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FULL PAPER

Helicobacter pylori-miRNA interaction in gastric cancer tissues: First prospective study from Turkey

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SUMMARY

Helicobacter pylori (*H. pylori*) is involved in the etiology of gastric cancer (GC). miRNAs are short RNAs that regulate gene expression by marking mRNAs for degradation. miRNAs are involved in tumorigenesis, metastasis, and cell proliferation. We aimed to investigate the miRNA expression profiles of tissues from *H. pylori* (+) and (-) GC patients. Forty GC patients, 20 *H. pylori* (+) and 20 *H. pylori* (-), and a healthy control group were included. The miRNA expression levels were investigated by microarrays and quantitative RT-PCR. We detected 9 upregulated and 4 downregulated miRNAs by microarray. We selected 5 upregulated and 5 downregulated miRNAs for the quantitative RT-PCR assay. The relative fold changes of miRNAs in the cancerous tissue and non-tumor mucosa specimens of *H. pylori* (+) GC patients for hsa-miR-194 were 4.24- and 3.83-fold higher, respectively, whereas the hsa-miR-145 expression levels were downregulated 0.33-fold and 0.43-fold, respectively, in the same group. The presence of *H. pylori* significantly upregulated hsa-miR-194 and downregulated hsa-miR-145 expression levels in *H. pylori* (+) GC cases, compared to *H. pylori* (-) GC cases. Regional differences in the virulence of *H. pylori* strains may also be involved in the up- or downregulation of miRNA expression levels.

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INTRODUCTION

Gastric cancer (GC) is amongst the most common cancers and is still a significant public health issue, despite the decreased global incidence of GC in recent years. GC is considered the fifth-most common malignancy and the third leading cause of cancer-related deaths worldwide (Ferlay *et al.*, 2015). One of the spiral-shaped human gastrointestinal pathogens, *Helicobacter pylori* (*H. pylori*), has been confirmed as the microorganism that is responsible for the pathogenesis of peptic ulcer disease, chronic gastritis, gastric mucosa-associated lymphoid tissue lymphoma, and GC (Zhou *et al.*, 2016). After the discovery of *H. pylori*'s role in gastric diseases and malignancies through many clinical studies, *H. pylori* infection is considered the inducer of GC. As a common consensus, the prevalence of *H. pylori* infections is significantly higher in GC patients than in normal control patients (Kato *et al*., 2004). On the other hand, Yoon *et al*. (2011) reported that at least 5.4% of GC cases in South Korean patients were *H. pylori* infection (-), though they used a very stringent definition of *H. pylori* infection status. Therefore, it is incorrect to focus on *H. pylori* as the sole etiological factor in GC pathogenesis. In recent years, molecular studies in this area have accelerated and promising biomarkers, such as microRNAs (miRNAs), have been extensively studied for the early diagnosis of and/or as therapeutic targets for cancer treatment (Wang *et al*., 2017).

miRNAs are short RNAs (approximately 17 to 24 nucleotides) that regulate gene expression by binding to their target messenger RNAs (mRNAs) within the 3'-untranslated region and marking those mRNAs for degradation (He and Hannon, 2004). miRNAs are involved in tumorigenesis, invasion, apoptosis, cell migration, metastasis, and cell proliferation (Zhu *et al*., 2014; Yu *et al*., 2015). There is growing interest in and convincing evidence for the role of miRNAs in nearly every tissue type and cellular process (Love and Dave, 2013). Aberrant miRNA expression has been linked to the development and progression of various types of human cancers, including GC (Zhang *et al*., 2018; Kang *et al*., 2018). miRNA expression patterns differ in human GC between cancerous tissue and non-cancerous adjacent tissue (Wang *et al*., 2017). Numerous reports suggest that miRNAs are effective biomarkers for the diagnosis and prognosis of various human cancers (Wilmott *et al*., 2011). Researchers believe that miRNAs may be prom-

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ising therapeutic targets for cancer treatment; specifically, since miRNAs were suggested to play a role in the pathogenesis of *H. pylori*-associated GC, they may also serve as therapeutic targets in GCs (Kashyap *et al*., 2018).

In this study, we aimed to investigate the miRNA expression profiles of tissues from *H. pylori* (+) and (-) GC patients and *H. pylori* (+) and (-) healthy controls by microarrays. We also investigated the miRNA profiles of tissues from patients with renal cell carcinomas. Our other aim was to further investigate 5 upregulated and 5 downregulated miRNAs that were detected in the tissues of *H. pylori* (+) GC patients by quantitative real-time PCR (qRT-PCR). We sought to understand the complex mechanisms behind the interaction between miRNAs and *H. pylori* infection in the development of GC cases in Turkey.

MATERIALS AND METHODS

Study groups and sample collection for miRNA expression profiling using microarrays and quantitative real-time PCR (qRT-PCR)

This study was conducted from January 2017 to January 2018. The study population was comprised of 40 GC patients, specifically 20 *H. pylori* (+) GC patients (13 males, 7 females, mean age 57.1 years and age range of 31-79) and 20 *H. pylori* (-) GC patients (11 males, 9 females, mean age 5.1 years and age range 38-73), a control group of 10 patients with renal carcinoma (7 males, 3 females, mean age 55.8 years and age range 43-63), a healthy control group of 10 individuals infected with *H. pylori* (6 males, 4 females, mean age 55.1 years and age range 49-62), and 10 individuals without *H. pylori* infection (6 males, 4 females, mean age 56.3 years and age range 49-63). For all groups, 120 biopsy specimens from the antrum and corpus of the stomach and lesions of the kidney and normal tissues (located >5 cm away from the cancerous tissue) were obtained from 70 study participants. We excluded patients who were under 18 years of age, had previous gastric surgery and *H. pylori* eradication treatment, or had a history of therapy with antibiotics, antisecretory drugs, bismuth salts, or sucralfate in the month prior to sampling. Clear cell renal cell carcinoma was identified by hematoxylin and eosin staining in kidney sections according to WHO criteria (Moch *et al.*, 2016). All samples were verified by pathological examination. The patients' demographic characteristics are shown in *Table 1*.

To determine the miRNA expression profiles in *H. pylori*-associated GC, we used a microarray chip to measure miRNA expression levels in 8 pairs of cancerous tissues and adjacent non-tumor mucosa tissues (2 from each group of GC and renal cell carcinoma patients and 2 from healthy controls, both with and without *H. pylori* infection). These 16 samples were included in the microarray studies. In total, 120 samples from 70 people (two samples from each cancer patient, one cancerous tissue and one non-tumor mucosa tissue) were examined via qRT-PCR. Tissue samples were immediately snap-frozen in liquid nitrogen and stored in a refrigerator at –80°C. This study was approved by the ethics committee of the Cerahpasa Medical Faculty (No. 07/09/2016-327307) and written informed consent was obtained from all patients included in the study. Other clinical characteristics were obtained from clinical records with patient permission.

miRNA isolation

Tissue samples were homogenized with a Magna Lyser instrument (Roche Diagnostics, Mannheim, Germany). TRIzol Reagent (Thermo Fisher Scientific, USA) was used for total RNA extraction. Low-molecular-weight RNAs were separated from total RNA using a mirVana miRNA purification column (Thermo Fisher Scientific, USA) in accordance with the manufacturer's instructions. The RNA purity was measured by a NanoDrop 2000 (Thermo Fisher Scientific, USA) spectrophotometer and the RNA integrity number (RIN) was detected by an Agilent 2000 analyzer (Agilent Technologies, USA).

miRNA expression profiling using microarrays

One hundred nanograms of miRNA from each of 16 samples were analyzed using a human microRNA microarray v.21.0 (Agilent Technologies, USA). For dephosphorylation and ligation, calf intestine alkaline phosphatase (GE Healthcare, Amersham, USA) and T4 RNA ligase (Thermo Fisher Scientific, USA) were used. Each sample was hybridized on a Human miRNA Microarray 8x60K slide (cat: G4872A, Agilent Technologies, USA) containing probes for 2549 human miRNAs. Slides were scanned using an Agilent G2565CA microarray scanner system (Agilent Technologies, USA) and the resulting images were processed. The average fluorescence intensity for each spot was cal-

Table 1 - *Demographic properties and GC and renal carcinoma stages of study groups.*

		HP+GC	*HPN-GC*	*RCC*	*HP+HCG*	*HP-HCG*	*Total*
Sex	Female	7 (35)	9 (45)	3 (30)	4 (40)	4 (40)	27 (38.5)
	Male	13 (65)	11 (55)	7 (70)	6 (60)	6 (60)	43 (61.5)
Age	Min.	31	38	43	49	49	
	Max.	79	73	63	62	63	
	Mean	57.1	56.1	55.8	55.1	56.3	
	Std Dev.	12.03023	8.71719	6.57943	4.62961	4.34741	
Grade	I	1 (5)	4 (20)				
	II	5 (25)	6 (30)	6 (60)			
	III	14 (70)	10 (50)	2 (20)			
	IV	0 (0)	0 (0)	2 (20)			
Lymph Node involvement	Yes	15 (75)	14 (70)				
	No	5 (25)	6 (30)				

Abbreviations: HP+GC: *H. pylori* (+) Gastric Cancer Patients, HPN-GC: *H. pylori* (-) Gastric cancer patients, RCC: renal cell carcinoma, HP+HCG: *H. pylori* (+) healthy control group, HP-NGC: *H. pylori* (-) healthy control group.

culated and the local background was subtracted. Data visualization and analysis were performed using GeneSpring GX 7.3 software (Agilent Technologies) (Chang *et al*., 2015).

Pathway analysis of miRNA targets by DIANA miR-Path
We utilized DIANA miRPath v2.0, a web server (http://snf-515788.vm.okeanos.grnet.gr/) established for the identification of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways corresponding to the networks of miRNA targets by numerous miRNA-target relationships, for the merging and meta-analysis algorithm. This program predicts miRNA targets with high accuracy based on the DIANA-microT-CDS algorithm, which considers the evolutionary conservation of miRNA-binding sites (Vlachos *et al*., 2012). KEGG (www.genome.jp/kegg/kegg_ja.html) is a publicly accessible knowledge database composed of 364,925 manually curated pathways that cover a wide range of metabolic, genetic, environmental, and cellular processes and human diseases (Satoh *et al*., 2015).

qRT-PCR
To confirm that some of these miRNAs were differentially expressed, miRCURY LNA miRNA PCR Assays (Qiagen GmbH, Hilden Germany) and the miScript Primer Assays (Qiagen GmbH, Hilden Germany) for hsa-miR-34c (cat: MS00003332), hsa-miR-194 (cat: MS00006727), hsa-miR-22 (cat: MS00003220), hsa-miR-223 (cat: MS00003871), hsa-miR-31 (cat: MS00003290), hsa-miR-203a (cat: MS00003766), hsa-miR-378a (cat: MS00006909), hsa-miR-145 (cat: MS00003528), hsa-miR-375 (cat: MS00031829), and hsa-miR-335 (cat: MS00003976) were used. The Hs_SNORD61_11 (SNORD61 small nucleolar RNA, C/D box 61) miScript Primer Assay (Qiagen GmbH, Hilden Germany) was used as a reference gene for the normalization of the qRT-PCR results. The qRT-PCR gene expression studies were performed with a LightCycler 480 probe master kit using a LightCycler 480 II instrument (Roche Diagnostics, Germany) according to the manufacturer's instructions. The relative fold changes were calculated by the $2^{-\Delta\Delta Ct}$ method published by Livak and Schmittgen (Livak and Schmittgen, 2001) and described in our previous study (Karakullukcu *et al*., 2017).

Statistical analysis
Analyses were performed using SPSS software, v25 (IBM, USA). The Kruskal-Wallis and Mann-Whitney U tests were used to evaluate differences in miRNA expression between the groups and for the comparison of miRNA ratios, respectively. Data were considered statistically significant at $P<0.05$.

RESULTS

The participants' demographic characteristics and GC and renal carcinoma stages are shown in *Table 1*. Seventy percent of *H. pylori* (+) GC cases were classified as Grade III GC and 50% of the *H. pylori* (-) GC cases were classified as Grade III GC; however, 60% of patients with renal cell carcinomas were classified as having Grade II cancers (*Table 1*).
The heat map image of miRNAs from the cancerous tissue specimens of *H. pylori* (+) GC patients showed aberrant miRNA signal intensities, compared to the *H. pylori* (-) GC's non-tumor mucosa samples (microarray heat map analysis). miRNAs with fold changes ≥2 were considered significantly upregulated and those with fold changes <0.5 were considered significantly downregulated. Nine miRNAs were upregulated (fold changes ≥2) and, in contrast, 27 miRNAs were downregulated in the cancerous tissue specimens of *H. pylori* (+) GC patients, compared to the *H. pylori* (-) GC's non-tumor mucosa samples (fold change<0.5). We also compared the cancerous tissue specimens of *H. pylori* (+) GC patients with the cancerous tissue specimens of *H. pylori* (-) GC patients and the mucosa specimens of healthy controls with and without *H. pylori*. (*Figure 1, Table 2a, 2b*). Additionally, the cancerous tissue specimens of *H. pylori* (-) GC patients were also compared to the non-tumor mucosa tissue specimens of *H. pylori* (+) GC patients and mucosa tissue specimens of the *H. pylori* (+) healthy control group. We selected the 5 most upregulated and 5 most downregulated miRNAs with statistical significance ($p<0.05$) for further study via qRT-PCR. The 5 most upregulated miRNAs were hsa-miR-34c, hsa-miR-194, hsa-miR-22, hsa-miR-223, and hsa-miR-31 and the 5 most downregulated miRNAs were hsa-miR-203, hsa-miR-378, hsa-miR-145, hsa-miR-375, and hsa-miR-335 in *H. pylori* (+) GC tissue specimens, compared to non-tumor mucosa tissue specimens of *H. pylori* (-) GC patients. In *H. pylori* (+) GC patients, even if their ranks or fold changes differed, the candidate upregulated or downregulated miRNAs were not changed in any of the comparisons (*H. pylori* (+) GC tissue specimens, compared to the cancerous and non-tumor mucosa tissue specimens of *H. pylori* (-) GC patients and the mucosa specimens of healthy controls with or without *H. pylori*).
In *H. pylori* (-) GC patients, when the non-tumor mucosa tissue specimens of *H. pylori* (+) healthy controls were compared to *H. pylori* (-) GC patients' cancerous tissue samples, different miRNAs (upregulated hsa-miR-139, hsa-miR-

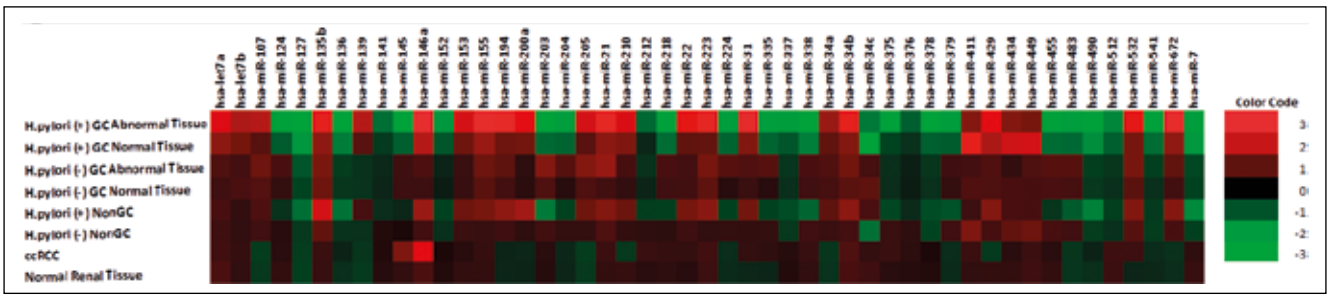

Figure 1 - *Differentially expressed miRNAs in H. pylori-positive, H. pylori-negative gastric cancer cancerous tissues, renal cell carcinoma cancerous tissues. The heat map image of miRNAs from the cancerous tissue specimens of H. pylori (+) GC patients showed aberrant miRNA signal intensities (microarray heat map analysis. Colors indicate relative signal intensities.*

Table 2 - *(a) Upregulated miRNAs with altered expression in cancerous tissue specimens of H. pylori (+) GC patients compared with the cancerous and non-tumor mucosa tissue specimens of H. pylori (-) GC patients and the mucosa specimens of healthy controls with or without H. pylori. (b) Downregulated miRNAs with altered expression in cancerous tissue specimens of H. pylori (+) GC patients compared with the cancerous and non-tumor mucosa tissue specimens of H. pylori (-) GC patients and the mucosa specimens of healthy controls with or without H. pylori.*

Table 2a

Upregulated miRNAs	*HP+GC_CT/ HPN-GC_NT*	*HP+GC_CT/HPN-GC_CT Fold Change*	*HP+GC_CT/ HP+GC_NT*	*HP+GC_CT/ HP+HCG*	*HP+GC_CT/ HP-HCG*
hsa-miR-34c	2.81	2.2	1.56	1.14	2.24
hsa-miR-194	2.76	1.79	1.65	1.32	1.74
hsa-miR-22	2.48	1.96	1.41	1.5	1.63
hsa-miR-223	2.36	1.85	1.62	1.38	1.76
hsa-miR-31	2.25	1.76	1.51	1.27	1.64
hsa-miR-155	2.21	1.44	1.32	0.58	1.54
hsa-miR-146a	2.2	1.41	1.14	0.79	1.41
hsa-miR-672	2.07	1.43	1.32	0.54	1.53
hsa-miR-139	2.01	1.46	0.89	1.01	1.04
hsa-miR-21	1.83	1.16	1.27	1.11	1.36
hsa-miR-34b	1.78	1.37	1.22	0.65	1.13
hsa-miR-200a	1.76	1.46	1.15	1.05	1.11
hsa-miR-210	1.58	1.4	1.01	0.49	1.09
hsa-miR-429	1.55	1.45	1.36	0.55	1
hsa-let-7a	1.52	1.38	0.81	0.1	1.42
hsa-miR-135b	1.52	1.3	1.11	1.02	1.32
hsa-miR-532	1.52	1.21	1.22	0.39	1.13
hsa-miR-205	1.5	1.21	1.18	0.52	1.08
hsa-miR-153	1.46	1.46	1.01	0.45	1.01
hsa-let-7b	1.15	1.28	1.25	0.02	1.13
hsa-miR-107	1.1	1.17	1.09	0.12	1.48
hsa-miR-34a	0.86	1.36	1.35	0.32	1.24

Table 2b

Downregulated miRNAs	*HP+GC_CT/ HPN-GC_NT*	*HP+GC_CT/ HPN-GC_CT Fold Change*	*HP+GC_CT/ HP+GC_NT*	*HP+GC_CT/ HP+HCG*	HP+GC_CT/ HP-HCG
hsa-miR-203	0.11	0.08	0.1	0.04	0.1
hsa-miR-378	0.14	0.07	0.14	0.04	0.09
hsa-miR-145	0.15	0.04	0.12	0.05	0.11
hsa-miR-375	0.17	0.07	0.1	0.02	0.13
hsa-miR-335	0.19	0.08	0.16	0.03	0.08
hsa-miR-212	0.2	0.36	0.36	0.19	0.24
hsa-miR-224	0.21	0.09	0.19	0.16	0.29
hsa-miR-379	0.22	0.2	0.28	0.14	0.2
hsa-miR-204	0.23	0.15	0.25	0.13	0.25
hsa-miR-124	0.24	0.1	0.18	0.22	0.16
hsa-miR-337	0.26	0.27	0.3	0.18	0.3
hsa-miR-338	0.26	0.15	0.18	0.17	0.15
hsa-miR-141	0.27	0.36	0.25	0.17	0.23
hsa-miR-376	0.28	0.36	0.25	0.17	0.23
hsa-miR-455	0.28	0.17	0.3	0.11	0.16
hsa-miR-490	0.29	0.24	0.33	0.09	0.24
hsa-miR-136	0.3	0.19	0.29	0.07	0.21
hsa-miR-127	0.31	0.21	0.24	0.22	0.19
hsa-miR-152	0.31	0.36	0.3	0.14	0.28
hsa-miR-7	0.31	0.31	0.3	0.23	0.25
hsa-miR-218	0.32	0.16	0.22	0.14	0.17
hsa-miR-483	0.34	0.28	0.3	0.16	0.29
hsa-miR-512	0.34	0.36	0.25	0.17	0.23
hsa-miR-541	0.37	0.23	0.26	0.21	0.21
hsa-miR-434	0.45	0.41	0.42	0.26	0.25
hsa-miR-411	0.49	0.43	0.41	0.21	0.42
hsa-miR-449	0.49	0.39	0.39	0.11	0.36

Abbreviations: HP+GC_NT: *H. pylori* positive gastric cancer patients, non-tumor mucosa specimens; HP+GC_CT: *H. pylori* positive gastric cancer patients, cancerous tissue specimens; HPN-GC_NT: *H. pylori* negative gastric cancer patients, non-tumor mucosa specimens; HP-GC_CT: *H. pylori* negative gastric cancer patients, cancerous tissue specimens; HP+HCG: *H. pylori* positive healthy controls, normal tissue; HP-HCG: *H. pylori* negative healthy controls, normal tissue.

Table 3 - *Pathway analysis of miRNA targets by DIANA miR-Path.*

Pathways	*p values*	*#Total affected genes*	*hsa-miR-203*	*hsa-miR-378*	*hsa-miR-145*	*hsa-miR-375*	*hsa-miR-335*	*hsa-miR-34c*	*hsa-miR-194*	*hsa-miR-22*	*hsa-miR-223*	*hsa-miR-31*	*#miRNAs*
Ubiquitin mediated proteolysis (hsa04120)	2.42E-21	52	5	3		5	36	4		3	6	3	8
PI3K-Akt signaling pathway (hsa04151)	1.54E-20	103	5	4	1	10	79	5	3	13	8	2	10
Transcriptional misregulation in cancer (hsa05202)	1.48E-11	59	3	6		8	40	4		8	12	2	8
Focal adhesion (hsa04510)	3.35E-11	61	5	4		3	47	6	1	7	1	2	9
ErbB signaling pathway (hsa04012)	5.32E-11	32	2	2		2	22	6		4	2	1	8
Pathways in cancer (hsa05200)	1.17E-10	97	5	10		8	62	7	2	14	9	5	9
MAPK signaling pathway (hsa04010)	8.53E-10	73	10	2		10	44	11	5	12	7	1	9
RNA transport (hsa03013)	5.23E-06	42	3	6		5	26	2	2	4	3	1	9

Table 4 - *Distribution of miRNA gene expression mean fold changes between groups assessed by qRT-PCR.*

		HP+GC_NT	*HP+GC_CT*	*HPN-GC_NT*	*HPN-GC_CT*	*RCC_NT*	*RCC_CT*	*HP+HCG*	*HP-HCG*
hsa-miR-34c	Mean fold change	1.45	1.70	1.25	1.20	1.04	1.05	1.32	1.04
	SD	0.11	0.22	0.24	0.13	0.11	0.008	0.13	0.008
	p[1]	0.000	0.000	0.002	0.002	0.912	0.631	0.000	–
hsa-miR-194	Mean fold change	3.83	4.24	1.68	1.85	1.01	1.01	1.79	1.01
	SD	0.28	0.46	0.20	0.11	0.08	0.06	0.14	0.17
	p[1]	0.000	0.000	0.000	0.000	0.684	0.631	0.000	–
hsa-miR-22	Mean fold change	1.57	1.75	1.18	1.14	1.02	0.99	1.35	1.01
	SD	0.11	0.27	0.08	0.11	0.06	0.04	0.11	0.11
	p[1]	0.000	0.000	0.000	0.004	0.769	0.684	0.000	–
hsa-miR-223	Mean fold change	2.23	2.86	1.21	1.64	1.01	1.01	1.70	1.02
	SD	0.11	0.68	0.13	0.14	0.09	0.05	0.17	0.16
	p[1]	0.000	0.000	0.005	0.002	1.000	0.853	0.000	–
hsa-miR-31	Mean fold change	1.97	2.21	1.52	1.53	1.01	1.00	1.43	1.01
	SD	0.10	0.20	0.09	0.10	0.08	0.08	0.10	0.14
	p[1]	0.000	0.000	0.000	0.002	0.796	0.796	0.000	–
hsa-miR-203	Mean fold change	0.59	0.57	0.88	0.87	1.00	1.02	0.69	1.03
	SD	0.09	0.23	0.04	0.09	0.08	0.12	0.07	0.10
	p[1]	0.000	0.000	0.000	0.002	0.579	1.000	0.000	–
hsa-miR-378	Mean fold change	0.66	0.58	0.89	0.92	1.02	1.00	0.72	1.06
	SD	0.07	0.26	0.06	0.07	0.08	0.10	0.07	0.12
	p[1]	0.000	0.000	0.000	0.001	0.436	0.143	0.0000	–
hsa-miR-145	Mean fold change	0.43	0.33	0.87	0.90	1.01	1.02	0.54	1.01
	SD	0.06	0.14	0.04	0.07	0.06	0.09	0.09	0.08
	p[1]	0.000	0.000	0.000	0.003	1.000	0.796	0.000	–
hsa-miR-375	Mean fold change	0.49	0.66	0.88	0.87	0.99	0.98	0.71	0.94
	SD	0.09	0.19	0.05	0.08	0.05	0.06	0.05	0.07
	p[1]	0.000	0.000	0.013	0.055	0.165	0.393	0.000	
hsa-miR-335	Mean fold change	0.44	0.38	0.88	0.86	1.03	1.01	0.50	0.99
	SD	0.06	0.12	0.05	0.07	0.08	0.07	0.10	0.12
	p[1]	0.000	0.000	0.000	0.001	0.247	0.436	0.000	–

[1]All group compared with HP-NGC group and p values measured with Mann-Whitney U test.
Abbreviation: HP+GC_NT: *H. pylori* positive gastric cancer patients, non-tumor mucosa specimens; HP+GC_CT: *H. pylori* positive gastric cancer patients, cancerous tissue specimens; HPN-GC_NT: *H. pylori* negative gastric cancer patients, non-tumor mucosa specimens; HP-GC_CT: *H. pylori* negative gastric cancer patients, cancerous tissue specimens; RCC_NT: Renal Cell carcinoma non-tumor mucosa tissue specimens; RCC_CT: renal cell carcinoma, cancerous tissue specimens; HP+HCG: *H. pylori* positive healthy controls, normal tissue; HP-HCG: *H. pylori* negative healthy controls, normal tissue.

200a, hsa-miR-135b, and hsa-miR-146a, and downregulated hsa-miR-7, hsa-miR-127, and hsa-miR-145) were prominent as upregulated and downregulated miRNAs. In the selection of candidate miRNAs, almost all the miRNAs failed to reach the ≥2-fold change threshold for being considered significantly upregulated. In *H. pylori* (-) GC patients, when the non-tumor mucosa tissue specimens of *H. pylori* (+) GC patients were compared to *H. pylori* (-) GC patients' cancerous tissue samples via microarray, only hsa-miR-34c showed fold changes ≥2. Also, when the mucosa tissue specimens of *H. pylori* (+) healthy controls were compared to *H. pylori* (-) GC patients' cancerous tissue samples, hsa-miR-139 changed 1.42-fold and the other miRNAS changed less than 2-fold. For all the aforementioned reasons, we decided to study only the candidate miRNAs that were significantly upregulated and downregulated in *H. pylori* (+) GC patients. Comparisons other than *H. pylori* (+) GC patients, such as to the *H. pylori* (-) healthy control group's mucosa samples, did not show significantly upregulated miRNAs (i.e., ≥2-fold change) (*Table 2a, 2b*). On the other hand, downregulated miRNAs were present in *H. pylori* (-) GC patients (fold changes <0.5), but we did not study them due to limited resources.

The related pathways of these selected 10 up- or downregulated miRNAs were controlled by the DIANA miR-Path web software in silico. We observed that these 10 miRNAs are related to the PI3K-Akt signal pathway and that they can affect 103 different genes in that pathway. In another pathway (pathway in cancer), 9 of these 10 miRNAs can also affect 97 different genes (*Table 3*).

We studied the miRNA expression levels of 120 tissue specimens from 70 study participants by qRT-PCR. All the miRNA assays examined the fold changes in the absolute expression levels of the candidate miRNAs of each sample. We observed that the expression levels of hsa-miR-34c, hsa-miR-194, hsa-miR-22, hsa-miR-223, and hsa-miR-31 (the upregulated miRNAs) from the cancerous specimens of *H. pylori* (+) GC patients were very significantly higher than those of the *H. pylori* (-) healthy control group's mucosa samples, as assessed by the delta delta Ct method. On the other hand, the expression levels of hsa-miR-203, hsa-miR-378, hsa-miR-145,

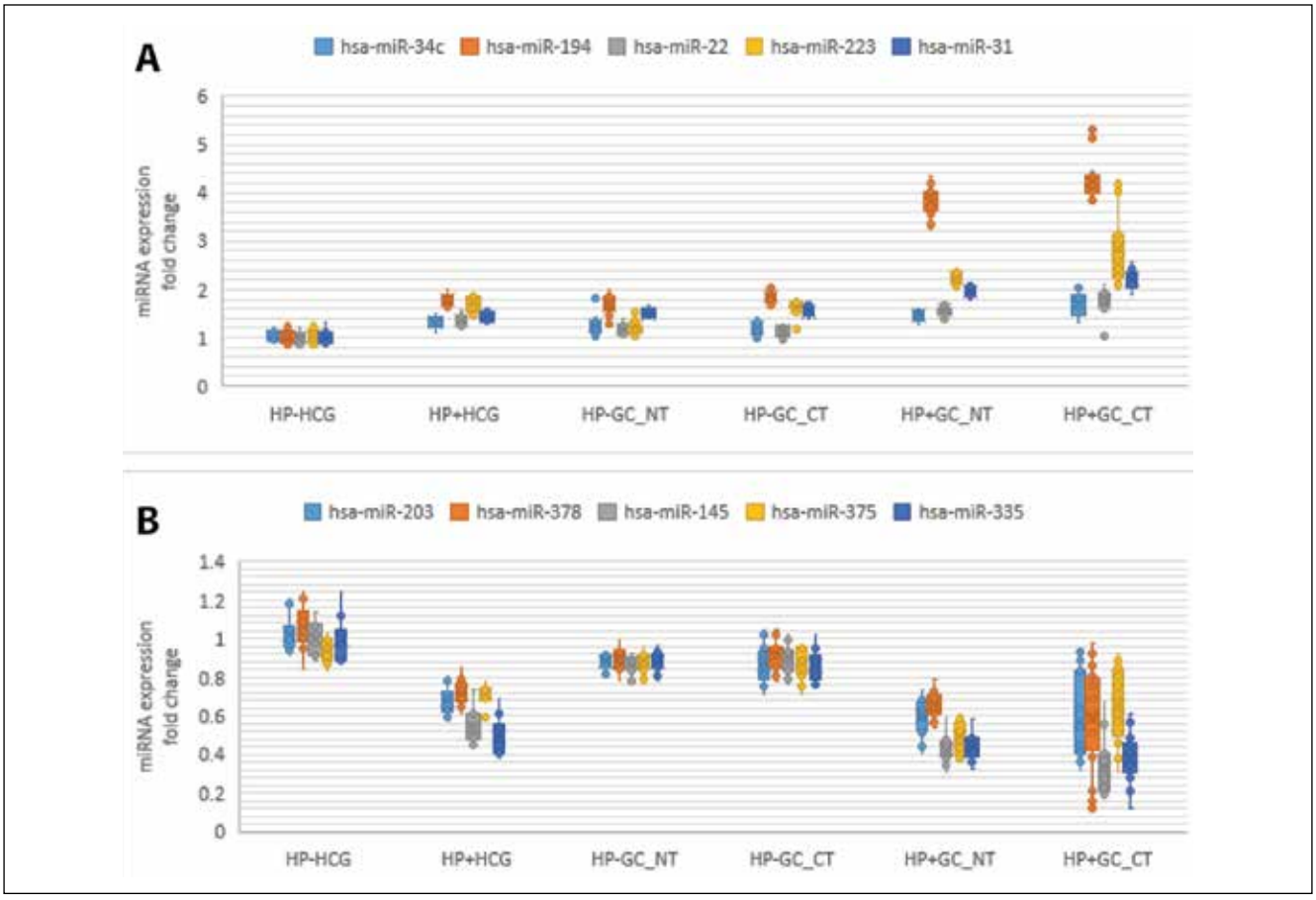

Figure 2 - *(A): The miRNA expression levels of hsa-miR-34c, hsa-miR-194, hsa-miR-22, hsa-miR-223, and hsa-miR-31 (the upregulated miRNAs) from cancerous tissues of H. pylori (+) GC patients when compared with H. pylori (-) healthy control group's mucosa tissues by qRT-PCR. (B): The miRNA expression levels of hsa-miR-203, hsa-miR-378, hsa-miR-145, hsa-miR-375, and hsa-miR-335 (the downregulated miRNAs) from cancerous specimens of H. pylori (+) GC patients when compared with H. pylori (-) healthy control group' mucosa samples by qRT-PCR.*

Abbreviations: HP+GC_NT: H. pylori positive gastric cancer patients, non-tumor mucosa specimens; HP+GC_CT: H. pylori positive gastric cancer patients, cancerous tissue specimens; HPN-GC_NT: H. pylori negative gastric cancer patients, non-tumor mucosa specimens; HP-GC_CT: H. pylori negative gastric cancer patients, cancerous tissue specimens; RCC_NT: Renal Cell carcinoma non-tumor mucosa tissue specimens; RCC_CT: renal cell carcinoma, cancerous tissue specimens; HP+HCG: H. pylori positive healthy controls, normal tissue; HP-HCG: H. pylori negative healthy controls, normal tissue.

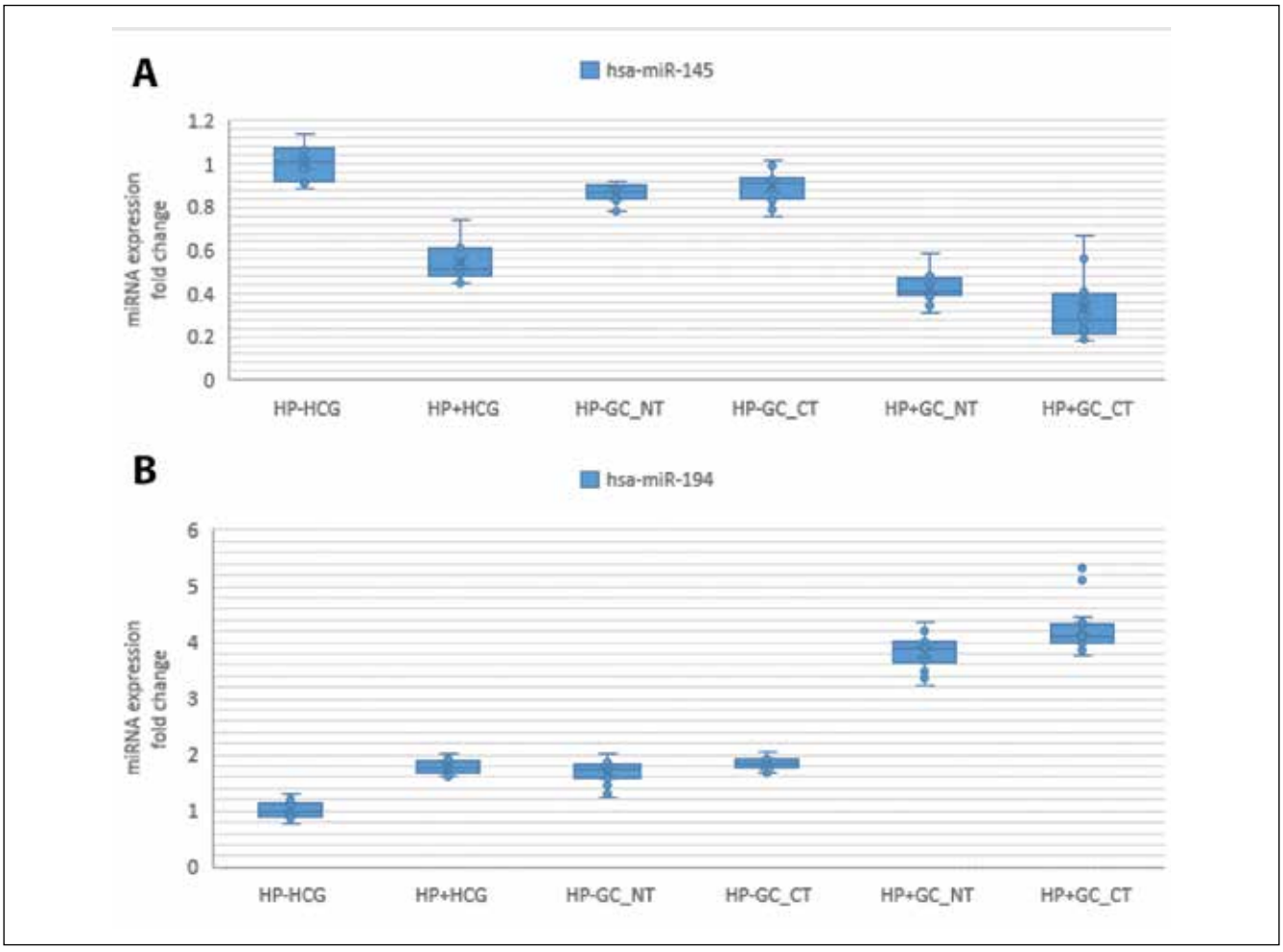

Figure 3 - *(A): miRNA expression levels of hsa-miR-145 in the cancer and control groups. (B): miRNA expression levels of hsa-miR-194 in the cancer and control groups.*
Abbreviations: HP+GC_NT: H. pylori positive gastric cancer patients, non-tumor mucosa specimens; HP+GC_CT: H. pylori positive gastric cancer patients, cancerous tissue specimens; HPN-GC_NT: H. pylori negative gastric cancer patients, non-tumor mucosa specimens; HP-GC_CT: H. pylori negative gastric cancer patients, cancerous tissue specimens; HP+HCG: H. pylori positive healthy controls, normal tissue; HP-HCG: H. pylori negative healthy controls, normal tissue.

Table 5 - *Receiver operating characteristic (ROC) curve analysis of hsa-miR-34c, hsa-miR-194, hsa-miR-22, hsa-miR-223, hsa-miR-31, hsa-miR-203, hsa-miR-378, hsa-miR-145, hsa-miR-375, hsa-miR-335 for distinguishing the GC (gastric cancer) patients from the healthy controls.*

Area Under the Curve								
Test Result Variable(s)	*Area*	*Std. Error*[a]	*Asymptotic Sig.*[b]	*Asymptotic 95% Confidence Interval*		*Cutoff values*		
				Lower Bound	*Upper Bound*	*Positivity*	*Sensitivity*	*Specificity*
hsa-miR-34c	0.739	0.06	0.001	0.621	0.857	0.975	0.988	0.9
hsa-miR-194	0.875	0.04	0	0.796	0.954	0.825	1	0.95
hsa-miR-22	0.703	0.062	0.005	0.582	0.824	0.865	1	0.95
hsa-miR-223	0.775	0.053	0	0.672	0.878	0.83	1	0.95
hsa-miR-31	0.938	0.028	0	0.883	0.994	0.85	1	0.95
hsa-miR-203	0.329	0.072	0.018	0.187	0.471	0.395	0.95	1
hsa-miR-378	0.352	0.075	0.042	0.205	0.5	0.3	0.95	1
hsa-miR-145	0.305	0.065	0.007	0.178	0.433	0.185	0.988	1
hsa-miR-375	0.373	0.067	0.079	0.241	0.504			
hsa-miR-335	0.358	0.073	0.051	0.216	0.501	0.165	0.988	1

The test result variable(s): hsa-miR-34c, hsa-miR-194, hsa-miR-22, hsa-miR-223, hsa-miR-31, hsa-miR-203, hsa-miR-378, hsa-miR-145, hsa-miR-375, hsa-miR-335 has at least one tie between the positive actual state group and the negative actual state group. Statistics may be biased.
[a]Under the nonparametric assumption.
[b]Null hypothesis: true area = 0.5.

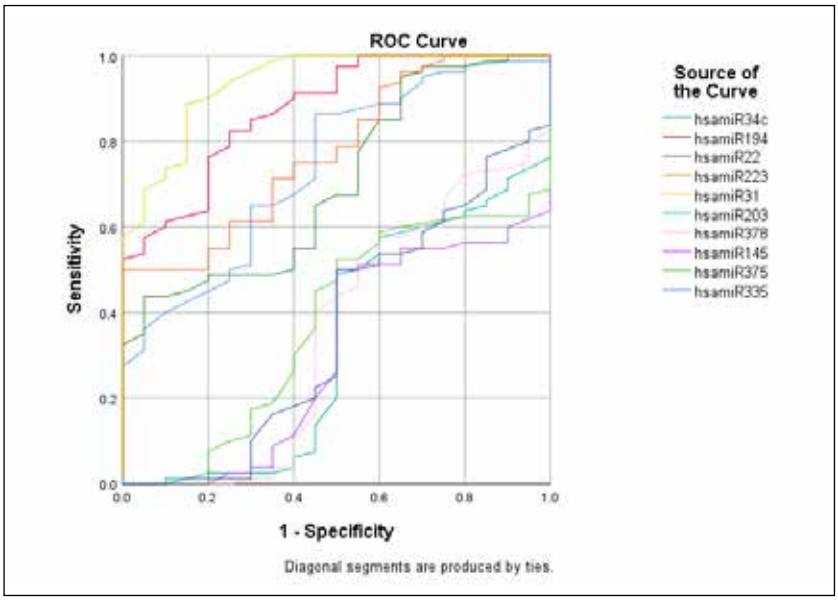

Figure 4 - *Receiver operating characteristic (ROC) curve analysis of hsa-miR-34c, hsa-miR-194, hsa-miR-22, hsa-miR-223, hsa-miR-31, hsa-miR-203, hsa-miR-378, hsa-miR-145, hsa-miR-375, hsa-miR-335 for distinguishing the GC (gastric cancer) patients from the healthy controls.*

hsa-miR-375, and hsa-miR-335 (the downregulated miRNAs) from the cancerous specimens of *H. pylori* (+) GC patients were significantly lower than those of the *H. pylori* (-) healthy control group's mucosa samples. The distribution of mean fold changes for the miRNA gene expression levels were calculated between the groups by qRT-PCR (*Table 4, Figure 2a, 2b*). The miRNA levels of renal cell carcinoma cancerous tissue specimens showed no statistically significant difference from the *H. pylori* (-) healthy control group's mucosa samples. In *H. pylori* (+) GC patients, however, hsa-miR-194 was upregulated 4.24-fold and 3.83-fold in the cancerous tissue specimens and non-tumor mucosa specimens, respectively, when compared with the mucosa specimens of the *H. pylori* (-) healthy control group. Meanwhile, hsa-miR-145 was downregulated 0.33-fold and 0.43-fold in the cancerous tissue and non-tumor mucosa specimens of *H. pylori* (+) GC patients, respectively, when compared with the mucosa specimens of the *H. pylori* (-) healthy control group.

The presence of *H. pylori* significantly upregulated hsa-miR-194 and downregulated hsa-miR-145 expression levels in *H. pylori* (+) GC cases when compared to the *H. pylori* (-) GC cases (*Table 4, Figure 3a, 3b*).

When the miRNA expression values of cancerous tissue specimens from GC patients were compared with the non-tumor mucosa of healthy control individuals by ROC curve analyses, the miRNAs had reasonable cut-off values, which may be accepted as a promising indicator for GC. The sensitivity and specificity of hsa-miR-194 were 100% and 95%, respectively, whereas the same parameters were 98.8% and 100% for hsa-miR-145, respectively (*Table 5, Figure 4)*.

When the target was narrowed for the ROC curve analyses to *H. pylori* (+) GC patients and *H. pylori* (+) healthy controls, the sensitivity and specificity of hsa-miR-194 were 100% and 90%, respectively (similar to *Table 5*), whereas the same parameters were 97.5% and 100% for hsa-miR-145, respectively (*Table 6, Figure 5*).

Table 6 - *Receiver operating characteristic (ROC) curve analysis of hsa-miR-34c, hsa-miR-194, hsa-miR-22, hsa-miR-223, hsa-miR-31, hsa-miR-203, hsa-miR-378, hsa-miR-145, hsa-miR-375, hsa-miR-335 for distinguishing the H. pylori (+) GC (gastric cancer) patients from the H. pylori (+) healthy controls.*

Area Under the Curve								
Test Result Variable(s)	*Area*	*Std. Error*[a]	*Asymptotic Sig.*[b]	*Asymptotic 95% Confidence Interval*		*Cutoff values*		
				Lower Bound	*Upper Bound*	*Positivity*	*Sensitivity*	*Specificity*
hsa-miR-34c	0.840	0.061	0.001	0.720	0.960	1.11	1.000	0.900
hsa-miR-194	1.000	0.000	0.000	1.000	1.000	1.625	1.000	0.900
hsa-miR-22	0.906	0.045	0.000	0.818	0.994	1.075	0.975	1.000
hsa-miR-223	1.000	0.000	0.000	1.000	1.000	1.465	1.000	0.900
hsa-miR-31	1.000	0.000	0.000	1.000	1.000	1.315	1.000	0.900
hsa-miR-203	0.271	0.070	0.026	0.135	0.408	0.34	0.975	1.000
hsa-miR-378	0.316	0.079	0.075	0.161	0.472			
hsa-miR-145	0.109	0.045	0.000	0.020	0.198	0.185	0.975	1.000
hsa-miR-375	0.239	0.065	0.011	0.111	0.366	0.37	0.950	1.000
hsa-miR-335	0.279	0.086	0.032	0.110	0.447	0.165	0.975	1.000

The test result variable(s): hsa-miR-34c, hsa-miR-194, hsa-miR-22, hsa-miR-223, hsa-miR-31, hsa-miR-203, hsa-miR-378, hsa-miR-145, hsa-miR-375, hsa-miR-335 has at least one tie between the positive actual state group and the negative actual state group. Statistics may be biased.

[a]Under the nonparametric assumption.

[b]Null hypothesis: true area =0.

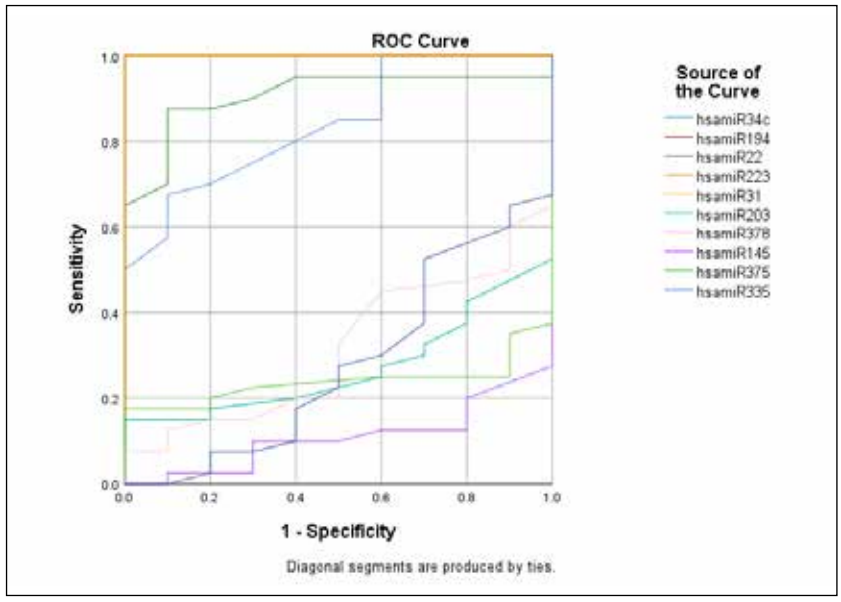

Figure 5 - *Receiver operating characteristic (ROC) curve analysis of hsa-miR-34c, hsa-miR-194, hsa-miR-22, hsa-miR-223, hsa-miR-31, hsa-miR-203, hsa-miR-378, hsa-miR-145, hsa-miR-375, hsa-miR-335 for distinguishing the H. pylori (+) GC (gastric cancer) patients from the H. pylori (+) healthy controls.*

DISCUSSION

It seems that early detection of GCs will continue to be a long-awaited hope until the discovery of useful, noninvasive biomarkers for routine population screening. In the general sense, while tumor-suppressing miRNAs usually repress oncogenes, oncogenic miRNAs (oncomiRs) usually silence tumor suppressor genes. Overexpressed oncomiRs in GC promote cancer cell growth and also inhibit apoptosis by silencing growth inhibition- associated genes (Wu *et al*., 2014). In 2008, Zhang *et al.* (2008) were the first to suggest that the upregulation of some miRNAs, such as miR-21 in GC, may be related to *H. pylori* infection.

In this study, we detected 9 miRNAs as upregulated and 27 miRNAs as downregulated in the cancerous tissue specimens of *H. pylori* (+) GC patients, compared to the *H. pylori* (-) healthy control group's mucosa samples, by microarrays, with changes greater than two-fold and smaller than <0.5-fold considered differential changes. Comparisons other than between the *H. pylori* (+) GC patients and the *H. pylori* (-) GC's non-tumor mucosa samples did not show significantly upregulated miRNAS (i.e., ≥2-fold change). We decided to use miRNAs with changes that were ≥2-fold as candidate miRNAs for later analysis. Wang *et al*. (2017) found a total of 14 miRNAs to be upregulated and 39 miRNAs to be downregulated, with a greater than two-fold change being considered differential, in 42 GC patients when they compared 21 *H. pylori* (+) and 21 *H. pylori* (-) patients. Meanwhile, in the study by Chang *et al.* (2015), 19 miRNAs were found to be upregulated in *H. pylori* (-) cancerous tissue ($p<0.05$) and 18 miRNAs were found to be upregulated in *H. pylori* (+) cancerous tissue. Our limited number of upregulated and downregulated miRNAs probably resulted from the initial low number of specimens (8 patients and their 16 specimens) for miRNA expression profiling using microarrays.

We selected 5 upregulated miRNAs (hsa-miR-34c, hsa-miR-194, hsa-miR-22, hsa-miR-223, and hsa-miR-31) and five downregulated miRNAs (hsa-miR-203, hsa-miR-378, hsa-miR-145, hsa-miR-375, and hsa-miR-335) from the significantly increased and decreased miRNAs detected in the cancerous tissue specimens of *H. pylori* (+) GC patients by microarray. qRT-PCR studies were performed for the aforementioned 10 miRNAs. In particular, hsa-miR-194 was upregulated 4.24-fold and 3.83-fold in the cancerous tissue specimens and non-tumor mucosa specimens of *H. pylori* (+) GC patients, respectively, when compared with the mucosa specimens of the *H. pylori* (-) healthy control group, whereas hsa-miR-145 was downregulated 0.33-fold and 0.43-fold in the cancerous tissue and non-tumor mucosa specimens of *H. pylori* (+) GC patients, respectively, when compared with the mucosa specimens of the *H. pylori* (-) healthy control group. Therefore, we focused the remainder of our study on hsa-miR-194 and hsa-miR-145. In an extensive literature search, Sousa *et al.* (2016) showed synergistic miRNA alterations in metaplastic lineages, specifically the downregulation of the miR-30 family and the upregulation of miR-194. They suggested that this synergy contributed to the expression of intestinal transcripts that are characteristic of intestinal metaplasia through the regulation of HNF4γ and NR2F2. miR-30 family members are tumor suppressor miRNAs and are downregulated in different cancers, including GC. Sousa *et al.* (2016) also showed that NR2F2, a known co-regulator of HNF4, is downregulated during metaplasia progression and is a direct target of miR-194. Our study results for miR-194 are similar to those of Sousa *et al.* (2016) (our 4.24-fold change versus their 1.9-fold change for miR-194). In another study, Shiotani *et al.* (2012) detected miR-21, miR-194, and miR-196 at significantly higher levels in the gastric mucosa of the cancer group than in the controls, and *H. pylori* eradication resulted in a significant decrease in oncomiR expression only in the controls. They suggested that the expression of miR-194 was induced by *H. pylori* infection, but they could not define the target of miR-194 and suggested that this miRNA is a tumor-suppressor miRNA. They also suggested that *H. pylori* eradication improves miRNA deregulation and that the long-term colonization of *H. pylori* may induce epigenetic modifications in gastric mucosal genes, including the promoters of tumor suppressor miRNAs, but this is not completely reversible by bacterial eradication alone (Nishizawa and Suzuki, 2013).

In contrast, in the study of Zhang *et al*. (2017), miR-194

was dramatically downregulated in gastric cancer tissues when compared with the adjacent normal tissues. They showed that the upregulation of miR-194 significantly suppressed GC cell proliferation and induced apoptosis. They showed that gamma-glutamylcyclotransferase (GGCT), which is involved in many kinds of cancer, is a potential target of miR-194. Similarly, Li *et al*. (2014) suggested that miR-194 expression inhibited cell migration and invasion in GC cells and that miR-194 acted as a tumor inhibitor through its targeting of FoxM1. RING box protein1 (RBX1) is a component of SCF E3 ubiquitin ligases and is involved in GC. Chen *et al.* reported that miR-194 was significantly downregulated and RBX1 upregulated in GC tissues (Chen *et al.,* 2015). As seen above, there are conflicting results related to the effects of miR-194, but the studies that claim a protective role against GC do not include the involvement of *H. pylori*; therefore, we suggest that persistent *H. pylori* infections may play a role in upregulating this miRNA, which should be extensively investigated. The study of Shiotani *et al*. (2012) matches our study the best because it is among the few studies to include *H. pylori* and our results are in parallel with their study.

Lee *et al.* (2017) reported that hsa-miR-145-5p decreased in cancer tissue, compared to non-cancerous gastric mucosa, in a *H. pylori* (-) group. In our study, hsa-miR-145 was significantly downregulated in *H. pylori* (+) and (-) GC cancerous tissue specimens, but was obvious in *H. pylori* (+) GC cancerous tissue specimens. It was suggested that depressed hsa-miR-145-5p activity was related to the increased rate of epithelial-mesenchymal transitions (EMTs) and that the EMT may have an important role in GC carcinogenesis and progression. Cytotoxin-associated gene A (CagA) was suggested to trigger an EMT through the activation of several transcription factors. Lee *et al.* (2017) suggested adding hsa-miR-145-5p to the list of miRNAs related to the EMT (hsa-miR-335, hsa-miR-21, hsa-miR-31, hsa-miR-205, and hsa-miR-10b.19). In our study, the presence of *H. pylori* had a downregulating effect on hsa-miR-145-5p, probably by inducing EMTs by CagA.

In GCs, Takagi *et al.* (2009) showed that miR-145 was generally downregulated and that overexpression of miR-145 suppressed cell growth in a human cancer cell line. Morphological examination after transfection with miR-145 revealed enlarged, flat cells, suggesting that the silencing of B-actin stopped cell growth; in the downregulation of miR-145, active B-actin will induce cell growth. Gao *et al.* (2013) identified a novel mechanism in which miR-145 suppressed the invasion-metastasis cascade in GC by inhibiting the protein translation of its direct target gene, N-cadherin, which may then indirectly downregulate its downstream effector, MMP9. The fact that miR-145 functions as a tumor suppressor identifies it as a novel therapeutic target for cancer therapy. Bibi *et al.* (2016) reported that GC-related miRNAs, such as miRNA-145-5p, are downregulated in GC tissues and regarded as tumor suppressor miRNAs. In the above studies, only the study of Lee *et al.* (2017) included *H. pylori*, but they detected it only in the *H. pylori* (-) group. However, our results are in parallel with the results of other studies related to the downregulation of miRNA-145-5p as a tumor suppressor miRNA. The miRNA expression of renal cell carcinoma specimens, both normal and tumor tissue, showed no statistically significant difference between the miRNA levels of *H. pylori* (-) GC non-tumor mucosa specimens and those of healthy controls. Therefore, we did not evaluate the results related to renal cell carcinoma tissue specimens.

H. pylori may persist in the stomach at high density for years. miRNAs target cytokines and other mediators of the immune response. For example, miR-21 is a regulator of *H. pylori*-induced inflammation and targets the receptor of the TGFβ signaling pathway. This miRNA's expression is increased in both GC and *H. pylori*-infected gastric tissue (Olivieri *et al*., 2012). The mature form of this miRNA also shows increased expression in both GC and *H. pylori*-infected gastric tissue (Zhang *et al*., 2008). Also, hsa-miR-155 and hsa-miR-146a target the MyD88 complex and the adaptor proteins (IRAK-1 and TRAF6) of the TLRs signaling cascade, resulting in decreased NF-κB activation. Other relationships between inflammatory mediators and miRNAs are described elsewhere (Cadamuro *et al*., 2014).

When the miRNA expression values of tissues from GC patients were compared to the normal tissues of healthy control individuals by ROC curve analyses, the sensitivity and specificity of hsa-miR-194 were detected as 100% and 95%, respectively, whereas the same parameters were 98.8% and 100% for hsa-miR-145, respectively. However, when the target for the ROC curve analyses was narrowed to *H. pylori* (+) GC patients and *H. pylori* (+) healthy controls, the sensitivity and specificity of hsa-miR-194 were detected, similar to former analyses, as 100% and 90%, respectively, and the same parameters were 97.5% and 100% for hsa-miR-145, respectively. Their high sensitivity and specificity values increase our hope that these miRNAs can be used as potential biomarkers.

This study has some limitations. We used a limited number of samples for microarrays. In the qRT-PCR studies, we only compared miRNAs from cancerous specimens of *H. pylori* (+) GC patients with the miRNA expression levels of mucosa specimens of *H. pylori* (-) healthy controls. Considering the presence of miRNAs in the circulatory system, we suggest that every tissue may have been induced to up- or downregulate their miRNAs by these circulating miRNAs as well as *H. pylori* virulence factors. To address this bias, we used the mucosa specimens of *H. pylori* (-) healthy controls as a reference point for comparison. We compared all the miRNA expression levels with those of the *H. pylori* (-) healthy controls. The most up- and downregulated miRNA expression levels were seen in *H. pylori* (+) GC cancerous specimens. The impact of the presence of *H. pylori* was obvious in the GC patients' cancerous tissues. There may be other unexplored mechanisms behind the up- or downregulation of miRNA expression in the cancerous tissues of *H. pylori* (+) GC patients. Considering the low specimen numbers in this study, it is not plausible to conclude that these miRNAs are perfectly predictive biomarkers for GC with *H. pylori* infection.

In conclusion, we detected the upregulation in hsa-miR-194 expression and downregulation in hsa-miR-145 expression in *H. pylori* (+) GC cancerous tissues for the first time in Turkey. The presence of *H. pylori* significantly upregulated hsa-miR-194 and downregulated hsa-miR-145 expression levels in *H. pylori* (+) GC cases when compared to *H. pylori* (-) GC cases. We did not investigate the targets of these miRNAs and the mechanisms behind their effects for GC pathogenesis or the intervention of *H. pylori* virulence factors. Geng and Zhang (2017) suggested that *H. pylori* may affect the PI3K/AKT/GSK3β signal pathways in GC. Our 10 miRNAs are also related to the PI3K-Akt signal pathway and can affect 103 different genes in that

pathway. Regional differences in the virulence factors of *H. pylori* strains may also play a role in the up-or downregulation of these miRNA expression levels. We also suggest that discrepancies in our results with other studies may be the result of complex mechanisms that need to be explored with other expanded studies.

There are many pathways and targets for miRNAs in GC carcinogenesis. On the other hand, there are conflicting results in this area and so far there is no consensus for their usage as potential biomarkers or therapeutic purposes. Comprehensive, serial studies are still needed to explore their important role as diagnostic or therapeutic tools.

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