The metastasis associated microRNA miR-516a-3p is a novel therapeutic target for inhibiting peritoneal dissemination of human scirrhous gastric cancer

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Abstract

Although aberrant microRNA (miRNA) is expressed in different types of human cancer tissues, its pathophysiological role and the relevance of tumorigenesis and metastasis are still largely unknown. Here we defined miRNAs involved in cancer metastasis (metastamirs) using an established mouse model for peritoneal dissemination of human scirrhous gastric carcinoma cells. Highly metastatic derivatives (44As3 cells) were derived from the parental cells originally isolated from patients (HSC-44PE cells). Using microarray analysis to identify differentially expressed miRNAs in 44As3 and HSC-44PE cells, we focused on miR-516a-3p as a candidate anti-metastatic miRNA (anti-metastamir) whose functions in cancer had not been studied. We confirmed attenuated expression of miR-516a-3p in 44As3 cells compared with HSC-44PE cells by Northern blot analysis and qRT-PCR. Stable ectopic overexpression in 44As3-miR-516a-3p cells permitted identification of Sulfatase1 as a direct target of the miRNA, through use of the isobaric tagging reagent iTRAQ and the QSTAR Elite Hybrid LC-MS/MS system. Sulfatase1 is known to remove 6-O-sulfates from heparan-sulfate proteoglycans on the cell surface, causing release of membrane-bound Wnt ligands from cells. Consistent with this function, Western blot analyses revealed high levels of Wnt3a, Wnt5a, and nuclear beta-catenin accumulation in 44As3 cells but relatively reduced levels in 44As3-miR-516a-3p cells. Notably, orthotopic inoculation of nude mice with 44As3-miR-516a-3p cells yielded significantly longer survival periods compared to mice inoculated with control 44As3 cells. Through atelocollagen-mediated delivery of an miR-516a-3p expression vector into orthotopic 44As3 tumors we documented its feasibility as a treatment agent. Our findings define the microRNA miR-516-3p as an anti-metastamir with potential therapeutic applications in blocking metastatic dissemination of gastric cancers.

Precis

This study defines an anti-metastatic microRNA and offers an initial preclinical proof of concept for its possible use in blocking dissemination of metastatic gastric cancers.
Introduction

MicroRNAs (miRNAs) are a class of short, conserved, noncoding RNAs that play important regulatory roles in post-transcriptional repression (1-3). miRNAs are fundamental gene regulators that control proliferation, differentiation, and apoptosis during development (1-3). They target a large number of mRNAs and induce mRNA degradation or inhibition of translation by targeting the 3’ untranslated regions (2). These broad functions suggest that miRNAs may contribute to a range of diseases including cancers, and recent studies indicate that miRNAs can function as tumor suppressors and oncogenes (1, 4). The development of microarray platforms for the analysis of miRNA expression revealed that multiple miRNAs are aberrant or lacking in human malignancies (5, 6).

Gastric cancer is a major cause of deaths from cancer, particularly on the basis of peritoneal metastasis (7). Scirrhous gastric carcinoma, a diffusely infiltrating type of poorly differentiated gastric carcinoma also known as linitis plastica-type carcinoma, is characterized by rapid cancer cell infiltration and proliferation accompanied by extensive stromal fibrosis (8). Common features of scirrhous gastric cancer include rapidly progressive invasion and a high frequency of metastasis to the peritoneum (9), which distinguishes it from intestinal types of gastric cancer (10). Scirrhous carcinoma, which represents approximately 10% of gastric carcinomas, shows a 5-year survival rate of 0 to 17%, compared with 35% to 70% for other types of gastric carcinomas (11). Various treatments, including chemotherapy, hormonal therapy, hyperthermia, and immunotherapy, have been tested for effectiveness in peritoneal metastasis from scirrhous gastric carcinoma, but the results have been unsatisfactory (12). Accordingly, novel therapies based on the characteristic biologic behavior of scirrhous gastric cancer are urgently required. However, the mechanisms responsible for proliferation, particularly the mechanism underlying peritoneal metastasis, remain unclear. To elucidate these mechanisms, we need to make a reliable scirrhous gastric carcinoma model in mouse. We thus recently established a cell line, HSC-44PE, from patients with gastric scirrhous carcinomas (13). We have also isolated a cell line, 44As3, with high metastatic characteristics from the parental cells, HSC-44PE, by repeating cycles of orthotopic transplantation in nude mice,
gathering cells from ascites, and further transplantation (14). 44As3, when orthotopically transplanted in the stomach wall of nude mice, will spontaneously occur peritoneal metastasis disseminated over the great omentum, mesenterium, and parietal peritoneum with 100% incidence (14). This suggests that this animal model is reliable to use. The model also causes fatal cancerous peritonitis and bloody ascites in about 100% of cases (14).

Here, we identified several miRNAs (metastamirs) involved with peritoneal dissemination of scirrhous gastric cancers via an miRNA array (HSC-44PE versus 44As3). Among the miRNAs, we focused on hsa-miR-516a-3p, whose expression is significantly lower in 44As3 compared with HSC-44PE. Moreover, using proteomics approaches (15, 16) we showed that the miRNA targets extracellular sulfatase1 (SULF1) and the other unknown proteins in 44As3, and that the enzyme also regulates the Wnt signaling pathway in human scirrhous gastric cancer. Further, we showed the feasibility of the miRNA for antimetastasis therapy of scirrhous gastric carcinomas in vivo.
Materials and Methods

Cells. HSC-44PE cells were previously established from a patient with scirrhous gastric cancer (13). Orthotopic implantation of HSC-44PE cells into BALB/c nude mice led to the dissemination of the tumor cells to the greater omentum, mesenterium, peritoneum, and so on, and caused ascites in a small number of animals. Cycles of isolation and orthotopic inoculation of the ascitic tumor cells were repeated in turn in the animals (12 cycles). We obtained a cell line (44As3) that possessed a high metastatic potential, with a strong capability of inducing ascites (14). All cells were cultured in RPMI 1640 with 10% FBS (13, 14).

Isolation of miRNA from the cells and miRNA microarray analysis. Total RNA was extracted from the cells using TRIzol® Reagent (Invitrogen). Each total RNA (50 μg) was fractionated and cleaned up with the flashPAGE™ fractionator system (Ambion) to isolate miRNA fractions (17). Chemically synthesized oligoribonucleotides or purified miRNAs were labeled with a mirVana™ miRNA labeling kit (Ambion, Austin, TX). Hybridization buffer (Ambion) was added to the fluorescently labeled miRNAs, and the solution was heated at 95ºC for 3 min. Slides were hybridized for 16 h at 42 ºC in sealed cassettes using a water bath. The slides were washed and dried prior to a high-resolution scan on a GenePix 4000B (Axon Instruments, Union City, CA). Raw data were normalized and analyzed using Array-Pro Analyzer Version 4.5 (Media Cybernetics, Silver Spring, MD).

Northern blot analysis. The analysis was performed according to a previous report (18) with some modifications. Total RNA (20 μg) was separated on 12.5% denaturing (7 M urea) polyacrylamide gel and then transferred onto Biodyne® Nylon Transfer Membranes (0.2 μm, Pall Corp., Port Washington, NY) using a vacuum blotting system (GE Healthcare, Piscataway, NJ). The membrane was dried and UV cross-linked. The probes modified with locked nucleic acid (LNA, Exiqon, Denmark) were labeled with ³²P (18, 19). The blots were prehybridized at 35 ºC for 30 min in hybridization buffer (GE Healthcare), and then each ³²P-labeled LNA probe was added and incubated at 60 ºC for 2 h. The membranes were washed twice in 2xSSC with 0.1%
SDS at 60 ºC for 10 min. The probe sequences are as follows: hsa-miR-516a-3p, 5’-ACCCCTCTGAAAGGAAGCA -3’; and U6 (Control), 5’- CACGAATTTGCGTGTCATCCTT -3’.

Total RNA from normal stomach was obtained from the FirstChoice® Total RNA Human Normal Tissue Set (ABI).

**Quantitative real-time RT-PCR.** TaqMan® MicroRNA Assays were used to detect the expression levels of mature miRNAs. Ten nanograms of total RNA was reverse-transcribed with a TaqMan® microRNA RT kit (ABI). All of the PCRs were run in triplicate, and miRNA expression, relative to small nuclear RNA (RNU6B), was calculated by the comparative ΔCt method (20).

**Stable overexpression of hsa-miR-516a-3p in 44As3 cells.** The hsa-miR-516a-3p expression vector (1 μg, Takara Bio, Shiga, Japan) was transfected in 44As3 cells using a Lipofectamine-plus reagent as reported previously (21). Then, the cells were selected with Geneticin® (100 μg/ml) to acquire a stable clone with overexpression of the miRNA (called 44As3-miR 516a-3p).

**Proliferation, migration, and invasion analysis in vitro.** The 44As3-miR-516a-3p was seeded onto a 96-well plate (0.5×10^4 cells/well) in RPMI 1640 with 10% FBS. On the next day, the cells were washed once with serum-free RPMI 1640. Cell proliferation was monitored with a cell counting kit (Dojin, Kumamoto, Japan) as reported previously (22). Migration and invasion analysis was performed with BD BioCoat™ Angiogenesis System-Endothelial Cell Migration, and BD BioCoat™ Fluoroblok™ Tumor Invasion System (BD Biosciences) according to the manufacturer’s instructions.

**Primary tumor growth and survival rate in nude mice.** The 44As3-miR-516a-3p (1.5×10^6 cells in 50 μl of RPMI 1640) was orthotopically inoculated into the middle wall of the greater curvature of the glandular portion of the stomach using a 29-gauge needle (Nipro,
Tokyo, Japan) in BALB/c nude mice (6-week-old females) as described previously (14). Twenty days later, the primary tumors were removed and weighed. Peritoneal dissemination to the omentum, mesenterim, and ascites was examined.

Animal experiments in the present study were performed in compliance with the guidelines of the Institute for Laboratory Animal Research at the National Cancer Center Research Institute or at Nagoya University Graduate School of Medicine.

**iTRAQ sample preparation and QSTAR® Elite Hybrid LC-MS/MS.** The analysis was performed according to previous reports (15, 16) with some modifications. The cells were washed with PBS twice on the dish, collected with a cell scraper, and centrifuged. The pellet was vigorously washed with PBS. After centrifugation (15,000 rpm for 30 min at 4 °C), the clarified supernatant was transferred to fresh microtubes and the total protein concentration was determined using the DC Protein Assay (Bio-Rad Laboratories, Hercules, CA). Each sample (100 μg protein) was digested with 0.2 ml of trypsin solution (50 μg/ml) at 37 °C and labeled with the iTRAQ tags (113, 115, and 118: ABI) as summarized in Supplementary Fig. S1. After we combined the three labeled samples, QSTAR® Elite Hybrid LC-MS/MS and DiNa System driven by Analyst® QS 2.0 software (Applied Biosystems-MDS SCIEX Ins, Foster City, CA) gave us the MS or MS/MS spectrum data. Finally we determined the candidate proteins with ProteinPilot™ Software 2.0.1 (Applied Biosystems-MDS SCIEX Ins).

**Isolation of cytoplasmic and nuclear proteins from the cells.** Cytoplasmic and nuclear proteins were separately isolated using a ProteoJET™ Cytoplasmic and Nuclear Protein Extraction kit (Fermentas Life Sciences, York, UK).

**β-catenin ELISA.** The total amount of β-catenin was determined using a DuoSet® IC human total β-catenin ELISA kit (R&D Systems, Minneapolis, MN).
TCF/LEF reporter assay. To monitor the activity of the Wnt signal transduction pathway in the cells, a Cignal™ TCF/LEF Reporter Assay kit was used (SABiosciences, Frederick, MD). Thus, each cell was transfected with the TCF/LEF reporter using a Lipofectamine-plus kit (21). Sixteen hours later, the medium was changed to assay medium (Opti-MEM with 0.5% FBS, 0.1 mM NEAA, and 1 mM sodium pyruvate). At this time, we added LiCl (50 mM, a GSK inhibitor) to the medium of HSC-44PE cells as a positive control. Dual-Luciferase® assay (Promega, Madison, WI) was performed 48 hours after the transfection. Promoter activity values are expressed as arbitrary units using a Renilla reporter for internal normalization (23).

Plasmid DNA vector/atelocollagen complex preparation. Atelocollagen was prepared from calf dermal collagen (Koken, Tokyo, Japan) (24, 25). Atelocollagen is a liquid at 4°C and a gel at 37°C, when it is at a high concentration (≥ 0.5%). This biochemical feature of atelocollagen contributes to the controlled release of siRNA or plasmid DNA when injected directly into the tumor (24, 25). Further, atelocollagen contributes to the increased stability of siRNA or plasmid DNA injected in tumors (21, 24, 25, 28). To prepare the plasmid DNA/atelocollagen complex, equal volumes of atelocollagen in PBS and plasmid DNA solution were mixed by rotation for 20 min at 4 °C (25).

Antimetastasis therapy procedure. 44As3 cells (1.5×10^6 cells in 50 μl of RPMI 1640) were orthotopically inoculated into the stomach wall of 6-week-old BALB/c nude mice (CREA) (14). After 10 days (day 0), we performed an intratumor (it) injection of the miR-516a-3p expression vector/atelocollagen complex or empty vector/atelocollagen complex under anesthesia. The final concentration of each vector/atelocollagen was 25 μg/20 μl per tumor in 0.5% atelocollagen. Five days later (on day 5), we repeated the injection. On day 15, we sacrificed the mouse and excised the primary tumor. Total RNA or tissue lysate was prepared for further study. For the controls, HSC-44PE was orthotopically inoculated using the above-described method, but without any therapy, and then on day 15 the tumors were excised. The overall survival rate after the therapy was also examined (n=20 mice in each group).
Western blot analysis. The analysis was performed according to our previous reports (22, 26-28). The following primary antibodies were used (Cell Signaling Japan, Tokyo, Japan): rabbit anti-human Wnt3a antibody, rabbit anti-human Wnt5a antibody, rabbit anti-human β-catenin antibody, α-tubulin, mouse anti-human α-tubulin antibody, and mouse anti-human histone H3 antibody, respectively. To detect SULF1, we used mouse anti-human sulfatase1 antibody (Abcam). We used appropriate secondary antibody with horseradish peroxidase (Jackson ImmunoResearch Laboratories, West Grove, PA). Finally, the peroxidase activity was detected with an enhanced chemiluminescence detection system (GE Healthcare).

Clinical samples. The studies of clinical samples were conducted with institutional review board approval from Nagoya University School of Medicine. After obtaining appropriate informed consent from each patient, in accordance with the Declaration of Helsinki, tumor specimens were harvested. Detail of the patients’ background was summarized in Supplementary Table 1. Normal stomach tissues were purchased from BioChain Institute, Inc. (Hayward, CA). Each total RNA was extracted from the tissues, and quantitative real-time RT-PCR for miR-516a-3p was performed.

Statistical analysis. The data were analyzed using the Mann-Whitney U test. Probability values of less than 0.05 were considered to indicate significant differences.
Results

High frequency of peritoneal dissemination of scirrhous cancer results in miRNAome alteration. We performed miRNA microarray profiling between HSC-44PE and 44As3 (Table 1). As a result, 12 miRNAs were significantly upregulated and 24 miRNAs downregulated in 44As3 (the threshold was 1.5-fold up- or downregulation). These alterations of the miRNA levels were often observed in the human chromosomal location of 19q13.42 or 14q32.2. Among the miRNAs, we focused on miR-516a-3p because the roles of the miRNA in both metastasis and tumorigenesis have not yet been elucidated at all. We performed both Northern blot analysis and quantitative RT-PCR to confirm the microarray (Fig. 1A, B). The miRNA in 44As3 was 3-fold downregulated compared with HSC-44PE. In the normal stomach, the miRNA showed 3-fold higher expression compared with HSC-44PE.

We have a cell-pair from another scirrhous gastric cancer patient (called HSC-58, parental cell; and 58As9, high metastatic cell) (Ref. 14). According to the miRNA array, we obtained a similar result as showing significant decreased expression level of miR-516a-3p in 58As9, compared with HSC-58 (Supplementary Table 2). These consistent results from two cell-pairs potently prompted us to further examine about miR-516a-3p.

Stable overexpression of miR-516a-3p in 44As3 reduced their proliferation rate, migration, and invasion in vitro. We prepared a stable cell line with overexpression of miR-516a-3p from 44As3 (called 44As3-miR-516a-3p). Northern blot analysis and quantitative RT-PCR revealed the successful overexpression of the miRNA (Fig. 1C, D). 44As3-miR-516a-3p demonstrated lower cell proliferation, migration, and invasion compared with a 44As3-empty vector stably transfected one (Fig. 2A). In the other cell-pair (HSC-58 and 58As9), we also prepared a stable cell line with overexpression of the miRNA from 58As9 (called 58As9-miR-516a-3p). 58As9-miR-516a-3p gave similar results such as lower cell proliferation, migration, and invasion compared with an empty vector-transfected control cell (Supplementary Figure S2).

In Fig. 2B, we orthotopically inoculated these four lines in the stomach wall of nude mice.
Twenty days later, we observed the reduced tumor formation ratio there. Markedly, 44As3-miR-516a-3p showed that only 3 of the 12 mice that had formed tumors harbored ascetic fluid. We consistently observed the reduction of disseminated metastasis, i.e., omentum or mesenterium, in the mice inoculated with the line (Fig. 2B). The line showed significantly lighter tumors than those of 44As3 or 44As3-empty vector. The mice inoculated with 44As3-miR-516a-3p survived significantly longer than those of 44As3 and those of the 44As3-empty vector group.

**Extracellular sulfatase 1 (SULF1) is a direct target of miR-516a-3p.** Proteomics analysis using an iTRAQ reagent and QSTAR® Elite Hybrid LC-MS/MS simultaneously identified 11 candidate proteins as the targets of miR-516a-3p (Fig. 3B). On the target prediction (TargetScan) in silico, 5 candidates did not appear, suggesting these might be indirect targets of the miRNA. We focused on SULF1 as an attractive target, and then performed a luciferase reporter assay. Fig. 3C showed that SULF1 is an actual target of the miRNA. The nucleotide sequences of SULF1 in the 3’ untranslated regions, which were predicted as a binding site of the miRNA, were highly conserved broadly beyond species (Supplementary Fig. S3). Western blot analysis (Fig. 3D) revealed that SULF1 expression was simply the inverse of that of miR-516a-3p by Northern blot analysis (Fig. 1). In the normal stomach, SULF1 protein was undetectable (Supplementary Fig. S4), probably as a result of regulation by the miRNA.

**miR-516a-3p indirectly regulates the Wnt pathway by targeting SULF1.** SULF1 is an extracellular sulfatase (29). It acts on internal glucosamine-6-sulfate (6S) removal within heparan sulfate proteoglycans (HSPGs) and thereby modulates HSPG interactions with various signaling molecules (30-32). Indeed, SULF1 promotes Wnt signaling (30-32). We found increased secretion of both Wnt3a and Wnt5a in 44As3, but 44As3-miR-516a-3p revealed decreased secretion of the ligands (Fig. 4A). In the cytoplasmic fraction prepared from 44As3, the amount of β-catenin was increased, and thus β-catenin accumulated in the nucleus of 44As3. In contrast, 44As3-miR-516a-3p harbored a lesser amount of β-catenin in both the
cytoplasmic and nuclear fraction compared with 44As3 (Fig. 4B, C). Among the cells, mRNA levels of Wnt3a, Wnt5a, and β-catenin did not change at all (Supplementary Fig. S5). Both mRNA and protein levels of Frizzled 4 and 5 also did not change (Fig. 4A and Supplementary Fig. S5). These results suggested that the miRNA did not alter the expression levels of those proteins. Finally we showed activated TCF/LEF in 44As3 but decreased TCF/LEF activity in 44As3-miR-516a-3p, consistent with the series of results showing activated Wnt pathway (Fig. 4D).

Antimetastasis therapy against 44As3 orthotopic tumors. We showed that decreased miR-516a-3p expression in 44As3 orthotopic tumors is associated with the promotion of peritoneal dissemination of scirrhous gastric cancers. To assess the therapeutic effect of the miRNA, intratumoral treatment with miR-516a-3p vector with atelocollagen was examined (Fig. 5A). Atelocollagen was facilitated to deliver the plasmid DNA inside the cell (25). Compared with the empty vector control, the expression of the miRNA was higher via the intratumoral injection of the miR-516a-3p vector/atelocollagen complex, as shown by Northern blot analysis and qRT-PCR (Fig. 5B). Further, SULF1 protein levels in the treated tumors were decreased (Fig. 5C), and significantly less ascitic fluid was pooled in the treated group. Finally, the overall survival period was longer in the treated group than in the control (Fig. 5D). In Supplementary Fig. S6, we observed green fluorescence from the pAcGFP1-N1 vector with atelocollagen injected into the tumor in the same manner, confirming that our delivery method was functional.

Expression level of miR-516a-3p in clinical samples. Quantitative real-time RT-PCR revealed significant decreased expression level of miR-516a-3p in primary gastric cancer tissues from the patients with peritoneal dissemination, compared with that from the patients without peritoneal dissemination (Fig. 6). In stomach tissues from normal donors, we observed high expression level of the miRNA.
Discussion

The major causes of deaths from cancer are complications arising from metastasis (33). Therefore, effective treatments targeting metastatic disease will improve morbidity and mortality among cancer patients. Despite many advances in knowledge from more than a century of researching metastasis, its molecular mechanisms are still not completely understood. Several reports have described the involvement of a recently discovered class of noncoding regulatory RNA, i.e., miRNA, in the regulation of cancer (oncomir; refs. 34). More recently, a specialized family of miRNA, which Hurst et al. call metastamir (33), has been shown to have pro- and antimetastatic effects (35, 36). In the present article, we described many kinds of metastamirs, which are potently associated with peritoneal disseminations of scirrhous gastric carcinomas. Among the miRNAs, we focused on miR-516a-3p, whose expression was significantly lower in 44As3 than in HSC-44PE (Fig. 1). A clone cell with stable overexpression of the miRNA demonstrated that the miRNA dramatically regulates cell mobility rather than cell proliferation. Upon orthotopic inoculation of 44As3-miR 516a-3p in the stomach wall in nude mice, we hardly observed ascitic fluids; this lack of fluids prolonged the survival of the mice. On the other hand, orthotopically inoculated mice with 44As3 rapidly pooled a large volume of ascitic fluids without exception, consistent with our previous report (14). These results strongly suggested that the miRNA is a genuine metastamir exhibiting antimetastatic effects (‘antimetastamir’).

To identify the target protein of the miRNA, a proteomic analysis was performed by means of isobaric tagging for relative quantification using multidimensional liquid chromatography tandem mass spectrometry (LC-MS/MS). iTRAQ-based LC-MS/MS provides a valuable platform for simultaneously identifying and quantifying target proteins of miRNAs. Indeed, we identified and quantified 11 proteins, including some whose functions are unknown. Unlike the conventional method (37), i.e., two-dimensional gel electrophoresis, and the peptide mass fingerprint method via MALDI-TOF MS, the iTRAQ-based LC-MS/MS methods are much more accurate at identifying proteins and at enabling high-throughput tasks.

Here, we focused on SULF1 as a target of the miRNA, because extracellular sulfatases are...
crucial elements of the Wnt signaling pathway in cancers (32). SULF1 removes 6-O sulfates from HSPG chains to liberate Wnt ligands, and thus the liberated ligands enable binding to Frizzled receptors (30). Wnt signaling plays an important role in cancers, as reported many times (38). In patients with gastric cancers, Wnt5a is very frequently expressed (39); Wnt5a is stained in tumor cells but not in stromal cells. In addition, the 5-year survival rate is significantly low in patients with Wnt5a-positive gastric cancers (39). The expression of Wnt5a in gastric cancer cells could be critical for the migration and invasion of cancer cells from primary regions (40). Taken together, the previous and present results suggest that the expression of Wnt5a is correlated with the aggressiveness and poor prognosis of gastric cancer (39, 40). In the present study, secreted Wnt5a was increased in 44As3; in contrast, the secretion level was decreased in 44As3-miR-516a-3p. Wnt3a and Wnt5a activate the typical canonical pathway (the β-catenin pathway) in the cells (38, 41, 42). However, regarding Wnt5a in 44As3, we have another pathway to examine: the PCP (non-canonical) pathway (40), which should be initially proved by us in peritoneal dissemination of scirrhous gastric cancers. Overall, decreased miR-516a-3p upregulates SULF1, after which Wnt ligands, which were liberated by SULF1, activate at least the Wnt canonical pathway, finally leading to peritoneal dissemination. In that case, miR-516a-3p triggers a series of reactions. Of course, not only the other proteins determined in Fig. 3B, but also the other miRNAs in Table 1, must be involved in and regulate peritoneal dissemination. To elucidate how strong is the influence of the other target proteins besides SULF1, we are generating the miR-516a-3p-resistant SULF1 construct by cloning the SULF1 cDNA from the start codon until the stop codon, thereby excluding the 3’-UTR region. It is our subsequent urgent task to determine the phenotypes such as proliferation, or in vivo peritoneal dissemination using the cell with overexpression of both miR-516a-3p and the above construct.

To elicit a therapeutic outcome, we indicated the feasibility of using the miR-516a-3p plasmid vector complexed with a biomaterial delivery reagent, atelocollagen, to inhibit peritoneal dissemination in a mouse model. Atelocollagen is a natural product prepared from bovine dermal collagen (24, 25). Atelocollagen shows neither antigenicity nor toxicity in
animals, since antigenic telopeptides are eliminated by pepsin digestion (25). Atelocollagen is soluble at a lower temperature (below 10 °C) but solidifies to refibrillation upon injection into an animal body (37 °C). When plasmid DNA is embedded in atelocollagen, the quantity and period of gene expression are well controlled in vivo (25). Atelocollagen can protect plasmid DNA, antisense oligodeoxynucleotides, and siRNAs from degradation by several nucleases, proteases, and antibodies, thereby prolonging the half-life of embedded nucleic acids (21, 25, 26). In the present study, we successfully demonstrated that intratumoral injection of miR-516a-3p vector with atelocollagen revealed its constitutive active expression in primary 44As3-tumors orthotopically inoculated in the stomach in nude mice. These findings demonstrate that miR-516a-3p, a novel antimetastasmir, can be a target for inhibiting peritoneal dissemination of scirrhous gastric cancer. However, much works remains before it can be applied clinically. First, synthetic miRNA instead of the plasmid vector should be examined; second, a systemic delivery system of synthetic miRNA specific to tumors should be developed. Toward these ends we have acquired promising results, because we already have much knowledge how to systemically deliver synthetic small RNA molecules, i.e., siRNA, specifically to tumors (22). The systemic therapeutic effect by the synthetic double-stranded RNA mimicking miR-516a-3p complexed with atelocollagen will be described elsewhere in the near future.

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Figure legends

Figure 1. Decreased expression of miR-516a-3p in 44As3 compared with HSC-44PE. A, Northern blot analysis. Each lane is explained in a box. U6 is used as a loading control. Densitometric analysis normalized with U6 is also shown. B, real-time qRT-PCR was performed with the same samples in A. C, Northern blot analysis showed successful stable expression level of miR-516a-3p in 44As3-miR-516a-3p cells. D, real-time qRT-PCR was performed with the same samples as in C. All of the bars in the figures show mean±SD (n=3). *, p<0.05; **, p<0.01.

Figure 2. Phenotypes of 44As3-miR-516a-3p in vitro and in vivo. A, Cell proliferation, migration, and invasion are analyzed. Each bar shows mean±SD (n=4). *, p<0.05; **, p<0.01. B, Each cell was orthotopically inoculated in the stomach wall in nude mouse (15 mice each). Twenty days later, the tumor formation ratio, ascites, and disseminated metastasis were examined. The numbers of mice forming tumors were as follows: HSC-44PE, 11; 44As3, 15; 44As3-empty vector, 15; and 44As3-miR-516a-3p, 12. Primary tumor weight was measured (*, p<0.05). The survival rate (n=15 each on day 0) was examined by the same orthotopic model. **, p<0.01.

Figure 3. Simultaneous identification of target proteins of miR-516a-3p by proteomics analysis using an iTRAQ reagent and QSTAR® Elite Hybrid LC-MS/MS. A, a strategy for the analysis. We aimed to identify one or more proteins whose expression levels were higher in 44As3 (sample 2) than in HSC-44PE (sample 1), and at the same time were lower in 44As3-miR-516a-3p (sample 3) than in 44As3 (sample 2). B, A list of proteins identified. *, Each total context score of a prediction program, TargetScan is shown. NS**, no scores appear in TargetScan. C, Reporter assay. miR-516a-3p inhibits wild-type but not mutated SULF1-3’-UTR reporter activity. 44As3-miR-516a-3p cells were transiently transfected with the indicated plasmids. Forty-eight hours later, cell lysates were subjected to dual-luciferase...
Figure 4. miR-516a-3p regulates Wnt pathway via sulfatase1 expression. A, Western blot analysis of Wnt3a and Wnt5a ligands. Frizzled 4 and 5 were also examined. β-actin was used as a loading control. Each lane was explained in a box. B, Western blot analysis of β-catenin of cytoplasmic and nuclear fraction. The bands on the blot were normalized with α-tubulin or Histone H3, respectively. The density ratios are shown in the graphs. C, Total β-catenin amount was determined by a specific ELISA. The bars show means±SD (n=4). *, p<0.05; ***, p<0.001. D, TCF/LEF reporter assay. The bars show means±SD (n=4). ***, p<0.001. LiCl (50 mM) for positive controls as a GSK inhibitor was added to the culture of HSC-44PE cells. Firefly and Renilla luciferase activities were determined using a Dual-Luciferase Reporter Assay System, with firefly luciferase activities calculated as means±SD after being normalized by Renilla luciferase activities.

Figure 5. Antimetastasis therapy using miR-516a-3p expression vector via atelocollagen-mediated delivery method. A, a therapy procedure. Nude mice bearing orthotopic 44As3-tumors were injected (intratumorally) with an empty vector or miR-516a-3p vector (25 μg) with atelocollagen (day 0). Five days later, the same therapy was repeated. On day 15, we excised the tumors for further study. B, Northern blot analysis (left), and real-time qPCR (right) were examined. Each lane is explained in a box. All of the bars in B show means±SD (n=4). **, p<0.01. C, Western blot analysis of human SULF1. Ratios of density, individually normalized with β-actin, are shown beneath the blot. D, Volume of ascites (left). The bars show mean±SD (n=4). **, p<0.01. Overall survival (right) of each of 20 mice was determined.

Figure 6. miR-516a-3p expression levels in gastric cancer patients with or without peritoneal dissemination. Real-time qRT-PCR was performed with the clinical samples as summarized in Supplementary Table 1. Stomach tissues from normal donors were also examined. All of the
PCRs were run in triplicate, and miRNA expression, relative to small nuclear RNA (RNU6B), was calculated. All of the bars in the figure show mean±SD (n=3). **, p<0.01; ***, p<0.001.
Table 1. Summary of significantly differentially expressed miRNAs in 44As3, compared with HSC-44PE

### Increased expression

<table>
<thead>
<tr>
<th>Rank</th>
<th>miRNA</th>
<th>Fold change*</th>
<th>p-value</th>
<th>Chromosomal location</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>hsa-miR-498</td>
<td>1.96</td>
<td>0.013</td>
<td>19q13.42</td>
</tr>
<tr>
<td>2</td>
<td>hsa-miR-505</td>
<td>1.95</td>
<td>0.014</td>
<td>Xq27.1</td>
</tr>
<tr>
<td>3</td>
<td>hsa-miR-519c-3p</td>
<td>1.93</td>
<td>0.021</td>
<td>19q13.42</td>
</tr>
<tr>
<td>4</td>
<td>mmu-miR-291b-3p†</td>
<td>1.88</td>
<td>0.022</td>
<td>7A1 (mouse)</td>
</tr>
<tr>
<td>5</td>
<td>hsa-miR-376b</td>
<td>1.88</td>
<td>0.030</td>
<td>14q32.2</td>
</tr>
<tr>
<td>6</td>
<td>hsa-miR-520d-5p</td>
<td>1.83</td>
<td>0.036</td>
<td>19q13.42</td>
</tr>
<tr>
<td>7</td>
<td>hsa-miR-522</td>
<td>1.78</td>
<td>0.031</td>
<td>19q13.42</td>
</tr>
<tr>
<td>8</td>
<td>mmu-miR-292-3p †</td>
<td>1.75</td>
<td>0.043</td>
<td>7A1 (mouse)</td>
</tr>
<tr>
<td>9</td>
<td>hsa-miR-514</td>
<td>1.65</td>
<td>0.037</td>
<td>Xq27.3</td>
</tr>
<tr>
<td>10</td>
<td>hsa-miR-95</td>
<td>1.62</td>
<td>0.035</td>
<td>4p16.1</td>
</tr>
<tr>
<td>11</td>
<td>hsa-miR-432</td>
<td>1.59</td>
<td>0.044</td>
<td>14q32.2</td>
</tr>
<tr>
<td>12</td>
<td>hsa-miR-502-5p</td>
<td>1.52</td>
<td>0.034</td>
<td>Xp11.23</td>
</tr>
</tbody>
</table>

### Decreased expression

<table>
<thead>
<tr>
<th>Rank</th>
<th>miRNA</th>
<th>Fold change*</th>
<th>p-value</th>
<th>Chromosomal location</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>hsa-miR-183</td>
<td>-3.02</td>
<td>0.008</td>
<td>7q32.2</td>
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<tr>
<td>2</td>
<td>hsa-miR-23a §</td>
<td>-2.88</td>
<td>0.010</td>
<td>19p13.13</td>
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<tr>
<td>3</td>
<td>hsa-miR-125a-5p</td>
<td>-2.74</td>
<td>0.010</td>
<td>19q13.42</td>
</tr>
<tr>
<td>4</td>
<td>hsa-miR-516a-3p</td>
<td>-2.73</td>
<td>0.009</td>
<td>19q13.42</td>
</tr>
<tr>
<td>5</td>
<td>hsa-miR-33a</td>
<td>-2.72</td>
<td>0.011</td>
<td>22q13.2</td>
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<tr>
<td>6</td>
<td>hsa-miR-16</td>
<td>-1.97</td>
<td>0.013</td>
<td>13q14.2</td>
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<tr>
<td>7</td>
<td>hsa-miR-106b §</td>
<td>-1.92</td>
<td>0.018</td>
<td>7q22.1</td>
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<tr>
<td>8</td>
<td>hsa-miR-140-5p</td>
<td>-1.89</td>
<td>0.029</td>
<td>16q22.1</td>
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<tr>
<td>9</td>
<td>hsa-miR-345</td>
<td>-1.87</td>
<td>0.019</td>
<td>14q32.2</td>
</tr>
<tr>
<td>10</td>
<td>hsa-miR-93 §</td>
<td>-1.82</td>
<td>0.024</td>
<td>7q22.1</td>
</tr>
<tr>
<td>11</td>
<td>hsa-miR-34a</td>
<td>-1.82</td>
<td>0.022</td>
<td>1p36.22</td>
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<tr>
<td>12</td>
<td>hsa-miR-370</td>
<td>-1.81</td>
<td>0.039</td>
<td>14q32.2</td>
</tr>
<tr>
<td>13</td>
<td>hsa-miR-129-3p</td>
<td>-1.78</td>
<td>0.020</td>
<td>11p11.2</td>
</tr>
<tr>
<td>14</td>
<td>hsa-miR-17</td>
<td>-1.77</td>
<td>0.036</td>
<td>13q31.3</td>
</tr>
<tr>
<td>15</td>
<td>hsa-miR-27a §</td>
<td>-1.71</td>
<td>0.021</td>
<td>19p13.13</td>
</tr>
<tr>
<td>16</td>
<td>hsa-miR-450a</td>
<td>-1.66</td>
<td>0.037</td>
<td>Xq26.3</td>
</tr>
<tr>
<td>17</td>
<td>hsa-miR-100</td>
<td>-1.61</td>
<td>0.033</td>
<td>11q24.1</td>
</tr>
<tr>
<td>18</td>
<td>hsa-miR-31</td>
<td>-1.61</td>
<td>0.039</td>
<td>9p21.3</td>
</tr>
<tr>
<td>19</td>
<td>hsa-miR-193b</td>
<td>-1.60</td>
<td>0.036</td>
<td>16p13.12</td>
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<tr>
<td>20</td>
<td>hsa-miR-330-5p</td>
<td>-1.59</td>
<td>0.048</td>
<td>19q13.32</td>
</tr>
<tr>
<td>21</td>
<td>hsa-miR-200c</td>
<td>-1.56</td>
<td>0.032</td>
<td>12p13.31</td>
</tr>
<tr>
<td>22</td>
<td>hsa-miR-27b</td>
<td>-1.56</td>
<td>0.038</td>
<td>9q22.32</td>
</tr>
<tr>
<td>23</td>
<td>hsa-miR-337-3p</td>
<td>-1.56</td>
<td>0.045</td>
<td>14q32.2</td>
</tr>
<tr>
<td>24</td>
<td>hsa-miR-526b</td>
<td>-1.53</td>
<td>0.046</td>
<td>19q13.42</td>
</tr>
</tbody>
</table>

*Only those miRNAs whose expression levels showed >1.5-fold increase or decrease in 44As3 cells are listed.

†, ‡, §: miRNAs clustered with each other
Figure 1

A Northern blot analysis

Lane 1: Normal stomach
Lane 2: HSC-44PE
Lane 3: 44As3
Lane 4: MKN7

miR-516a-3p

Densitometric analysis

miR-516a-3p levels normalized with U6

B real-time qRT-PCR

miR-516a-3p / RNU6B

Densitometric analysis

miR-516a-3p levels normalized with U6

C Northern blot analysis

Lane 1: HSC-44PE
Lane 2: 44As3
Lane 3: 44As3-empty vector#
Lane 4: 44As3-miR-516a-3p#

#, stably overexpressed cell lines

D real-time qRT-PCR

miR-516a-3p / RNU6B

**

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Figure 2

A. **in vitro study**

- **Cell proliferation**
  - HSC-44PE
  - 44As3
  - 44As3-empty vector
  - 44As3-miR-516a-3p

- **Cell migration**
  - HSC-44PE
  - 44As3
  - 44As3-empty vector
  - 44As3-miR-516a-3p

- **Cell invasion**

B. **in vivo study**

**Characteristics of the formed tumors (20 days after inoculation)**

<table>
<thead>
<tr>
<th>Types of cells</th>
<th>Tumor formation</th>
<th>Ascites</th>
<th>Disseminated metastasis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Omentum</td>
</tr>
<tr>
<td>HSC-44PE</td>
<td>11/15</td>
<td>2/11</td>
<td>1/11</td>
</tr>
<tr>
<td>44As3</td>
<td>15/15</td>
<td>15/15</td>
<td>15/15</td>
</tr>
<tr>
<td>44As3-empty vector</td>
<td>15/15</td>
<td>14/15</td>
<td>14/15</td>
</tr>
<tr>
<td>44As3-miR-516a-3p</td>
<td>12/15</td>
<td>3/12</td>
<td>3/12</td>
</tr>
</tbody>
</table>

**Primary tumor growth (20 days later)**

- **Survival**

*Data represents the percentage of mice with tumors of each type.*

**Absorbance at 450 nm**

**Fluorescence intensity**

**Survived days**

**Tumor wet weight (g)**
### A

<table>
<thead>
<tr>
<th>Target gene(s)</th>
<th>Accession No.</th>
<th>Ratio (sample1 VS sample2)</th>
<th>Ratio (sample2 VS sample3)</th>
<th>Target prediction*</th>
</tr>
</thead>
<tbody>
<tr>
<td>FLJ5219</td>
<td>IPI00168494</td>
<td>2.934</td>
<td>0.519</td>
<td>-0.53</td>
</tr>
<tr>
<td>Keratin, type II cytoskeletal 1 (KRT1)</td>
<td>IPI00220327</td>
<td>2.428</td>
<td>0.868</td>
<td>NS**</td>
</tr>
<tr>
<td>FLJ78679</td>
<td>IPI00329791</td>
<td>1.794</td>
<td>0.595</td>
<td>-0.41</td>
</tr>
<tr>
<td>Transmembrane protein 165</td>
<td>IPI00514663</td>
<td>1.683</td>
<td>0.538</td>
<td>-0.37</td>
</tr>
</tbody>
</table>

* TargetScan: total context score
** NS, no score in TargetScan

### B

**Determined proteins**

<table>
<thead>
<tr>
<th>Sample 1</th>
<th>Sample 2</th>
<th>Sample 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSC-44PE</td>
<td>44As3</td>
<td>44As3-miR-516a-3p#</td>
</tr>
<tr>
<td><strong>up</strong></td>
<td><strong>down</strong></td>
<td><strong>up</strong></td>
</tr>
</tbody>
</table>

### C

**Luciferase activity (arbitrary units)**

- pGL3 alone (mock)
- pGL3-SULF1-3'-UTR
- pGL3-SULF1-3'-UTR(MUT)

### D

**Luciferase activity (arbitrary units)**

- Lane 1: HSC-44PE
- Lane 2: 44As3
- Lane 3: 44As3-empty vector#
- Lane 4: 44As3-miR-516a-3p#

# stably overexpressed cell lines
Figure 5

Takei Y et al

**A**
Orthotopic inoculation of 44As3 cells

Start therapy

Day -10

0

5

10

15

Sacrifice:

- Northern blot analysis
- Real-time qRT-PCR
- Western blot analysis

Day 0

Day 5

Day 10

Day 15

Lane 1: Empty vector (25 microgram/site) with atelocollagen
Lane 2: miR-516a-3p vector (25 microgram/site) with atelocollagen

**B**

**Northern blot analysis**

miR-516a-3p

U6

miR-516a-3p levels normalized with U6

Densitometric analysis

miR-516a-3p / RNU6B

**real-time qRT-PCR**

Lane 1: Empty vector
Lane 2: miR-516a-3p vector
Lane 3: HSC-44PE was inoculated i.t. delivered via atelocollagen

**C**

**Western blot analysis**

SULF1 (density)

beta-actin

**D**

**Day 15**

Volume of ascites (ml)

Overall survival rate

- Empty vector
- miR-516a-3p vector
- HSC-44PE (inoculation only, without therapy)
Figure 6

1: Normal stomach tissues (n=8 donors)
2: Gastric cancer tissues without peritoneal dissemination (n=12 patients)
3: Gastric cancer tissues with peritoneal dissemination (n=12 patients)

miR-516a-3p / RNU6B

1 2 3

0.0 0.2 0.4 0.6 0.8 1.0 1.2

** ***

1: Normal stomach tissues (n=8 donors)
2: Gastric cancer tissues without peritoneal dissemination (n=12 patients)
3: Gastric cancer tissues with peritoneal dissemination (n=12 patients)
The metastasis associated microRNA miR-516a-3p is a novel therapeutic target for inhibiting peritoneal dissemination of human scirrhouss gastric cancer

Yoshifumi Takei, Misato Takigahira, Keichiro Mihara, et al.

Cancer Res  Published OnlineFirst December 17, 2010.

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