**Hsa-miR-21 and Hsa-miR-29 in Tissue as Potential Diagnostic and Prognostic Biomarkers for Gastric Cancer**

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**Key Words**

Hsa-miR-21 • Hsa-miR-29 • Diagnosis • Prognosis • Gastric cancer

**Abstract**

**Background/Aims:** Gastric cancer (GC) is the fourth most common cancer and the second most common cause of cancer deaths worldwide. Endoscopic examination is the most used method to detect the GC nowadays, whereas this method is expensive and invasive. MicroRNAs (miRNAs) are a group of recently discovered small non-protein-coding RNAs. They regulate the expression of hundreds of target genes; thereby control a wide range of tumorigenic processes. In this study, we selected two miRNAs, hsa-miR-21 and hsa-miR-29, as the targets to assess their diagnostic and prognostic value for GC. **Methods:** A total of 50 GC patients including 24 females and 26 males were recruited. Tumor and adjacent non-tumor tissue samples were collected from all these participants during the endoscopic examination. RNAs were extracted from these samples, then quantified via qRT-PCR and normalized with RNU43 as the internal control. Statistical analyses were conducted using the GraphPad Prism 5.0. **Results:** We discovered a higher expression of hsa-miR-21 and a relatively lower expression of hsa-miR-29 in the tumor tissue than in the adjacent non-tumor tissue. Moreover, both the two miRNAs showed moderate diagnostic performance (hsa-miR-21: AUC = 0.75, sensitivity = 0.70, specificity = 0.78; hsa-miR-29: AUC = 0.73, sensitivity = 0.70, specificity = 0.68). In the follow-up research, we found that higher tissue hsa-miR-21 level was related to a lower overall survival rate, whereas higher tissue hsa-miR-29 level was associated with the higher overall survival rate. These results indicated that both hsa-miR-21 and hsa-miR-29 had the potential to be the biomarkers for GC prognosis. **Conclusion:** In summary, we verified the diagnostic and prognostic value of tissue hsa-miR-21 and hsa-miR-29 in GC. Both of them can be potentially applied as novel and non-invasive biomarkers for GC.
Introduction

Despite advances in surgical treatment, gastric cancer (GC) still poses a deadly threat to human health. It is the fourth most common cancer and the second most common cause of cancer deaths worldwide, with a total of 989,600 new cases and 738,000 deaths estimated in 2008 [1]. Due to the lack of specific symptoms and efficient early detection methods, gastric cancer is usually diagnosed at an advanced stage. In the United States, the prognosis of GC patients is depressing if the neoplastic cells invade the muscularis propria, with reported 5-year survival rates of around 20% in the United States [2]. Endoscopic examination is the mostly used method to detect the GC nowadays. However, this method is expensive and invasive, highlighting an urgent need for more sufficient biomarkers with higher specificity and sensitivity for GC diagnosis.

MicroRNAs (miRNAs) are a group of recently discovered small non-protein-coding RNAs [3]. They regulate the expression of hundreds of target genes; thereby control a wide range of tumorigenic processes including cellular proliferation, differentiation, and apoptosis [4]. For example, the has-miR-15a/has-miR-16-1 cluster has been demonstrated to promote prostate cancer by targeting genes related to a multitude of oncogenic activities, and p53-mediated transactivation of has-miR-34a has been shown to influence the expression of genes related to apoptosis. The down-regulation of miR-141 promoted the invasion of gastric cancer by targeting STAT4 [5].

MicroRNAs (miRNAs) also function as endogenous silencers of numerous target genes [6]. Many miRNA genes are expressed in a tissue-specific manner and play important roles in cell proliferation, apoptosis, and differentiation. The deregulation of cell cycle progression is a sign of malignancy [7]. And this progression is regulated by cyclin-dependent kinase (CDK) or SUZ12 [8]. MiRNA deregulation promotes cell cycle progression by up-regulating cyclin expression or down-regulating expression of CDK inhibitors [9].

Nowadays, aberrantly expressed miRNAs in the cancer patients have attracted a lot of attention among the researchers [10-14]. And accumulative studies have been conducted to confirm whether some specific miRNAs are suitable as biomarkers for GC. Hsa-miR-21 is one of the most studied miRNAs, which had a higher expression in breast cancer, related to poor disease-free survival [15]. For pancreatic cancer cells, hsa-miR-21 could regulate cell proliferation and invasion [16]. Meanwhile, Chan et al. demonstrated that 92% (34/37) of gastric cancer samples were over-expressed of hsa-miR-21 [17]. Although several researches have focused on the relationship of the GC and hsa-miR-21 expression in the serum [18-20], plasma [21] and gastric juice [22], no previous studies have ever investigated tissue hsa-miR-21 and GC. As for hsa-miR-29, researchers have reported that it was down-regulated in some tumor types and essential with tumor suppressor functions [23], and hsa-miR-29 can modulate the expression of many oncogenes, such as MCL-1, TCL-1, CDC42 and DNMT3b [24, 25]. Although, there were several studies investigating its application in human cancers detection [26-29], no published studies assess the diagnostic performance of hsa-miR-29 in GC detection. Thus, we selected hsa-miR-21 and hsa-miR-29 as our target miRNAs to investigate whether they can be diagnostic and prognostic biomarkers for GC. In this study, we hypothesized that the expression levels of the two miRNAs would differ significantly between tumor and the pair-matched adjacent non-tumor tissue.

Materials and Methods

Subjects and samples

A total of 50 patients including 24 females and 26 males were recruited from the Fourth Hospital of Hebei Medical University. Informed consent was signed by all participants involved in this study. This study was performed in accordance with the ethical guidelines of the Declaration of Helsinki and approved by the ethics committee of the Fourth Hospital of Hebei Medical University. All the participants had been diagnosed pathologically and were considered sporadic with no family histories of cancer. Patients with
previous history of chemotherapy and radiotherapy were excluded from our study. Tissue samples were collected from all these participants during the endoscopic examination. Two samples from tumor tissue and pair-matched adjacent non-tumor tissue (about 10 mg) from each patient were collected.

**RNA extraction**

Total RNA was extracted from the tissue sample using mirVana PARIS Kit (Ambion), and finally eluted into 100 μL of pre-heated (95°C) Elution Solution according to the manufacturer’s protocol.

**Quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR)**

For qRT-PCR, the total RNA was reverse transcribed to complementary DNA by using the TaqMan miRNA RT Kit and stem-loop RT primers (Applied Biosystems, USA). The quantitative detection of miRNA was performed using the TaqMan PCR kit as implemented in the ABI 7900 Real-Time PCR System (Applied Biosystems, USA). The reactions were initiated with a 384-well optical plate at 95°C for 5 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min [30]. Equal amount of tumor tissue and adjacent non-tumor tissue was assigned in each plate and the expression levels of target miRNAs and internal control miRNA (RNU43) were measured simultaneously. All reactions were performed in triplicate. The relative expression levels of target miRNAs were calculated with 2^{-ΔΔCT}, where CT means cycle threshold and its values were obtained from Bio-Rad iQ5 2.1 Standard Edition Optical System Software.

**Statistical analysis**

Statistical analysis was performed with the help of GraphPad Prism 5.0 (Graphpad Software Inc., California). Data was presented in the form of mean ± SD, and we used the student’s t test and one-way ANOVA (Analysis of Variables) test to determine the difference of the expression level of miRNAs between these groups classified by the characteristics of the patients (gender, age, TNM stage, metastatic status, tumor size, T classification and differentiation grade). Before doing any comparison, all samples were tested by using Shapiro-Wilk to see whether samples follow a Gaussian distribution, with the result of p > 0.05 using R software. Tumors were staged according to the seventh edition of tumor, node, and metastasis (TNM) classification system issued by the Union for International Cancer Control. The patients are classified as stage I, II, III and IV based on the evaluation of metastatic status (yes or no), tumor size (≤ 5cm or >5 cm), differentiation grade (low, middle and high) and T classification (T1, T2, T3 or T4).

Receiver operating characteristic (ROC) curves were constructed and the area under the curve (AUC) was calculated to assess the feasibility of using hsa-miR-21 and hsa-miR-29 as a diagnostic tool to discriminate gastric tissue from adjacent no-tumor tissue. In addition, we used the Youden index to identify the optimal cut-off point [31]. A P value less than 0.05 was considered as a sign of statistical significance. According to definition, the survival period referred to the duration from the time of surgery to death, or to the last follow-up day, and the survival analysis was conducted using the Kaplan-Meier method, which is one of the best ways to be used to measure the survival for a certain amount of time after treatment, involving computing of probabilities of occurrence of event at a certain point of time and multiplying these successive probabilities by any earlier computed probabilities to get the final estimate [32]. By using the Log-rank test, we performed univariate analysis upon the two groups. From the result, we selected those factors with P values less than 0.1 for the multivariate analysis by using the Cox’s proportional hazard regression model. The hazard ratio (HR) value and Log-rank P value were calculated.

**Results**

**Characteristics of study subjects**

From June 2013 to November 2014, 50 pathologically proven GC patients were recruited continuously for this study. The characteristics of these patients are shown in Table 1. Among these 50 patients, 24 were female and 26 were male. The age ranged from 34 to 67 with 23 patients younger than 60 and 27 elder than that. All the patients were divided into 2 groups according to the TNM stage. Earlier stage (I+II) was verified in 30 patients, and advanced stage (III+IV) in other 20 patients. By examination we found tumor metastasis in 21 cases and no metastasis in the rest. Given that tumor size is always correlated with the
differentiation degree of tumor cells, we also classified them into 2 groups in terms of tumor diameter, 35 patients had relatively smaller tumor (< 5 cm), and 15 patients have bigger ones (≥ 5 cm). Furthermore, according to the T classification, which is related to tumor size and direct extent of primary tumor, 16 patients are diagnosed as T1 and T2; 23 diagnosed as T3 and 11 diagnosed as T4. In addition, we divided the patients into groups according to the differentiation degree of the primary tumor. Lower differentiation was detected in 28 patients, and middle and higher differentiation degree was found in 22 patients.

**Evaluation of hsa-miR-21 and hsa-miR-29 as potential biomarkers for GC detection**

To investigate whether tissue hsa-miR-21 and hsa-miR-29 can be used to detect GC, we performed a series of paired t tests on relative expression of target miRNAs from the 50 tumor and adjacent non-tumor tissues. As for hsa-miR-21, its expression in tumor tissue was higher than that in adjacent non-tumor tissue \((P < 0.001, t = 4.743)\); on the contrary, hsa-
mir-29 expression in tumor tissue was much lower than that in adjacent non-tumor tissue \((P < 0.001, t = 4.428)\). The results indicated that both miRNAs had the potential value as the biomarkers for GC detection. Scatter dot plots of the levels of hsa-miR-21 and hsa-miR-29 was plotted in Fig. 1a and 2a.

To further evaluate the diagnostic value of the two miRNAs, we constructed the ROC curves and calculated the AUC values. As shown in Fig. 1b and 2b, both the two miRNAs generated moderate diagnostic performance \((\text{hsa-miR-21: AUC} = 0.75, \text{sensitivity} = 0.70, \text{specificity} = 0.78; \text{hsa-miR-29: AUC} = 0.73, \text{sensitivity} = 0.70, \text{specificity} = 0.68)\).

| Characteristics         | Sample size (n) | Relative expression of hsa-miR-21 | P value | Relative expression of hsa-miR-29 | P value |
|-------------------------|-----------------|-----------------------------------|---------|-----------------------------------|---------|
| Gender                  |                 |                                   |         |                                   |         |
| female                  | 24              | 7.45±3.25                         | 0.357a  | 4.34±2.12                         | 0.115a  |
| male                    | 26              | 8.35±3.56                         |         | 3.56±1.23                         |         |
| Age                     |                 |                                   |         |                                   |         |
| <60                     | 23              | 9.32±3.54                         | 0.331a  | 4.12±1.76                         | 0.568a  |
| ≥60                     | 27              | 10.34±3.76                        |         | 3.85±1.56                         |         |
| TNM stage               |                 |                                   |         |                                   |         |
| I+II                    | 30              | 7.87±2.89                         | 0.044a  | 4.68±1.89                         | 0.033a  |
| III+IV                  | 20              | 10.67±3.65                        |         | 3.56±1.56                         |         |
| Metastatic status       |                 |                                   |         |                                   |         |
| yes                     | 21              | 10.56±5.12                        | 0.025a  | 3.45±1.23                         | 0.023a  |
| no                      | 29              | 7.85±3.13                         |         | 4.45±1.65                         |         |
| Tumor size              |                 |                                   |         |                                   |         |
| ≥5cm                    | 15              | 11.34±5.87                        | 0.036a  | 2.89±1.34                         | 0.007a  |
| <5cm                    | 35              | 8.11±4.35                         |         | 4.34±1.79                         |         |
| T classification        |                 |                                   |         |                                   |         |
| T1+T2                   | 16              | 8.56±3.21                         | 0.328b  | 4.86±2.02                         | 0.222b  |
| T3                      | 23              | 9.67±4.23                         |         | 4.23±1.67                         |         |
| T4                      | 11              | 11.24±6.45                        |         | 3.67±1.45                         |         |
| Differentiation degree  |                 |                                   |         |                                   |         |
| low                     | 28              | 7.68±3.42                         | 0.019a  | 4.58±1.94                         | 0.024a  |
| middle/high             | 22              | 10.34±4.35                        |         | 3.45±1.32                         |         |
To further investigate the diagnostic value of tissue hsa-miR-21 and hsa-miR-29 as biomarkers for GC detection, we collected their specific clinical data and assessed the difference of the corresponding miRNA expression according to their clinical status.

**Fig. 1.** Diagnostic performance of hsa-miR-21 in differentiating tumor tissues and normal tissues from gastric cancer patients. (a) Relative expression levels of hsa-miR-21 in tumor tissues and normal tissues. (b) ROC curve analysis of hsa-miR-21 in differentiating tumor tissues and normal tissues from gastric cancer patients.

**Fig. 2.** Diagnostic performance of hsa-miR-29 in differentiating tumor tissues and normal tissues from gastric cancer patients. (a) Relative expression levels of hsa-miR-29 in tumor tissues and normal tissues. (b) ROC curve analysis of hsa-miR-29 in differentiating tumor tissues and normal tissues from gastric cancer patients.

**Fig. 3.** Survival analyses of gastric cancer patients in different groups according to the relative expression of hsa-miR-21 and hsa-miR-29. (a) Survival curve of gastric cancer patients in high/low hsa-miR-21 expression groups. (b) Survival curve of gastric cancer patients in high/low hsa-miR-29 expression groups.

Hsa-miR-21 and hsa-miR-29 expressions in GC patients with different clinical status

To further investigate the diagnostic value of tissue hsa-miR-21 and hsa-miR-29 as biomarkers for GC detection, we collected their specific clinical data and assessed the difference of the corresponding miRNA expression according to their clinical status. All the
results were listed in the Table 1. Norm-Finder, which is a direct measure for the estimated expression variation enabling the user to evaluate the systematic error introduced when using the gene/miRNA for normalization [33], was used to calculate the stability value for hsa-miR-21 and hsa-miR-29. Results showed that endogenous control of hsa-miR-21 and hsa-miR-29 varied little. And as the table shows, we found no statistical significance in gender and age, which indicated that the development of GC might have little relation with the two characteristics.

However, relatively higher expression of tissue hsa-miR-21 and lower expression of tissue hsa-miR-29 were found in the patients at TNM stage III and IV than those in the stage I and II (P = 0.044 for hsa-miR-21 and P = 0.033 for hsa-miR-29). Expression of tissue hsa-miR-21 was increased, while the tissue hsa-miR-29 expression was decreased in the patients with metastasis (P = 0.025, P = 0.023, respectively). In terms of tumor size, expression of hsa-miR-21 was much higher in patients with tumor diameter ≥5cm than in those with smaller tumor size (P = 0.036); while hsa-miR-29 expression was lower in patients with tumor diameter ≥5cm (P = 0.007). However, even though the expression of both miRNAs varied with different T classification, we discovered no statistical significance (P = 0.328 for hsa-miR-21, and P = 0.222 for hsa-miR-29), which might be resulted from the limited samples. In addition, in terms of differentiation degree, we observed a higher tissue hsa-miR-21 expression and a relatively lower tissue hsa-miR-29 expression in patients of middle and high differentiation degree than in those of lower differentiation degree (P = 0.019 for hsa-miR-21, P = 0.024 for hsa-miR-29).

**Correlation between hsa-miR-21 and hsa-miR-29 and the prognosis of the GC patients**

We measured the overall survival (OS) in accordance with the time when the tissue samples were acquired until the date of death or date of last follow-up. The patients who were alive at the time of analysis were censored by using the time between the sample assessment and their most recent follow-up evaluations.

By using the Kaplan-Meier method, we performed an OS analysis. All the patients were divided into 2 groups according to the target miRNAs’ expression levels (high/low). As shown in Fig. 3, higher tissue hsa-miR-21 level was related to a lower OS rate, whereas higher tissue hsa-miR-29 level was associated with the higher OS rate. The prognostic value of the two miRNAs’ expression was evaluated by using the log-rank test. Kaplan-Meier survival curves analysis did reveal an association between hsa-miR-21 (P = 0.0096, HR = 1.89 [1.17-3.07]) or hsa-miR-29 (P = 0.0005, HR = 2.23 [1.34-3.65]) levels and OS.

**Discussion**

MiRNAs are now becoming a hot spot among the researchers for its implication in cancer. It was reported that they are frequently over-expressed or under-expressed in human cancers [34-36]. There are several studies concerning hsa-miR-21 and hsa-miR-29 in human cancers. Asangani et al. found that the tumor suppressor protein Programmed Cell Death 4 (PDCD4) is regulated by hsa-miR-21 in breast cancer cells [37]. Zhang et al. discovered that hsa-miR-21 down-regulates the expression of tumor suppressor PTEN and stimulates growth and invasion in non-small cell lung cancer [38]. Wu et al. reported that hsa-miR-29a, a member of the hsa-miR-29 family, plays an important role in inhibiting growth of breast cancer cells and arresting cells at G0/G1 phase [39]. In addition, several researchers have also studied the possibility of using hsa-miR-21 as biomarker for GC diagnosis. For instance, Shiotani et al. discovered that hsa-miR-21 could provide a novel and stable marker of increased risk for early gastric cancer after H. pylori eradication [18]; Cui et al. found that hsa-miR-21 level in gastric juice of GC patients were significantly higher than that of normal people [22]; Li et al. also discovered that plasma levels of hsa-miR-21 were significantly higher in GC patients than in healthy controls [21]. Even with those previous published researches, our study, to our knowledge, is still the first to demonstrate the potential role of tissue hsa-miR-21 and
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hsa-miR-29 in both diagnosis and prognosis of GC.

In this study, we investigated the diagnostic and prognostic values of hsa-miR-21 and hsa-miR-29 for GC. Compared with the expression of the two miRNAs in adjacent non-tumor tissue, level of hsa-miR-21 is over-expressed with 1.39-fold change and hsa-miR-29 is under-expressed with 0.69-fold change in the tumor tissue, both suggesting that these two miRNAs are potential biomarkers for GC diagnosis and prognosis.

A crucial step of the study is the selection of appropriate internal control. In this study, we used the small nucleolar RNU43 as an internal control, an RNA which is considered as one of the most widely used references in miRNA expression studies [40]. RNU43, which is lower in tumors with a poorer prognosis (although not statistically significant) is mapped to an intronic region of *H. sapiens* ribosomal protein L3 (RPL3). Alternate transcriptional splice variants, encoding different isoforms, have been characterized [40].

Accumulative reports indicated that miRNAs are detectable in tissue and blood samples. And miRNAs that can be used as biomarkers must demonstrate different hallmark characteristics. First of all, they must be stable and readily quantifiable in clinical samples; secondly, they are expressed by cancer cells at significantly different levels from those by normal cells; thirdly, they should provide predictive or prognostic clinical information; finally, they should exhibit biological functions on tumor progression [41].

There are two main discoveries in our research. First of all, both miRNAs are aberrantly expressed (*hsa-miR-21* is over-expressed and *hsa-miR-29* is under-expressed, respectively) in the tumor tissue from GC patients, while the expression profile of both *hsa-miR-21* and *hsa-miR-29* a in healthy gastric antrum were 240 read counts and 444 read counts, respectively [42]. Over-expressed of *hsa-miR-21* could change biological processes of human gastric cancer cells such as proliferation, apoptosis, migration, and invasion, probably through regulating *RECK* (a known tumor suppressor in gastric cancer) and other critical target genes [43]. Under-expression of *hsa-miR-29* served as tumor suppressor gene by accommodating critical oncogenic targets [44]. These changed values were closely related to the GC diagnosis and associated with the GC stage, indicating the potential of using it as biomarkers for the GC diagnosis and staging. Secondly, the expression levels of both the two miRNAs provided prognostic information for patients with GC independent of a comprehensive panel of other established clinical predictors.

However, there are still several limits in our study. First of all, the number of the patients involved in this study was relatively small; meanwhile, we didn't recruit healthy controls so that we could not make a more accurate comparison between the patients and healthy controls. Moreover, all eligible subjects were from Asian population; the result of the study may not be applicable to Caucasian and African ethnicities.

In the future investigation, we may include more GC samples of different stages and appropriate number of healthy controls to comprehensively evaluate the role of tissue *hsa-miR-21*, *hsa-miR-29* or other tissue miRNAs associated with GC.

**Conclusion**

In summary, we verified the diagnostic and prognostic value of tissue *hsa-miR-21* and *hsa-miR-29* in gastric cancer. Both of them might be applied as novel a biomarkers for gastric cancer. Further studies on detailed mechanisms and population from other ethnicities are recommended.

**Disclosure Statement**

The corresponding author had full access to all data in the study and had final responsibility for the decision to submit for publication. Authors have no conflict of interest to declare.
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