Circulating MicroRNA-92b-3p as a Novel Biomarker for Monitoring of Synovial Sarcoma

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The lack of useful biomarkers is a crucial problem for patients with soft tissue sarcomas (STSs). Emerging evidence has suggested that circulating microRNAs (miRNAs) in body fluids have novel impact as biomarkers for patients with malignant diseases, but their significance in synovial sarcoma (SS) patients remains unknown. Initial global miRNA screening using SS patient serum and SS cell culture media identified a signature of four upregulated miRNAs. Among these candidates, miR-92b-3p secretion from SS cells was confirmed, which was embedded within tumour-derived exosomes rather than argonaute-2. Animal experiments revealed a close correlation between serum miR-92b-3p levels and tumour dynamics. Clinical relevance was validated in two independent clinical cohorts, and we subsequently identified that serum miR-92b-3p levels were significantly higher in SS patients in comparison to that in healthy individuals. Moreover, serum miR-92b-3p was robust in discriminating patients with SS from the other STS patients and reflected tumour burden in SS patients. Overall, liquid biopsy using serum miR-92b-3p expression levels may represent a novel approach for monitoring tumour dynamics of SS.

Synovial sarcoma (SS) is a high-grade soft tissue sarcoma (STS) that accounts for 10% to 20% of STSs in adolescents and in the young adult population1,2. The incidence is estimated to be 2 per 100,000 people3. Reported 5-year survival rates for SS range from 36% to 76% and reported 10-year survival rates range from 20% to 63%4,5. The difference in the 5- and 10-year survival rates reflects the relatively high incidence of late metastases. Metastatic lesions develop in about half of cases, most commonly to the lung, followed by the lymph nodes and the bone marrow. With adequate surgical excision or with adjunctive therapy, the recurrence rate has been reported to be less than 40%. In most cases the recurrent growth manifests within the first 2 years after initial therapy1. Since the outcomes are far worse for SS patients who present with local recurrence or metastasis, the early diagnosis of tumour, recurrence, metastasis, and even drug response is crucial for better management. Indeed, this tumour is characterized by the SS18-SSX fusion gene, and the presence of this chromosomal translocation is clinically useful as a diagnostic marker. However, it does not reflect disease progression and is only evaluated using tumour specimens surgically resected4. To date, there are no useful non-invasive biomarkers for tumour monitoring of SS.

microRNAs (miRNAs) are small non-coding RNAs involved in post-transcriptional regulation of gene expression in the cytoplasm, and they can influence a variety of biological processes, including development, proliferation and differentiation7. Accumulating evidence indicates that miRNAs may function as either tumour suppressors or oncogenes that regulates growth and apoptosis7. Recent reports have demonstrated that they exist...
with remarkable stability in body fluids as cell-free miRNA that originates from primary tumour cells embedded within tumour-derived exosomes or argonoutate-2 (Ago2). Circulating cell-free miRNA is attracting attention as a target of liquid biopsy, including as circulating cell-free DNA or in circulating tumour cells\(^4\)–\(^12\). While evidence on circulating miRNAs has been accumulated in various cancers, there has been little involving the soft tissue sarcomas, which to date lack useful circulating biomarkers.

We investigated the expression profiles of serum cell-free miRNAs using blood samples from SS patients as well as in other STS patients, compared to controls, followed by the evaluation of biological and clinical significance using in vitro and in vivo experimental procedures and involving independent patient cohorts.

**Results**

Global miRNA microarray profiling of SS patient serum and SS cell culture media. Microarray profiling analysis was performed on nine pairs of serum samples obtained from SS-patients, age-matched benign tumour patients, and from healthy individuals, as well as culture media of SYO-1 and HS-SY-II SS cell lines. Characteristics of this cohort are shown in Supplementary Tables S1 and S2. Following hierarchical clustering, candidates were narrowed down to the upregulated miRNAs (fold change >1.5) with statistical significance \((p < 0.05)\). Forty-nine serum miRNAs were significantly upregulated in SS patients compared with controls, and eight among these 49 miRNAs were markedly reduced at post-operative status compared to pre-operative status. Of the eight miRNAs, 5 were also highly expressed in culture media of SS cell lines (Fig. 1A,B). Then, miR-92b-3p, miR-150-3p, miR-147-1p, miR-4701-5p and miR-4728-3p, for which qPCR reporter probes were available, were selected for the further detailed analysis. Each of these candidates was highly expressed in SS patients compared to controls (Fig. 1C).

Identification of miRNAs secreted from SS cells. The miRNA candidates were analyzed for their expression levels in the tumour cells and in the culture media of SYO-1, HS-SY-II, and YaFuSS cell lines. RT-qPCR revealed that these candidates were evidently expressed in all SS cell lines, and miR-4701-5p expression was significantly upregulated in all SS cell lines compared with that observed in control hMSCs \((p < 0.05)\), Fig. 1D). On the other hand, miR-92b-3p expression in the culture media of all SS cell lines examined and miR-150-3p expression in the culture medium of YaFuSS was significantly higher than that of hMSCs \((p < 0.05)\), Fig. 1E), suggesting that miR-92b-3p and miR-150-3p are abundantly secreted from SS-cells.

Next, the secretion of these candidate miRNAs from SS cells was evaluated using established SS cell lines. Expression levels of miR-92b-3p in culture media of each SS cell line increased with the number of tumour cells and duration of the incubation, whereas expression levels in culture media did not correlate with that observed in control hMSCs

Serial monitoring of miRNAs in SS tumour-bearing mice. To evaluate whether serum miR-92b-3p levels could be used to monitor tumour dynamics in vivo, we evaluated possible correlations between tumour growth and serum miR-92b-3p expression levels using SYO-1-bearing mice (Fig. 2A). After subcutaneous transplantation of SYO-1 cells into mouse hind quarters, serum miR-92b-3p and miR-150-3p levels were investigated and their identified elevation in association with the growing tumour volume was established (Fig. 2B,C). We observed statistical significance between tumour size and serum miR-92b-3p levels \((R = 0.776, p < 0.05)\), while serum miR-150-3p levels were partially correlated with tumour growth \((R = 0.486, p < 0.05)\), Fig. 2D). Furthermore, serum miR-92b-3p levels significantly decreased after tumour resection (Fig. 2E). These results suggested that serum levels of miR-92b-3p, rather than of miR-150-3p, reflect tumour burden in SYO-1-bearing mice.

Correlation of serum miR-92b-3p with tumour burden in SS patients. Next, we analyzed the serum miR-92b-3p and miR-150-3p levels in a validation cohort of SS patients, age-matched benign tumour patients, and healthy individuals \((n = 12, each)\). The demographics and clinical characteristics of patients and healthy individuals of the validation cohort are described in Supplementary Table S1 and S2. There were no significant differences in age or gender between groups. The expression levels of serum miR-92b-3p were significantly higher in SS patients than in age-matched benign tumour patients and healthy individuals \((p < 0.05)\), Fig. 3A). Receiver operation characteristic (ROC) analysis revealed that serum miR-92b-3p levels contributed to the capacity to distinguish patients with SS from age-matched controls and healthy individuals, with area under the curve (AUC) value of 0.77 (95% confidence interval \((CI) = 0.61–0.94)\) (Fig. 3B). The sensitivity and specificity of serum miR-92b-3p levels were 81.8% and 63.6%, respectively. On the other hand, the AUC value of ROC analysis based on serum miR-150-3p levels was 0.94 (95% CI = 0.86–1.0) for control individuals (Supplementary Figure 1A and B). Although there was no correlation with the expression levels of both miR-92b-3p and miR-150-3p or age, gender, tumour location, and presence of lung metastasis at diagnosis, univariate analysis demonstrated that serum miR-92b-3p tended to correlate with tumour size (Supplementary Table S3).

Serum miR-92b-3p expression levels for clinical tumour monitoring. To further investigate the clinical utility of serum miR-92b-3p for tumour monitoring, we evaluated serum miR-92b-3p and miR-150-3p levels, as well as white blood cell (WBC) counts and hemoglobin (Hb) levels in SS patients from whom we could obtain a series of serum samples during multimodal treatment. Relative expression levels of miRNAs were evaluated by standardization to the initial expression levels of each subject. Case 1 was an 11 year-old male with SS arising in his lower back (Fig. 3C). Serum miR-92b-3p levels decreased after tumour resection and adjuvant chemotherapy, whereas serum miR-150-3p levels did not. Case 2 was a 39 year-old female with SS arising in the groin region (Fig. 3D). Serum miR-92b-3p levels decreased after tumour resection and adjuvant chemotherapy, but gradually increased after local recurrence, whereas serum miR-150-3p levels did not reflect tumour dynamics. Case 3 was a 61 year-old female with SS in her proximal thigh, with lung metastasis at diagnosis (Fig. 3E). Serum miR-92b-3p levels decreased after tumour resection, and slightly increased with the growth of the lung metastasis, and finally
Figure 1. Identification and experimental validation of circulating/secretory miRNAs in SS-patient serum. (A) Schematic representation of the approach used for the detection of circulating/secretory miRNAs in SS-patients. (B) Heatmap and the hierarchical clustering of miRNA microarray analysis using patient serum in preoperative state as well as in cell culture media of SS cells. (C) Serum levels of the candidate markers in SS patients, age-matched benign tumour patients, and healthy individuals. *p < 0.05; Student’s t test. (D) Expression levels of candidate miRNAs in SS cells and human mesenchymal stem cells (hMSCs). Data are presented as mean ± S.D. (n = 3 in each group) *p < 0.05; Student’s t test. (E) Expression levels of candidate miRNA biomarkers in culture media of SS cells (SYO-1, YaFuSS, and HS-SY-II) and hMSCs. Data are presented as mean ± S.D. (n = 3 in each group) *p < 0.05; Student’s t test. (F) The expression dynamics of miR-92b-3p and miR-150-3p in the culture media of SS cell lines according to cell number and the culture duration. Data are presented as mean ± S.D. (n = 3 in each group) *p < 0.05; Student’s t test.
exhibited further increase with progressive disease. In contrast, serum miR-150-3p levels did not correlate with disease progression. Case 4 involved a 21 year-old male with SS in his knee joint (Fig. 3F). In this instance, the response to neo-adjuvant chemotherapy based on adriamycin and ifosfamide was poor. Serum levels of miR-92b-3p and miR-150-3p increased after neo-adjuvant chemotherapy, but these parameters decreased gradually after tumour resection and adjuvant chemotherapy by gemcitabine and docetaxel. In all cases, measures of WBC and Hb did not correlate with miR-92b-3p levels, suggesting that this miRNA was not secreted from hematocytes. Overall, serum miR-92b-3p levels could be useful for tumour monitoring in SS patients.

Secreted miR-92b-3p expression levels from SS and other soft tissue sarcoma cells. To investigate whether cell-free miR-92b-3p was specifically secreted from SS cells compared to other STS cell types, we evaluated expression levels of miR-92b-3p in culture media of SS and other STS cell lines described. We identified that miR-92b-3p expression levels in cells and culture media were significantly higher in SS cells than in other STS cells (p < 0.001, Fig. 4A,B). On the other hand, miR-150-3p expression levels were also significantly higher in SS cells but did not show statistically significant difference in culture media of SS and other STS cell lines described (Fig. 4A,B).

Differential diagnosis of SS and other STS patients by serum miR-92b-3p quantification. To assess whether quantification of serum miR-92b-3p levels could be used for differential diagnosis, we examined levels in patients with SS (n = 12) and other STS patients (n = 24). Demographics and histological classification of the other STS patients are described in Supplementary Table S4. Serum miR-92b-3p levels demonstrated a significant upregulation in SS patients compared with the other STS patients at time of diagnosis (p < 0.0001, Fig. 4C). ROC curve depiction reveals the diagnostic significance of serum miR-92b-3p quantification differentiating SS from other STS with AUC value of 0.87 (p < 0.0001, 95% CI = 0.72–1.0, Fig. 4D). ROC curve analysis revealed sensitivity and specificity of miR-92b-3p determination as 84.6% and 80.0%, respectively. In contrast,
Figure 3. Serum miR-92b-3p expression levels and dynamics in synovial sarcoma patients in the validation cohort. (A) Serum miR-92b-3p expression levels in SS patients and control individuals in the validation cohort ($p < 0.05$) with one-way ANOVA with Holm-Sidak's multiple comparison test. (B) Receiver operating characteristic (ROC) curve analysis. ROC curve analysis indicated the AUC of 0.77 (95% confidence interval = 0.61–0.94) discriminating SS from age-matched benign tumour patients and healthy individuals. (C–F) Tumour monitoring of serum miR-92b-3p levels during multimodal therapies. Four SS patients including an 11 year old male with lower back involvement (C), 39 year old female (groin region) (D), 61 year old female (proximal thigh) (E), and 21 year old male (knee joint) (F) could be evaluated during the treatment. Abbreviations: WBC = white blood cell; Hb = hemoglobin; OP = operative surgery; Cx = chemotherapy; Rec = recurrence; Mets = metastasis; SD = stable disease; PD = progressive disease.
Figure 4. Differential diagnosis of synovial sarcoma from other soft tissue sarcomas by circulating/secreted miR-92b-3p. (A, B) The expression of miR-92b-3p and miR-150-3p in cells (A) and cell culture media (B) of SS (SYO-1, YaFuSS, HS-SY-II, and Yamato-SS) and the other STS cell lines (HT1080, NMS2, and UPS2023). The Mann-Whitney U test was used for comparison between groups. (C) Serum miR-92b-3p levels in SS patients and other STS patients (p < 0.001). The Mann-Whitney U test was used for comparison between groups. (D) ROC curve analysis of miR-92b-3p discriminating SS patients from other STS patients. AUC was 0.88 (95% confidence interval = 0.72–1.0). (E) Serum miR-150-3p expression levels in SS patients and other STS patients. The Mann-Whitney U test was used for comparison between groups. (F) ROC curve analysis of miR-150-3p discriminating SS patients from other STS patients. AUC was 0.54 (95% confidence interval = 0.33–0.75).
the expression levels of serum miR-150-3p did not reveal any significant difference between SS and the other STS patients, and AUC numerics were poor (Fig. 4E,F).

**Secrete miR-92b-3p in tumour-derived exosomes.** To investigate the basis of miR-92b-3p stability in the extracellular environment, we evaluated levels of this miRNA in exosomes derived from SS cell lines. The collected exosomes were identified using SEM as essentially homogeneous vesicles of 40–200 nm in diameter (Fig. 5A), which was confirmed by employing the NS300 NanoSight® (Fig. 5B). Western blotting revealed that the isolated exosomes were positive for CD9 and negative for cytochrome-c (Fig. 5C) and the SS18-SSX fusion gene was separately detected in the tumour-derived exosomes (Fig. 5D). Moreover, the expression levels of miR-92b-3p were higher in exosomes derived from SS cells than in hMSCs (Fig. 5E). These results suggested that miR-92b-3p is loaded in exosomes derived from SS cells.

**Differential expression of miR-92b-3p in exosomes and Ago2 derived from SS-patient serum.** To further identify how miR-92b-3p is stable during circulation in SS patients, we evaluated miR-92b-3p levels in both exosomes and Ago2 from sera of SS patients. The serum samples were fractionated by using EV-Second®, followed by IP with anti-Ago2 antibody. CD9 expression differentially the exosomes from Ago2-positive fractions, which was confirmed by western blot analysis (Fig. 5F,G). Our investigations identified that miR-92b-3p levels were significantly elevated in exosomes than in Ago2-positive fractions of SS-patient serum (Fig. 5H), indicating that this miRNA circulates in SS patients and is loaded on tumour-derived exosomes. Although we additionally evaluated serum SS18-SSX fusion gene transcript, it was not detected in SS-patient serum or exosome fractions within the serum (Supplementary Figure S6 and Supplementary Tables SS, 6). These observations offer immediate tumour surveillance and future potential therapeutic avenues.

**Discussion**

To date, miRNA dysregulation in SS cells or tissue specimens has been reported by several groups (Supplementary Table S7)3–18. The representative functional miRNAs in SS cells includes the upregulated miR-17-5p, miR-18a, miR-125a31, miR-18331, and the downregulated miR-143/145. Our global miRNA profiling analysis using SS patient serum and SS cell culture media demonstrated dissimilar patterns, compared to the reported miRNA dysregulation in SS cells, and miR-92b-3p has never been identified. Similarly, several investigators have also demonstrated the dissimilarity between cellular miRNAs and secreted miRNAs in various cancers, such as breast cancer. These dissimilarities in the miRNA expression pattern may indicate the existence of molecular mechanisms regulating secretion of miRNAs, which has been suggested by several researchers. Therefore, our approach of global miRNA analysis based on patient blood samples, rather than focusing on dysregulated miRNA within tumour cells or tissue specimens, could be a suitable method for investigation of clinically important circulating miRNA. Importantly, researchers have to pay attention to the evidence that a variety of circulating miRNAs, reported as circulating cancer biomarkers, reflect a secondary effect on patient blood cells rather than a tumour-specific origin. miR-92b-3p has not been reported as a hematocyte-derived miRNA and we confirmed here that hematocytes were not associated with serum miR-92b-3p levels.

Recent investigations have demonstrated that exosomes are enriched in bioactive molecules, contain nucleic acid and protein, and are secreted into the extracellular environment. Furthermore, some reports indicate cell-free miRNAs are stable not only within exosomes, but also in a complex with RNA-binding proteins which include Ago. One recent study has reported that endogenous miR-92b-3p is associated with the RNA-induced silencing complex including Ago protein, although this study did not evaluate miRNA within exosomal fractions. In the present study, we demonstrated that cell-free miR-92b-3p is stable and contained within exosome fractions, rather than bound to Ago2. These results were supported by the presence of SS18-SSX within exosomes, confirming a recent report showing that exosomes derived from SS harbor the SS18-SSX fusion gene. Importantly, exosomal miRNAs have been suggested to play important roles in intercellular communication. We hypothesize that cell-free miR-92b-3p contributes to cell-cell communication, resulting in SS progression, and which we will next investigate.

The origin of SS is not clear, despite the name of this tumour. Previous reports have suggested the possibility of a neuroectodermal origin, by use of genome-wide analysis of gene expression in SS tissues using a cDNA microarray. Further, the miR-92b-3p inhibitor has been shown to promote glioma cell apoptosis, by targeting Dkk3 and blocking the Wnt/beta-catenin signaling pathway. miR-92b-3p has also been reported to specifically overexpressed in primary brain tumours and to regulate the development of intermediate cortical progenitors in embryonic mouse brain. Therefore, our results demonstrating that miR-92b-3p is abundantly secreted from SS cells also support the possibility that this tumour is of neuroectodermal origin.

In conclusion, the potential clinical significance of serum miR-92b-3p for tumour monitoring of SS was demonstrated through use of experimental procedures and a validation study based on independent patient cohorts. Although further studies in large patient cohorts would determine the significance of serum miR-92b-3p as a non-invasive biomarker of SS, this methodology could also be a novel approach to detect other soft tissue sarcomas that lack useful circulating biomarkers, and help clinicians to determine treatment strategies.

**Materials and Methods**

**Serum Collection.** The Institutional Review Board of Okayama University Hospital, National Cancer Center Hospital, Kochi Health Sciences Center and Chiba Cancer Center Hospital approved this study protocol. Written informed consent was obtained from all patients after study approval. All experimental methods were carried out in accordance with relevant guidelines and regulations. Whole blood samples were obtained from patients with SS, alveolar soft part sarcoma, clear cell sarcoma, dedifferentiated liposarcoma, leiomyosarcoma, malignant peripheral nerve sheath tumour, myxofibrosarcoma, solitary fibrous tumour, undifferentiated pleomorphic

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**Note:** The text continues with further detailed content, which is not shown here due to the constraints of the example. This excerpt highlights the importance of miRNA dysregulation in SS, the role of exosomes in tumor surveillance, and the significance of circulating miRNAs as potential biomarkers.
sarcoma, age-matched benign soft tissue tumour, and healthy individuals at the three major sarcoma institutes in Japan; Okayama University hospital, National Cancer Center Hospital, and Chiba Cancer Center Hospital. These blood samples were obtained at the time of diagnosis, post-operative, post-chemotherapeutic status, or disease progression stage. Murine blood was obtained by cardiac puncture at the indicated time points. Sera were fractionated from whole blood samples by centrifugation at 3,500 rpm for 15 min at 4 °C. The collected serum was
centrifuged at 20,000 × g for 15 min at 4 °C, and supernatants were collected and passed through a 0.22-µm-pore filter (Merck Millipore, Billerica, MA, USA), then stored at −80 °C.

**Cell lines and cell culture.** Four human SS cell lines, SYO-1, HS-SY-II, YaFuSS, and Yamato-SS were used in this study. SYO-1 was previously established in our laboratory38, YaFuSS, HS-SY-II, and Yamato-SS were kindly provided by J. Toguchida, H. Sonobe, and N. Naka39,40. The human myxofibrosarcoma cell line NMFH-1 was generously provided by A. Ogose41. The human undifferentiated pleomorphic sarcoma cell line UPS2023 was established in our laboratory. Human mesenchymal stem cells (hMSC) were purchased from Lonza (Walkersville, USA). The human human fibrosarcoma cell line HT1080 was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). A human malignant peripheral nerve sheath tumour cell line NMS2 was available from the cell bank of RIKEN BioResource Center (Ibaraki, Japan). Cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Gibco Laboratories, Grand Island, NY) or Roswell Park Memorial Institute media (RPMI, Gibco)-1640 or MSC-BM (Invitrogen, Carlsbad, CA) supplemented with 10 or 15% fetal bovine serum (FBS, Hyclone), 100 units/ml of penicillin G and 100 µg/ml of streptomycin (NACALAI TESQUE, Inc., Kyoto, Japan). Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO₂.

**Preparation of conditioned medium.** The conditioned medium (CM) was changed to FBS-free CM at 24 hours after seeding of cells, and then collected at 24 hours after CM exchange. Collected culture medium was centrifuged at 3,500 rpm for 15 min at 4 °C. The CM supernatant was collected and centrifuged at 20,000 × g for 15 min at 4 °C, and supernatants were collected and passed through a 0.22-µm-pore filter (Merck Millipore), then stored at −80 °C.

**Animal experiments.** Animal experiments were performed in accordance with the Animal Care and Use Committee, Okayama University. All animal studies were approved by this committee. BALB/c nu/nu female mice were purchased from CLEA Japan Inc. (Tokyo, Japan) at 4 weeks of age, and given at least 1 week to adapt to their new environment prior to tumour transplantation in a specific-pathogen-free environment. On day 0, the mice were anesthetized with 2% isoflurane, and transplanted in their right hind-quarters with SYO-1 cells (5 × 10⁶ cells/mouse in 100 µL total volume with DMEM suspension). Tumour growth was monitored once each week. Tumour resection was performed 3 weeks after transplantation. Blood samples were taken by cardiac puncture and collected into CAPIJECT® micro collection tubes (TERUMO, Tokyo, Japan) under anesthesia with isoflurane.

**miRNA array.** Whole circulating miRNA profiling was performed using a miRNA microarray manufactured by Agilent Technologies (Tokyo, Japan). Two nanograms of extracted RNA were used for each microarray experiment. The results of miRNA microarray analysis were processed using Agilent Feature Extraction software (v10.7.3.1) and analyzed using GeneSpring 12.6.1 software (Agilent Technologies, Tokyo, Japan).

**RNA extraction and RT-qPCR analysis.** Total RNA was isolated from cells collected after 24-hrs cell culture using miRNAeasy mini Kits (Qiagen, Valencia, CA, USA) according to manufacturer’s instructions. For serum samples and culture media, total RNA was extracted from 200 µL of serum supernatant or concentrated medium using the same extraction kits. RNA samples were reverse transcribed using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). The products were mixed with 5.0 µL of TaqMan 2 × Universal PCR Master mix and 0.50 µL of each primer for qPCR using Agilent Mx3000 P (Agilent Technologies, Santa Clara, CA, USA) instrumentation. Data obtained from RT-qPCR were analyzed using the 2−ΔΔCt method42. The miRNA expression levels were normalized using cel-miR-39 for serum and culture media, and RNU6B for tumour cells. Endogenous mir-16 was used as the normalizer for circulating miRNA quantification. Differences between the groups are presented as ∆Ct, indicating differences between Ct values of miRNAs of interest and Ct values of normalizer miRNAs.

**Exosomes isolation from cell culture medium.** Exosomes were purified from the culture medium supernatant as previously reported43 with partial modification. Each cell line was grown to 60–70% confluence, and then CM was exchanged to FBS-free. The CM samples were collected 24 hrs after medium exchange. The CM was centrifuged at 3,500 rpm for 15 min at 4 °C, followed by further centrifugation at 9,000 × g for 30 min at 4 °C and supernatant was passed through a 0.22-µm-pore filter (Merck Millipore) to remove apoptic bodies, micovesicles, and cell debris. The collected CM supernatant was concentrated to approximately 1 ml using 100 kDa MWCO ultrafiltration membranes (Fisher Scientific, Loughborough, UK) at 4 °C. The sample was then ultracentrifuged (Optima TL-100 (Beckman Coulter, Fullerton, CA, USA) at 100,000 g for 70 min at 4 °C). The resulting pellet was rinsed with PBS, followed by further ultracentrifugation at 100,000 × g for 70 min at 4 °C. Finally, the supernatant was discarded, with exosomes concentrated in the pellet. The obtained exosomes were authenticated by scanning electron microscopy (SEM) and by NS300 Nanosight® nanoparticle analyzer (Malvern Instruments Ltd. Worcestershire, UK).

**Exosomes isolation from human serum.** Exosomes were purified from human serum samples by size exclusion chromatography on drip using EV-second® (GL sciences, Tokyo, Japan) in a low-temperature environment. The column was initially equilibrated with 700 µL of PBS twice, followed by a blocking step using 700 µL of PBS. After repeating the wash steps six times with 700 µL of PBS, 200 µL of the collected human serum sample was loaded onto this column followed by collection of 12 consecutive fractions in 100 µL of PBS. CD9 expression in these fractions was analyzed by western blotting and CD9-positive fractions were recognized as the exosome-rich portion44.
Immunoprecipitation and immuno-blot analysis. Immunoprecipitation (IP) for analytical separation of Ago2 from patient serum samples was performed using Protein G Sepharose 4 Fast Flow® (GE Healthcare, Amersham, UK) with anti-Ago2 monoclonal IgG antibody (Wako, Osaka, Japan) according to the product manual. Total protein from cells (10 μg) and exosomes (1 μg) was fractionated using an electrophoretic gradient across Mini-PROTEAN® tris-glycine extended gels (BIO-RAD, Richmond, CA, USA). Loading samples were normalized according to protein concentrations quantified using the Bradford assay. The gels were then transferred onto the Immun-Blot® PVDF membrane (BIO-RAD) under wet electrophoretic conditions. The blotted protein was blocked for 1 hr at room temperature with Odyssey® blocking buffer in PBS (LI-COR, Lincoln, NE, USA) and was followed by incubation overnight at 4°C with the following primary antibodies: 1:1000 anti-CD9 mouse monoclonal antibody (Abcam, Cambridge, MA, USA); 1:1000 anti-cytokriochrome-c mouse monoclonal antibody (Abcam); and 1:10000 anti-β-actin mouse monoclonal antibody (Sigma-Aldrich, St. Louis, MO, USA). Thereafter, IRDye® 800CW anti-rabbit IgG and IRDye® 680RD anti-mouse IgG secondary antibodies (LI-COR) were incubated with the protein-blotted membrane for 1 hr at room temperature. Fluorescence was then detected on the Odyssey® imaging system (LI-COR).

Statistical Analysis. Results were depicted as the mean ± standard deviation or the median with a 25–75% range. Differences in patient demographics and clinical characteristics were measured by the unpaired t-test. Statistical differences in quantified miRNA levels were determined by unpaired t-test or Analysis of Variance (ANOVA) followed by Holm-Sidak’s multiple comparisons test. Correlations between miRNA and tumour size in animal experiments were assessed with Pearson’s correlation coefficient. ROC curve analysis was performed to examine the diagnostic potential of serum miRNA expression levels. A two-sided p-value of less than 0.05 was considered statistically significant. Statistical analysis was carried out using GraphPad Prism version 6.0 h (GraphPad Software, San Diego, CA, USA) and R (version 3.3.1).

Data Availability. All data generated or analyzed during this study are included in this published article and its Supplementary Information files.

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### Author Contributions

T. Fujiwara contributed to the conception and design of the experiment. K. Uotani performed the experimental work and wrote the draft of the manuscript with assistance of T. Fujiwara. A. Yoshida, T. Morita, M. Kiyono, and S. Yokoo helped with the experimental work and data analysis. S. Iwata, K Numoto, Y. Nezu, T. Kunisada, K. Takeda, J. Hasei, T. Yonemoto, T. Ishii, A. Kawai carried out administrative, technical, or material support. T. Ozaki and T. Ochiya provided helpful discussion. K. Uotani and T. Fujiwara finalized the manuscript with the assistance of all authors.

### Additional Information

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### Competing Interests

The authors declare that they have no competing interests.

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