Reduced expression of exosomal miR-29s in peritoneal fluid is a useful predictor of peritoneal recurrence after curative resection of gastric cancer with serosal involvement

HIDEYUKI OHZAWA[1], AKIRA SAITO[2], YUKO KUMAGAI[2], YUKI KIMURA[2], HIRONORI YAMAGUCHI[2,3], YOSHINORI HOSOYA[2], ALAN KAWARAI LEFOR[2], NAOHIRO SATA[2] and JOJI KITAYAMA[1,2]

[1]Center for Clinical Research; Departments of [2]Gastrointestinal Surgery and [3]Clinical Oncology, Jichi Medical University, Shimotsuke, Tochigi 329-0498, Japan

Abstract. The peritoneal surface is the most frequent site of metastasis disease in patients with gastric cancer. Even after curative surgery and adjuvant chemotherapy, peritoneal recurrences often develop. Exosomes play pivotal roles in tumor metastasis via the transfer of microRNAs (miRNAs). In the present study, exosomes were isolated from peritoneal lavage fluid or ascites in 85 patients with gastric cancer and the relative expression levels of miR-29s were examined. The expression of miR-29a-3p, miR-29b-3p and miR-29c-3p in peritoneal exosomes were all downregulated in patients with peritoneal metastases (PM) compared to those without PM. In 30 patients who underwent curative gastrectomy with serosa-involved (T4) gastric cancer, 6 patients exhibited recurrence in the peritoneum within 12 months. The expression levels of miR-29s at gastrectomy tended to be lower in these 6 patients than in the other 24 patients with significant differences in miR-29b-3p (P=0.003). When the patients were divided into two groups based on median levels of miR-29s, peritoneal recurrence developed more frequently in patients with low expression of miR-29b-3p, and lower expression of miR-29s were related with worse overall survival. miR-29s are thought to play a suppressive role in the growth of disseminated peritoneal tumor cells. Reduced expression of miR-29b in peritoneal exosomes is a strong risk factor of developing postoperative peritoneal recurrence.

Introduction

Gastric cancer is the fifth leading cause of cancer-related deaths worldwide (1), and peritoneal metastases (PM) are the most life-threatening form of recurrence (2,3). Although various approaches have been used to treat PM such as extended surgery, chemotherapy, and heated intraperitoneal chemotherapy, the prognosis of patients with PM is still very poor (4-6). Even after curative surgery, approximately half of patients with gastric cancer with serosal involvement (T4) have been reported to develop peritoneal recurrences (7-9). Therefore, intensive adjuvant treatment to suppress peritoneal recurrence in high risk patients is a key factor to improve the survival of patients with advanced gastric cancer. However, no reliable biomarkers are available to accurately predict postoperative peritoneal recurrence in these patients, although molecular detection of CEA mRNA in peritoneal lavage fluid may have prognostic value (10-12).

Exosomes are small membrane-covered vesicles released from many different cell types and are present in various biological fluids. Recent studies have revealed that exosomes play pivotal roles in cell-to-cell communication via the transfer of protein, lipids, and nucleic acids, such as DNA, messenger RNA, microRNA (miRNA), and other non-coding RNAs (13). Accumulating evidence suggests that dysregulated expression of miRNAs with oncogenic or tumor-suppressing activities is often detected in patients with malignancies (14,15). Recently, it has become evident that secretory exosomes contain numerous miRNAs and serve as an efficient vehicle for intercellular transfer of miRNA (16). Aberrant expression of exosomal miRNAs in serum can be used as potential biomarkers of tumor progression and metastasis (17,18).

The miR-29 family is one of the most intensively examined groups of miRNAs. Previous studies have revealed that miR-29 plays an important role in various biological processes such as cell proliferation, apoptosis, invasion and angiogenesis and that downregulation of the miR-29 family is associated with aging, pathogenesis of neurodegenerative or fibrotic diseases, as well as tumor development and progression (19,20). However, there is little information regarding miRNAs in peritoneal fluid. In this study, exosomes were isolated from peritoneal lavage fluid from patients with gastric cancer and it was determined that the relative expression levels of miR-29s in peritoneal exosomes were strongly associated with the development of PM.

Correspondence to: Professor Joji Kitayama, Department of Gastrointestinal Surgery, Jichi Medical University, Yakushiji 3311-1, Shimotsuke, Tochigi 329-0498, Japan
E-mail: kitayama@jichi.ac.jp

Materials and methods

Patients and sample collection. The study protocol was approved by the Bioethics Committee for Clinical Research A, Jichi Medical University Hospital (Approval #A15-163), and written informed consent was obtained from all participants. All patients had pathologically confirmed gastric cancer in biopsy specimens obtained endoscopically from a primary lesion. Peritoneal fluid samples were collected from 85 patients (median age 69, range, 35-86; 49 male and 36 female patients) who were treated at Jichi Medical University Hospital between January 2016 and June 2019. Peritoneal lavage fluid was obtained from patients who underwent open surgery just after laparotomy. All of the patients from whom peritoneal lavage fluids were collected had fairly good performance status and received elective surgery. Patients who received urgent surgery, such as gastrointestinal perforation and acute peritonitis, were excluded from this study. In some patients with PM, malignant ascites were obtained by paracentesis or laparoscopic examination under general anesthesia. The present study was performed in accordance with the precepts established by the Helsinki Declaration.

Isolation of extracellular vesicles and RNA extraction. Peritoneal fluid samples were centrifuged at 2,000 x g for 10 min to remove floating cells. Supernatants were filtered through an 800-nm filter (EMD Millipore) to remove cell debris. For exosome preparation, filtered samples were ultracentrifuged at 150,000 x g for 70 min at 4°C. The size distribution and number of exosomes were determined using Nanosight LM10 (Malvern Panalytical, Ltd.). RNA extraction was performed using an miRNeasy Mini kit (Qiagen GmbH) according to the manufacturer's instructions. Total RNA in the samples was verified for quantity and quality using an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc.).

Western blot analysis. Protein concentration of exosome fraction was determined using a Qubit Protein Assay Kit with Qubit 2.0 Fluorometer (Invitrogen; Thermo Fisher Scientific, Inc.). The exosome fractions (1 μg of protein) were directly mixed with loading buffer and heated at 70°C for 10 min as previously described (21) and loaded and separated using 10% Novex Bis-Tris Protein Gels (Invitrogen; Thermo Fisher Scientific, Inc.), and then transferred onto PVDF membranes (Invitrogen), and immunoblotted with the following primary antibodies (incubated overnight at 4°C): CD9 (dilution 1:200; cat. no. sc-59140; Santa Cruz Biotechnology, Inc.) and CD63 (dilution 1:1,000; cat. no. MEX002-3; Medical & Biological Laboratories). Anti-mouse IgG, peroxidase-linked antibody (dilution 1:50,000; cat. no. NA931; GE Healthcare) was used as a secondary antibody (incubated for 1 h at room temprature). Chemiluminescence was detected using Amersham ECL Prime Western Blotting Detection Reagents (GE Healthcare Bio-Sciences) and imaged using LAS-3000 mini (Fujifilm Life Sciences).

miRNA expression analysis. In the initial screening phase, pooled samples from patients with or without PM were analyzed using miScript SYBR Green PCR Kit and the Human miRNome miScript miRNA PCR Array (Qiagen GmbH). A custom miScript miRNA PCR Array (Qiagen GmbH) was constructed based on the results. The miRNA expression profiles in each sample were evaluated using the custom PCR array. Using the NormFinder algorithm (22), miR-30d-5p and miR-10a-5p were identified as the most stably expressed miRNAs, which were selected as internal controls. Then, 8 miRNAs and 3 miRNAs (miR29a-3p, miR-29b-3p, and miR29c-3p) were significantly up- and downregulated in samples with PM, respectively. The miR-29 family was selected for further validation since all three miRNAs were downregulated in samples with PM.

For the quantitative PCR reaction, complementary DNA (cDNA) templates were prepared from 2 ml of total RNA using TaqMan Advanced miRNA cDNA Synthesis Kit (Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. A quantitative PCR reaction was subsequently performed in triplicate with a 1:10 dilution of cDNA using TaqMan Advanced miRNA Assays (Thermo Fisher Scientific, Inc.) on a ViiA7 Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). Relative expression levels of miR-29a-3p, miR-29b-3p, and miR-29c-3p were determined using relative quantification analysis module on Thermo Fisher Cloud (Thermo Fisher Scientific, Inc.). All miRNA data were calculated relative to the average of miR-10a-5p and miR-30d-5p performed on the same reaction plate.

Statistical analysis. The difference between categorical variables was evaluated using chi-square and Fisher's exact test. Relative expression values obtained from each PCR experiment were compared with linear regression analysis and the Mann-Whitney or Kruskal-Wallis test. Survival curves were calculated using the Kaplan-Meier method and differences were evaluated using the log-rank test. Patients were divided into miR-29s 'high' and 'low' groups based on the median expression values. Statistical analysis was conducted using GraphPad Prism 8.2.0 (GraphPad Software, Inc.), and differences were considered statistically significant with $P<0.05$.

Results

Characteristics of exosomes isolated from peritoneal fluid. Peritoneal fluid was collected from 33 patients with PM and 52 patients without PM, and exosomes were isolated by ultracentrifugation. Nanotracking analysis revealed that the isolated exosomes were mostly between 100 and 200 nm in size and western blotting revealed the presence of CD9 and CD63 (Fig. 1). Although the expression levels of the exosome markers varied among samples possibly due to the different protein composition in peritoneal fluids, these results clarified that we were working with exosomes. The protein concentration of the exosome fractions was higher in samples from patients with PM compared to those without PM (protein concentration (mg/ml), median=4.66, range: 0.16-20.6 vs. median=0.71, 0.17-1.52). However, the number as well as the size distribution of the exosomes were almost the same in both groups [particle size (nm), median=136.5, range: 93.0-175.0 vs. median=157.9, range: 120.7-252.9, number of particles (x10^{10} particles/ml), median=30.3, range: 14.3-112.2 vs. median=23.5, range: 2.2-104.0].
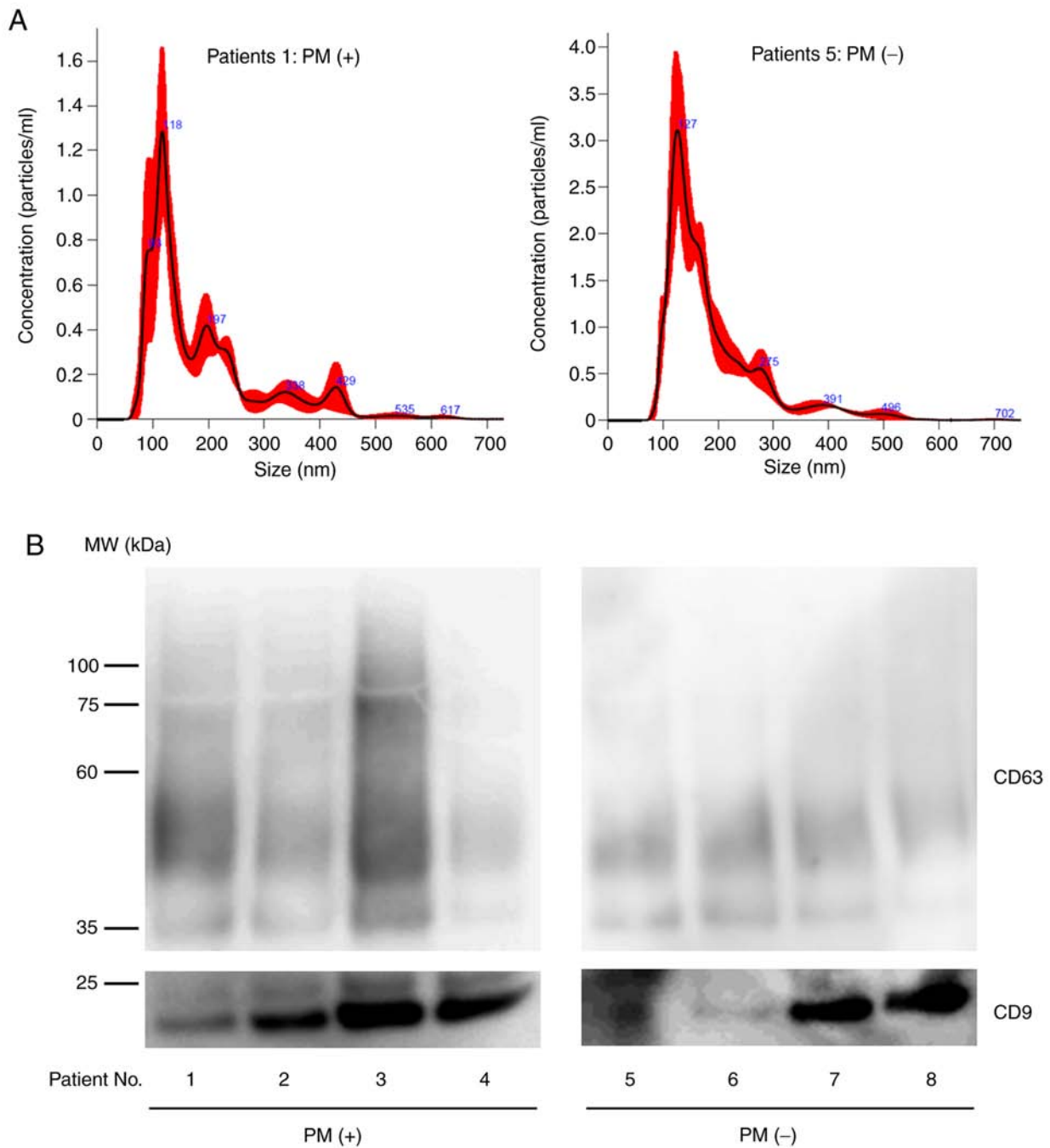
Figure 1. Representative features of exosomes purified from peritoneal fluid obtained from patients with and without peritoneal metastases. (A) Nanoparticle tracking analysis (B) Western blotting for CD9 or CD63. PM, peritoneal metastases.

The miR-29 family is downregulated in exosome in peritoneal fluids with PM. Relative expression levels of miR-29a-3p, miR-29b-3p and miR-29c-3p extracted from the exosome fractions were evaluated against an internal control, which revealed a positive correlation among the 3 miRs (Fig. 2). As revealed in Fig. 3, in patients without PM, the relative expression levels of these miR-29s were not different among tumors with serosal exposure (T4) and less invasive tumors (T1-T3) (miR-29a-3p, median=2.55, range: 1.69-8.29 vs. median=2.53, range: 1.35-4.42; miR-29b-3p, median=4.34, range: 2.49-12.76 vs. median=3.73, range: 1.48-8.55; miR-29c-3p, median=3.02, range: 1.31-9.58 vs. median=2.99, range: 0.92-6.67). However, their expression levels were significantly reduced in samples from patients with PM compared with those from patients without PM (miR-29a-3p, median=1.75, range: 0.53-5.07, P<0.001; miR-29b-3p, median=2.02, range: 0.32-4.56, P<0.001; miR-29c-3p, median=1.37, range: 0.24-2.84, P<0.001).

Low miR-29b-3p expression in peritoneal exosomes is associated with peritoneal recurrence in patients with T4 gastric cancer. Next, the expression of the miR-29 family and outcomes of the 30 patients with T4 tumors with serosal involvement were examined. Among them, 24 patients did not develop peritoneal recurrence with a median follow-up of 24 months, whereas peritoneal recurrence was observed with radiologic
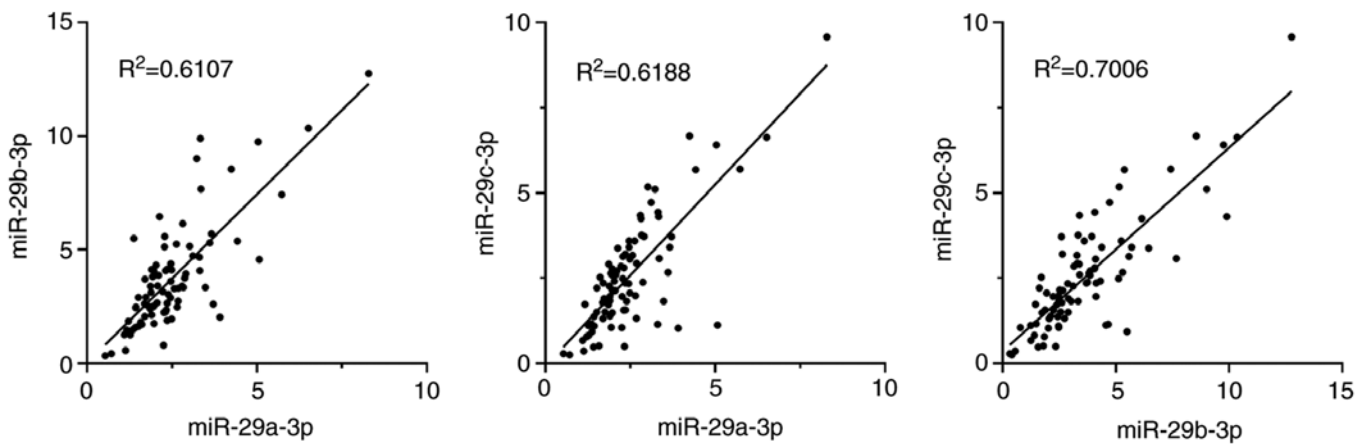
Figure 2. Correlation of the expression levels of miR-29a-3p, miR-29b-3p and miR-29c-3p in exosomes derived from the peritoneal fluid of all 85 patients. R^2 and P-values were examined using Spearman's correlation analysis.
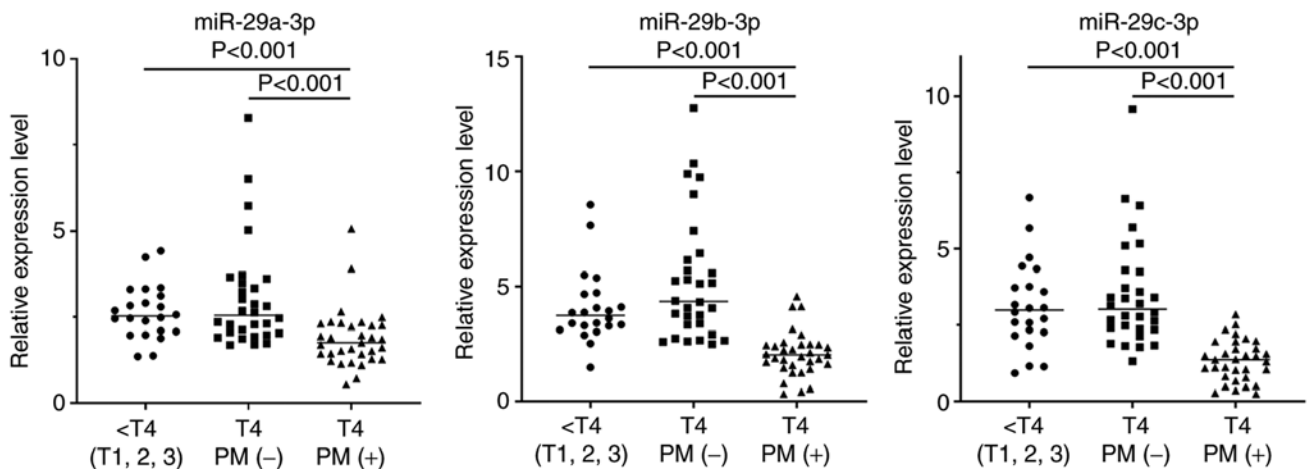

Figure 3. Expression levels of miR-29a-3p, miR-29b-3p and miR-29c-3p in 33 patients with PM as well as 52 patients without PM which were divided into T1-3 (n=22) and T4 (n=30) tumors. P-values were analyzed by the Kruskal-Wallis test. Relative expression levels were calculated relative to an average of miR-10a-5p and miR-30d-5p. PM, peritoneal metastases.

findings in the other 6 patients within 12 months after curative gastrectomy. Clinical and pathological factors revealed no significant differences comparing patients with and without recurrent disease (Table I). However, the expression levels of miR-29b-3p and miR-29c-3p in peritoneal exosomes obtained at surgery were significantly lower in the 6 patients with peritoneal recurrence compared with the other 24 patients (miR-29b-3p, median=2.82, range: 2.59-3.75 vs. median=5.19, range: 2.49-12.76, P=0.003; miR-29c-3p, median=2.13, range: 1.31-3.71 vs. median=3.29, range: 1.81-9.58, P=0.038) (Fig. 4). The level of miR-29a-3p also exhibited a similar trend although the differences were not statistically significant. When the patients were divided into two groups based on median levels of miR-29s, the peritoneal recurrence-free survival of patients with low expression of miR-29b-3p were significantly worse than those of patients with high expression of miR-29b-3p (P=0.014). The overall survival of patients with low expression of miR-29a-3p, miR-29b-3p, and miR-29c-3p was significantly worse than those of patients with high expression of these miRNAs, respectively. (miR-29a-3p, P=0.038; miR-29b-3p, P=0.031; miR-29c-30, P=0.021) (Fig. 5).

Discussion

miRNAs are non-coding RNAs with <25 nucleotides which have important roles in a wide range of pathophysiological processes through post-transcriptional gene repression of target RNA transcripts. In particular, miRNAs in exosomes circulating in blood or body fluids are resistant to enzymatic degradation by RNase and play pivotal roles in tumor metastasis formation (14,15). Emerging evidence has demonstrated that dysregulation of miRNAs has been revealed to be related to tumor development and progression (17,18), and that exosomes and exosomal miRNAs in circulating blood can be used as non-invasive biomarkers for early diagnosis and evidence of tumor progression in various malignancies (23-26), including gastric cancer (27,28).

However, little information is available regarding the miRNA profile in peritoneal exosomes, although 5 miRNAs have been revealed to be upregulated in malignant ascites, which could be candidate miRNA species related to peritoneal dissemination in gastric cancer (29). In patients with PM, however, tumor nodules were directly exposed to peritoneal

Table I. Characteristics of patients with pT4 gastric cancer with or without peritoneal recurrence.

Variables	Peritoneal recurrence (+)	Peritoneal recurrence (-)	P-values
Age			N.S.
Median (min-max)	70 (57 to 82)	69 (35 to 86)	
Sex			N.S.
Male	3	14	
Female	3	10	
Location			N.S.
Upper	3	9	
Middle	1	7	
Lower	2	8	
Macroscopic type			N.S.
2	3	4	
3	2	16	
4	1	3	
Other	0	1	
Histologic type			N.S.
Differentiated	2	16	
Undifferentiated	4	8	
Venous invasion			N.S.
+	6	24	
-	0	0	
Lymphatic invasion			N.S.
+	5	24	
-	1	0	
pT			N.S.
pT4a	6	23	
pT4b	0	1	
pN stage			N.S.
pN0	0	1	
pN1	0	4	
pN2	3	7	
pN3	3	12	
pStage			N.S.
IIB	0	1	
IIIA	0	5	
IIIB	3	7	
IIIC	3	11	
CEA (ng/ml)			N.S.
Median (min-max)	5.0 (0.5-357.3)	2.3 (1.0-29.5)	
CA19-9 (U/ml)			N.S.
Median (min-max)	13.0 (2.0-616.0)	13.5 (2.0-155.0)	
Operative procedure			N.S.
Total gastrectomy	4	15	
Partial gastrectomy	2	9	
Adjuvant therapy			N.S.
+	5	16	N.S.
-	1	8	

N.S., not significant; CEA, carcinoembryonic antigen.

surface and numerous tumor cells were contained in peritoneal fluids, at least, more than circulating blood. Therefore, it is supposed that large amounts of exosomes containing tumor specific molecules including functional miRNAs are released in the peritoneal cavity before the formation of PM, and thus analysis of the miRNA profile in peritoneal exosomes should provide important information to develop the adequate treatment of PM.

The number as well as size distribution of the exosomes were almost the same between PM(+) and (-) patients, although the protein concentration of the exosome fractions was higher in samples from PM(+) patients, possibly due to the contaminated protein complexes in peritoneal fluids. However, the present results clearly revealed that exosomal miR-29s in peritoneal fluid were significantly downregulated in patients with PM. Moreover, patients with T4 tumors with serosal involvement who had low levels of miR-29b-3p in peritoneal fluid obtained at gastrectomy recurred frequently in the peritoneal space. In the present study, although the sample size was not large, this was the only significant predictor of peritoneal recurrence since other clinical and pathological factors did not have a positive correlation.

The expression levels of miR-29b-3p and miR-29c-3p were even lower in patients with PM compared with recurred patients with non-PM. Patients with PM had macroscopic metastasis, and recurred patients with non-PM may have residual tumor cells which cannot be detected either by macroscopic observation or conventional cytology. Therefore, the expression levels of miR-29s in exosomes may reflect the tumor burden in peritoneum.

miR-29s predominantly function as tumor suppressors (30,31). In gastric cancer, miR-29 has been revealed to downregulate CCND2 and MMP-2 (32) or DNMT3A (33) which are critically involved in carcinogenesis and tumor progression, and their expression levels have been revealed to be reduced in gastric cancer tissues as compared with adjacent non-malignant tissue (32,34,35). Moreover, Zhang et al revealed that miR-29a/c derived from cell-derived microvesicles suppressed angiogenesis and inhibited the growth of gastric cancer (36). Serum levels of miR-29s have been revealed to be reduced in glioma (37) and hepatocellular carcinoma (38). The data in the present study were consistent with those results, and suggest that miR-29s in peritoneal exosomes have biological relevance for the development of PM.

Recently, Wei et al also revealed that exosomes derived from human malignant ascites converted mesothelial cells to carcinoma-associated fibroblasts and induced peritoneal fibrosis and promoted PM (39). Deng et al revealed that gastric cancer cells produced exosomes which promoted the formation of PM by disrupting the mesothelial barrier and inducing fibrosis (40). These facts suggest that exosomes in malignant ascites may promote peritoneal fibrosis which appears to be a favorable microenvironment for disseminated tumor cells. In contrast, it is well known that miR-29s target multiple genes encoding extracellular matrix components such as collagen and fibronectin, and that loss of miR-29 contributes to fibrosis in various organs (41-43). In fact, Yu et al demonstrated in a murine model that in vivo gene transfer of miR-29b using an ultrasound-microbubble technique effectively inhibited dialysis-related peritoneal fibrosis through the blockade of the Sp1-TGF-b/Smad pathway (44). These facts
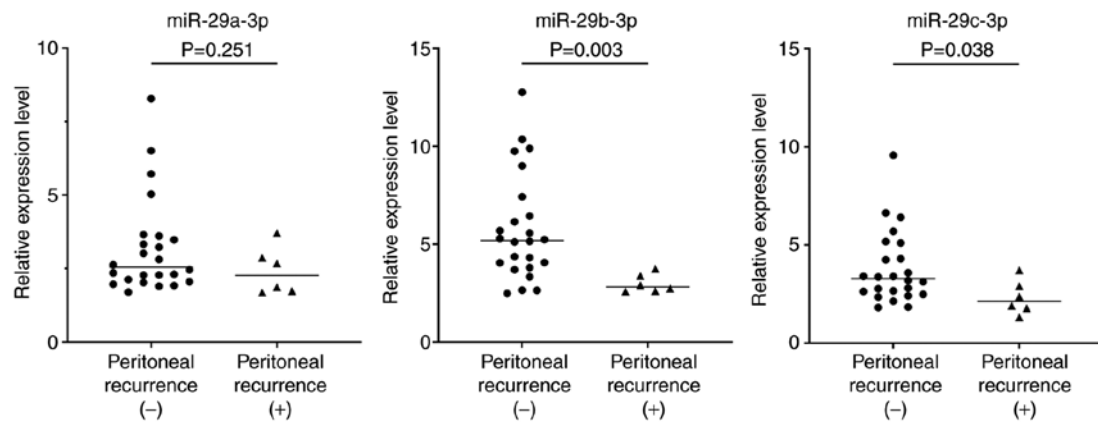
Figure 4. Expression levels of miR-29a-3p, miR-29b-3p and miR-29c-3p in 30 patients with T4 tumors who both had peritoneal recurrences (n=6) after curative resection and who did not (n=24). P-values were evaluated with the Mann-Whitney test. Relative expression levels were calculated relative to an average of miR-10a-5p and miR-30d-5p.
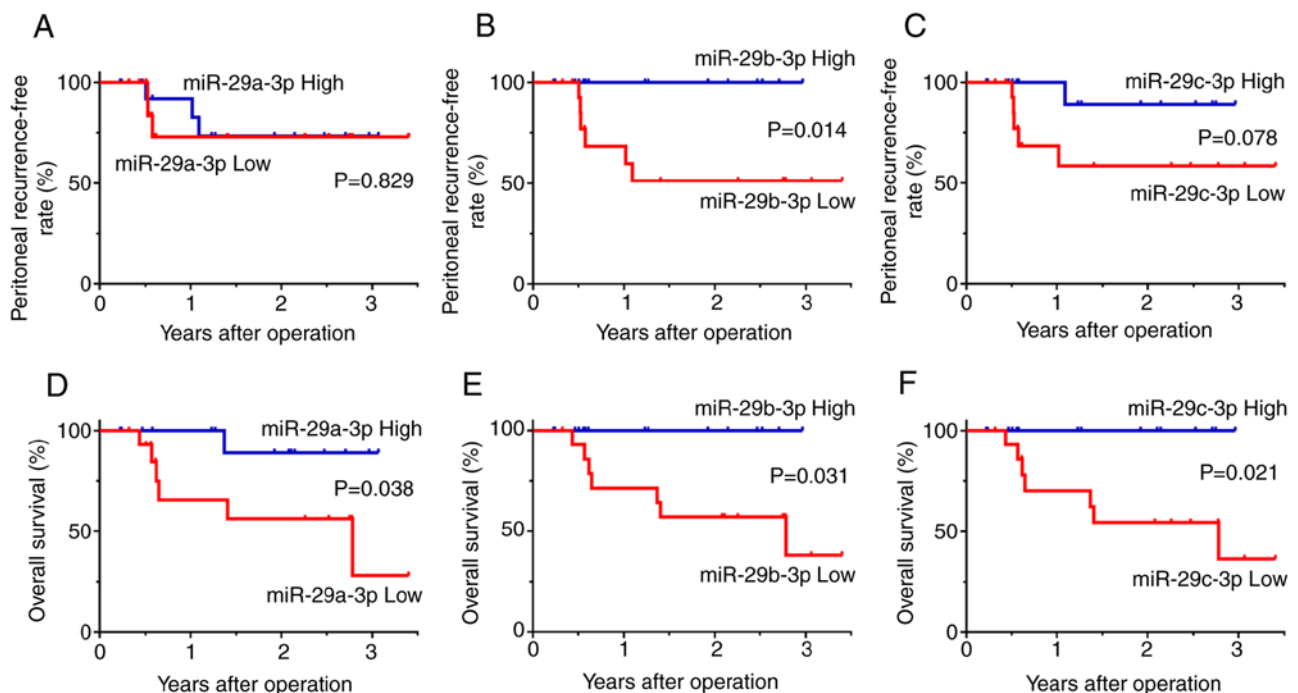

Figure 5. Clinical outcomes of patients with T4 tumors who underwent curative gastrectomy of the T4 tumor. Patients were divided into miR-29s 'high' and 'low' groups based on the median values. Survival rates were calculated using the Kaplan-Meier method and differences were evaluated using the log-rank test. Peritoneal (A-C) recurrence-free survival and (D-F) overall survival in patients with low and high expression of miR-29a-3p, miR-29b-3p, miR-29c-3p respectively.

indicated that miR-29s in peritoneal exosomes have the opposite effect and act as a defender against the progression of peritoneal fibrosis which favors the pro-metastatic niche. Further studies will be undertaken to clarify the mechanism of miR-29s and peritoneal metastasis.

There are some limitations to this study that should be addressed. First, the sample size was relatively small and examined at a single institution. Second, CD9 and CD63, which were generally used as exosome markers, were confirmed with western blotting, but other subpopulation markers for extracellular vesicles, such as TyA and C1a for ectosomes, were not verified in this study. The presence of other larger vesicles could not be excluded.

In summary, it was revealed that the level of miR-29s in peritoneal lavage-derived exosomes was a reliable biomarker to predict peritoneal recurrence in patients who underwent curative surgery for T4 gastric cancer. Reduction of miR-29 levels in peritoneal fluid may be a crucial event for the development of PM. In Asia, postoperative administration of S-1 or capecitabine and oxaliplatin is standard adjuvant treatment for curatively resected stage II/III gastric cancer (45,46). However, these adjuvant therapies do not adequately suppress peritoneal recurrence, especially in patients with T4 tumors, and more intense adjuvant treatment should be considered for selected patients in the high-risk group. Recent advances in nanotechnology suggest that exosomes could be used as an

effective tool for gene delivery and targeted therapy (47,48). Collectively, these data indicated that miR-29s are good therapeutic targets in patients with PM and that replacement of exosomal miR-29b-3p in the peritoneal cavity may be a novel strategy to prevent peritoneal recurrence in patients with low expression.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

HO and JK designed the project. HO, AS, YKu and YKi performed the experiments. HY, YH and NS contributed to the analysis and interpretation of the data. HO drafted the work. AKL and JK wrote the paper and revised it critically. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The study protocol was approved by the Bioethics Committee for Clinical Research A, Jichi Medical University Hospital (Approval #A15-163), and written informed consent was obtained from all participants.

Patient consent for publication

Not applicable.

Competing interests

All authors declare that they have no competing interests.

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