Multiple-to-Multiple Relationships between MicroRNAs and Target Genes in Gastric Cancer

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Abstract

MicroRNAs (miRNAs) act as transcriptional regulators and play pivotal roles in carcinogenesis. According to miRNA target databases, one miRNA may regulate many genes as its targets, while one gene may be targeted by many miRNAs. These findings indicate that relationships between miRNAs and their targets may not be one-to-one. However, many reports have described only a one-to-one, one-to-multiple or multiple-to-one relationship between miRNA and its target gene in human cancers. Thus, it is necessary to determine whether or not a combination of some miRNAs would regulate multiple targets and be involved in carcinogenesis. To find some groups of miRNAs that may synergistically regulate their targets in human gastric cancer (GC), we re-analyzed our previous miRNA expression array data and found that 50 miRNAs were up-regulated on treatment with 5-aza-2’-deoxycytidine in a GC cell line. The “TargetScan” miRNA target database predicted that some of these miRNAs have common target genes. We also referred to the GEO database for expression of these common target genes in human GCs, which might be related to gastric carcinogenesis. In this study, we analyzed two miRNA combinations, miR-224 and -452, and miR-181c and -340. Over-expression of both miRNA combinations dramatically down-regulated their target genes, DPYSL2 and KRAS, and KRAS and MECP2, respectively. These miRNA combinations synergistically decreased cell proliferation upon transfection. Furthermore, we revealed that these miRNAs were down-regulated through promoter hypermethylation in GC cells. Thus, it is likely that the relationships between miRNAs and their targets are not one-to-one but multiple-to-multiple in GCs, and that these complex relationships may be related to gastric carcinogenesis.

Introduction

MicroRNAs (miRNAs), a class of small non-protein-coding RNAs, have been identified as a new type of gene regulator that bind to the 3’-untranslated regions (UTRs) of target mRNA, thereby resulting in mRNA degradation or the blockade of mRNA translation [1]. It is generally known that miRNA alterations are associated with tumorigenesis [1]. According to miRNA target databases, one miRNA may regulate many genes as its targets, while one gene may be targeted by many miRNAs. However, numerous studies revealed a one-to-one relationship between miRNA and its target gene. It has also been reported that multiple or a cluster of miRNAs co-operatively regulate a gene, which is related to carcinogenesis [2–4]. On the other hand, multiple genes are targeted by one miRNA [3,4]. Even multiple-to-multiple relationships between miRNAs and target genes have been reported by using computational analyses [5,6]. However, there have been only a few papers experimentally validating multiple-to-multiple relationships in cancer cells.

Gastric cancer is the fourth most common human malignant disease and the second most frequent cause of cancer-related death worldwide, with an estimated one million new cases per year [7]. Gastric cancers are histologically classified into two major types, the intestinal and diffuse types [8]. Diffuse-type GC is often intractable and exhibits a poor patient prognosis. Recently, we demonstrated that loss of the Cdh1 and Trp53 functions induces diffuse type GC using mice models [9]. Although this finding will help us to develop new human gastric cancer therapies, further investigations on the molecular mechanisms underlying gastric carcinogenesis are necessary to develop other approaches for targeted therapy.

Promoter CpG island hypermethylation is one of the most common mechanisms by which tumor suppressor genes are inactivated in human cancers [10]. Recently, it has become apparent that some miRNAs are also targets of epigenetic silencing in cancers [11]. Our and other groups have previously shown that pharmacologic or genetic disruption of DNA methylation in cancer cell lines induces up-regulation of substantial numbers of miRNAs [12–15]. These data led to identification of candidate tumor-suppressive miRNAs whose silencing is associated with CpG island methylation. So far, methylation of miR-124 family members has been identified in colorectal and in tumors of other organs [11]. In addition, the miR-34b/c cluster has a typical CpG island, and is down-regulated through frequent methylation in colorectal and gastric cancers [12]. Similarly, we found that miR-181c methylation is associated with gastric carcinogenesis via regulation of oncogenic genes KRAS and NOTCH1 [13]. Down-regulation of many miRNAs through methylation simultaneously occurs in cancer cells, and may enhance multiple-to-multiple relationships between miRNAs and targets.

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In this study, to validate multiple-to-multiple relationships between miRNAs and targets in cancer cells, we demonstrated that two pairs of multiple miRNAs, miR-224 and -452, and miR-181c and -340, had multiple target genes and synergistically decreased cell proliferation through regulation of their targets in human GC cells.

Results

50 miRNAs were up-regulated in a GC Cell Line, KATO-III, after 5-aza-2'-deoxycytidine Treatment

To identify candidate miRNAs that synergistically affect their target genes, we reanalyzed our previous microarray data (GEO accession No. GSE16006) [13]. We considered that mRNA levels were up-regulated on 5-aza-2'-deoxycytidine (5-aza-CdR) treatment when the net intensities of particular 3 spots were more than 1.5-fold. Furthermore, we also selected miRNAs of which the averages were found to be increased more than 3-fold on microarray analysis. Based on these new criteria, we found that 50 miRNAs were up-regulated in a GC cell line, KATO-III (Table S1). We confirmed the up-regulation of 5 of 6 representative precursor miRNAs, that is, miR-145, -148a, -152, -224 and -340, after 5-aza-CdR treatment by RT-PCR (Figure 1A).

TargetScan Predicted Common Target Genes of the Candidate miRNAs

To determine whether or not epigenetically regulated miRNAs have common target genes, we searched the TargetScan database (version 5.1) for their common targets. According to TargetScan, 50 miRNAs could be classified into 46 groups based on their seed sequences. TargetScan also showed that these 46 groups can target 6,460 genes. Among these target genes, we selected 13 of which expression was reported to be increased in GC in the GEO database (GEO accession No. GSE26083) or to be related to gastric carcinogenesis (Table S2). Here, we focus on four miRNAs, miR-152, -181c, -224 and -340, because we have already reported that miR-181c is epigenetically down-regulated in GC [13] and CpG islands are located in the upstream regions of three other miRNAs (Figure 2A and C, and Figure S1). Figure 2A also reveals that miR-452 is clustered with miR-224. TargetScan predicted that the five miRNAs can regulate common targets. For instance, DIPSL2 (dihydrolipomdiamidase-like 2; also known as collapsing response mediator protein 2, CRMP2) is targeted by miR-224, -452, and -181c, KRAS by miR-224, -452, -181c, -340 and -132, and MECP2 (methyl CpG binding protein 2) by miR-101c and -340, respectively (Figure 3).

Expression of miR-224, -452, -152 and -340 Decreased on DNA Hypermethylation in GC Cell Lines

We examined the involvement of epigenetic changes in downregulation of the miRNAs. The expression of miR-224, -340 and -152 was increased by 5-aza-CdR treatment in several GC cell lines (Figure 1B). We quantitatively analyzed mature miR-224 expression in 9 GC cell lines and a colorectal cancer (CRC) cell line. No expression of miR-224 was detected in 7 of 10 cancer cell lines (Figure 1C). We also analyzed the expression change of the miR-224 cluster in KATO-III cells treated with a low dose of 5-aza-CdR (0.2 μM/l), a histone deacetylase inhibitor, trichostatin A (TSA, 0.3 μM/l), or a combination of these two drugs. KATO-III cells with low-dose 5-aza-CdR treatment exhibited up-regulation of the miR-224/−452 cluster, whereas TSA alone did not cause up-regulation. The miR-224/−452 cluster was synergistically up-regulated in KATO-III cells with combined 5-aza-CdR and TSA treatment (Figure 1D). These results indicate that miR-224 and miR-452 may be down-regulated through DNA methylation in GC cell lines as the same transcription unit.

It has been reported that intronic miRNAs are regulated through promoter methylation of their host genes [14,15]. According to the results of computational analysis, the miR-224/−452 cluster and miR-340 are located in intron 6 in GABRE and intron 2 in RAF130, respectively, both of which contain dense CpG islands only in the promoter regions of their host genes (Figure 2A and C). We investigated the relationship between the expression levels of these miRNAs and the methylation status of their host genes in GC cell lines by MSP analysis. GC cell lines without miR-224 expression exhibited only methylation signals, whereas expression-positive cell lines exhibited strong unmethylation patterns (Figure 2B). A similar relationship was detected for miR-340 in GC cell lines (Figure 2D). These data indicate that expression of these miRNAs in GC cell lines may be silenced through promoter methylation of their host genes.

Epigenetically Regulated miRNAs Might be Related to GC Cell Proliferation

To determine whether epigenetically regulated miRNAs are tumor-suppressive or not, we evaluated the effect of 5-aza-CdR in siDICER1-transfected KATO-III and DICER1 KO HCT116 (D1KO) cells (Figure 4A). The untreated KATO-III and parental HCT116 cells showed accelerated proliferation after treatment with 5-aza-CdR. On the other hand, in siDICER1-transfected KATO-III and D1KO cells, the effect of 5-aza-CdR treatment on cell proliferation became weak in both cases. These results suggest that the effect of 5-aza-CdR was decreased in low or null DICER1 cells, and that epigenetically regulated miRNAs play an important role in GC and CRC cells.

In order to analyze the relationship between these miRNAs and 13 target genes shown in Table S2, we transfected KATO-III cells with siDICER1 and/or treated with 5-aza-CdR. Although, expression of 3 (DIPSL2, KRAS and MECP2) of the 13 genes were decreased after 5-aza-CdR treatment, the expression of these genes did not change on 5-aza-CdR treatment followed by transfection of siDICER1 (Figure 4B).

Combinational Transfection of miR-224 and -452 Repressed GC Cell Proliferation

We transfected GC cell lines with miR-224 and/or -452 mimics as a representative of miRNA clusters, or a negative control, and then carried out water-soluble tetrazolium-8 (WST-8) assays. Seventy-two hours after transfection, we observed that ectopic expression of miR-224 or -452 suppressed the growth of two cell lines, KATO-III and AGS (Figure 5A). Notably, combinational transfection of the miR-224 and -452 mimics extensively decreased the growth of the two GC cell lines (Figure 5A).

The miR-224/−452 Cluster Co-operatively Decreased Expression of DIPSL2 and KRAS

To investigate whether or not the miR-224/−452 cluster is actually related to the regulation of DIPSL2 and KRAS, we analyzed the expression of DIPSL2 after transfection of KATO-III and AGS cells with the miR-224/−452 cluster alone or together. We carried out RT-PCR and Western blot analyses. The DIPSL2 and KRAS mRNA levels were decreased after transfection with the miR-224 or miR-152 mimic (Figure 5B). Interestingly, in the case of combinational transfection with miR-224 and -452, expression of these target genes was further down-regulated (Figure 5B). The down-regulation of DIPSL2 was also observed at the protein level.
Figure 1. Expression patterns of human miRNAs in GC cell lines after 5-aza-CdR treatment. (A) RT-PCR analyses of precursor miRNAs in KATO-III cells untreated (U) or treated (A) with 5-aza-CdR. GAPDH mRNA expression was used as a loading control. (B) RT-PCR analyses of precursor miRNAs in human GC cell lines untreated (U) or treated (A) with 5-aza-CdR (5 μmol/l), and normal gastric mucosae. GAPDH mRNA expression was used as a loading control. (C) Quantitative real-time RT-PCR analysis of mature miR-224 expression in 9 GC cell lines and a CRC cell line, HCT116. (D) Quantitative real-time RT-PCR analysis of the mature miR-224 and -452 levels in KATO-III cells untreated (U) and treated with 0.2 μmol/l 5-aza-CdR (A), 0.3 μmol/l TSA (T), or a combination of the two drugs (A/T). M, mock.
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in the two cell lines (Figure 5C). We also examined the expression levels of other five genes, MECP2, MYC, JUNB, MUC1 and SETDB1, which were not shown as targets for miR-224 or -452 by TargetScan. As expected, no expressional changes of these five genes were found in KATO-III cells after transfection with miR-224 or -452 (Figure S2). These data suggest that miR-224 and -452 specifically down-regulated DPYSL2 and KRAS.

**DPYSL2 was Associated with GC Cell Proliferation**

We examined the effect of knockdown of DPYSL2, which was shown to be a target of the miR-224/-452 cluster, on cell proliferation. The transfection of DPYSL2 siRNA clearly decreased the levels of the DPYSL2 transcripts (Figure 5D), and inhibited the growth of AGS and KATO-III cells 72 hours after the knockdown of DPYSL2 (Figure 5E), indicating that DPYSL2 has an oncogenic activity.

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**Figure 2.** MSP analysis of miR-224/-340 in GC, CRC cell lines and normal stomach. (A), (C) Schematic representation of the miRNAs and their host genes. Filled boxes represent the exons of the host genes and blank boxes denote the untranslated regions of the host genes. The bent arrows indicate the transcription start sites of the host genes. The vertical arrows indicate the locations of miRNAs. The vertical thick lines indicate CpG sites. Arrowheads indicate the regions examined for MSP. (B), (D) MSP analyses of miR-224 and miR-340, respectively, in GC cell lines. The bands in the ‘Mt’ lanes are PCR products obtained with methylation-specific primers, and those in the ‘Un’ lanes were obtained with unmethylation-specific primers; PBL, peripheral blood lymphocyte. The expression of miRNAs is indicated under the photographs of the MSP results.
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**Figure 3.** A diagram of the relationship between up-regulated miRNAs after 5aza-CdR treatment and their candidate target genes. Red lines, conserved site; blue line, conserved site in mammals; yellow lines, poorly conserved site.
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Combinational Transfection of miR-340 and -181c Repressed GC Cell Proliferation, and Induced Downregulation of KRAS and MECP2 Expression

As a second example of multiple-to-multiple relationships between microRNAs and target genes, we analyzed the relationship between miR-340/miR-181c and KRAS/MECP2. When miR-340 and miR-181c were transfected into KATO-III cells, proliferation was synergistically down-regulated by two miRNAs (Figure 6A). To determine whether or not epigenetically regulated miR-340 and miR-181c co-operatively affect their targets, we analyzed the mRNA levels of KRAS and MECP2. On RT-PCR analysis, KRAS and MECP2 were found to be down-regulated by miR-340 and miR-181c alone, or combinational transfection in KATO-III cells (Figure 6B). As for the four genes, MYC, JUNB, MUC1, and SETDB1, showing no predicted sites for these two miRNAs by TargetScan, the expression levels were not changed in this study. Representative data are shown in Figure 6B. Thus, the effects of miR-340 and -181c may be specific to their common target genes as well as miR-224 and -452.

Expression and Methylation Status of miR-224 and -340 in Primary GC Cases

We examined the methylation status of miR-224 and -340 in primary GC cases. Methylated patterns of miR-224 were detected in 15 of 26 (57.7%) primary GC tissues (Figure 7A and Table 1). Paired non-cancerous gastric mucosae hardly exhibited a methylation pattern of miR-224. Next, we quantitatively examined the miR-224 levels in primary GC tissues and corresponding non-cancerous gastric mucosae. The paired t-test was used to compare the values for the test and control samples. A value of $P < 0.05$ was taken as significant.

**Figure 4. Changes of candidate target genes expression and cell proliferation after decreased DICER1 expression and/or 5-aza-CdR treatment.** (A) The growth curves of KATO-III, HCT116 and DICER1 KO HCT116 cells. KATO-III cells were transfected with 20 nmol/l of siDICER1 or scrambled siRNA. The paired t-test was used to compare the values for the test and control samples. A value of $P < 0.05$ was taken as significant. (B) Representative results of target gene expression on the RT-PCR analysis. doi:10.1371/journal.pone.0062589.g004
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cancerous mucosa by TaqMan RT-PCR. A significant reduction of miR-224 expression in GC tissues was observed in methylation-positive cancer cases compared with in methylation-negative ones and non-cancerous gastric mucosa (Figure 7C).

We further analyzed the DPYSL2 mRNA levels in comparison with the methylation status of miR-224 in GC tissues: GCs with miR-224 methylation (Ca miR-224 Mt), GCs with miR-224 unmethylation (Ca miR-224 Un), and non-cancerous tissues with unmethylation (N miR-224 Un). The DPYSL2 mRNA level in the “Ca miR-224 Mt” group was significantly higher than those in the “N miR-224 Un” and “Ca miR-224 Un” groups, p = 0.049 and p = 0.035, respectively (Figure 7D). Thus, there is a correlation between the methylation status of miR-224 and DPYSL2 expression in GC tissues.

The miR-340 methylation frequency was relatively low in the gastric cancerous tissues tested (4 of 26, 15.4%) (Figure 7B and Table 1), whereas none of 26 paired non-cancerous gastric mucosae exhibited apparent methylation patterns of miR-340. As for miR-152 methylation analysis, we tried three primer sets designed in the upstream region of miR-152 containing CpG islands (Figure S1), but none of them completely matched miR-152 expression on MSP analyses (data not shown).

**Discussion**

Although it has been reported that the expression of some miRNAs is decreased in several cancers through DNA methylation, most of the reports described that the relationship between aberrant expression of miRNAs and its target genes was one-to-one, one-to-multiple or multiple-to-one. To examine the possibility of multiple-to-multiple relationships between miRNAs and targets in cancer cells, we focused on two combinations of miRNAs in GC cells, the miR-224/-452 cluster, and miR-181c and -340, in this study. We found that the two sets of miRNAs, miR-224 and -452, and miR-181c and -340, had multiple target genes, DPYSL2 and KRAS, and KRAS and MECP2, respectively, and synergistically decreased cell proliferation in human GC cell lines. It is notable that an oncogene, KRAS [16], was found to be targeted by four miRNAs, although candidate binding sites of the four miRNAs are different in the 3'-UTR of KRAS (TargetScan). We previously reported that miR-181c down-regulated NOTCH4 too [13]. Thus, multiple-to-multiple relationships between miRNAs and targets were indicated not only by database analyses but also by transfection experiments involving human cells.

miR-224- and/or -340-expression-negative GC cell lines exhibited hypermethylation signals on MSP analysis and the expression of miR-224 and -340 restored on demethylating agent treatment. Furthermore, hypermethylation of miR-224 and -340 was more frequently observed in primary GCs than corresponding noncancerous mucosa. In miR-224 methylation-positive cases, expression of miR-224 was significantly lower than in methylation-negative ones. These data strongly indicate that aberrant DNA methylation is one of the key mechanisms underlying down-regulation of miR-224 and -340 in GC cells.

We showed that inhibition of miRNA processing by siDICER1 transfection or using DICER1 knockout cells decreased the effect of 5-aza-CdR, that is, decreased cell proliferation and down-regulation of target genes, in GC and CRC cells. It has been reported that the abundance of DICER1, the enzyme that catalyzes the final step of miRNA maturation, is directly associated with tumor progression [17]. These results suggest that aberrant regulation of miRNA maturation contributes to GC and CRC formation.

We found that the miR-224/-452 cluster was aberrantly down-regulated in GCs through hypermethylation. Aberrant expression of miR-224 has also been reported in other tumors. Expression of miR-224, let-7f and miR-516a is decreased in ovarian cancer, and they synergistically regulate expression of kallikrein-related peptidase 10 (KLK10) [18]. miR-224 is down-regulated in methotrexate-resistant CRC cell lines compared with in sensitive cells [19]. Here we also revealed that the methylation status of miR-224 was correlated with the DPYSL2 level in human GCs. Taken together,
Figure 7. Methylation analysis of miR-224/340 and expression of DPYSL2 in human gastric cancer tissues. Representative results of miR-224 (A) and miR-340 (B) MSP analyses in primary GC tissues. The bands in the 'Mt' lanes are PCR products obtained with methylation-specific primers, and those in the 'Un' lanes were obtained with unmethylation-specific primers. 'Ca' and 'N' denote primary GCs and paired non-cancerous tissues, respectively. 'F' and 'M' denote female and male. Black stars indicate samples in which the aberrant hypermethylation of CpG islands was detected. (C) Comparison of the miR-224 methylation status and miR-224 levels in gastric tissues. The relative levels of miR-224 expression in gastric tissues were determined by real-time RT-PCR and then compared with the miR-224 methylation status in gastric tissues: N miR-224 Un (non-cancerous without miR-224 methylation; n = 20), Ca miR-224 Un (GC tissues without miR-224 methylation; n = 10), and Ca miR-224 Mt (GC tissues with miR-224 methylation; n = 15). The two-sample t-test was performed to examine the difference between two groups. (D) Comparison of the miR-224 methylation status and DPYSL2 mRNA levels in GC tissues. The relative levels of DPYSL2 expression in gastric tissues were determined by real-time RT-PCR.
miR-224 plays an important role as a tumor-suppressive miRNA in GC as well as in several other cancers. In contrast, miR-224 is up-regulated in hepatocellular carcinomas [20] and medulloblastomas [21] compared with in the normal tissues. Thus, further studies are required to clarify the role of miR-224 in carcinogenesis.

We investigated the common targets of epigenetically down-regulated miRNAs. DPYSL2 was shown to be down-regulated by miR-224 and -452. DPYSL2 plays an important role in the establishment of neuronal polarity [22]. DPYSL2 is also involved in pathways that regulate the proliferation of non-neuronal cells through its phosphorylation by regulatory proteins. DPYSL2 undergoes dynamic phosphorylation changes in response to contact inhibition-induced quiescence and hyperphosphorylation of DPYSL2 occurs in a tumor [22]. Although the role of DPYSL2 in GCs is not clear, our siRNA-based knockdown of DPYSL2 expression induced a reduction of proliferation in the two GC cell lines, suggesting oncogenic activity of DPYSL2.

In summary, our findings indicate that multiple-to-multiple relationships between miRNAs and target genes really exist in GC.

It is likely that aberrant methylation decreases the expression of multiple tumor-suppressive miRNAs, such as miR-224, -452, -340 and -181c, which then induce over-expression of multiple oncogenic genes, like KRAS, DPYSL2, and MECP2. These abnormal multiple-to-multiple relationships between miRNAs and targets would be one of the important mechanisms underlying gastric carcinogenesis. It is, therefore, highly possible that epigenetic drugs may normalize the expression of not only tumor-suppressive genes but also multiple tumor-suppressive miRNAs, resulting in decreases of the abnormal multiple-to-multiple relationships between miRNAs and targets, and thus may become excellent therapeutic drugs against cancer.

**Materials and Methods**

**Ethics Statement**

Written informed consent was obtained from all subjects, and the ethics committee of Tokyo Medical and Dental University School of Medicine approved this research.

**Cell Lines and Tissue Samples**

We studied 9 GC cell lines (KATO-III, MKN45, AGS, MKN74, TGBCI1TKB, HSC59, HSC43, HSC58 and GCIY), 2 CRC cell lines (HCT116 and DICER1 knock out HCT116 [23]), and 26 primary GC cases. MKN45, MKN74, TGBCI1TKB and GCIY were purchased from RIKEN cell bank, and KATO-III and AGS were from ATCC (American Type Cell Collection). HSC59, HSC43 and HSC58 were obtained from Dr. Kazuyoshi Yanagihara [24,25]. KATO-III, MKN45, MKN74, HSC59, HSC43 and HSC58 were grown in RPMI 1640, and AGS, TGBCI1TKB, GCIY and the two CRC cell lines in Dulbecco’s modified Eagle’s medium, minimal essential medium or McCoy’s 5A, supplemented with 10% fetal bovine serum. Surgically resected specimens from 26 primary GC patients were randomly obtained from the Affiliated Hospital of School of Medicine, Tokyo Medical and Dental University.

**In silico Analysis**

We referred to the GEO database for miRNA and gene expression profiles (GEO accession No. GSE16006 and GSE2685). We also used miRNA target database “TargetScan”.

**Drug Treatment of Cells and RNA Extraction**

For demethylation studies, cells were daily treated with 5 μmol/l 5-aza-CdR (Sigma–Aldrich, St Louis, MO) for 72 h. We also treated cells with 0.3 μmol/l TSA alone, and with a combination of 0.2 μmol/l 5-aza-CdR and TSA. Total RNA was isolated by using Trizol reagent (Invitrogen, Carlsbad, CA) or a miRNasy mini kit (Qiagen, Hilden, Germany).

**Quantitative Real-time Reverse Transcription–polymerase Chain Reaction**

Real-time reverse transcription-polymerase chain reaction (RT-PCR) analyses were carried out using a StepOne Real-time PCR System (Applied Biosystems, Foster City, CA), EagleTaq Master Mix with ROX (Roche, Mannheim, Germany), a TaqMan Reverse Transcription kit (Applied Biosystems), and TaqMan miRNA assays (Applied Biosystems), according to the manufac-

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**Table 1. The results of MSP analysis in primary GCs.**

| Case # | miR-224/452 | miR-340 |
|--------|-------------|---------|
| 1      | U           | M       |
| 2      | M           | U       |
| 3      | M           | U       |
| 4      | M           | U       |
| 5      | M           | U       |
| 6      | U           | U       |
| 7      | U           | U       |
| 8      | U           | M       |
| 9      | M           | U       |
| 10     | M           | U       |
| 11     | M           | U       |
| 12     | U           | U       |
| 13     | U           | U       |
| 14     | M           | U       |
| 15     | U           | U       |
| 16     | M           | U       |
| 17     | M           | U       |
| 18     | U           | U       |
| 19     | U           | U       |
| 20     | U           | U       |
| 21     | M           | U       |
| 22     | M           | U       |
| 23     | M           | U       |
| 24     | U           | U       |
| 25     | U           | M       |
| 26     | U           | U       |

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Molecular Technologies, Inc., Mashikimachi, Japan), according to the number of cells with cell proliferation reagent WST-8 (Dojindo was evaluated on days 1–4 after transfection by determining the Cell Proliferation Assay.

Synthetic miRNA Transfection

KATO-III and AGS cells were transfected with a Precursor Molecule mimicking miR-224, -452, -340 or -181c, or scrambled sequence miRNA (Sigma) to give a final concentration of 25-50 nmol/I by using an electroporator, Neon (Invitrogen), according to the manufacturer’s instructions. At 24–72 h after transfection, cells were harvested for RT-PCR or Western blot analysis.

Cell Proliferation Assay

miR-mimic-transfected KATO-III and AGS cells were plated at 1 × 10^5 or 1 × 10^4 cells per well on 96-well plates. Cell proliferation was evaluated on days 1–4 after transfection by determining the number of cells with cell proliferation reagent WST-8 (Dojindo Molecular Technologies, Inc., Mashikimachi, Japan), according to the manufacturer’s instructions.

miRNA Target Prediction and Western Blotting

The predicted targets of miRNAs and their target sites were analyzed using TargetScan. The miRNA expression levels of the predicted targets in transiently transfected cells were analyzed 24 h after transfection by RT-PCR. Western blot analyses were performed as described previously [26]. The primary antibody used was rabbit anti-DPYSL2 (1:1000; Bio-Rad Laboratories, Hercules, CA). Blots were probed with phosphatase-conjugated anti-rabbit IgG and anti-mouse IgG (YY). The secondary antibodies used were alkaline phosphatase-conjugated anti-rabbit IgG and anti-mouse IgG (Bio-Rad Laboratories, Hercules, CA). Blots were developed with ImmunoStar AP Substrate (Bio-Rad Laboratories).

Supporting Information

Figure S1: Schematic representation of the COPZ2 region containing mir-152. FITC Filled boxes represent the exons of COPZ2A and a blank box denotes the untranslated region of COPZ2. A bent arrow indicates the transcription start site of COPZ2. A vertical arrow indicates the location of mir-152. Vertical lines indicate CpG sites. Arrowheads indicate the regions examined for MSP.

Figure S2: Effects of transfection of miR-224 and -452 in KATO-III cells. RT-PCR analyses after transfection with miR-224 and/or -452. The expression of target genes was analyzed 48h later by RT-PCR. miR-224 and -452 specifically down-regulated DPYSL2 and KRAS, but not other five genes examined, which are consistent with the results of database analysis (Figure 3).

Table S1: Expression profiling of human miRNAs in KATO-III cells after 5-aza-CdR treatment.

Table S2: List of target genes of which expression we examined by RT-PCR after treatment with or without 5-aza-CdR, and transfection with 20 nmol/L of siDICER1 or scrambled siRNA.

Table S3: Sequences of primers used in this study.

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Author Contributions

Conceived and designed the experiments: YH YY. Performed the experiments: YH YA. Analyzed the data: YH YA. Wrote the paper: YH YY.

References

1. Calin GA, Sevignani C, Dumitru CD, Hyslop T, Noch E, et al. (2004) Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancer. Proc Natl Acad Sci U S A 101: 2999–3004.
2. Kim YK, Yu J, Han TS, Park SY, Namkoong B, et al. (2009) Functional links between clustered microRNAs: suppression of cell-cycle inhibitors by microRNA clusters in gastric cancer. Nucleic Acids Res 37: 1672–1681.
3. Bondi D, Cogpala V, Musumeci M, Addario A, Giuffrida R, et al. (2008) The miR-15a-miR-16–1 cluster controls prostate cancer by targeting multiple oncogenic activities. Nat Med 14: 1271–1277.
4. Sirota M, Page DB, Shin J, Marth JD, Nguyen QB, et al. (2010) Integrated analyses of microRNAs demonstrate their widespread influence on gene expression in high-grade serous ovarian carcinoma. PLoS One 7: e34546.
5. Manavalan KJ, Van Der Meulen J, Wolfe AL, Liu X, Mert E, et al. (2011) A cooperative microRNA-tumor suppressor gene network in acute T-cell lymphoblastic leukemia (T-ALL). Nat Genet 43: 673–678.
6. Ooi CH, Oh HK, Wang HZ, Tan AL, Wu J, et al. (2011) A densely interconnected genome-wide network of microRNAs and oncogenic pathways revealed using gene expression signatures. PLoS Genet 7: e1002113.
7. Jain A, Center MM, DeSanctis C, Ward EM (2010) Global patterns of cancer incidence and mortality rates and trends. Cancer Epidemiol Biomarkers Prev 19: 1893–1907.
8. Yuasa Y (2003) Control of gut differentiation and intestinal-type gastric carcinogenesis. Nat Rev Cancer 3: 592–600.
9. Shimada S, Mimata A, Sekine M, Mogushi K, Akiyama Y, et al. (2012) Synergistic tumour suppressor activity of Ecadherin and p53 in a conditional mouse model for metastatic diffuse-type gastric cancer. Gut 61: 344–353.
10. Egger G, Liang G, Aparicio A, Jones PA (2004) Epigenetics in human disease and prospects for epigenetic therapy. Nature 429: 457–463.
11. Lujambio A, Ropero S, Ballerstein E, Fraga MF, Cerrato C, et al. (2007) Genetic unmasking of an epigenetically silenced microRNA in human cancer cells. Cancer Res 67: 1424–1429.
12. Toyama M, Suzuki H, Sasaki Y, Matsuyama R, Imai K, et al. (2008) Epigenetic silencing of microRNA-34b/c and B-cell translocation gene 4 is associated with CpG island methylation in colorectal cancer. Cancer Res 68: 4123–4132.
13. Hashimoto Y, Akiyama Y, Otsubo T, Shimada S, Yuasa Y (2010) Involvement of epigenetically silenced microRNA-181c in gastric carcinogenesis. Carcinogenesis 31: 777–784.
14. Cheung HH, Davis AJ, Lee TL, Pang AL, Nagrani S, et al. (2011) Methylation unmasking of an intronic microRNA in human cancer cells. Cancer Res 71: 2319–2327.
15. Grady WM, Parkin RK, Mitchell PS, Lee JH, Kan YH, et al. (2006) Epigenetic silencing of the intronic microRNA hsa-miR-342 and its host gene EVI1 in colorectal cancer. Oncogene 27: 3808–3818.
16. Jimeno A, Messersmith WA, Hirsch FR, Franklin WA, Eckhardt SG (2009) KRAS mutations and sensitivity to epidermal growth factor receptor inhibitors in colorectal cancer: practical application of patient selection. J Clin Oncol 27: 1130–1136.
17. Merritt WM, Bar-El M, Sood AK (2010) The dicey role of Dicer: implications for RNAi therapy. Cancer Res 70: 2571–2574.
18. White NM, Chow TF, Mejia-Guerrero S, Diamandis M, Rofael Y, et al. (2010) Three dysregulated miRNAs control kallikrein 10 expression and cell proliferation in ovarian cancer. Br J Cancer 102: 1244–1253.

19. Mencia N, Selga E, Noel V, Ciafalai CJ (2011) Underexpression of miR-224 in methotrexate resistant human colon cancer cells. Biochem Pharmacol 82: 1372–1382.

20. Wang Y, Lee AT, Ma JZ, Wang J, Ren J, et al. (2008) Profiling microRNA expression in hepatocellular carcinoma reveals microRNA-224 up-regulation and apoptosis inhibitor-5 as a microRNA-224-specific target. J Biol Chem 283: 13205–13213.

21. Gokhale A, Kunder R, Goel A, Sarin R, Moiyadi A, et al. (2010) Distinctive microRNA signature of medulloblastomas associated with the WNT signaling pathway. J Cancer Res Ther 6: 521–529.

22. Tahimic CG, Tomimatsu N, Nishigaki R, Fukuhara A, Toda T, et al. (2006) Evidence for a role of Collapsin response mediator protein-2 in signaling pathways that regulate the proliferation of non-neuronal cells. Biochem Biophys Res Commun 349: 1244–1250.

23. Cummins JM, He Y, Leary RJ, Pagliarini R, Diaz LA Jr, et al. (2006) The colorectal microRNAome. Proc Natl Acad Sci U S A 103: 3697–3692.

24. Yanagihara K, Tanaka H, Takigahira M, Ino Y, Yamaguchi Y, et al. (2004) Establishment of two cell lines from human gastric scirrhous carcinoma that possess the potential to metastasize spontaneously in nude mice. Cancer Sci 95: 575–582.

25. Yanagihara K, Kamada N, Tsumuraya M, Amano F (1993) Establishment and characterization of a human gastric scirrhous carcinoma cell line in serum-free chemically defined medium. Int J Cancer 54: 200–207.

26. Wen XZ, Miyake S, Akiyama Y, Yuasa Y (2004) BMP-2 modulates the proliferation and differentiation of normal and cancerous gastric cells. Biochem Biophys Res Commun 316: 100–106.