microRNAs is variable.

Wang *et al* (27) identified different profiles of microRNAs present in *H. pylori*-positive gastritis and intestinal metaplasia samples, supporting the use of microRNAs as biomarkers for the progression of gastric lesions (27). However, despite several different lines of evidence implicating numerous microRNAs as potential biomarkers for GC, hsa-miR-21 expression is the only microRNA that has been consistently identified to exhibit altered expression in GC (15). The present study also identified hsa-miR-21 to be overexpressed, but only in 2 of the GC samples (Fig. 2). By contrast, the remaining 4 GC samples demonstrated similar values to those of the non-atrophic gastritis samples. Therefore, this microRNA did not fulfill the objective of this study to identify microRNAs that are differentially expressed between patients with non-atrophic gastritis and those with GC.

Considering the differential expression and the stability of circulating microRNAs, it has been recommended to establish the normalizing microRNAs within the set of samples being tested (16). In the present study, using the NormFinder algorithm, improved stability values were observed for hsa-miR-18a-5p and hsa-miR-29a-3p compared with the snoRNA/snRNA panel provided by the array. Data normalized with these stable microRNAs were more narrowly distributed, and allowed the identification of downregulated expression of hsa-miR-200c-3p and hsa-miR-26b-5p in all GC samples studied, compared with the non-atrophic gastritis samples. These microRNAs have been reported to be dysregulated in GC and other types of cancer in previous studies (28-41). Therefore, the present study also supports normalization against the most stable microRNAs within the set of samples analyzed.

hsa-miR-200c belongs to the microRNA-200 family, which includes hsa-miR-200a, hsa-miR-200b, hsa-miR-200c, hsa-miR-141 and hsa-miR-429, whose increased expression has been associated with negative regulation of the epithelial-to-mesenchymal transition (EMT) via the downregulation of master regulators of EMT, zinc finger E-box binding homeobox (ZEB) 1 and 2 (28). In agreement with this, it has been observed that the overexpression of hsa-miR-200c can suppress metastasis following the orthotopic xenotransplantation of liver cancer cells (28). In a clinical colorectal cancer study by Hur *et al* (29), miR-200c underexpression was observed at the invasive front of the primary tumors, whereas its overexpression as detected in liver metastases. A study revealed a complex pattern of expression of this family of microRNAs in which lower levels of expression may promote EMT, facilitating cell invasion and metastasis, whilst higher levels of them may promote the reversion of EMT and the colonization of distant organs (30). A similar axis of regulation between microRNA-200, ZEB transcription factors and EMT has been previously reported in GC cell lines (31). The underexpression of hsa-miR-200c has also been reported in GC samples derived from aggressive tumors and in invasive GC-derived cell lines with higher cell growth capacity (32). The reported targets of hsa-miR-200b and hsa-miR-200c are DNA methyltransferase (DNMT) 3A, *DNMT3B* and *SP1* (a transactivator of the *DNMT1* gene); thus, the loss of these microRNAs has been associated with increased global DNA methylation, and their re-expression associated with concomitant re-expression of genes encoding E-cadherin and p16 through promoter hypomethylation (32). The present data agree with these studies, as they also support the finding of a loss of hsa-miR-200c during the progression from non-atrophic gastritis to GC. However, the overexpression of hsa-miR-200c has also been reported in the sera of patients with GC when compared with sera from healthy controls, and the same study also reported that high levels of hsa-miR-200c were markers of poor prognosis (33). The reasons for this discrepancy of data, and the question of how a suppressor of EMT could be associated with poor prognosis, is not currently understood.

microRNAs are therefore divided into two types, oncogenic microRNA (onco-microRNA) and tumor-suppressive microRNA. microRNA-26 has also been documented as being a critical regulator of tumor initiation and progression (onco-microRNA) and as a tumor suppressor microRNA. microRNA-26 has been observed to be downregulated in several types of cancer, including bladder, breast and lung cancer, as well as in hepatocellular carcinoma (34). In prostate cancer hsa-miR-26b was underexpressed and acted as a tumor suppressor by regulating the oncogene La ribonucleoprotein domain family member 1 together with hsa-miR-26a (35). By contrast, two studies have observed an overexpression of hsa-miR-26b-5p in GC tissue samples when compared with non-tumor adjacent tissue (42,43). However, neither of these studies associated hsa-miR-26b expression with clinical features. It has been observed that hsa-miR-26a serves a critical role in the carcinogenesis of different tissues by regulating various processes, including

angiogenesis, invasion, cellular proliferation, metastasis and energy metabolism (37). Additionally, the therapeutic use of hsa-miR-26b has been suggested, since its overexpression is associated with decreased cellular proliferation, migration and invasion in hepatocellular carcinoma (38), osteosarcoma (39), epithelial ovarian carcinoma (40), prostate (35) and lung cancer (41). Qiu *et al* (14) also reported the downregulation of hsa-miR-26a in the plasma of patients with GC, identifying this microRNA as an interesting candidate for use as a GC biomarker.

In the present study, the DIANA miRPath and KEGG analyses revealed that hsa-miR-200c-3p and hsa-miR-26b-5p microRNAs were associated with cancer pathways, including p53 signaling, hippo signaling, transforming growth factor-β signaling, and glycosaminoglycan biosynthesis-chondroitin sulfate/dermatan sulfate. Cooney *et al* (44) described that chondroitin sulfate glycosaminoglycans serve an important role in metastasis of breast cancer cells, and proposed that these polysaccharides could be targets for novel anti-metastatic therapies (44).

A critical limitation of the present study is the low number of samples tested. Despite this, the two microRNAs identified have been previously suggested to be involved in cancer, and the *in silico* analysis supported the involvement of hsa-miR-200c-3p and hsa-miR-26b-5p in cancer pathways, confirming these microRNAs as interesting candidates to be evaluated as potential biomarkers for GC progression in larger scale studies. The present study therefore serves as a pilot study, providing an initial exploration of circulating microRNAs that may mirror oncogenic processes occurring in the gastric mucosa. Although an extension of this study is planned, we consider the observations regarding the instability of the array normalization targets to be a valuable contribution for researchers aiming to quantify microRNA levels. There are already reports demonstrating that normalization based on the values of the snoRNA/snRNA of the array is not precise (16-18,20-23). The present study has provided a strategy to overcome those problems, and this method of analysis based on a stability test allowed reliable data to be obtained, since the two microRNAs identified to be downregulated in GC patients have previously been reported in other GC studies of circulating and tissue microRNAs. In addition to increasing the number of samples analyzed, it is important to perform *in silico* and *in vitro* analyses of the target genes of hsa-miR-200c and hsa-miR-26b, in order to improve the understanding of their participation in gastric carcinogenesis.

In conclusion, the present data supports the use of a test to identify the most stable microRNAs within a set of samples. Using a stability test, hsa-miR-18a-5p and hsa-miR-29a-3p were identified as the most stable microRNAs across all the samples. hsa-miR-18a-5p and hsa-miR-29a-3p microRNAs were subsequently used to normalize samples derived from patients with gastric lesions, which led to the finding that the expression levels of hsa-miR-200c-3p and hsa-miR-26b-5p were downregulated in sera from GC patients compared with sera from patients with symptomatic non-atrophic gastritis. This finding suggests that hsa-miR-200c-3p and hsa-miR-26b-5p may be lost throughout the progression of gastric lesions.

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