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miRNA-155-5p and miRNA-let-7a-5p expression controlled by *Helicobacter pylori*

Santos Mônica P[1]; Pereira Jéssica N[1]; Almeida Laine A[1]; Labio Roger W[1]; Smith Marilia AC[2]; Barbosa Mônica S[3]; Payão Spencer LM[1]; Rasmussen Lucas T[1]*

[1]Faculdade de Medicina de Marília (FAMEMA), Marília, São Paulo, Brazil.

[2]Universidade Federal de São Paulo (UNIFESP), São Paulo, Brazil.

[3]Universidade Federal de Goiás (UFG), Goiânia, Goiás, Brazil.

***Corresponding Author: Lucas Trevizani Rasmusse**

Genetics laboratory, Lourival Freire, 240, Bairro Fragata, CEP 17519-050, Marília, São Paulo, Brazil.

Tel: +55 14 34021856.

Email: lucasrasmussen@gmail.com

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Abstract

Introduction: Gastric diseases, including Gastric Cancer (GC), are frequently caused by *Helicobacter pylori* infection and its virulence factors, such as the *cag*A and *cag*T genes. Along with *H. pylori* infection, dysregulation of specific microRNAs (miRNAs) also appears to contribute to the development of this diseases. Therefore, this study investigated miRNA-155-5p and miRNA-let-7a-5p expression, taking into consideration virulence factors of *H. pylori* to understand their relationship with GC development.

Methods: Polymerase Chain Reaction (PCR) was employed to detect *H. pylori* and the virulence factors. Real-Time-qPCR was used to evaluate miRNA expression. A total of 208 gastric biopsy samples were obtained from patients with gastric symptoms.

Discussion: According to the histological analysis, they were divided into groups: Control, Gastritis, and Gastric Cancer. *H. pylori* was detected in 43.7%, and *cag*A/*cag*T genes in 30.7% and 38.4%, respectively. Further, *H. pylori* seems to influence the miRNA-155-5p expression, which showed an increased expression in the Gastritis ($p=0.0015$*) and GC ($p=0.0001$*) groups positive for *H. pylori.* The virulence factor *cag*T gene also promoted an increase expression of this miRNA in patients with GC ($p=0.0222$*). On the other hand, the GC group had a low expression of miRNA-let-7a-5p ($p<0.0001$*), considering or not *H. pylori* infection.

Conclusion: Our results indicated that H. *pylori* and virulence factor, specifically the *cag*T gene, influence the expression of miRNA-155-5p and miRNA-let-7a-5p, as an important mechanism involved in the inflammatory process, contributing to the development of Gastritis and GC. Furthermore, *H. pylori* infection increases the risk for the development of gastric diseases.

Keywords: gene expression; stomach neoplasms; miRNA-155a-5p; miRNA-let-7a-5p; *Helicobacter pylori.*

Introduction

Helicobacter pylori infection in the gastric epithelium is one of the most prevalent infections in the world. Generally, the infection is acquired in early childhood and prevails in approximately half of the world population, which may or may not have symptoms [1,2]. *H. pylori* is characterized as a gram-negative and microaerophilic bacterium, which colonizes the gastric epithelium and activates the host's immune system, leading to the expression of proinflammatory cytokines. The recruitment of immune cells and the high expression of these cytokines lead to an intense inflammatory process, favoring a possible carcinogenic process [3,4]. Moreover, *H. pylori* has specific characteristics, such as virulence factors, like *cag*A and *cag*T, which seem to further contribute to the development of pathologies associated with its colonization. The *cag*A gene is located in a segment of DNA, cag pathogenicity island (PAI) in region 5' (*cag*I) and was the first specific gene identified in the bacterium and encodes the *cag*A protein, frequently studied for its pathogenic effects. The cagT gene is located in the cagII region, on *cag* pathogenicity island, and its protein acts mainly in inflammatory processes. Thus, *cag*T has been associated with poor prognosis. Taking these factors into account, *H. pylori* has been associated with the development of several gastric diseases, including GC [5,6].

GC is a multifactorial and complex disease. Although its exact etiology has not been clarified yet, colonization of the gastric epithelium by *H. pylori*, as well as environmental factors such as diet and smoking along with genetic factors seem to contribute to its development. Among genetic factors, dysregulation of specific microRNAs (miRNAs) may be associated with GC development [7-9]. miRNAs are classified as oncogenic or tumor suppressor. These small non-coding RNAs regulate gene expression and participate in important processes in tumor development, including proliferation, invasion, and angiogenesis, besides acting in the cell growth process. All of these factors indicate their importance in cancer development and progression [7,10]. Furthermore, studies have shown that dysregulation of specific miRNAs could be further influenced by colonization of *H. pylori* in the gastric mucosa [11,12].

miRNA-155-5p is one of the miRNAs that play important roles in different cardiovascular and inflammatory diseases as well as cancer [13,14]. Nevertheless, it has considerable actions on the immune system, being able to control the intensity of the inflammatory response, and is important in vital biological processes. In the gastrointestinal tract, abnormal levels of this miRNA expression have been demonstrated, especially during *H. pylori* infection, and are still considered an important factor in the regulation of T cells, as an attempt to eradicate the bacterium. According to Wan et al. (2016) [15], miRNA-155-5p is highly expressed in T cells, macrophages, and gastric epithelial cells during bacterium colonization. On the other hand, miRNA-let-7a-5p has been identified as a tumor suppressor. Studies have shown that high miRNA-let-7a expression can inhibit tumor cell invasion, proliferation, and migration in the GC [16,17]. At high levels of expression, it is capable of inhibiting the tumor cell cycle, although generally its expression is low in GC. In addition, Matsushima et al. (2011) [18] reported a decrease in miRNA-let-7a expression in *H. pylori*-induced inflammatory process.

As previously mentioned, *H. pylori* and miRNAs have been widely studied for their participation in the development of different types of cancer. However, few studies in the literature have analyzed the association between these two miRNAs and the virulence factors, especially *cag*A and *cag*T of *H. pylori,* in the development of CG. Therefore, in this study, we evaluated miRNA-155-5p and miRNA-let-7a-5p expression, which are considered important in inflammation and the carcinogenic process, considering or not the presence of *H. pylori* and its virulence factors, *cag*A and *cag*T, to understand their importance in the development of GC.

Materials and methods

Gastric biopsy samples and patients

Two hundred and eight samples of gastric biopsies were obtained from the region of the antrum of the stomach from patients with peptic diseases who had undergone an endoscopic investigation at two hospitals located in the interior of São Paulo (Brazil) and at the Federal University of Goiás and Federal University of São Paulo. All samples were submitted to histopathological analysis based on the Sydney and Lauren's classification System [19], for division into groups: Control, Gastritis, and GC. We analyzed 60 patients (12♂/38♀, mean age 56 ± 15 years) with healthy gastric mucosa (Control group); 101 (46♂/55♀, mean age 54 ± 17 years) with Chronic Gastritis (Gastritis group), and 47 (16♂/31♀, mean age 56 ± 9 years) with GC. However, for the analysis of the expression of miRNA-155-5p, only one hundred and ninety seven samples were analyzed, following the same standard established for miRNA-let-7a-5p. Patients who received antimicrobial or anti-inflammatory treatment for at least 30 days prior to the examination were excluded from the study. 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.

DNA extraction and *H. pylori* detection

DNA extraction was performed using the Qiagen QiaAmp Kit (Cat. No. 51304; Qiagen, Germany) following the manufacturer's instructions. PCR was employed to detect *H. pylori* and virulence factors from the *cagA* and *cagT* genes. Details about the oligonucleotides and specific conditions are described in (Table 1). The diagnosis of *H. pylori* and its virulence factors was obtained by electrophoresis. All fragments were viewed on 2.5% agarose gel stained with ethidium bromide and photographed in a transilluminator on the α Imager 2200 image capture system [2].

RNA extraction, cDNA synthesis, and real-time-qPCR

The biopsy fragments collected for RNA extraction were stored in RNAlater® Tissue Collection (Ambion, Woodlands, TX, USA) and kept at -20°C. The miRNeasy® Mini Kit 50 (Qiagen, cat. No 217004) was used for extraction. The quantification of the extracted RNA was performed using Nanodrop ND-1000 (Thermo Fisher Scientific, Waltham, MA, USA). The Complementary DNA (cDNA) synthesis for miRNA analysis was performed using the TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems™, USA). Both techniques were performed following the manufacturer's instructions. Expression levels was determined

by the qPCR technique in the ABI Prism 7500 Fast Sequence Detection System using the assays hsa-miR-155-5p (002623) and hsa-let-7a-5p (000377). The RNU6B (Hs001093) and RNU48 (Hs001006) were employed for normalization, and all measurements were made in duplicate. Relative quantification was calculated using the 2-ΔΔCt method [20].

Table 1: Information about *H. pylori* Detection and *cagA* and *cagT* genes.

Primers	Genes	Primer sequence (5' - 3')	Amplicons	PCR Conditions	Reference
Hpx1	16SrRNA	CTGGAGARAC-TAAGYCCTCC	150pb	40 cycles: 1 min, 94 °C; 1 min 59 °C; 1 min 72 °C	[38]
Hpx2		GAGGAATACT-CATTGC-GAAGGCGA			
Cag1	*cag*A	ATGACTAAC-GAAACTATT-GATC	232pb	40 cycles: 1 min 94 °C; 1 min 53 °C; 1 min 72 °C	[2]
Cag2		CAGGATTTTT-GATCGCTTTATT			
cagT - F	*cag*T	CCATGTTTATAC-GCCTGTGT	301pb	35 cycles: 1 min 94 °C, 1 min 57 °C 1 min 72 °C	[39]
cagT - R		CATCACCA-CACCCTTTTGAT			

R = A or G and Y = C or T.

Table 2: *H. pylori* detection frequency in the Control, Gastritis, and Gastric Cancer groups.

	Control (n = 60)	Gastritis (n = 101)	GC (n = 47)
Hp -	49 (81.9%)	52 (51.5%)	16 (34.1%)
Hp +	11 (18.1%)	49 (48.5%)	31 (65.9%)
OR (95% CI), *p*		23.8 (0.11-0.51), *p=0.0002* * [1]	0.11 (0.04-0.28), *p<0.0001* * [2]

Hp: *Helicobacter pylori*; GC: Gastric Cancer; n: number of samples; * *statistically significant*; * 1: Control vs Gastritis; * 2: Control vs GC.

Table 3: Frequency of *cagA* and *cagT* Virulence Factors in patients infected by *H. pylori.*

Virulence Factor			Groups		
			Control (n = 11)	Gastritis (n = 49)	GC (n = 31)
	*cag*A	pos.	3 (27.3%)	17 (34.7%)	8 (25.8%)
		neg.	8 (72.7%)	29 (65.3%)	23 (74.2%)
Hp +					
	*cag*T	pos.	5 (45.4%)	23 (47%)	7 (22.6%)
		neg.	6 (54.6%)	19 (53%)	23 (77.4%)

Hp: *Helicobacter pylori*; GC: Gastric Cancer; n: number of samples.

Statistical analysis

Results with p<0.05 were considered significant. The GraphPad Prism 5 program was used for data analysis. ANOVA, Two-Tailed Student's t-test, and Fisher's Exact Test were employed.

Results

The first analysis performed in this work was to detect *H. pylori*, which was found in 91 samples (43.7%). Among the positive samples, the *cagA* gene was detected in 28 (30.7%) and the *cagT* gene in 35 (38.4%). The frequency of detection of the *H. pylori*, ORs, the 95% CI, and the p value as well as the frequency of the virulence factors in Control, Gastritis, and GC groups are described in detail in (Tables 2 and 3), respectively. In agreement with previous studies, the bacterium is found most prevalently in patients with chronic gastritis and GC. The results seems to confirm the influence of *H. pylori* presence and the development of gastric diseases [21,22].

For both miRNAs, the analyses were performed in two parts: in the first, the groups were analyzed without the presence of *H. pylori* being taken into account. Then, we performed a more refined analysis, in which the three groups were subdivided for the presence or absence of *H. pylori* (Positive and Negative groups). The Control negative group consisted only of patients with healthy gastric mucosa and no colonization by the bacterium. For miRNA-155-5p, as described above, in the first analysis (Control vs. Gastritis vs. GC), in which the presence of *H. pylori* was disregarded, no statistically significant difference was found (Figure 1). However, when considering the presence of *H. pylori*, significant differences were found comparing the Control negative group with: Control, Gastritis, and GC positives for *H. pylori* groups, and compared to the Cancer negative group. We also found statistically significant differences between the Gastritis negative and positive groups (Figure 1).

The miRNA-155-5p expression increased mainly in the groups: Control (median RQ: 2.170) and Gastritis (median RQ: 2.780) as well as in the Cancer group (median RQ: 1.640) colonized by *H. pylori*, in relation to the Control negative group (median RQ: 0.9500). This increase in expression is probably influenced by the presence of *H. pylori*, since this increase occurs even in the Control positive group. The same analyzes were performed for miRNA-let-7a-5p. However, when *H. pylori* was not considered, different results were observed for this miRNA. Statistically significant differences were found in the comparison among the three groups. (*p=0<0.0001**) (Figure 2). In this case, its expression decreased in the Gastritis group (median RQ: 0.8300) and the Cancer group (median RQ: 0.3500) compared to the Control group (median RQ: 1.050). In subsequent analyzes, similar results were found. When we compared the Control negative group with: Control positive, Gastritis negative and positive, and with GC negative and positive groups and the same in association: Gastritis positive and negative groups (Figure 2).

In this case, the significant decrease of miRNA-let-7a-5p expression in Gastritis and GC groups positive for *H. pylori*, in agreement with previous studies, highlighted its decrease in *H. pylori* induced inflammatory processes [17,18,24]. Another analysis was performed aiming to evaluate a possible association between cagA and cagT virulence factors from *H. pylori* with

changes in the miRNA-155-5p and miRNA-let-7a-5p expression. Both virulence factors were compared (*cagA* neg. vs *cagA* pos. and *cagT* neg. vs *cagT* pos.) for the expression of both miRNAs in each studied group. However, only the presence of the *cagT* gene seems to influence the expression levels of miRNA-155-5p in patients with GC (p=0.0222*; Figure 3).

Discussion

GC has been described as a public health problem in the world and, therefore, has gained increasing attention [25]. Although its etiology is multifactorial, miRNAs, which are known mainly for regulating gene expression, have also stood out. Due to this ability and their role in physiological and pathological processes, their dysregulation can affect multiple signaling pathways through target genes, contributing to the development of inflammatory diseases and cancer. Some studies also suggest that *H. pylori* infection may influence dysregulation of specific miRNAs [11,12,23,26]. *H. pylori* has been linked to gastric diseases since its discovery approximately 30 years ago. Its colonization in the gastric epithelium promotes an intense inflammatory process, which is known as the key to the early stage of GC [23,27].

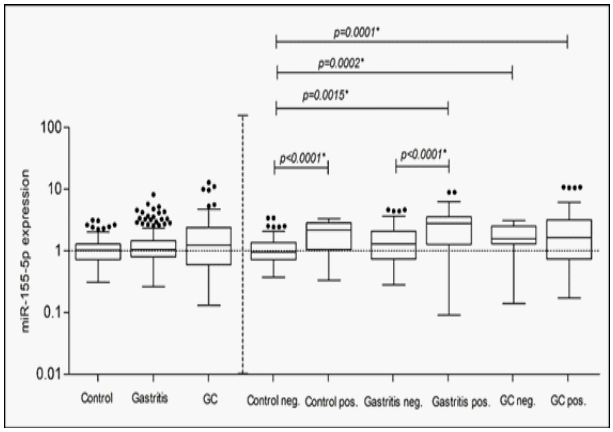

Figure 1: Analysis of miRNA-155-5p expression in groups: Control, Gastritis, and Gastric Cancer regardless of the presence of *H. pylori* as well as analysis of miRNA-155-5p expression in the same groups considering the presence of the bacterium. Legend: Control neg.: patients with healthy mucosa and negative for *H. pylori;* Control pos: patients with healthy mucosa but infected by *H. pylori;* Gastritis neg.: patients with gastritis and not infected by *H. pylori*; Gastritis pos: patients with gastritis and positive for *H. pylori*; GC neg.: patients with gastric cancer and not infected by *H. pylori*; GC pos: patients infected by *H. pylori* and diagnosed with Gastric Cancer.

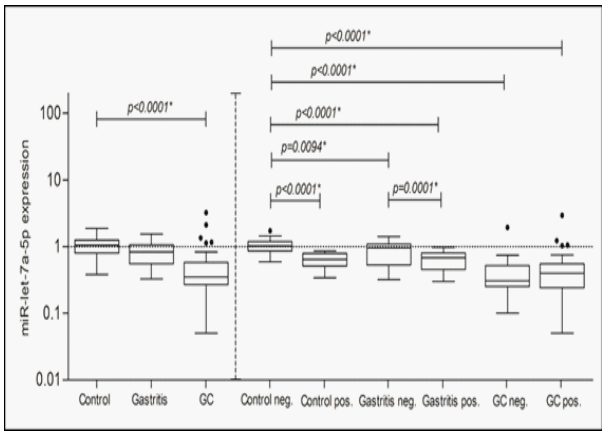

Figure 2: Analysis of miRNA-let-7a-5p expression in groups: Control, Gastritis, and Gastric Cancer regardless of the presence of *H. pylori* as well as analysis of miRNA-let-7a-5p expression in the same groups considering the presence of the bacterium. Legend: Control neg.: patients with healthy mucosa and negative for *H. pylori*; Control pos.: patients with healthy mucosa but infected by *H. pylori*; Gastritis neg.: patients with gastritis and not infected by *H. pylori*; Gastritis pos.: patients with gastritis and positive for *H. pylori*; GC neg.: patients with gastric cancer and not infected by *H. pylori*; GC pos.: patients infected by *H. pylori* and diagnosed with Gastric Cancer.

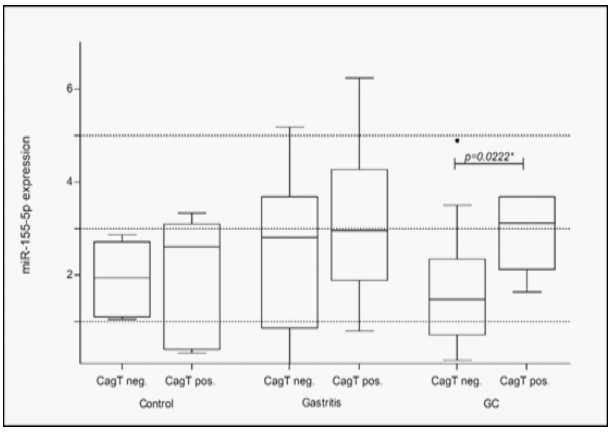

Figure 3: Analysis of miRNA-155-5p expression and cagT gene. Legend: GC: Gastric Cancer; *statistically significant.

miRNA-155-5p originates from a conserved region of the MIR155HG gene located in the 21q21.3 region of chromosome 21. Characterized as an important regulatory factor in normal immune response and inflammatory process, it appears to act in regulating responses to a vast network of stimuli. Studies on miRNA-155-5p deficient mouses have found that they are immunodeficient, suggesting that this miRNA is necessary for the development of B and T cells and dendritic cells, which are indispensable for an effective immune response [28-31].

In this study, when the presence of the bacterium was considered in the analyzes, we found an increase of miRNA-155-5p expression in groups positive for *H. pylori,* suggesting that the bacterium is inducing this increase, since it occurs even in the Control positive group. Although no statistically significant differences were found disregarding the presence of the bacteria, we found an increase in its expression in the Gastritis and Gastric Cancer groups in relation to the Control group. Similar to our results, Mahesh and Biswas (2019) [14] found increased miRNA-155-5p expression in gastric cancer cells by signaling molecules that induce inflammation. In contrast, Ma et al. (2016) [29] observed reduced expression in the Gastric Cancer group, suggesting an anti-tumor effect of miRNA-155-5p. In addition, our results suggest that the *cagT* gene expression promotes increased expression of this miRNA in patients with GC. However, the relation between the virulence factors and miRNAs is still poorly described, and we believe that other analyzes could promote a better understanding of the relationship between *cagT* gene and the miRNA-155-5p. At the same time, some studies have found that cagT is more expressed in patients with peptic ulceration and GC, making it an important prognostic marker. Therefore, *H. pylori* strains that express *cagT* trend to develop more critical symptoms due to the intense inflammatory process [32,33].

Studies suggests that miRNA-155-5p expression may be regulated by the presence of *H. pylori*, although the NF-kB and

AP-1 pathways are required for its expression in response to the pathogen. They also suggest that this miRNA may negatively modulate the release of some proinflammatory cytokines, such as IL-8 and GRO-a. Pathways such as IKK and FADD have been revealed as potential targets of miRNA-155 for the development of its mechanisms of action. The ability of miRNA-155-5p to control these pathways could interfere with activation of the NF-kB pathway, associated to the inflammatory process and regulation of immune genes, which consequently would decrease inflammation in response to *H. pylori* infection. However, despite the activation of these mechanisms to perform their action, the *H. pylori* colonization and secretion of virulence factors, specially cagT, continues to stimulate an inflammatory response, which appears as a cycle [10,14,31,34,35]. Some studies have also shown that miRNA-155-5p is usually overexpressed in solid tumors, suggesting an oncogenic action of this miRNA; however, this profile depends on tissue and cell type [14,36], although its exact role in gastric cancer is still not well understood.

miRNA-let-7a-5p was first identified as a gene capable of promoting the transition from the larval to adult state in Caenorhabditis elegans and acts as a new type of miRNA. it is characterized as a tumor suppressor in different types of tumors. Its action is through several signaling pathways, such as PKM2, associate to proliferation, invasion, and apoptosis in some types of cancer. Some researchers have shown that miRNA-let-7a expression levels are generally low in cancer patients [16,18].

Here, when the presence of *H. pylori* was not taken into account, we found a statistically significant difference among the three groups studied, as previously mentioned. When we considered the presence of *H. pylori*, we demonstrated a decrease in patients with gastritis and cancer, especially in positive groups. In line with our results, Tang et al. (2016) [17] also demonstrated decreased miR-let-7a expression in CG patients, and that this loss was associated with increased tumor cell proliferation, invasion, and worse disease prognosis. Similar to the other miRNA studied here, miRNA-let-7a-5p also appears to have its expression regulated by the presence of *H. pylori* through the NF-kB pathway, along with the MAPK pathway. *H. pylori* virulence factors activate the NF-kB pathway, suggesting that the presence of *H. pylori* promotes continuous activation of NF-kB, intensifying the inflammatory process and influencing in miRNA-let-7a-5p expression by activating these pathways [18]. Johnson et al. (2007) [37] found a high expression of miRNA-let-7a-5p in healthy cells, as well as its ability to directly regulate some stages of the cell cycle, such as RAS and CDK6, and thus control cell proliferation. Two further hypotheses are suggested by them that the alteration of miRNA is a consequence of the disease or that this miRNA is itself contributing to the development of the tumor.

Taken together, our results demonstrated an increased expression of miRNA-155-5p and a decreased in miRNA-let-7a-5p expression in patients with Gastritis and GC, both of which appear to be influenced by the presence of *H. pylori.* Moreover, *cag*T gene of *H. pylori* promoted an increased miRNA-155-5p expression in GC group. Therefore, due to the importance of these miRNAs in cancer development, both could become future therapeutic targets and contribute to the development of biomarkers for early detection of Gastric Cancer.

Declarations

Conflicts of Interest: No conflicts of interest to declare.

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Data availability statement: The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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