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의학석사 학위논문

Different microRNA expressions in gastric cancer depending on *Helicobacter pylori* infection

Helicobacter pylori 감염 여부에 따른 위암 내 microRNA 발현 차이 연구

2014년 2월

서울대학교 대학원

의학과 분자유전체전공

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Different microRNA expressions in gastric cancer depending on *Helicobacter pylori* infection

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지도 교수 김 나 영

이 논문을 의학석사 학위논문으로 제출함

2013 년 **10** 월

서울대학교 대학원

의학과 분자유전체전공

장 현

장현의 의학석사 학위논문을 인준함

2013 년 **12** 월

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학위논문 원문제공 서비스에 대한 동의서

본인의 학위논문에 대하여 서울대학교가 아래와 같이 학위논문 제공하는 것에 동의합니다.

1. 동의사항

① 본인의 논문을 보존이나 인터넷 등을 통한 온라인 서비스 목적으로 복제할 경우 저작물의 내용을 변경하지 않는 범위 내에서의 복제를 허용합니다.

② 본인의 논문을 디지털화하여 인터넷 등 정보통신망을 통한 논문의 일부 또는 전부의 복제.배포 및 전송 시 무료로 제공하는 것에 동의합니다.

2. 개인(저작자)의 의무

본 논문의 저작권을 타인에게 양도하거나 또는 출판을 허락하는 등 동의 내용을 변경하고자 할 때는 소속대학(원)에 공개의 유보 또는 해지를 즉시 통보하겠습니다.

3. 서울대학교의 의무

① 서울대학교는 본 논문을 외부에 제공할 경우 저작권 보호장치(DRM)를 사용하여야 합니다.

② 서울대학교는 본 논문에 대한 공개의 유보나 해지 신청 시 즉시 처리해야 합니다.

논문제목 : Different microRNA expressions in gastric cancer depending on *Helicobacter pylori* infection

학위구분 : 석사 □ · 박사 □
학 과 : 의학과
학 번 : 2012-21754
연 락 처 :
저 작 자 : 장 현 (인)

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서울대학교총장 귀하

Abstract

Background and aim: *Helicobacter pylori* (*H. pylori*) infection increases the risk of gastric cancer through inducing aberrant gene expression regulation of cell. The microRNA (miRNA) regulate downstream target gene which can control cell proliferation and differentiation. The expressions of miRNA are usually analyzed with microarray and FFPE (formalin fixed paraffin embedded) sample can be used for microarray demanding high quality RNA. The study was undertaken to identify microRNAs differently expressed by *H. pylori* infection in patients with intestinal type of gastric cancer using miRNA microarray, and to confirm the candidate miRNAs expression levels.

Methods: Total RNA was extracted from cancerous region and non-cancerous regions in formalin fixed paraffin embedded tissues of intestinal type gastric cancer patients who were *H. pylori*-positive (n=8) or -negative (n=8). The RNA was analyzed with a 3,523 miRNA profiling microarray based on the Sanger miRBase. Validation analysis was performed using TaqMan miRNA assays.

Results: 219 miRNAs in the aberrant miRNA profiles across the miRNA microarray showed at least two-fold changed different expression in *H. pylori*-positive and -negative cancer tissue. Seven statistically significant candidate miRNAs were selected with online miRNA databases; miRWalk and HMDD. TaqMan miRNA assays confirmed that three miRNAs (*miR-99b-3p, miR-564* and *miR-638*) were significantly increased in *H. pylori*-positive cancer than *H. pylori*-negative. In addition, four miRNAs (*miR-204-5p, miR-*

338-5p, miR-375 and *miR-548c-3p*) were significantly increased in *H. pylori*-negative cancer than *H. pylori*-positive.

Conclusion: The miRNA expression in the intestinal type of gastric cancer depending on *H. pylori* infection suggest that different gastric cancer pathogenesis could be exist between *H. pylori*-positive and -negative gastric cancer. FFPE specimens can be used for investigating the miRNA expression patterns. Further study which focused on miRNA function in gastric carcinogenesis is needed.

Keywords: Gastric carcinoma; *Helicobacter pylori*; Microarray; MicroRNA; TaqMan miRNA assays

Student Number: **2012-21754**

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Introduction

About twenty length nucleotides of non-protein 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 fresh tissue specimens is recommended to reduce collateral damage of miRNAs. However it is hard to get fresh tissue specimens right after operations, so 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* (*H. 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 different genetic

background in gastric mucosa depending on *H. pylori* infection and 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

Study 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. Cancerous and adjacent non-cancerous regions of cancer FFPE samples were prepared as per patient, respectively. 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 sixteen 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 MALT 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 (IRB No. B-1301-186-111). All participants provided their written informed consent to participate in this study.

Helicobacter pylori **testing**

Three types of *H. pylori* testing (histology, 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, South 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.

RNA isolation and miRNA microarray analysis

After manual dissection under microscopic guidance avoiding the contamination of inflammatory cells and stromal cells, H&E stained sections 50 μm in thickness from cancerous and adjacent non-cancerous 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

Table 1. Characteristics of the miRNA microarray and TaqMan assay study subjects

miRNA microarray				
Study subjects	*Hp*[*]- GC[†] (n = 8)	*Hp*+ GC (n = 8)		
Age (years, mean±SE[§])	69.3±1.7	67.8±3.4		
Male Gender, n (%)	6 (75)	5 (62.5)		
Intestinal type histology, n (%)	8 (100)	8 (100)		
TaqMan miRNA assay				
Study subjects	*Hp*- Cont.[‡] (n = 24)	*Hp*+ Cont. (n = 24)	*Hp*- GC (n = 24)	*Hp*+ GC (n = 28)
Age (years, mean±SE[§])	60.1±11.0	61.4±8.5	66.5±8.8	67.3±8.1
Male Gender, n (%)	17 (70.8)	15 (62.5)	19 (79.2)	18 (64.3)
Intestinal type histology, n (%)	-	-	24 (100)	28 (100)

"*Hp*+" means current active *H. pylori* infection if any one of these endoscopy-based tests show positive (CLO-test, culture and histology). "*Hp*-" means the absence of *H. pylori* both on endoscopy-based tests and on serology testing.

[*]*Hp*, *Helicobacter pylori;* [†]GC, gastric cancer patients; [‡]Cont. non-cancer controls; [§]SE, standard error.

extracted using a RecoverAll™ Total Nucleic Acid Isolation kit (Life Technologies, 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℃ for 20 hrs at 20 rpm. The hybridization images were analyzed using a DNA microarray scanner (Agilent Technologies, 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 cut-off measurements were <0.01.

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*-value<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 nineteen miRNAs which were highly expressed in *H. pylori*-negative gastric cancer and eighteen 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.

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, 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). The assays were carried out on 96-well optical reaction plates in duplicate using following miRNAs (assay ID indicated in brackets): *hsa-miR-99b-3p* (002196); *hsa-miR-204-5p* (000508); *hsa-miR-338-5p* (002658); *hsa-miR-375* (000564); *hsa-miR-548c-3p* (001590); *hsa-miR-564* (001531); *hsa-miR-638* (001582); and RNU6B (001093) as the control. 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 water. Amplification was performed using the 7500/7500 Fast PCR system (Applied Biosystems) at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 60 sec. Amplification signals were computed with 7500 software v2.0.6 (Applied Biosystems). Relative miRNA expression levels are presented as $2^{-\Delta\Delta Ct}$ method.

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 ANOVA (*P*-value<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's

correlation tests. All statistical analyses were performed using the Statistical Package for the Social Sciences version 13.0 (SPSS, IL, USA).

Results

RNA isolation and miRNA microarray data analysis

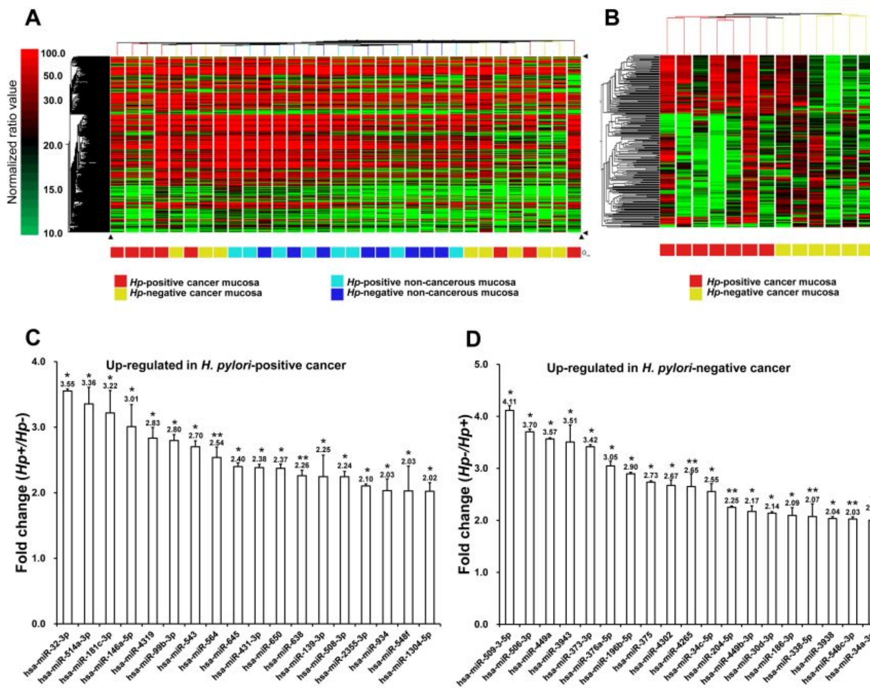
Agilent 2100 Bioanalyzer was used for analyzing quantity and quality of RNA from FFPE specimens. The average RNA concentration is 527.74 ng/ul, average $OD_{260/280}$ value is 1.99 and RNA integrity number (RIN) is 2.26. The RIN algorithm provides information about the RNA integrity and the low RNA Integrity Number (around 2.0) is typical for FFPE extractions (3).

In all FFPE individual samples, the low intensity hybridization 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 non-cancerous region, and *H. pylori*-negative cancer or non-cancerous region), unsupervised hierarchical clustering of hybridization values showed clustering trends of the cancerous and non-cancerous 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 Figure 1B shows 219 miRNAs which displayed at least a two-fold changed expression between *H. pylori*-positive and -negative cancerous tissues. Among them, one-way ANOVA showed 19 miRNAs which were up-regulated in *H. pylori*-negative cancerous tissue (*P*-value<0.05) and 18 miRNAs which were up-regulated in *H. pylori*-positive cancerous tissue (*P*-value<0.05) (Fig. 1C and 1D, Table 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 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 miRWalk 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.

Figure 1. Unsupervised hierarchical clustering analysis of gastric cancer FFPE tissue



A. A total of 1,781 miRNA probes were identified as differentially expressed between cancerous region and non-cancerous region of gastric cancer patients. The higher normalized ratio value denotes more expression level of miRNAs. B. Two subclasses; *H. pylori*-positive (n = 7) and -negative (n = 6) showed clustering results of 219 miRNAs that exhibited a two-fold change in gastric cancer samples. C. A fold-change graph of 18 miRNAs up-regulated in *H. pylori*-positive cancer. D. A fold-change graph of 19 miRNAs up-regulated in *H. pylori*-negative cancer. * *P*-value <0.05; ** *P*-value <0.01; *Hp*, *Helicobacter pylori*

Table 2. Different expression of 37 miRNAs between *H. pylori*-positive and -negative gastric cancer

	miRNA	Fold change*	*P*†		miRNA	Fold change	*P*
Up in	*miR-32-3p*	3.55	<0.02	Up in	*miR-509-3-5p*	4.11	<0.02
Hp+ GC‡	*miR-514a-3p*	3.36	<0.02	*Hp*- GC§	*miR-506-3p*	3.70	<0.02
	miR-181c-3p	3.22	<0.02		*miR-449a*	3.57	<0.02
	miR-146a-5p	3.01	<0.03		*miR-3943*	3.51	<0.05
	miR-4319	2.83	<0.02		*miR-373-3p*	3.42	<0.01
	miR-99b-3p	2.80	<0.05		*miR-376a-5p*	3.05	<0.03
	miR-543	2.70	<0.03		*miR-196b-5p*	2.90	<0.02
	miR-564	2.54	<0.01		*miR-375*	2.73	<0.02
	miR-645	2.40	<0.04		*miR-4302*	2.67	<0.05
	miR-431-3p	2.38	<0.03		*miR-4265*	2.65	<0.04
	miR-650	2.37	<0.04		*miR-34c-5p*	2.55	<0.01
	miR-638	2.26	<0.01		*miR-204-5p*	2.25	<0.01
	miR-139-3p	2.25	<0.03		*miR-449b-3p*	2.17	<0.03
	miR-508-3p	2.24	<0.04		*miR-30d-3p*	2.14	<0.04
	miR-2355-3p	2.10	<0.04		*miR-186-3p*	2.09	<0.03
	miR-934	2.03	<0.03		*miR-338-5p*	2.07	<0.05
	miR-548f	2.03	<0.03		*miR-3938*	2.04	<0.03
	miR-1304-5p	2.02	<0.04		*miR-548c-3p*	2.03	<0.01
					miR-34a-3p	2.00	<0.05

*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 value of one-way ANOVA *P*-value was less than 0.05. ‡Up in *Hp*+ GC, up-regulated in cancerous region of *H. pylori*-positive cancer; §Up in *Hp*- GC, up-regulated in cancerous region of *H. pylori*-negative cancer.

Finally we used seven commercially available primers in TaqMan miRNA assays to confirm the miRNA microarray results (Table 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*-value=0.004, Fig. 2A) with *H. pylori*-positive and -negative control groups, there was a considerably high correlation (R^2=0.878; *P*-value=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 non-cancerous 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). The 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'.

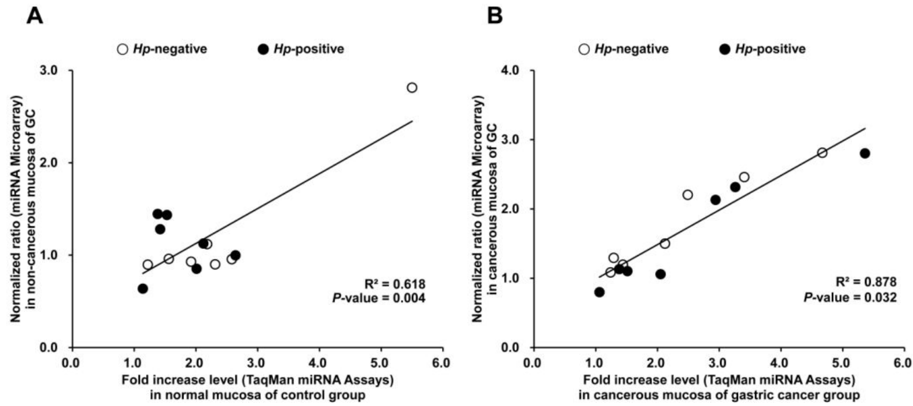
These seven miRNAs were presently validated with a good correlation (R^2=0.878; *P*-value=0.032) between the microarray and TaqMan miRNA assays. Similarly, a few studies also reported similar correlation tendency between Agilent miRNA microarrays and TaqMan miRNA assays, with R^2>0.9 and R^2=0.83 (20, 21). Interestingly, this good correlation between

Table 3. List of the validation assay targets

	Stomach Neoplasms*		
	miRWalk	HMDD	Conserved miRNAs†
Up in *Hp*+ GC	*miR-32-3p*	*miR-99b-3p*	*miR-99b-3p*
	miR-99b-3p	*miR-146a-5p*	*miR-564*
	miR-139-3p	*miR-181c-3p*	*miR-638*
	miR-564	*miR-564*	
	miR-638	*miR-638*	
	miR-650		
Up in *Hp*- GC	*miR-34a-3p*	*miR-30d-3p*	*miR-204-5p*
	miR-34c-5p	*miR-186-3p*	*miR-338-5p*
	miR-196b-5p	*miR-204-5p*	*miR-375*
	miR-204-5p	*miR-338-5p*	*miR-548c-3p*
	miR-338-5p	*miR-375*	
	miR-373-3p	*miR-376a-5p*	
	miR-375	*miR-449a*	
	miR-548c-3p	*miR-449b-3p*	
		miR-548c-3p	

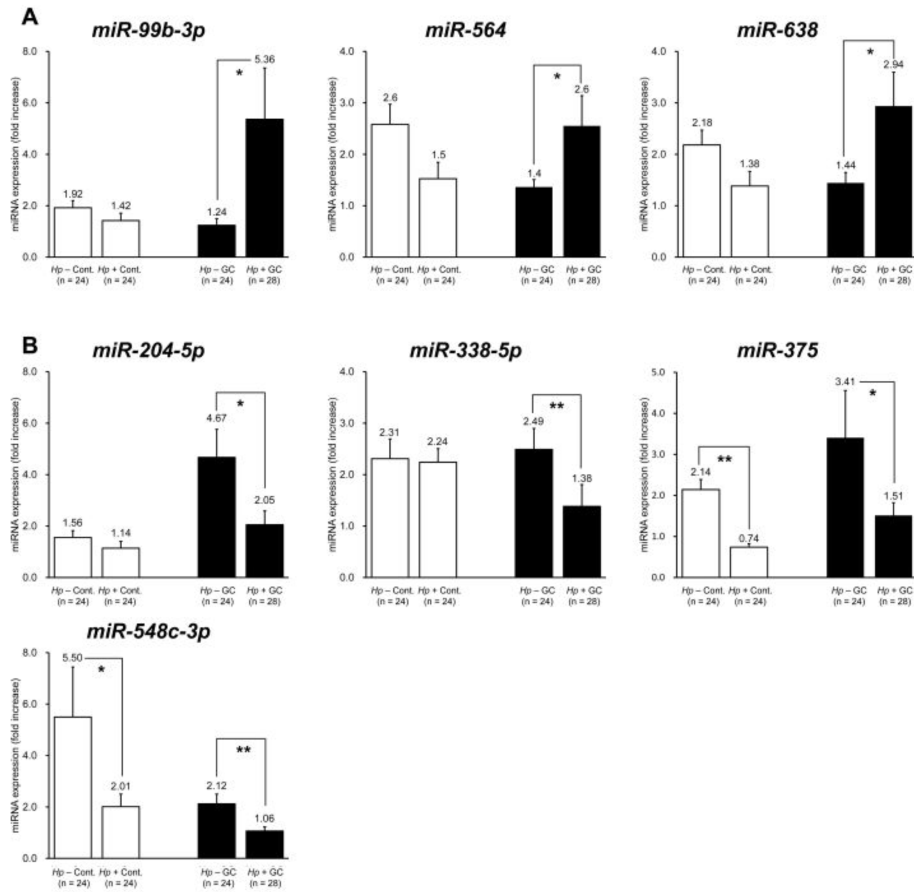
*The miRNA list of 'Stomach Neoplasms' denotes validated target of two databases, miRWalk and HMDD. Next validation study was undertaken with seven conserved miRNAs. †'Conserved miRNAs' denotes overlapped miRNAs in the screened 37 miRNAs and miRNA list of miRWalk and HMDD.

Figure 2. A correlation analysis representing the corresponding fold-increase level of TaqMan miRNA assays for the same miRNAs in the miRNA microarray



A. between non-cancerous mucosa of *H. pylori*-positive and -negative gastric cancer and normal mucosa of *H. pylori*-positive and -negative control groups. B. between cancerous mucosa of *H. pylori*-positive cancer and -negative cancer groups. R^2=Spearman's correlation coefficient; *P*-value=value of paired Student's t-test

Figure 3. miRNA expression level of seven miRNAs in cancer and control groups



A. The miRNA expression level of three miRNAs over-expressed in *H. pylori*-positive cancer group. B. The miRNA expression level of four miRNAs over-expressed in *H. pylori*-negative cancer group. * *P*-value <0.05; ** *P*-value <0.01; Cont, non-cancer control groups; GC, Gastric cancer group; *Hp* -, *H. pylori*-negative; *Hp* +, *H. pylori*-positive

microarray assay and TaqMan assay was not observed in normal mucosa of control group. The miRNA profile of non-cancerous mucosa in cancer patients was not same as that of control patients (22, 23) and we also got different hybridization signal between cancerous- and non-cancerous 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 TGF-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 down-regulated expression has caused to over-expression 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. According 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 non-cancer control patients.

This study has a few weaknesses. First, microarray experiments were performed in the only sixteen 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. Second, 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 and the high cost. So we decided to use commercially affordable RNA extraction kit for FFPE specimens and followed manufacturer's instructions thoroughly. Then we screened miRNAs displaying more than 2-fold altered expression level between cancerous and non-cancerous 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 miRNAs. 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.

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국문 초록

Helicobacter pylori 감염 여부에 따른 위암 내 microRNA 발현 차이 연구

서론: *Helicobacter pylori* (*H. pylori*)의 감염은 세포 내 비정상적 gene regulation을 유발하여 위암의 발생위험을 증가시킨다. microRNA (miRNA)는 세포의 생장 및 분화를 제어하는 하부단계의 표적 유전자를 조절한다. miRNA 발현 분석에 주로 사용되는 microarray에는 높은 순도의 RNA가 필요하며 이에 FFPE (formalin fixed paraffin embedded) 시료가 사용될 수 있다. 이에 본 연구는 FFPE 시료를 사용해 장형 위암 조직에서 *H. pylori* 감염 여부에 따라 miRNA의 발현이 microarray 상에서 다른지 찾아보고, 동결 보존된 조직에서 후보 miRNA의 발현량을 확인하고자 하였다.

방법: *H. pylori* 양성 (8명) 및 음성 (8명)인 장형 위암 수술 환자의 위암 FFPE 시료에서 암 중심부과 비위암성 변연부 조직으로부터 RNA를 채취하였다. Sanger miRBase 기준 총 3,523개의 miRNA를 대상으로 microarray를 수행하였다. 각 miRNA의 발현량 입증 시험은 TaqMan miRNA assays를 이용하였다.

결과: 총 219 개의 miRNA 가 microarray 에서 *H. pylori* 양성과 음성 위암에서 miRNA 발현량이 서로 2 배 이상 나타났다. 통계적으로

유의한 총 7 개의 후보 miRNA 를 miRWalk 와 HMDD 데이터베이스 검색을 통해 얻었다. TaqMan miRNA assays 에서는 3 개의 miRNA (*miR-99b-3p, miR-564*, *miR-638*)가 *H. pylori* 양성 위암 군에서 음성 군보다 더 많이 발현함을 확인했다. 또한, 4 개의 miRNA (*miR-204-5p, miR-338-5p, miR-375*, *miR-548c-3p*)가 *H. pylori* 음성 위암 군에서 양성 군보다 유의하게 더 많이 발현했다.

결론: *H. pylori* 감염 여부에 따른 장형 위암 내 miRNA 의 발현은 *H. pylori* 양성 및 음성 위암간 서로 다른 발암기전이 존재할 가능성을 제시한다. 이러한 miRNA 발현 양상을 조사하기 위해 FFPE 시료가 유용하게 사용될 수 있다. 향후 후보 miRNA 의 위암 발암기전 대한 추가실험이 더 필요하다.

주요어: 위암; *Helicobacter pylori*; Microarray; MicroRNA; TaqMan miRNA assays

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