

MicroRNA-148a is downregulated in gastric cancer, targets MMP7, and indicates tumor invasiveness and poor prognosis

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Gastric cancer (GC) remains a major public health issue as the fourth most common cancer and the second leading cause of cancer mortality worldwide.⁽¹⁾ Gastric cancer is assumed to originate from a sequential accumulation of molecular and genetic alterations to stomach epithelial cells.⁽²⁾ A deeper understanding of the pathogenesis and biological features of GC is necessary to further inform and enhance early detection and treatment methods.

MicroRNAs (miRNAs) are non-protein-coding small RNAs in the size range of 19–25 nucleotides, which play important regulatory roles in posttranscriptional repression and fine-tune various essential biological processes.^(3–5) Recent data clearly shows that miRNAs are abnormally expressed in various cancers, indicating novel roles as oncogenes or tumor suppressors in different tumor types.⁽⁶⁾ Currently, miRNA target-prediction algorithms and publicly available databases are undergoing rapid and dramatic evolution. However, precision and sensitivity of state-of-the-art algorithms were estimated as ~50% and 12%, respectively, when tested against proteomics-supported miRNA targets,⁽⁷⁾ highlighting the necessity for mass experimental miRNA target validation.⁽⁸⁾ As for GC, although there are several

Gastric cancer (GC) develops through deregulation of gene expression and accumulation of epigenetic abnormalities, leading to tumor cell acquisition of malignant features. MicroRNAs (miRNAs) play a critical role in cancer development where they can act as oncogenes or oncosuppressors. To identify miRNAs that are associated with some clinicopathologic features of GC and/or participate in tumor progression, miRNA expression in 20 GC tissues and five corresponding non-neoplastic gastric mucosa was examined by miRNA microarray. Oligonucleotide array analysis was carried out for miRNA target prediction. The functions of candidate miRNAs and their target genes were also analyzed by quantitative RT-PCR, Western blotting, reporter gene assay, and cell invasion assay. Comparison of miRNA expression profiles revealed that downregulation of miR-148a was identified in most of the GC tissues. Downregulation of miR-148a was significantly correlated with an advanced clinical stage, lymph node metastasis, and poor clinical outcome. Custom oligonucleotide array analysis revealed that *MMP7* expression was markedly downregulated in miR-148a-overexpressing GC cells; *MMP7* was found to be a direct and functional target of miR-148a, participating in cell invasion. These results suggest that miR-148a contributes to the maintenance of homeostasis in normal stomach tissue and plays an important role in GC invasion by regulating *MMP7* expression.

reports of aberrant expression of specific miRNAs, target predictions of identified miRNAs were carried out by several online databases in each study.^(9,10) Each new finding of deregulated miRNA in GC and its target gene is a welcome addition to the body of knowledge; however, there is no denying the possibility that genuine targets can be overlooked using online algorithms.

In the present study, we examined miRNA expression in 20 GC cases and five corresponding non-neoplastic gastric mucosa using miRNA microarray. By comparing these expression profiles, we found downregulation of miR-148a in the majority of GC tissues. The correlation between the miR-148a expression level and clinicopathologic parameters of GC was also examined and we identified that lower expression levels of miR-148a correlate with worse clinicopathologic characters. To predict gene targets of miR-148a, we used an oligonucleotide array to generate a list of genes whose expression was deregulated by altered expression of miR-148a, and found that expression of *MMP7* was suppressed by upregulation of miR-148a. It was confirmed that *MMP7* was a direct and functional target of miR-148a using quantitative RT-PCR (qRT-PCR), Western blot analysis, and reporter gene assay. We also carried

out target prediction of miR-148a using representative online databases. However, no lists of miR-148a target genes contained *MMP7*, thus validating the reliability of the conventional laboratory-based experimental strategy for miRNA target prediction, by which crucial target genes were detected-genes, which online target prediction could not detect.

Materials and Methods

MicroRNA microarray hybridization. Total RNA was isolated from frozen tissue with Isogene (Nippon Gene, Tokyo, Japan). Short-strand RNA was purified from total RNA with the RNeasy MinElute Cleanup Kit (Qiagen, Hilden, Germany). For the oligonucleotide array we used Genopal-MICH07 DNA chips (Mitsubishi Rayon, Tokyo, Japan) comprising 188 oligonucleotide DNA probes. Details are described in Data S1.

In situ hybridization for miR-148a. Biotin 3'-labeled locked nucleic acid-incorporated miRNA probe (miR-CURY LNA detection probe; Exiqon, Woburn, MA, USA) was used for visualization of miR-148a. Details are described in Data S1.

Cell lines. Nine cell lines derived from human GC were used. All cell lines were maintained as described previously.⁽¹¹⁾ Details are described in Data S1.

Oligonucleotide array construction, hybridization, detection, and data analysis. The oligonucleotide array, Genopal (Mitsubishi Rayon), was prepared as described previously.⁽¹²⁾ Details of oligonucleotide array probes, total RNA isolation, RNA quantification and assessment of integrity, hybridization, detection, and data analysis were carried out as described previously.⁽¹³⁾

Quantitative RT-PCR and Western blot analysis. Quantification of *MMP7* mRNA levels was carried out using real-time fluorescence detection as described previously.⁽¹⁴⁾ For analysis of miR-148a and U6B, expression levels of miR-148a and RNU6B were measured and quantified as described previously.⁽¹⁵⁾ Western blot analysis was carried out as described previously.⁽¹⁶⁾ Details are described in Data S1.

CpG island determination, genomic DNA extraction, and bisulfite genomic DNA sequencing. A 20-kb genomic DNA fragment encompassing the miRNA coding sequences was analyzed with CpG plot software (<http://bioweb2.pasteur.fr>). We used the parameter values to report a CpG island as described previously.⁽¹⁷⁾ Thermal cycling conditions were as described previously.⁽¹⁸⁾ Methylation status of PCR products were confirmed as described previously.⁽¹⁹⁾ Details are described in Data S1.

Plasmids. For constitutive expression of the *MMP7* gene, commercially available *MMP7* cDNA construct (Invitrogen, Carlsbad, CA, USA) was purchased. pCMV-MIR (Origene, Rockville, MD, USA) was used for constitutive expression of miR-148a. Details are described in Data S1.

Cell transfection. Transfection of cells was carried out with Lipofectamine RNAiMAX Reagent (Invitrogen) according to the manufacturer's instructions. Details are described in Data S1.

Reporter gene assay. Reporter gene assay was carried out as described previously.⁽²⁰⁾ Details are described in Data S1.

Tissue samples. A total of 81 primary tumor samples were collected from patients diagnosed with GC. Details are described in Data S1.

Cell growth and in vitro invasion assay. For the MTT assay, cells were seeded at a density of 2000 cells per well in 96-well plates. Cell growth was monitored after 1, 2, and 4 days by

MTT assay.⁽²¹⁾ Modified Boyden chamber assays were used to examine invasiveness as described previously.⁽²⁰⁾

Statistical methods. Statistical differences between miRNA levels in non-neoplastic gastric mucosa and GC were evaluated using the Mann-Whitney *U*-test and Wilcoxon matched pairs test. The correlation between expression levels of miR-148a and clinicopathologic parameters was analyzed with Fisher's exact test. Univariate and multivariate Cox regression was used to evaluate the associations between clinical covariates and cancer-specific mortality. Hazard ratios and 95% confidence intervals were estimated from Cox proportional hazard models. *P*-values < 0.05 were considered to be statistically significant.

Results

MicroRNA microarray analysis. To identify GC-related miRNA, we carried out miRNA microarray profiling to compare between 20 cancer tissue and five corresponding non-neoplastic gastric mucosa. Through the comparison between these two profiles, six miRNAs showed significantly higher expression in corresponding non-neoplastic gastric mucosa than in cancer tissue (Table 1). Among six miRNAs, we focused on miR-148a because its expression was most downregulated in GC tissue. Further analysis of the same GC tissues by qRT-PCR also showed a significant downregulation of miR-148a (Fig. S1).

Clinicopathologic characteristics of GC cases in which miR-148a is downregulated. To confirm the localization of miR-148a expression, *in situ* hybridization (ISH) was carried out using GC tissues. We could detect the miR-148a expression only in fundic glands and pyloric glands. However, neither cancer cells nor stromal tissues expressed miR-148a (Fig. 1a). Then, to further understand the relationship between miR-148a expression and clinicopathologic parameters, we examined the miR-148a expression levels in 61 formalin-fixed paraffin-embedded samples of primary GC and their corresponding non-neoplastic gastric mucosa by qRT-PCR. In this sample set, miR-148a expression levels were also significantly downregulated in GC tissue compared with corresponding non-neoplastic mucosa (Fig. S2). The expression level of miR-148a was evaluated using the tumor : normal ratio of miR-148a. The relationship

Table 1. Summary of significantly downregulated miRNAs in gastric cancer tissue compared with non-neoplastic mucosa

| miRBase ID | Mature accession | Intensity | | Fold change | <i>P</i> -value |
|----------------|------------------|-----------------------|-------|-------------|-----------------|
| | | Non-neoplastic mucosa | Tumor | | |
| hsa-miR-148a | MIMAT0000243 | 892 | 279 | -0.31 | 0.0178 |
| hsa-miR-29a | MIMAT0000086 | 1626 | 932 | -0.57 | 0.0395 |
| hsa-miR-30a-3p | MIMAT0000088 | 681 | 412 | -0.60 | 0.0246 |
| hsa-miR-302b | MIMAT0000715 | 953 | 581 | -0.61 | 0.0207 |
| hsa-miR-30a-5p | MIMAT0000087 | 648 | 402 | -0.62 | 0.0443 |
| hsa-miR-125b-1 | MIMAT0000423 | 1257 | 804 | -0.64 | 0.0315 |

miRBase ID codes are available from www.mirbase.org.

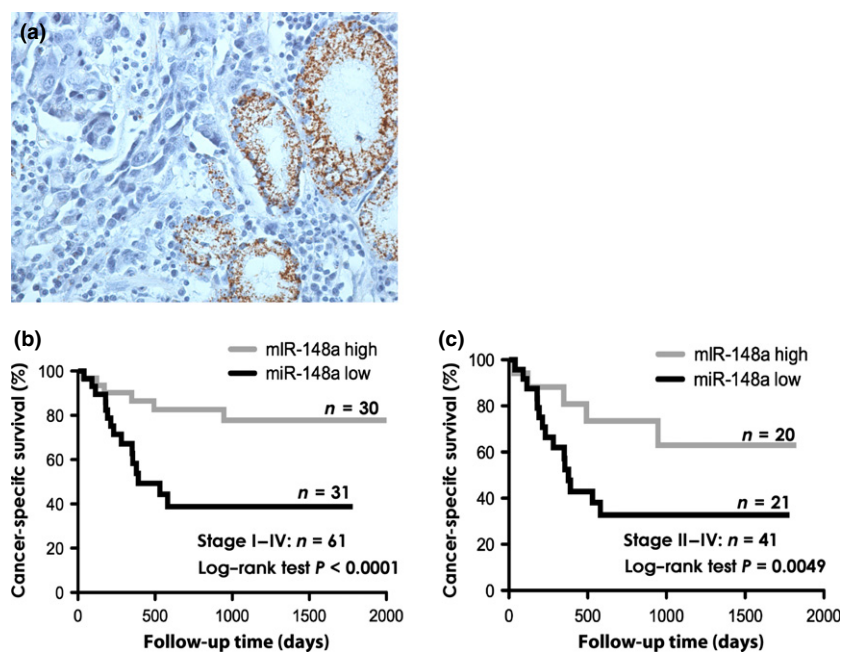


Fig. 1. *In situ* hybridization for microRNA-148a (miR-148a) and relationship between miR-148a expression and gastric cancer (GC) patient prognosis. (a) MiR-148a expression in GC tissue detected by *in situ* hybridization. (b) Log-rank test and Kaplan–Meier plots were constructed for the miR-148a high and miR-148a low groups. Cancer-specific survival of 61 patients with GC based on the expression levels of miR-148a (cut-off line = median miR-148a expression level). (c) Cancer-specific survival of 41 patients with advanced (stage II–IV) GC based on the expression levels of miR-148a (cut-off line = median miR-148a expression level in this group).

Table 2. Univariate and multivariate analysis of factors influencing survival in 61 patients with gastric cancer

| Parameter | Univariate analysis | | | Multivariate analysis | | |
|---------------------|---------------------|--------------|---------|-----------------------|---------------|---------|
| | HR | 95% CI | P-value | HR | 95% CI | P-value |
| Sex | | | | | | |
| Female | Ref. | | | | | |
| Male | 1.271 | 0.702–1.532 | 0.5896 | NA | NA | NA |
| Age, years | | | | | | |
| <60 | Ref. | | | | | |
| ≥60 | 1.122 | 0.442–1.483 | 0.8593 | NA | NA | NA |
| Mucin phenotype | | | | | | |
| I and N type | Ref. | | | | | |
| G and GI type | 0.903 | 0.214–3.802 | 0.1003 | NA | NA | NA |
| Histology† | | | | | | |
| Intestinal | Ref. | | | | | |
| Diffuse | 1.601 | 0.254–1.473 | 0.2805 | NA | NA | NA |
| T grade‡ | | | | | | |
| T1/2 | Ref. | | | Ref. | | |
| T3/4 | 4.886 | 1.136–3.225 | 0.0030 | 13.22 | 3.371–50.747 | <0.0010 |
| N grade‡ | | | | | | |
| N0 | Ref. | | | Ref. | | |
| N1/2/3 | 9.234 | 1.388–16.667 | <0.0010 | 5.063 | 0.931–94.529 | 0.0625 |
| Stage‡ | | | | | | |
| 0/I/II | Ref. | | | Ref. | | |
| III/IV | 8.460 | 2.083–11.111 | <0.0010 | 34.579 | 6.293–222.270 | <0.0010 |
| miR-148a expression | | | | | | |
| High | Ref. | | | Ref. | | |
| Low | 10.820 | 1.785–20.000 | 0.0041 | 3.076 | 1.216–8.776 | 0.0169 |

†Histology was determined according to the Japanese Classification of Gastric Cancer (14th edition). ‡Tumor stage was classified according to the criteria of the International Union Against Cancer TNM classification of malignant tumors. CI, confidence interval; HR, hazard ratio; NA, not applicable; Ref., reference.

between the miR-148a expression level and clinicopathologic parameters of GC is summarized in Table S1. We also evaluated the association between miR-148a expression levels and cancer-specific mortality. In all cases and in 41 advanced GC patients, the GC tissues with lower expression levels of miR-148a showed significantly worse prognosis than those with high expression (Fig. 1b,c). In order to further evaluate

the association of miR-148a expression with cancer-specific mortality, we used both univariate and multivariate Cox proportional hazards analyses (Table 2). According to the univariate analysis, T grade, N grade, stage, and miR-148a expression were significantly associated with cancer-specific mortality. According to the multivariate model, expression of miR-148a was an independent prognostic classifier of cancer-specific

mortality. These results indicate that loss of miR-148a may play important roles in GC progression.

Analysis of *MIR148A* methylation in GC cell lines. To pursue the cause of miR-148a downregulation, we examined the methylation status in CpG islands of the *MIR148a* promoter region in GC cell lines. Expression levels of miR-148a measured by qRT-PCR are shown in Figure S3(a), and we carried out bisulfite sequencing of genomic DNAs from two GC cell lines with high miR-148a expression and two cell lines with low miR-148a expression. Results of bisulfite sequencing are shown in Figure S3(b). An inverse correlation was observed between frequency of methylation in *MIR148a* promoter-region CpG islands and expression levels of miR-148a. In addition, we ascertained that miR-148a expression was restored in five aza-dc-treated MKN-45 and MKN-74 cells (Fig. S3c). These results suggest that DNA methylation of CpG islands of *MIR148A* play an important role in transcriptional inactivation in GC cell lines.

Effect of deregulation of miR-148a expression on cell growth and invasive activity. To investigate the biological significance of miR-148a in GC, we carried out an MTT assay 4 days after altering miR-148a expression. In this experiment, we used MKN-1 cells transfected with miR-148a precursor and HSC-57 cells transfected with miR-148a inhibitor. MKN-1 and HSC-57 were selected as they possessed the lowest and highest endogenous miR-148a expression among nine GC cell lines, respectively. Successful overexpression and suppression of miR-148a in each treated cell line were confirmed by qRT-PCR (Fig. 2a). Cell growth of GC cells with deregulated miR-148a expression did not differ from that of cells transfected with control miRNA up to day 4 (data not shown). Next, a Transwell invasion assay was carried out. The invasiveness of miR-148a-overexpressing MKN-1 cells was reduced compared with the negative control miRNA-transfected MKN-1 cells (Fig. 2b). In contrast, the invasiveness of miR-148a-suppressed HSC-57 cells was greater than that of the negative control miRNA-transfected HSC-57 cells (Fig. 2c). These results are consistent with those of a previous report,⁽²²⁾ and indicate that miR-148a regulates some genes which in turn affect tumor invasion in GC cells.

MicroRNA-148a target prediction using custom oligonucleotide array. Although some reports have identified the target gene of miR-148a, these studies used online databases to derive predicted targets.^(22–25) To identify novel GC invasion-related genes whose expression was directly regulated by miR-148a, we analyzed gene expression profiles from four GC cell lines showing deregulation of miR-148a alongside corresponding non-treated cells, using a custom oligonucleotide array. In each cell line, either overexpression or suppression of miR-148a was induced, according to their endogenous miR-148a expression level. The list of genes with expression altered more than twofold in miR-148a-deregulated cells is summarized in Table 3. Among these candidates, *MMP7* was markedly downregulated by miR-148a overexpression both in MKN-45 and MKN-74 cell lines. Although target prediction for miR-148a was carried out using online databases including TargetScan, PicTar, and miRanda, these databases did not list *MMP7* as a candidate miR148a target gene (data not shown). Therefore, further study of the correlation between miR-148a and *MMP7* was undertaken.

To confirm the regulation of *MMP7* by miR-148a, MKN-45 and MKN-74 cells were transfected to induce miR-148a overexpression, then examined for *MMP7* expression level by Western blotting. In both cell lines, *MMP7* expression was significantly downregulated; levels were similar to that of *MMP7*-specific

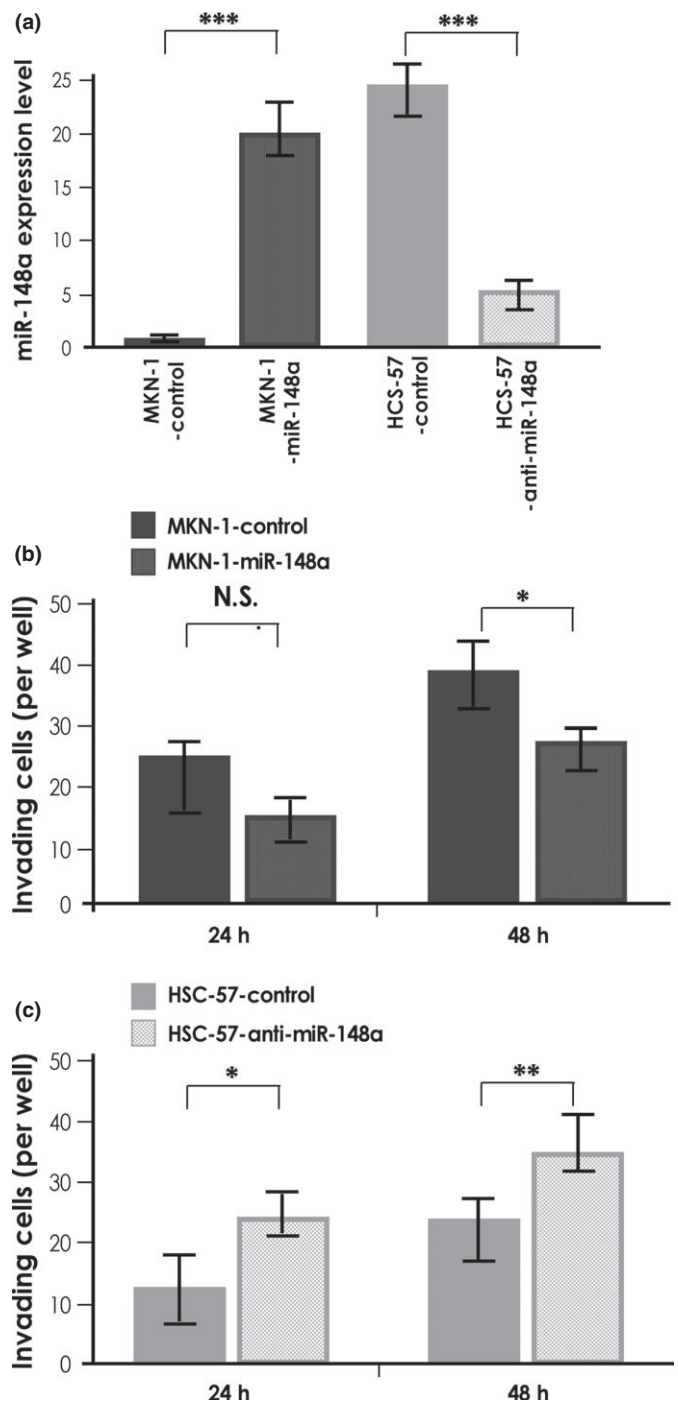


Fig. 2. MicroRNA-148a (miR-148a) expression and functional analysis. (a) Quantitative RT-PCR analysis of miR-148a in MKN-1 gastric cancer cells transfected with pre-miR-148a and HSC-57 cells transfected with anti-miR-148a. (b) Effect of miR-148a downregulation on cell invasion of MKN-1 cells. MKN-1 transfected with negative control miRNA and pre-miR-148a were incubated in Boyden chambers. (c) Effect of miR-148a downregulation on cell invasion of HSC-57 cells. HSC-57 transfected with negative control miRNA and anti-miR-148a were incubated in Boyden chambers. Results are mean \pm SD of triplicate measurements. * P < 0.05; ** P < 0.01; *** P < 0.001. N.S., not significant.

siRNA treated cells (Fig. 3a). The *MMP7* mRNA levels determined by qRT-PCR were consistent with the *MMP7* protein levels determined by Western blotting (Fig. 3b). It was also

Table 3. Genes downregulated by microRNA-148a (miR-148a) deregulation

| Symbol | Description | GenBank | Intensity | | Fold change |
|---------------|------------------------------|-------------|-----------|----------|-------------|
| | | | Control | miR-148a | |
| MKN-1 | | | | | |
| <i>KRTHB1</i> | Keratin 86 | NM_000584.2 | 168 | 72 | -2.33 |
| <i>CDH17</i> | Cadherin 17, LI-cadherin | NM_001912 | 148 | 72 | -2.09 |
| <i>APOE</i> | Apolipoprotein E | NM_145804.1 | 118 | 57 | -2.03 |
| MKN45 | | | | | |
| <i>MMP7</i> | Matrix metalloproteinase 7 | NM_002423.3 | 730 | 132 | -5.55 |
| <i>MIA</i> | Melanoma inhibitory activity | NM_006533.3 | 649 | 244 | -2.63 |
| MKN-74 | | | | | |
| <i>MMP7</i> | Matrix metalloproteinase 7 | NM_002423.3 | 291 | 32 | -9.99 |
| <i>NEIL</i> | Nei endonuclease VIII-like 1 | NM_024608.3 | 2004 | 831 | -2.43 |
| <i>MMP1</i> | Matrix metalloproteinase 1 | NM_002421.3 | 455 | 208 | -2.17 |
| HSC-57 | | | | | |
| <i>MMP10</i> | Matrix metalloproteinase 10 | NM_002425.2 | 766 | 381 | -2.00 |

found that upregulation of *MMP7* was inversely associated with miR-148a expression in GC cases analyzed in Figure 1(b) (Table S2). In addition, we examined the effect of miR-148a deregulation on the expression of epithelial–mesenchymal transition-related molecules. However, no significant alterations of these molecules were detected in miR-148a deregulated cells (Fig. S4a). As shown in Figure 2(b), overexpression of miR-148a repressed invasion of MKN-1 cells. However, *MMP7* expression was not detected in MKN-1 cells (data not shown). It was reported that ROCK1 is a direct target of miR-148a and could be involved in GC invasion.⁽²²⁾ We checked expression of ROCK1 in MKN-1 and the alteration of its expression in miR-148a-deregulated cells. Actually, we detected that expression of ROCK1 was downregulated by miR-148a overexpression in MKN-1 cells (Fig. S4b). Furthermore, to prove that *MMP7* is a direct target of miR-148a, we used a 3'-UTR sequence of *MMP7* cloned into a reporter vector downstream of the luciferase complementary DNA (Fig. 3c). Transfection of this construct into three GC cell lines with high endogenous miR-148a expression levels led to suppression of *MMP7* reporter activity, and mutation of the miR-148a binding site abolished the inhibitory effect of miR-148a on reporter activity (Fig. 3d). Conversely, transfection of this construct into three GC cell lines with low endogenous miR-148a expression levels did not lead to suppression of *MMP7* reporter activity. However, cotransfection of this construct with miR-148a precursor into these cells showed that miR-148a suppressed the *MMP7* reporter activity (Fig. S5). These results imply that miR-148a downregulates *MMP7* expression by directly targeting its 3'-UTR.

Several reports show that miR-148a regulates cancer cell invasion through suppressing its target genes.^(22,23) To confirm the cellular level at which *MMP7* contributes to GC cell invasion, the MKN-45 cell line was stably transfected with vector expressing miR-148a alone, and cotransfected with vectors expressing miR-148a and *MMP7*. MKN-45 cells were selected because they express high levels of *MMP7* and low levels of miR-148a. In MKN-45-miR-148a, which stably expressed miR-148a, *MMP7* expression was significantly suppressed, and in MKN-45-miR-148a/-*MMP7*, which stably expressed miR-148a and *MMP7*, the expression of *MMP7* was 50% less than that of empty vector-transfected MKN-45 cells (Fig. 3e). Next, the invasive properties of these cells were examined by Transwell invasion assay. As shown in Figure 3(f), the inva-

siveness of MKN-45-miR-148a was approximately 50% less than that of MKN-45-empty vector at all endpoints. However, the invasiveness of MKN-45-miR-148a/-*MMP7* was only approximately 10–20% less than that of the MKN-45-empty vector. These observations suggest that *MMP7* plays a central role in GC invasion among miR-148a target genes.

Discussion

In this study, we showed frequent downregulation of miR-148a in GC tissue that was significantly correlated with lymph node metastasis, advanced clinical stage, and poor clinical outcome. We also established that *MMP7* is a direct and functional target of miR-148a. This is the first report to identify miRNA that regulates *MMP7* expression. Further studies revealed that overexpression of miR-148a suppressed GC cell invasion *in vitro*, and cotransfection of miR-148a and *MMP7* restored invasiveness of GC cells. It has been reported that *MMP7* plays a key role in invasive properties of various cancer cells, and *MMP7* is well known as a representative cancer invasion-related gene whose expression is correlated with worse clinicopathologic characters.^(26,27) Together with previous reports, our results suggest that miR-148a plays important roles in suppressing tumor progression in GC, and that the induction of *MMP7* expression by downregulation of miR-148a contributes to GC progression.

MicroRNA microarray analysis and qRT-PCR revealed that miR-148a was significantly downregulated in GC tissue, and we also confirmed that hypermethylation of *MIR148A* CpG islands is correlated with suppression of miR-148a in GC cell lines. It has been reported that hypermethylation of *MIR148A* CpG islands is induced by DNA methyltransferase-1, however DNA methyltransferase-1, in turn, is conversely downregulated by miR-148a overexpression.⁽²⁸⁾ In healthy stomach tissue, next-generation sequencing reportedly confirms overexpression of miR-148a,⁽²⁹⁾ and downregulation of miR-148a has been reported in various cancers including GC.^(30–32) In relation to miR-148a, almost all of its target genes were implicated in promoting cancer progression. These results suggest that miR-148a may play essential roles in maintaining homeostasis of normal stomach tissue, and when downregulated, sequential induction of some oncogenes may contribute to the development of gastric neoplasia. However, the points at which downregulation of miR-148a occurs in the process of carcinogenesis

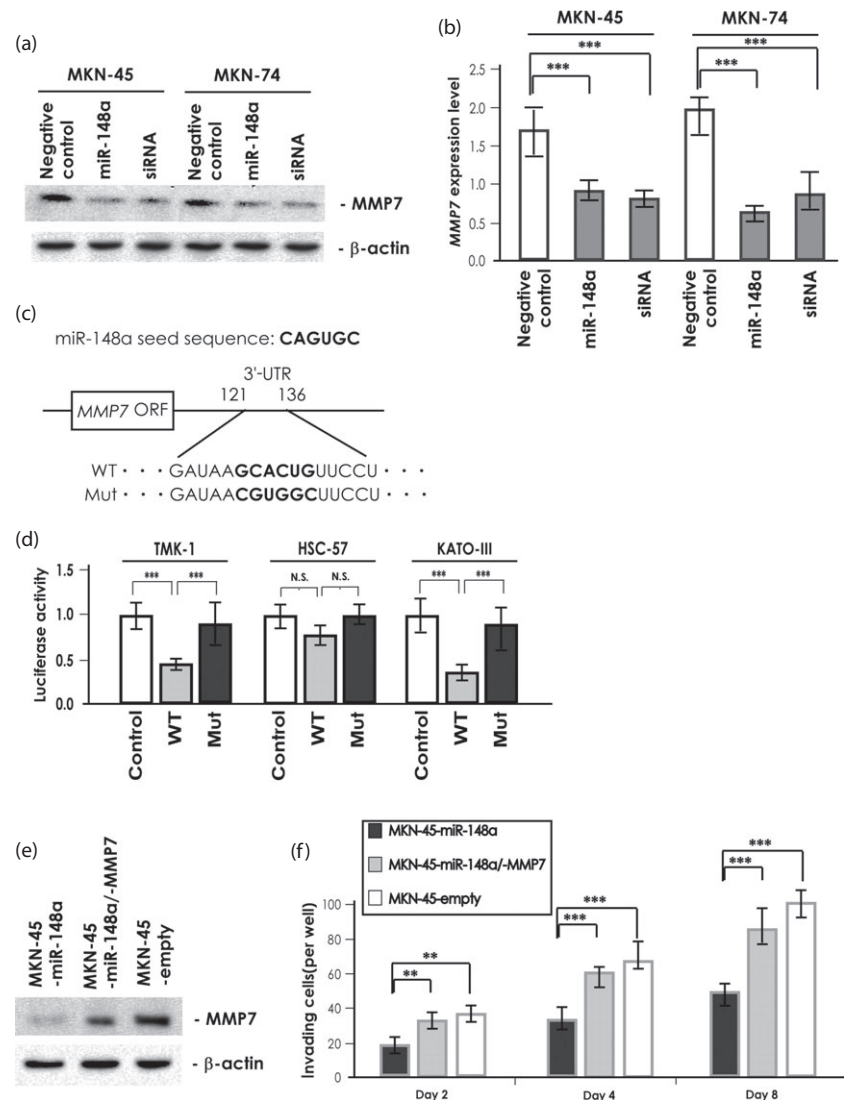


Fig. 3. Effect of microRNA-148a (miR-148a) on *MMP7* expression and gastric cancer (GC) cell invasiveness. (a) Western blot analysis of *MMP7* in MKN-45 and MKN-74 cell lysates after treatment with negative control miRNA, pre-miR-148a, or *MMP7*-specific siRNA. A β -actin blot served as loading control. (b) Quantitative RT-PCR analysis of *MMP7* in MKN-45 and MKN-74 cells after treatment with negative control miRNA, pre-miR-148a, or *MMP7*-specific siRNA. Results are mean \pm SD of triplicate measurements. *** P < 0.001. (c) Schematic representation of miR-148a seed sequence and *MMP7* 3'-UTR, showing the putative miR-148a-binding site. The seed region is in bold text and the seed region is mutated in the reporter construct. (d) Luciferase activity of three GC cell lines cotransfected with reporter vector containing either control vector, wild-type (WT), or mutant miR-148a (Mut) 3'-UTR. Results are mean \pm SD of triplicate measurements. *** P < 0.001. N.S., not significant. (e) Western blot analysis of *MMP7* in MKN-45 cell lysates after stable transfection with the miR-148a construct, both miR-148a and *MMP7* construct, or empty vector. β -Actin served as loading control. (f) Effect of miR-148a-stable transfection and restoration of *MMP7* on cell invasion of MKN-45 cells. Boyden chambers were used for incubation of MKN-45 cells after stable transfection with miR-148a construct, both miR-148a and *MMP7* construct, or empty vector. Results are mean \pm SD of triplicate measurements. ** P < 0.01; *** P < 0.001.

or tumor progression have not been fully elucidated. In GC, there is no report that has examined the timing of miR-148a downregulation. It is our current important task to determine when miR-148a is downregulated in gastric neoplasia.

A number of miRNA target-prediction databases have been developed and tested for accuracy and precision using both computational and laboratory techniques. However, when results from laboratory techniques were compared to those of computational approaches, analysis showed that most gene lists generated using computational target prediction contained high false-negative and false-positive results.^(7,33) In addition, various miRNA target prediction programs, which use different algorithms of targeting, produce different lists of predicted targets. It is documented that these differences can arise from the source of 3'-UTR sequences. In the present study, using custom oligonucleotide array-based target prediction, we found that *MMP7* expression is downregulated by miR-148a overexpression. Moreover, this was confirmed by qRT-PCR, Western blotting, and reporter gene assays. We determined the 3'-UTR of *MMP7* by referring to the Gene database available from the National Center for Biotechnology Information (NCBI), and found that the 3'-UTR of *MMP7* contains a putative binding site that is complementary to the miR-148a seed sequence. We

compared the sequence of *MMP7* coding region from the Gene database (NCBI: <http://www.ncbi.nlm.nih.gov>) with that from Ensembl (<http://useast.ensembl.org>) or UCSC (<http://genome.ucsc.edu>). Although slight differences were observed among these sequences, including the definition of the start point, most were almost completely matched. This result suggests that the extant online miRNA target prediction database did not detect *MMP7* as a candidate target gene of miR-148a, due to a deficiency in their chosen algorithm. These considerations point to a need for further refinement of online databases, in order to effectively identify real target genes for any given miRNA. At this point in time, conventional laboratory techniques remain very valuable methods for effective identification of miRNA target genes.

In summary, we have shown that downregulation of miR-148a is an independent prognostic classifier in patients with GC. Furthermore, this study identified *MMP7* as a direct target of miR-148a in GC cells and its restoration by downregulation of miR-148a in these cells increases tumor invasive activity. The present findings provide evidence for the contribution of miR-148a to the maintenance of homeostasis in normal stomach tissue and suggest an additional role for miR-148a in GC invasion through regulation of *MMP7* expression. Elucidation

of a fuller picture of the molecular mechanisms through which miR-148a is involved in gene deregulatory oncogenic processes could improve our understanding of carcinogenesis, particularly tumor progression in GC.

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Disclosure Statement

The authors have no conflict of interest.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1. Comparison between microarray and quantitative RT-PCR data for microRNA-148a (miR-148a) expression levels in the same gastric cancer (GC) sample set.

Fig. S2. Comparison between microRNA-148a (miR-148a) expression levels in non-neoplastic gastric mucosa and gastric cancer (GC).

Fig. S3. Quantitative RT-PCR and bisulfite genomic DNA sequencing analysis of *MIR148A* in gastric cancer (GC) cell lines.

Fig. S4. Effect of microRNA-148a (miR-148a) on epithelial–mesenchymal transition-related molecules and ROCK1 expression in gastric cancer cell lines.

Fig. S5. Effect of microRNA-148a (miR-148a) on MMP7 expression in gastric cancer cell lines.

Table S1. Correlation between microRNA-148a (miR-148a) expression level and clinicopathologic characters.

Table S2. Correlation between *MMP7* and microRNA-148a (miR-148a) expression in gastric cancer tissue.

Data S1. Materials and methods.