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ORIGINAL ARTICLE

Expression analysis of miRNA-155 level in *Helicobacter pylori* related inflammation and chronic gastritis

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ABSTRACT

Background and Objectives: *Helicobacter pylori*, is a major etiologic agent associated with gastritis. There is more evidence of noncoding microRNAs (miRs) dysregulation in gastrointestinal diseases, including inflammation caused by *Helicobacter pylori.* Also, the classification of gastrointestinal malignancies using the miRs profile is better than the protein profile. MiRNA-155(*miRNA-155*) among other miRs plays an important role in control of inflammation and gastric malignancy, so it can be remarkable prognosis marker of gastric cancer in the phase of chronic gastritis. The aim of this study was to compare the expression of miRNA-155 in gastric biopsy and serum samples of adult patients with chronic gastritis.

Materials and Methods: Biopsy and blood samples were collected from endoscopy candidates at Taleghani hospital, Tehran, during 2019. *H. pylori* infection was detected using histology, culture and molecular PCR methods. Based on *cagA* and *vacA* genotyping, the toxicity of *H. pylori* isolates were determined. After RNA extraction, the expression rate of miRNA-155 was evaluated by real-time polymerase chain reaction (RT-PCR) in gastric tissue and serum of adults infected by *H. pylori* (n = 30) compared with control group without infection (n = 20). RNU6 housekeeping miRNA were used as endogenous control and statistical analyses were performed using SPSS, ANOVA and Student's t-test.

Results: *miRNA-155* expression in *H. pylori* infected adult patients increased significantly by 5.61 and 10.11 fold in serum and tissue respectively, compared to that observed in the control group. Evaluation of *miRNA-155* expression pattern in relation to bacterial virulence factors showed that the increase in *miRNA-155* expression is independent of CagA and VacA toxins.

Conclusion: According to the differential expression patterns of *miRNA-155* in serum samples of the infected adult patients, *miRNA-155* has the potential to evaluate as chronic gastritis marker.

Keywords: *Helicobacter pylori*; Gastritis; Serum marker; MicroRNA

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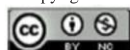

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INTRODUCTION

Helicobacter pylori (H. pylori) is a Gram-negative bacterium that causes gastritis, peptic ulcers, and gastric adenocarcinoma and infects about half of the world's population (1). Inflammation is thought to be a major determinant of both peptic ulceration and gastric cancer in both acute and chronic *H. pylori* infections (2). However, the regulatory mechanisms that control *H. pylori*-induced inflammation have remained unknown. The progression of *H. pylori* infections is highly diverse, and depending on how long the inflammation persists, this might result in acute or chronic active gastritis. Patients with *H. pylori* infection experience acute gastritis, which can resolve. This kind of gastritis is linked to hypochlorhydria and neutrophil infiltration. Acute gastritis, on the other hand, can progress to chronic active gastritis, which is marked by the infiltration of mononuclear cells, primarily lymphocytes, plasma cells, and macrophages. Acute gastritis can also lead to multifocal chronic gastritis, which has a variety of causes (genetics, age of acquisition, and bacterial strain virulence), and patients are frequently asymptomatic (3).

The bacterial cag pathogenicity island (cagPAI) is one of the virulence factors that affects the severity of the disease. This gene region codes for a type 4 secretory system and an effector protein called cytotoxin associated gene A (CagA), a toxin that enters the secretory system and is phosphorylated by kinase enzymes after binding to cellular phosphatases like SHP-2, causing epithelial cell death (4).

MicroRNAs (miRNAs) are single-stranded non-coding RNA molecules with 19-24 nucleotides that influence gene expression at post transcriptional level. There are thousands of miRNAs in the human genome that target, an average, 45% of genes encoded by the genome (5). MicroRNAs are significant regulators of a wide range of physiological processes, and their disruption has been linked to human diseases, including cancer, as well as immunological and inflammatory disorders (6). MiRNAs play a vital regulatory function in the innate immune response to pathogens and stimuli, according to mounting evidence (7). Among the miRNAs involved in the development and prognosis of gastric malignancy and the inflammation immune response, over expression of *miRNA-155* consider as an important prognosis biomarker of chronic inflammation in people at gastric cancer risk. Due to the pathological manifestations which followed by overexpression of miR-155, reducing the expression level during *H. pylori* infection may prevents the development of chronic gastritis to cancer (8). Various studies have shown an increase in *miRNA-155* expression in cells and tissues infected with *H. pylori*, and some key target genes for miRNA in the pathogenesis of this bacterium, such as MYD88 and IL-6, have been identified (9, 10). Furthermore, multiple algorithms for *miRNA-155* can predict thousands of target genes that may be involved in host immunological responses. As a result, identifying potential new targets and pathways related with *miRNA-155* will aid in clarifying and raising knowledge of the disease's etiology, as well as exploiting them as diagnostic, prognostic, and therapy techniques.

Expression pattern of *miRNA-155* were discussed in this study to investigate the relationship between *H. pylori* infection and interactions between *miRNA-155* and bacterial CagA and VacA toxins. Due to the importance of *miRNA-155* in immune function in response to *H. pylori* infection, this study was conducted on analysis of expression level of *miRNA-155* in gastric tissue and serum specimens in adults with chronic gastritis.

MATERIALS AND METHODS

Gastric biopsy and blood samples collection. Gastric biopsy and blood samples were selected from 50 adult candidates undergoing upper gastrointestinal endoscopy at Taleghani hospital, Tehran during 2019. All samples were received from the adult patients with an age range of 30-70 years old. Patient group included 30 whom diagnosed with *H. pylori* (14 male and 16 female) and controls included 20 patients without *H. pylori* gastritis (12 male and 8 female). Sample size calculated after formulation based on the percent of *H. pylori* infection using the formula:

$N= z(1-z\alpha/2)^2 (p) (q)/d^2$ where N is sample size, z is stands for level of confidence and d is margin of error.

Four biopsy, specimens were prepared from the gastric antrum section of each patient, one of which was sent to the pathology laboratory in formalin solution for histological examination. A biopsy specimen was placed immediately in a transfer medium containing 1.3 g / l agar (Merck, Germany) for culture. The other two samples were sent to the microbiology lab for

H. pylori molecular detection, genotyping, and RNA extraction in order to analyze the expression of the genes under investigation in specific transfer conditions. The patients' blood samples were stored at room temperature for 30 minutes before being centrifuged at 2000 rpm for 10 minutes to separate the serum. The extracted DNA and RNA sample were kept at -20°C and - 80°C until the experiment, respectively. Patients who had not had an antibiotic or *H. pylori*-specific treatment for at least 4 weeks prior to endoscopy were included. A summary of patients' history is provided in Table 1.

Histology. Gastric biopsy samples of candidates in formalin solution were sent to the pathology laboratory of Taleghani hospital, Tehran, Iran. Tissue samples were stained using Giemsa and haematoxylin-eosin technique and were independently observed and evaluated by an experienced pathologist. *H. pylori* colonization rate, neutrophil and mononuclear cell growth, glandular atrophy, and intestinal metaplasia are among the markers that have been evaluated. These parameters were assigned to null, mild, moderate and severe, respectively.

Isolation of *H. pylori* by culture method. Gastric biopsy specimens were cultured after being crushed in BHI broth in Brucella agar-specific medium containing 7% sheep blood, 10% FCS (fetal calf serum) and Campylobacter selective supplement (vancomycin 2 mg, polymyxin B 0.05 mg and trimethoprim 1 mg and amphotericin B 2.5 mg / l). The cultured plates were incubated in the CO_2 incubator (Innova co-170, New Braunswick Scientific, USA) for 3 to 7 days. *H. pylori* isolates were, gray colonies and were confirmed by urease, catalase, and oxidase tests.

Extraction of DNA and RNA. Gastric biopsy samples were stored in RNA LATER solution (Qiagene, Germany) at -20°C until RNA extraction. DNA extraction from amplified pure colonies as well as from gastric biopsy was performed according to the manufacturer's instructions with FavorPrep tissue genomic DNA extraction kit (Favorgen, Taiwan). The extracted DNA samples were stored at -20°C until use. Another sample was used to extract RNA. First, tissue (weighing approximately 40 mg) was precipitated in liquid nitrogen and then total RNA was extracted with Trizol solution (Biobasic, Canada) followed by phenol: chloroform extraction steps. The quality and amount of RNA and DNA extracts was evaluated using Nano-drop spectrophotometer (DeNovix, USA).Total RNA was extracted from patient's serum samples by the same method.

Detection of H. pylori by PCR method. As mentioned above, the DNA extracted from bacterial culture was used for molecular detection of *H. pylori*. In culture-negative samples, PCR was performed on DNA extracted of gastric biopsy specimens. The presence of *H. pylori* was assessed by 16S rRNA and *glmM* genes. PCR reaction was performed for each gene in a volume of 25 µl (Eppendorf, Germany) which included 1 µl of each of the forward and reverse primers (1pmol), 10 µl of Master Mix (Biofact, Korea), 1 µl of bacterial DNA (approximately 200 ng) and 12 µl of distilled water. The PCR product was run in 1.8% agarose gel electrophoresis at V90 voltage. To confirm the specificity and sensitivity of primers, J99 *H. pylori* standard strain (CCUG471167) and no template reaction were used as control positive and control negative respectively.

Table 1. Pathological characteristic of patients

Sample (total= 50)	Gender	Age (year old)	Chronic Inflammation grade	*H. pylori* infection	*H. pylori* colonization
	Male (26)	<50 (12)	0 (7)	N (20)	Mild (12)
	Female (24)	>50 (18)	1 (21)	P (30)	Moderate (15)
			2 (22)		Sever (3)
			3 (0)		

Chronic inflammation grade; null (0), mild (1) moderate (2) and sever (3)
H. pylori infection; N (negative), P (positive)

Determination of *H. pylori* genotype by PCR. PCR genotyping was performed for *vacA* (1s /2s), *VacA* (1m /2m) and *cagA* genes under the following conditions: 30 cycles, one minute 94 degrees, 45 seconds 58 degrees and one minute 72 degrees. PCR products were electrophoresed on 1% agarose gel at V90 voltage. The reaction volume for each gene was 25 µl including 1 µl of each of the forward and reverse primers, 10 µl of Master Mix X, 1 µl of bacterial DNA and 12 µl of distilled water performed in the X system (Table 2).

Investigation of *miRNA-155* expression by Real time PCR. The expression of *miRNA-155* was evaluated in *H. pylori* infected and control groups by stem loop RT-PCR method. The RNU6 gene was used as endogenous control to normalize the data. The sequence of primers used in this study is given in Table 3. Due to the short length of miRNAs in the cDNA synthesis stage using the BIOFACT RT series kit (Biofact, Korea) in this method, the specific *miRNA-155*RT primer (designed by gene runner in this study) and RNU6 with stem loop structure were used. This primer provides the ability to detect them by RT-PCR and increasing the length of miRNAs. In the following step, the expression of miR genes was investigated using the specific forward primer of each gene and the UNIVERSAL REVERSE primer. These 15 µl reactions were performed according to the following steps in Rotor-Gene thermocycler (Qiagen, Germany) and Rotor-Gene Q series software: 2 minutes' incubation at 50°C and 10 minutes' incubation at 95°C (for polymerase activation) and this is followed by 40 cycles of denaturation at 95°C for 20 seconds, alignment at 45°C for 30 seconds and extension at 72°C for 20 seconds.

Statistical analysis. To evaluate the expression of genes in positive samples with control group, t test and One Way ANOVA tests were used. Data analysis was performed using GraphPad Prism 6.0 and SPSS 20. Differences with a p value of < 0.05 were considered.

RESULTS

A total of 50 gastric biopsy and serum samples were used in this study. There were 26 men and 24 women

Table 2. Primer sequences used for verification and genotyping of clinical isolates

Target gene	Oligonucleotide sequence	PCR product (bp)	Annealing temperature (°C)	Reference
16S rRNA	F: GGCTATGACGGGTATCCGGC R: GCCGTGCAGCACCTGTTTTC	764	58	(25)
glmM	F: GGATAAGCTTTTAGGGGTGTTAGGGG R: GCTTACTTTCTAACACTAACGCGC	296	56	(26)
cagA	F: AATAGAATTCATAGCCTATCGTCTCAG R: AATACACCAACGCCTCCAAG	400	52	(27)
vacA s1/s2	F: ATGGAAATACAACAAACACAC R: CTGCTTGAATGCGCCAAAC	259/286	57	(28)
vacA m1/m2	F: CAATCTGTCCAATCAAGCGAG R: GCGTCAAAATAATTCCAAGG	570/645	57	(29)

Table 3. Primer sequences used in Real-Time PCR assay

Target gene	Oligonucleotide sequence	Reference
miRNA-155	Forward primer ACACTCCATCTGGGTTAATGCTAATCGTG	this study
RNU6	Forward primer ACACTCCATCTGGGTCGTGAAGCGTTC Universal reverse primer TGGTGTCGTGGAGTCGGCAATTCAGTTG	this study

in this group, with an average age of 50 years. Thirty of the fifty samples were positive for *H. pylori* gastritis, while the other twenty cases were negative for the *H. pylori* infection which was used as a control group. Control samples in this study included patients with normal gastric tissue without gastritis or inflammation or those with negative gastritis for *H. pylori* infection.

Diagnosis of *H. pylori* infection. Culture, histology, and specific PCR were used to diagnose *H. pylori* infection in patient biopsy specimens (16S rRNA and *glmM*). Samples with three positive tests were classified as infected. According to histopathological results, out of 30 samples with *H. pylori* gastritis, 14 were classified as mild chronic gastritis and 16 with moderate chronic gastritis. Culture and PCR results were positive for both genes specific for all *H. pylori* positive patients (Table 1).

Differential expression of *miRNA-155* in tissue and serum samples of patients with *H. pylori* infection. The expression of *miRNA-155* in *H. pylori* gastritis tissue and serum samples differed from the control group. The mean and standard deviation of *miRNA-155* expression in tissue samples of control subjects was 15.10 ± 3.52 and in the group of patients was 25.21 ± 6.28, which indicates a 10.11-fold increase in expression level in the patients group compared to the control group (P <0.0001) (Table 4). Mean and standard deviation of *miRNA-155* expression in serum samples of control subjects was 14.52 ± 1.87 and in patients group was 20.14 ± 4.86, which indicates a 5.617-fold increase in expression level in patients compared to the control group (P <0.0002) (Table 5).

Relationship between *miRNA-155* expression and *cagA* and *vacA* genetic markers in isolates isolated from patients. The presence of CagA toxin and subtyping of VacA were determined by PCR. Nineteen of the 30 isolates obtained from patient tissue samples were CagA negative, while 11 were CagA positive. When compared to the samples without toxin, the expression of *miRNA-155* in tissue samples from individuals positive for CagA toxin rose by 2.08 times. The expression of *miRNA-155* in toxin-positive serum samples of patients increased by 1.58 times compared to the group without the toxin. Nevertheless, the differences in *miRNA-155* expression in both tissue and serum samples was not statistically significant compared to the control group. So, the presence of CagA toxin was not related to *miRNA-155* expression in this study. Out of 30 samples with *H. pylori* gastritis, 12 had s1m1 genotype in terms of *vacA* genetic index, 8 had s1m2 genotype, 2 had s2m2 genotype and 8 had s2m1 genotype. In terms of the *vacA* genetic index, *miRNA-155* expression in tissue samples from the negative group (s2m2, s2m1) compared to the positive samples in terms of this genetic index (s1m1, s1m2), was 0.34 times statistically significant. The expression of *miRNA-155* in serum samples increased by 0.99 times in the positive toxin group compared to the negative toxin group, but the difference was not statistically significant.

DISCUSSION

Helicobacter pylori infection mainly occurs in childhood and may remain in the host until puberty. The clinical isolates of *H. pylori* adapt rapidly to their human host. In this way, they acquire the ability of long-term colonization in the gastric epithelium cells, which can cause different gastric disorders in

Table 4. *miRNA-155* expression in gastric tissues of infected and control groups

Groups	N	Mean ± SD	Mean Difference vs Controls	P value	
Controls	20	15.10 ± 3.52			
Chronic gastritis	30	25.21 ± 6.28	-10.11	<0.0001	***

Table 5. *miRNA-155* expression in serum samples of infected and control groups

Groups	N	Mean ± SD	Mean Difference vs Controls	P value	
Controls	20	14.52 ± 1.87			
Chronic gastritis	30	20.14 ± 4.86	-5.617	0.0001	***

humans. The chronic nature of this infection leads to the stimulation of microorganisms to evolve in the host, which is the reason for the difference in the severity of the disease caused by this pathogen in different people (11). The prevalence of infection in developing countries such as Iran is higher than in developed countries and the infection rates of this bacterium is on the rise in Iran, ranging from 60% to 70% in different regions (12). Given that *H. pylori* infection is known as a risk factor for gastric cancer, examining the factors involved in the process of infection with this bacterium can provide a clearer path to rapid and more precise diagnosis and prevention of malignancy.

H. pylori infection induces a wide range of responses in gastric epithelial cells and the human immune system play a significant role in regulating or perpetuating the infection. Hence, despite severe immune responses, the infection in some people is not completely cleared and remains in the host for a long time. Thus, the persistence of infection in individuals depends significantly on host-related factors, especially factors involved in the immune system, as well as the severity of bacterial pathogenesis (13). Among mediators regulating the response to *H. pylori* infection, miRNA molecules can play a potential role in host-bacterial communication. Various studies have suggested the role of miRNAs in *H. pylori* gastritis and gastric cancer. In fact, the role that these molecules play in the process of disease is the results of the imbalance between the miRNAs involved in the inflammatory process and the inflammatory regulatory processes. However, various factors are involved in upsetting this balance, many of which have yet to be identified (14).

In another study by Lario et al., they investigated the expression profile of miRNA by microarray method in tissue samples from people with *H. pylori* positive duodenal ulcer, non-ulcer *H. pylori* infection, and healthy people, and found that there was no significant difference in *miRNA-155* expression between the two groups with duodenal ulcer and the control group, but they found a threefold increase in expression in patients with chronic *H. pylori* gastritis (15). As a result, according to the study of Lario et al., the presence of bacteria in any situation of gastrointestinal diseases has no effect on *miRNA-155* expression. Another study examining the regulation of inflammatory responses in *H. pylori* infection by *miRNA-155* in *H. pylori*-infected macrophages as well as in the blood samples of patients with *H. pylori*-induced gastritis concluded that *miRNA-155* expression has increased in comparison to the control group, leading to the macrophages resistance to apoptosis (16). In the present study, we evaluated the expression of *miRNA-155* in tissue and serum samples for each patient.

The aims of our study were to determine whether infection of gastric epithelial cells in the presence of *H. pylori* could lead to the alteration in miRNAs expression in comparison with the control group. The results of this investigation demonstrated that the expression level of *miRNA-155* in adult serum samples with *H. pylori* infection was 5.61 times higher than in non-infected adult serum samples. Infected adult tissues had a 10.11 unit increase in *miRNA-155* expression compared to non-infected adult's tissues. The presence of *H. pylori* in adults resulted in a significant increase in expression in blood and serum samples compared to the control group. *H. pylori* has several virulence factors that play a considerable role in triggering inflammation. The most important of these factors include the pathogen coding for type 4 secretory system (cagPAI), the cytotoxin VacA that induces vacuolar induction, induction of apoptosis, and the enzyme gamma glutamyl transferase. Given that *miRNA-155* is one of the most prominent molecules that differs significantly after *H. pylori* infection in most studies. It is important to investigate the effect of these virulence factors on the expression of this key molecule. In our experiment, we investigated the expression of this molecule in patients with chronic gastritis in the presence and absence of *H. pylori*. Various studies have examined the expression pattern of *miRNA-155* in the presence of bacteria and in different conditions, including cell culture, as well as in patient samples. In one study the miRNA profile of *H. pylori*-infected and non-infected T-lymphocytes was investigated. Based on RT-PCR results, they found an overexpression of 2.5 to 3 times in *miRNA-155* in infected T cells, but there was no relationship between the presence of cagA and the expression of *miR-155* in the presence of VacA toxin (17). In another research on the expression of *miR-155* in macrophages of mice infected with *H. pylori,* they concluded that the increased expression of this molecule during infection depends on the secretory system of type 4 bacteria and the NF-ĸB transmission pathway, but has no relationship with the presence of CagA (18). In a study of normal tissue samples, *H.*

pylori infected tissues, and *H. pylori* wounded tissue, it was concluded that *H. pylori* cagA+ strains in wound samples induces the IL-6 expression. However, by adding *miRNA-155* to infected cells, a significant decrease in IL-6 expression occurred in infected cells induced by CagA- strains (19). In other study the association between *H. pylori* inflammation and *miR-155* expression was evaluated and they found that expression of this molecule in patients with chronic gastritis could be up to 4-fold higher than that in controls (20). In the study by Huang et al on the diagnosis value of *miRNA-155* and *miR-203* in blood samples of children with chronic gastritis they found direct relationship between *miRNA-155* and IL-6 expression and also between *miRNA-155* and TNF-α expression in context of *H. pylori* infection. Hence, *miR-155* might introduce as an indicator of chronic gastritis and *H. pylori* infection in children (21).

Comparison of *miRNA-155* expression between the groups with duodenal ulcer, chronic gastritis and the control group showed that there was no significant difference between the control group and the ulcer group, but in gastritis patients there was a 3-fold increase in *miRNA-155* expression which was in accordance to our result (15). In this study, the evaluation of *miRNA-155* expression pattern in relation to bacterial virulence factors showed that the increase in *miRNA-155* expression in cagA+ samples is not statistically significant with CagA toxin. Furthermore, no significant difference was observed in increasing the expression of *miRNA-155* among isolates with different vacA subtypes. In addition, animal model studies have shown that *miRNA-155* expression is involved in the development of infection and stimulation the ability of bacteria to adapt to the host in a *H. pylori* infection model (2). According to the results of several studies on the effect of *H. pylori* infection on the expression pattern of *miRNA-155,* this molecule can be used as an indicator of chronic infection and severity of *H. pylori* diseases. Moreover, various studies have shown different results on the expression of *miRNA-155* in the gastric cancer phase. In some studies, investigating the expression of this molecule in cancer cells compared to healthy cells, represented a decrease in its expression in the cancer phase, but others have indicated an increase in its expression (22-24). However, in a study of patients with *H. pylori* infection with various pathological indicators, they showed that *miRNA-155* is not expressed in IM patients, which is known as the precancerous level. Therefore, simultaneous study of *miRNA-155* expression pattern in the presence of bacteria in different situations such as chronic gastritis, ulcers and gastric cancer can play a significant role in identifying potential of *miRNA-155* as a prognostic or diagnostic biomarker.

CONCLUSION

In conclusion *miRNA-155* expression level can changed beyond *H. pylori* infected gastric tissue and serum samples. Due to increased level of *miRNA-155* in serum of patients, it can be suggested as a potential diagnostic indicator of chronic gastritis in adults. However, the exact role of abnormal expression of *miRNA-155* in *H. pylori* related inflammation can identify by further studies.

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