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Overexpression of miR-146a and miR-155 are Potentially Biomarkers and Predict Unfavorable Relationship between Gastric Cancer and Helicobacter pylori Infection

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Gastric Cancer (GC) is one of the most dangerous malignancies in the world. This study aims to evaluate the relationship between miR-146a and miR-155 in patients with *H. pylori* infections with GC compared to *H. pylori*-infected patients and healthy subjects. Forty patients with *H. pylori* and GC positive diagnoses and 40 patients with *H. pylori* positive and GC negative diagnoses, and 40 healthy persons were selected. The expression of miR-146a and miR-155 genes in the whole blood was examined using qRT-PCR. Moreover, ROC curves were drawn to represent the sensitivity and specificity of miR-146a and miR-155 expression as biomarkers. The results showed the expression of miR-146a and miR-155 in the whole blood of patients with *H. pylori* and GC positive diagnoses are significantly higher than in healthy individuals and are non-significantly enhanced compared to *H. pylori* positive and GC negative. Also, the results stated miR-146a and miR-155 expression in the whole blood of patients who are *H. pylori* positive and GC negative are significantly increased compared to healthy individuals. Furthermore, the ROC curve analysis of miR-146a and miR-155 RNA level demonstrated the two miRNAs have an appropriate sensitivity and specificity for diagnostic goals. In conclusion, *H. pylori* infection may increase the expression of miR-146a and miR-155 in patients with *H. pylori* and GC positive diagnoses, which can be effective in the curbing the progression of GC. For this reason, up-regulation of miR-146a and miR-155 along with *H. pylori* infection might contribute to the pathogenesis of GC, and also can be suggested as biomarkers for GC diagnosis and treatment.

Key Words: *Stomach Neoplasms; MicroRNAs; Helicobacter pylori; Biomarkers*

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INTRODUCTION

Gastric Cancer (GC) is one of the most dangerous malignancies in the world, especially in the eastern regions of Asia.[1,2] Each year, the numbers of diagnosed people are estimated to be around 950,000 in the world, accounting for almost half of the prevalence in East Asia.[1,2] Although the prevalence and mortality rate is reduced by this malignancy, GC is the fifth most common cancer and the forth highest cause of cancer-associated deaths all over the world.[1,2] Most patients with GC are identified in the advanced stages. Although specific diagnostic and treatment methods for GC have been used, the survival rate of these patients is about 5 years, which is a very low survival rate.[1-3] Today, certain tumor markers are engaged, such as

CA-19.9 and carcinoma embryonic antigen (CEA) to diagnose GC, but they have no high specificity to diagnose this malignancy at early stages.[3]

Helicobacter pylori (*H. pylori*) is a gram-negative, motile, microaerophilic, and spiral shape bacterium.[4] The relationship between *H. pylori* infection and the pathogenesis of GC has been well analyzed.[4] Based on the relevant studies, 70 to 90% of people are *H. pylori* carriers before the age of 10 years old, in developing countries, but, the prevalence of infection differs from 25 to 50% in developed countries.[5] *H. pylori* is responsible for more than 60% of GC all over the world. As before mentioned, GC is responsible for more than 8.2% of all cancer deaths worldwide.[5] The WHO has categorized *H. pylori* as a group 1 carcinogen for the pathogenesis of GC.[5] Today, *H. pylori* can be diagnosed by serological test, a rapid urease test (RUT), histological examination of biopsy specimens, and polymerase chain reaction (PCR) technique.[5]

There is no doubt that microRNAs (miRNAs) are known factors in regulating gene expression, which alter the production of various proteins in the cell in the post-transcriptional phase by using the mechanism that affects the stability of mRNAs.[6] MicroRNAs are a group of small RNAs with a regulatory role by binding to the non-translational region of the 3 'end of each mRNA, which can increase or decrease the stability of mRNAs and affect the expression of various genes in the cell. MicroRNAs can be introduced as either tumor suppressors or oncogenes, depending on the types of proteins that are regulated.[6,7]

miR-146a and miR-155 are the most pivotal microRNAs that have been studied in various cancers, such as breast, ovarian, lung, liver, prostate, thyroid, and oesophagus cancer. These two microRNAs can be involved in the pathogenesis of neoplasm through different cellular and molecular pathways, but no studies have been performed on the relationship between these microRNAs and GC. Also, the association of the microRNAs and *H. pylori* infection has not been evaluated in the development of GC.[8-11] This study aimed to investigate the relationship between miR-146a and miR-155 gene expression in patients with *H. pylori* and GC positive in comparison with *H. pylori* positive and GC negative and healthy individuals.

MATERIALS AND METHODS

1. Patients and specimens

In this case-control study, which was conducted in Tehran, Iran, patients who were referred to Imam Khomeini and Shariati hospitals provided samples. Biopsy samples were taken from all patients for bacterial culture to confirm the presence of bacteria in subjects, and 1 cc of whole blood was taken to investigate the expression of the genes.

The specimens were divided into three groups:

Group 1: 40 patients with *H. pylori* and GC positive who had not received any treatment (*H. pylori* and GC positive).

Group 2: 40 people infected with *H. pylori* who did not have any cancer or diseases (*H. pylori* positive and GC negative).

Group 3: 40 people who were neither infected with *H. pylori* nor had GC (*H. pylori* and GC negative).

All specimens and consents were collected. All samples were divided into two groups. The first part was a biopsy sample and sent to the microbiology department to confirm the presence of bacteria. The second part was immediately placed in liquid nitrogen and used for RNA extraction, cDNA generation, and qRT-PCR technique to determine the expression of the related microRNAs genes.

2. RNA extraction and qRT-PCR

Total RNA was isolated from the samples using RNX-Plus solutions (Cinnagen, Tehran, Iran) according to the instructions of the manufacturer. The extracted RNA was quantitatively and qualitatively evaluated by a nano-drop spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, USA) and 0.8% agarose gel. cDNA was prepared by a First Aid Reverse Transcription Kit (Fermentas). Real-time PCR was performed by SYBR Green master mix (Amplicon, Denmark) in the LightCycler96 instruments (Roche Life Science Deutschland GmbH, Sandhofer, Germany). Primer sequences were designed and synthesized by Takapou Zist Company. The primer sequences are shown in Table 1: The differences in gene expression in comparison with the reference gene was determined by the $2^{-\triangle\triangle Ct}$ formula.[12,13]

TABLE 1. Primer sequences of the genes

Genes	Primer type	Primer sequence
miR-146a	RT	5'-AACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGATACATACTTC-3'
	F	5'-TCCATGGGTTGTGTCAGTGT-3'
	R	5'-GACTCTGCCTTCTGTCTCCA-3'
miR-155	RT	F:5'-GTCGTATCCAGTGCGTGTTCCGGATCTTAGAATTGCACTGGATACGACACCCCTA-3'
	F	5'-TCATCCTCTGAGTGCTGAAGG-3'
	R	5'-CATGTGAATGCTAGTAACAGGC-3'
RnU6	RT	5'-AACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGTGCAGCACTA-3'
	F	5'-TCCAATTTTAGTATATGTGC-3'
	R	5'-CGATACAGAGAAGATTAGC-3'

3. Data analysis

Statistical analysis was carried out using the Statistical Package for Social Sciences version 16 (SPSS Inc., Chicago, IL). To compare the expression ratio of these genes in two groups, the distribution of data using the Kolmogorov-Smirnov test was examined according to the proportions of the results of parametric and non-parametric tests (Student t-tests and Mann Whitney). In this study, the significance level p was less than 0.05. The Pearson and Spearman tests were used to examine the relationship between variables in terms of parametric and non-parametric proportions. Receiver operating characteristic (ROC) curves were drawn for the evaluation of the possibility of miRNAs for GC detection.

RESULTS

1. Characteristics of the participants

Properties of the patients are illustrated in Table 2. All study participants were Iranian, and the average age was 51 years (45 to 65). In the *H. pylori* and GC positive group, 19 (47.5%) were male patients, and 21 (52.5%) were female patients. In the *H. pylori* positive and GC negative group, 20 (50%) were male patients, and 20 (50%) were female patients. In *H. pylori* and GC negative group, 28 (70%) were male, and 12 (30%) were female. Morevoer, in TNM staging, patients with low stage (I and II) were 12 (30%) and with high stage were 28 (70%). Furthermore, patients with grade I, grade II, grade III, and grade IV were 2 (5%), 6 (15%), 26 (65%), and 6 (15%), respectively.

2. Quantitative RT-PCR analysis

1) miR-146a expression: In this study, we evaluated miR-146a expression in the blood samples of the *H. pylori* and GC positive group (n=40) and the *H. pylori* positive and GC negative group (n=40) and observed that the miR-146a RNA levels non-significantly increased in the *H. pylori* and GC positive group compared to the *H. pylori* positive and GC negative group (p=0.094, Fig. 1A).

We measured miR-146a expression in the 40 blood samples of the *H. pylori* and GC positive group (n=40) and the *H. pylori* and GC negative group (n=40) and observed that the miR-146a RNA levels significantly increased in the *H. pylori* and GC positive group compared to the *H. pylori* and GC negative group (p=0.004, Fig. 1B).

Also, we analyzed miR-146a expression in the 40 blood samples of *H. pylori* positive and GC negative group (n=40) and *H. pylori* and GC negative group (n=40), then we ob-

TABLE 2. The demographic characteristics of participants

Characteristic	Categorization	N (%)
Age, years		
H. pylori and GC positive group	≥51	23 (57.5)
	<51	17 (42.5)
H. pylori positive and GC negative group	≥51	21 (52.5)
	<51	19 (47.5)
H. pylori and GC negative group	≥51	20 (50)
	<51	20 (50)
Gender		
H. pylori and GC positive group	Female	21 (52.5)
	Male	19 (47.5)
H. pylori positive and GC negative group	Female	20 (50)
	Male	20 (50)
H. pylori and GC negative group	Female	12 (30)
	Male	28 (70)
TNM stage		
H. pylori and GC positive group	Low (I, II)	12 (30)
	High (III, IV)	28 (70)
Grade		
H. pylori and GC positive group	Grade I	2 (5)
	Grade II	6 (15)
	Grade III	26 (65)
	Grade IV	6 (15)

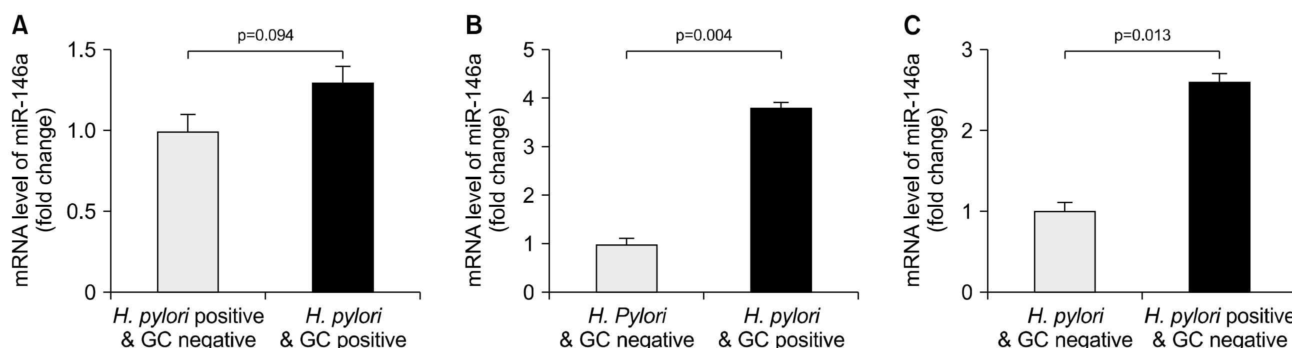

FIG. 1. The level of mRNA folding change miR-146a gene in (A) *H. pylori* and GC positive group and *H. pylori* positive and GC negative group, (B) *H. pylori* and GC positive group and *H.pylori* and GC negative group, (C) *H. pylori* positive and GC negative group and *H. pylori* and GC negative group. $p<0.05$ is considered for mRNA folding change. As shown in the figure, (A) the level of mRNA folding change non-significantly increased in *H. pylori* and GC positive group compared to *H. pylori* positive and GC negative group, (B) the level of mRNA folding change significantly increased in *H. pylori* and GC positive group compared to *H. pylori* and GC negative group, and (C) the level of mRNA folding change increased in *H. pylori* positive and GC negative group compared to *H. pylori* and GC negative group.

served that the miR-146a RNA levels significantly increased in the *H. pylori* positive and GC negative group compared to the *H. pylori* and GC negative group (p=0.013, Fig. 1C).

2) miR-155 expression: In addition, we evaluated miR-155 expression in the blood samples of the *H. pylori* and GC positive group (n=40) and the *H. pylori* positive and GC negative group (n=40) and observed that the miR-155 RNA levels non-significantly increased in the *H. pylori* and GC positive group compared to the *H. pylori* positive and GC negative group (p=0.132, Fig. 2A).

We measured miR-155 expression in 40 blood samples of the *H. pylori* and GC positive group (n=40) and the *H. pylori* and GC negative group (n=40) and observed that the miR-155 RNA levels significantly increased in the *H. pylori* and GC positive group compared to the *H. pylori* and GC negative group (p=0.033, Fig. 2B).

Also, we analyzed miR-155 expression in the 40 blood samples of the *H. pylori* positive and GC negative group (n=40) and *H. pylori* and GC negative group (n=40), then we observed that the miR-155 RNA levels significantly increased in the *H. pylori* positive and GC negative group compared to the *H. pylori* and GC negative group (p=0.036, Fig. 2C).

3. ROC curve analysis

ROC curve analysis was performed to find out whether the expression levels of miR-146a and miR-155 might be considered as potential tumor markers for GC. The area under curve (AUC) of ROC analysis for miR-146a as plotted for the *H. pylori* and GC positive group and the *H. pylori* positive and GC negative group was obtained as (0.6753 [95% CI, 0.5552 to 0.7955]), the *H. pylori* and GC positive group and the *H. pylori* and GC negative group was obtained as (0.8703 [95% CI, 0.7917 to 0.9489]), the *H. pylori* positive and GC negative group and the *H. pylori* and GC negative group was obtained as (0.7756 [95% CI, 0.6752 to 0.8761]) as shown in Fig. 3A-C. Similarly, the AUC of miR-155 as plotted for the *H. pylori* and GC positive group and the *H. pylori* positive and GC negative group was obtained as (0.6466 [95% CI, 0.5245 to 0.7686]), the *H. pylori* and GC positive group and the *H. pylori* and GC negative group was obtained as (0.6984 [95% CI, 0.5834 to 0.8135]), the *H. pylori* positive and GC negative group and the *H. pylori* and GC negative group was obtained as (0.8203 [95% CI, 0.7270 to 0.9137]) as shown in Fig. 3D-F.

DISCUSSION

There is no denying that GC is one of the most dangerous and fatal cancers in the world, which is ranked fifth in terms the deadliest among all types of malignancies. Different cognitive ways have been expressed to diagnose GC at early stages. However, extensive studies are required to identify and cure this malignancy.[12]

miRNAs are non-coding RNAs which have 19 to 25 nucleotides that play pivotal tasks in modulating gene expression. Most miRNAs are transcribed from DNA sequences into primary miRNAs and precursor miRNAs and finally processed into mature miRNAs.[14] miRNAs interact with the 3' untranslated regions (3' UTR) of mRNAs to activate mRNA degradation and translational suppression. However, miRNA interaction with other sequences, including gene promoters, the 5' UTR, and coding sequence have also been identified. miRNAs may also induce translation or transcription.[14] miRNA interaction with their target genes and mRNAs is dynamic and dependent on many agents, for example, the affinity of miRNA-mRNA interactions, the cellular location of miRNAs, and the abundance of miRNAs and target genes. Moreover, miRNAs have been proven to be involved in the pathogenesis of many diseases, especially neoplasm.[14] As previously mentioned, less than thousands of miRNAs mechanisms have been discovered. Recently, two miRNAs have been identi-

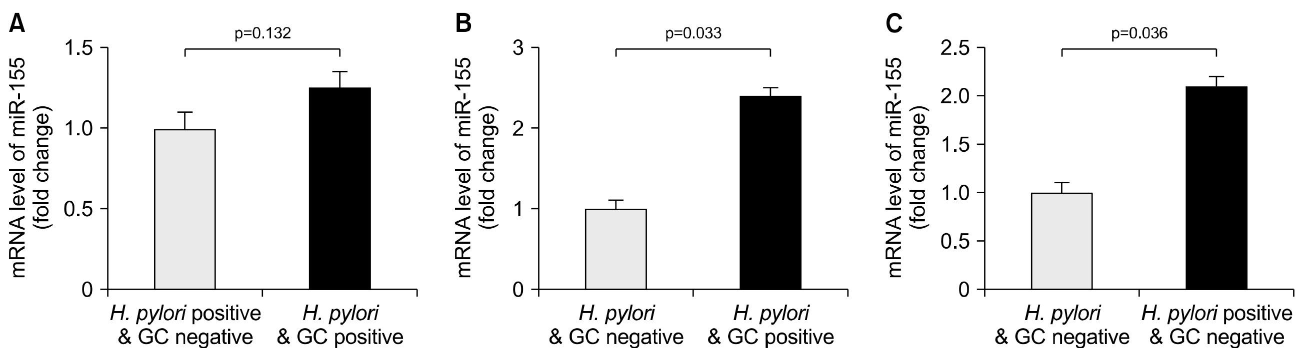

FIG. 2. The level of mRNA folding change miR-155 gene in (A) *H. pylori* and GC positive group and *H. pylori* positive and GC negative group, (B) *H. pylori* and GC positive group and *H. pylori* and GC negative group, (C) *H. pylori* positive and GC negative group and *H. pylori* and GC negative group. $p < 0.05$ is considered for mRNA folding change. As shown in this figure, (A) the level of mRNA folding change non-significantly increased in *H. pylori* and GC positive group compared to *H. pylori* positive and GC negative group, (B) the level of mRNA folding change significantly increased in *H. pylori* and GC positive group compared to *H. pylori* and GC negative group, and (C) the level of mRNA folding change increased in *H. pylori* positive and GC negative group compared to *H. pylori* and GC negative group.

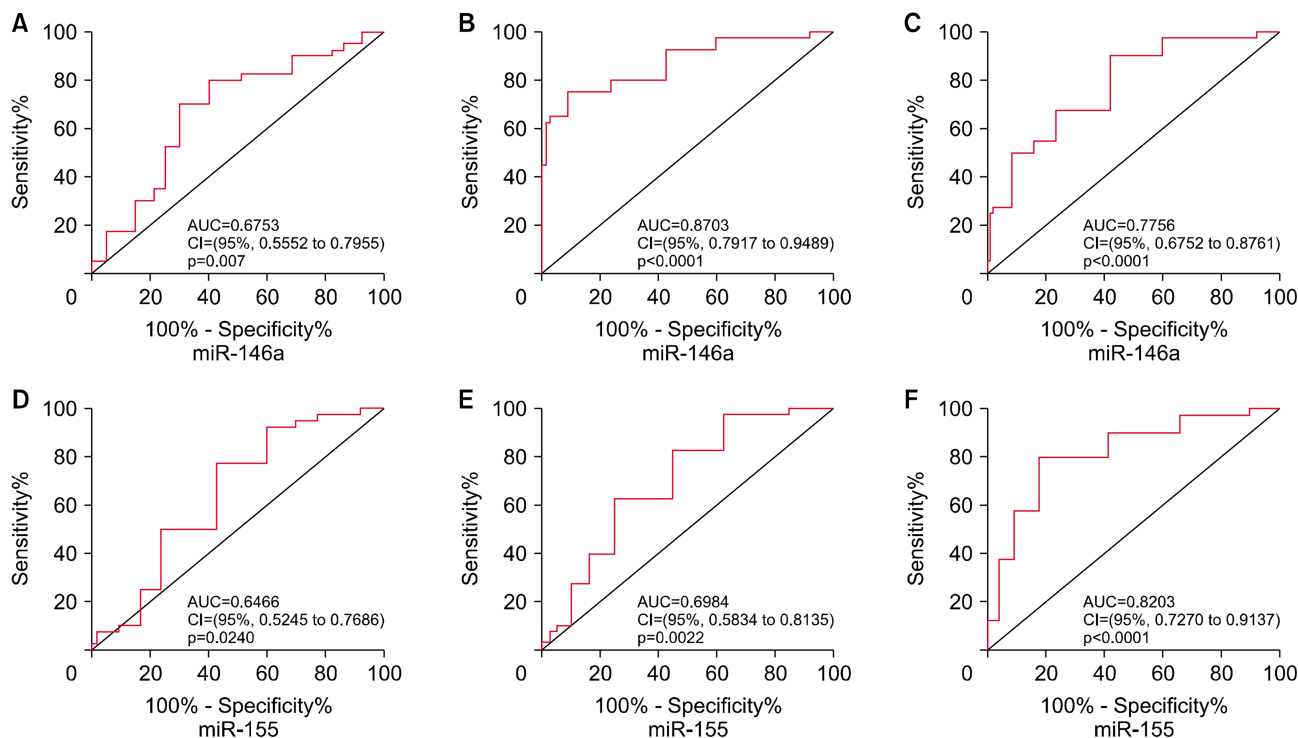

FIG. 3. ROC of miR-146a and miR-155 RNA levels for GC detection in different states. (A) ROC of miR-146a RNA levels for detection between *H. pylori* and GC positive group vs. *H. pylori* positive and GC negative group. (B) ROC of miR-146a RNA levels for detection between *H. pylori* and GC positive group vs. *H. pylori* and GC negative group. (C) ROC of miR-146a RNA levels for detection between *H. pylori* positive and GC negative group vs. *H. pylori* and GC negative group. (D) ROC of miR-155 RNA levels for detection between *H. pylori* and GC positive group vs. *H. pylori* positive and GC negative group. (E) ROC of miR-155 RNA levels for detection between *H. pylori* and GC positive group vs. *H. pylori* and GC negative group. (F) ROC of miR-155 RNA levels for detection between *H. pylori* positive and GC negative group vs. *H. pylori* and GC negative group.

fied, including miR-146a and miR-155 which have been subjected to limited studies on their action mechanism in *H. pylori* infection and cancer, especially GC.

The miR-146a gene is located on human chromosomes 5, 10, and 19. Many miRNAs, like miR-146, although having the same sequence, are transcripted by several positions or locus in the human genome.[15] miR-146a is an abundantly expressed miRNA in different mammalian cells. miR-146a has several studies on its involvement in differentiation, inflammation, and function of adaptive and innate immune cells.[15] The expression of miR-146a is activated upon stimulation of T-cell receptor and, in T lymphocyte cells for triggering this miRNA transcription, the binding sites of NF-κB are needed. Furthermore, the role of miR-146a as an oncomir in various cancers has been investigated.[15] Considering the multiple functions of miR-146a and its various target genes, pro-apoptotic target genes of miR-146a are PTEN, RECK, TPM1, and pdcd4, which are down-regulated by this miRNA, on the other hand, the anti-apoptotic target gene of miR-146a is Bcl2 which is up-regulated via this miRNA.[9,15]

The ROC curve findings showed a relatively appropriate specificity and sensitivity for the miR-146a RNA level in discriminating in different states including, the *H. pylori* and GC positive group vs. the *H. pylori* positive and GC negative group, the *H. pylori* and GC positive group vs. the *H. pylori* and GC negative group, the *H. pylori* positive and GC negative group vs. the *H. pylori* and GC negative group, indicating that the miR-146a expression level may be used for diagnosis goals (Fig. 3A-C).

On the other hand, one of the most important miRNAs that play a critical role in the cell is miR-155. According to the studies, miR-155 was the first miRNA to be identified as up-regulated in tumors. We will discuss miR-155 in cancer.[16] BIC (also known as MIR155HG) is the non-coding RNA host gene of miR-155, which was discovered before miR-155 itself. Up-regulation of BIC coordinated with Myc over-expression in the onset of avian lymphoma.[16] It is hypothesized to perform as a non-coding RNA because of the absence of long, conserved open reading frames and extensive structure. Some studies have proven that miR-155 is processed from BIC, hence BIC over-expression results in miR-155 up-regulation.[16] One common mechanism of oncogenesis by miR-155 is induction by viral gene products in Epstein Barr virus (EBV) infected cells. Also, tumors may promote by miR-155 when it is up-regulated pending inflammation or in response to other TNF-alpha activity.[16]

Previous studies have shown that the dysregulation of some tumor suppressors in driving tumor production as an important target of miR-155. Some of the most pivotal pro-

tein targets for miR-155 are PU.1, MLH1, MSH6, and FOXO3a. miR-155 inhibits DNA stability by interacting with MLH1, MSH6, and FOXO3a. In addition, miR-155 interacts with several other proteins, such as PU.1 to block differentiation, and through these interactions, miR-155 can play oncogenic roles and cause tumor growth and development.[16]

Furthermore, The ROC curve results showed a relatively suitable specificity and sensitivity for miR-155 RNA levels in discriminating different states including, the *H. pylori* and GC positive group vs. the *H. pylori* positive and GC negative group, the *H. pylori* and GC positive group vs. the *H. pylori* and GC negative group, *H. pylori* positive and GC negative group vs. *H. pylori* and GC negative group, indicating that the miR-146a expression level may be used for diagnosis goals (Fig. 3D-F).

In this study, to evaluate the expression of miR-146a and miR-155 genes, subjects were divided into the following groups, group 1: *H. pylori* and GC positive, group 2: *H. pylori* positive and GC negative, and group 3: *H. pylori* and GC negative.

According to Figs. 1A and 2A and the results of miR-146a and miR-155 genes expression in the *H. pylori* and GC positive group, and the *H. pylori* positive and GC negative group, it was found that the expression of miR-146a and miR-155 genes increased in the first group compared to the second group (respectively p=0.094 and p=0.132), but the increased expression of genes was not significant.

On the other hand, based on the results and Figs. 1B and 2B, miR-146a and miR-155 gene expression significantly increased in the first group compared to the third group (respectively p=0.004 and p=0.033).

In addition, based on the findings and Figs. 1C and 2C, our results showed that miR-146a and miR-155 gene expression are considerably increased in the *H. pylori* positive and GC negative group compared to the *H. pylori* and GC negative (respectively p=0.013 and p=0.036).

Based on the results of our study, it is suggested that miR-146a plays a vital role in the development of cancer cells and tumorigenesis, and inhibition of miR-146a can be a useful method for cancer treatment, especially GC.

In 2015, Gerloff and colleagues conducted a study on patients with Acute Myeloid Leukemia (AML). In vitro and in vivo, they showed that NF-κB, STAT5, and miR-155 are related to each other. In this research, they proved that the FLT3-ITD signalling pathway induces the expression of miR-155. Therefore, according to the findings of their study, it was found that the FLT3-ITD signalling route, by inducing NF-κB and STAT5, can increase the expression of the miR-155 gene, which ultimately leads to cancer cell proliferation and tumor progression.[17]

According to our results, it was found that in the *H. pylori* and GC positive group, and the *H. pylori* positive and GC negative group, the expression of miR-146a and miR-155 genes increased insignificantly. However, in the groups of *H. pylori* and GC positive, and *H. pylori* positive and GC negative in comparison to *H. pylori* and GC negative, the expression of miR-146a and miR-155 genes were significantly over-expressed. Based on the findings, this increased expression of the genes may be caused by the *H. pylori* infection. However, the results of gene expression in patients with GC showed that miR-146a and miR-155 can cause tumor growth and cancer progression, which requires further studies to prove this hypothesis. Furthermore, our results showed that in patients with *H. pylori*, the over-expression of the miR-146a and miR-155 genes could be due to infection with this bacterium, which might activate signalling pathways related to miR-146a and miR-155. Over-expression of miR-146a and miR-155 genes will eventually affect the tumor cells and cause GC progression.

DISCUSSION

According to the results, patients with *H. pylori* and GC (group 1) have considerably greater levels of miR-146a and miR-155 expression in their whole blood compared to healthy subjects (group 3), and the increased expression of the genes is not significantly higher than those of *H. pylori* positive but GC negative patients (group 2). To sum up, upregulation of miR-146a and miR-155 in these patients may influence some downstream tumor suppressor genes and knock them down. According to the theory, *H. pylori* infection might launch the expression of these microRNAs and affect the pathogenesis of GC. For this reason, it seems that the analysis of miR-146a and miR-155 along with *H. pylori* infection could be a useful and helpful biomarker to predict, diagnose and ultimately cure patients. However, further studies are warranted to unravel the precise association between miR-146a, miR-155 and *H. pylori* infection with GC to pave the way.

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CONFLICT OF INTEREST STATEMENT

None declared.

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