(Agilent Technologies) according to the manufacturer's instructions. Subsequently, labeled DNA was hybridized on the miRNA promoter microarray (Fig. 2).

2.4. ChIP-PCR

Quantitative analysis of ChIP products was performed by real-time PCR with the CYBR Premix Ex Taq (Takara Bio, Ohtsu, Japan) using the Thermal Cycler Dice Real-Time System (Takara Bio). The sequences of the primers used were as follows: *miR-9-1* Forward: 5′-CTCAAGGAGAGAAGGAAACAGC-3′, *miR-9-1* Reverse: 5′-TCACAACCCTGGGTGATCTC -3′; *miR-9-3* Forward: 5′-GCTAGAT CTACTGCAAGTGCTG-3′, *miR-9-3* Reverse: 5′-GGACCATCAGAGTTT GG GAG-3′.

The fraction of immunoprecipitated DNA was calculated as follows: (immunoprecipitated DNA with each antibody – nonspecific antibody control (NAC))/(input DNA – NAC).

2.5. Tissue specimens of gastric cancers

Tissue specimens from advanced gastric cancers and the surrounding non-tumor gastric mucosae were obtained from materials surgically resected from 13 patients at the National Cancer Center Hospital (Tokyo, Japan). This study was approved by the Ethics Committee of the National Cancer Center and was performed in accordance with the 1964 Declaration of Helsinki. Written informed consent was obtained from all patients.

2.6. Quantitative RT-PCR of miR-9

Levels of miRNA expression were analyzed by quantitative RT-PCR using the TaqMan microRNA assay for *miR-9* (Applied Biosystems, Foster City, CA) in accordance with the manufacturer's instructions. Expression levels were normalized to those of U6 RNA.

3. Results

3.1. Identification of candidates of epigenetically regulated miRNAs by ChIP-on-chip assay with a novel miRNA promoter array

We designed a custom oligo microarray covering from −10 to +2.5 kb surrounding the genomic positions of pre-miRNAs in the human genome (Fig. 1). This microarray covers 541 miRNAs with 125 base spacing between the probes on average, and each miRNA is covered with approximate 100 probes (60 mer) over its 12.5-kb genomic position, that includes predicted TSSs.

To investigate miRNAs, which are regulated by epigenetic alterations, we treated AGS cells with the DNA-methylation inhibitor 5-Aza-CdR and the HDAC inhibitor PBA. Histone H3 acetylation and histone H3-K4 methylation are enriched at transcriptionally active gene promoters. Fig. 2 shows a schematic of the experimental procedure for ChIP-on-chip assay using the miRNA promoter microarray. After crosslink and sonication, chromatin was immunoprecipitated using antibodies for histone H3 acetylation and histone H3-K4 methylation, and immunoprecipitated DNA was hybridized on the miRNA promoter microarray. We considered miRNAs to be candidates for epigenetic regulation when five or more of their probes showed increased level ($>2^{0.5}$) of both histone H3 acetylation and histone H3-K4 methylation after epigenetic treatment. Table 1 summarizes the results of our miRNA ChIP-on-chip assay using the miRNA promoter array. Fifty-three miRNAs (9.8%; 53 of 541 miRNAs) showed increased levels of both histone H3 acetylation and histone H3-K4 methylation. Among these 53 miRNAs, 19 miRNAs were located in the intronic regions of their host genes, and 12 miRNAs were located near neighboring genes. Twenty-one miRNAs were located near CpG islands. We suggest that these miRNAs may be regulated by epigenetic alterations, such as acetylation and methylation of histone H3, in gastric cancer cells.

Table 1
miRNAs immunoprecipitated with antibodies for both histone H3 acetylation and histone H3-K4 methylation.

miRNAs	Host genes	Genes near miRNAs	CpG islands
let-7c	C21orf34		
miR-1-1		FLJ30313	■
miR-7-3	PGSF1		
miR-9-1	C1orf61		■
miR-9-3			■
miR-29b-2			
miR-30a			
miR-30b			
miR-33b	SREBF1		■
miR-34a			■
miR-99a	C21orf34		
miR-100			
miR-124a-1			
miR-125b-2	C21orf34		
miR-129-1			
miR-129-2			■
miR-135b			
miR-138-2		NUP93	
miR-142		BZRAP1	
miR-143			
miR-147b	C15orf48		■
miR-148b	COPZ1		
miR-149	GPC1		■
miR-183			
miR-195		BCL6B	
miR-196a-1			■
miR-196a-2		HOXC10	
miR-196b		HOXA10	
miR-202		ADAM8	
miR-205			
miR-296			
miR-328		LRRC29	
miR-335	MEST		■
miR-337			
miR-365-2			
miR-370			
miR-375		CRYBA2	■
miR-424			■
miR-455	COL27A1		
miR-483	IGF2		■
miR-497		BCL6B	
miR-511-2	MRC1		
miR-532			■
miR-548c	RASSF3		■
miR-549	KIAA1199		■
miR-566	SEMA3F		■
miR-612			
miR-636		MFSD11	■
miR-769		PGLYRP1	
miR-770			■
miR-933	ATF2		■
miR-1233	GOLGA8A		
miR-1237	RPS6KA4		■

3.2. Downregulation of miR-9 in gastric cancer tissues and miR-9 activation by chromatin-modifying drugs

Our ChIP-on-chip assay identified *miR-9-1* (chr1q22) and *miR-9-3* (chr15q26) as candidates for epigenetic regulation in gastric cancer cells. Recent studies have shown that *miR-9* is regulated by epigenetic alterations in human cancer metastasis [8], and that *miR-9-1* is epigenetically inactivated in human breast cancer [9]. We therefore selected *miR-9* for validation as a candidate epigenetically controlled miRNA in human gastric cancer cells. ChIP-PCR for

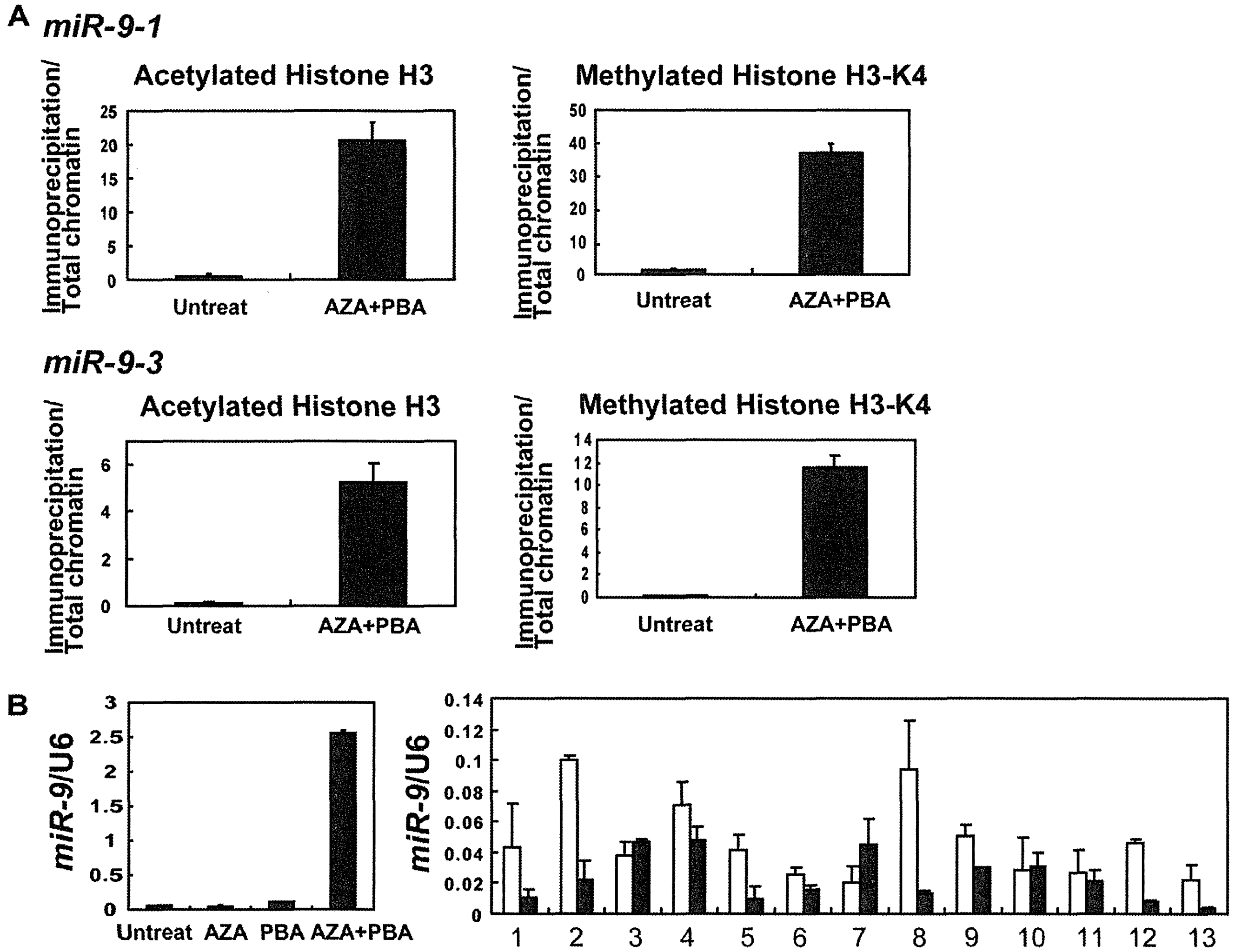

Fig. 3. Confirmation of the results of ChIP-on-chip assay with the miRNA promoter microarray. (A) ChIP-PCR for *miR-9-1* and *miR-9-3* in AGS cells treated with 5-aza-CdR and PBA. The levels of histone H3 acetylation and histone H3-K4 methylation around the promoter regions of *miR-9-1* and *miR-9-3* significantly increased after epigenetic treatment of AGS cells. (B) Quantitative RT-PCR for *miR-9* expression in AGS cells and human gastric cancer samples. The expression level of *miR-9* significantly increased in AGS cells after treatment with 5-aza-CdR and PBA. Expression levels of *miR-9* reduced in 77% (10 of 13 cases) of gastric cancer tissues (filled bar) compared with the levels in the corresponding non-tumor gastric mucosae (open bar). The average levels of *miR-9* expression were significantly lower in gastric cancer tissues than in the corresponding non-tumor gastric mucosae ($p < 0.05$).

miR-9-1 and *miR-9-3* (Fig. 3A) showed that the levels of histone H3 acetylation and histone H3-K4 methylation around the promoter regions of *miR-9-1* and *miR-9-3* significantly increased after epigenetic treatment of AGS cells. Because histone H3 acetylation and histone H3-K4 methylation are active chromatin marks associated with increased level of gene expression, we examined expression levels of *miR-9* in AGS cells after treatment with 5-aza-CdR and PBA. The expression level of *miR-9* significantly increased after combination treatment with 5-aza-CdR and PBA, whereas *miR-9* expression was not induced by either 5-aza-CdR alone or PBA. We next examined *miR-9* expression levels by quantitative RT-PCR in tissue specimens of gastric cancers. Expression levels of *miR-9* reduced in 77% (10 of 13 cases) of gastric cancer samples compared with the levels in the corresponding non-tumor gastric mucosae (Fig. 3B). The average levels of *miR-9* expression were significantly lower in gastric cancer tissues than in the corresponding non-tumor gastric mucosae ($p < 0.05$).

4. Discussion

Using a custom miRNA promoter microarray, we performed comprehensive ChIP-on-chip analysis of histone modifications in predicted human miRNA promoter regions. Because recent studies have shown that the majority of TSSs occur within 10 kb upstream of the 5′-end of pre-miRNAs [10,11], we designed microarray probes from 10 kb upstream to 2.5 kb downstream (relative to the TSS) of each pre-miRNAs to comprehensively analyze histone modifications. We identified miRNAs as candidates for epigenetic regulation when five or more of their probes showed increased levels ($>2^{0.5}$) of both histone H3 acetylation and histone H3-K4 methylation after epigenetic treatment. Our microarray platform contains approximately 100 probes per miRNA with an average spacing of 125 bases. Enrichment of five probes therefore monitors histone modification events over at least 500 bp of sequence, which is considered sufficient to change chromatin structure.

Our assay identified miRNAs regulated by histone H3 acetylation and histone H3-K4 methylation after epigenetic treatment of AGS cells. Because DNA-methylation inhibitors and HDAC inhibitors synergistically induce open chromatin structure associated with active gene expression [12], we treated AGS cells with the DNA-methylation inhibitor 5-Aza-CdR and the HDAC inhibitor PBA. Our results indicate that chromatin structural changes resulting from acetylation and methylation of histone H3 by epigenetic treatment can affect the expression of a substantial number of miRNAs. Approximately 10% of miRNAs on the array showed increased levels of both histone H3 acetylation and histone H3-K4 methylation after epigenetic treatment, indicating that these miRNAs are regulated by histone modification.

Gastric cancer is the second most common cause of cancer-related death worldwide, and systemic chemotherapy is the only treatment available for advanced gastric cancer. Because epigenetic alterations due to *Helicobacter pylori* infection or various exogenous antigen exposures are frequently observed in the stomach, chromatin-modifying drugs such as DNA-methylation inhibitors and HDAC inhibitors may have an inhibitory effect on gastric cancer growth. In our ChIP-on-chip assay using AGS cells with epigenetic treatment, *miR-9* was identified as a miRNA showing increased levels of both histone H3 acetylation and histone H3-K4 methylation at 2 different genomic positions corresponding to *miR-9-1* and *miR-9-3*. Expression levels of *miR-9* in gastric cancer tissues were significantly decreased compared with the levels in the corresponding non-tumor gastric mucosae, and combination treatment with 5-Aza-CdR and PBA markedly activated *miR-9* expression. These findings indicate that *miR-9* is a potential tumor suppressor miRNA in gastric cancer and that its expression is regulated by chromatin-modifying drugs, suggesting that *miR-9* may be a potential target for epigenetic therapy of gastric cancer. Recent studies have also shown that *miR-9* is a potential tumor suppressor miRNA that is inactivated by epigenetic mechanisms in human cancers [8,9]. Besides *miR-9*, a number of other important miRNAs have been identified, such as *miR-34a*, which has been identified as a target of p53, and which induces G(1) cell cycle arrest, senescence and apoptosis [13,14]. Recent studies have shown that *miR-34a* is a tumor suppressor miRNA that is silenced in several types of cancer because of aberrant CpG methylation in its promoter region [15].

Our novel miRNA promoter array can be used to carry out ChIP-on-chip assays to identify miRNAs, which are regulated by other epigenetic marks, such as histone H3-K27 methylation, as well as to determine the specific transcription factors, which bind to miRNA promoters. Further studies are necessary to gain comprehensive understanding of the regulatory mechanism of miRNA expression and to identify critical miRNAs as therapeutic targets for epigenetic therapy of human cancer.

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Are Solifenacin and Ramosetron Really Ideal to Treat Irritable Bowel Syndrome?:
Author's Reply

TO THE EDITOR: We appreciate the thoughtful comments from Chang[1] in regards to our recent paper.[2] The overall improvement was assessed on the basis of the patients' subjective reports during their interview regarding whether the symptoms had improved, compared to before administering solifenacin. Since the overall improvement is an established index often used in determining the therapeutic efficacy of irritable bowel syndrome (IBS), we do not think that the extremely high efficacy of solifenacin in our paper[2] have been caused by the setting of overall improvement as a primary endpoint. Differences between the enrolled populations could be a possible cause. Most of the previous studies on IBS have been "tertiary-care-hospital-based," however, this study was "health clinic-based." Most of the patients had not received any treatment, despite the presence of IBS symptoms. In addition, because no placebo group was established and participants were aware that the medication, they were taking, was the actual drug, the placebo effect might be stronger than previously reported. For this reason, the actual therapeutic efficacy of solifenacin in the treatment of IBS will have to be verified using a placebo-controlled study.

As Chang[1] mentioned, setting up a washout period and evaluating IBS symptoms before and after administration of ramosetron has allowed more accurate understanding of the effects of ramosetron. Meanwhile, solifenacin has a half-life of 45 to 68 hours.[3] Because the score after the 4-week administration of ramosetron reflected the symptoms from the third week to the fourth week after administration of ramosetron, it can be considered that the score is unlikely to be affected by the residual pharmacological effect of solifenacin.

Since Figure 2A to 2F (including 2C) demonstrate the average scores of the subscales of IBS-symptom severity scale, the y-axis was labeled as "scores."

As constipation has been pointed out as a side effect of solifenacin, precaution for constipation must be taken when using solifenacin in the medical treatment of overactive bladder.[4] Although the mechanism that solifenacin was effective for the treatment of diarrhea predominant IBS could not be elucidated, it would be reasonable to believe that the improvement of diarrhea might have been attributable to the same mechanism as that by which solifenacin caused constipation. In the future, when using solifenacin in the treatment of diarrhea predominant IBS, it would be appropriate to conduct the same dose adjustment as we performed, after administering the dose for 1 to 2 weeks.

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The tumor suppressor *microRNA-29c* is downregulated and restored by celecoxib in human gastric cancer cells

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MicroRNAs (miRNAs) are small noncoding RNAs that function as endogenous silencers of target genes and play critical roles during carcinogenesis. The selective cyclooxygenase-2 (COX-2) inhibitor celecoxib has been highlighted as a potential drug for treatment of gastrointestinal tumors. The aim of this study was to investigate the role of miRNAs in gastric carcinogenesis and the feasibility of a new therapeutic approach for gastric cancer. miRNA expression profiles were examined in 53 gastric tumors including gastric adenomas (atypical epithelia), early gastric cancers and advanced gastric cancers and in gastric cancer cells treated with celecoxib. miRNA microarray analysis revealed that *miR-29c* was significantly downregulated in gastric cancer tissues relative to nontumor gastric mucosae. *miR-29c* was significantly activated by celecoxib in gastric cancer cells. Downregulation of *miR-29c* was associated with progression of gastric cancer and was more prominent in advanced gastric cancers than in gastric adenomas and early gastric cancer. In addition, expression of the oncogene *Mcl-1*, a target of *miR-29c*, was significantly increased in gastric cancer tissues relative to nontumor gastric mucosae. Activation of *miR-29c* by celecoxib induced suppression of Mcl-1 and apoptosis in gastric cancer cells. These results suggest that downregulation of the tumor suppressor *miR-29c* plays critical roles in the progression of gastric cancer. Selective COX-2 inhibitors may have clinical promise for the treatment of gastric cancer via restoration of *miR-29c*.

MicroRNAs (miRNAs) are small noncoding RNAs that function as endogenous silencers of various target genes. Hundreds of human miRNAs have been identified in the human genome, being expressed in a tissue-specific manner and playing important roles in cell proliferation, apoptosis and differentiation during mammalian development.[1] Links between miRNAs and the development and progression of human malignancies are becoming increasingly apparent, especially with regard to aberrant expression of miRNAs.[2,3] We have recently reported that some miRNAs are regulated by epigenetic alterations such as DNA methylation and histone modification at their CpG island promoters and that epigenetic activation of tumor suppressor miRNAs may be a novel therapeutic approach for human cancers.[4–7] We have also reported that miRNAs may play important roles in the pathogenesis of not only malignancies but also functional gastrointestinal disorders such as functional dyspepsia.[8]

Gastric cancer is the second most common cause of cancer-related death worldwide.[9,10] Advanced gastric cancer is defined as adenocarcinoma with invasion to the muscularis propria or deeper gastric wall. Although patients who are diagnosed as having gastric cancer at an advanced stage undergo surgical resection and systemic chemotherapy, their prognosis is generally poor. However, early gastric cancer is defined as adenocarcinoma confined to the mucosa or submucosa of the stomach and can be treated using endoscopic submucosal dissection (ESD), which is an advanced therapeutic technique that can offer extremely promising outcomes.[11,12] Examination of miRNA expression profiles has revealed that specific miRNAs are aberrantly expressed in various human cancers.[2,13,14]

Cyclooxygenase (COX) is a critical enzyme involved in prostaglandin production and has two isoforms: COX-2,

Key words: microRNA, *miR-29c*, gastric cancer, *Mcl-1*, celecoxib
Abbreviations: ChIP: chromatin immunoprecipitation; COX: cyclooxygenase; ESD: endoscopic submucosal dissection; *H. pylori: Helicobacter pylori*; HE: hematoxylin-eosin; ISH: *in situ* hybridization; miRNA: microRNA
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What's new?

MicroRNAs (miRNAs) play critical roles in carcinogenesis and may be valuable therapeutic targets for malignant disease. Here, the miRNA tumor suppressor miR-29c was found to be significantly downregulated in gastric cancer cells and to be reactivated by the selective cyclooxygenase-2 (COX-2) inhibitor celecoxib. Reactivation led to miR-29c-induced suppression of the anti-apoptotic protein Mcl-1. The data reveal that selective COX-2 inhibitors may have clinical promise for the treatment of gastric cancer via restoration of miR-29c.

which is commonly overexpressed in solid tumors including gastric cancer, and COX-1, which is expressed constitutively in normal tissues, suggesting that inhibition of COX-2 may reduce the risk of cancer development. Clinical trials have revealed that the selective COX-2 inhibitor celecoxib is effective for prevention of colorectal adenomas.[15,16] Recent studies have also shown that celecoxib has a preventive effect against *Helicobacter pylori*-associated gastric cancer.[17,18] Therefore, celecoxib has been suggested to have promise for the treatment of gastrointestinal tumors. However, the molecular mechanisms underlying the chemopreventive effects of selective COX-2 inhibitors are not fully understood.

To identify miRNAs that play critical roles in the development and progression of gastric cancer, we examined the miRNA expression profiles of gastric tumors including gastric adenomas and both early and advanced gastric cancers. We also investigated miRNAs that are targeted by celecoxib. Our findings revealed that *miR-29c* was downregulated in gastric cancers and activated by celecoxib. Recent studies have shown that *miR-29c* is downregulated in various human malignancies including gastric cancer and acts as a tumor suppressor.[19–25] Herein, we show that the putative tumor suppressor *miR-29c* is an important miRNA in gastric carcinogenesis and is a potential therapeutic target for gastric cancer.

Material and Methods

Patients and tissue specimens

A total of 53 clinical samples of gastric tumors were examined. The clinicopathological features of the patients are shown in Supporting Information Table 1. The average age of the patients was 67.7 years (male/female, 39/14). Thirty patients with gastric adenomas (atypical epithelium) or early gastric cancers underwent ESD at Keio University Hospital (Tokyo, Japan). This study was approved by the research ethics committee of Keio University School of Medicine (#19-68-5) and registered with the UMIN Clinical Trials Registry (UMIN 000001057). Informed consent was obtained from all patients before the examinations. Tissue specimens from gastric adenomas and early gastric cancers and the surrounding nontumor gastric mucosae were obtained by endoscopic biopsy and kept in RNAlater (Ambion, Austin, TX) at −80°C until RNA extraction. Tissue specimens from advanced gastric cancers and the surrounding nontumor gastric mucosae were obtained from materials surgically resected from 23 patients at the National Cancer Center Hospital (Tokyo, Japan). This study was approved by the Ethics Committee of the National Cancer Center and performed in accordance with the 1964 Declaration of Helsinki. All patients gave their informed consent for inclusion in this study.

Cell lines and treatment with celecoxib

The human gastric cancer cell lines AGS and MKN45 were used in this study. AGS was obtained from the American Type Culture Collection (Rockville, MD), and MNK45 was obtained from the Japan Health Science Foundation (Osaka, Japan). Cells were cultured in RPMI1640 medium supplemented with 10% fetal bovine serum and seeded at 1×10^5 cells per 100-mm dish 24 hr prior to treatment. Cells were treated with celecoxib (Pfizer, New York, NY) at 30 μM for 48 hr.

RNA extraction and microarray analysis

Total RNAs were extracted from both tissue specimens of gastric tumors resected by ESD and gastric cancer cell lines using the mirVana miRNA isolation kit (Ambion). Total RNA of tissue specimens from advanced gastric cancers and matched nontumor gastric mucosae was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA).

Five hundred nanograms of total RNA from each of ten advanced gastric cancers were pooled, and the same was done with 10 matched nontumor gastric mucosae. miRNA microarray analysis of samples of advanced gastric cancer was conducted by Toray Industries (www.toray.com: Tokyo, Japan). miRNA microarray analysis of AGS cells treated with celecoxib was conducted by LC Sciences (www.lcsciences.com: Houston, TX). All data were submitted to the ArrayExpress database, under the accession numbers E-MEXP-2230 (gastric cancer) and E-MEXP-2231 (celecoxib). These microarray chips contain probe regions that detect 724 (Toray Industries) and 711 (LC Sciences) miRNA transcripts listed in Sanger miRBase Release 10.0 (http://www.sanger.ac.uk). These chips contain multiple probes for each miRNA (2 probes in the Toray Industries chip, 5 probes in the LC Sciences chip), and the average values of their signal intensities are shown in Tables 1 and 3.

Quantitative RT-PCR of miR-29c

Levels of miRNA expression were analyzed by quantitative RT-PCR using the TaqMan microRNA assay for *miR-29c* (Applied Biosystems, Foster City, CA) in accordance with the manufacturer's instructions. Expression levels were normalized to that of U6 RNA.

Table 1. Summary of the most downregulated miRNAs in advanced gastric cancers relative to non-tumorous gastric mucosae

No.	miRNAs	Non-tumor	Tumor	Fold change
1	*miR-193b*	1133.8	563.0	0.5
2	*miR-768–3p*	756.8	372.1	0.5
3	*miR-140–3p*	1438.6	690.7	0.5
4	*miR-923*	43834.8	20277.2	0.5
5	*miR-939*	3780.0	1655.2	0.4
6	*miR-487b*	173.0	72.2	0.4
7	*miR-378**	95.3	39.6	0.4
8	*miR-29c*	1733.9	641.1	0.4
9	*miR-133b*	424.6	155.0	0.4
10	*miR-768–5p*	1499.5	518.1	0.3

Note: Data for Non-tumor and Tumor are average values of the signal intensities in microarray analysis. Fold change represents the ratio of the signal intensities for Tumor/Non-tumor.

In situ hybridization (ISH) of miR-29c

Locked nucleic acid (LNA)-modified probes for *miR-29c* were used (miRCURY-LNA detection probe, Exiqon, Vedbaek, Denmark). *In situ* hybridization was performed using the RiboMap *in situ* hybridization kit (Ventana Medical Systems, Tucson, AZ) on the Ventana Discovery automated *in situ* hybridization instrument (Ventana Medical Systems). *In situ* hybridization steps after deparaffinization were performed based on the standard protocol provided in the manufacturer's RiboMap application note (http://www.ventanamed.com). Hematoxylin-eosin (HE) was used for counterstaining.

Immunohistochemical examination of Mcl-1

Formalin-fixed and paraffin-embedded tissues were deparaffinized and rehydrated. For antigen retrieval, the sections were treated for 10 min at 105°C in an autoclave, and nonspecific reactions were blocked with a blocking reagent (Protein Block Serum-Free, Dako Cytomation, Glostrup, Denmark). The sections were incubated with the rabbit anti-human Mcl-1 polyclonal antibody (S-19, Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C followed by horseradish peroxidase labeled anti-rabbit IgG (Histofine, Simple stain MAX-PO, Nichirei, Tokyo, Japan) for 30 min at room temperature. Then, the sections were treated with 3.3′-diaminobenzidine tetrahydrochloride solution. All sections were counterstained with hematoxylin.

Immunoreactivity of Mcl-1 was confirmed in the germinal centers as described previously,[26] and this was defined as the internal positive control. The intensity of Mcl-1 immunoreactivity in each sample was graded as 0 (less than the internal positive control), 1 (equal to the internal positive control) and 2 (more than the internal positive control). Mcl-1 expression in randomly selected fields, two in gastric cancer tissues and two in nontumor gastric mucosal tissues, was examined in gastric adenomas and early ($n = 30$) and advanced ($n = 21$) gastric cancers at high magnification (×400). The Mcl-1 expression score was determined as the product of the Mcl-1 immunoreactivity grade and the percentage of Mcl-1-positive cells.

Chromatin immunoprecipitation (ChIP) assay

The ChIP assay was performed as described previously.[4] An antibody against C/EBPα (sc-61, Santa Cruz Biotechnology) was used. Quantitative analysis was performed by real-time PCR with the CYBR Premix Ex Taq (Takara Bio, Ohtsu, Japan) using the Thermal Cycler Dice Real-Time System (Takara Bio). The sequences of the primers used were as follows; Forward: 5′-CTAAGAGCAGACTGATGGTGTC-3′, Reverse: 5′-CTATTTCTGTTGACTCCTAGCAGC-3′.

The fraction of immunoprecipitated DNA was calculated as follows: [immunoprecipitated DNA with C/EBPα antibody–nonspecific antibody control (NAC)]/(input DNA - NAC).

Western blotting

Protein extracts were separated by SDS/polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. The membranes were hybridized with a rabbit anti-human Mcl-1 polyclonal antibody (S-19, Santa Cruz Biotechnology), and β-actin was used as the internal control.

Transfection of miR-29c precursor molecules and the specific miR-29c inhibitor

The *miR-29c* precursor molecules, the specific *miR-29c* inhibitor and negative control precursor miRNAs were purchased from Ambion. They were transfected into AGS and MKN45 cells at a final concentration of 100 nM each using oligofectamine (Invitrogen) in accordance with the manufacturer's instructions. Forty-eight hours after transfection, the cells were collected and their expression of Mcl-1 was analyzed by Western blotting as described above.

Apoptosis assay

The levels of apoptosis in AGS and MKN45 cells transfected with *miR-29c* and treated with celecoxib were evaluated using Annexin V kit (Beckman Coulter, Brea CA) in accordance with the manufacturer's instructions.

Statistics

Data were analyzed using the SPSS statistics 17.0 software package. Differences in miRNA expression levels and apoptosis levels between groups were analyzed using unpaired t test. Differences at $p < 0.05$ were considered significant.

Results

Downregulation of miR-29c is associated with progression of gastric cancer

To investigate the miRNA expression profile in gastric cancer, we compared the miRNA expression of gastric cancer tissues with that of the corresponding nontumorous gastric mucosae. The microarray data shown in Table 1 revealed that the putative tumor suppressor *miR-29c* was significantly

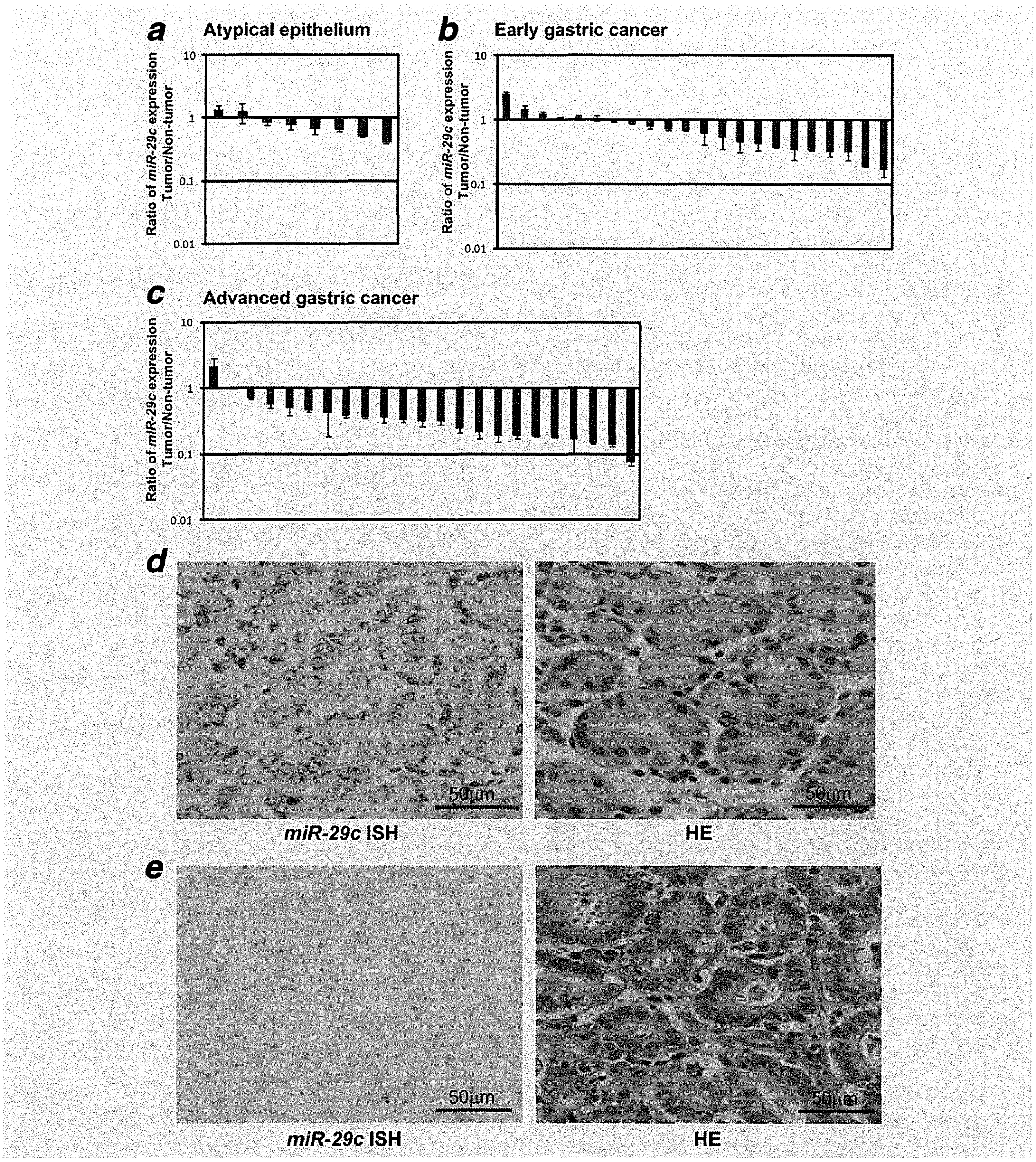

Figure 1. The expression levels of *miR-29c* in the 53 clinical gastric tumor samples and matched nontumorous gastric mucosae. All reactions were performed in duplicate with U6 as the internal control. The graph shows the ratio of *miR-29c* expression of tumor/nontumor on a logarithmic scale (mean ± SD). (*a*) Gastric adenomas (atypical epithelium, $n = 8$). There was no significant association of *miR-29c* expression between tumor and nontumor. (*b*) Early gastric cancers ($n = 22$). The expression levels of *miR-29c* in early gastric cancers were significantly lower than those in the corresponding nontumorous gastric mucosae ($p < 0.005$). (*c*) Advanced gastric cancers ($n = 23$). The expression levels of *miR-29c* in advanced gastric cancers were significantly lower than those in the corresponding nontumorous gastric mucosae ($p < 0.0005$). (*d*) A representative example of ISH for *miR-29c* in normal gastric tissue. *miR-29c* is expressed in the cytoplasm of normal gastric epithelial cells (blue staining) but not detected in other cells such as fibroblasts and muscle cells. HE was used for counterstaining. (*e*) A representative example of ISH for *miR-29c* in gastric cancer tissue. *miR-29c* expression was not detected in gastric cancer cells. HE was used for counterstaining.

downregulated in gastric cancer tissues relative to nontumorous gastric mucosae. To confirm the microarray data, we examined the levels of *miR-29c* expression in tissue specimens of gastric adenomas (atypical epithelium), early gastric cancers and advanced gastric cancers by quantitative RT-PCR. As shown in Figure 1*a*, the expression level of *miR-29c* was reduced in 75% (6 of 8) of gastric adenomas compared with the corresponding nontumor gastric mucosae. There was no significant difference in the average level of *miR-29c* expression between gastric adenomas and the corresponding nontumor gastric mucosae. The expression level of *miR-29c* was reduced in 73% (16 of 22) of early gastric cancers compared with the corresponding nontumor gastric mucosae (Fig. 1*b*), and the expression levels of *miR-29c* in early gastric cancers were significantly lower than those in the corresponding nontumorous gastric mucosae ($p < 0.005$). Furthermore, the expression level of *miR-29c* was reduced in 91% (21 of 23) of advanced gastric cancers relative to the corresponding nontumorous gastric tissues (Fig. 1*c*), and this reduction was shown to be significant ($p < 0.0005$). The relative expression levels of *miR-29c* in nontumorous gastric mucosa and gastric tumors and the ratio of *miR-29c* expression on a linear scale are shown in Supporting Information Figure 1.

To confirm the localization of *miR-29c* in gastric cancer tissues, ISH for *miR-29c* was performed. Figures 1*d* and 1*e* show representative examples of ISH for *miR-29c*. *miR-29c* is expressed in the cytoplasm of normal gastric epithelial cells (blue staining) but not detected in other cells such as fibroblasts and muscle cells. *miR-29c* expression was not detected in gastric cancer cells. These results are consistent with the data for quantitative RT-PCR of *miR-29c*.

We also examined the association between the ratio of *miR-29c* expression and clinicopathological features such as tumor differentiation, tumor location, *H. pylori* infection and clinical stage. We found that downregulation of *miR-29c* was more prominent in undifferentiated than in differentiated advanced gastric cancers (Table 2, Supporting Information Fig. 1). There was no significant association between the ratio of *miR-29c* expression and other clinicopathological features such as tumor location, *H. pylori* infection and clinical stage (Table 2).

Table 2. Association between the ratio of *miR-29c* expression and clinicopathological features of patients with gastric cancer

	N	Ratio of *miR-29c* expression (T/N)	*p*-value
a) Early gastric cancer			
Tumor differentiation			
Differentiated	19	0.75 ± 0.13	$p = 0.98$
Undifferentiated	3	0.76 ± 0.27	
Tumor location			
Upper and Middle portion	3	0.98 ± 0.14	$p = 0.23$
Lower portion	19	0.72 ± 0.13	
H.pylori infection			
Positive	10	0.88 ± 0.23	$p = 0.51$
Negative	10	0.71 ± 0.11	
b) Advanced gastric cancer			
Tumor differentiation			
Differentiated	14	0.54 ± 0.14	$p = 0.04^1$
Undifferentiated	9	0.22 ± 0.04	
Tumor location			
Upper and Middle portion	13	0.45 ± 0.15	$p = 0.65$
Lower portion	10	0.37 ± 0.08	
T stage			
T1, T2	9	0.58 ± 0.21	$p = 0.25$
T3, T4	14	0.31 ± 0.05	
Clinical stage			
I, II, III	13	0.30 ± 0.05	$p = 0.22$
IV	10	0.56 ± 0.19	

Note: The ratios of *miR-29c* expression (Tumor/Non-tumor) are expressed as mean ± SE. Differences between groups were analyzed by unpaired t test.

[1] There was a significant correlation between the ratio of *miR-29c* expression and tumor differentiation.

Increased expression of Mcl-1, a target gene of miR-29c, in gastric cancer tissues

We next investigated the target genes of *miR-29c*. Mott *et al.*[19] have demonstrated that Mcl-1, encoding an antiapoptotic Bcl2 family protein, is one of the targets of *miR-29* miRNAs, and that *miR-29* miRNAs regulate apoptosis by targeting Mcl-1. The levels of Mcl-1expression in gastric cancer tissues were examined by immunohistochemistry. Immunoreactivity of the Mcl-1 antibody was confirmed by staining of the germinal centers, as described previously (Fig. 2*a*).[26] Prominent staining of Mcl-1 was observed in the cytoplasm of gastric cancer cells. Expression of Mcl-1 was markedly increased in advanced gastric cancer (Figs. 2*c* and 2*d*), whereas in nontumorous gastric tissues only faint staining was evident (Fig. 2*b*). Mcl-1 expression was also increased in early gastric cancer (Fig. 2*e*).

The degree of Mcl-1 overexpression was represented as the ratio of the Mcl-1 expression score between the tumor and nontumorous mucosa (*T*/*N*). The average value of the ratio of Mcl-1 expression (*T*/*N*) in advanced gastric cancers was significantly higher than that in gastric adenomas and early gastric cancers (Fig. 2*f*).

miR-29c is activated by the selective COX-2 inhibitor celecoxib and suppresses Mcl-1

To identify miRNAs that are differentially expressed upon treatment of AGS gastric cancer cells with the selective COX-2 inhibitor celecoxib, we performed miRNA microarray

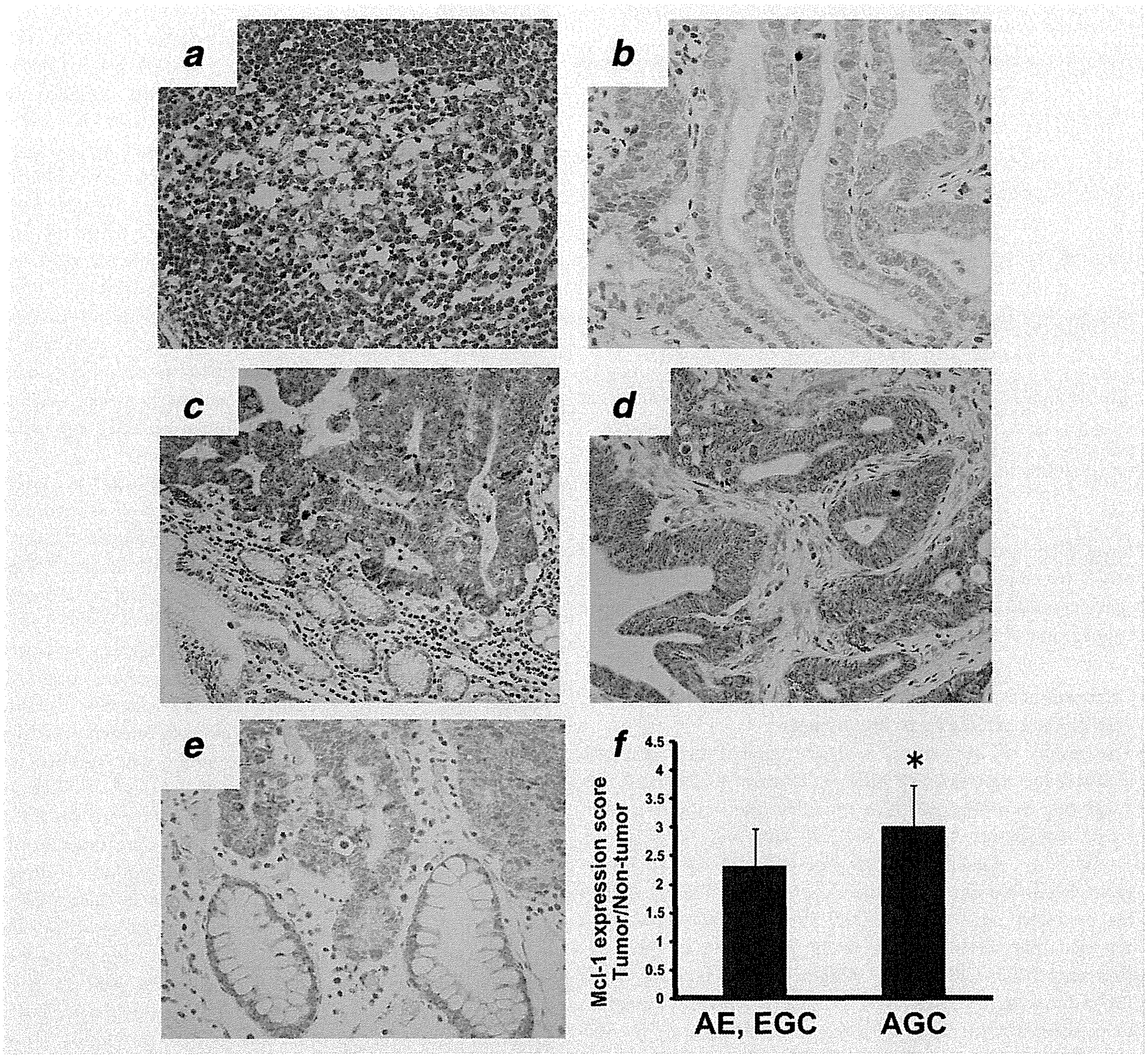

Figure 2. Expression levels of Mcl-1 in gastric cancer tissues examined by immunohistochemistry. (*a*) Immunoreactivity of the Mcl-1 antibody was confirmed by staining of the germinal centers as described previously.[26] Immunohistochemical examinations of Mcl-1 in nontumorous gastric tissues (*b*), advanced gastric cancer (*c*, *d*) and early gastric cancer (*e*) are shown. (*f*) The average levels of the ratio of the Mcl-1 expression score (*T*/*N*) in gastric cancer. AE: atypical epithelium; AGC: advanced gastric cancer; ECG: early gastric cancer. $^*p < 0.05$.

analysis. As shown in Table 3, *miR-29c*, which is downregulated in gastric cancer tissues, was significantly activated after treatment with celecoxib.

Figure 3*a* shows the expression levels of *miR-29c* in AGS and MKN45 gastric cancer cells treated with celecoxib. The expression levels of *miR-29c* in AGS and MKN45 cells treated with celecoxib were significantly higher than those in untreated cells ($p < 0.05$), being consistent with the microarray data. We also examined the expression levels of *miR-29c* in AGS and MKN45 cells after treatment with celecoxib in the presence of the specific *miR-29c* inhibitor. We confirmed that increased expression of *miR-29c* by celecoxib was cancelled after transfection with the specific *miR-29c* inhibitor.

We next examined the expression levels of Mcl-1, a target of *miR-29c*, by Western blotting in AGS and MKN45 cells after transfection with the *miR-29c* precursor molecules and treatment with celecoxib with or without the specific *miR-29c* inhibitor. We measured the expression levels of *miR-29c* after its transfection and confirmed that *miR-29c* expression was markedly increased (over 100-fold) after transfection. The results of Western blotting showed that the expression levels of Mcl-1 in AGS and MKN45 cells treated with celecoxib and transfected with the *miR-29c* precursor molecules were

Table 3. Summary of the most upregulated miRNAs in AGS cells treated with celecoxib

No.	miRNAs	Control	Celecoxib	Fold change
1	*miR-663*	206.8	935.1	4.5
2	*miR-181b*	90.4	395.9	4.4
3	*miR-29c*	497.0	1972.0	4.0
4	*miR-141*	122.5	410.3	3.3
5	*miR-149**	285.8	894.4	3.1
6	*miR-98*	392.9	1113.9	2.8
7	*miR-26b*	593.4	1340.3	2.3
8	*miR-195*	279.4	626.5	2.2
9	*miR-148a*	333.1	707.6	2.1
10	*miR-638*	3682.7	7073.3	1.9

Note: Data for Control and Celecoxib are average values of the signal intensities in microarray analysis. Fold change represents the ratio of the signal intensities for Celecoxib/Control.

lower than in the control. We confirmed that suppression of Mcl-1 by celecoxib was cancelled after transfection with the specific *miR-29c* inhibitor, suggesting that celecoxib-induced suppression of Mcl-1 is mediated by *miR-29c* (Fig. 3*b*)

Celecoxib activates miR-29c expression by enhancing the DNA binding of C/EBPα to the promoter

The results of microarray analysis revealed that celecoxib activates the expression of *miR-29c*, but not that of *miR-29b*, suggesting that celecoxib may modulate the binding of transcriptional factors between *miR-29b* and *miR-29c* (Fig. 3*c*). Using the database (www.gene-regulation.com/pub/programs.html), we found that there are C/EBPα binding sites in the promoter region of *miR-29c*. Moreover, Wu *et al.* have reported that celecoxib induces the expression of the invasion-suppressor CRMP-1 by enhancing the binding of C/EBPα DNA to the promoter.[27] These findings prompted us to perform a ChIP assay with the C/EBPα antibody in AGS and MKN45 cells treated with celecoxib. As shown in Figure 3*c*, the results of the ChIP assay showed that immunoprecipitation with the C/EBPα antibody was significantly increased in AGS and MKN45 cells treated with celecoxib, indicating that celecoxib activates the expression of *miR-29c* by enhancing the binding of C/EBPα DNA to the promoter.

Celecoxib and miR-29c induce apoptosis in gastric cancer cells

Since Mcl-1 is an antiapoptotic factor and suppressed by *miR-29c* in gastric cancer cells, we performed annexin V-FITC apoptosis assay in AGS and MKN45 cells. Fluorescence microscopy and flow cytometry analyses demonstrated that annexin V staining (green staining on the cell surface membrane) was markedly increased in AGS and MKN45 cells 48 hr after *miR-29c* transfection and celecoxib treatment, indicating that *miR-29c* and celecoxib induce apoptosis in gastric cancer cells (Fig. 4).

Discussion

We focused our study on *miR-29c*, because it was the only miRNA that was highlighted by the results of the two microarray analyses. Although the extent of these analyses was intrinsically limited, the results suggested that *miR-29c* is downregulated in gastric cancer and reactivated by celecoxib.

Gastric adenomas and early gastric cancers are clinically similar gastric neoplasms, and sometimes they are difficult to diagnose only by endoscopic findings even with biopsied pathological examination. Therefore, the significant reduction of *miR-29c* expression in early gastric cancers, but not in gastric adenomas, suggests that *miR-29c* could be a novel molecular marker of early gastric cancer. Moreover, reduction of *miR-29c* expression is more prominent in advanced gastric cancers than in gastric adenomas and early gastric cancers. These findings suggest that reduction of *miR-29c* expression is critical for the progression of gastric cancer.

Downregulation of *miR-29c* has been reported in various human malignancies including nasopharyngeal carcinoma,[20] bladder transitional cell carcinoma,[21] esophageal cancer,[22] chronic lymphocytic leukemia[23,24] and gastric cancer.[25] Mott *et al.*[19] have demonstrated that Mcl-1, encoding an antiapoptotic Bcl2 family protein, is one of the targets of *miR-29* miRNAs, and that *miR-29* miRNAs regulate apoptosis by targeting Mcl-1. Mcl-1 is an antiapoptotic protein originally isolated from the ML-1 human myeloid leukemia cell line during cell differentiation.[28] The biological relevance of Mcl-1 as an antiapoptotic protein promoting cell survival has been reported in various human malignancies.[29–31] Elevated expression of Mcl-1 and its association with poor prognosis have been reported in gastric cancer.[32,33] In accordance with these reports, our results demonstrate that *miR-29c* is downregulated in gastric cancers and suppresses its target oncogene, *Mcl-1*. In addition, downregulation of *miR-29c* and overexpression of Mcl-1 are associated with the progression of gastric cancer. These findings suggest that *miR-29c* functions as a tumor suppressor by suppressing Mcl-1 in gastric epithelial cells. We and other groups have previously shown that other miRNAs such as *miR-512-5p* and transcription factors also regulate *Mcl-1* expression.[7] Further studies are necessary to examine other miRNAs and/or transcription factors that regulate Mcl-1 expression in gastric epithelial cells.

Our data from this study showed that the selective COX-2 inhibitor celecoxib activated the expression of *miR-29c* and suppressed its target oncogene *Mcl-1*, resulting in induction of apoptosis in gastric cancer cells. We confirmed that suppression of Mcl-1 by celecoxib was cancelled after transfection with the specific *miR-29c* inhibitor. These results suggest that suppression of Mcl-1 and induction of apoptosis in gastric cancer cells by celecoxib are mediated by *miR-29c*. Since celecoxib is a selective COX-2 inhibitor frequently used for treatment of pain, fever and inflammation, it can prevent side effects of other anticancer drugs. Combination treatment with celecoxib and other anticancer drugs may be beneficial for reducing side effects and achieving more potent activity against gastric cancer.

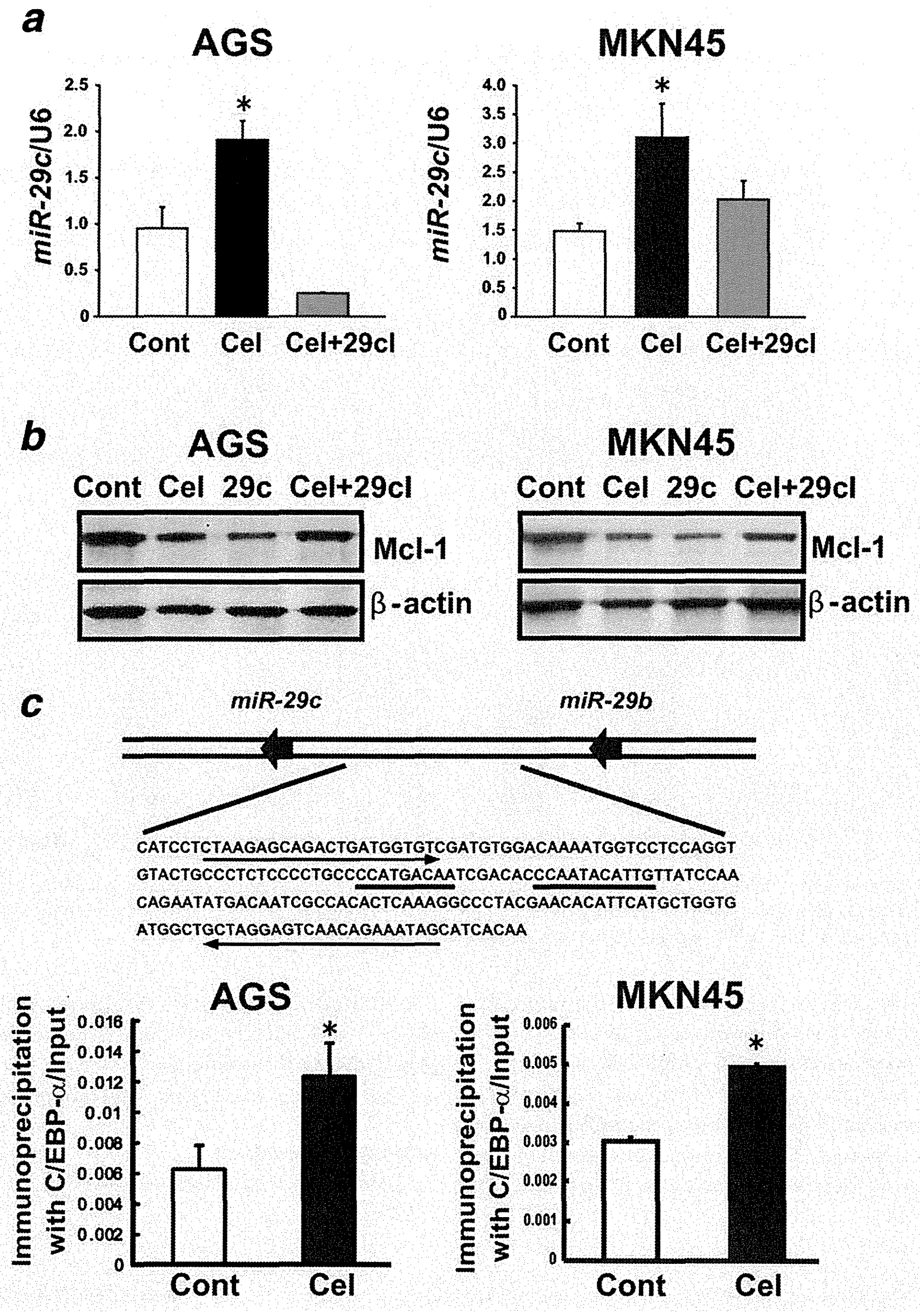

Figure 3. *miR-29c* is activated by celecoxib and suppresses Mcl-1. (*a*) The expression levels of *miR-29c* in AGS and MKN45 cells treated with celecoxib with or without the specific *miR-29c* inhibitor. Cont: control; Cel: celecoxib; Cel+29cl: celecoxib with *miR-29c* inhibitor. *$p < 0.05$ compared with control. (*b*) Western blotting for expression levels of Mcl-1 in AGS and MKN45 cells after transfection with the *miR-29c* precursor molecules and treatment with celecoxib with or without the specific *miR-29c* inhibitor. 29c: miR-29c; Cel+29cl: celecoxib with miR-29c inhibitor; Cel: celecoxib; Cont: control. β-actin was used as the internal control. (*c*) ChIP assay with the C/EBPα antibody in AGS and MKN45 cells treated with celecoxib. The database shows that there are C/EBPα binding sites in the promoter region of *miR-29c* (www.gene-regulation.com/pub/programs.html). Arrows show the primers used for the ChIP assay. Underlining shows the C/EBPα binding sites. Immunoprecipitation with the C/EBPα antibody was significantly increased in AGS and MKN45 cells treated with celecoxib (*$p < 0.05$).

The miRNA expression profiles shown in this study, and in other reports, have demonstrated that *miR-29b* and *miR-29c* have different expression patterns, suggesting that *miR-29c* has a unique promoter in gastric cancer cells. The results of ChIP assay indicated that celecoxib activates *miR-29c* expression by enhancing the binding of C/EBPα DNA at the promoter. Selective COX-2 inhibitors may activate the tumor suppressor *miR-29c* via C/EBPα and induce apoptosis of gastric cancer cells.

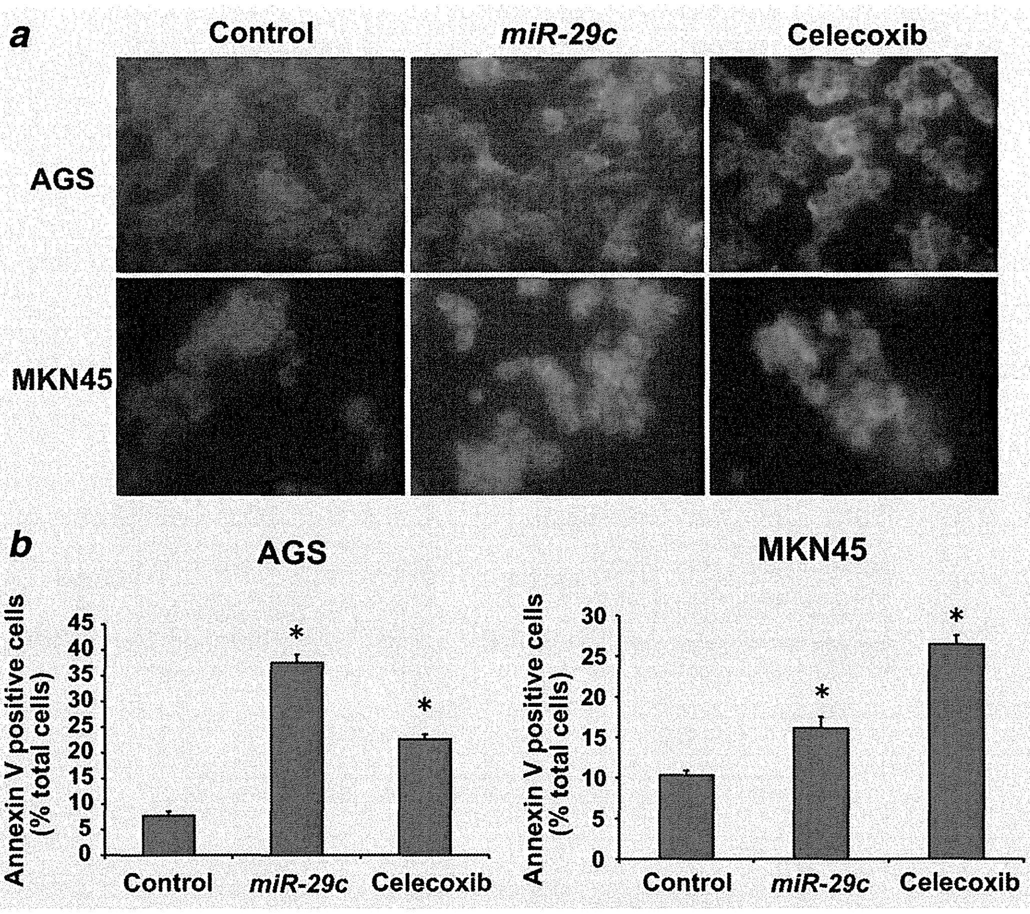

Figure 4. Celecoxib and *miR-29c* induce apoptosis in gastric cancer cells. Annexin V-FITC staining in AGS and MKN45 cells was analyzed by fluorescence microscopy (*a*) and flow cytometry (*b*). Annexin V staining (green staining on the cell surface membrane) was markedly increased in AGS and MKN45 cells 48 hr after *miR-29c* transfection and celecoxib treatment ($*p < 0.05$).

Further investigations will be needed to clarify the molecular mechanism underlying the recruitment of C/EBPα at the *miR-29c* promoter region by selective COX-2 inhibitors.

In conclusion, the tumor suppressor *miR-29c* is downregulated with overexpression of its target oncogene Mcl-1 in gastric cancers and plays important roles in cancer progression. Treatment of gastric cancer cells with the selective COX-2 inhibitor celecoxib activates *miR-29c* expression, resulting in suppression of Mcl-1 and induction of apoptosis in the cells. Administration of selective COX-2 inhibitors may have promise for the treatment of gastric cancer via restoration of *miR-29c*.

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Overexpression of *miR-142-5p* and *miR-155* in Gastric Mucosa-Associated Lymphoid Tissue (MALT) Lymphoma Resistant to *Helicobacter pylori* Eradication

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Abstract

microRNAs (miRNAs) are small non-coding RNAs that can function as endogenous silencers of target genes and play critical roles in human malignancies. To investigate the molecular pathogenesis of gastric mucosa-associated lymphoid tissue (MALT) lymphoma, the miRNA expression profile was analyzed. miRNA microarray analysis with tissue specimens from gastric MALT lymphomas and surrounding non-tumor mucosae revealed that a hematopoietic-specific miRNA *miR-142* and an oncogenic miRNA *miR-155* were overexpressed in MALT lymphoma lesions. The expression levels of *miR-142-5p* and *miR-155* were significantly increased in MALT lymphomas which do not respond to *Helicobacter pylori* (*H. pylori*) eradication. The expression levels of *miR-142-5p* and *miR-155* were associated with the clinical courses of gastric MALT lymphoma cases. Overexpression of *miR-142-5p* and *miR-155* was also observed in *Helicobacter heilmannii*-infected C57BL/6 mice, an animal model of gastric MALT lymphoma. In addition, *miR-142-5p* and *miR-155* suppress the proapoptotic gene *TP53INP1* as their target. The results of this study indicate that overexpression of *miR-142-5p* and *miR-155* plays a critical role in the pathogenesis of gastric MALT lymphoma. These miRNAs might have potential application as therapeutic targets and novel biomarkers for gastric MALT lymphoma.

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Introduction

Extranodal marginal zone B-cell lymphoma of mucosa-associated lymphoid tissue (MALT) is a low-grade lymphoma characterized by histological features such as lymphoepithelial lesions (LELs). The stomach is the most common site of MALT lymphoma, accounting for almost half of all cases. Gastric MALT lymphomas are sometimes associated with chronic inflammation triggered by chronic infection with *Helicobacter pylori* (*H. pylori*), suggesting that the proliferation of MALT lymphoma cells may depend on immune responses to antigens. Indeed, *H. pylori* eradication therapy leads to complete remission in 60–80% of cases of gastric MALT lymphoma and has been used as a first-line treatment [1–3]. However, 20–40% of cases do not respond to *H. pylori* eradication therapy, and predictors of the response to antibiotic treatment as well as the appropriate length of the observation period before second-line treatment remain controversial.

The *API2-MALT1* fusion gene, which results from a t(11;18)(q21;q21) translocation, has been identified as the most frequent chromosome translocation in MALT lymphoma cells [4,5], and Liu *et al.* [6] have reported that it is a potential predictor of resistance to *H. pylori* eradication therapy. Since *API2-MALT1* fusion transcripts lead to inhibition of apoptosis [7,8], they may confer a survival benefit on MALT lymphoma cells. Despite these reports, the molecular mechanism underlying the initiation and progression of gastric MALT lymphoma is not fully understood.

Many previous studies have focused mainly on aberrant expression of protein-coding genes in the pathogenesis of MALT lymphoma [9]. However, it has recently become apparent that non-coding genes including microRNAs (miRNAs) play important roles as tumor suppressor genes and oncogenes during human carcinogenesis. miRNAs are small (20–25 nucleotides) non-coding RNAs that function as endogenous silencers of target genes. miRNAs are expressed in a tissue-specific manner and play critical roles in cellular proliferation, apoptosis and differentiation [10]. It has been shown that aberrant expression of miRNAs contributes to the development of human malignancies, and that miRNA expression signatures are associated with prognostic factors of human diseases [11–15]. Moreover, we have recently proposed that epigenetic regulation of tumor suppressor miRNAs could be a novel therapeutic approach for the treatment of human malignancies [16–19].

Table 1. Clinicopathological and molecular features of gastric MALT lymphoma cases.

			API2-MALT1	*H.pylori*	infection	Remission after	Months after
No.	**Sex**	**Age**	**fusion**	**before Tx**	**after Tx**	**eradication**	**eradication**
1	M	74	+	+	–	NC	74
2	M	74	+	+	–	NC	32
3	M	61	+	–	–	NC	10
4	F	75	+	–	*	NC	*
5	F	59	+	–	*	NC	*
6	F	57	+	–	*	NC	*
7	M	77	–	+	–	NC	45
8	F	68	–	–	–	NC	3
9	F	58	–	+	–	CR	24
10	F	46	–	+	–	CR	10
11	F	66	–	+	–	CR	10
12	F	52	–	+	–	CR	8
13	M	64	–	+	–	CR	6
14	F	71	–	+	–	CR	4
15	F	70	–	+	–	CR	4
16	M	60	–	+	–	CR	4
17	F	56	–	+	–	CR	4
18	M	54	–	+	–	CR	3
19	F	68	–	+	–	CR	2
20	F	40	–	+	–	CR	1

CR, complete remission; NC, no change.
*Eradication therapy not used.
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Although recent studies have shown that *miR-155*, a potential oncogenic miRNA, is highly expressed in diffuse large B cell lymphoma (DLBCL) [20,21] and overexpression of *miR-155* is correlated with a poor outcome in patients with DLBCL [20], the miRNA expression profiles of low-grade MALT lymphoma have not yet been described. In the present study, therefore, the miRNA expression profiles and potential miRNA target genes of gastric MALT lymphoma were analyzed to clarify the molecular pathogenesis of this malignancy.

Methods

Patients and tissue specimens

Twenty patients with primary low-grade gastric MALT lymphomas who were diagnosed and treated at Keio University Hospital (Tokyo, Japan) were enrolled. This study was approved by the ethics committee of Keio University School of Medicine (No. 18-96-3) and was registered with the Clinical Trials Registry (UMIN 000000858). Written informed consent was obtained from all the patients before examination. The clinicopathological and molecular features of the patients are shown in Table 1. The *H. pylori* infection status was identified using the ^{13}C-urea breath test. Some cases were confirmed by serological or histological examination in addition to the ^{13}C-urea breath test. Tissue specimens from gastric MALT lymphomas and the corresponding non-tumor gastric mucosae were obtained from patients during an endoscopic biopsy and were stored in RNAlater (Ambion, Austin, TX) at −80°C until RNA extraction.

Fluorescence in situ hybridization (FISH) analysis

To detect the chromosome translocation t(11;18)(q21;q21) and the *API2-MALT1* fusion gene arising from it, FISH analysis using the LSI API2-MALT1 t(11;18)(q21;q21) Dual Color, Dual Fusion Translocation Probe (Vysis/Abbot Laboratories Ltd., Maidenhead, Berkshire, United Kingdom) was performed by Mitsubishi Chemical Medience Corporation (Tokyo, Japan).

RNA extraction and microarray analysis

Total RNA, including small RNA, was extracted using a mirVana miRNA isolation kit (Ambion). The total RNAs from each of three gastric MALT lymphomas were pooled, as were the total RNAs from each of three matched samples of non-tumor gastric mucosa. The miRNA microarray analysis was performed by LC Sciences (www.lcsciences.com, Houston, TX). RNA from the MALT lymphomas was labeled using Cy5, while the RNA from the non-tumor gastric mucosa was labeled using Cy3. The microarray chip contains probe regions that detect 711 miRNA transcripts listed in Sanger miRBase Release 10.0 (http://www.sanger.ac.uk) and 5 probes for each miRNA. Detected signals greater than background plus 3 times the standard deviation were derived for each color channel; the mean and the co-variance (CV = standard deviation × 100/replicate mean) of each probe having a detected signal was calculated. For two color experiments, the ratio of the two sets of detected signals and *p*-values of the *t*-test were calculated. Differentially detected signals were accepted as true when the *p*-values of the ratios were less than 0.01. The average values of their signal intensities are shown in

Table 2. miRNAs differentially expressed between MALT lymphomas and non-tumor gastric mucosae.

No.	microRNA	Non-tumor (Cy3 signal)	MALT (Cy5 signal)	Fold change
1	*miR-142-5p*	56	1236.3	22.1
2	*miR-142-3p*	25.4	440.5	17.3
3	*miR-223*	574.1	5330.9	9.3
4	*miR-138*	40.9	200.8	4.9
5	*miR-155*	3093.2	13503.9	4.4
6	*miR-572*	1581.7	368.2	0.2
7	*miR-146b-5p*	1029.1	4249.4	4.1
8	*miR-141*	6525.1	1588.8	0.2
9	*miR-146a*	2187.8	8374.4	3.8
10	*miR-378*	3218.7	933.6	0.3

The miRNA microarray analysis was performed by LC Sciences (www.lcsciences.com, Houston, TX). All data were submitted to the ArrayExpress database; the accession number was E-MEXP-1898.

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Table 2. All the data were submitted to the ArrayExpress database under the accession number E-MEXP-1898.

Quantitative RT-PCR of miRNAs

Levels of miRNA expression were analyzed using quantitative RT-PCR and the TaqMan microRNA assay for *miR-142-5p* and *miR-155* (Applied Biosystems, Foster City, CA), in accordance with the manufacturer's instructions. The expression levels were normalized to that of U6 RNA and expressed as the mean ± standard deviation.

Infection of C57BL/6 mice with *H. heilmannii*

Ten months prior to the experiment, six-week-old C57BL/6 mice were inoculated with gastric mucosal homogenates from *H. heilmannii*-infected mice, as described previously [22]. Stool DNA was extracted using a QIAamp DNA Stool Mini Kit (Qiagen, Tokyo, Japan), and infection with *H. heilmannii* was confirmed by real-time PCR of stool DNA using specific primers (HeilF, 5′-AAGTCGAACGATGAAGCCTA-3′ and HeilR, 5′-ATTTGGTATTAATCACCATTTC-3′). RNA extraction and histological examination were performed using tissue specimens from the stomachs of *H. heilmannii*-infected mice and control mice. This study was approved by the Keio University Animal Research Committee (No. 08080).

Western blotting

Protein extracts were separated using SDS/polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The membranes were hybridized with the rabbit anti-human TP53INP1 polyclonal antibody (LifeSpan Biosciences, Seattle, WA). This antibody shows cross-reactivity with mouse TP53INP1. The signal intensities were analyzed using ImageJ software.

Immunohistochemistry

Formalin-fixed and paraffin-embedded tissues were deparaffinized and rehydrated. For antigen retrieval, the sections were treated for 20 min at 100°C in an autoclave and non-specific reactions were blocked with a blocking reagent (Protein Block Serum-Free, Dako Cytomation, Glostrup, Denmark). The sections were incubated with the rabbit anti-human TP53INP1 polyclonal antibody (diluted 1:200; LifeSpan Biosciences) overnight at 4°C followed by horseradish peroxidase (HRP)-labeled anti-rabbit IgG (Histofine, Simple stain MAX-PO; Nichirei, Tokyo, Japan) for 30 min at room temperature. Then, the sections were treated with 3, 3′-diaminobenzidine tetrahydrochloride solution. All the sections were counterstained with HE.

Luciferase assay

Luciferase constructs were made by ligating oligonucleotides containing the wild-type or mutant target site of the *TP53INP1* 3′ untranslated region (UTR) into the *Xba* I site of the pGL3-control vector (Promega, Madison, WI). The AGS human gastric cancer cell line was used in this study. AGS was obtained from the American Type Culture Collection (Rockville, MD). Cells were cultured in RPMI1640 medium supplemented with 10% fetal bovine serum. AGS cells were transfected with 0.4 μg of firefly luciferase reporter vector containing the wild-type or mutant target site and 0.02 μg of the control vector containing *Renilla* luciferase pRL-CMV (Promega) using lipofectamine 2000 (Invitrogen, Carlsbad, CA) in 24-well plates. The *miR-142-5p* and *miR-155* precursor molecules and negative control precursor miRNAs were purchased from Ambion. The molecules were transfected into AGS cells at a final concentration of 100 nM each. The luciferase assays were performed 24 hours after transfection using the Dual Luciferase Reporter Assay System (Promega). The activity of firefly luciferase was normalized to that of *Renilla* luciferase.

Statistics

Differences in the miRNA levels between the groups were analyzed using paired t test and unpaired t test. Differences at $p<0.05$ were considered significant.

Results

Overexpression of *miR-142-5p* and *miR-155* in gastric MALT lymphoma

To identify miRNAs that play critical roles in the development of gastric MALT lymphoma, we performed miRNA microarray analysis using RNAs obtained from three gastric MALT lymphomas and three matched samples of non-tumor gastric mucosa. The miRNAs that were differentially expressed between non-tumor gastric mucosae and MALT lymphoma lesions are listed in Table 2. The miRNA expression profile revealed that a hematopoietic-specific miRNA, *miR-142* [23,24], and an oncogenic miRNA, *miR-155* [12,25], were overexpressed in MALT lymphoma lesions, relative to the levels of expression in the corresponding non-tumor mucosae.

To validate the microarray data, we performed quantitative RT-PCR for *miR-142-5p* and *miR-155* in 20 cases of gastric MALT lymphoma. The clinicopathological and molecular features of the 20 patients are shown in Table 1. All of the gastric MALT lymphoma cases in the present study were stage I_E according to the Ann Arbor staging system [26]. The average age of the patients was 62.5 years (male/female: 7/13). As shown in Figure 1, the expression levels of *miR-142-5p* and *miR-155* in the gastric MALT lymphoma lesions were significantly higher than those in the corresponding non-tumor gastric mucosae ($p<0.05$ and $p<0.05$, respectively).

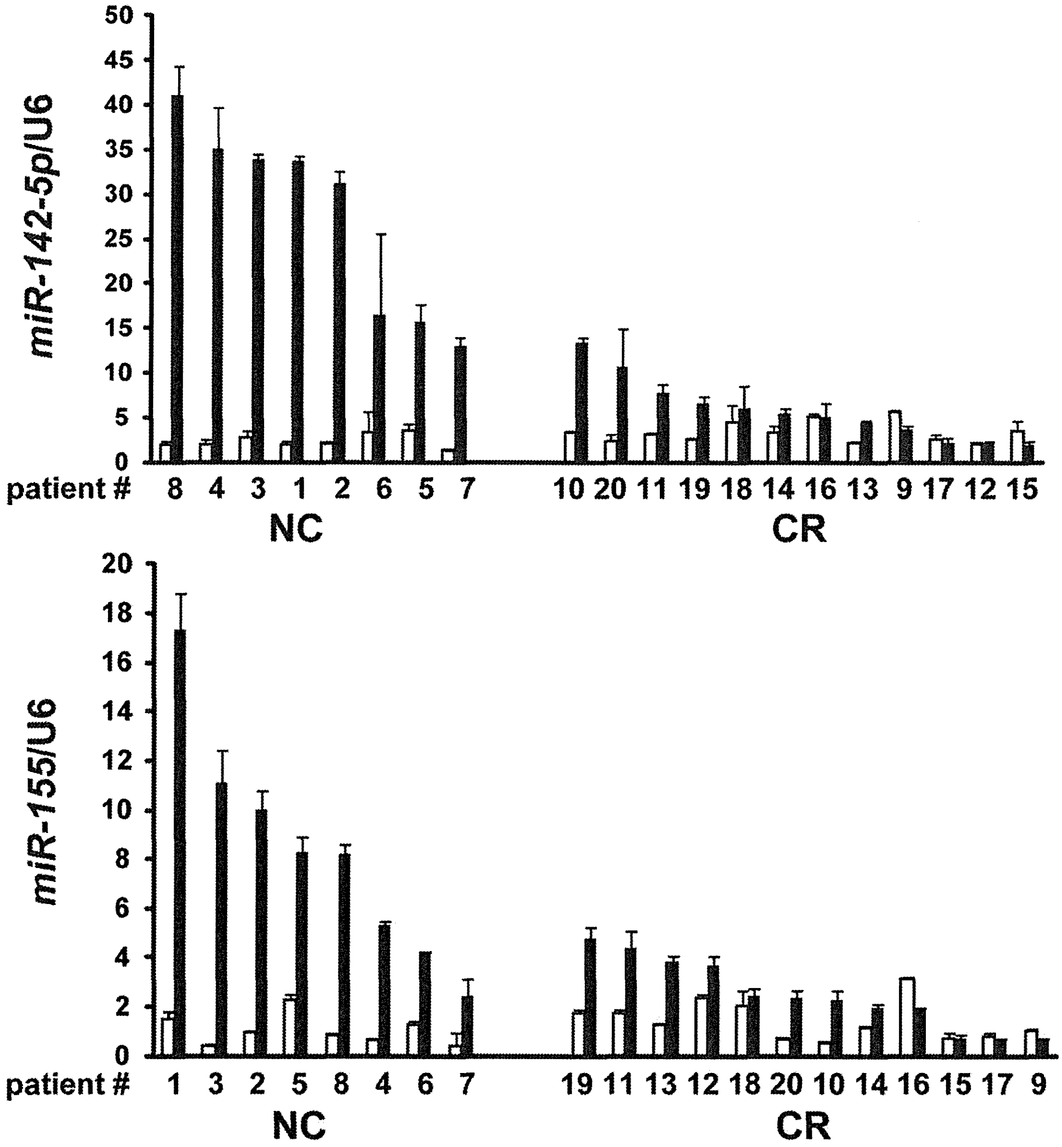

Figure 1. Expression levels of *miR-142-5p* and *miR-155* and responses to *H. pylori* eradication therapy in gastric MALT lymphoma cases. The levels of *miR-142-5p* and *miR-155* expression were evaluated using quantitative RT-PCR and normalized to the expression of U6 RNA. The filled bars and blank bars indicate MALT lymphoma lesions and non-tumor gastric mucosae, respectively. The levels of *miR-142-5p* and *miR-155* expression in gastric MALT lymphoma lesions were significantly increased relative to the corresponding non-tumor gastric mucosae ($p<0.05$ and $p<0.05$, respectively). The levels of *miR-142-5p* and *miR-155* expression in MALT lymphoma lesions which confers resistance to *H. pylori* eradication were significantly higher than those in lesions lacking the *API2-MALT1* fusion gene, which exhibited CR after *H. pylori* eradication ($p<0.0001$ and $p<0.005$, respectively). CR, complete remission; NC, no change.
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miR-142-5p and *miR-155* as novel biomarkers of gastric MALT lymphoma

The patients with gastric MALT lymphoma were divided into two groups according to their response to *H. pylori* eradication therapy (Table 1). All the patients who were positive for *H. pylori* underwent eradication therapy and were subsequently confirmed to be *H. pylori*-negative. The use of *H. pylori* eradication therapy for patients with gastric MALT lymphoma who are *H. pylori*-negative is controversial. Nakamura *et al.* have recently reported the long-term clinical outcome of patients with gastric MALT lymphoma after *H. pylori* eradication [27]. In their study, 44 *H. pylori*-negative gastric MALT lymphoma patients underwent *H. pylori* eradication therapy, and 6 (14%) responded to *H. pylori* eradication. Akamatsu *et al.* have also reported that 1 out of 9 (11%) *H. pylori*-negative patients showed complete regression of gastric MALT lymphoma [28]. On the other hand, Fischbach *et al.* have reported that most patients with histological residuals of gastric MALT lymphoma after successful *H. pylori* eradication (treatment failure cases) had a favorable disease course without additional treatment (Fischbach *et al.* Gut 2007; 56: 1685–7). For these patients, a watch and wait strategy with regular endoscopic examinations and biopsies appears to be safe. Our policy of *H. pylori* eradication therapy for gastric MALT lymphoma patients who were *H. pylori*-negative was decided in accordance with the preference of individual patients.

We also analyzed the chromosome translocation t(11;18)(q21;q21) with the *API2-MALT1* fusion gene by FISH

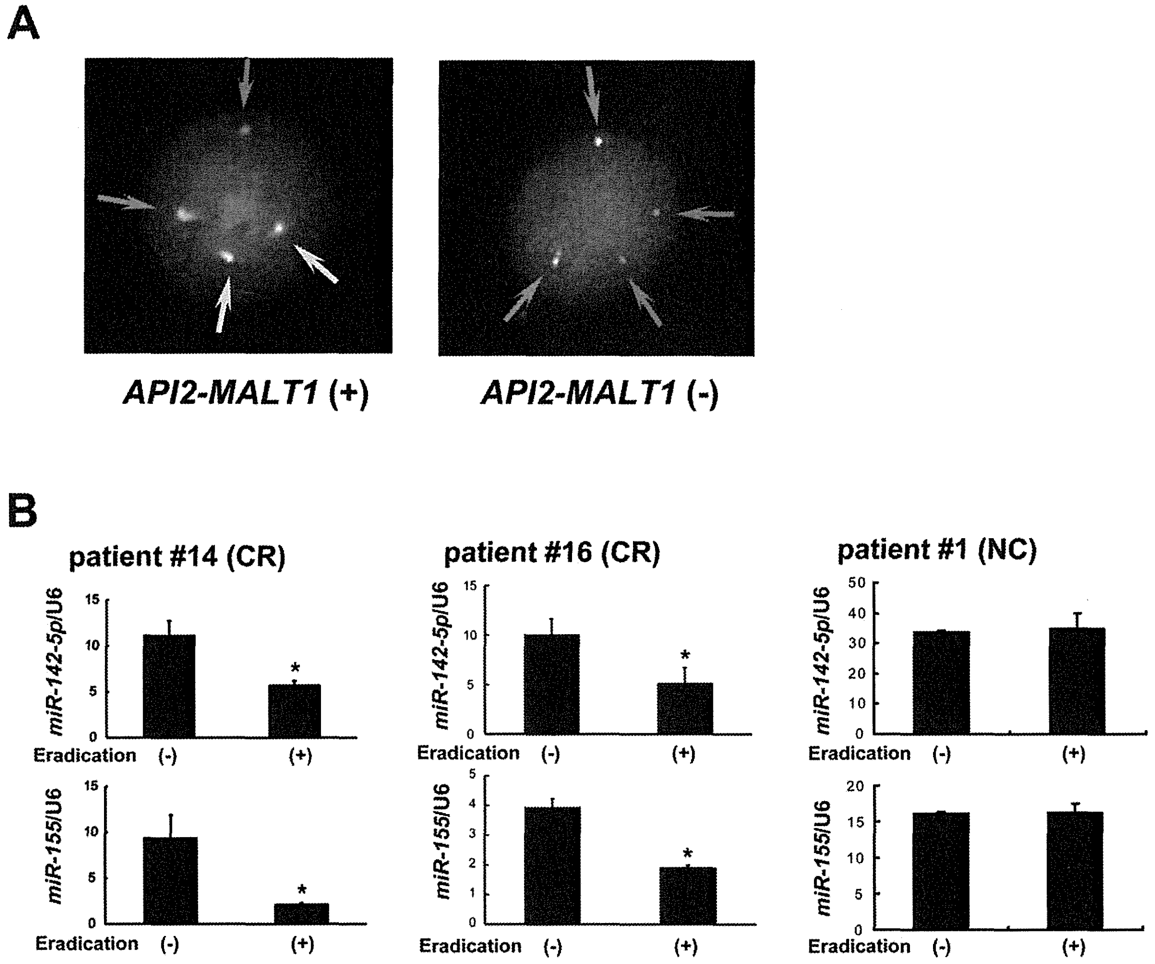

Figure 2. Expression levels of *miR-142-5p* and *miR-155* in gastric MALT lymphoma cases before and after *H. pylori* eradication therapy. (A) Representative cases of the FISH analyses for the detection of the *API2-MALT1* fusion gene are shown. The probes for *API2* and *MALT1* were labeled with green and red signals, respectively. The *API2-MALT1* fusion gene produces a yellow signal. (B) Patients #14 and #16, positive for *H. pylori* and negative for the *API2-MALT1* fusion gene. These patients received *H. pylori* eradication therapy and attained CR. The expression levels of *miR-142-5p* and *miR-155* were significantly lower after *H. pylori* eradication. * $p<0.05$. Patient #1, positive for *H. pylori* and positive for the *API2-MALT1* fusion gene. This patient was resistant to *H. pylori* eradication therapy (NC). The expression levels of *miR-142-5p* and *miR-155* showed no significant differences.
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analysis. The probes for *API2* were labeled with green signals, and the probes for *MALT1* were labeled with red signals. In positive cases, the *API2-MALT1* fusion genes resulting from the chromosome translocation produced yellow signals (Figure 2A).

As shown in Figure 1, the levels of expression of *miR-142-5p* and *miR-155* in MALT lymphoma lesions that were resistant to *H. pylori* eradication were significantly higher than in cases showing complete remission (CR) after *H. pylori* eradication ($p<0.0001$ and $p<0.005$, respectively). Although the *API2-MALT1* fusion gene has been identified as a potential predictor of resistance to *H. pylori* eradication therapy, patients #7 and #8 with gastric MALT lymphoma lacking the *API2-MALT1* fusion gene were resistant to *H. pylori* eradication. These patients showed increased expression levels of *miR-142-5p* and *miR-155* (Figure 1).

In addition, we investigated the correlation between the levels of *miR-142-5p* and *miR-155* expression and the clinical courses of gastric MALT lymphoma cases. The expression data for patients #14 and #16 with gastric MALT lymphoma who achieved CR after *H. pylori* eradication therapy showed that the levels of *miR-142-5p* and *miR-155* expression were significantly reduced after eradication (Figure 2B, $p<0.05$ and $p<0.05$, respectively). On the other hand, in patient #1 with gastric MALT lymphoma harboring the *API2-MALT1* fusion gene that was resistant to *H. pylori* eradication, there was no significant difference in the expression levels of *miR-142-5p* and *miR-155* (Figure 2B). Although the *H. pylori* infection was eradicated by antibiotic treatment, histological examination showed no regression of the MALT lymphoma lesion. This patient was followed up by observation without treatment, and no marked changes in the endoscopic or histological findings were observed. These findings indicate that the levels of *miR-142-5p* and *miR-155* expression have potential applicability as novel biomarkers of gastric MALT lymphoma.

Overexpression of *miR-142-5p* and *miR-155* in an animal model of gastric MALT lymphoma

To further confirm the molecular pathogenesis of gastric MALT lymphoma, we examined the expression levels of *miR-142-5p* and *miR-155* in an animal model of gastric MALT lymphoma. Infection with *H. heilmannii* induces gastric LELs that are consistent with low-grade MALT lymphomas in C57BL/6 mice [22].

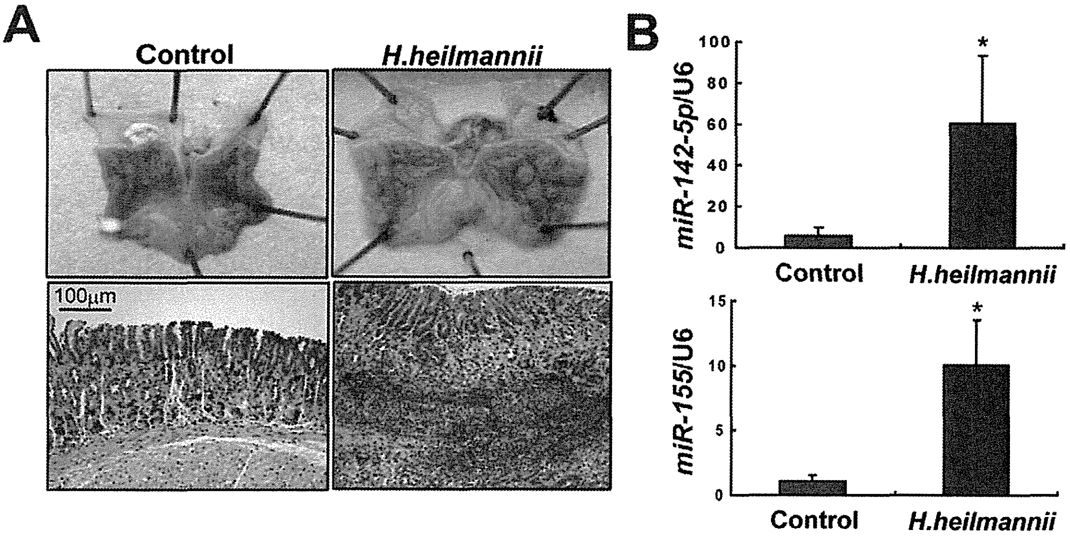

Figure 3. Expression levels of *miR-142-5p* and *miR-155* in an animal model of gastric MALT lymphoma. (A) Macroscopic and light microscopic findings with HE staining of the stomach of control and *H. heilmannii*-infected mice (10 months after infection). Protrusive lesions in the fundic stomach of *H. heilmannii*-infected mice were observed. Light microscopic observations using HE staining revealed the presence of LELs consistent with low-grade MALT lymphoma. (B) Expression levels of *miR-142-5p* and *miR-155* in the stomach of control and *H. heilmannii*-infected mice. The levels of *miR-142-5p* and *miR-155* expression normalized to the level of U6 RNA were significantly increased in *H. heilmannii*-infected C57BL/6 mice. * $p<0.05$.
doi:10.1371/journal.pone.0047396.g003

Infection with *H. heilmannii* was confirmed using quantitative PCR analysis of stool DNA (data not shown). Ten months after infection with *H. heilmannii*, control (n = 3) and *H. heilmannii*-infected C57BL/6 mice (n = 4) were dissected, and this revealed protruding lesions in the gastric fundus in all of the latter. Light microscopic observations using hematoxylin and eosin (HE) staining revealed the presence of LELs consistent with low-grade MALT lymphomas (Figure 3A). Quantitative RT-PCR analyses showed that the levels of expression of *miR-142-5p* and *miR-155* were significantly higher in *H. heilmannii*-infected C57BL/6 mice ($p<0.05$ and $p<0.05$, respectively), being similar to the results for human gastric MALT lymphomas (Figure 3B).

miR-142-5p and *miR-155* suppress the proapoptotic gene *TP53INP1* as their target

Identification of miRNA target genes is essential for determining miRNA function. Recent studies have indicated that a single miRNA may regulate more than 200 target genes. A database for predicting target genes, TargetScan (http://www.targetscan.org), revealed that both *miR-142-5p* and *miR-155* are able to bind to the 3′ UTR of the mRNA of the proapoptotic gene *TP53INP1* (Tumor Protein P53 Inducible Nuclear Protein 1). Moreover, *miR-155* has been shown to repress *TP53INP1*, which inhibits the development of pancreatic tumors [29]. Therefore, we focused on *TP53INP1* as a common target of *miR-142-5p* and *miR-155*.

To confirm the target specificity of *miR-142-5p* and *miR-155* for *TP53INP1*, we performed a luciferase reporter assay using a vector containing the putative *TP53INP1* 3′ UTR target sites downstream of the luciferase reporter gene, which was transfected into AGS cells. The base pairing between *miR-142-5p* and *miR-155* and the wild-type (WT) or mutant (MUT) target sites in the 3′ UTR of *TP53INP1* mRNA is shown in Figure 4A. The luciferase activities of the AGS cells transfected with the *TP53INP1*-WT construct were significantly lower after transfection with *miR-142-5p* and *miR-155* ($p<0.05$ and $p<0.005$, respectively), whereas those of cells transfected with the *TP53INP1*-MUT construct and the pGL3 control vector (empty vector) showed no significant differences (Figure 4B). It has been shown that conserved perfect 6- to 8-bp matches between the 5′ end of the mature miRNA and the 3′ UTR of the predicted target mRNA (called 'seed' matches) are the most important factor determining miRNA targets [30]. As shown in Fig. 4A, 'seed' matches between the 5′ end of the miRNAs and the 3′ UTR of *TP53INP1* were stronger in *miR-155* than in *miR-142*, suggesting that *miR-155* may have a more profound effect on suppression of TP53INP1.

We next examined the expression levels of TP53INP1 by Western blot analysis in *H. heilmannii*-infected mice and immunohistochemistry in human gastric MALT lymphoma-like lesions. The expression of TP53INP1 was suppressed in *H. heilmannii*-infected mice, relative to control mice (Figure 4C). We examined the expression levels of TP53INP1 in human gastric tissues by immunohistochemistry. The gastric tissue shown in Fig. 4D contains both MALT lymphoma and non-MALT lymphoma tissue. The infiltrating lymphocytic lesion shown by HE staining is consistent with MALT lymphoma. TP53INP1 staining is negative or faint in the MALT lymphoma lesion, whereas normal gastric glands around the MALT lymphoma show sufficient staining for TP53INP1. Thus TP53INP1 staining was markedly reduced in MALT lymphoma lesions (Figure 4D). These findings suggest that *TP53INP1* is a common target of *miR-142-5p* and *miR-155* and is suppressed by overexpression of both *miR-142-5p* and *miR-155* in gastric MALT lymphoma lesions.

Discussion

A recent study has demonstrated that miRNA expression profiles can be used to classify the developmental lineages and differentiation stages of tumors, and are more accurate for tumor classification than conventional mRNA profiles [31]. Furthermore, miRNA expression signatures are associated with prognostic factors and disease progression in chronic lymphocytic leukemia [11] and lung cancer [32]. Thus miRNA expression is clinically promising as both a diagnostic tool and a prognostic marker for