ORIGINAL ARTICLE

Different MicroRNA Expression Levels in Gastric Cancer Depending on *Helicobacter pylori* Infection

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Background/Aims: This study was conducted to identify microRNAs (miRNAs) that are differentially expressed in *Helicobacter pylori*-infected patients with an intestinal type of gastric cancer using miRNA microarray and to confirm the candidate miRNA expression levels. **Methods:** Total RNA was extracted from the cancerous and noncancerous regions of formalin-fixed, paraffin-embedded tissues of *H. pylori*-positive (n=8) or *H. pylori*-negative (n=8) patients with an intestinal type of gastric cancer. RNA expression was analyzed using a 3,523 miRNA profiling microarray based on the Sanger miRBase. Validation analysis was performed using TaqMan miRNA assays. **Results:** A total of 219 miRNAs in the aberrant miRNA profiles across the miRNA microarray showed at least a 2-fold change differential expression in *H. pylori*-positive and *H. pylori*-negative cancer tissues. After candidate miRNAs were selected using online miRNA databases, TaqMan miRNA assays confirmed that three miRNAs (*miR-99b-3p*, *miR-564*, and *miR-638*) were significantly increased in three *H. pylori*-positive cancer tissues compared to the *H. pylori*-negative cancer tissues. Additionally, four miRNAs (*miR-204-5p*, *miR-338-5p*, *miR-375*, and *miR-548c-3p*) were significantly increased in *H. pylori*-negative cancer tissues compared to *H. pylori*-positive cancer tissues. **Conclusions:** miRNA expression in the intestinal type of *H. pylori* infection-dependent gastric cancer suggests that different gastric cancer pathogenesis mechanisms could exist between *H. pylori*-positive and *H. pylori*-negative gastric cancer. Additional functional studies are required. **(Gut Liver, 2015;9:188-196)**

Key Words: Gastric carcinogenesis; *Helicobacter pylori*; Microarray; MicroRNAs; TaqMan miRNA assays

INTRODUCTION

About 20 length nucleotides of nonprotein coding microRNA (miRNA) regulates gene expression by hybridizing to the 3' untranslated region of specific messenger RNA (mRNA) targets. Up to now, approximately 1,600 human miRNAs have been studied and the relationship of miRNAs with human diseases is being intensively studied.[1,2]

Microarray-based hybridization profiling is a powerful technique for screening and use of fresh tissue specimens recommended to reduce collateral damage of miRNAs. However, it is hard to get fresh tissue specimens right after operations. Therefore, it is helpful to use the miRNA preserving formalin-fixed paraffin-embedded (FFPE) tissue for miRNA screening analysis. It has been used for extraction of miRNAs and a few studies have examined the correlation with clinical data.[3,4] In addition, a strong correlation in global miRNA expression between fresh frozen and FFPE human cancer samples has been revealed.[5]

Epidemiological studies have implicated that colonization of the stomach by *Helicobacter pylori* is a risk for various development of gastric diseases including gastric cancer.[6] Given the possible pathogenesis of gastric cancer, abnormal miRNA expression after *H. pylori* infection might cause unregulated inflammation response and immune system disruption.[7]

The occurrence of gastric cancer still remains very high in much of Asia and especially, à Laurens' classification divided gastric cancer into two histological main types; intestinal-type and diffuse-type,[8,9] in which the number of intestinal-type gastric cancer patients in South Korea is more prevalent than the other type.[10]

On the other hand, 5.3% of gastric cancer patients were not infected with *H. pylori* in Korea.[11] Few articles noted that dif-

ferent genetic background in gastric mucosa depending on *H. pylori* infection and a recent study has reported that miRNA expression patterns in *H. pylori*-infected and -uninfected gastric normal mucosa were different.[12-14] Thus different miRNAs could be involved in gastric carcinogenesis depending on *H. pylori* as well. However, no study has ever reported regarding distinct miRNA profiles in intestinal type of gastric cancer FFPE specimens depending on *H. pylori* infection. Furthermore, it has not been determined whether miRNA expression patterns which are different in this gastric cancer depending on *H. pylori* infection are different or not in normal gastric mucosa.

From this background, the present study was undertaken to identify whether miRNA expression profiles differ between *H. pylori*-positive and -negative FFPE specimens in the intestinal type of gastric cancer patients using miRNA microarray. The microarray results were confirmed by TaqMan miRNA assays.

MATERIALS AND METHODS

1. Subjects

Sixteen gastric cancer patients matched for age, sex, and *H. pylori* status, who received curative operation at Seoul National University Bundang Hospital, were included for microarray study. Table 1 shows the baseline characteristics of the study subjects. All subjects of this study received gastroscopy for gastric cancer screening and conformation of histological gastric adenocarcinoma diagnosis. For TaqMan miRNA assays, gastric cancer tissue was retrieved from gastric cancer patients who received endoscopic submucosal dissection with current *H. pylori* (n=28) or without any *H. pylori* infection evidence at all (n=24), including 16 patients in whom microarray experiments were performed. In addition, gastric body tissue was obtained from controls with current *H. pylori* (n=24) or without any *H. pylori* infection evidence at all (n=24). The subjects who underwent gastroscopy and *H. pylori* gastric cancer screening but did not show any significant gastroduodenal diseases, such as gastric cancer, dysplasia, mucosa-associated lymphoid tissue lymphoma, esophageal cancer or peptic ulcer disease were enrolled into the control group. The study protocol was approved by the Ethics Committee of Seoul National University Bundang Hospital

(Institutional Review Board number: B-1301-186-111). All participants provided their written informed consent to participate in this study.

2. *H.pylori* testing

Three types of *H. pylori* testing (histology, *Campylobacter*-like organism [CLO]-test, and culture) were conducted in both the antrum and the body as previously described.[15] If one of the three examinations was positive, the patients were *H. pylori*-positive. If all three tests were negative then *H. pylori* serology test was performed using anti-*H. pylori* immunoglobulin G in an enzyme-linked immunosorbant assay (Green Cross Medical Science, Eumsung, Korea). Intestinal metaplasia (IM) was graded according to the modified Sydney system in hematoxylin and eosin stained tissue.[16] All of the 48 *H. pylori*-negative cases (24 controls and 24 patients with gastric cancer) were sero-negative and IM grade was absent.

3. RNA isolation and miRNA microarray analysis

After manual dissection under microscopic guidance avoiding the contamination of inflammatory cells and stromal cells, hematoxylin and eosin stained sections 50 μm in thickness from cancerous and noncancerous regions of intestinal type of gastric cancer FFPE samples were reviewed by one pathologist (H.S.L.). Each section was incubated in xylene and total RNA was extracted using a RecoverAll™ Total Nucleic Acid Isolation kit (Life Technologies, Carlsbad, CA, USA). Each 400 ng RNA was dephosphorylated with 15 units of calf intestine alkaline phosphatase, followed by RNA denaturation with 40% dimethylsulfoxide. Dephosphorylated RNA was ligated with pCp-Cy3 mononucleotide and resuspended in Gene Expression Blocking Reagent and Hi-RPM Hybridization buffer. The denatured, labeled samples were pipetted onto assembled Agilent Human miRNA microarray Release 16.0 platform and hybridized at 55°C for 20 hours at 20 rpm. The hybridization images were analyzed using a DNA microarray scanner (Agilent Technologies, Palo Alto, CA, USA). The average fluorescence intensity for each spot was calculated and local background was subtracted. Data visualization and analysis were performed with GeneSpring GX 7.3 software (Agilent Technologies). Signal cutoff measurements

Table 1. Characteristics of the Subjects Used in the MicroRNA Microarray and TaqMan Assay Analyses

	miRNA microarray		TaqMan miRNA assay			
	Hp- GC (n=8)	*Hp*+ GC (n=8)	*Hp*- Cont. (n=24)	*Hp*+ Cont. (n=24)	*Hp*- GC (n=24)	*Hp*+ GC (n=28)
Age, yr	69.3±1.7	67.8±3.4	60.1±11.0	61.4±8.5	66.5±8.8	67.3±8.1
Male sex	6 (75)	5 (62.5)	17 (70.8)	15 (62.5)	19 (79.2)	18 (64.3)
Intestinal type histology	8 (100.0)	8 (100.0)	-	-	24 (100.0)	28 (100.0)

Data are presented as mean±standard error or number (%). *Hp*+, current active *Helicobacter pylori* infection if any one of these endoscopy-based tests are positive (*Campylobacter*-like organism [CLO] test, culture, and histology); *Hp*-, the absence of *H. pylori* by both endoscopy-based tests and serological testing.
miRNA, microRNA; *Hp*, *Helicobacter pylori*; GC, gastric cancer group; Cont., noncancer controls.

were <0.01.

4. Selection of miRNA candidates

The microarray showed 219 miRNAs which were at least 2-fold change between the *H. pylori*-positive and -negative cancer. Only 37 miRNAs were statistical significance (p<0.05) and those miRNAs were compared with miRNAs of Validated Targets associated with gastric cancer in miRWalk (http://www. umm.uni-heidelberg.de/apps/zmf/mirwalk)[17] and HMDD v2.0 (http://202.38.126.151/hmdd/tools/hmdd2.html).[18] Specifically, 19 miRNAs which were highly expressed in *H. pylori*-negative gastric cancer and 18 miRNAs which were highly expressed in *H. pylori*-positive gastric cancer were compared with target miRNAs of "stomach neoplasms" in each site. At last, 2-fold changed and statistically significant seven miRNAs which have been reported to be associated with gastric cancer were selected for next validation assay.

5. TaqMan miRNA validation assay

miRNA was extracted from the frozen gastric cancer tissue in gastric cancer patients and gastric body in control cases, which had been obtained during gastroscopy and had been kept at -80°C, with a mirVana™ miRNA Isolation Kit (Invitrogen, Carlsbad, CA, USA). Reverse transcription was performed using 5 μL of miRNA and TaqMan MicroRNA Reverse Transcription Kit and miRNA-specific stem-loop primers (Applied Biosystems, Foster City, CA, USA). The assays were carried in duplicate. The 20 μL reaction mixture contained reverse transcription reaction product, TaqMan Universal PCR Master Mix without uracil-N-glycosylase, TaqMan miRNA assay mix, and nuclease-free wa-
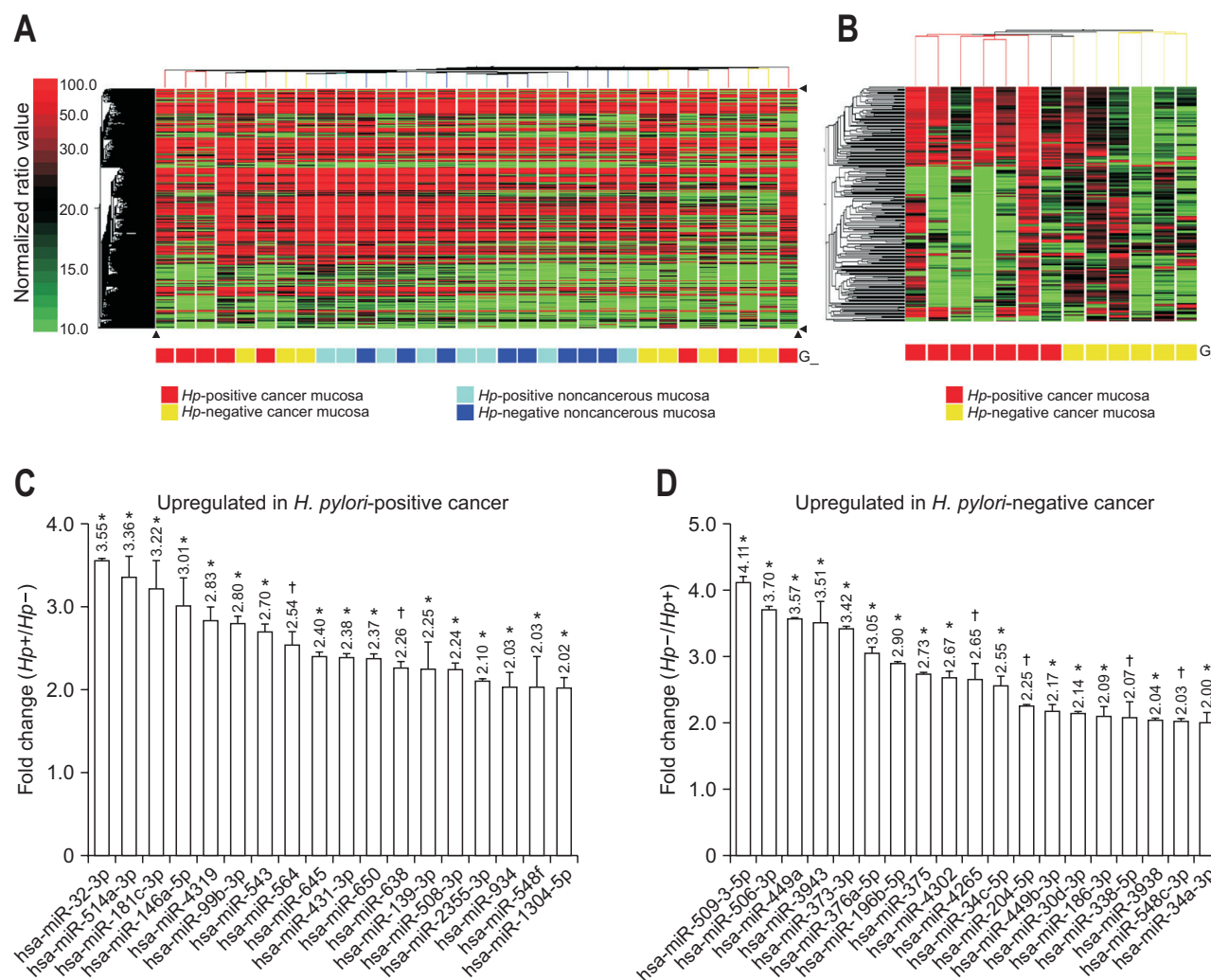
Fig. 1. Unsupervised hierarchical clustering analysis of gastric cancer formalin-fixed paraffin-embedded tissues. (A) A total of 1,781 microRNA (miRNA) probes were identified as differentially expressed between the cancerous and noncancerous regions of patients with gastric cancer. The higher normalized ratio value denotes higher expression levels of the miRNAs. (B) Two subclasses, *Helicobacter pylori* (*Hp*)-positive (n=7) and *H. pylori*-negative (n=6), showed clustering results of 219 miRNAs that exhibited 2-fold changes in gastric cancer samples. (C) A fold-change graph of 18 miRNAs upregulated in *H. pylori*-positive cancer. (D) A fold-change graph of 19 miRNAs upregulated in *H. pylori*-negative cancer. *p<0.05; †p<0.01.

ter. Amplification was performed using the 7500/7500 Fast PCR system (Applied Biosystems) at 50°C for 2 minutes and 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 60 seconds. Amplification signals were computed with 7500 software v2.0.6 (Applied Biosystems). Relative miRNA expression levels are presented as $2^{-\Delta\Delta Ct}$ method.

6. Statistical analysis

No interarray normalization was applied on the array, because the similarity between matched normal and cancer sample arrays was unknown. To identify distinct miRNAs hybridization signals, one-way analysis of variance (ANOVA) (p<0.05) and multiple testing correction (Benjamini and Hochberg false discovery rate) were employed for microarray clustering analysis. Relationship between two assays was evaluated by calculation of Spearman correlation tests. All statistical analyses were performed using the SPSS version 13.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

1. miRNA microarray data analysis

In all FFPE individual samples, the low intensity hybridiza-

tion signals (<0.01) between miRNAs and probes were filtered out and 1,781 of 3,523 (50.55%) miRNA probes remained as the dataset and were used for further analysis. Among the four subdivided groups (current H. pylori-positive cancer or noncancerous region, and H. pylori-negative cancer or noncancerous region), unsupervised hierarchical clustering of hybridization values showed clustering trends of the cancerous and noncancerous groups (Fig. 1A). Especially, no interarray normalization on normalized ratio and multiple testing corrections were used for getting rid of discordant three samples' hybridization signals and finally, clustering result of Fig. 1B shows 219 miRNAs which displayed at least a 2-fold changed expression between H. pylori-positive and -negative cancerous tissues. Among them, one-way ANOVA showed 19 miRNAs which were upregulated in H. pylori-negative cancerous tissue (p<0.05) and 18 miRNAs which were upregulated in H. pylori-positive cancerous tissue (p<0.05) (Table 2, Fig. 1C and D).

2. Selection of promising candidate miRNAs

Next, miRNA candidates which might be associated with gastric cancer were selected for validation assay. When 18 highly expressed miRNAs in H. pylori-positive cancer were compared

Table 2. Differential Expression of 37 MicroRNAs between the H. pylori-Positive and H. pylori-Negative Gastric Cancer Tissue Samples

	miRNA	Fold change*	p-value[†]		miRNA	Fold change	p-value
Up in Hp+ GC[‡]	hsa-miR-32-3p	3.55	<0.0213	Up in Hp- GC[§]	hsa-miR-509-3-5p	4.11	<0.0102
	hsa-miR-514a-3p	3.36	<0.0193		hsa-miR-506-3p	3.70	<0.0193
	hsa-miR-181c-3p	3.22	<0.0168		hsa-miR-449a	3.57	<0.015
	hsa-miR-146a-5p	3.01	<0.0279		hsa-miR-3943	3.51	<0.0458
	hsa-miR-4319	2.83	<0.0236		hsa-miR-373-3p	3.42	<0.0085
	hsa-miR-99b-3p	2.80	<0.0446		hsa-miR-376a-5p	3.05	<0.0244
	hsa-miR-543	2.70	<0.0227		hsa-miR-196b-5p	2.90	<0.0169
	hsa-miR-564	2.54	<0.0055		hsa-miR-375	2.73	<0.0182
	hsa-miR-645	2.40	<0.0322		hsa-miR-4302	2.67	<0.0406
	hsa-miR-431-3p	2.38	<0.0268		hsa-miR-4265	2.65	<0.0368
	hsa-miR-650	2.37	<0.0312		hsa-miR-34c-5p	2.55	<0.0049
	hsa-miR-638	2.26	<0.006		hsa-miR-204-5p	2.25	<0.0076
	hsa-miR-139-3p	2.25	<0.0273		hsa-miR-449b-3p	2.17	<0.024
	hsa-miR-508-3p	2.24	<0.0309		hsa-miR-30d-3p	2.14	<0.0366
	hsa-miR-2355-3p	2.10	<0.0394		hsa-miR-186-3p	2.09	<0.0233
	hsa-miR-934	2.03	<0.021		hsa-miR-338-5p	2.07	<0.0445
	hsa-miR-548f	2.03	<0.0292		hsa-miR-3938	2.04	<0.0237
	hsa-miR-1304-5p	2.02	<0.0363		hsa-miR-548c-3p	2.03	<0.0078
					hsa-miR-34a-3p	2.00	<0.0428

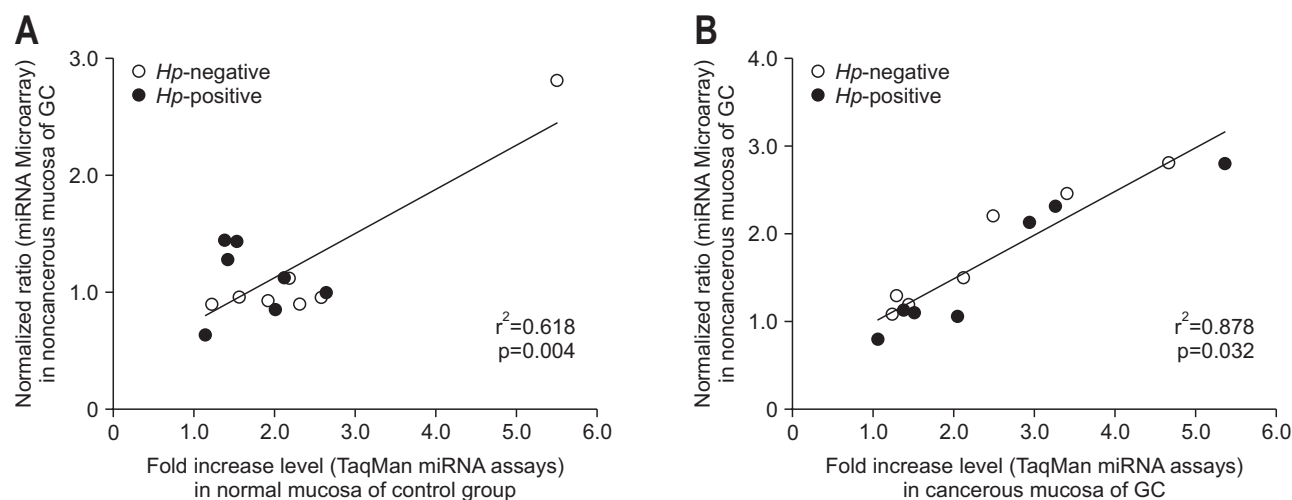miRNA, microRNA; Hp, *Helicobacter pylori*; GC, gastric cancer group.
*Fold change denotes reciprocal ratio, Hp+ GC/Hp- GC and Hp- GC/Hp+ GC. More than 2-fold changed hybridization signals were reserved for candidate miRNAs; [†]Differences in fold-change were considered statistically significant if the p-value of the one-way analysis of variance was <0.05; [‡]Up in Hp+ GC, upregulated in the cancerous regions of *H. pylori*-positive cancer; [§]Up in Hp- GC, upregulated in the cancerous regions of *H. pylori*-negative cancer.

Table 3. List of the Validation Assay Targets

	Stomach neoplasms*			Assay ID[‡]	Sequence
	miRWalk	HMDD	Conserved miRNAs[†]		
Up in *Hp*+ GC	*hsa-miR-32-3p*	*hsa-miR-99b-3p*	*hsa-miR-99b-3p*	002196	CAAGCUCGUGUCUGUGGGUCCG
	hsa-miR-99b-3p	*hsa-miR-146a-5p*	*hsa-miR-564*	001531	AGGCACGGUGUCAGCAGGC
	hsa-miR-139-3p	*hsa-miR-181c-3p*	*hsa-miR-638*	001582	AGGGAUCGCGGGCGGGUGGCGGCCU
	hsa-miR-564	*hsa-miR-564*			
	hsa-miR-638	*hsa-miR-638*			
	hsa-miR-650				
Up in *Hp*- GC	*hsa-miR-34a-3p*	*hsa-miR-30d-3p*	*hsa-miR-204-5p*	000508	UUCCCUUUGUCAUCCUAUGCCU
	hsa-miR-34c-5p	*hsa-miR-186-3p*	*hsa-miR-338-5p*	002658	AACAAUAUCCUGGUGCUGAGUG
	hsa-miR-196b-5p	*hsa-miR-204-5p*	*hsa-miR-375*	000564	UUUGUUCGUUCGGCUCGCGUGA
	hsa-miR-204-5p	*hsa-miR-338-5p*	*hsa-miR-548c-3p*	0001590	CAAAAAUCUCAAUUACUUUUGC
	hsa-miR-338-5p	*hsa-miR-375*			
	hsa-miR-373-3p	*hsa-miR-376a-5p*			
	hsa-miR-375	*hsa-miR-449a*			
	hsa-miR-548c-3p	*hsa-miR-449b-3p*			
		hsa-miR-548c-3p			

miRNA, microRNA; *Hp*, *Helicobacter pylori*; GC, gastric cancer group.
*The miRNA list of stomach neoplasms denotes the validated targets of two databases, miRWalk and HMDD. The validation study was conducted using seven conserved miRNAs; [†]Conserved miRNAs denotes overlapping miRNAs in the screened 37 miRNAs and the miRNA list from miRWalk and HMDD; [‡]ID of TaqMan miRNA assays in miRBase version 19.0. RNU6B was used as an endogenous control.


Fig. 2. Correlation analysis representing the corresponding fold-increase levels from the TaqMan miRNA assays for the same microRNAs (miRNAs) in the miRNA microarray. (A) Between noncancerous mucosa from *Helicobacter pylori* (*Hp*)-positive and *H. pylori*-negative gastric cancer groups and normal mucosa of *H. pylori*-positive and *H. pylori*-negative control groups. (B) Between cancerous mucosa of *H. pylori*-positive and *H. pylori*-negative cancer groups. r^2, Spearman correlation coefficient; p, value of paired Student t-test.
GC, gastric cancer group.

with "disease target miRNAs," 6 and 5 miRNAs were in agreement with target miRNAs of "stomach neoplasms" in miRWalk and HMDD, respectively. Then duplicated three miRNAs; *hsa-miR-99b-3p*, *hsa-miR-564*, and *hsa-miR-638*, were conserved (Table 3). In the same manner, among 19 highly expressed miRNAs in *H. pylori*-negative cancer, 8 and 9 miRNAs were matched with target miRNAs of "stomach neoplasms" in miR-

Walk and HMDD v2.0. Overlapped four miRNAs; *hsa-miR-204-5p*, *hsa-miR-338-5p*, *hsa-miR-375*, and *hsa-miR-548c-3p*, were conserved.

Finally, we used seven commercially available primers in TaqMan miRNA assays to confirm the miRNA microarray results (Table 3).
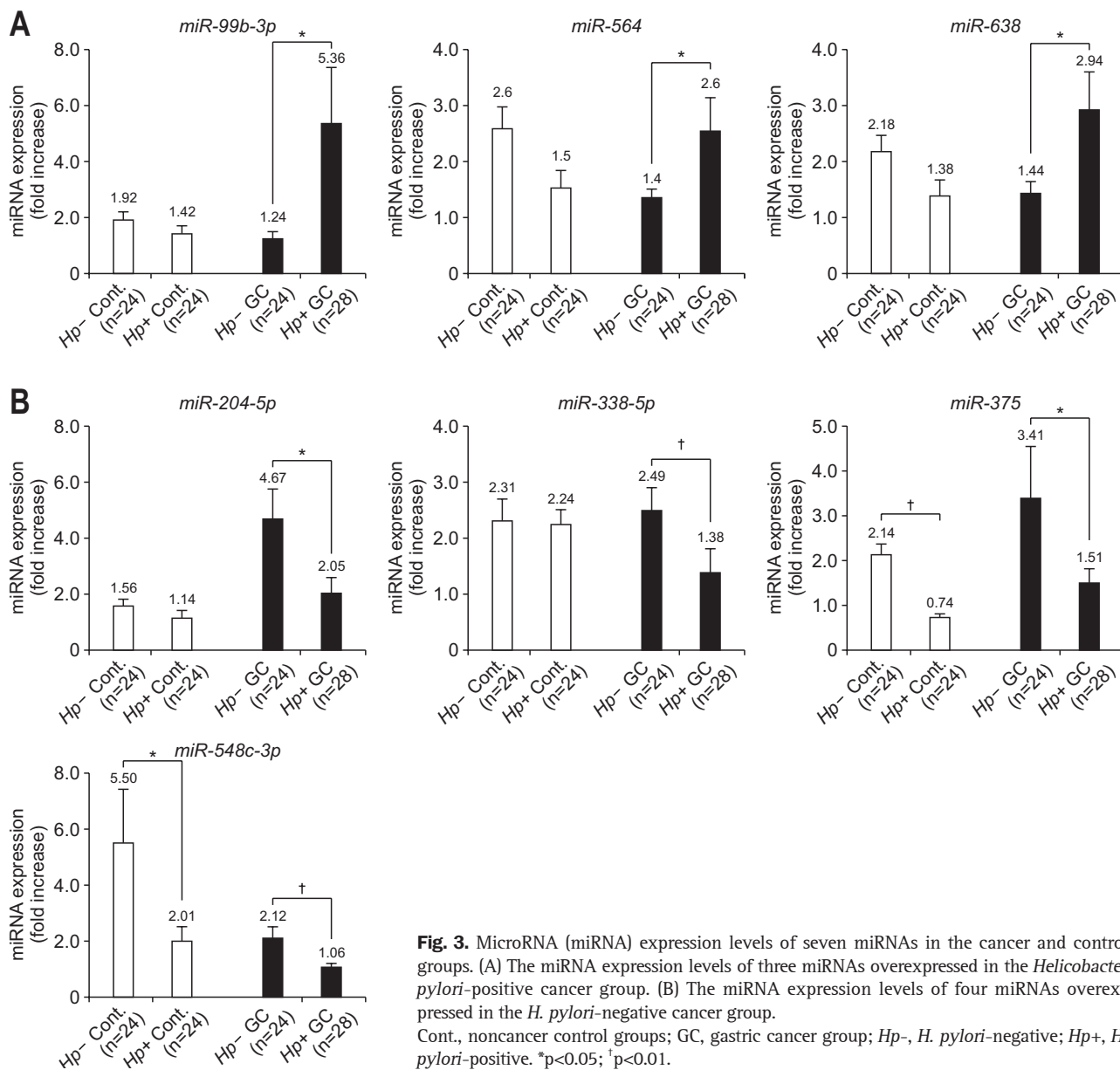
Fig. 3. MicroRNA (miRNA) expression levels of seven miRNAs in the cancer and control groups. (A) The miRNA expression levels of three miRNAs overexpressed in the *Helicobacter pylori*-positive cancer group. (B) The miRNA expression levels of four miRNAs overexpressed in the *H. pylori*-negative cancer group.
Cont., noncancer control groups; GC, gastric cancer group; *Hp*-, *H. pylori*-negative; *Hp*+, *H. pylori*-positive. *p<0.05; †p<0.01.

3. Correlation of candidate miRNAs and validation study by TaqMan miRNA assay

All TaqMan miRNA assays examined the fold-change of absolute expression levels of candidate miRNAs of each sample. To identify the correlation between the two assays, we compared the normalized ratio of the miRNA microarray hybridization signal and the fold-increase levels of the TaqMan miRNA assay. Although these two assays showed a low correlation (r^2=0.618, p=0.004) (Fig. 2A) with *H. pylori*-positive and -negative control groups, there was a considerably high correlation (r^2=0.878, p=0.032) (Fig. 2B) in cancer groups regardless of *H. pylori*-positive or -negative status. The miRNA expression in control subjects was not significantly different, except for *miR-375* and *miR-548c-3p*, irrespective of *H. pylori* infection. However, the

expression level of *miR-99b-3p*, *miR-564*, and *miR-638* in the *H. pylori*-positive cancer increased 4.32-, 2.53-, and 2.04-fold compared to -negative cancer, respectively (Fig. 3A). Also, the expression level of *miR-204-5p*, *miR-338-5p*, *miR-375*, and *miR-548-3p* increased 2.28-, 1.81-, 2.25-, and 2.00-fold, respectively, in the *H. pylori*-negative cancer group compared to the-positive cancer group (Fig. 3B).

DISCUSSION

There are some concerns about the integrity of miRNA from FFPE specimens and its suitability in the microarray assay because RNA in the FFPE tissue is fragmented and might be modified in the chemical reaction.[19] However, it is reported that miRNA is so small (about 20 nucleotides) that it cannot be degraded

in FFPE preparation.[3,4] And commercially available microarray platforms help to profile miRNA expression. Furthermore, there were a report which has shown a strong correlation in global miRNA expression between fresh frozen and FFPE human cancer samples using miRNA microarray platforms.[3-5] Supporting miRNA preserving characteristics of FFPE samples, our FFPE samples showed biologically-useful RNA integrity and optical density value (RIN=2.26±0.04 and $OD_{260/280}$=1.99±0.01, data not shown) in all eight paired RNAs. We found different result of cancerous region from noncancerous regions in the FFPE samples, and screened 219 miRNAs depending on *H. pylori* infection. For the clear results, multiple testing corrections were employed to minimize the probability of happening of these clustering results by chance originated from FFPE samples.

The miRWalk and HMDD v2.0 were employed to select candidate RNAs from 2-fold changed statistically significant 37 miRNAs. These two databases were created to offer a platform to scrutinize the mechanisms of miRNAs in disease.[17,18] miRWalk has a comprehensive database of miRNAs on experimentally validated miRNA targets associated with 549 diseases.[17] Also HMDD v2.0 provides experimentally supported miRNA and 397 diseases association data.[18] Because the aim of our study was screening and selecting specific miRNAs in gastric cancer before functional study, we found seven candidate miRNAs after comparing screened results with validated miRNA lists of "stomach neoplasms."

These seven miRNAs were presently validated with a good correlation (r^2=0.878, p=0.032) between the microarray and TaqMan miRNA assays. Similarly, a few studies also reported similar correlation tendency between Agilent miRNA microarrays and TaqMan miRNA assays, with r^2>0.9 and r^2=0.83.[20,21] Interestingly, this good correlation between microarray assay and TaqMan assay was not observed in normal mucosa of control group. The miRNA profile of noncancerous mucosa in cancer patients was not same as that of control patients[22,23] and we also got different hybridization signal between cancerous and noncancerous mucosa in the cancer patients. This less correlation in the normal mucosa of controls may be attributed to different molecular background. Anyway, in contrast to the different expression of microRNA between cancer tissue of *H. pylori*-positive and -negative cancer patients the miRNA expression in control subjects was not different, except for *miR-375* and *miR-548c-3p*, irrespective of *H. pylori* infection. These results might support the specificity of the different expression of seven miRNAs in the cancer tissue depending on *H. pylori* infection.

All of the seven miRNA candidates have been previously identified as potential regulator in gastric cancer aside from *H. pylori* infection. Interestingly, we found that three miRNAs (*miR-99b-3p*, *miR-564*, and *miR-638*) are strongly expressed in the *H. pylori*-positive gastric cancer tissue than *H. pylori*-negative. *miR-99b-3p* has been reported to be overexpressed in gastric cancer and a possible link between *miR-99b-3p* and the

epithelial-to-mesenchymal transition (EMT) induced by transforming growth factor β has been suggested.[24,25] As this miRNA was enhanced, especially, in the *H. pylori*-positive gastric cancer tissue in the present study *miR-99b-3p* might play a role in the *H. pylori*-positive gastric cancer tissue in terms of metastasis of gastric cancer by means of EMT. Now, we are investigating the role of *miR-99b-3p* on the EMT in the *H. pylori*-positive gastric cancer. Another candidate *miR-564* was reported to control malignant phenotypes of gastric cancer cells.[26] And its significantly suppressed expression levels in tumor material and in blood samples of gastric cancer patients has suggested that dysfunction of this miRNA may lead to gastric cancer.[27,28] Our result that *miR-564* has been overexpressed especially in the *H. pylori*-infected gastric cancer suggests that *H. pylori* infection somehow provokes *miR-564* resulting to gastric cancer. In case of *miR-638* its relationship with gastric cancer was not certain, so far. That is, the *miR-638* expression level of gastric cancer mucosa was lower than normal mucosa.[29] However, *miR-638* has been reported to be a specific oncomir in gastric cancer.[30] The present study result strongly supports a relationship between *miR-638* and *H. pylori*-positive gastric cancer. Further functional analyses are necessary to uncover the relationships between these miRNAs and gastric carcinogenesis depending on *H. pylori* infection.

Other four miRNAs (*miR-204-5p*, *miR-338-5p*, *miR-375*, and *miR-548c-3p*) were strongly expressed in the *H. pylori*-negative gastric cancer tissue than *H. pylori*-positive. In case of *miR-204-5p* its downregulated expression has caused to overexpression of ezrin target and Ras activation, which promoted development of gastric cancer.[31] Interestingly, it increased in intestinal metaplastic gland tissue after *H. pylori* eradication, suggesting a negative relationship with *H. pylori* infection.[32] And our study result could support this report. However, it also needs functional study. Recent study has identified a miRNA signature of *miR-338-5p* for overall survival and relapse-free survival of gastric cancer patients.[33] Deregulated *miR-338* in drug resistant cell and its higher expression in gastric cancer patients show a possible involvement of *miR-338* in gastric cancer pathogenesis.[29,34] *miR-338-5p* overexpression could be related with poor prognosis of *H. pylori*-negative cancer than *H. pylori*-positive although it needs further study.[11,35]

Especially, a significant inverse correlation between *miR-375* expression and JAK2 protein level in gastric cancer and a suppression of potent antiapoptotic 14-3-3ζ, PDK1 and Akt phosphorylation in *miR-375* transfected cell were reported.[36,37] These data suggest that it may function as a tumor suppressor. Unexpectedly, as *miR-375* has been overexpressed in the *H. pylori*-negative gastric cancer in the present study it is rather puzzling and challenging. In the future it also needs functional study. Especially, *miR-548c-3p* overexpression was reported in blood of gastric cancer patients.[24,38,39] However, this report did not classify this result depending on *H. pylori*-positivity. Accord-

ing to our study, this higher expression of *miR-548c-3p* might be more definite in case of *H. pylori*-negative gastric cancer. Taken together all of these results suggest that miRNAs might be differently involved in gastric carcinogenesis depending on *H. pylori* infection. Also, to the best of our knowledge, the present study is the first to address the different expression patterns of these seven miRNAs in terms of *H. pylori* infection in gastric cancer and noncancer control patients.

This study has a few limitations. First, microarray experiments were performed in the only 16 cancer patients. However, our data showed good correlation between the microarray and TaqMan miRNA assay. In addition, we tried to leave little sample-to-sample variation in this validation assay by distinguishing the patients according not only to the *H. pylori* infection but also in an intestinal metaplasia and all cases were intestinal type of cancer. Secondly, normal control groups were not included in the miRNA microarray in this study. The main reason was the difficulty of FFPE gastric tissue of normal control. Instead, we screened miRNAs displaying more than 2-fold altered expression level between cancerous and noncancerous tissues in the same host. Then, we included normal gastric mucosa in the validation study step to reinforce microarray results, in comparison to miRNA expression patterns with cancer tissue and normal mucosa of the control group depending on *H. pylori* infection. Interestingly, there was a significant difference in cancer tissue between *H. pylori*-positive and -negative, but not in the normal mucosa, with the exception of *miR-375* and *miR-548c-3p*.

In conclusion, with the findings of microRNA microarray using FFPE specimens, the results of this study demonstrated that there are somewhat different miRNA expression patterns in cancerous region of intestinal type gastric cancer depending on *H. pylori* infection. And validation assay with selected seven candidate miRNAs confirmed the different expression of miR-NAs. Functional study is in progress to identify how these seven miRNAs could lead to different gastric carcinogenesis between *H. pylori*-positive and -negative cancer and what target genes would be regulated.

CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

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REFERENCES

1. Lu J, Getz G, Miska EA, et al. MicroRNA expression profiles classify human cancers. Nature 2005;435:834-838.

2. Iorio MV, Croce CM. microRNA involvement in human cancer. Carcinogenesis 2012;33:1126-1133.

3. Li J, Smyth P, Flavin R, et al. Comparison of miRNA expression patterns using total RNA extracted from matched samples of formalin-fixed paraffin-embedded (FFPE) cells and snap frozen cells. BMC Biotechnol 2007;7:36.

4. Glud M, Klausen M, Gniadecki R, et al. MicroRNA expression in melanocytic nevi: the usefulness of formalin-fixed, paraffin-embedded material for miRNA microarray profiling. J Invest Dermatol 2009;129:1219-1224.

5. Zhang X, Chen J, Radcliffe T, Lebrun DP, Tron VA, Feilotter H. An array-based analysis of microRNA expression comparing matched frozen and formalin-fixed paraffin-embedded human tissue samples. J Mol Diagn 2008;10:513-519.

6. Nomura AM, Perez-Perez GI, Lee J, Stemmermann G, Blaser MJ. Relation between *Helicobacter pylori* cagA status and risk of peptic ulcer disease. Am J Epidemiol 2002;155:1054-1059.

7. Belair C, Darfeuille F, Staedel C. Helicobacter pylori and gastric cancer: possible role of microRNAs in this intimate relationship. Clin Microbiol Infect 2009;15:806-812.

8. Parsonnet J, Friedman GD, Vandersteen DP, et al. Helicobacter pylori infection and the risk of gastric carcinoma. N Engl J Med 1991;325:1127-1131.

9. Lauren P. The two histological main types of gastric carcinoma: diffuse and so-called intestinal-type carcinoma. An attempt at a histo-clinical classification. Acta Pathol Microbiol Scand 1965;64:31-49.

10. Jeong O, Park YK. Clinicopathological features and surgical treatment of gastric cancer in South Korea: the results of 2009 nationwide survey on surgically treated gastric cancer patients. J Gastric Cancer 2011;11:69-77.

11. Yoon H, Kim N, Lee HS, et al. Helicobacter pylori-negative gastric cancer in South Korea: incidence and clinicopathologic characteristics. Helicobacter 2011;16:382-388.

12. Ruzzo A, Graziano F, Pizzagalli F, et al. Interleukin 1B gene (IL-1B) and interleukin 1 receptor antagonist gene (IL-1RN) polymorphisms in Helicobacter pylori-negative gastric cancer of intestinal and diffuse histotype. Ann Oncol 2005;16:887-892.

13. Ye H, Liu H, Raderer M, et al. High incidence of t(11;18)(q21;q21) in Helicobacter pylori-negative gastric MALT lymphoma. Blood 2003;101:2547-2550.

14. Matsushima K, Isomoto H, Inoue N, et al. MicroRNA signatures in Helicobacter pylori-infected gastric mucosa. Int J Cancer 2011;128:361-370.

15. Shin CM, Kim N, Jung Y, et al. Role of Helicobacter pylori infection in aberrant DNA methylation along multistep gastric carcinogenesis. Cancer Sci 2010;101:1337-1346.

16. Kim N, Park RY, Cho SI, et al. Helicobacter pylori infection and

development of gastric cancer in Korea: long-term follow-up. J Clin Gastroenterol 2008;42:448-454.

17. Dweep H, Sticht C, Pandey P, Gretz N. miRWalk-database: prediction of possible miRNA binding sites by "walking" the genes of three genomes. J Biomed Inform 2011;44:839-847.

18. Lu M, Zhang Q, Deng M, et al. An analysis of human microRNA and disease associations. PLoS One 2008;3:e3420.

19. Liu A, Xu X. MicroRNA isolation from formalin-fixed, paraffin-embedded tissues. Methods Mol Biol 2011;724:259-267.

20. Ach RA, Wang H, Curry B. Measuring microRNAs: comparisons of microarray and quantitative PCR measurements, and of different total RNA prep methods. BMC Biotechnol 2008;8:69.

21. Git A, Dvinge H, Salmon-Divon M, et al. Systematic comparison of microarray profiling, real-time PCR, and next-generation sequencing technologies for measuring differential microRNA expression. RNA 2010;16:991-1006.

22. Volinia S, Calin GA, Liu CG, et al. A microRNA expression signature of human solid tumors defines cancer gene targets. Proc Natl Acad Sci U S A 2006;103:2257-2261.

23. Ando T, Yoshida T, Enomoto S, et al. DNA methylation of microRNA genes in gastric mucosae of gastric cancer patients: its possible involvement in the formation of epigenetic field defect. Int J Cancer 2009;124:2367-2374.

24. Guo J, Miao Y, Xiao B, et al. Differential expression of microRNA species in human gastric cancer versus non-tumorous tissues. J Gastroenterol Hepatol 2009;24:652-657.

25. Turcatel G, Rubin N, El-Hashash A, Warburton D. MIR-99a and MIR-99b modulate TGF-β induced epithelial to mesenchymal plasticity in normal murine mammary gland cells. PLoS One 2012;7:e31032.

26. Inui M, Martello G, Piccolo S. MicroRNA control of signal transduction. Nat Rev Mol Cell Biol 2010;11:252-263.

27. Kim CH, Kim HK, Rettig RL, et al. miRNA signature associated with outcome of gastric cancer patients following chemotherapy. BMC Med Genomics 2011;4:79.

28. Shah AA, Leidinger P, Backes C, et al. A set of specific miRNAs is connected with murine and human gastric cancer. Genes Chromosomes Cancer 2013;52:237-249.

29. Yao Y, Suo AL, Li ZF, et al. MicroRNA profiling of human gastric cancer. Mol Med Rep 2009;2:963-970.

30. Krutovskikh VA, Herceg Z. Oncogenic microRNAs (OncomiRs) as a new class of cancer biomarkers. Bioessays 2010;32:894-904.

31. Lam EK, Wang X, Shin VY, et al. A microRNA contribution to aberrant Ras activation in gastric cancer. Am J Transl Res 2011;3:209-218.

32. Shiotani A, Iishi H, Uedo N, et al. Evidence that loss of sonic hedgehog is an indicator of Helicobater pylori-induced atrophic gastritis progressing to gastric cancer. Am J Gastroenterol 2005;100:581-587.

33. Li X, Zhang Y, Zhang Y, Ding J, Wu K, Fan D. Survival prediction of gastric cancer by a seven-microRNA signature. Gut 2010;59:579-585.

34. Wu XM, Shao XQ, Meng XX, et al. Genome-wide analysis of microRNA and mRNA expression signatures in hydroxyc-amptothecin-resistant gastric cancer cells. Acta Pharmacol Sin 2011;32:259-269.

35. Marrelli D, Pedrazzani C, Berardi A, et al. Negative Helicobacter pylori status is associated with poor prognosis in patients with gastric cancer. Cancer 2009;115:2071-2080.

36. Ding L, Xu Y, Zhang W, et al. MiR-375 frequently downregulated in gastric cancer inhibits cell proliferation by targeting JAK2. Cell Res 2010;20:784-793.

37. Tsukamoto Y, Nakada C, Noguchi T, et al. MicroRNA-375 is downregulated in gastric carcinomas and regulates cell survival by targeting PDK1 and 14-3-3zeta. Cancer Res 2010;70:2339-2349.

38. Kozomara A, Griffiths-Jones S. miRBase: integrating microRNA annotation and deep-sequencing data. Nucleic Acids Res 2011;39:D152-D157.

39. Li Y, Xie J, Xu X, et al. MicroRNA-548 down-regulates host antiviral response via direct targeting of IFN-lambda1. Protein Cell 2013;4:130-141.