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Predicting the presence of breast cancer using circulating small RNAs, including those in the extracellular vesicles

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Abstract

Emerging evidence indicates that small RNAs, including microRNAs (miRNAs) and their isoforms (isomiRs), and transfer RNA fragments (tRFs), are differently expressed in breast cancer (BC) and can be detected in blood circulation. Circulating small RNAs and small RNAs in extracellular vesicles (EVs) have emerged as ideal markers in small RNA-based applications for cancer detection. In this study, we first undertook small RNA sequencing to assess the expression of circulating small RNAs in the serum of BC patients and cancer-free individuals (controls). Expression of 3 small RNAs, namely isomiR of miR-21-5p (3′ addition C), miR-23a-3p and tRF-Lys (TTT), was significantly higher in BC samples and was validated by small RNA sequencing in an independent cohort. Our constructed model using 3 small RNAs showed high diagnostic accuracy with an area under the receiver operating characteristic curve of 0.92 and discriminated early-stage BCs at stage 0 from control. To test the possibility that these small RNAs are released from cancer cells, we next examined EVs from the serum of BC patients and controls. Two of the 3 candidate small RNAs were identified, and shown to be abundant in EVs of BC patients. Interestingly, these 2 small RNAs are also more abundantly detected in culture media of breast cancer cell lines (MCF-7 and MDA-MB-231). The same tendency in selective elevation seen in total serum, serum EV, and EV derived from cell culture media could indicate the efficiency of this model using total serum of patients. These findings indicate that small RNAs serve as significant biomarkers for BC detection.

KEYWORDS
biomarker, breast cancer, extracellular vesicle, serum, small RNA

Abbreviations: AUC, area under the receiver operating characteristic curve; BC, breast cancer; CA, carbohydrate antigen; CEA, carcinoembryonic antigen; ER, estrogen receptor; EV, extracellular vesicle; HER2, human epidermal growth factor receptor 2; IHC, immunohistochemistry; isomiR, microRNA isoform; miRNA, microRNA; N, control; ncRNA, noncoding RNA; NGS, next-generation sequencing; PR, progesterone receptor; RPM, reads per million; TEM, transmission electron microscope; tRF, transfer RNA fragment.

1 | INTRODUCTION

Breast cancer is one of the most common cancers among women worldwide.[1] Although mammography is commonly used for BC screening,[2] it is hampered by the occurrence of false-positive results, which might lead to additional imaging or tumor biopsy.[3,4] In addition, the sensitivity of mammography is decreased in younger women or women with dense breast tissue.[5,6] Asian women have relatively high breast density and are of particular need of methods for BC detection that overcome these problems.[7]

Extracellular vesicles are membranous vesicles secreted from cells to the extracellular environment that contain nucleic acid, proteins, and lipids.[8] Extracellular vesicles can be classified into various types according to size and mode of biogenesis.[9] Exosomes are nanosized EVs (30-150 nm) originating from the endocytic pathway.[10] Extracellular vesicles, especially exosomes, have been found to play essential roles in intercellular communication through their cargo. Extracellular vesicle components differ by releasing cell type and have therefore been investigated as markers for disease, including cancer. One example is EV-encapsulated small RNAs (EV small RNAs).[8]

Small RNAs consist of multiple classes of short ncRNA molecules that include miRNAs, tRFs, and other RNA fragments generated from Y RNAs, miscellaneous RNAs, and others.[11] Recent results have shown that miRNAs can be divided into canonical miRNAs and isomiRs; this latter type is differentiated from canonical miRNAs by variations in length and/or sequence through the addition or deletion of nucleotides at the 5′- or 3′-end, or both.[12,13] Small RNAs circulate stably in blood (circulating small RNAs) and can be isolated from blood samples.[14,15]

Small RNAs play essential roles in gene regulation at the post-transcriptional level.[16,17] It is generally accepted that small RNAs are crucial to biological processes and that their dysregulation results in disease development.[15,18] Indeed, aberrant expression of miRNAs is associated with various cancers,[19,20] and expression profiles of circulating miRNA or EV miRNAs have been recently investigated as potential biomarkers for BC detection.[21-25] Furthermore, the distribution and abundance of isomiRs, tRFs, and other small RNA fragments have been shown to differ nonrandomly depending on disease status.[18,26-29] Significant levels of these small RNA fragments as well as miRNAs can serve as precise biomarkers for cancer detection. Surprisingly, however, almost no investigation of these fragments in patients with BC has yet been reported.

In this study, we used NGS to identify biomarkers by profiling multiple classes of small RNAs in serum from BC patients. By comparing the small RNA signature with that of cancer-free individuals, we aimed to detect specific circulating small RNAs that can indicate the presence of BC. In addition, we also attempted to construct an NGS-based diagnostic model using small RNAs for BC detection. Finally, we evaluated the expression pattern of EV small RNAs in serum from BC patients and in cell culture media of BC cell lines to investigate the relation between circulating small RNA and EV small RNAs.

2 | MATERIALS AND METHODS

2.1 | Ethics statement

This study was approved by the Institutional Review Board of Hiroshima University Hospital. Written informed consent was obtained from all participants before enrollment.

2.2 | Participants

Seventy-eight patients with newly diagnosed BC at clinical stage 0-III at Hiroshima University Hospital from November 2016 to August 2017 were included in the BC group. In parallel, 72 cancer-free female individuals were recruited in the N group. Participants of the N group had undergone screening mammography or breast ultrasound sonography within the previous 2 years at Okimi-Hamai Hospital to ensure their BC-free status before enrollment. We excluded patients with distant metastasis in order to focus on finding biomarkers for early detection. Participants with a previous history of any cancer or with a current inflammatory condition were also ineligible.

Carcinoembryonic antigen and CA15-3, which are traditional serum markers for BC monitoring, were assessed in the BC group before the start of treatment. All BCs were histopathologically diagnosed using primary tumor specimens, with staging done using the 8th edition of the TNM classification of malignant tumors.[30] For invasive BC tumors, we evaluated the expression of ER, PR, and HER2 by IHC. Tumors were considered to be ER- or PR-positive when 1% or more of cells were stained. The HER2 expression was scored according to the guidelines of the American Society of Clinical Oncology/College of American Pathologists.[31] Scores of 2 or higher were further assessed using FISH. We defined HER2 positivity as either IHC 3 or higher, or IHC 2 or higher and *HER2* gene amplification by FISH (HER2/CEP ratio greater than 2.0).

2.3 | Serum sample collection and small RNA extraction

Serum was isolated by centrifugation of the whole blood twice at 2330 *g* at room temperature (25°C) for 10 minutes followed by 12 000 *g* at 4°C for 10 minutes. Serum samples were transferred to RNase-free tubes and stored at −80°C until further use.

Circulating small RNAs and EV small RNAs were extracted from 250 μL serum and EV samples using a miRNeasy Mini Kit (Qiagen) in accordance with the manufacturer's protocol. Quantification and integrity of the obtained small RNAs were assessed with an Agilent 2100 Bioanalyzer (Agilent Technology) using RNA 6000 pico chips in accordance with the manufacturer's instructions to confirm the amount of starting extracted small RNAs was sufficient for library construction.

2.4 | Isolation of EVs

Extracellular vesicles were isolated from 100 μL serum and 10 mL cell supernatant using a Total Exosome Isolation kit (Invitrogen) in accordance with the manufacturer's protocol. Pellets containing EVs were dissolved in 50 μL PBS that had been filtered once through a 0.22-μm pore filter (Steriflip SCGP00525; Millipore). To evaluate small RNA profiles within serum-derived EVs, 32 samples in the BC group and 20 samples in the N group were randomly selected. Analysis of EV samples from cell culture media was undertaken in duplicate.

2.5 | Characterization of EVs

Following EV isolation, we examined the size distribution and concentration of particles using qNANO (Izon) and analyzed the data with software provided by Izon (version 3.2). Protein concentrations were determined using a Micro BCA Protein Assay Kit (Thermo Fisher Scientific). The presence of exosome markers CD9 (CAC-SHI-EXO-M01, clone 12A12; Cosmo Bio) and TSG-101 (5377-1, clone EPR7131(B); Abcam) and lipoprotein marker antiapolipoprotein B-100 (SC-13538; Santa Cruz Biotechnology) was confirmed by western blot analysis. Transmission electron microscopy analysis was undertaken for morphological characterization of EVs as described previously.[32] Carbon-film grids that EVs applied to were examined with a Hitachi H-7600 TEM (Hitachi High-Technologies) at Hanaichi UltraStructure Research.

2.6 | Cell culture

Human BC cell lines MCF7 and MDA-MB-231 and normal human breast epithelial telomerase immortalized cells 184-h TERT were cultivated to isolate EVs. Breast cancer cells were cultured in DMEM supplemented with 10% FBS (Gibco/Thermo Fisher Scientific) and 1% penicillin/streptomycin (Corning/Mediatech) in accordance with the suppliers' recommendations. After the cells were washed with PBS, the culture medium was replaced with FBS-free advanced DMEM and collected after 48 hours of incubation for EV isolation. 184-h TERT cells were cultivated in serum-free Mammary Epithelial Basal Medium (Lonza) supplemented with Mammary Epithelial Cell Growth Medium SingleQuots Kit (Lonza) in accordance with the supplier's recommendations. After the cells were washed with PBS, the culture medium was collected after 48 hours of incubation. Before isolation of EVs, collected cell supernatant was centrifuged at 2000 *g* for 30 minutes at 4°C and at 12 000 *g* for 30 minutes at 4°C to remove dead cells and cellular debris and large vesicles were discarded. After centrifugation, the supernatant was filtered through a 0.22-μm pore filter. All cells were incubated in a humidified chamber at 37°C and 5% CO_2.

2.7 | Library preparation and small RNA sequencing

Libraries for sequencing analysis were generated from 4 μL small RNA per sample. The barcoded libraries were constructed using an Ion Total RNA-Seq Kit version 2 (Thermo Fisher Scientific) and an Ion Xpress RNA-Seq Barcode 01-16 Kit (Thermo Fisher Scientific) in accordance with the manufacturer's instructions. All libraries were size-selected using the Blue Pippin system (Sage Science) to reduce low molecular weight contamination and collect tight size distribution of cDNA fragments to improve the quality of analysis. We used a 3.0% agarose gel and set the range of selection from 88 to 112 base pairs to isolate cDNA fragments on the system. Quantification and integrity of the amplified cDNA were assessed with an Agilent 2100 Bioanalyzer (Agilent Technology) using high sensitivity chips in accordance with the manufacturer's instructions. The libraries were subsequently equalized to 100 pmol/L. The Ion Chef System (Thermo Fisher) was used to deliver template preparation and chip loading. The libraries were delivered up to 400 bp. Sequencing was undertaken on an ION 540 chip for 160 cycles using an Ion 540 Kit-Chef (Life Technologies). All samples were sequenced on an Ion S5XL system (Thermo Fisher) in accordance with the manufacturer's instructions.

2.8 | Sequencing analysis

The obtained sequence data were imported to a CLC Genomic Workbench 7 (CLCbio) and analyzed. To normalize the measured reads, the resultant values were modified to RPM for the respective library. The normalized reads were annotated to miRBase version 21 with high priority.[33] Remaining reads were annotated to GtRNAdb and GRCh38.p12 as references.[34-36]

Selection criteria for small RNA as biomarker candidates were as follows: (i) found in more than 90% of each sample; (ii) mean RPM in the BC group was above 1000; and (iii) presence of a significant fold change (more than 2.0-fold change for upregulated small RNAs) compared to the N group.

2.9 | Statistical analysis

Statistical analysis was done using the JMP 12 software program (SAS Institute), with the data presented as mean RPM. Group comparisons were undertaken using the *t* test. Prediction performance of small RNAs was assessed using receiver operating characteristic curves and AUC. The diagnostic accuracy was defined as the proportion of true positive with disease and true negative without disease in total participants. Significance threshold was set at $P < .05$.

3 | RESULTS

3.1 | Participant characteristics

Figure 1 shows the flowchart for this study. Participants from each group were divided into 2 independent cohorts (BC [n = 39] vs N [n = 36]), one cohort for the selection of small RNA biomarker candidates (screening phase) and the second cohort for testing of

biomarker candidates (validation phase). Characteristics of participants are summarized in Table 1.

3.2 | Selection of circulating small RNA biomarker candidates

To determine differentially expressed circulating small RNAs in serum, we undertook small RNA sequencing using samples from the screening phase and compared the expression level of each small RNA identified between the BC and N group. The expression of 11 circulating small RNAs was significantly upregulated in the BC group compared to the N group and met the cut-off criteria that we set (Table 2). These small RNAs were then further evaluated in the validation phase as biomarker candidates.

3.3 | Validation of circulating small RNA candidates

The differential expression of small RNA candidates was confirmed by NGS profiling analysis of the independent cohort used for the validation phase. Among the 11 small RNA candidates, 3 achieved reproducibility of our selection criteria with significant *P* values, as shown in Figure 2. The expression levels of 1 canonical miRNA (miR-23a-3p) and 1 isomiR (isomiR of miR-21-5p [3′ addition C]) were significantly upregulated in the BC group compared to the N group. In addition, expression of tRF-Lys (TTT) was also significantly higher in the BC group. As no specific abbreviations have been established, we used temporary labels for the sake of convenience in this study, with tRF-Lys (TTT) for tRF having the sequence 5′-GCCCGGAUAGCUCAGUCGGUAGAGCAUCAGAC-3′.

3.4 | MicroRNA isoform abundance

Expression levels of canonical miRNA and isomiRs differed between the BC and N groups. Figure 3 shows the top 5 expression levels of the canonical miRNA and isomiRs of miR-21-5p and miR-23a-3p (Figure 3). For miR-21-5p, isomiR of miR-21-5p (3′ addition C) was more abundant in serum samples from the BC group, and expression differed between the BC and N groups. For miR-23a-3p, in contrast, canonical miR-23a-3p showed the greatest difference in abundance between 2 groups.

3.5 | Diagnostic accuracy of small RNAs

The diagnostic accuracy, including sensitivity and specificity, of each small RNA was assessed. As shown in Table 3, the combination of 3 small RNAs had the best diagnostic accuracy. Next, we established a discriminant model for BC detection with multiple regression analysis using the data obtained from the screening phase. The model was calculated as follows: Score = −44.23 + 1.407 * log2(RPM of isomiR of miR-21-5p) + 1.191 * log2(RPM of miR-23a-3p) + 1.396 * log2(RPM of tRF-Lys (TTT)). The cut-off point was set at 0. The potential of our model was evaluated using data from total participants. Receiver operating characteristic curves revealed an AUC value for the combination model of these 3 small RNAs of 0.92 (Figure 4A). Moreover, the diagnostic accuracy of this combination model could also successfully discriminate stage 0 breast cancer from the control group (Figure 4B). In addition, our model could detect BC irrespective of breast cancer subtype (Figure 4C). Immunohistochemical staining did not reveal any significant difference for discrimination of our model, with or without stain positivity (Figure 4D). Among 78 BC patients, the number of patients with CEA and CA15-3 levels above the normal limit was 1 (1.3%) and 3 (3.8%), respectively. In contrast, our constructed model showed a positivity of 92.0% (Figure 4C).

3.6 | Evaluation of EV characteristics

The TEM analysis revealed the isolated EVs had an exosome-like structure in the size of approximately 150 nm, within the expected range from the results of size distribution (Figure 5A). The isolated EVs had detectable CD9 and TSG101 as indicated by western blot analysis (Figure 5B). The size distribution of EVs is presented in Figure 5C; mode diameter was 89.7 nm (range, 75.9-403.7) in the BC samples and 87.8 nm (range, 76.1-368.6) in the N samples, suggesting that the main component of isolates were exosomes. The concentration of EVs (×10^{11} particles/mL) was 8.05 in the BC group and 3.07 in the N group (Figure 5D). Lipoproteins for potential contamination were also detected in isolated EVs (Figure 5E).

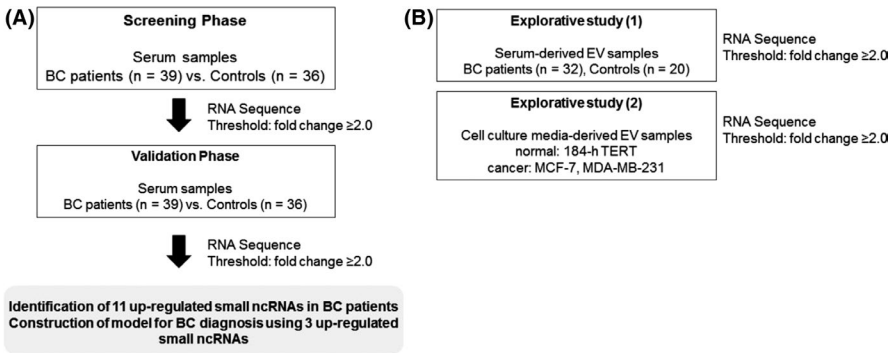

FIGURE 1 Schematic overview of this study into the diagnostic utility of circulating small RNAs for detection of breast cancer (BC). ncRNA, noncoding RNA

TABLE 1 Clinicopathologic characteristics of breast cancer patients (BC) (n = 39) and cancer-free individuals (N) (n = 36)

	Screening phase (n = 75)		Validation phase (n = 75)	
	BC (n = 39)	N (n = 36)	BC (n = 39)	N (n = 36)
Median age (range), years	57 (40-80)	65 (42-80)	50 (26-78)	62 (25-80)
Stage				
0	6 (15.4)	—	5 (12.8)	—
I	13 (33.3)	—	15 (38.5)	—
II	17 (43.6)	—	14 (35.9)	—
III	3 (7.7)	—	5 (12.8)	—
Histopathologic type				
Ductal carcinoma in situ	6 (15.4)	—	5 (12.8)	—
Invasive ductal carcinoma	32 (82.0)	—	34 (87.2)	—
Invasive lobular carcinoma	1 (2.6)	—	0 (0.0)	—
ER				
Positive	28 (84.8)	—	29 (85.3)	—
Negative	5 (15.2)	—	5 (14.7)	—
PR				
Positive	26 (78.8)	—	26 (76.5)	—
Negative	7 (21.2)	—	8 (23.5)	—
HER2				
Positive	2 (6.1)	—	3 (8.8)	—
Negative	31 (93.9)	—	31 (91.2)	—
Breast cancer subtype				
ER+HER2−	26 (78.8)	—	26 (78.8)	—
ER+HER2+	2 (6.1)	—	2 (6.1)	—
ER−HER2+	0 (0.0)	—	2 (6.1)	—
ER−HER2−	5 (15.2)	—	3 (8.8)	—

Note: Data are shown as n (%) unless otherwise indicated.

—, not applicable; ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; PR, progesterone receptor.

3.7 | Small RNA expression levels in serum EVs and EVs from cell culture media

To evaluate the expression levels of small RNAs within EVs, we analyzed small RNA profiling in EVs derived from serum. Among the 3 circulating small RNAs we identified as significantly upregulated in the serum of BC patients, 2 miRNAs were contained in EVs at a significantly high level in the BC group (all, $P < .01$). In contrast, tRF-Lys (TTT) showed no significant difference in expression (Figure 6).

To identify enriched small RNAs in EVs from cells, we profiled small RNA expression in EVs derived from the cell culture media. Extracellular vesicles from BC cells contained all 3 small RNAs. Compared to small RNA expression levels in EVs from 184-h TERT cell media, 2 miRNAs were more enriched in EVs from the BC cell media. Furthermore, tRF-Lys (TTT) levels were lower in EVs from the BC cell media than those from 184-h TERT cells (Figure 6).

4 | DISCUSSION

In this study, we identified discriminative small RNA expression in serum samples from BC patients using NGS. Comprehensive analysis revealed that the expression levels of 3 circulating small RNAs, namely isomiR of miR-21-5p (3′ addition C), miR-23a-3p, and tRF-Lys (TTT), were significantly upregulated in BC patients compared to cancer-free individuals, and might accordingly serve as novel diagnostic biomarkers for BC detection. Our constructed model using these 3 small RNAs provided high diagnostic performance with an AUC of 0.92. In addition, we evaluated EV small RNAs from serum

TABLE 2 Significantly upregulated small RNAs in serum of breast cancer patients (BC group) compared to controls (N group) in both screening and validation phases

			Screening phase				Validation phase			
Sequence	L	Small RNA	BC mean reads	N mean reads	FC	*P* value	BC mean reads	N mean reads	FC	*P* value
miRNAs										
UAGCUUAUCAGACUGAUGUUGAC	**23**	**IsomiR of miR-21-5p (3′ addition C)**	**1835**	**708**	**2.59**	**<.01**	**1722**	**841**	**2.05**	**<.01**
AUCACAUUGCCAGGGAUUUCC	**21**	**miR-23a-3p**	**2635**	**1248**	**2.11**	**<.01**	**2669**	**1077**	**2.48**	**<.01**
UUCAAGUAAUCCAGGAUAGGCU	22	miR-26a-1//miR-26a-2	1011	466	2.17	<.01	848	602	1.41	.02
GUCAGUUUGUCAAAUACCCCA	21	IsomiR of miR-223-3p (5′ deletion U)	2573	952	2.7	<.01	2752	1726	1.59	.02
GUCAGUUUGUCAAAUACCCCAA	22	IsomiR of miR-223-3p (5′ deletion U and 3′ addition A)	6644	2699	2.46	.01	7131	5012	1.42	.05
UGUCAGUUUGUCAAAUACCCCAA	23	IsomiR of miR-223-3p (3′ addition A)	22 652	11 286	2.01	.04	24 779	16 169	1.53	.02
tRFs										
GCGCCGCUGGUGUAGUGGUAUCAUGCAAGA	30	tRF-Gly (CCC)	62 251	28 598	2.18	<.01	59 129	57 599	1.03	.92
GCGCCGCUGGUGUAGUGGUAUCAUGCAAGAU	31	tRF-Gly (CCC)	48 196	7331	6.57	<.01	53 153	34 470	1.54	.07
GCGCCGCUGGUGUAGUGGUAUCAUGCAAGAUU	32	tRF-Gly (CCC)	78 465	12 471	6.29	<.01	85 170	53 442	1.59	.02
AGCAGAGUGGCGCAGCGGAAGCGUGCUGGGC	31	tRF-iMet (CAT)	37 525	11 628	3.23	<.01	40 779	29 984	1.36	.04
GCCCGGAUAGCUCAGUCGGUAGAGCAUCAGAC	**32**	**tRF-Lys (TTT)**	**19 234**	**6941**	**2.77**	**<.01**	**22 138**	**10 059**	**2.2**	**<.01**

Bold values indicate small RNAs which met our selection criteria (described in method section; sequence analysis) both screening and validation phase and thus have chosen them as biomarkers for BC detection.

Abbreviations: isomiR, miRNA isoform; miR, microRNA, miRNA, microRNA; tRF, transfer RNA fragment.

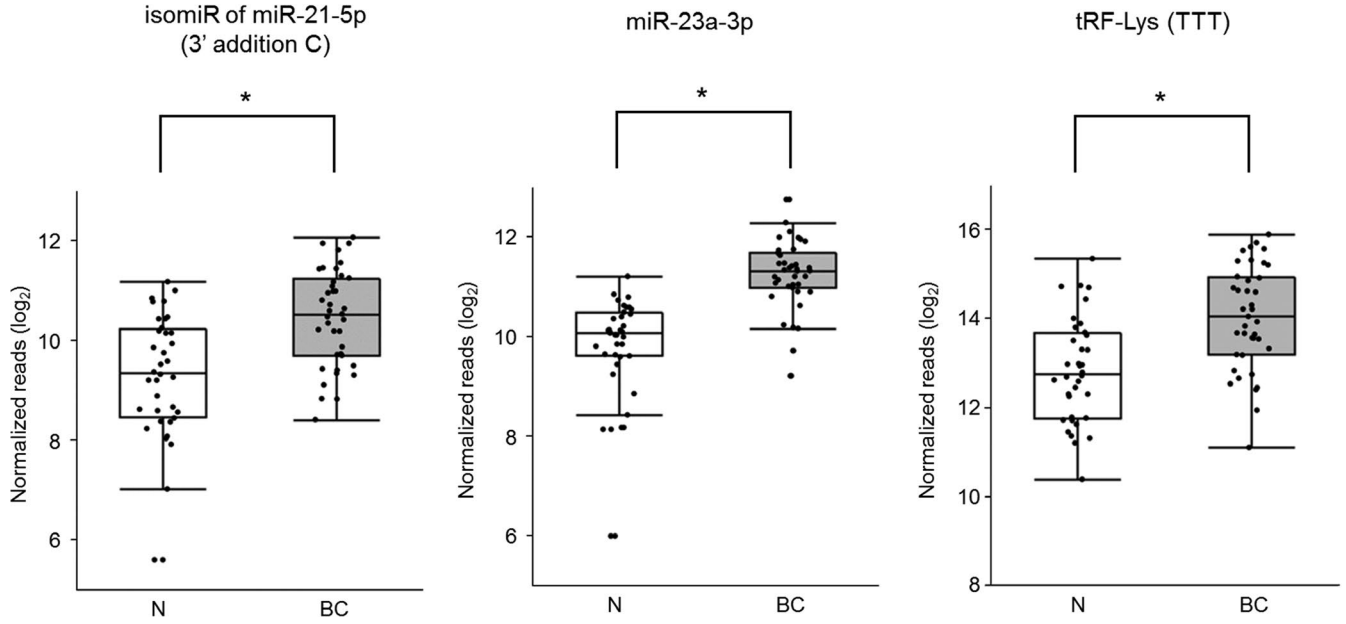

FIGURE 2 Comparison of normalized reads of 3 significantly upregulated small RNAs in the validation cohort (*P < .01) of breast cancer patients (BC, n = 39) and cancer-free individuals (N, n = 36). isomiR, microRNA isoform; miR, microRNA; tRF, transfer RNA fragment

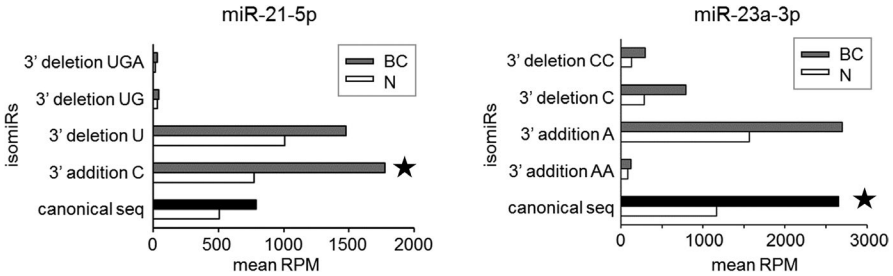

FIGURE 3 Comparison of microRNA isoform (isomiR) abundance in serum from breast cancer patients (BC, n = 78) and cancer-free individuals (N, n = 72). The 5 most abundant forms of microRNA (miR) are presented in the *y*-axis. A, miR-21-5p. B, miR-23a-3p. RPM, reads per million; seq, sequence

and cell culture media to identify the abundance of each RNA in EVs. Two of the 3 small miRNAs were more abundantly included in EVs of serum and cell culture media from BC compared to those from cancer-free individuals.

The association between dysregulation of miRNAs with cancer development is well established.[37] The use of miRNAs as cancer biomarkers is supported not only by their cancer-related biogenesis, but also by their high stability in peripheral blood. The use of a biofluid such as blood has the distinct advantages that it can be obtained non-invasively and repeatedly, and does not require organ-specific examinations such as mammography. Although the serum markers CEA and CA15-3 are clinically useful for BC monitoring, they are not recommended for BC detection because of their low sensitivity and specificity.[38] These factors warrant exploration for possible blood biomarkers for BC detection and explain the interest shown in circulating miRNAs.[39-42]

Recent studies have revealed that isomiRs are generated from miRNA processing of canonical miRNAs and that canonical miRNA is not always expressed as abundantly as isomiRs of the same family.[27] Moreover, several studies have reported that isomiRs have active roles and thus should not be ignored.[43-45] Attempts to evaluate the expression of individual isomiRs using hybridization methods such as microarray and PCR are made difficult by the shortness of isomiR sequences and their similarity to canonical miRNA.[46] In contrast, the NGS platform is not affected by these characteristics and facilitates the identification of isomiRs. Indeed, our study showed that isomiR abundance differed from that of the canonical miRNA and that expression levels in serum also differed between BC patients and cancer-free individuals.

MicroRNA-21-5p has been reported as an oncogenic miRNA related to cell proliferation and tumor invasion.[47] Increased expression of circulating miR-21-5p has been observed in various cancers, including BC, and is associated with lymph node metastasis or prognosis.[48,49] Although few studies have evaluated the expression of isomiRs, aberrant expression of the isomiR of miR-21-5p (3′ addition C) in tissue of colon cancer was recently reported.[46] The isomiR of miR-21-5p (3′ addition C) might not be BC-specific; however, our data raise the possibility that the significant difference in the isomiR of miR-21-5p (3′ addition C) in serum can serve as a precise biomarker for BC detection and warrants further functional investigation.

In addition to using increases in specific isomiR as a biomarker, we also found that canonical miRNA, miR-23a-3p in our study, is expressed abundantly in BC and also has biomarker potential.

TABLE 3 Diagnostic accuracy, sensitivity, and specificity in discriminating breast cancer (BC) patients from cancer-free individuals

	Screening phase			Validation phase		
Small RNA	Accuracy (%)	Sensitivity (%)	Specificity (%)	Accuracy (%)	Sensitivity (%)	Specificity (%)
IsomiR of miR-21-5p (3′ addition C)	80	76.9	83.3	69.3	53.8	86.1
miR-23a-3p	78.7	69.2	88.8	89.3	82.1	97.2
tRF-Lys (TTT)	77.3	74.4	80.6	72	71.8	72.2
Combination of 3 small RNAs above	89.3	92.3	86.1	89.3	79.5	100

Abbreviations: isomiR, miRNA isoform; miR, microRNA, tRF, transfer RNA fragment.

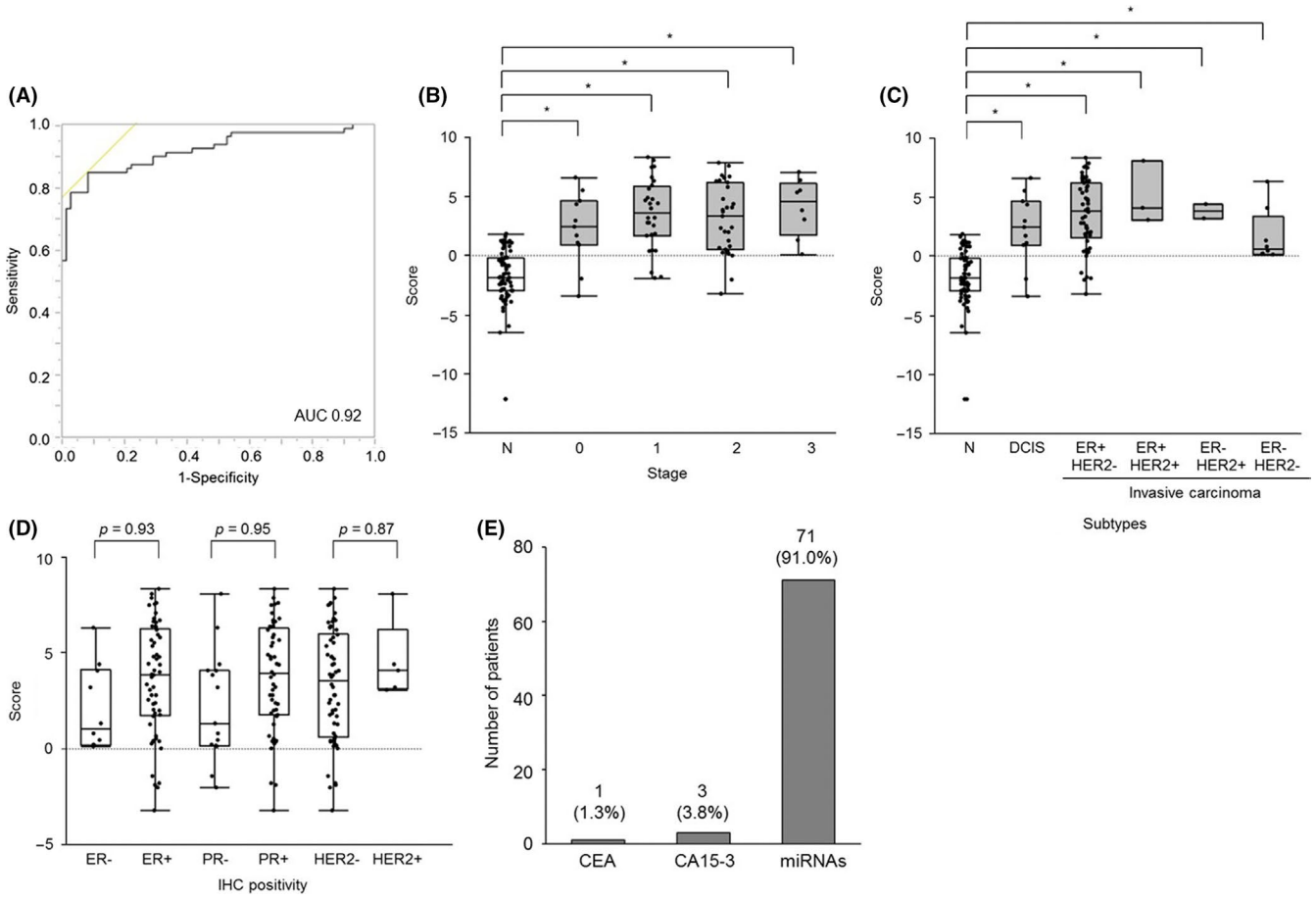

FIGURE 4 Diagnostic utility of 3 small RNAs for breast cancer (BC) detection. A, Analysis of the area under the receiver operating characteristic curve (AUC). B, Relative levels using small RNAs in BC patients and cancer-free individuals (N) by BC stage. *$P < .01$. C, Relative levels using small RNAs in BC patients and N by BC subtype. *$P < .01$. D, Relative levels using small RNAs in BC patients by immunohistochemical (IHC) analysis in patients with invasive BC. E, Comparison of diagnostic values among serum markers carcinoembryonic antigen (CEA) and carbohydrate antigen (CA)15-3, and our constructed model (C). DCIS, ductal carcinoma in situ; ER, estrogen receptor; HER2, human epidermal growth factor receptor 2; miRNA, microRNA; PR, progesterone receptor.

Upregulated miR-23a-3p has been observed in BC, and plays a role in cancer progression through the MAPK pathway.[50,51] Although the mechanism by which individual isomiRs work in the gene regulation network remains poorly understood, our results suggest the importance of comprehensive analysis of miRNA expression, including isomiRs, as a way to provide more accurate biological information than analysis of canonical miRNAs alone.

In addition, we also identified the importance of tRF-Lys (TTT) as a small RNA that displayed significantly high expression levels in BC patients. Transfer RNA fragments are a novel class of small RNAs that play regulatory roles in several biological processes and have been associated with cancer.[52] With the development of high-throughput sequencing technology, aberrant expression of tRFs has been identified in tissue and blood as well as miRNAs.[29,53] Of note, tRFs are the

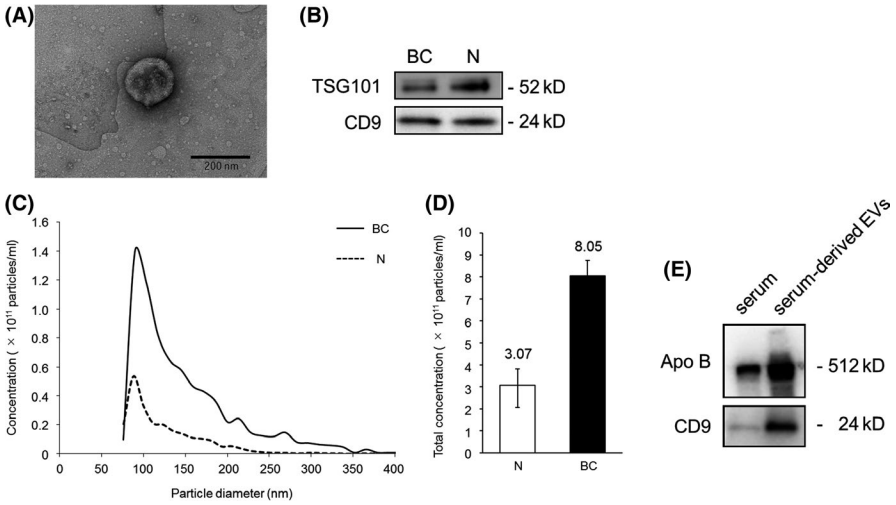

FIGURE 5 Characterization of extracellular vesicles (EVs) isolated with an exosome isolation kit. A, Transmission electron microscopy analysis of EVs. B, Western blot analysis of exosomal markers CD9 and TSG-101 in samples from breast cancer patients (BC) and cancer-free individuals (N). C, D, Contribution of nanoparticles of EVs (C), and total concentration of each EV (D). E, ApoB as a marker of lipoproteins by western blot analysis

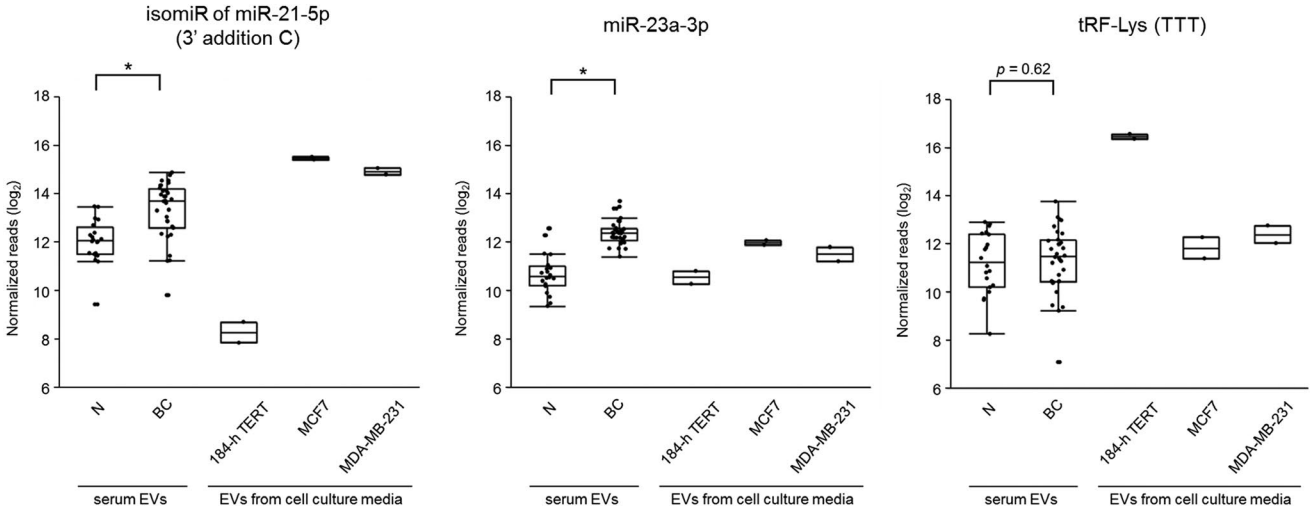

FIGURE 6 Expression of identified small RNAs in extracellular vesicles (EVs) derived from serum of 32 breast cancer patients (BC) and 20 cancer-free individuals (N), and from cell culture media of 184-h TERT, MCF7, and MDA-MB-231 cell lines. *$P < .01$

most abundant small RNA in serum, more than miRNAs.[54] Indeed, expression levels of circulating tRFs were more abundant than miRNAs and other ncRNA fragments in our study. Our study is the first to report the increased expression of tRF-Lys (TTT) in the serum of BC patients, and the significant difference in expression in these patients introduces tRF-Lys (TTT) as a novel actor that requires further exploration.

An important aspect of our study is its evaluation of small RNA profiles within EVs from serum and cell culture media. Considering EVs function as intercellular messengers, an increasing number of studies have evaluated EV miRNAs rather than miRNA in whole serum or plasma as cancer biomarkers.[55] Extracellular vesicles are released from various normal and cancer cells, and those in blood circulation are accordingly derived from various cell types.[56] Importantly, the composition of EVs are known to be origin-specific, providing the potential advantage of biomarkers.[57] We observed high concentrations of serum-derived EVs in BC patients compared to controls, as shown in Figure 5C. This result is supported by the finding that showed the increased number of EVs released from tumor cells into the bloodstream.[58] In addition, we found increased expression levels of the isomiR of miR-21-5p (3′ addition C) and miR-23a-3p in serum EVs from BC patients, albeit that we did not analyze EV small RNA in all serum samples. In this study, we calculated RPM values for the NGS analysis and thus we could not conclude whether the contributor of upregulated expression of 2 miRNAs in serum-derived EVs was due to an increase of loaded miRNAs per particle and/or an increased amount of EV secretion. It might be considered as an ideal biomarker for cancer detection that holds the concept of highly encapsulated small RNAs within EVs involved in oncogenesis at high concentration level; however, it must be taken into consideration that the occupancy level of small RNAs in EVs required to mediate communication remains unclear.[59] Considering the utility of diagnostic biomarkers using total serum, this might not impact on our findings that suggest the intriguing ability of 2 miRNAs as biomarkers for BC detection. Although we could not exclude the possibility that our identified small RNAs were not directly related to BC using serum EV samples,

our additional approach for small RNA profiling of EVs derived from cell culture media could complement the availability of 2 miRNAs based on their high expression level in BC cell lines. In contrast, our results also show that tRF-Lys (TTT) are not aberrantly included in EVs but circulate in serum. Further studies of small RNA expression in supernatant excluding EVs could help to describe the discrepant findings for tRF-Lys (TTT) between serum and EVs so that the different circulating form might be considered as the possible cause of inconsistency. However, we were unable to do this additional study due to the lack of serum sample volume and the technical inability to obtain supernatant after using the EV isolation kit.

Although the use of EV small RNAs in serum for BC detection appears warranted, practical adoption is hampered by the lack of uniform standards for the definition and isolation of EVs. Methods for EV isolation vary among studies, which could cause discrepant results and lack of reproducibility. Furthermore, practical application will require a simple and efficient system that can detect cancer without an additional EV isolation step. Clinical use therefore requires the construction of a distinctive model using circulating small RNAs, as in our study.

Several limitations of our study warrant mention. First, participant numbers were small and did not include patients with benign breast tumors. Confirmation of biomarker accuracy and the clinical utility of our diagnostic model therefore requires larger studies undertaken in multiple centers, including patients with benign breast tumors. Second, a study using both serum and tissue samples would be desirable, but we could not obtain tissue samples from participants suitable for experimentation. Further prospective studies are needed to evaluate the association between small RNA in serum samples and tissues. Additionally, functional studies of small RNAs could be further investigated to improve the robustness of our results. Finally, we used the commercially available isolation kit for EV isolation in our study. Ultracentrifugation is commonly used for EV isolation as the gold standard method in research setting[60]; however, we could not obtain sufficient blood samples for ultracentrifugation. Thus, we used the alternative method that works with small sample volumes for higher recovery of EVs. An important factor that should be considered for EV characterization isolated using the kit is lipoprotein, which might affect EV purification.[60] In our study, lipoprotein was detected in serum-derived EVs that could interfere the analysis of EV small RNAs. We acknowledge our method requires improvement for analysis of serum-derived EVs; however, as the TEM analysis and positive markers identified by western blotting revealed the presence of EVs and EVs derived from cell culture media were not affected by lipoproteins, our identified small RNAs in serum serve as novel biomarkers for BC detection.

In conclusion, this study found that small RNA expression in serum samples significantly differed between BC patients and cancer-free individuals. Comprehensive analysis using NGS identified 3 circulating small RNAs that showed significantly upregulated expression in BC, suggesting a novel biomarker for BC detection. Our constructed classifier based on expression levels of circulating small RNAs can distinguish BC with high diagnostic accuracy. The robustness of our diagnostic model was enhanced by further investigation of EV small RNAs in serum. Even with limited data from only 2 BC cell lines, analysis of EV expression in cells allowed us to construct a reliable model comprising circulating small RNAs.

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CONFLICT OF INTEREST

Professor Hidetoshi Tahara is representative director of a university-originated venture, MiRTeL Co. A family member, Kanoko Tahara, is an employee of MiRTeL Co. The remaining authors declare no potential conflict of interest.

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