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Expression Analysis of MicroRNAs, miR-20a, miR-30a, miR-210, and miR-874 in *Helicobacter pylori*-infected patients with or without Gastric Cancer

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ABSTRACT

Introduction. *Helicobacter pylori* infection is one of the primary etiological factors associated with gastric carcinogenesis. This study investigated the expression of microRNAs, miR-20a, miR-30a, miR-210, and miR-874, in *H. pylori*-infected patients with and without gastric cancer (GC) compared to healthy subjects. **Methods.** Total RNA was extracted from the plasma of *H. pylori*-infected GC patients (n=40), *H. pylori*-infected patients (n=40), and healthy individuals (n=12). The expression of microRNAs was analyzed using a reverse transcription-quantitative real-time PCR (RT-qPCR) technique. **Results.** The miR-20a, miR-30a, miR-210, and miR-874 exhibited higher overexpression in *H. pylori*-infected GC patients than healthy persons (P=0.004, 0.033, 0.023, 0.024). The *H. pylori*-positive patients without GC also had higher miR-20a, miR-30a, and miR-210 levels than the healthy individuals (P= 0.013, 0.036, 0.032). There were no statistical differences between *H. pylori*-infected GC patients and *H. pylori*-infected patients without GC **Conclusion.** The microRNAs overexpression in *H. pylori*-infected patients with GC might be linked to *H. pylori* rather than GC. Therefore, these microRNAs can be helpful in *H. pylori* infection diagnosis rather than predicting GC. in *H. pylori*-infected patients.

INTRODUCTION

Gastric cancer (GC) is one of the most severe malignancies that leads to cancer-related deaths worldwide [1]. Approximately 1%-3% of *Helicobacter pylori*-infected individuals develop GC with a 5-year survival rate. In other words, *H. pylori*-infected individuals have an increased risk of GC tumorigenesis compared to uninfected individuals [2]. Therefore, *H. pylori* might be one of the primary etiological factors associated with multifocal chronic gastritis that induces gastric carcinogenesis [3]. *H. pylori* are Gram-negative, microaerophilic organisms with a lipopolysaccharide cell wall responsible for mucosal integrity disorder [4]. These bacteria release several harmful factors associated with an increased risk of severe gastritis, ulcer disease, and GC [5]. For instance, CagA (cytotoxin-associated gene A) can affect epithelial cells by several mechanisms and contribute to GC development [6].

MicroRNAs (miRNAs) are endogenous, non-coding RNAs (18 to 24 nucleotides) that regulate genes expression through various biological processes [7]. The miRNAs expression is altered in numerous human diseases, such as cancer, indicating their potential roles in tumorigenesis [8]. They also play essential roles in host-parasite interaction and the host's innate immune response. Furthermore, *H. pylori* can directly affect the host's miRNAs expression and induce epigenetic deregulation of oncogenes and tumor suppressor genes [9-11].

Previous studies evaluated the expression profiles of different miRNAs in GC patients and tried to introduce them as diagnostic biomarkers [12]. The miRNAs, including mir-20a [13], miR 30-a [14, 15], miR-210 [16, 17], were studied before and were shown to have altered expressions in cancer patients. The mir-874 is a newly

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identified miRNA that inhibits cell proliferation and induces apoptosis in GC cells [18].

Considering the regulatory function of miRNAs in *H. pylori* infection and *H. pylori*-related GC, in the current study, we compared the plasma levels of miR-20a, miR-30a, miR-210, and miR-874 among *H. pylori* patients with GC, *H. pylori* patients without GC, and healthy individuals using a reverse transcription-quantitative real-time PCR (RT-qPCR). The selected miRNAs are a collection of old and new ones that may provide a prognostic package for individuals infected with *H. pylori*.

MATERIALS AND METHODS

Patients' recruitment. Gastric cancer patients infected with *H. pylori* admitted to a referral hospital in Tehran, Iran, were enrolled in the study. The *H. pylori*-infected GC patients comprised 50 cases, from which 40 patients had received no treatments in any manner and therefore considered new cases. These individuals were defined as the *H. pylori*$^{+}$/ GC^{+} group. Also, 40 *H. pylori*-infected patients without any cancer were included in the study as the *H. pylori*$^{+}$/G.C.$^{-}$ group. Among the participants, 12 individuals with neither *H. pylori* infection nor GC were invited to participate in the study as controls. The absence of *H. pylori* infection was assessed by serological tests using an enzyme-linked immunosorbent assay kit (Pishtaz Teb Co., Iran). The hospital doctors did confirm both GC and *H. pylori* infection. Nevertheless, they did not provide us with the patients' clinical and pathological data.

Three ml of peripheral venous blood sample were collected from all participants into an EDTA-containing tube; then, the plasma was separated by centrifugation at 3,000 rpm, 4 °C for 10 min. The plasma samples were aliquoted and kept at −80 °C until tested.

The study was approved by the Islamic Azad University, Tehran Medical Branch Committee of Biomedical Research. Written informed consent was obtained from each participant after fully explaining the purpose and nature of all procedures used.

Table 1. The primers used for qPCR measurements*

miRNA	Primer	Sequence
miR-20a-5p	Stem-loop RT primer	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCTACCT
	Forward primer	CGCAGTAAAGTGCTTATAGTG
miR-30a-5p	Stem-loop RT primer	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACCTTCCAG
	Forward primer	GCAGTGTAAACATCCTCGAC
miR-210-3p	Stem-loop RT primer	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCAGCCG
	Forward primer	GGTCCTGTGCGTGTGACAG
miR-874-3p	Stem-loop RT primer	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCGGTC
	Forward primer	ATTACTGCCCTGGCCCGA
Universal Reverse Primer for qPCR		CCAGTGCAGGGTCCGAGGTA

* The authors designed the primers

RNA Extraction. According to the manufacturer's protocol, total RNA was extracted from 200 µL of thawed plasma using Trizol reagent (Invitrogen, Carlsbad, CA, USA), then RNA concentration was determined using the NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Waltham, and Mass).

RT-qPCR. According to the manufacturer's protocol, total RNA (1 µg) was reverse transcribed using the microRNA cDNA synthesis kit (PrimeScript RT Reagent Kit, Takara, Japan) and stored at -20 °C for later analysis. Quantitative real-time PCR was performed in 25 µL volumes using SYBR Green Premix Kit (Takara, Japan) in a Roche LightCycler instrument (Roche, Germany) with an amplification program described by others [19], and the primers designed in this study (Table 1). Relative quantitation of miRNAs' expression was done using the comparative cycling threshold (Ct) method. U6 snRNA was used as an internal control to normalize miRNAs expression levels.

Statistical Analysis. The expression of miRNAs was calculated using the $2^{-\Delta\Delta Ct}$ method [20]. The differences among groups were analyzed using the one-way analysis of variance and the Tukey post hoc test. Data were analyzed using SPSS 20 (Chicago, IL, USA). $P < 0.05$ was considered as a cut-off for statistical significance.

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Table 2. Demographic characteristics of the participants in the study.

Parameter	*H. pylori* $^{+}$/GC $^{+}$	*H. pylori* $^{+}$/ GC $^{-}$	*H. pylori* $^{-}$/ GC $^{-}$ *
Patient number	40	40	12
Gender			
Male	22	22	6
Female	18	18	6
Age (mean±SD)	52.35±5.2	47.23±4.2	50.25±3.22

GC; gastric cancer; *, Control

RESULTS

Demographic characteristics. Table 2 shows the demographic characteristics of participants. Of 40 *H. pylori*$^{+}$/GC^{+} patients, 22 (55%) were male, and 18 (45%) were female. The number of males and females in the *H. pylori*$^{+}$/GC^{-} group was the same as *pylori*$^{+}$/GC^{+}. The number of males and females in the healthy group was equal, i.e., six for each gender. The mean age of the *H. pylori*$^{+}$/GC^{+} group was significantly different from the *H. pylori*$^{+}$/G.C.$^{-}$ group (P<0.0001) but not significantly different from the healthy group (P=0.1929). The difference in mean age between *H. pylori*$^{+}$/GC^{-} and healthy individuals (*H. pylori*$^{-}$/GC^{-}) was also significant (P=0.0262).

MiRNAs expression. The expression of microRNAs, miR-20a, miR-30a, miR-210, and miR-874, in *H. pylori*$^{+}$/GC^{+} group, compared to the *H. pylori*$^{+}$/GC^{-} group and healthy individuals (*H. pylori*$^{-}$/GC^{-}) are presented in Fig. 1 and Table 3.

Table 3. MiRNAs expression levels in three groups

miRNA	Group 1 *H. pylori*$^{+}$ / GC^{+}	Group 2 *H. pylori*$^{+}$/ GC^{-}	Group 3* *H. pylori* $^{-}$/ GC^{-}	ANOVA P-Value	Tukey HSD	P-Value
miR-20a	3.644±0.417	2.669±0.393	0.976±0.309	0.030	Group 1 vs. Group 2	0.094
					Group 1 vs. Group 3	0.004
					Group 2 vs. Group 3	0.013
miR-30a	2.148±0.479	1.846±0.361	0.976±0.309	0.007	Group 1 vs. Group 2	0.132
					Group 1 vs. Group 3	0.033
					Group 2 vs. Group 3	0.036
miR-210	2.238±0.479	1.756±0.361	0.985±0.309	0.001	Group 1 vs. Group 2	0.114
					Group 1 vs. Group 3	0.023
					Group 2 vs. Group 3	0.032
miR-874	1.647±0.316	0.661±0.037	0.271±0.017	0.027	Group 1 vs. Group 2	0.066
					Group 1 vs. Group 3	0.024
					Group 2 vs. Group 3	0.724

GC; gastric cancer, *, Control

Our study showed that all four miRNAs were significantly overexpressed in *H. pylori*$^{+}$/GC^{+} and *H. pylori*$^{+}$/GC^{-} groups than in healthy individuals (*H. pylori*-/GC-). However, no statistical difference was observed between *H. pylori*$^{+}$/GC^{+} and *H. pylori*$^{+}$/GC^{-} groups.

DISCUSSION

In the present study, the plasma levels of miR-20a, miR-30a, miR-210, and miR-874 were compared in three groups consisting of 40 *H. pylori*$^{+}$/GC^{+} patients, 40 *H. pylori*$^{+}$/GC^{-} patients, and 12 healthy individuals. The results showed that the expression of all four miRNAs increased significantly among *H. pylori*$^{+}$/GC^{+} and H. *pylori*$^{+}$/GC^{-} patients than healthy subjects, whereas the levels of these miRNAs exhibited no significant difference between *H. pylori*$^{+}$/GC^{+} and H. *pylori*$^{+}$/GC^{-} groups.

Several studies have reported alterations in microRNAs expression in GC patients; however, data on the four miRNAs including miR-20a, miR-30a, miR-210, and miR-874 in *H. pylori*-associated GC in comparison to only *H. pylori*-positive individuals and healthy persons is limited. The miR-20a is a member of the miR-17-92 cluster [21], commonly upregulated in cancer such as non-small cell lung cancer [22], hepatoma [23], colorectal [24], and prostate cancers [25].

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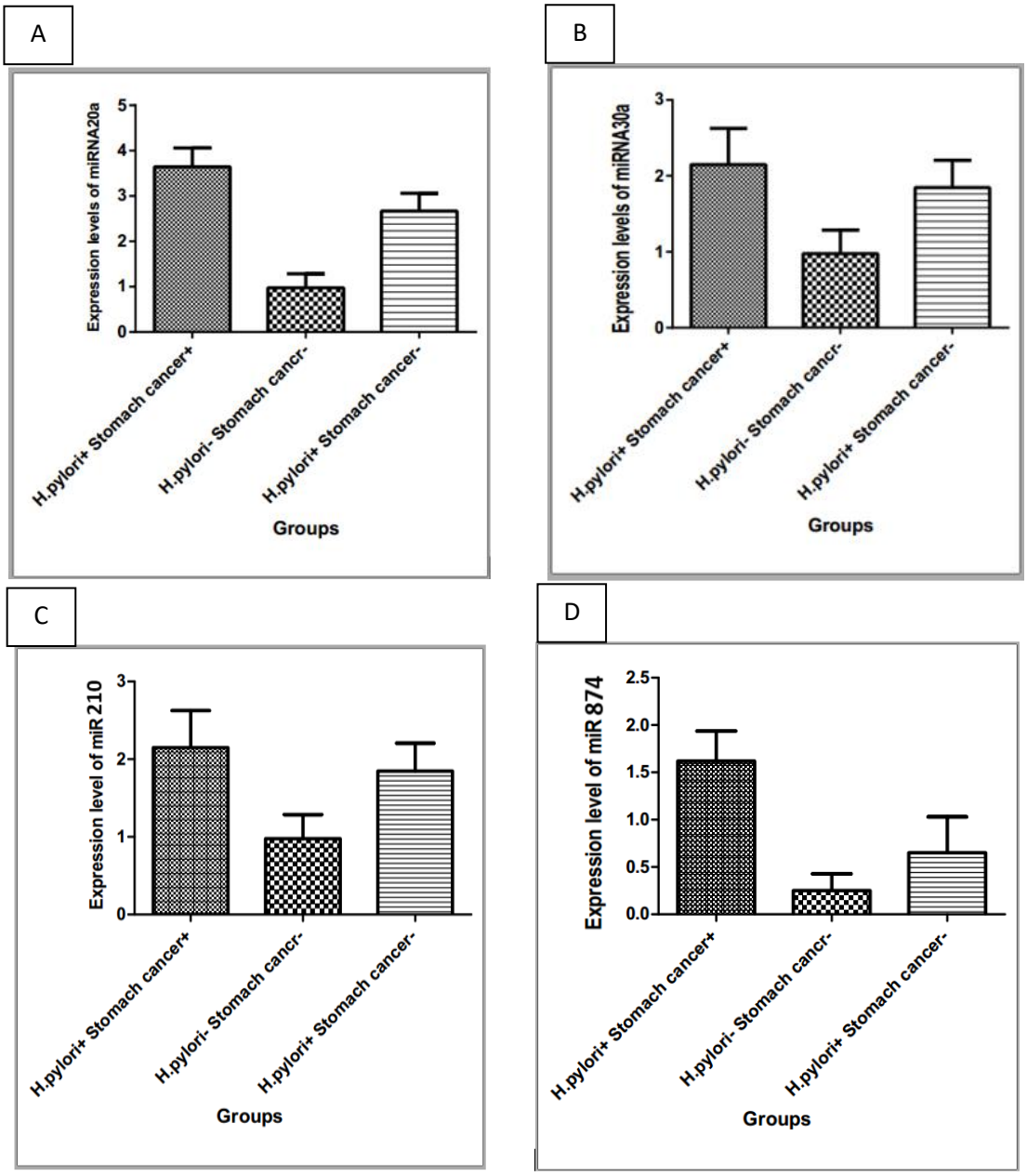


Fig. 1. Plasma levels of miR-20a (A), miR-30a (B), miR-210 (C), and miR- 874 (D) in studied groups (Details are available in Table 3)

Recently, Yang *et al.* [26] reported that miR-20a was downregulated following surgery in patients with GC and found that serum miR-20a level correlated with age and pathological characteristics of GC patients. They concluded that miR-20a could be a promising serum biomarker for GC diagnosis, evaluating therapeutic efficacy and prognosis [26]. In the present study, the expression level of circulating miR-20a was significantly higher in patients within *H. pylori*$^{+}$*/GC*$^{+}$ and *H. pylori*$^{+}$/GC^{-} groups than in healthy individuals. Since there was an upregulated level of miR-20a in both *H. pylori*$^{+}$/GC^{+} and *H. pylori*$^{+}$/GC^{-} patients, an increased miR-20a level may be a warning sign of *H. pylori* infection.

High miR-30a expression, together with let-7a and miR-126, has been reported as the protective miRNAs signature of GC patients [27]. Liu *et al.* [28] showed that miR-30a targeted COX-2 and BCL9 in *H. pylori*$^{+}$ GC cell lines, resulting in cell proliferation and metastasis control. They found that alterations of miR-30a level lead to aberrant expression of target mRNAs of miR-30a, which correlated with GC progression [28]. In our study, elevated levels of miR-30a were significantly higher in both *H. pylori*$^{+}$/GC^{+} and *H. pylori*$^{+}$/GC^{-} patients.

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This finding suggests that this microRNA may be associated with *H. pylori* infection rather than cancer.

The miR-210 is a crucial controller in gastric cell growth and directly targets potential oncogenes such as STMN1 and DIMT1 [29]. Kiga *et al.* [29] showed that decreased miR-210 in *H. pylori*$^{+}$ gastric mucosa was associated with STMN1 and DIMT1 upregulation, possibly leading to a molecular mechanism initiating chronic gastrointestinal diseases like GC during *H. pylori* infection [29]. However, the current study results showed upregulations of miR-210 in both *H. pylori*$^{+}$/GC^{+} and *H. pylori*$^{+}$/GC^{-} patients. Our findings are compatible with those reported by Yu *et al.* [30], who demonstrated that miR-210 was often highly overexpressed in GC patients and was associated with *H. pylori* infection [30].

MiR-874 is a newly identified miRNA that is often downregulated in certain types of cancer, including GC [31]. Jiang *et al.* [32] showed that ectopic miR-874 expression suppressed the growth, migration, invasion, and tumorigenicity of GC cells, whereas miR-874 knockdown promoted these phenotypes [32]. Sun *et al.* [18] confirmed the reduced miR-874 expression in GC tissues and cells. They demonstrated that miR-874 overexpression suppressed GC cell proliferation and promoted apoptosis [18]. Contrary to these findings, our results showed miR-874 upregulation in *H. pylori*$^{+}$ patients with or without GC. The expression of this molecule may be associated with infection, not cancer. However, more investigations are required to determine the role of mir-874 and its downstream mediators in developing and GC progression in patients with *H. pylori* infection. The expression of miRNAs levels in different sample types should be interpreted with caution.

In conclusion, our results indicated the upregulation of miR-20a, miR-30a, miR-210, and miR-874 in *H. pylori*$^{+}$ patients with or without GC compared to healthy controls. The increased levels of these miRNAs are linked to *H. pylori* infection rather than GC. Therefore, these molecules have the diagnostic biomarker potential for *H. pylori* infection and cannot predict GC in *H. pylori*-infected patients. Confirmation of these findings requires further studies.

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CONFLICT OF INTEREST

Saba Hariri Monfared and Mina Yaghoub Kazemi equally contributed to the study as the first authors. The authors declare no conflicts of interest associated with this manuscript.

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