

## Basic Study

***hsa-miR-29c* and *hsa-miR-135b* differential expression as potential biomarker of gastric carcinogenesis**

Amanda Ferreira Vidal, Aline MP Cruz, Leandro Magalhães, Adenilson L Pereira, Ana KM Anaissi, Néllisson CF Alves, Paulo JBS Albuquerque, Rommel MR Burbano, Samia Demachki, Ândrea Ribeiro-dos-Santos

Amanda Ferreira Vidal, Aline MP Cruz, Leandro Magalhães, Adenilson L Pereira, Ana KM Anaissi, Pós-Graduação em Genética e Biologia Molecular, Universidade Federal do Pará, Cidade Universitária Prof. José Silveira Netto, Belém, PA 66075-970, Brasil

Amanda Ferreira Vidal, Aline MP Cruz, Leandro Magalhães, Adenilson L Pereira, Ana KM Anaissi, Ândrea Ribeiro-dos-Santos, Laboratório de Genética Humana e Médica, Instituto de Ciências Biológicas, Universidade Federal do Pará, Belém, PA 66075-970, Brasil

Néllisson CF Alves, Pós-Graduação em Neurociências e Biologia Molecular, Universidade Federal do Pará, Belém, PA 66075-970, Brasil

Néllisson CF Alves, Rommel MR Burbano, Laboratório de Citogenética Humana- Instituto de Ciências Biológicas, Universidade Federal do Pará, Belém, PA 66075-970, Brasil

Paulo JBS Albuquerque, Hospital São Camilo e São Luís - Rua Dr. Marcelo Cândia, Macapá, AP 68901-901, Brasil

Rommel MR Burbano, Samia Demachki, Ândrea Ribeiro-dos-Santos, Núcleo de Pesquisas em Oncologia, Hospital Universitário João de Barros Barreto, Universidade Federal do Pará, Belém, PA 66075-970, Brasil

Samia Demachki, Instituto de Ciências da Saúde, Universidade Federal do Pará, Belém, PA 66075-970, Brasil

**Author contributions:** Vidal AF performed the experiments, analyzed the data and wrote the manuscript; Cruz AMP performed the experiments; Magalhães L performed the statistical analysis and reviewed the manuscript; Anaissi AKM, Alves NCF, Albuquerque PJBS, Burbano RMR and Demachki S selected samples and obtained the clinical and pathological informations; Ribeiro-dos-Santos A conceived of the study, and participated in its design and coordination; all authors approved the final version of manuscript.

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**Correspondence to:** Dr. Ândrea Ribeiro-dos-Santos, Laboratório de Genética Humana e Médica, Instituto de Ciências Biológicas, Universidade Federal do Pará, Av. Augusto Corrêa 01, Belém, PA 66075-970, Brasil. [akelyufpa@gmail.com](mailto:akelyufpa@gmail.com)  
**Telephone:** +55-91-32017843  
**Fax:** +55-91-32017843

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## Abstract

**AIM:** To investigate the expression profiles of *hsa-miR-29c* and *hsa-miR-135b* in gastric mucosal samples and their values as gastric carcinogenesis biomarkers.

**METHODS:** The expression levels of *hsa-miR-29c* and *hsa-miR-135b* in normal gastric mucosa, non-atrophic chronic gastritis, intestinal metaplasia and intestinal-type gastric adenocarcinoma were analysed using quantitative real-time PCR. The difference between *hsa-miR-29c* and *hsa-miR-135b* expression profiles in the grouped samples was evaluated by ANOVA and Student's *t*-test tests. The results were adjusted for multiple testing by using Bonferroni's correction. *P* values  $\leq 0.05$  were considered statistically significant. To evaluate *hsa-miR-29c* and *hsa-miR-135b* expressions as potential biomarkers of gastric carcinogenesis, we performed a receiver operating characteristic curve analysis and the derived area under the curve, and a Categorical Principal Components Analysis. *In silico* identification of the genetic targets of *hsa-miR-29c* and *hsa-miR-135b* was performed using different prediction tools, in order to identify possible genes involved in gastric carcinogenesis.

**RESULTS:** The expression levels of *hsa-miR-29c* were higher in normal gastric mucosal samples, and decreased progressively in non-atrophic chronic gastritis samples, intestinal metaplasia samples and intestinal-type gastric adenocarcinoma samples. The expression of *hsa-miR-29c* in the gastric lesions showed that non-atrophic gastritis have an intermediate profile to gastric normal mucosa and intestinal-type gastric adenocarcinoma, and that intestinal metaplasia samples presented an expression pattern similar to that in intestinal-type gastric adenocarcinoma. This microRNA (miRNA) has a good discriminatory accuracy between normal gastric samples and (1) intestinal-type gastric adenocarcinoma; and (2) intestinal metaplasia, and regulates the *DMNT3A* oncogene. *hsa-miR-135b* is up-regulated in non-atrophic chronic gastritis and intestinal metaplasia samples and down-regulated in normal gastric mucosa and intestinal-type gastric adenocarcinoma samples. Non-atrophic chronic gastritis and intestinal metaplasia are significantly different from normal gastric mucosa samples. *hsa-miR-135b* expression presented a greater discriminatory accuracy between normal samples and gastric lesions. This miRNA was associated with *Helicobacter pylori* presence in non-atrophic chronic gastritis samples and regulates the *APC* and *KLF4* tumour suppressor genes.

**CONCLUSION:** Our results provide evidence of epigenetic alterations in non-atrophic chronic gastritis and intestinal metaplasia and suggest that *hsa-miR-29c* and *hsa-miR-135b* are promising biomarkers of gastric carcinogenesis.

**Key words:** Gastric cancer; Gastric lesions; MicroRNA; Biomarker; Carcinogenesis

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**Core tip:** The miRNAs *hsa-miR-29c* and *hsa-miR-135b* were reported as potential biomarkers of intestinal-type gastric adenocarcinoma. We evaluated and compared the expression profile of these miRNAs in gastric mucosal samples, including normal gastric mucosa, non-atrophic chronic gastritis, intestinal metaplasia and intestinal-type gastric adenocarcinoma. Our results provided evidence of epigenetic alterations in non-atrophic chronic gastritis and intestinal metaplasia and suggest that *hsa-miR-29c* and *hsa-miR-135b* are promising biomarkers of gastric carcinogenesis.

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## INTRODUCTION

Since the middle of the last century, the histological classification of adenocarcinomas has been largely based on the criteria proposed by Lauren<sup>[1]</sup>. According to this classification, there are three gastric adenocarcinoma types: intestinal, diffuse and undifferentiated, which is also classified as indeterminate<sup>[1]</sup>. Intestinal-type and diffuse-type gastric adenocarcinomas have their own characteristics and specific risk factors<sup>[2]</sup>.

In 1988, Pelayo Correa proposed a paradigm for intestinal-type gastric adenocarcinoma carcinogenesis, which became known as the Correa cascade. According to this cascade, a subset of patients who develop intestinal-type gastric adenocarcinomas undergo a multi-stage and complex process of carcinogenesis, initiated by (1) chronic superficial gastritis, also called non-atrophic chronic gastritis; followed by (2) chronic atrophic gastritis; then (3) intestinal metaplasia; and finally (4) dysplasia<sup>[3,4]</sup>.

*Helicobacter pylori* (*H. pylori*) infection is the major risk factor among all the main risk factors involved in chronic gastritis, intestinal metaplasia, dysplasia and intestinal-type gastric adenocarcinoma. For this reason, in 1994, this bacterium was classified as a type 1 carcinogen by the World Health Organization<sup>[5]</sup>.

Despite the fact that the different inflammatory stages of the Correa cascade are pathologically well defined, the molecular signatures of these stages have not been well explored and the mechanisms that lead to carcinogenic progression are still unknown<sup>[6]</sup>.

The pre-cancerous or pre-malignant lesions are defined as those that precede invasive cancers which many of the molecular changes and phenotypic

characteristics of invasive cancer are present but not fully expressed<sup>[7]</sup>. Thus, it is assumed that the changes found in intestinal-type gastric adenocarcinomas may also be present in different stages of gastric lesions, such as chronic gastritis, intestinal metaplasia and dysplasia.

Several studies have shown that gastric cancer is a complex disease involving changes in oncogenes, tumour suppressor genes, DNA repair regulatory genes, cell cycle and cell adhesion, as well as numerous epigenetic changes<sup>[8]</sup>. A class of small non-coding RNAs, called microRNAs (miRNAs), have emerged as key agents in these epigenetic changes<sup>[9]</sup>.

MiRNAs are short (approximately 22 nucleotides in length), endogenous, noncoding RNAs that regulate the expression of target mRNAs at a post-transcriptional level<sup>[10]</sup>. Based on the results obtained in studies by Ribeiro-dos-Santos *et al.*<sup>[11]</sup>, Moreira *et al.*<sup>[12]</sup>, Gomes *et al.*<sup>[13]</sup> and Darnet *et al.*<sup>[14]</sup>, *hsa-miR-29c* and *hsa-miR-135b* were reported as potential biomarkers of intestinal-type gastric adenocarcinoma. However, more studies are needed to confirm and validate *hsa-miR-29c* and *hsa-miR-135b* as potential biomarkers.

The objective of this study was to investigate the expression profiles of *hsa-miR-29c* and *hsa-miR-135b* in gastric mucosal samples, including normal gastric mucosa, non-atrophic chronic gastritis, intestinal metaplasia and intestinal-type gastric adenocarcinoma, and their values as gastric carcinogenesis biomarkers. Additionally, *in silico* prediction was performed to identify potential driver genes involved in the carcinogenic mechanism<sup>[15]</sup> regulated by these miRNAs.

## MATERIALS AND METHODS

### Patient tissues

This study comprised randomly selected frozen tissue samples of normal gastric mucosa ( $n = 20$ ), FFPE samples of non-atrophic chronic gastritis ( $n = 20$ ) and of intestinal metaplasia ( $n = 10$ ) from patients undergoing endoscopic gastric biopsy samples, and gastric intestinal adenocarcinoma frozen tissue samples obtained from patients undergoing gastrectomies ( $n = 14$ ). All cases investigated in this study were reviewed and confirmed by a pathologist.

Histological processing was performed using glass slide-mounted 3  $\mu\text{m}$ -thick rotary microtome slices (Leica 2125RT). These preparations were deparaffinised, stained with haematoxylin-eosin (HE) and analysed by light microscopy. After histological processing, manual microdissection was performed to increase the accuracy of histopathological characterisation.

Non-atrophic chronic gastritis samples were defined by the presence of lymphocytes and plasmocytes in the lamina propria. The presence or absence of neutrophils permeating the glandular and the surface epithelia, and the presence or absence of lymphoid follicles were also evaluated<sup>[16]</sup>.

Samples of intestinal metaplasia were histopathologically diagnosed by the replacement of the surface and glandular gastric columnar epithelial cells by metaplastic cells of intestinal morphology, such as absorptive and goblet cells.

Fresh tissue samples were immediately stored in *RNAlater Solution* (Ambion) at  $-80\text{ }^{\circ}\text{C}$  until total RNA extraction. Only samples with a pure tumour area occupying at least 80% of the slide were used. Pathological TNM staging was evaluated according to the 2010 criteria of The American Joint Committee on Cancer.

The histological sections of gastric mucosal biopsy were stained with HE and cresyl fast violet to perform the *H. pylori* detection.

Samples were obtained from the Hospital Universitário João de Barros Barreto - Federal University of Pará (Belém, Pará, Brazil) and from the Hospital São Camilo e São Luís (Macapá, Amapá, Brazil). Informed consent was obtained from all individual participants included and the study protocol was approved by the Local Ethics Committee (Protocol number: 657 666) in accordance with the Helsinki Declaration of 1964.

### Total RNA isolation and quantification

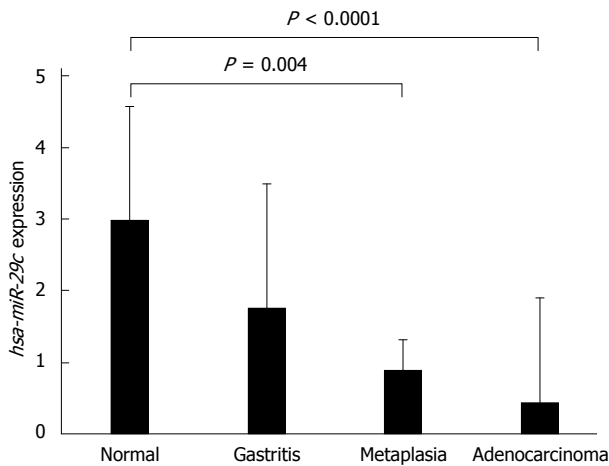
The total RNA was extracted using the High Pure Kit miRNA Isolation Kit (Roche Diagnostics) according to manufacturer's protocol, and stored at  $-80\text{ }^{\circ}\text{C}$  to avoid degradation. The total RNA concentrations were determined by the Qubit<sup>®</sup> 2.0 Fluorometer (Life Technologies) using the Qubit RNA HS Assay kit (Life Technologies). The samples were diluted to the final concentration of 4 ng/ $\mu\text{L}$ .

### Quantitative real-time polymerase chain reaction

Assays for measuring the miRNAs expression were performed using TaqMan MicroRNA Assays (Applied Biosystems) according to the manufacturer's instructions. Initially, 10 ng of total RNA was subjected to reverse transcription polymerase chain reaction using the TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems) according to manufacturer's protocol. The thermocycling conditions were: 30 min at  $16\text{ }^{\circ}\text{C}$ , followed by 30 min at  $42\text{ }^{\circ}\text{C}$ , 5 min at  $85\text{ }^{\circ}\text{C}$  and 5 min at  $4\text{ }^{\circ}\text{C}$ .

The quantitative real-time polymerase chain reaction (qRT-PCR) was performed using TaqMan Universal PCR Master Mix Kit (Applied Biosystems) according to the manufacturer's protocol and the equipment 7500 Real-Time PCR System (Applied Biosystems). The reactions were performed in triplicate and incubated in optical 96-well reaction plates. The thermocycling conditions were:  $95\text{ }^{\circ}\text{C}$  for 10 min, and 40 cycles of 15 s at  $95\text{ }^{\circ}\text{C}$ , followed by 1 min at  $60\text{ }^{\circ}\text{C}$ .

After finalization of the qRT-PCR experiments, the average values of the cycle threshold (Ct) of the reactions in triplicate were determined. The comparative Ct method was adopted, and Z30 was used as an



**Figure 1** Expression values of *hsa-miR-29c* in samples of normal gastric mucosa, non-atrophic chronic gastritis, intestinal metaplasia and intestinal-type gastric adenocarcinoma, respectively (values in log scale). *P* values were obtained by ANOVA test (adjusted by using Bonferroni's correction).

endogenous control. The relative amount of miRNA expression was normalized by the average values of  $Ct_{z30}$  and calculated by the equation  $2^{-\Delta Ct}$ , where  $\Delta Ct = Ct_{miRNA} - Ct_{z30}$ .

#### ***In silico* prediction of *hsa-miR-29c* and *hsa-miR-135b* target genes**

In order to identify genes that may be involved in gastric carcinogenesis process, we compared the driver genes ranked by Vogelstein *et al.*<sup>[15]</sup> with the target genes of *hsa-miR-29c* and *hsa-miR-135b*.

*In silico* identification of the target genes was performed using miRecords (<http://mirecords.biolead.org>) (which integrates 11 prediction tools), TargetCompare (<http://54.187.40.156:8080/targetcompare/>), miRTarBase (<http://mirtarbase.mbc.nctu.edu.tw>), MicroCosm/miRBase (<http://ebi.ac.uk>), miRDB (<http://mirdb.org>), miRo (<http://ferrolab.dmi.unict.it>) and miRNAmap (<http://mirmamap.mbc.nctu.edu.tw>). We considered target genes the ones that were observed in no less than 10 tools.

We also used the miRTarBase database to check which miRNA target genes have already been validated experimentally. The mRNA sequences of target genes were obtained from NCBI.

#### **Statistical analysis**

The pattern of distribution of the data was determined by the Shapiro-Wilk test. The difference between *hsa-miR-29c* and *hsa-miR-135b* expression profiles in the grouped samples was evaluated by ANOVA and Student's t-test tests. The results were adjusted for multiple testing by using Bonferroni's correction. *P* values  $\leq 0.05$  were considered statistically significant.

To evaluate *hsa-miR-29c* and *hsa-miR-135b* as potential biomarkers of gastric carcinogenesis, we performed a receiver operating characteristic (ROC)

curve analysis and the derived area under the curve (AUC), and a Categorical Principal Components Analysis (CATPCA). Statistical tests and graphics were performed using IBM SPSS Statistics software (version 20), GraphPad Prism (GraphPad Software), MATLAB® 8.3 (Release 2014a) and RStudio (version 0.98.1103).

## **RESULTS**

### **Expression levels of *hsa-miR-29c* and *hsa-miR-135b***

To determine and compare the expression levels of *hsa-miR-29c* and *hsa-miR-135b* in gastric mucosal samples, we performed qRT-PCR.

We used the ANOVA test to compare the miRNAs expression levels between normal gastric mucosa, non-atrophic chronic gastritis, intestinal metaplasia and intestinal-type gastric adenocarcinoma samples. The results were adjusted for multiple testing by using Bonferroni's correction.

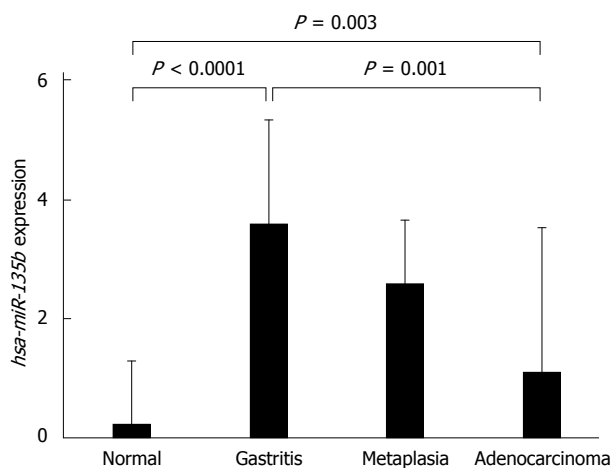
The expression levels of *hsa-miR-29c* were higher in normal gastric mucosa samples, and decreased progressively in non-atrophic chronic gastritis, intestinal metaplasia and intestinal-type gastric adenocarcinoma samples (Figure 1). The decrease in the expression of *hsa-miR-29c* as the Correa cascade stages progress is molecular evidence for the Correa cascade pathogenesis.

ANOVA test showed that non-atrophic chronic gastritis samples have an intermediate *hsa-miR-29c* profile to gastric normal mucosa and intestinal-type gastric adenocarcinoma. Furthermore, intestinal metaplasia *hsa-miR-29c* expression is significantly different to its in normal gastric mucosa samples ( $P = 0.004$ ) but is similar to its in intestinal-type gastric adenocarcinoma. There is no difference between non-atrophic chronic gastritis and intestinal metaplasia expression profiles (Figure 1).

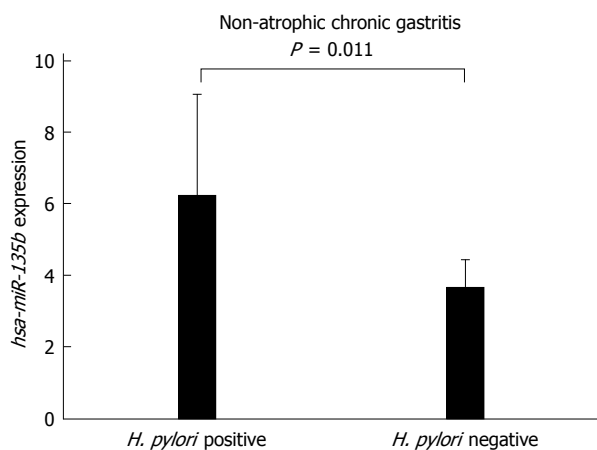
The expression levels of *hsa-miR-135b* showed higher values in the non-atrophic chronic gastritis and the intestinal metaplasia samples and lower values in the normal gastric mucosa and intestinal-type gastric adenocarcinoma. ANOVA test showed non-atrophic chronic gastritis ( $P < 0.0001$ ) and the intestinal metaplasia ( $P = 0.003$ ) are different to normal gastric mucosa, but similar to each other. Non-atrophic chronic gastritis showed a significant difference in expression of *hsa-miR-135b* in comparison to intestinal-type gastric adenocarcinoma ( $P = 0.001$ ) (Figure 2).

### **Expression levels of *hsa-miR-29c* and *hsa-miR-135b* and its relationship to *H. pylori* infection**

To determine whether the presence of *H. pylori* affects the expression of *hsa-miR-29c* and *hsa-miR-135b*, a Student's t-test was used to compare the *H. pylori*-positive and *H. pylori*-negative non-atrophic chronic gastritis samples. No significant difference was found in the expression of *hsa-miR-29c* ( $P = 0.0939$ ) between those groups, however, for *hsa-miR-135b*, there was a significant difference ( $P = 0.011$ ). Samples of *H.*



**Figure 2** Expression values of *hsa-miR-135b* in samples of normal gastric mucosa, non-atrophic chronic gastritis, intestinal metaplasia and intestinal-type gastric adenocarcinoma, respectively (values in log scale). *P* values were obtained by ANOVA test (adjusted by using Bonferroni's correction).



**Figure 3** Comparison of the expression values of *hsa-miR-135b* between samples of *Helicobacter pylori*-positive non-atrophic chronic gastritis and *Helicobacter pylori*-negative non-atrophic chronic gastritis (*P* = 0.011). *H. pylori*: *Helicobacter pylori*.

*pylori*-positive non-atrophic chronic gastritis have a higher expression of *hsa-miR-135b* than the *H. pylori*-negative non-atrophic chronic gastritis samples (Figure 3), indicating that this miRNA may be involved in the immune response modulation in association with *H. pylori* infection.

**Evaluation of *hsa-miR-29c* and *hsa-miR-135b* as potential biomarkers**

To evaluate *hsa-miR-29c* and *hsa-miR-135b* expression as potential biomarker of gastric carcinogenesis, ROC curve analysis and the discriminatory accuracy by AUC values were performed. As shown in Figure 4, *hsa-miR-29c* expression presented a greater discriminatory accuracy between normal gastric samples and (1) intestinal-type gastric adenocarcinoma (Figure 4A); and (2) intestinal metaplasia (Figure 4C). Otherwise,

*hsa-miR-135b* expression presented a greater discriminatory accuracy between normal samples and gastric lesions (Figure 4G-I).

To provide a global view of *hsa-miR-29c* and *hsa-miR-135b* expression in all samples groups studied simultaneously, CATPCA analysis was performed. The CATPCA analysis of *hsa-miR-29c* expression resulted in two dimensions (first component: Cronbach's alpha = 0.778 and eigenvalue = 2.401; second component: Cronbach's alpha = 0.422 and eigenvalue = 1.463). According to the angles between the vectors (Figure 5), the *hsa-miR-29c* expression was able to distinguish each group of samples. It was not possible to construct a three-dimensional graphic due to the negative value of the third component's Cronbach's alpha.

The CATPCA analysis for *hsa-miR-135b* expression resulted in three dimensions (first component: Cronbach's alpha = 0.938 and eigenvalue = 8.051; second component: Cronbach's alpha = 0.829 and eigenvalue = 4.424; third component: Cronbach's alpha = 0.81 and eigenvalue = 4.101). Figure 6 shows the same CATPCA in a three-dimensional space in two different angles (Figure 6). According to the angles between the vectors, *hsa-miR-135b* expression was able to distinguish the gastric normal mucosa samples to *H. pylori*-positive non-atrophic chronic gastritis and intestinal metaplasia samples.

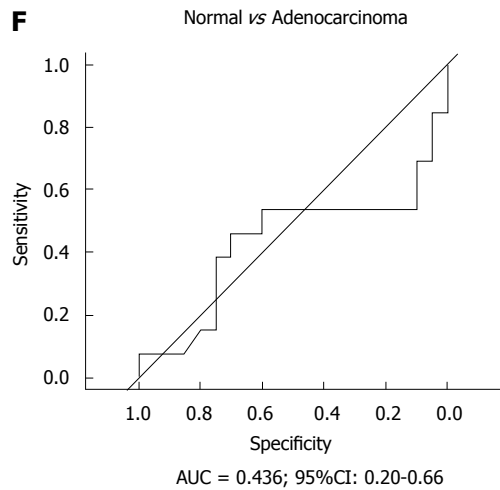
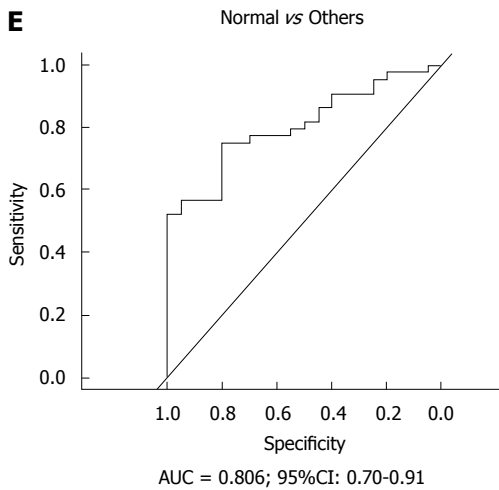
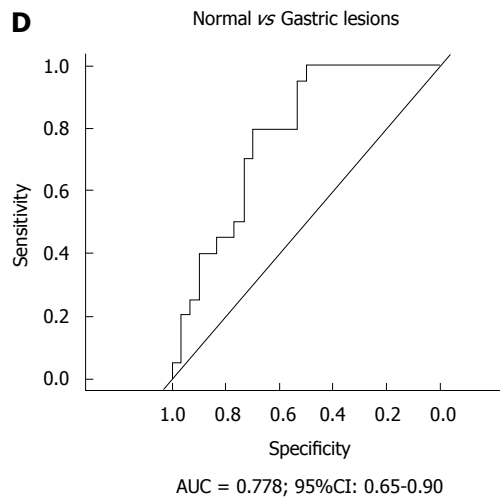
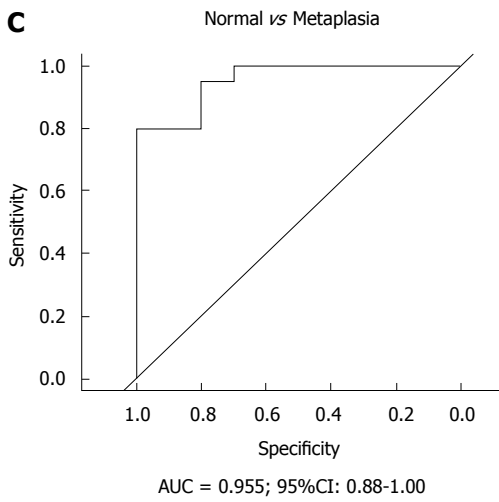
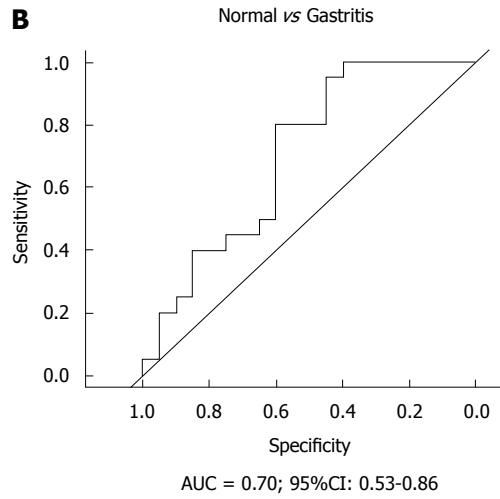
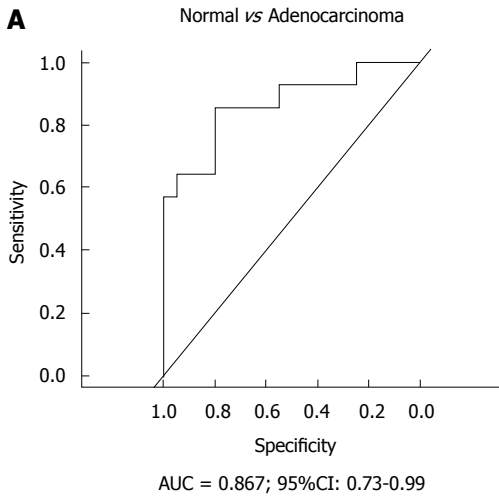
**In silico prediction of *hsa-miR-29c* and *hsa-miR-135b* target genes**

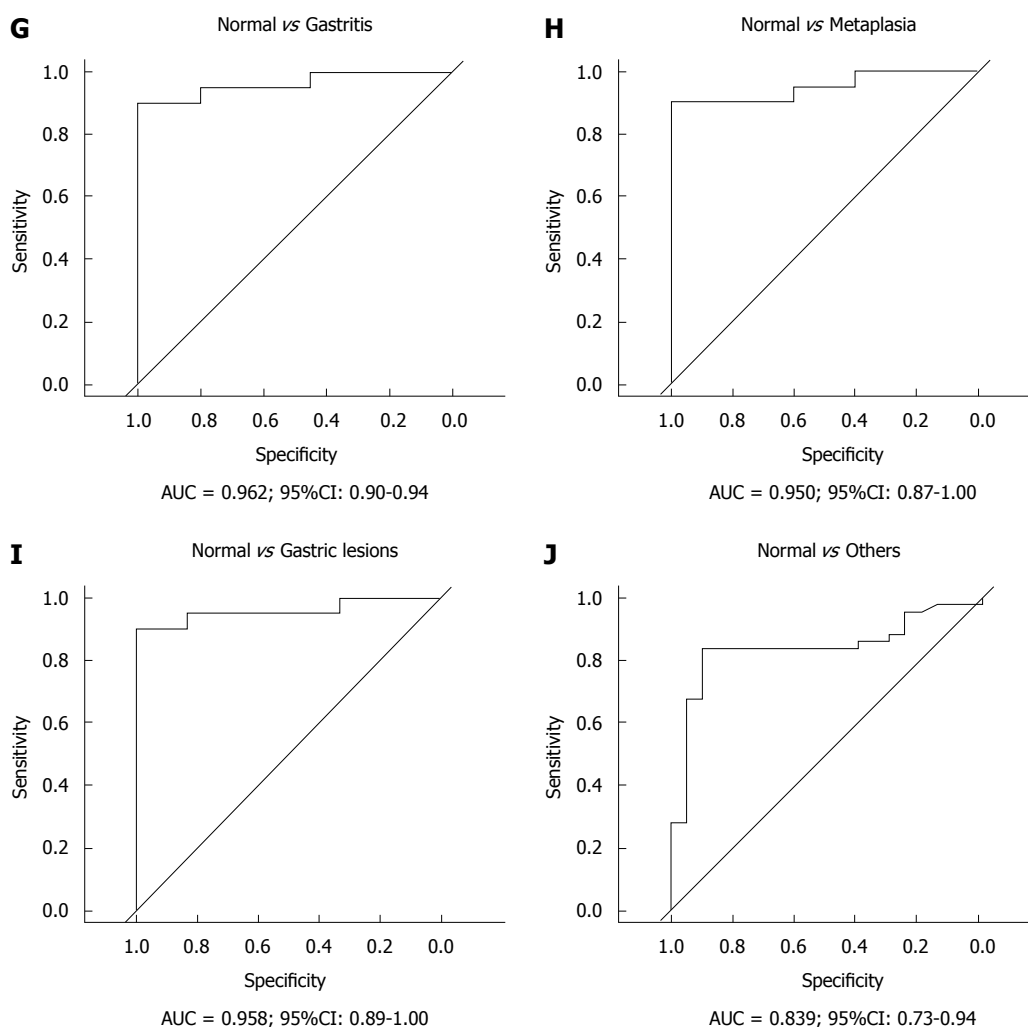
To predict the target genes of *hsa-miR-29c* and *hsa-miR-135b* and identify possible genes involved in gastric carcinogenesis, we used 17 different tools and compared the results with the driver genes list ranked by Vogelstein *et al*<sup>[15]</sup>. The *DNMT3A* driver gene is a validated target of *hsa-miR-29c*, and the *APC* and *KLF4* driver genes are validated targets of *hsa-miR-135b* (Table 1).

**DISCUSSION**

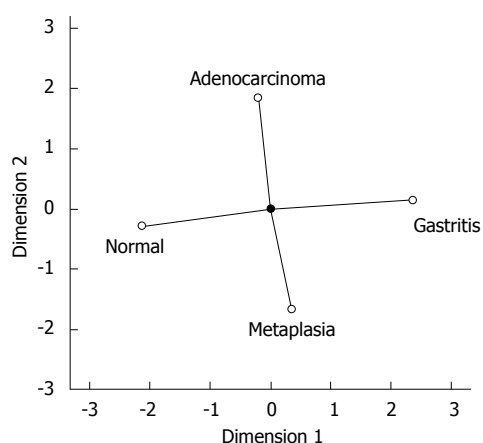
This study compared the expression levels of two miRNA candidates for gastric cancer biomarkers (*hsa-miR-29c* and *hsa-miR-135b*) between normal gastric mucosa, gastric lesions (non-atrophic chronic gastritis and intestinal metaplasia) and intestinal-type gastric adenocarcinoma.

The results showed a progressive down-regulation of *hsa-miR-29c* in normal gastric mucosa, non-atrophic chronic gastritis, intestinal metaplasia and intestinal-type gastric adenocarcinoma, providing evidence for the pathogenesis of the Correa cascade. The expression of this miRNA in the gastric lesions showed that non-atrophic gastritis have a intermediate profile to gastric normal mucosa and intestinal-type gastric adenocarcinoma samples, and that intestinal metaplasia samples presented an expression pattern similar to that in intestinal-type gastric





**Figure 4** Receiver operating characteristic curve analysis of *hsa-miR-29c* (A-E) and *hsa-miR-135b* (F-J) expression. Gastric lesions: Non-atrophic chronic gastritis and intestinal metaplasia; Others: Non-atrophic chronic gastritis, intestinal metaplasia and intestinal-type gastric adenocarcinoma; AUC: Area under the curve.



**Figure 5** Categorical principal components analysis of *hsa-miR-29c* expression in two dimensions. Vectors making 180-degree indicate they are closely and negatively related. Vectors making a 90-degree angle indicate they are not related.

adenocarcinoma.

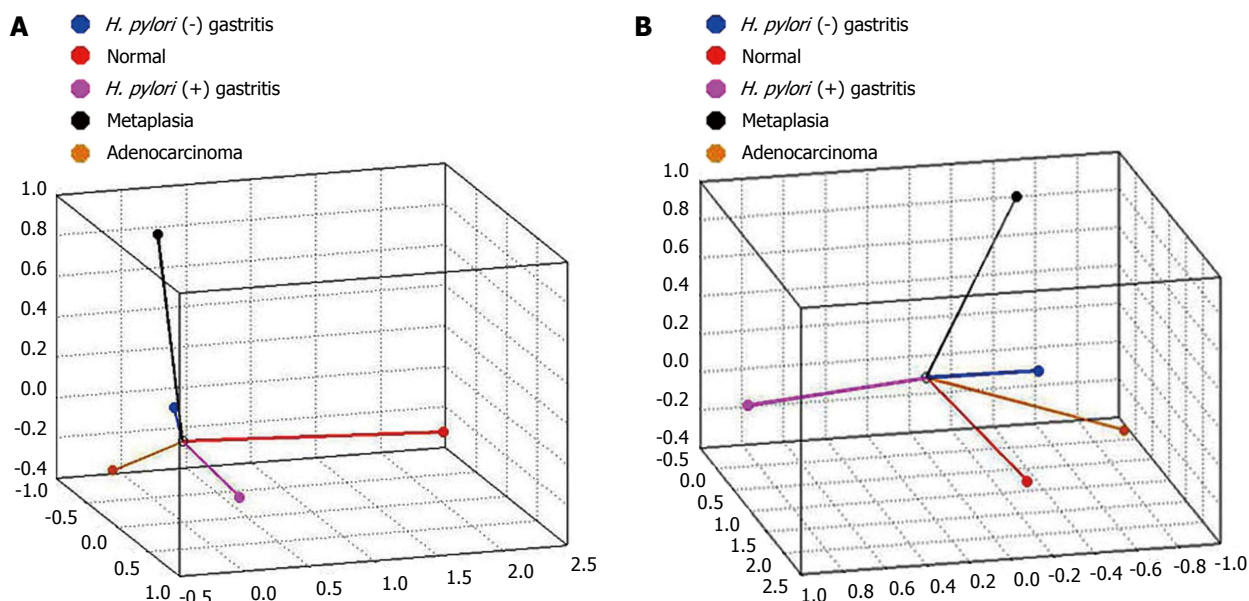
Furthermore, we observed a significantly difference between *hsa-miR-29c* profiles in normal gastric

mucosa and intestinal-type gastric adenocarcinoma. Different studies analysed the expression of *hsa-miR-29c* in gastric cancer and found results consistent with those of this investigation<sup>[17-23]</sup>.

The down-regulation of *hsa-miR-29c* has been reported in several human malignancies, including nasopharyngeal carcinoma<sup>[24]</sup>, bladder carcinoma cells<sup>[25]</sup>, lung cancer<sup>[26]</sup>, esophageal cancer<sup>[27,28]</sup>, chronic lymphocytic leukaemia<sup>[29,30]</sup> and melanoma<sup>[31]</sup>.

Considering the expression profile of *hsa-miR-29c* in gastric cancer, it is suggested that *hsa-miR-29c* acts as a TS-miR. Therefore, down-regulation of this miRNA can lead to overexpression of oncogenes, such as *DNMT3A*.

The *DNMT3A* gene encodes the DNA methyltransferase 3A, an enzyme responsible for the dynamics of DNA methylation during embryogenesis and pathogenesis. The de-regulation of DNA methylation patterns can shut-down or cripple the normal transcriptional activity and is considered an early event in tumour development<sup>[32]</sup>. This gene was validated to be a target of *hsa-miR-29c* in three different papers<sup>[32-34]</sup>.



**Figure 6** Categorical principal components analysis of *hsa-miR-135b* expression in three dimensions in two different angles (A and B). Vectors making 180-degree indicate they are closely and negatively related. Vectors making a 90-degree angle indicate they are not related. *H. pylori*: *Helicobacter pylori*.

**Table 1** Validated target genes of *hsa-miR-29c* and *hsa-miR-135b* according to the miRTarBase database

MicroRNA	Target genes	Ref. Seq. RNA	Validation methods					No. of papers
			Reporter assay	Western blot	qPCR	Microarray	Other	
<i>hsa-miR-29c</i>	<i>DNMT3A</i>	NM_022552	×	×	×	×	×	3
		NM_153759						
		NM_175629						
		NM_175630						
<i>hsa-miR-135b</i>	<i>APC</i>	NM_000038	×		×		×	1
		NM_001127510						
		NM_001127511						
	<i>KLF4</i>	NM_004235	×		×	×	×	1

*DNMT3A*: DNA methyltransferase 3 alpha; *APC*: adenomatous polyposis coli; *KLF4*: Kruppel-like factor 4 (gut); Ref.Seq.RNA: Reference Sequence of messenger RNA; qPCR: Quantitative real-time polymerase chain reaction.

According to these papers, in normal conditions, highly expressed *hsa-miR-29c* may control *DNMT3A* through a conserved function. Therefore, expression of this miRNA in tumour cells can lead to reduced global DNA methylation, restoring expression of tumour suppressor genes and inhibiting tumourigenicity both *in vivo* and *in vitro*<sup>[32-34]</sup>.

Indeed, overexpression of *DNMT3A* was reported not only in gastric cancer itself but also in gastric lesions<sup>[35-37]</sup>. Hyper-methylation of the *hMLH1*, *P16*, *DAP-kinase*, *THBS1* and *TIMP-3* genes was detected in chronic gastritis and intestinal metaplasia samples<sup>[38]</sup>.

It is possible that *hsa-miR-29c* inhibits tumourigenicity both *in vivo* and *in vitro*<sup>[32]</sup> by restoring expression of tumour suppression genes involved in the control of cell proliferation. Indeed, Matsuo *et al*<sup>[18]</sup> demonstrated that *hsa-miR-29c* is involved in regulating the S phase of the cell cycle in gastric cancer. Furthermore, Wang *et al*<sup>[23]</sup> find that *hsa-miR-29c* act as metastasis suppressor in gastric

cancer. These findings suggested that this miRNA not only functioned as TS-miR in gastric cancer but also might serve as effective predictors for gastric cancer prevention<sup>[23]</sup>.

Up-regulated expression of *hsa-miR-135b* was observed in gastric lesions compared to normal gastric mucosa and intestinal-type gastric adenocarcinoma samples. Non-atrophic chronic gastritis and intestinal metaplasia are significantly different from normal gastric mucosa samples.

To date, the expression of *hsa-miR-135b* has been analysed in gastric cancer in a few studies<sup>[17,21]</sup>. This miRNA has been most extensively studied in other types of human cancer, such as colon<sup>[39]</sup>, breast<sup>[40]</sup>, cutaneous squamous cell carcinoma<sup>[41]</sup> and lung cancer<sup>[42,43]</sup>. In all of these cases, its overexpression points to the hypothesis that this miRNA acts as oncomiR in the process of carcinogenesis.

*In silico* analysis showed that *hsa-miR-135b* has two validated target genes, *KLF4*<sup>[44]</sup> and *APC*<sup>[45]</sup>, which



are both tumour suppressor genes.

The *KLF4* gene encodes a zinc-finger transcription factor, which is involved in mediating pro-inflammatory responses and regulating cell proliferation and differentiation<sup>[46,47]</sup>. Down-regulation of this gene is reported in gastric cancer, indicating its participation in the regulation of homeostasis and maintenance of the gastric mucosa. In addition, restoration of *KLF4* expression was able to inhibit tumour growth *in vivo* and *in vitro* by inducing apoptosis in gastric cancer cells. Thus, altering *KLF4* expression plays a critical role in gastric cancer development and progression<sup>[48]</sup>.

The *APC* gene encodes a protein that binds to the transcription factor  $\beta$ -catenin and results in degradation of  $\beta$ -catenin. The loss of function of this gene causes the nuclear accumulation of APC-free  $\beta$ -catenin, which stimulates the Wnt signalling pathway and leads to de-regulated cell growth and adhesion<sup>[45]</sup>. Mutations in *APC* have been identified in patients with gastric adenocarcinoma, especially in those with the intestinal type<sup>[49-52]</sup>. These results suggest that the loss of *APC* expression plays an important role during gastric carcinogenesis.

Several studies have suggested that miRNAs represent a bridge between chronic gastritis and gastric cancer development<sup>[53-56]</sup>. This study showed that *hsa-miR-29c* expression has a good discriminatory accuracy between normal gastric samples and (1) intestinal-type gastric adenocarcinoma; and (2) intestinal metaplasia; *hsa-miR-135b* expression presented a greater discriminatory accuracy between normal samples and gastric lesions.

In conclusion, our results suggest that *hsa-miR-29c* and *hsa-miR-135b* are promising biomarkers of gastric carcinogenesis and provide evidence of epigenetic alterations in non-atrophic chronic gastritis and intestinal metaplasia, indicating that better understanding of these gastric lesions is required for the prevention of gastric cancer.

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## COMMENTS

### Background

The molecular signatures of the gastric pre-cancerous lesions, such as non-atrophic chronic gastritis and intestinal metaplasia, have not been well explored and the mechanisms that lead to carcinogenic progression are still unknown. microRNAs (miRNAs) were reported as potential biomarkers of intestinal-type gastric adenocarcinoma. *hsa-miR-29c* and *hsa-miR-135b* are promising biomarkers of gastric carcinogenesis and provide evidence of epigenetic alterations in non-atrophic chronic gastritis and intestinal metaplasia.

### Research frontiers

A subset of patients who develop intestinal-type gastric adenocarcinomas undergo a multi-stage and complex process of carcinogenesis, initiated

by chronic gastritis and intestinal metaplasia. *Hsa-miR-29c* and *hsa-miR-135b* expressions are altered in non-atrophic chronic gastritis and intestinal metaplasia, indicating that better understanding of these gastric lesions is required for the prevention of gastric cancer.

## Innovations and breakthroughs

Previous studies have shown that *hsa-miR-29c* and *hsa-miR-135b* are potential biomarkers of intestinal-type gastric adenocarcinoma. This study investigated the expression profiles of *hsa-miR-29c* and *hsa-miR-135b* in gastric mucosal samples, including normal gastric mucosa, non-atrophic chronic gastritis, intestinal metaplasia and intestinal-type gastric adenocarcinoma. The expression of *hsa-miR-29c* in the gastric lesions showed that non-atrophic gastritis have an intermediate profile to gastric normal mucosa and intestinal-type gastric adenocarcinoma, and that intestinal metaplasia samples presented an expression pattern similar to that in intestinal-type gastric adenocarcinoma. This miRNA regulates the *DMNT3A* oncogene. *Hsa-miR-135b* is up-regulated in non-atrophic chronic gastritis and intestinal metaplasia samples and down-regulated in normal gastric mucosa and intestinal-type gastric adenocarcinoma samples. Non-atrophic chronic gastritis and intestinal metaplasia are significantly different from normal gastric mucosa samples. *Hsa-miR-135b* expression presented a greater discriminatory accuracy between normal samples and gastric lesions. This miRNA was associated with *Helicobacter pylori* presence in non-atrophic chronic gastritis samples and regulates the *APC* and *KLF4* tumour suppressor genes.

## Applications

This study showed that *hsa-miR-29c* expression has a good discriminatory accuracy between normal gastric samples and (1) intestinal-type gastric adenocarcinoma; and (2) intestinal metaplasia; *hsa-miR-135b* expression presented a greater discriminatory accuracy between normal samples and gastric lesions. These results suggest that *hsa-miR-29c* and *hsa-miR-135b* are promising biomarkers of gastric carcinogenesis, indicating that better understanding of the gastric lesions is required for the prevention of gastric cancer.

## Terminology

According to the Correa cascade, a subset of patients who develop intestinal-type gastric adenocarcinomas undergo a multi-stage and complex process of carcinogenesis, initiated by (1) non-atrophic chronic gastritis; followed by (2) chronic atrophic gastritis; then (3) intestinal metaplasia; and finally (4) dysplasia. *hsa-miR-29c* and *hsa-miR-135b* are miRNAs, a class of noncoding RNAs, that regulate the expression of target mRNAs at a post-transcriptional level. These miRNAs are related to the gastric carcinogenesis and may be potential biomarkers.

## Peer-review

The authors used gastric tissues derived from normal, gastritis, metaplasia, and carcinoma to investigate expression of 2 miRNAs and evaluate their potential to be biomarkers to distinguish these 4 groups. The concept is clear and the flow of data presentation is straightforward.

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