


**ORIGINAL ARTICLE**

# Exosomal microRNA in peritoneal fluid as a biomarker of peritoneal metastases from gastric cancer

Hideyuki Ohzawa<sup>1</sup> | Yuko Kumagai<sup>2</sup> | Hironori Yamaguchi<sup>2,3</sup> | Hideyo Miyato<sup>2</sup> |  
 Yasunaru Sakuma<sup>2</sup> | Hisanaga Horie<sup>2</sup> | Yoshinori Hosoya<sup>2</sup> | Alan Kawarai Lefor<sup>2</sup> |  
 Naohiro Sata<sup>2</sup> | Joji Kitayama<sup>1,2</sup> 

<sup>1</sup>Center for Clinical Research, Jichi Medical University Hospital, Shimotsuke, Japan

<sup>2</sup>Department of Gastrointestinal Surgery, Jichi Medical University, Shimotsuke, Japan

<sup>3</sup>Department of Clinical Oncology, Jichi Medical University, Shimotsuke, Japan

**Correspondence**

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

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**Abstract**

**Aim:** Peritoneal metastases (PM) frequently occur in patients with gastric cancer and result in a poor prognosis. Exosomes play pivotal roles in tumor metastasis through the transfer of microRNAs (miRNAs). We examined the exosomal miRNA profile in peritoneal fluids to identify novel biomarkers to reflect tumor burden in the peritoneum.

**Methods:** Exosomes were isolated from peritoneal fluids of patients of gastric cancer with macroscopic (P1) or microscopic (POCY1) peritoneal metastasis (PM) and comprehensive miRNA expression analysis was carried out. Expressions of candidate miRNAs were then validated in all 58 samples using TaqMan Advanced miRNA Assays.

**Results:** In initial screening, we carried out comprehensive analysis of exosomal miRNA using peritoneal fluids from 11 and 14 patients with or without PM, respectively, and identified 11 dysregulated miRNAs in PM (+) samples. Validation analysis showed that four miRNAs (miR-21-5p, miR-92a-3p, miR-223-3p, and miR-342-3p) were significantly upregulated in 12 PM (+) samples, and their expression levels showed positive correlation with peritoneal cancer index. In contrast, miR-29 family were all downregulated in patients with PM (+) samples. Moreover, in 24 patients with pT4 tumor, miR-29 at gastrectomy tended to be lower in six patients with peritoneal recurrence with significant differences in miR-29b-3p ( $P = .012$ ).

**Conclusion:** Expression pattern of miRNAs in peritoneal exosomes well reflects the tumor burden in the peritoneal cavity and could be a useful biomarker in the treatment of PM.

**KEYWORDS**

biomarker, exosome, intraperitoneal chemotherapy, microRNA, peritoneal metastasis

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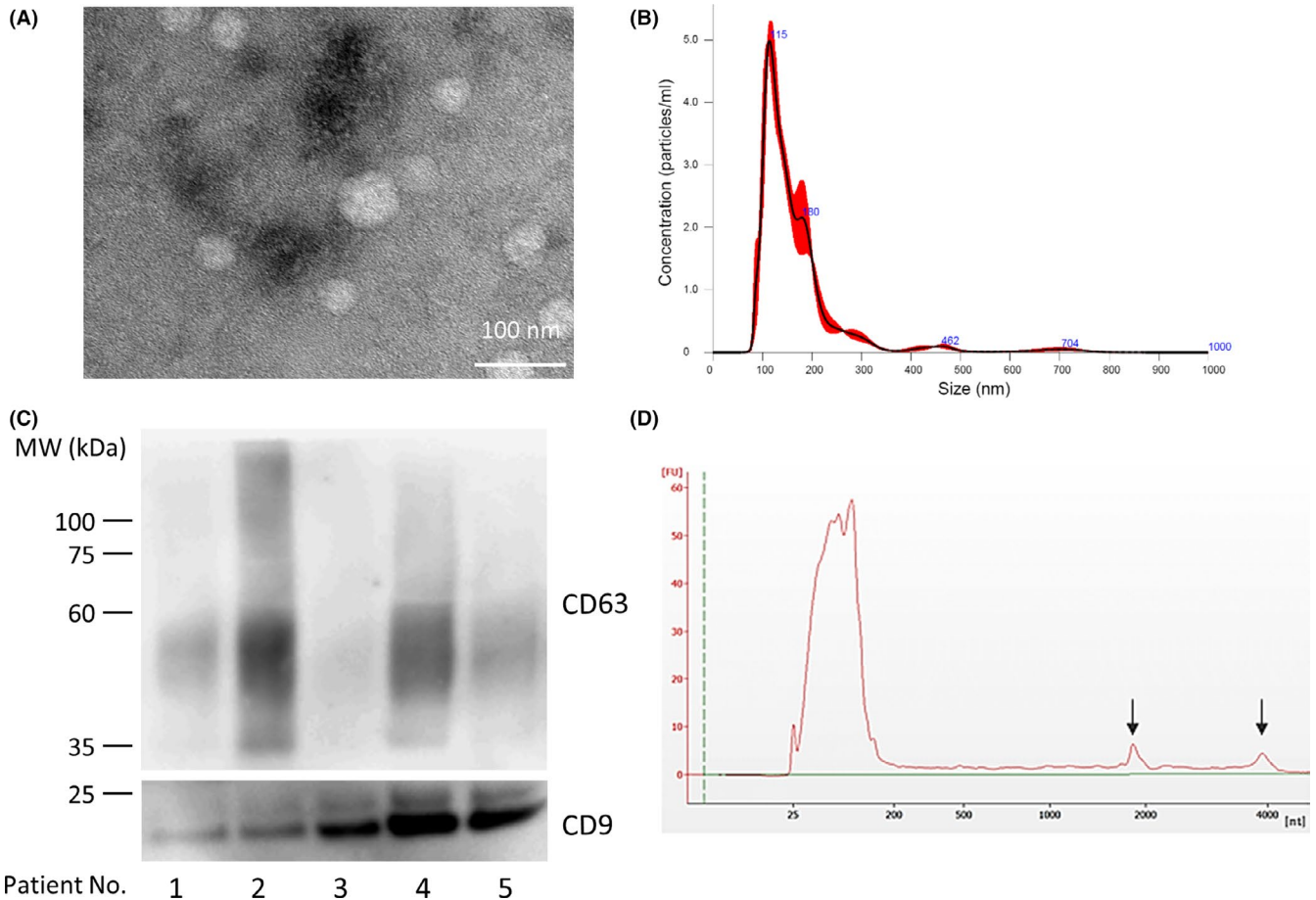
## 1 | INTRODUCTION

The peritoneal cavity is the most frequent site of metastasis in gastric cancer and the outcome of patients with peritoneal metastasis (PM) of gastric cancer is still very poor. Although a combination of platinum agents plus fluoropyrimidine, taxane, or anthracyclines are generally used as first-line therapy for unresectable gastric cancer,<sup>1</sup> the efficacy of these systemic chemotherapy drugs for patients with PM is limited.<sup>2,3</sup> Even after curative surgery, approximately half of patients with gastric cancer with serosal involvement (T4) have been reported to develop peritoneal recurrences.<sup>4</sup> 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.

Intraperitoneal chemotherapy (IPC) using taxane combined with systemic chemotherapy has shown marked clinical efficacy for gastric cancer with PM, and induction IPC followed by gastrectomy is supposed to be a promising strategy for this therapeutically challenging condition.<sup>3</sup> However, there is no reliable biomarker to assess the response of

peritoneal lesions, which makes it difficult to determine the appropriate timing of conversion surgery or transition to second-line treatment. Sensitivity of computed tomography (CT) scan for the detection of peritoneal metastases is limited even with modern techniques, because peritoneal metastases are often too small to be accurately evaluated for a reduction in size,<sup>5</sup> and thus accurate molecular biomarkers are necessary to assess tumor burden in the peritoneal cavity.

Exosomes are small membrane-covered extracellular vesicles (EV) released from many different cell types and are present in various biological fluids. Recent studies have shown that exosomes play an important role in cell-to-cell communication through the transfer of protein, lipids, and nucleic acids, such as DNA, messenger RNA, microRNA, and other non-coding RNAs.<sup>6</sup> Among them, microRNAs are short noncoding RNAs, ranging in length from 20 to 25 nucleotides, which regulate protein synthesis at the post-transcriptional level by binding to the 3' untranslated region of mRNAs. Numerous reports have shown that miRNAs in exosomes circulating in blood or body fluids are resistant to enzymatic degradation by RNase and



**FIGURE 1** Representative features of exosomes purified from peritoneal fluid obtained from patients with peritoneal metastases (PM) of gastric cancer. A, Exosomes were detected using transmission electron microscopy. Representative image shows typical morphology and size. B, Concentration and size distribution of particles in exosome fractions were measured by nanotracking analysis. Histogram represents particle size distribution (exosomes  $1 \times 10^{10}$ /mL and size in nanometers). C, Exosome markers were confirmed by western blot analysis. CD9 and CD63 were detected in exosome fraction isolated from peritoneal fluids. Lane number indicates patient number. D, Quality and quantity of total RNA were evaluated by chip-based capillary electrophoresis. The electropherogram represents the size of distribution in nucleotides (nt) and fluorescence units (FU). RNA extracted from the exosome fraction mainly contains a small RNA fraction (< 200 nt) and a small amount of rRNA. Arrows indicate 18S and 28S rRNA subunits

play pivotal roles in tumor metastasis formation<sup>7</sup> and that dysregulation of miRNAs is related to disease progression of gastric cancer.<sup>8</sup> In fact, exosomal miRNAs in blood can be used as non-invasive biomarkers for early diagnosis and evidence of tumor progression in various malignancies.<sup>9</sup>

In contrast, there is little information regarding miRNAs in peritoneal fluid. However, it is believed that large amounts of exosomes containing functional miRNAs are released into the peritoneal cavity before the formation of PM, and thus analysis of the miRNA profile in peritoneal exosomes should provide important information to develop adequate treatment of PM. In the present study, therefore, we tried to identify novel biomarkers that can reflect the volume of PM using the expression analysis of miRNAs in exosomes derived from peritoneal fluids of patients with gastric cancer.

## 2 | MATERIALS AND METHODS

### 2.1 | Patients and sample collection

The study protocol was approved by the Bioethics Committee for Clinical Research A, Jichi Medical University Hospital (approval no. A15-163), and was carried out in accordance with the precepts established by the Helsinki Declaration. Written informed consent was obtained from all participants. All patients had pathologically diagnosed gastric cancer in biopsy specimens. Peritoneal lavage fluid was obtained at laparotomy between January 2016 and December 2017. Macroscopic and microscopic PM were diagnosed by laparoscopic observation (P1) or positive cytology of peritoneal lavage fluids (CY1) under general anesthesia, respectively. In cases with PM, peritoneal cancer index (PCI) score was determined by optical observation on laparotomy or investigative laparoscopy.

**TABLE 1** Differential expression of microRNAs (miRNAs) in peritoneal fluid from patients with and without peritoneal metastases

Mature miRNA ID	Regulation	P value	Fold change
hsa-miR-150-5p	Up	.04	12.374
hsa-miR-223-3p	Up	.002	5.989
hsa-miR-204-5p	Up	.005	4.635
hsa-miR-720	Up	.001	3.402
hsa-miR-92a-3p	Up	.012	3.291
hsa-miR-21-5p	Up	.003	2.629
hsa-miR-4301	Up	.002	2.421
hsa-miR-342-3p	Up	<.001	2.249
hsa-miR-29c-3p	Down	<.001	0.35
hsa-miR-29a-3p	Down	<.001	0.35
hsa-miR-29b-3p	Down	<.001	0.39

Up- and downregulated miRs in samples with peritoneal metastasis using a custom polymerase chain reaction array. miRs with  $P < .05$  are expressed.  
miR, microRNA.

**TABLE 2** Characteristics of patients with various stages of gastric cancer

Variable	Patients without PM, P0CY0 (46)	Patients with PM, P1 or P0CY1 (12)
Age, y		
Median (range)	70 (35-86)	62 (38-75)
Gender		
Male	26	4
Female	20	8
Location		
U	11	5
M	9	6
L	20	1
Other	6	0
Macroscopic type		
Type 4	3	7
Other	43	5
Histological type		
Differentiated	21	0
Undifferentiated	25	12
Serum CEA (ng/mL)		
Median (range)	2.5 (0.5-223.2)	1.5 (0.7-32.8)
Serum CA19-9 (U/mL)		
Median (range)	13 (2-2529)	32 (4-6093)
Operative procedure		
Total gastrectomy	20	0
Partial gastrectomy	26	0
Staging laparoscopy	0	12
Pathological findings of resected primary tumor		
Depth of invasion		
pT1	8	
pT2	7	
pT3	7	
pT4	24	
Lymph node metastasis		
pN0	13	
pN1	9	
pN2	10	
pN3a	10	
pN3b	4	
Venous invasion		
+	38	
-	8	
Lymphatic invasion		
+	36	
-	10	
Pathological stage		
IA	8	

(Continues)

**TABLE 2** (Continued)

Variable	Patients without PM, POCY0 (46)	Patients with PM, P1 or POCY1 (12)
IB	4	
IIA	2	
IIB	4	
IIIA	6	
IIIB	10	
IIIC	12	

CEA, carcinoembryonic antigen; PM, peritoneal metastasis.

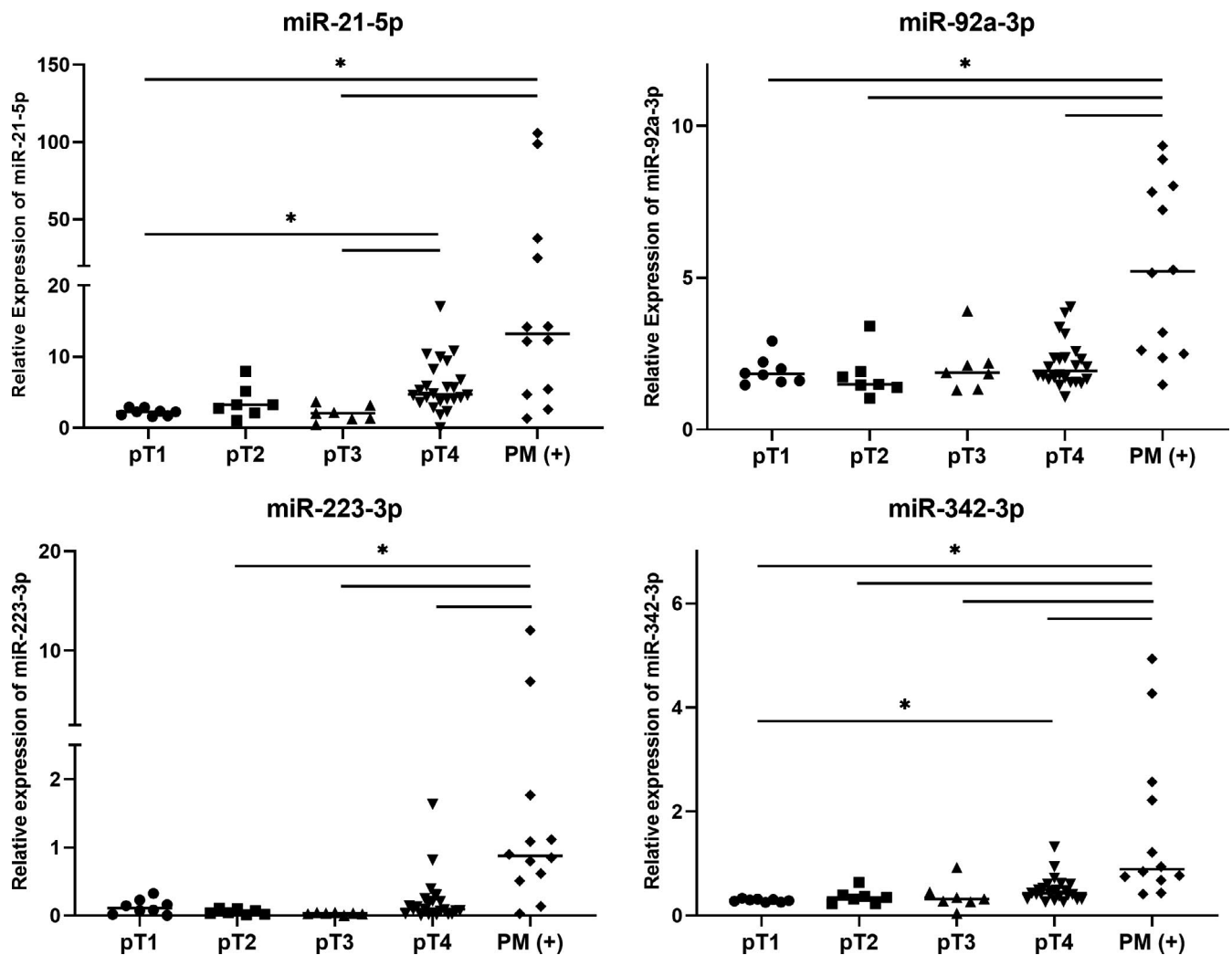
## 2.2 | Isolation of extracellular vesicles and RNA extraction

Peritoneal fluid samples were centrifuged at 2000 g for 10 minutes to remove floating cells. Supernatants were filtered through an 800-nm

filter (Millipore, Burlington, MA, USA) to remove cell debris and ultracentrifuged at 150 000 g for 70 minutes at 4°C. Presence of isolated EV were confirmed using an HT-7700 transmission electron microscope (Hitachi High-Technologies, Tokyo, Japan). Size distribution and number of EV were determined using NanoSight LM10 (Malvern, Amesbury, UK). RNA extraction was carried out using an miRNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Total RNA in the samples were assessed for quantities and quality using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

## 2.3 | Western blot analysis

Protein concentration of the exosome fraction was determined using a Qubit Protein Assay Kit with Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA). Exosome fractions (1 µg protein) were separated using 10% Novex Bis-Tris Protein Gels (Invitrogen),



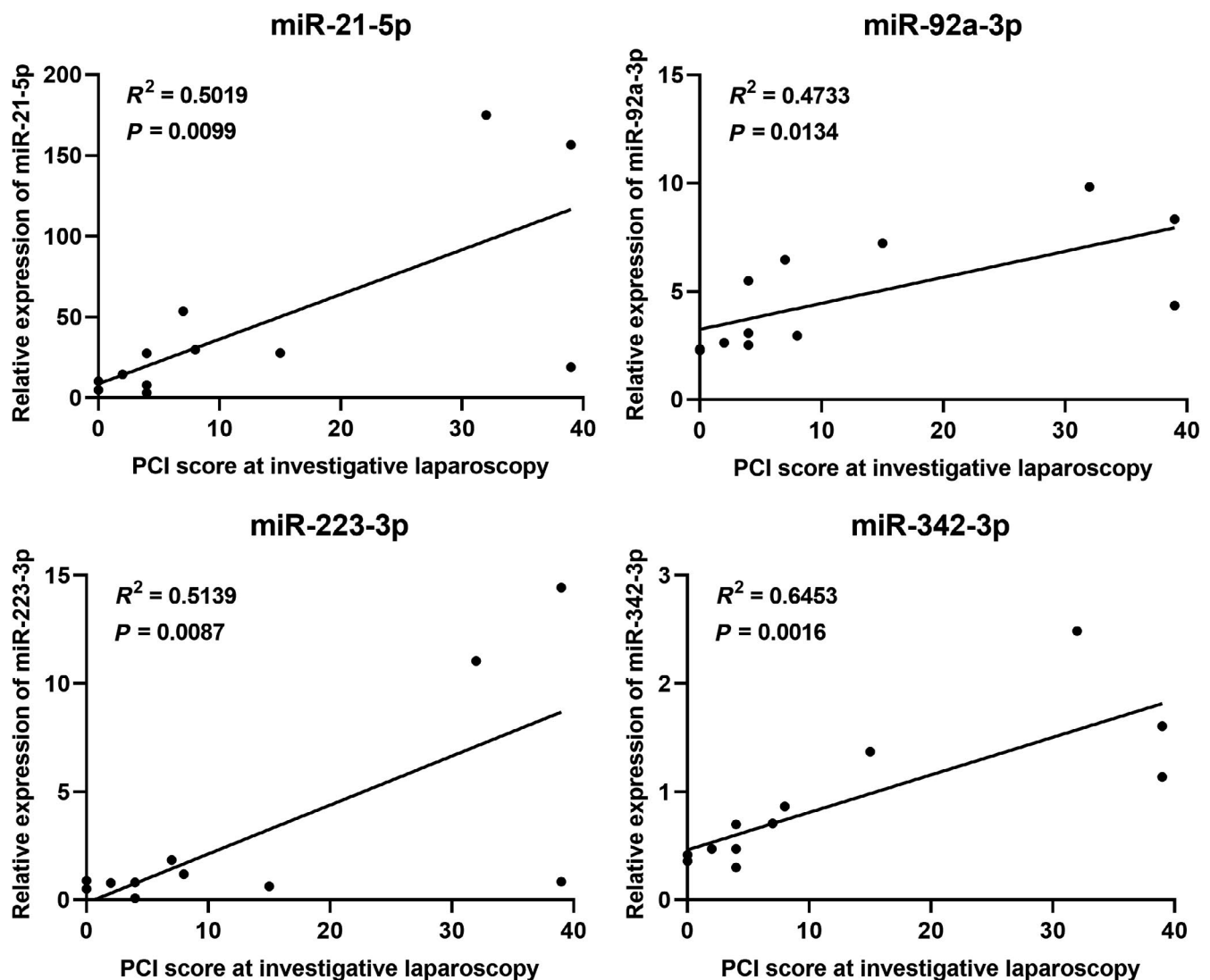
**FIGURE 2** Expression of microRNA (miR)-21-5p, miR-92a-3p, miR-223-3p, and miR-342-3p in exosomes derived from peritoneal fluid of patients with gastric cancer patients with various pathological T (depth of invasion) stages. Peritoneal metastases (PM) means cases with macroscopic (P1) or microscopic peritoneal metastasis (POCY1). Expression of miRNAs was determined with TaqMan Advanced miRNA assays and relative expression levels were calculated against average of miR-10a-5p and miR-30d-5p. \*P < .05

transferred to PVDF membranes (Invitrogen), and immunoblotted with the following primary antibodies: CD9 (1:200; Santa Cruz Biotechnology, Dallas, TX, USA) and CD63 (1:1000; Medical & Biological Laboratories, Nagoya, Japan). Antimouse immunoglobulin (Ig)G, peroxidase-linked antibody (GE Healthcare, Buckinghamshire, UK) was used as secondary antibody. Chemiluminescence was detected using Amersham ECL Prime Western Blotting Detection Reagents (GE Healthcare) and imaged using LAS-3000 mini (Fujifilm Life Science, Tokyo, Japan).

## 2.4 | miRNA expression analysis

To determine miRNAs expressed in peritoneal fluid, comprehensive miRNA expression analysis was carried out using miScript SYBR Green PCR Kit and the Human miRNome miScript miRNA PCR Array (Qiagen). Total RNA samples from 11 patients with PM and from

14 patients without PM were combined as pooled samples. Each pooled sample was reverse transcribed into cDNA using miScript II RT Kit according to the manufacturer's instructions. cDNA was diluted 10-fold with RNase-free water and quantitative PCR was carried out on a ViiA 7 system (Applied Biosystems, Foster City, CA, USA). Experimental data files were exported from the PCR tool, and then miRNA expression data were analyzed using relative quantification software on ThermoFisher Cloud (ThermoFisher Scientific, Waltham, MA, USA). Expressed miRNAs were selected based on Ct value <30 and a single, sharp melt peak. A custom miScript miRNA PCR Array (Qiagen) was constructed based on the results. The custom PCR array contained 56 miRNAs selected as the expressed miRNAs in prior analysis. The miRNA expression profiles in each sample were evaluated using the custom PCR array. Prior to quantitative PCR reaction, 500 pg RNA from each sample was used as a template, converted into cDNA and subsequently amplified using miScript



**FIGURE 3** Correlation between peritoneal cancer index (PCI) score and expression levels of microRNA (miR)-21-5p, miR-92a-3p, miR-223-3p, miR-342-3p in exosomes derived from peritoneal fluid of 12 patients with peritoneal metastases (PM).  $R^2$  and  $P$  values were examined by Spearman's correlation analysis

Single Cell qPCR system (Qiagen) according to the manufacturer's instructions. For data processing, a NormFinder algorithm<sup>10</sup> was used to explore the most stably expressed miRNAs in samples with or without PM. The Ct values were normalized with the miRNAs selected from the NormFinder algorithm and subsequently used for delta-delta-Ct calculation method.

Next, expressions of candidate miRNAs were validated in all 58 samples using TaqMan Advanced miRNA Assays (ThermoFisher Scientific). For the quantitative PCR reaction, 500 pg total RNA was used to prepare cDNA templates from miRNA followed by PCR amplification of the cDNA template using TaqMan Advanced miRNA cDNA Synthesis Kit (ThermoFisher Scientific). Quantitative PCR reactions were carried out, and the relative expression level was calculated using normalizer similar to the previous experiment.

## 2.5 | Statistical analysis

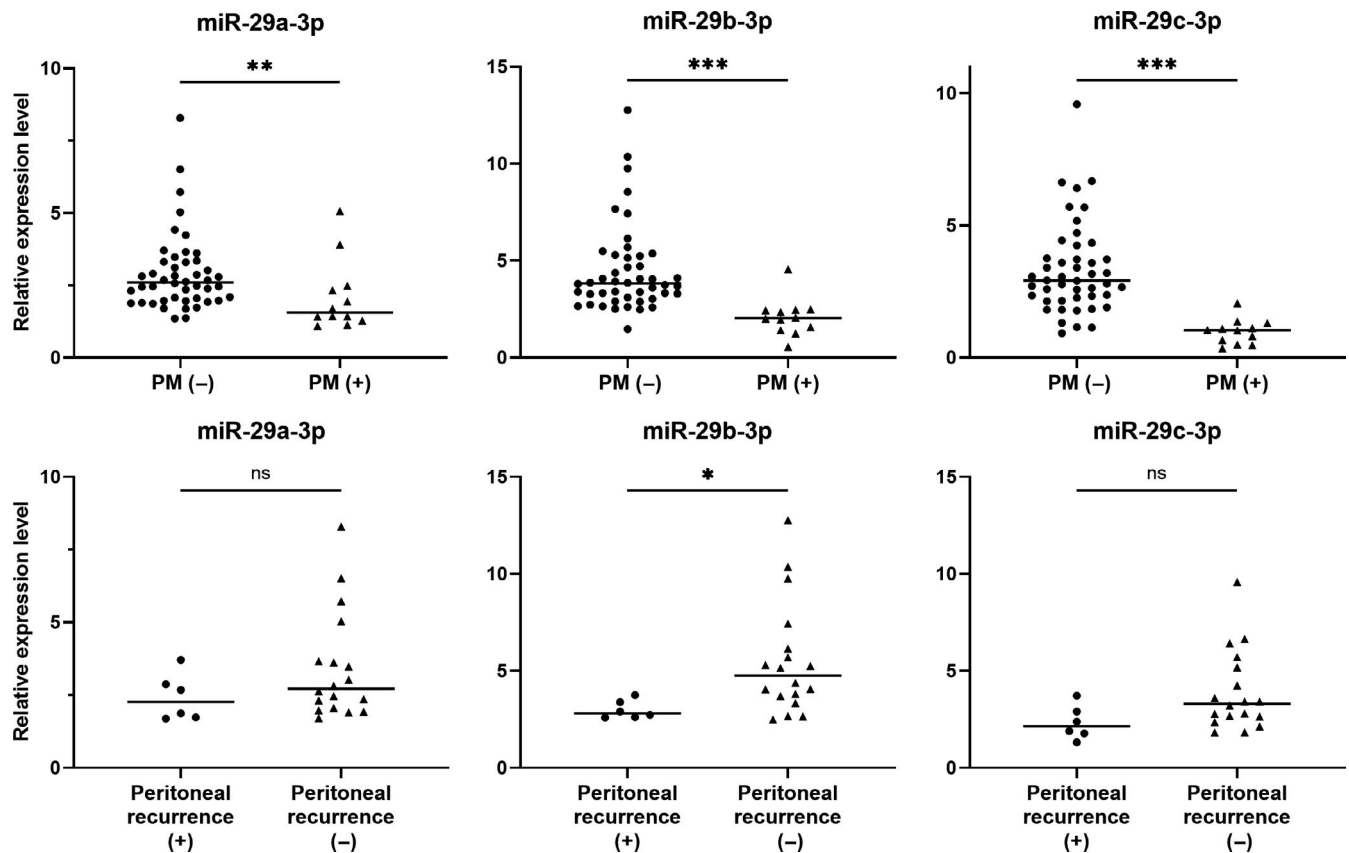
Difference between categorical variables was evaluated using chi-squared and Fisher's exact tests. Mann-Whitney *U* test was used to analyze continuous non-normal variables. Relative expression values obtained from the PCR experiment were compared with one-way analysis of variance. Analysis of correlation was made by Spearman's method. Differences were considered statistically significant at

$P < .05$ . Statistical analysis was conducted using GraphPad Prism 7 (GraphPad Software Inc.).

## 3 | RESULTS

### 3.1 | Isolation of exosome fraction and extraction of RNA

Peritoneal fluid was collected from 11 patients with PM and from 14 patients without PM. EV were isolated by the ultracentrifugation method. Transmission electron microscopy showed that many EV were  $<200$  nm in diameter (Figure 1A). Nanoparticle tracking analysis showed size distribution peaks of between 100 and 200 nm (Figure 1B), suggesting exosome fractions. Although the protein concentration of exosome fractions was significantly higher in samples from malignant ascites, number of particles in the exosome fraction was not different between samples with or without PM. Western blot analysis showed that CD9 and CD63, which were known exosome markers, were present in exosome fractions isolated from peritoneal fluids (Figure 1C). Total RNA extracted from the exosome fraction was evaluated for quality and quantity by chip-cased capillary electrophoresis which showed an abundance of small RNA fractions in both PM (-) and PM (+) samples with median of RNA concentrations (range) of 411.7



**FIGURE 4** Expression levels of microRNA (miR)-29a-3p, miR-29b-3p and miR-29c-3p in peritoneal metastases (PM) (+) and PM (-) patients (Upper) and in 24 patients with T4 tumors who showed peritoneal recurrences after curative gastrectomy ( $n = 6$ ) and in those who did not ( $n = 18$ ) (Lower). *P* values were evaluated with the Wilcoxon test. \* $P < .05$ , \*\* $P < .01$ , \*\*\* $P < .001$ ; ns, not significant

(138.5-1292.0) pg/ $\mu$ L and 687.2 (179.0-3133.1) pg/ $\mu$ L, respectively (Figure 1D).

### 3.2 | Identification of miRNAs expressed in peritoneal fluids

In the initial screening phase, we combined the 11 samples from patients with PM and the 14 samples from patients without PM, explored miRNA profiles using miRNome PCR array, and identified 56 and 54 stably expressed miRNAs, respectively (Data S1). Based on this result, a total of 56 miRNAs were selected as candidate miRNAs, and quantified in each sample using a custom PCR array. Using the NormFinder algorithm, we identified the most stably expressed miRNAs and miR-30d-5p and miR-10a-5p which were selected as internal controls. Then, eight and three miRNAs were significantly up- and downregulated in samples with PM, respectively (Table 1).

### 3.3 | Four miRNAs (miR-21-5p, miR-92a-3p, miR-223-3p, and miR-342-3p) reflect the progression of peritoneal metastasis

As validation analysis, we examined the expressions of these dysregulated miRNAs in a total of 58 patients with various stages of gastric cancer (Table 2). In 12 patients with PM, 10 patients showed macroscopic metastasis (P1) and two patients had microscopic metastasis (POCY1). A type 4 tumor with undifferentiated histology was more frequently included in the PM (+) group. Among the eight upregulated miRNAs, miR-21-5p, miR-92a-3p, miR-223-3p, and miR-342-3p were expressed significantly higher in 12 patients with PM compared to those without PM ( $P < .05$ ) (Figure 2). Among the patients without PM, levels of these miRNAs tended to be higher in cases with pathological serosal exposure (pT4) with significant difference in miR-21-5p ( $P < .05$ ) (Figure 2). Moreover, among the 12 patients with PM, their expression levels showed positive correlation with PCI score which is generally used to assess the extent of peritoneal cancer throughout the peritoneal cavity (Figure 3).

### 3.4 | miR-29 family is downregulated in exosomes in peritoneal fluids with PM and associated with peritoneal recurrence in patients with T4 gastric cancer

Next, we examined the miR-29 family which was downregulated in PM (+) patients in the screening phase. As shown in Figure 4 (upper panel), their expression levels were markedly reduced in 12 samples from patients with PM compared with those from patients without PM and the differences were especially prominent in miR-29b-3p and miR-29c-3p (miR-29a-3p:  $M = 1.57$ , 1.10-5.05,  $P < .01$ ; miR-29b-3p:  $M = 2.03$ , 0.55-4.56,  $P < .001$ ; miR-29c-3p:  $M = 10.3$ , 0.35-2.06,  $P < .001$ ).

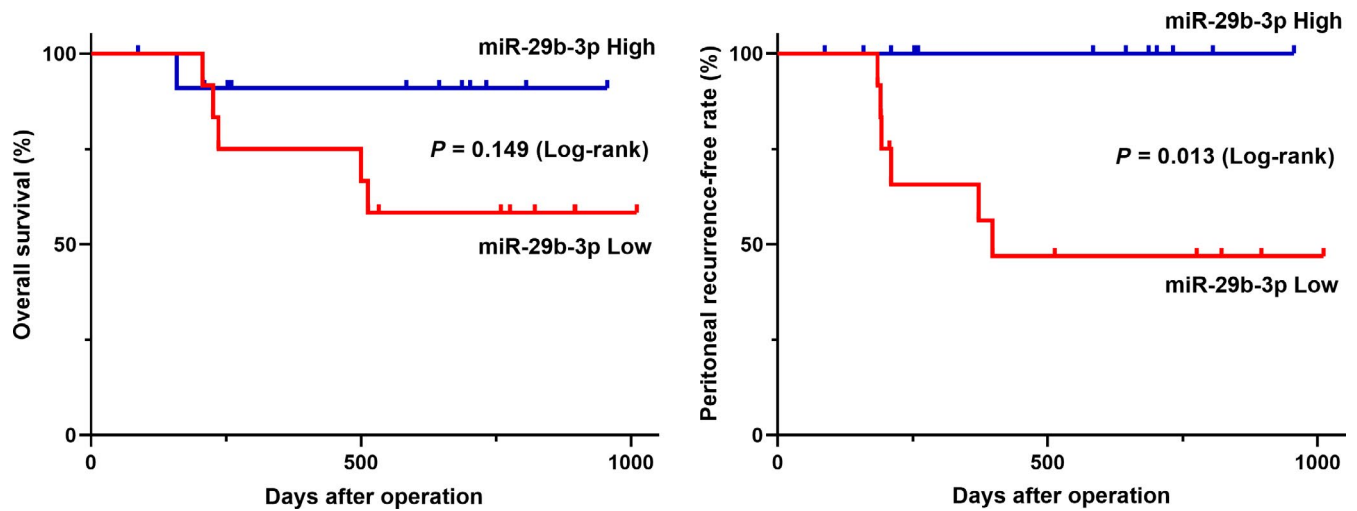
Among the 24 patients with T4 tumors with serosal involvement, six patients had recurrence in the peritoneum with radiological findings within 1 year, whereas peritoneal recurrence was not observed in the other 18 patients with a median follow up of 24 months. Clinical and pathological factors showed no

**TABLE 3** Characteristics of 24 patients with pT4 gastric cancer with or without peritoneal recurrence

Variable	Peritoneal recurrence (+)	Peritoneal recurrence (-)	P value
No. of cases	6	18	
Age, y			
Median (range)	70 (57-82)	69 (35-86)	NS
Gender			
Male	3	9	NS
Female	3	9	
Location			
U	2	6	NS
M	1	5	
L	2	7	
Other	1	0	
Macroscopic type			
Type 4	1	3	NS
Others	5	15	
Histological type			
Differentiated	2	6	NS
Undifferentiated	4	12	
Lymph node metastasis			
pN0	0	1	NS
pN1	0	3	
pN2	3	5	
pN3a	1	7	
pN3b	2	2	
Venous invasion			
+	6	6	NS
-	0	0	
Lymphatic invasion			
+	5	6	NS
-	1	0	
pStage			
IIIA	0	4	NS
IIIB	3	5	
IIIC	3	9	
CEA (ng/mL)			
Median (range)	4.4 (0.5-223.2)	2.6 (1.0-29.5)	NS
CA19-9 (U/mL)			
Median (range)	2 (13-423)	14 (2-155)	NS
Operative procedure			
Total gastrectomy	4	11	NS
Partial gastrectomy	2	7	

CEA, carcinoembryonic antigen; NS, not significant.

significant differences comparing patients with and without recurrence (Table 3). Interestingly, however, the expression levels of miR-29b-3p in peritoneal exosomes obtained at surgery were



**FIGURE 5** Overall survival (left) and peritoneal recurrence-free survival (right) of patients with T4 tumors who underwent curative gastrectomy. Patients were divided into microRNA (miR)-29b-3p high and low groups based on the median values. Survival rates were calculated using the Kaplan-Meier method and differences evaluated using the log-rank test

significantly lower in the six patients with peritoneal recurrence compared with the other 18 patients ( $M = 3.67, 3.31\text{--}4.82$  vs  $M = 5.94, 3.26\text{--}15.32, P < .05$ ) (Figure 4 lower panel). The levels of miR-29a-3p and miR-29c-3p also showed a similar trend although the differences were not statistically significant. When the patients were divided into two groups based on median levels of miR-29b-3p, patients with low miR-29b-3p expression showed significantly worse peritoneal recurrence-free survival ( $P < .05$ ) and worse trend in overall survival (Figure 5).

## 4 | DISCUSSION

Despite recent vigorous studies on exosomes, little information is available regarding the miRNA profile in peritoneal exosomes. In the present study, we tried to isolate exosomes from peritoneal fluid from patients with gastric cancer and found candidate miRNA species related to peritoneal dissemination. Morphological and nanotracking analysis showed that EV obtained by ultracentrifugation consisted mostly of exosomes, which is consistent with a previous study on various body fluids such as breast milk, serum, plasma, and urine.<sup>11</sup>

With comprehensive analysis using PCR arrays, we identified 11 exosomal miRNAs that were dysregulated in samples from patients with PM. As no reference miRNA is valid for every experimental design, it should be assessed in each experiment. In this study, we selected miR-10a-5p and miR-30d-5p as reference miRNAs among several stably expressed miRNAs based on NormFinder analysis in an exploratory data set.<sup>10</sup> Among them, we confirmed that the expression levels of four miRNAs (miR-21-5p, miR-92a-3p, miR-223-3p, and miR-324-3p) evaluated with TaqMan probe methods were significantly elevated in a validation series with 58 patients. Furthermore, in patients with PM, their expression levels

showed positive correlation with PCI score. This strongly suggests that these exosomal miRNAs are preferentially produced from tumor cells and thus reflect the total tumor burden in the peritoneal space.

Among the four miRNAs, miR-21-5p is the most commonly upregulated in various solid cancers and high expression of miR-21-5p was related to worse clinical outcomes.<sup>12,13</sup> In a systematic review, miR-21-5p was the most consistently reported to be upregulated in gastric cancer tissues compared with those in noncancerous gastric tissues.<sup>14</sup> Recently, Tokuhsa et al have used a similar approach and identified five miRNAs upregulated in malignant ascites.<sup>15</sup> In their study, miR-21-5p was significantly elevated in samples from patients with PM, and were significantly higher in patients with T4 tumor than in those with T1 to T3 tumors, which is totally consistent with our results.

Previous studies have shown that miR-223-3p and miR-92a-3p are also upregulated in gastric cancer tissue<sup>14,16</sup> and serum of patients with gastric cancer.<sup>17</sup> miR-342-3p expression has been shown to be upregulated in colon cancer tissue,<sup>18</sup> although no data are available on gastric cancer. More importantly, miR-21-5p and miR-223-3p have been reported to promote invasion of gastric cancer cells.<sup>19,20</sup> These results suggest a possibility that gastric cancer, once it has invaded the serosal surface, releases exosomes including these oncomiR into the peritoneal cavity, which may assist the progression of peritoneal tumor.

In contrast, the miR-29 family are significantly downregulated in peritoneal exosomes derived from patients with PM. Moreover, patients with T4 tumors with serosal involvement who had low levels of miR-29 in peritoneal fluid obtained at gastrectomy showed frequent recurrence in the peritoneal space. Indeed, the levels of miR-29b-3p in the six patients who had recurrence after curative surgery were almost the same as those of the 12 patients with macroscopic PM. Although the number of patients is not very large, this suggests that miR-29 may have a suppressive role for



peritoneal metastasis. Previous studies have provided substantial evidence that miR-29 predominantly function as tumor suppressors.<sup>21</sup> In gastric cancer, miR-29 has been shown to downregulate cyclin D2 (CCND2) and matrix metalloproteinase (MMP)-2<sup>22</sup> or DNA methyl transferase 3A (DNMT3A)<sup>23</sup> which are critically involved in carcinogenesis and tumor progression, and their expression levels have been shown to be reduced in gastric cancer tissues as compared with adjacent nonmalignant tissue.<sup>22,24</sup> Moreover, Zhang et al have shown that miR-29a/c derived from cell-derived microvesicles suppress angiogenesis and inhibit the growth of gastric cancer.<sup>25</sup> Data in the present study are basically consistent with those results, and suggest that miR-29 in peritoneal exosomes have biological relevance for the development of PM.

Recently, Wei et al have also shown that exosomes derived from human malignant ascites convert mesothelial cells to carcinoma-associated fibroblasts and induce peritoneal fibrosis and promote PM.<sup>26</sup> Deng et al have shown that gastric cancer cells produce exosomes which promote the formation of PM by disrupting the mesothelial barrier and inducing fibrosis.<sup>27</sup> These results 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-29 target multiple genes encoding extracellular matrix components such as collagen and fibronectin, and that loss of miR-29 contributes to fibrosis in various organs.<sup>28,29</sup> In fact, in a murine model, Yu et al showed 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-transforming growth factor- $\beta$ /Smad pathway.<sup>30</sup> This suggests that miR-29 in peritoneal exosomes function as defenders against the progression of peritoneal fibrosis, which is the pro-metastatic niche.

In summary, we examined exosomes contained in peritoneal fluids and identified four miRNAs which were upregulated and appeared to reflect tumor burden in the peritoneum. Periodical quantification of these exosomal miRNAs may be useful to determine the response to chemotherapy and select the appropriate treatment for patients with PM of gastric cancer. Reduced expression of miR-29 in peritoneal exosomes is a strong risk factor for developing post-operative peritoneal recurrence in patients who underwent curative surgery for T4 gastric cancer. miR-29b-3p might be a novel target to prevent peritoneal recurrence in these patients. The clinical relevance of these exosomal miRNAs should be validated in larger cohorts.

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## DISCLOSURE

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## ORCID

Joji Kitayama  <https://orcid.org/0000-0002-5708-7130>

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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