Detection of Circulating MicroRNAs with Ago2 Complexes to Monitor the Tumor Dynamics of Colorectal Cancer Patients during Chemotherapy

Tomokazu Fuji, Yuzo Umeda, Akihiro Nyuya, Fumitaka Taniguchi, Takashi Kawai, Kazuya Yasui, Toshiaki Toshima, Kazuhiro Yoshida, Toshiyoshi Fujiwara, Ajay Goel, and Takeshi Nagasaka

1Department of Gastroenterological Surgery, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, 700-8558, Japan.
2Department of Clinical Oncology, Kawasaki Medical School, 701-0192, Japan
3Center for Gastrointestinal Research; Center for Translational Genomics and Oncology, Baylor Scott & White Research Institute and Charles A. Sammons Cancer Center, Baylor University Medical Center, 75246, USA.

Corresponding Author:
Takeshi Nagasaka, M.D., Ph.D.
Department of Clinical Oncology Kawasaki Medical School
577 Matsushima, Kurashiki City, Okayama 701-0192, Japan
Phone: +81-86-462-1111; Fax: +81-86-464-1134
E-mail: takeshin@med.kawasaki-m.ac.jp

Keywords:
Liquid Biopsy, Chemotherapy, Circulating miRNA, Ago2, Colorectal Cancer

List of Abbreviations:
Novelty and Impact:

Most circulating miRNAs cofractionate with protein complexes, such as Argonaute2 (Ago2), rather than with exosome or microvesicles. We demonstrated an important feature of Ago2-associated miRNAs in the extracellular space, i.e., they originate in two ways: from passive release by cytolysis and from active export from viable cells. Next, we developed a methodology to detect circulating Ago2-miRNAs in plasma and demonstrated that measurement of their levels could be used to monitor tumor dynamics during chemotherapy.

Ago2-associated miRNAs can either be released by cytolysis or actively booted out of the cell. In this study, the authors showed which Ago2-complexed miRNAs are released by the different mechanisms. Because viable cancer cells release Ago-miRNA-21, it could serve as a biomarker to indicate the presence of cancer. Here, the authors used Ago-miRNA-21 to detect the presence of colorectal cancer. Conversely, Ago2-miR-200c appears in the bloodstream following cytolysis, and may indicate how well chemotherapy is killing tumor cells. The relationship between the two miRNAs could provide a window into tumor dynamics during treatment.
Abstract

Because of the different forms of circulating miRNAs in plasma, Argonaute2 (Ago2)-miRNAs and extracellular vesicles (EV-miRNAs), we examined the two forms of extracellular miRNAs in vitro and developed a unique methodology to detect circulating Ago2-miRNAs in small volumes of plasma. We demonstrated that Ago2-miR-21 could be released into the extracellular fluid by active export from viable cancer cells and cytolysis in vitro. As miR-21 and miR-200c were abundantly expressed in both metastatic liver sites and primary lesions, we evaluated Ago2-miR21 as a candidate biomarker of both active export and cytolysis while Ago2-miR-200c as a biomarker of cytolysis in plasma obtained from colorectal cancer (CRC) patients before treatment and in a series of plasma obtained from CRC patients with liver metastasis who received systemic chemotherapy. The measurement of Ago2-miR-21 allowed us to distinguish CRC patients from subjects without CRC. The trend in ∆Ct values for Ago2-miR-21 and -200c during chemotherapy could predict tumor response to ongoing treatment. Thus, capturing circulating Ago2-miRNAs from active export can screen patients with tumor burdens, while capturing them from passive release by cytolysis can monitor tumor dynamics during chemotherapy treatment.
Introduction

MicroRNAs (miRNAs) are small non-coding RNAs consisting of 18–24 nucleotides that mediate posttranscriptional regulation by binding to and repressing target messenger RNAs [1, 2]. They have a specific pattern of distribution within various organs and tumor types [3]. Previous studies have reported that mature miRNAs are exceptionally stable in blood plasma and that their expression profiles differ with the type of disease (e.g., diabetes or neoplasia) and the organ of origin (e.g., lung or pancreas) [4, 5]. Therefore, it has been proposed that patients with malignant neoplasia could be screened by capturing and profiling the miRNAs in plasma [6-9].

A fraction of circulating miRNAs resists degradation by ribonuclease [10]. Previous studies have demonstrated that most circulating miRNAs cofractionate with protein complexes rather than with exosomes or microvesicles [5-8]. A key effector protein of the miRNA-protein complex is Argonaute2 (Ago2), a protein component of the miRNA-induced silencing complex that binds to miRNA and that mediates the repression of messenger RNA [11]. Interestingly, it has been suggested that the miRNAs that cofractionate with Ago2 complexes (Ago2-miRNAs) predominantly originate from dead cells and that they stably persist within the extracellular space [5].

Another source of miRNAs is their release from cells in membrane-bound vesicles (EVs; extracellular vesicles). This mechanism is supported by the observation that cells growing in culture release EVs associated with miRNAs [12-15], and by the detection of miRNAs associated with EVs isolated from plasma and serum [16-20]. Vesicles are distinguished on the basis of size, surface antigen, or production mechanism as follows: 1) exosomes (size: 50–100 nm), 2) microvesicles (20–1000 nm), 3) membrane particles (50–80 nm and 600 nm), and 4) apoptotic bodies (1000–5000 nm) [21]. In contrast to Ago2-miRNAs, miRNAs in EVs are thought to be released from viable cells to engage in cell-to-cell communication [13, 22].

Because of the different forms of circulating miRNA in plasma, we hypothesized that the chemotherapeutic effects in patients with advanced cancer could be monitored by sequentially measuring Ago2-miRNAs and miRNAs in EVs during treatment. Therefore, in this study, we examined the release mechanisms of the two forms of miRNAs in vitro and then evaluated whether circulating miRNAs can serve as effective biomarkers for predicting and monitoring responses to anti-tumor therapies in advanced colorectal cancer (CRC).
Materials and Methods

All experiments were performed in accordance with relevant guidelines and regulations.

Patients

A cohort of 40 blood plasma samples was obtained from patients with stage I to IV CRC before treatment, and 20 blood plasma samples categorized as control subjects were obtained from patients who underwent curative resection for CRC and for whom computed tomography (CT) confirmed no recurrence at least one year later. Among the 20 plasma samples from control subjects, 11 samples were matched to the stage I to III CRC patients with curative resection. Primary tumors, normal colonic mucosa, and liver metastases for miRNA microarrays were obtained from two CRC patients with liver metastases. Blood plasma for the examination of the trend of clinical courses was obtained before the commencement of chemotherapy at each course from four CRC patients who were enrolled as research subjects in clinical trials (UMIN ID 8377, 9698, or 11954). These trials were conducted with patients treated between 2008 and 2014 at Okayama University Hospital, Japan.

Blood collection and plasma preparation

Peripheral blood was collected with an EDTA collection tube and processed for plasma isolation immediately after collection. The total blood was centrifuged at 3,000 rpm for 10 min at 4°C. The plasma was separated from the total blood and centrifuged again at 3,000 rpm for 10 min at 4°C to collect the supernatant from the plasma. Cell debris or large extracellular vesicles were removed from the samples by additional centrifugation at 14,400 rpm for 10 min followed by filtration through 0.22-μm filters (Merck Millipore, Merck KGaA, Darmstadt, Germany). Prepared plasma samples were stored at −80°C until use.

Cell culture medium assay

The human CRC cell line HT29 was purchased from American Type Culture Collections (Manassas, VA, USA) and cultured in McCoy medium supplemented with exosome-depleted-10% fetal bovine serum (System Biosciences, CA, USA) at 37 °C in 5% CO₂. One million cells per 10 ml of medium were seeded on a dish, and 750 μl of each cell culture supernatant was collected every
24 h after the initial seeding. The collected supernatant was centrifuged at 14,400 rpm for 10 min followed by filtration through 0.22-μm filters to remove cell debris. After collection at 48 h, 1 ml of medium containing 5-FU solution was added to each dish to reach a concentration of 0.5 mM.

Cell viability and toxicity assay

One hundred thousand cells in 100 μl of medium were seeded on a 96-well plate. The absorbance was determined at 0, 24, 48, 72, and 96 h after the initial seeding. The removing absorbance of cell-free medium was calculated as background subtraction. After the measurement at 48 h, 10 μl of medium containing 5-FU solution was added to reach a concentration of 0.5 mM. Cell viability was examined using a water-soluble tetrazolium salts (WST) assay. The absorbance determination was performed 1 h after the administration of 10 μl of Cell Proliferation Reagent WST-1 (Roche Diagnostics, Basel, Switzerland). Cell toxicity was examined using Cytotoxicity LDH Assay Kit-WST (DOJINDO, Japan) as manufactures protocol.

Immunoprecipitation

Immunoprecipitation of extracellular miRNAs with anti-Human Ago2 monoclonal antibody (WAKO, Osaka, Japan, 4G8; cat. 015-22031) was performed using a Pierce Classic IP Kit (Thermo Scientific, Waltham, MA, USA). Briefly, plasma samples (10–100 μl) were adjusted to a total volume of 200 μl with phosphate-buffered saline and incubated overnight with 20 μl of Pierce Protein A/G agarose (not combined with any antibodies) in a rotating wheel at 4°C (the preclear step). After centrifugation (at 1000 rpm for 1 min) for the collection of flow-through, the precleared plasma sample was incubated with 1 μg (1 μl) of anti-human Ago2 monoclonal antibody for 1 h in a rotating wheel at room temperature. As a negative control, we used 1 μg of monoclonal anti-β-actin antibody (Sigma-Aldrich, St. Louis, MO, USA). In a conditioned medium assay, 200 μl of medium was directly incubated with anti-human Ago2 antibody without the preclear step. The sample was incubated with 20 μl of new Pierce Protein A/G agarose for 1 h in a rotating wheel at room temperature to capture the immune complexes. After centrifugation, the flow-through was discarded or kept for a second Ago2 immunoprecipitation. The resin was washed three times with 200 μl of TBST; then, 200 μl of Qiazol reagent (Qiagen, Hilden, Germany) was added, and the solution was allowed to stand for 5 min. After centrifugation to collect the eluate, an additional 500 μl of Qiazol reagent was added to adjust the total volume to 700 μl.

Isolation of extracellular vesicles
Extracellular vesicles were isolated from 200 µl of plasma or medium using an exoRNeasy Serum/Plasma Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol.

**Isolation and reverse transcription of miRNA**

Total miRNAs were isolated from Ago2 immunoprecipitation products and EV isolation products using the miRNeasy Serum/Plasma kit (Qiagen) in accordance with the manufacturer's protocol. In total, 3.5 µl of cel-miR-39 (1.6 × 10^8 copies/µl) and 2 µl of yeast RNA (0.1 µg/µl), which were used as the normalization control and carrier RNA, respectively, were added to the samples before the addition of chloroform. First-strand complementary DNA synthesis was performed using 4 µl of extracted RNA template with the miScript II RT Kit (Qiagen) following the manufacturer's procedure.

**Quantitative real-time PCR**

The expression levels of miR-21 (hsa-mir-21-5p), miR-31 (hsa-mir-31-5p), miR-200c (hsa-mir-200c-3p), miR-16 (hsa-mir-16-5p), miR-let7a (hsa-let-7a-3p), miR-122 (has-mir-122-5p), miR-451 (hsa-mir-451a), and cel-miR-39 (cel-mir-39) were analyzed using miScript Primer Assays (Qiagen). Quantitative real-time PCR was performed using gene-specific primers and the miScript SYBR Green PCR Kit (Qiagen) in a LightCycler 480 (Roche Diagnostics). The quantitative value for a given sample of miRNA was calculated by subtracting the cycle threshold (Ct) value of cel-miR-39, which served as the external reference. Ct values from duplicate reactions were averaged, and \( \Delta Ct \) was calculated by subtracting the Ct of the added cel-miR-39 control from that of the Ct of the targeted miRNA. \( \Delta \Delta \text{Ct} \) was calculated by subtracting the \( \Delta \text{Ct} \) value of the reference sample from that of the control samples. The fold change for each miRNA was determined from the expression \( 2^{-\Delta \Delta \text{Ct}} \).

**Comprehensive analysis of the global expression status of Ago2-miRNAs and EV-miRNAs in plasma by miRNA PCR array**

The global expression ratios of Ago2-miRNAs and EV-miRNAs were evaluated using miScript miRNA PCR Arrays (Qiagen). One hundred µl of plasma was divided into 25-µl quantities and subjected to Ago2 immunoprecipitation procedures four times to maintain uniform sample volumes. Approximately 175 µl of Qiazol reagent was added to each A/G agarose resin at the elution step, and the eluates were mixed. MiRNA isolation with added cel-miR-39 as a control and reverse transcription using 1.5 µl of extracted RNA template was conducted as previously described. The
preamplification step was performed using a miScript PreAMP PCR Kit in combination with the miScript PreAMP Human miRNome Primer Mix (Qiagen). A miScript miRNA PCR Array Human miRNome was performed in the LightCycler 480, and ΔCt was calculated by subtracting the Ct of the added cel-miR-39 control from the Ct of the targeted miRNA. The miRNAs that were not detected were assigned Ct values of 45 (Ct > 45).

**SDS-PAGE and immunoblotting**

A/G agarose resin that had undergone the preclear step, Ago2 immunoprecipitation, the second Ago2 immunoprecipitation, or was untreated, was washed three times with 200 μl of TBST and eluted with 50 μl of a lane marker loading buffer (300 mM Tris-HCl at pH 6.8; 1% SDS; 10% glycerol; 20 mM DTT) at 100 °C for 10 min. Proteins were resolved on SDS-polyacrylamide gels and transferred to polyvinylidene fluoride membranes. After the proteins were transferred to the membranes, the gels were incubated with Oriole Fluorescent Gel Stain (Bio Rad, Hercules, CA, USA), and the membranes were incubated for 1 h at room temperature with anti-Human Ago2 monoclonal antibody or human IgG-Fc antibody (Bethyl Laboratories, Inc, Montgomery, TX, USA). After incubating the membranes with the HRP-conjugated secondary antibody, ECL was used to develop the blots.

**Comprehensive analysis of miRNA expression in the primary tumor, liver metastases, and corresponding normal tissues by miRNA microarray**

The total miRNA was isolated from frozen tissue specimens using a miRNeasy Mini Kit (Qiagen) and analyzed in an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer’s protocol. The SurePrint G3 Human miRNA Microarray Kit Release 21.0 (Agilent Technologies) contains 2,549 human microRNA probes. The expression level of each probe was the sum of 20 spots of raw intensity with the background subtracted. The target miRNAs that were not detected in any spots were represented as “undetected” and allocated an expression level of “0.1”. The data were normalized to the 90th percentile, and target miRNAs that were not detected in all samples were excluded.

**Statistical analysis**

Statistical analyses were performed using JMP software (version 10.0; SAS Institute Inc. Cary, NC, USA). The miRNA expression level was represented by Ct and ΔCt. To compare miRNA expression level in medium or relative expression indices, we used the t-test between 24 h and
The relative expression ratio was expressed as the mean ± standard deviation (SD). The Pearson product–moment correlation coefficient ($r^2$) was determined to examine the association between the ΔCt for Ago2-miR-451 and plasma volume. In addition to comparing differences in the Ct and ΔCt values of the Ago2-miRNAs in the plasma samples, we used the Wilcoxon signed-rank test to compare Ago2-miR-21 and -miR-200c expression in paired plasma samples. All reported $P$-values were from two-sided tests, and a $P$-value less than 0.05 was considered statistically significant.

Results

Assessment of the mechanism of release of extracellular miRNAs by a cytotoxic agent

We examined whether the two forms of circulating miRNAs could be detected in the extracellular fluid. Initially, we cultured cells from the HT29 CRC cell line and examined the expression of miR-21, miR-31-5p (miR-31) and miR-200c as examples of excreted and non-excreted miRNAs, respectively, in the culture medium, as described previously [9]. The HT29 CRC cell line, which abundantly expressed miR-21, miR-31-5p and miR-200c in the cytoplasm, was treated with 5-fluorouracil (5-FU) 48 h after seeding. A WST and a lactate dehydrogenase (LDH) assay showed that the HT29 cells continuously proliferated in normal culture conditions with minimal cell death. After 5-FU treatment, cell viability immediately decreased whereas cytolysis occurred after 24 hours (Fig. 1).

The culture medium was collected at 24, 48, 72, and 96 h. The Ago2-miRNAs and miRNAs encapsulated by EVs in the culture medium were independently measured by quantitative reverse transcription PCR (qRT-PCR) after Ago2-immunoprecipitation (IP) and by a conventional procedure for purifying miRNA from exosomes and other extracellular vesicles (EV-miRNAs) (Additional supporting information 1).

Using qRT-PCR, the expression ratios of miR-21, miR-31-5p and miR-200c in the culture medium at 48, 72, and 96 h were represented as fold changes based on the change in the threshold cycle value (ΔCt) in the medium obtained 24 h after seeding (Fig. 1c). In the control group, the expression ratio of Ago2-miR-31, Ago2-miR-200c and EV-miR-31, EV-miR-200c did not significantly increase over the same period. Interestingly, Ago2-miR-21 clearly increased, whereas EV-miR-21 slightly increased. This increases in Ago2-miR-21 was exponential from 24 to 96 h, suggesting that Ago2-miR-21 was actively exported into the medium from viable cancer cells. By contrast, in the 5-FU treatment group, the expression ratios of all Ago2-miRNAs and EV-miRNAs increased exponentially from 72 to 96 h coincident with cytolysis at the LDH assay. With respect
to the feature observed in the control group, Ago2-miR-31/200c and EV-miR-31/200c were released in the process of cytolysis into the media while Ago2-miR-21 (and possibly EV-miR-21) was released by cytolysis and actively exported into the medium from viable cancer cells.

Thus, our results suggest that the release mechanism for extracellular miRNAs differs with the type of miRNA and complex. Because Ago2-miR-21 can also be actively exported into the extracellular fluid from viable cancer cells, Ago2-miR-21 has the potential to be a robust biomarker for predicting the existence of viable cancer cells.

Identification of miRNAs associated with metastatic lesions

We tested our hypothesis that Ago2-miRNAs could be biomarkers of chemotherapeutic effects by attempting to identify miRNAs that were specifically expressed in metastatic lesions using a miRNA microarray for the primary tumors and liver metastatic tissues derived from two CRC patients with liver metastases. The global miRNA expression status was evaluated in the primary tumor, normal colonic mucosa, the liver metastatic lesion, and the normal liver tissue for each patient. The miRNA microarray allowed the examination of a total of 2,549 human miRNAs (see Availability of data and material). In this analysis, a strong correlation between expression in the primary tumor and in the metastatic liver site was observed for each patient (Fig. 2a). In particular, the expression level of miR-21-5p was the highest in both primary tumors and metastatic liver sites from the two patients. We also found that the expression level of miR-200c was quite higher in both tissues, while miR-31 was little.

Development of a new approach for the detection of circulating Ago2-miRNAs

Before examining the clinical behavior of Ago2-miR-21 and Ago2-miR-200c, we developed a novel approach for detecting circulating Ago2-miRNAs in a small quantity of plasma. Because many antibodies and proteins commonly exist in plasma and are likely to inhibit the binding of Ago2-miRNAs to protein A/G agarose beads, we adopted a preclearing step before Ago2-IP to reduce the amount of specific and nonspecific antibodies and proteins in the plasma by nonspecifically capturing protein with A/G agarose beads (Preclear method, Additional supporting information 2)[23]. By using this procedure, we confirmed Ago2 associated protein complexes in blood plasma (Additional supporting information 3).

Similar to previous studies [4, 24, 25], our analysis of the global expression status of Ago2-miRNAs and EV-miRNAs in plasma obtained from a healthy subject by miRNA PCR array showed that miR-451 interacted with Ago2 and existed as an Ago2-miR-451 complex and not as EV-miR-
451 in plasma (Additional supporting information 4 and 5). Therefore, we recovered miR-451 from Ago2 complexes in plasma obtained from healthy volunteers to optimize this method. The amount of miR-451 was represented by its relative expression level. Compared with a conventional Ago2-IP procedure (referred to here as the “non-preclean method”), the preclean method improved the relative expression level of miR-451 in Ago2-IP and reduced the residual miR-451 in the column flow-through. We then determined the volume of plasma required to capture the circulating miRNAs that were effectively bound to Ago2. With the non-preclean method, almost all miR-451 was found in the flow-through (Fig. 3a). Conversely, with the preclean method, the relative expression level of miR-451 recovered from the plasma increased linearly between 10 and 60 μl of plasma; a similar linear increase between 10 and 60 μl of plasma was observed in the flow-through (Fig. 3b). This result was confirmed by human IgG immunoblotting and SDS-PAGE (Additional supporting information 6). The relative expression level of miR-451 in 30 μl of plasma showed a strong correlation with plasma volume ($r^2=0.903$) (Fig. 3c). We used our procedures for the detection of Ago2- and EV-miRNAs to confirm the associations between the Ct values and various Ago2-miRNAs and EV-miRNAs (Fig. 3d). Similar to the result from the miRNA microarray, the relative expression level of miR-451 was dominant in Ago2-IP compared with the EV isolation procedure. Based on our results, we used 30 μl of plasma to capture Ago2-binding miRNAs. Ago2-miR-451 was used as the internal control for circulating Ago2-binding miRNAs in the subsequent experiments.

**Ago2-miRNAs in a liquid biopsy can predict the colorectal tumor burden in patients**

We confirmed whether the levels of Ago2-miR-21 and Ago2-miR-200c actually increased in blood obtained from patients with CRC by examining these miRNAs in plasma obtained from another 40 CRC patients and 20 control subjects (CRC patients who were under surveillance for recurrence after curative resection and for whom CT confirmed no metastatic lesion). In this study, because Ago2-miR-451 was used as a control to check the condition of the blood plasma, when a Ct value of Ago2-miR-451 was 40 or less, we considered the quality of the blood plasma to be adequate for the subsequent analyses. By this criterion, only one of 40 CRC patients displayed a Ct value for Ago2-miR-451 of greater than 40. Therefore, we excluded this sample from the subsequent analyses. Fig. 4a shows the actual Ct values of Ago2-miR-451, -21, and -200c obtained from 20 control subjects and 39 CRC patients. The mean Ct value of Ago2-miR-451 obtained from control subjects was the same as that from CRC patients. The mean Ct values of control subjects and CRC patients were 29.9 [the 95% confidence interval (CI) was 28.4-31.4] and 30.5 [29.5-31.5], respectively. The mean Ct value of Ago2-miR-21 obtained from CRC patients was significantly lower than that from control subjects (31.4 [31.0-31.9] and 33.0 [32.0-34.0], respectively, $P=0.0097$), whereas there was no difference between the mean Ct values for Ago2-miR-200c
obtained from CRC patients and control subjects (39.9 [39.5-40.2] and 41.6 [40.1-43.1], respectively), suggesting that Ago2-miR-21 was actively exported into the blood, whereas Ago2-miR-200c was not.

Next, we calculated the $\Delta$Ct ($miR-451$) values of Ago2-miR-21 and -200c by subtracting the $\Delta$Ct value of Ago2-miR-451. By this analysis, the mean $\Delta$Ct values ($miR-451$) of Ago2-miR-21 and -200c significantly decreased (i.e., the amounts of Ago2-miR-21 and -200c increased) in the plasma obtained from CRC patients compared with the values in that obtained from control subjects (Fig. 4b and 4d). Next, we analyzed paired pre- and postoperative plasma samples in a subset of 11 CRC patients who underwent curative surgical resection of their tumors and for whom CT confirmed that no metastatic lesions were present (Fig. 4c and 4e). The amounts of Ago2-miR-21 and -200c significantly plummeted after surgery in this subset of patients. Although we were unable to find any evidence of the active export of Ago2-miR-21 by these analyses, this miRNA was more abundant in the plasma from CRC patients.

Indeed, the mean $\Delta$Ct ($miR-451$) for Ago2-miR-21 was 3.12 (1.81–4.42) in control subjects and 0.96 (0.27–1.65) in CRC patients. We therefore defined the cut-off value of $\Delta$Ct ($miR-451$) for Ago2-miR-21 in plasma as 2.00, i.e., when $\Delta$Ct ($miR-451$) was 2.00 or below, this was taken to indicate the presence of a tumor burden. Similarly, the normal range of $\Delta$Ct ($miR-451$) for Ago2-miR-200c was defined as 10.00 or more because the mean value was 11.67 (9.72–13.62) in control subjects and 9.38 (8.51–10.2) in CRC patients.

Trend of Ago2-miRNAs in plasma obtained from advanced CRC patients receiving systemic chemotherapies

Finally, we reviewed Ago2-miR-21 and -200c in a series of plasma samples obtained from four patients while they were undergoing systemic chemotherapy.

Fig. 5a shows the clinical course of a patient with multiple unresectable liver metastases. This patient showed an effective response for 4 months before ultimately acquiring resistance to first-line chemotherapy. The clinical course of this patient could be divided into three phases according to the level of carcinoembryonic antigen (CEA): a shrinkage phase (day 1 to day 48), a stable phase (day 49 to day 113), and a progressive phase (day 113 to day 168). In this case, the trend of Ago2-miR-200c could predict tumor dynamics compared with that of Ago2-miR-21. The $\Delta$Ct ($miR-451$) for Ago2-miR-200c was constantly above the cut-off value of 10.00 from the shrinkage to the stable phase, whereas the $\Delta$Ct ($miR-451$) for Ago2-miR-21 entered the abnormal range after the second course of systemic chemotherapy. Although the CEA level reached its nadir (suggesting that the disease remained stable) in the stable phase, the $\Delta$Ct ($miR-451$) for Ago2-
miR-200c responded sensitively to the administration of the fourth course of chemotherapy. However, on the fifth course, the ΔCt (miR-451) for Ago2-miR-200c increased (i.e., the amounts of Ago2-miRNAs in the plasma decreased), suggesting that this regimen had failed. In the progressive phase, the CEA level increased linearly, whereas the ΔCt (miR-451) for Ago2-miR-200c increased above the cut-off value, suggesting that few viable cancer cells were destroyed by systemic chemotherapy.

Fig. 5b shows the clinical course of a patient who underwent adjuvant chemotherapy. After the first course, the ΔCt (miR-451) for the Ago2-miRNAs decreased (i.e., the amounts of Ago2-miRNAs increased), suggesting the existence of invisible micrometastasis. A liver metastasis was confirmed by CT at day 29. After commencing bevacizumab, when the CEA level decreased, the ΔCt (miR-451) for the Ago2-miRNAs also decreased (i.e., the amounts of Ago2-miRNAs in the plasma increased). Similarly, when the CEA level increased, the ΔCt (miR-451) for the Ago2-miRNAs also increased (i.e., the amounts of Ago2-miRNAs in the plasma decreased).

Fig. 5c shows the clinical course of a patient with multiple unresectable liver metastases who showed no effective response and acquired resistance to first-line chemotherapy. The clinical course of this patient was divided into two phases according to the CEA level: the stable phase (day 1 to day 84) and the progressive phase (day 85 to day 126). In this case, before treatment, the ΔCt (miR-451) for Ago2-miR-21 was lower than the cut-off value (i.e., the amount of Ago2-miR-21 in the plasma was abundant by active exportation). By contrast, the ΔCt (miR-451) for Ago2-miR-200c was higher than the cut-off value because it was not released by cytolysis. After the first course, the ΔCt (miR-451) for Ago2-miR-200c rapidly decreased (i.e., the amount of Ago2-miR-200c in the plasma increased by cytolysis), and then it increased over the cut-off value of 10.00, suggesting that no cytolysis occurred and that this regimen became ineffective.

Fig. 5d also shows the clinical course of a patient with multiple unresectable liver metastases who had surgical resection for the primary tumor before receiving systemic chemotherapy. After primary tumor resection, although the CEA level increased rapidly (3,542 ng/ml on day 49 before resection to 7,696 ng/ml on day 1 after resection), the ΔCt (miR-451) for Ago2-miRNAs was within the normal range. After the first course of chemotherapy, although the CEA level decreased rapidly, the ΔCt (miR-451) for Ago2-miR-21 decreased only after the first course (i.e., the amounts of Ago2-miR-21 possibly increased in the plasma by passive release) and immediately increased over the cut-off value of 2.00 throughout this regimen, suggesting that the effectiveness of this regimen was temporary.
Discussion

In this study, we demonstrated the potential of circulating miRNAs, particularly miRNAs cofractionated with an Ago2 complex, to be sensitive biomarkers for monitoring therapeutic response to anti-tumor therapy and as screening biomarkers for liquid biopsy detection in patients with CRC.

We first examined the different forms of circulating miRNA in plasma. Our initial hypothesis was that Ago2-miRNAs originated from cytolysis and that miRNAs originated from exosomes (EV-miRNAs in this study) released by viable cells. We evaluated this hypothesis by selecting miR-21, miR-31-5p and miR-200c, which are commonly expressed in CRC tissues [9, 26, 27], and measuring the two forms of these miRNAs in the culture medium of cells in vitro.

Previous studies have demonstrated that cells growing in culture release exosomes that are associated with miRNA [12-15]. In the present study, despite our use of a column method that could capture exosomes, other microvesicles, and membrane particles in the medium, and even though EV-miR-21 has the potential to be actively released from viable cells, the amount of EV-miR-21 was smaller than that of Ago2-miR-21. Additionally, we were not able to detect the active export of EV-miR-31/200c from viable cells into the cell culture medium.

Our results showed that both Ago2-miRNAs and EV-miRNAs were discharged into the medium in the process of cytolysis during 5-FU treatment which included not only cytolysis but also active export as response to drug-induced stress. Unexpectedly, only Ago2-miR-21 clearly showed the possibility of active export into the culture medium from viable cells. This specific feature of Ago2-miR-21 could be the reason why miR-21 increased in the blood plasma obtained from patients with adenoma and invasive cancers, as we demonstrated in a previous study [9].

It is controversial whether Ago proteins are associated with EVs or not [28]. Indeed, several studies reported EVs lack Ago2 while others demonstrated EVs contains significant amount of Ago2 and a transmembrane protein which consist of EVs directly interact with Ago2 [18, 29-33]. Therefore our Ago2 immunoprecipitation method has the possibility for capturing Ago2 which is associated with EVs as well as free from EVs.

We selected Ago2-miR-200c as additional candidate biomarkers of circulating miRNAs that could be used to evaluate a tumor’s response to anti-tumor therapy. We found that miR-200c was highly expressed in both primary tumors and metastatic liver sites and mainly discharged into the medium by cytolysis.

Finally, we tested our hypothesis that measuring Ago2-miRNAs could enable the evaluation of chemotherapeutic effects in patients with advanced cancer by selecting two miRNAs as candidate biomarkers: Ago2-miR-21, which is highly expressed in both primary tumors and
metastatic sites and has a unique release mechanism including active export; and Ago2-\textit{miR-200c}, which shows greater expression by metastatic sites than by the primary tumor and is mainly released by cytolysis.

Given the importance of Ago2-miRNAs, we attempted to develop a new procedure to measure Ago2-miRNAs in a small quantity of plasma. In developing this procedure, we adopted a preclear strategy used for the reduction of background noise in ribonucleoprotein immunoprecipitation [34]. Conventional strategies for the detection of circulating miRNAs require 80 to 400 µl of blood plasma or serum [5, 9, 24, 35]. However, using the preclear strategy, we could effectively recover Ago2-miRNAs from smaller volumes of plasma. Another problem was how to normalize miRNA expression when measuring circulating miRNAs in a liquid biopsy. Turchinovich et al. noted that normalization between the miRNAs themselves could be difficult if the miRNAs originated from different extracellular components (Ago2-bound versus exosomal) or different Ago-carriers [5]. Thus, the stability of miRNAs can differ. The authors suggested that the best way to overcome these problems was to implement the same standardized processing protocols for samples of different comparison groups and within study cohorts.

In agreement with previous studies [4, 24, 25], our results also demonstrated that \textit{miR-451} was the specific miRNA that cofractionated with the Ago2 complex and was stably expressed in plasma. Therefore, in addition to implementing the same standardized processing protocols, we selected \textit{miR-451} as the internal control for Ago2-miRNAs in blood plasma. Using our new procedure, we measured circulating Ago2-miRNAs in blood plasma obtained from patients with stage I, II, III, and IV CRC before treatment and in a series of plasma samples obtained from four additional CRC patients who received sequential systemic chemotherapy treatments. Our results demonstrated that the measurement of Ago2-miRNAs, especially Ago2-\textit{miR-21}, in plasma allowed us to screen CRC patients from subjects without CRC, suggesting that the higher level of Ago2-\textit{miR-21} in blood may be due to active exportation from viable cancer cells. On the other hand, because the amount of Ago2-miRNAs released by cytolysis was much greater than the amount that was actively exported into the fluid from viable cancer cells, plateaus or decreases in ∆Ct (\textit{miR-451}) values for Ago2-miRNAs, which are mainly released into the plasma by cytolysis, during systemic chemotherapy treatment suggested that the tumors had been nonresponsive to the ongoing treatment.

In general, cytotoxic drugs are administered with the assumption that the maximum clinical benefit is obtained by killing the largest possible number of cancer cells. Consistent with this premise, most systemic chemotherapies are applied at the maximum tolerated dose levels [30, 36], which has the theoretical benefits of killing the maximum number of cancer cells and minimizing the risk of resistant mutations. Recently, the traditional maximum dose density paradigm has been questioned on the basis of a theoretical model that views cancer therapy as
an evolutionary and ecological process [31, 32]. Indeed, in a breast cancer model, Enriquez-Navas et al. demonstrated that smaller drug doses could effectively maintain tumor progression after initial tumor control, which was achieved by intensive therapy with regular applications of the drug [28]. Interestingly, the four cases that achieved effective responses (shrinkage phases and stable phases in Figure 5) during a certain period after intensive chemotherapy displayed an instantaneous increase of Ago2-miRNAs resulting from cytolysis even after a few cycles of chemotherapy, suggesting that the initial doses would become overdoses after initial tumor control was achieved. Therefore, the dose of intensive chemotherapy could be potentially reduced before the appearance of adverse events, and monitoring Ago2-miRNAs via liquid biopsies could allow for a more informed decision about the timing of switching to intensive chemotherapy or to maintenance therapy.

We established the attractive features of circulating Ago2-miRNAs. The Ago2-miRNAs in the extracellular space originated from at least two mechanisms: passive release into the blood by cytolysis or active export from viable cells. We demonstrated that Ago2-miRNAs released by cytolysis could be used to monitor tumor dynamics during treatment with systemic chemotherapy. Although we were unable to directly show the active transport of Ago2-miRNAs, these complexes have potential use in predicting which patients will experience recurrence during adjuvant chemotherapy and as a robust screening biomarker for CRCs at earlier stages.
Declarations

Ethics approval and consent to participate
The study protocol was approved by the ethics committee of Okayama University Hospital and all patients provided written informed consent.

Consent for publication
Written consent for publication was obtained from all the patients involved in our study.

Availability of data and material
The datasets of the miRNA microarray analyzed during the current study are available in the Gene Expression Omnibus (GEO) repository, http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE73178. All data generated or analyzed during this study are included in this published article (and its Supplementary Information files) and available from the corresponding author on reasonable request.

Competing interests
The authors declare that they have no potential conflicts of interest associated with this manuscript.

Funding
This work was supported by consignment accounting findings.

Author’s contributions
TomF and TN: drafting of the manuscript; TN: study concept and design; TomF, YU, AN, FT, TK, KazuyY, TT, and KazuhY: acquisition of data; TosF and AG: analysis and interpretation of data; YU and TN: statistical analysis; and AG and TosF: material support.

Acknowledgments
The authors thank Mr. Toru Nakai and Mrs. Tae Yamanishi for technical assistance.
References

Figure legends

Figure 1. Assessment of the release mechanism of extracellular miRNAs by a cytotoxic agent (5-FU). Cells from a CRC cell line, HT29, were treated with 5-FU solution 48 h after seeding. The supernatants were collected at 24, 48, 72, and 96 h after seeding. (a) Cell proliferation and cell biological activities measured using a WST assay were plotted. (b) Cytotoxicity measured using a LDH assay were plotted. (c) The relative amount of miR-21 and miR-31 in the culture medium of the HT29 cell line was estimated by quantitative reverse transcription PCR. The expression ratio was represented by a fold change based on the change in threshold cycle value (ΔCt) obtained at 24 h after seeding. “Control” and “5-FU” denote the control group and the 5-FU treatment group, respectively. Each bar represents the mean and SD of five independent experiments. The yellow shaded areas represent the period of treatment with 5-FU. Abbreviations: 5-FU, 5-fluorouracil; Ago2, Argonaute2 protein; CRC, colorectal cancer; EVs, extracellular vesicles; miRNA, micro RNA; PCR, polymerase chain reaction; *p<0.05 (versus the base line at 24 h after seeding).

Figure 2. Global miRNA profiling of the primary tumors and liver metastases. The association of the global miRNA expression status of the primary tumor and the corresponding liver metastasis by miRNA microarray. CT1, 2 and LT1, 2 denote CRC and liver metastasis specimens from patients 1 and 2, respectively.

Figure 3. Development of a procedure for detecting circulating Ago2-miRNAs. (a) Ago2-miR-451 was recovered from Ago2-IP by quantitative reverse transcription PCR. Added ce-miR-39 was used as a normalization control for all samples. Data are presented as the relative fold changes in miRNA levels. (b) The expression levels of miR-16, miR-21, and miR-451 recovered from the first Ago2-IP and from a second Ago2-IP obtained from the flow-through. Anti-β-actin antibody was used as a negative control. Each bar represents the mean ± SD of three independent experiments. Data are presented as the relative fold changes in miRNA levels. (c) Scatter plot and correlation coefficient for miR-451 recovered from Ago2-IP and the plasma volume. Three independent experiments were performed for each volume. Data are presented as the relative fold changes in miRNA levels. (d) Scatter diagram of Ct values of each miRNA obtained from the Ago2-IP and EV isolation procedures using 30 μl of plasma. Each plot represents the mean of three independent experiments. Abbreviations: Ago2, Argonaute2 protein; Ct, threshold cycle value; EVs, extracellular vesicles; IP, immunoprecipitation; miRNA, micro RNA; PCR, polymerase chain reaction.
**Figure 4.** Ago2-**miR-451**, -21 and -200c levels in plasma samples. (a) Ct values of Ago2-**miR-451**, -21 and -200c in blood plasma obtained from control subjects (CS, n=20) and CRC patients (CRC, n=39). (b) △Ct (**miR-451**) values of Ago2-**miR-21** in blood plasma obtained from CS, CRC, and stages I, II, III and IV. (c) △Ct (**miR-451**) values for Ago2-**miR-21** in plasma obtained from 11 patients with CRC before surgery (Pre) and one year after the surgical removal of primary tumors (Post). (d) △Ct (**miR-451**) values of Ago2-**miR-200c** in blood plasma obtained from CS, CRC, and stages I, II, III and IV. (e) △Ct (**miR-451**) values for Ago2-**miR-200c** in plasma obtained from 11 patients with CRC before surgery (Pre) and one year after surgical removal of primary tumors (Post). The y-axes in (b), (c), (d), and (e) represent the △Ct values of Ago2-miRNAs normalized to Ago2-**miR-451**. Boxes represent the interquartile range, and the horizontal line across each box indicates the median value. Statistically significant differences were determined using the Wilcoxon test. NS, not statistically significant. Dotted lines represent the cut-off values for each Ago2-miRNA. △Ct (**miR-451**) values below the dotted line (beyond the dotted line on both Ago2-miRNAs) are considered abnormal values.

**Figure 5.** Examples of the expression levels of Ago2-**miR-21** and -200c during the clinical course of four patients receiving systemic chemotherapy. The numbers in circles represent the chemotherapy course number. Ago2-**miR-21** and -200c on the y-axes represent the △Ct values of Ago2-miRNAs normalized to △Ct values of Ago2-**miR-451**. Red arrows denote the time points at chemotherapy with blood collection for analyzing Ago2-miRNAs. Blue arrows denote chemotherapy alone and dashed arrow denote blood collection alone. Blue, yellow, and red shading represent the shrinkage phase, stable phase, and progressive phase, respectively. Dotted lines represent the cut-off values for each Ago2-miRNA. Black arrows denote the time point of image assessment for liver metastasis, and computed tomography or magnetic resonance imaging represents major lesions. Abbreviations: Ago2, Argonaute2 protein; Beva, bevacizumab; CRC, colorectal cancer; Ct, threshold cycle value; FOLFOX6, modified FOLFOX6 regimen; miRNA, micro RNA; SOX, TS1 with oxaliplatin regimen.