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Decreased expression of microRNA-629 in gastric cancer samples potentiated by the virulence marker of *H. pylori*, cagA gene

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HIGHLIGHTS

- Gastric cancer is associated with a drastic decrease in the expression of miR-629, a mechanism that may affect gastric carcinogenesis.
- The *H. pylori* virulence marker, cagA gene, somehow appears to modulate the expression of miR-629.
- The cagA gene, is associated with an intense reduction in the expression of miR-629 in gastric cancer samples.

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ABSTRACT – Background – *Helicobacter pylori* (*H. pylori*) is a gram-negative bacterium associated with the etiology of several gastrointestinal tract pathologies, and *cag*A-positive (*cag*A+) strains are found in populations with gastric ulcers and precancerous lesions, inducing pro-inflammatory responses. The development of neoplasms is related to microRNA (miRNA) dysregulation, indicating highly expressed *miRNA-629*. The article aims to correlate the expression level of *miRNA-629* with the presence of *H. pylori* and the pathogenicity marker *cag*A. **Methods** – 203 gastric biopsy samples were evaluated from individuals with normal gastric tissue (n=60), gastritis (n=96), and gastric cancer (n=47) of both genders and over 18 years old. The samples were subdivided according to the presence or absence of *H. pylori*, detected by polymerase chain reaction (PCR). RNA was extracted using a commercial kit and quantified. Complementary DNA (cDNA) was synthesized using commercial kits, and the relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method. **Results –** Individuals infected with *H. pylori* are nine times more likely to develop gastric cancer. Cancer patients appeared to have decreased expression of *miRNA-629*; however, the presence of the bacterium would not influence this reduction. Individuals in the cancer group showed lower *miRNA-629* expression when *cag*A+; however, in the control group, the expression was higher when *cag*A+. **Conclusion –** *H. pylori* is a factor involved in the etiology and progression of gastric diseases. Reduction in *miRNA-629* expression in cancer patients occurs independent of the presence of the bacterium, but when the *cag*A pathogenicity marker is present, it induces changes in the gene expression of the respective miRNA.

Keywords – *Helicobacter pylori*; gastric diseases; microRNA; virulence factors; chronic gastritis; inflammation.

INTRODUCTION

Helicobacter pylori (H. pylori) is a gram-negative, flagellated, spiral-shaped bacterium that produces urease, proteases, and phospholipases, which degrade the glycoproteins present in the gastric mucosa. It is associated with the etiology of various gastrointestinal (GI) tract pathologies, including chronic gastritis, peptic ulcers, mucosa-associated lymphoid tissue (MALT), and gastric neoplasia. Recognized by the World Health Organization as a Group I carcinogen, *H. pylori* is directly linked to the development of gastric neoplasia[1-7].

With the need to detect bacterial strains associated with gastric diseases, pathogenicity markers are important to research. Early studies discovered that not all strains of *H. pylori* expressed the *cag*A protein, a product of the *cag pathogenicity island (cagPAI)*, classified as a type IV secretion system. Translocated into gastric epithelial cells *cag*A is considered an oncoprotein and induces multiple signaling cascades[8-11].

Cancer is a generic term that characterizes a broad group of complex diseases causing cellular damage in different origins, involving genetic and epigenetic alterations. Its development can be induced by physical, chemical, or biological agents. Currently, gastric cancer is the fifth most common neoplasia and the leading cause of death in various countries in West Asia[12-20].

As mentioned before, for many years, it has been described that *H. pylori* infection can promote dysregulation in miRNAs expression, and this can influence the development of many gastric diseases, including gastric cancer[21-23].

In this context, this study focuses on *miR-629*, which is characterized as oncogenic genes; thus, its expression is usually increased in several types of cancer, including GC[24]. Studies show that this miRNA can affect important processes in carcinogenesis, such as proliferation, migration, and apoptosis. Moreover, *miR-629* is also playing an important role in lung, ovarian, pancreatic, renal, breast, osteosarcoma, and head and neck cancers[25-31].

Therefore, *miR-629* appears to be involved in different types of cancer. However, the specific mechanisms by which *H. pylori* infection is related to changes in *miR-629* expression in GC are still unclear. Besides, according to[23], CagA can be the main inducer of changes in expression of several cytokines in gastric cells, through the activation of NF-Kb. Thus, it would be very important to recognize the virulence factor of *H. pylori* that affects the expression level of *miR-629* in GC samples.

Considering this scenario, this study aimed to correlated the expression level of *miRNA-629* with the presence of *H. pylori* and the *cag*A pathogenicity marker in samples from patients with normal gastric mucosa, patients with gastritis, and patients with gastric cancer.

METHODS

Sample collection and inclusion criteria

We evaluated 203 gastric biopsy samples from dyspeptic patients of both genders and over 18 years old (113♀/90♂; mean age ± SD = 55±16.5 years). Among the 203 samples, 60 belonged to the control group (patients with intact gastric mucosa, without inflammatory process assessed by histology; 38♀/22♂; mean age ± SD = 56±16 years), 96 samples belonged to the gastritis group (53♀/43♂; mean age ± SD = 55±17 years), and 47 samples came from patients with gastric cancer group (22♀/25♂; mean age ± SD = 55±16 years). Patients who used antiparasitic and/or antibiotic and/or immunosuppressants and/or proton pump inhibitors treatments within the last 30 days were excluded from the study. Patients with infectious diseases were also excluded from the study.

The biopsies of fresh gastric tissues were also collected from antrum during endoscopic evaluation or gastric surgery in the Gastroenterology services of the *Hospital Estadual de Bauru (HEB), Hospital das Clínicas de Marília, and Santa Casa de Marília*. The samples from individuals with gastric cancer were obtained in collaboration with the *Universidade Federal de São Paulo* (UNIFESP) and *Universidade Federal de Goiás* (UFG).

After collection, all gastric tissue samples were stored in RNAlater (Ambion, Waltham, MA) according with manufacturer's protocol, transported to laboratory and stored at -20º until use. The samples were subdivided into groups (control, gastritis, and gastric cancer) according to the histopathological

analysis following the criteria of the updated Sydney System[32] and Lauren System[33] and only diffuse gastric cancer samples were used. Subsequently, the groups were also divided according to the presence or absence of *H. pylori* according to the Polymerase Chain Reaction (PCR) result.

DNA extraction, *H. pylori* detection, and *cagA* Gene

DNA extraction was performed according to the protocol established by the QiAmp® DNA Mini Kit from QIAGEN (Cat No. 51304). The PCR technique was employed to diagnose *H. pylori* and detect the *cag*A gene, as described in TABLE 1[34-35].

RNA extraction

For RNA extraction, approximately 40 mg of tissue was homogenized in a Precellys 24 tissue homogenizer (Bertin Corp., Rockville MD) and the total RNA was extracted using an miRNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The RNA samples were stored at 80°C and used for reverse transcription. RNA concentrations were measured and adjusted using the NanoDrop 2000 spectrophotometer (ThermoFisher Scientific, Waltham, MA, United States) and only samples with a ratio value between 1.85 and 2.2 were used.

cDNA Synthesis and Real-Time Quantitative PCR (qPCR)

The complementary DNA (cDNA) synthesis from miRNA was performed using the TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems™, USA), following the manufacturer's protocol.

The quantitative PCR (qPCR) reaction was carried out on the ABI Prism 7500 Fast Sequence Detection System, using TaqMan gene expression assay and specific probes. The relative quantification of expression was calculated using the $2^{-\Delta\Delta Ct}$ method, according to Livak and Schmittgen[36]. The constitutive genes were evaluated and validated in previous studies[37]. For miRNA, the hsa-*miR-629-5p* (478183 mir) assay was employed. The assays RNU6B (Hs001093) and RNU48 (Hs001006) (Applied Biosystems) were used as endogenous controls for the reactions.

Statistical analysis

Data were analyzed using GraphPad Prism 8 software. First, the results were analyzed using box-plot graphs to detect outliers. When necessary, the distribution was evaluated using the D'Agostino & Pearson tests. For the analysis of expression and association, the Wilcoxon Signed Rank, Kruskal-Wallis, Brown-Forsythe, Fisher's exact, and chi-squared tests were used, depending on the groups analyzed. A level of $P<0.05$ was considered statistically significant.

Ethics approval

All the patients who participated received and signed a consent form to participate and the study was approved by the Ethics Committee (Case Number 1.119.830) of the *Universidade do Sagrado* (USC), Bauru, SP, Brazil.

RESULTS AND DISCUSSION

Detection of *H. pylori*

H. pylori was detected in 91/203 (45%) of the analyzed samples. The results indicate an association between the presence of the bacterium and the development of gastric diseases. The analyses performed in relation to the *cag*A gene involved 91 samples, of which 30 were positive, 3 belonging to the control group, 17 to the gastritis group and 10 gastric cancer samples, as shown in TABLE 2.

TABLE 1. PCR reactions conditions for *H. pylori* and *cag*A gene diagnosis.

Gene	Primer	Sequence (5'>3')	Conditions	Amplicon
16SrRNA (*H. pylori*)	Hpx1	CTGGAGARACTAAGYCCTCC	40 cycles: 1 min 94 °C, 1 min 59 °C and 1 min 72 °C	150 bp
	Hpx2	GAGGAATACTCATTGCGAAGGCGA		
cagA	cagA1	ATGACTAACGAAACTATTGATC	40 cycles: 1 min 94 °C, 1 min 53 °C, and 1 min 72 °C	232 bp
	cagA2	CAGGATTTTTGATCGCTTTATT		

TABLE 2. Detection of *H. pylori* and *cagA* gene in gastric biopsy.

	Control n=60 (%)	Gastritis n=96 (%)	Gastric cancer n=47 (%)
H. pylori Pos	11 (18.3)	49 (51)	31 (65.9)
H. pylori Neg	49 (81.6)	47 (48.9)	16 (34)
OR (CI95%) *P*-valor	4.64 (2.180–9.948) <0.0001*[1]	1.858 (0.9041–3.861) 0.108[2]	8.63 (3.380–21.63) <0.0001*[3]
*cag*A Pos	3 (27.2)	17 (34.6)	10 (32.2)
*cag*A Neg	8 (72.7)	32 (65.3)	21 (677)
Total (n=91)	11	49	31

*statistically significant. [1]Control group vs gastritis group; [2]Gastritis group vs gastric cancer; [3]Control group vs cancer group.

Marshall and Warren discovered the presence of *H. pylori* in gastric mucosa in 1983. These researchers obtained tissue samples from patients through endoscopy and found an association between the presence of the bacterium and the etiology of gastrointestinal diseases, such as chronic gastric inflammation[38].

Kawai et al.[39] showed that infected patients have an increased, cumulative risk of developing gastric cancer, depending on the presence or absence of the bacterium. The risk of developing the disease was calculated from birth to 85 years of age among *H. pylori*-positive patients, resulting in 22.26% (95%CI, 20.63–23.21) for men and 8.74% (95%CI, 8.07–9.14) for women. These results were similar to those found in our samples, indicating a higher incidence of *H. pylori* in patients diagnosed with cancer.

In a retrospective study, Ddine et al.[40] investigated factors associated with the diagnosis of chronic gastritis and the presence or absence of *H. pylori* and found similar results for the group of patients diagnosed with gastritis, with a higher incidence of the bacterium. The study included 94 patients with the disease, evaluated through digestive endoscopy to identify the causative agents of the pathology. The results elucidated that 56.6% (54 individuals) carried the bacterium as the etiological agent, while only 43.6% (40 individuals) did not present a specific agent.

Gastritis is considered the starting point for analyzing the changes caused by *H. pylori* in infected individuals. The inflammation is provoked by the activation of the immune system caused by the installation of the bacterium. In cases where treatment is not properly carried out, it can progress to precancerous lesions[41].

Expression of *miRNA-629*

The obtained results about miRNA expresson, were realized in two stages. Initially, the values obtained between the control, gastritis, and cancer groups were analyzed without considering the presence of *H. pylori*. Subsequently, the groups were subdivided considering the presence and absence of the bacterium, and then they were subjected to new statistical tests for comparison. The results are showed in FIGURE 1.

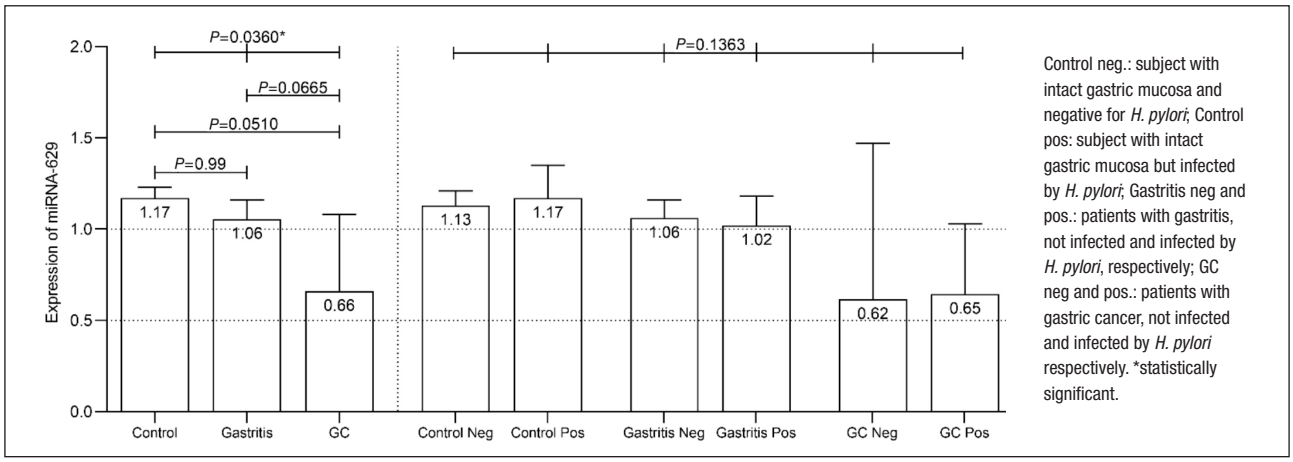

FIGURE 1. Analysis of *miRNA-629-5p* expression in groups: control, gastritis, and gastric cancer regardless and considering the presence of *H. pylori*.

The expression of *miRNA-629* was compared among the three studied groups (control, gastritis, and cancer). The results was statistically significant (P=0.036), with a progressive reduction in the expression of this miRNA in patients diagnosed with cancer compared to the other two groups. Furthermore, the analysis indicates no significant differences when performing paired comparisons between groups (control vs gastritis, gastritis vs cancer, and control vs cancer). subsequently, subgroups (negative control, positive control, negative gastritis, positive gastritis, negative cancer, and positive cancer) were compared, and the results showed that *miRNA-629* expression did not present significant differences between groups, with or without *H. pylori*.

Shin et al.[42] demonstrated the use of a platform to search for expressed miRNAs in the plasma of patients diagnosed with gastric cancer. After selection, they found that patients with a diagnosis of gastric neoplasia have a higher expression of *miRNA-627*, *miRNA-629*, and *miRNA-652* than healthy individuals. This is the first study to demonstrate a ten-fold higher expression of *miRNA-627* in patients diagnosed with gastric cancer, compared to individuals unaffected by diseases, warranting further detailed investigation in other tissues.

Another study by Hashemi et al.[43] linked the *BCL2* rs1016860 gene with *miRNA-629*, concluding that this association could be a potent biomarker for gastric and breast cancer.

Li et al.[44] found that *miRNA-629* promotes tumor cell invasion and endothelial cell permeability in lung cancer. Li et al.[45] found the same miRNA overexpressed in prostate cancer, suggesting that it contributed to tumor progression. In both cases, the miRNA played a role of an oncogene. In gastric cancer, the actual role of this gene remains unclear. According to our results, *H. pylori* does not influence its expression, whereas, in gastric cancer, it appears to down regulate, suggesting no relation to this specific condition.

miRNA-629 expression and *cagA* gene

As illustrated in FIGURE 2, two groups with statistically significant results (P<0.5) were observed: the control group (P=0.0364) and the cancer group (P=0.001). Individuals in the cancer group with a positive *cagA* gene presented lower expression of *miRNA-629* than the same group with a negative *cagA* gene. Conversely, the control group had opposite results, demonstrating that individuals with a positive *cagA* gene had higher *miRNA-629* expression than those with a negative *cagA* gene.

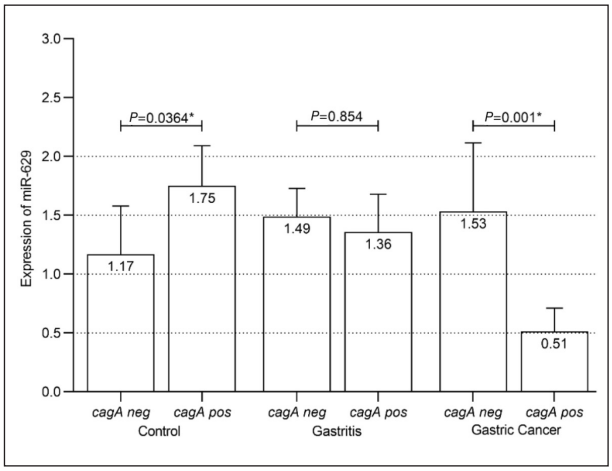

FIGURE 2. Analysis of *miRNA-629* expression and *cag*A gene. *statistically significant.

Considering the association between cancer and the virulence marker, individuals with *cagA*+ exhibited lower *miRNA-629* expression. This association, either alone or in conjunction with the disease, could be related to the presence of the positive *cagA* gene. The opposite was found in the control group, indicating that individuals with intact gastric mucosa and *cagA*+ had increased *miRNA-629* expression.

The Gastritis group presented no statistically significant difference related to the presence or absence of the *cagA* gene, suggesting that the virulence marker does not modulate *miRNA-629* expression when there is a gastric inflammatory process. This miRNA has been poorly studied, and this was the first study to investigate the correlation between *miRNA-629* and *H. pylori*, more precisely, the *cagA* virulence marker.

Nguyen et al.[46] conclude that *cagA* carriers had a higher risk of ulcerations than patients with *cagA*- (OR:325/CI:1.37–17.71) / P=0.008). Their study involved 268 children diagnosed with *H. pylori*, of which 185 cases (69%) were found to have the presence of *cagA* virulence marker. Fraga et al.[47] evaluated 225 individuals diagnosed with chronic gastritis and peptic ulcers (gastric and duodenal) and found 141 (62.7%) cases of *H. pylori* infection. They concluded that the presence of *cagA* and *babA2* genes and the combination of *cagA/oipA* genes increased the risk

of developing gastric inflammation. Additionally, the *cagA/iceA1* genes and the *cagA/oipA* combination increased the likelihood of individuals presenting lymphoid follicular hyperplasia.

Our results indicated that *cagA* negatively modulated the *miRNA-629* expression. Considering that this happened in the Cancer group, the gastric environmental conditions may have resulted from the influence *of cagA* because this virulence marker has an oncogenic profile[48]. Yang et al.[49] found that *miRNA-233-3p* expression was significantly increased in the presence of *H. pylori* and *cagA*+, but their results suggest that *H. pylori* infection induced *miRNA-233* expression independently of the *cagA* gene. This miRNA showed significantly increased expression in cancerous gastric tissue cells, and its presence was significantly higher when the bacterium was detected. Therefore, we may conclude that *cagA* does not depend on miRNAs or work with them to establish a neoplastic process.

CONCLUSION

H. pylori is involved in the etiology and progression of gastric diseases. In this work, we found that *miRNA-629* expression is reduced in gastric cancer patients, independent of the presence of the bacterium. On the other hand, the *cagA* gene appears to modulate the expression of *miRNA-629* in both cancer and normal patients. In the Cancer group, individuals with the presence of the virulence marker exhibit lower expression of *miRNA-629*, whereas in the control group, we observed higher expression of this microRNA, suggesting that neoplastic transformation may also play a modulating role in the expression of this microRNA.

Authors' contribution

Rasmussen LT and Payão SLM conceived and designed the experiments; Soares CRR, Silva LMV, Bianca Reis Almeida, Pereira JN, Santos MP collected the material, extracted the genetic material and performed the experiments, Barbosa MS and Rasmussen LT analyzed and interpreted the data; Smith MAC and Barbosa MS contributed with the collection of samples/reagents/materials and analysis tools; Soares CRR, Silva LMV, Almeida BR, Santos MP and Rasmussen LT drafted the manuscript and revised it. All authors contributed to manuscript preparation and read, commented on, and approved the manuscript.

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RESUMO – Contexto – *Helicobacter pylori* (*H. pylori*) é uma bactéria gram-negativa associada à etiologia de várias patologias do trato gastrointestinal, e cepas positivas para *cag*A (*cag*A+) são encontradas em populações com úlceras gástricas e lesões pré-cancerígenas, induzindo respostas pró-inflamatórias. O desenvolvimento de neoplasias está relacionado à desregulação do microRNA (miRNA), indicando *miRNA-629* altamente expresso. O artigo tem como objetivo correlacionar o nível de expressão do *miRNA-629* com a presença de *H. pylori* e o marcador de patogenicidade cagA. **Métodos –** Foram avaliadas 203 amostras de biópsia gástrica de indivíduos com tecido gástrico normal (n=60), gastrite (n=96) e câncer gástrico (n=47) de ambos os sexos e com mais de 18 anos. As amostras foram subdivididas de acordo com a presença ou ausência de *H. pylori*, detectado por reação em cadeia da polimerase (PCR). O RNA foi extraído usando um kit comercial e quantificado. O DNA complementar (cDNA) foi sintetizado usando kits comerciais, e a expressão relativa foi calculada usando o método 2-ΔΔCt. **Resultados –** Indivíduos infectados com *H. pylori* têm nove vezes mais chances de desenvolver câncer gástrico. Pacientes com câncer parecem ter diminuição da expressão do *miRNA-629*; no entanto, a presença da bactéria não influenciaria essa redução. Indivíduos no grupo do câncer apresentaram menor expressão do *miRNA-629* quando *cag*A+; no entanto, no grupo controle, a expressão foi maior quando *cag*A+. **Conclusão –** *H. pylori* é um fator envolvido na etiologia e progressão das doenças gástricas. A redução na expressão do *miRNA-629* em pacientes com câncer ocorre independentemente da presença da bactéria, mas quando o marcador de patogenicidade *cag*A está presente, induz mudanças na expressão gênica do respectivo miRNA.

Palavras-chave – *Helicobacter pylori*; doenças gástricas; MicroRNA; fatores de virulência; gastrite crônica; inflamação.

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