Oncogenic miRNAs Identified in Tear Exosomes From Metastatic Breast Cancer Patients

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Abstract. *Background/Aim:* Exosomes are produced by normal and cancer cells. Exosomes are found in the serum of cancer patients and have been used for diagnosis and prognosis. Recently tears from non-cancer patients have been found to contain exosomes. In the present report we describe tears from advanced breast-cancer patients. *Materials and Methods:* We found oncogenic miRNAs in the exosomes isolated from tear fluids obtained from five patients with metastatic breast cancer and compared them with tear exosomes from eight healthy volunteers. *Results:* Tear exosomes had a significantly higher quantity of exosome markers than serum exosomes (CD9, CD63). Tear exosomes were subjected to quantitative reverse-transcription polymerase reaction (qRT-PCR), and western blot analysis to elucidate the status of miRNAs, previously reported in serum from patients with metastatic breast cancer. qRT-PCR and western-blot analysis revealed that breast-cancer-specific miR-21 and miR-200c were highly expressed in tear exosomes from metastatic breast cancer patients in contrast to tear exosomes from healthy volunteers. *Conclusion:* Tear exosomes can be a potential source of diagnostic and prognostic biomarkers for metastatic breast cancer, and possibly other cancers or diseases.

Exosomes are nanometer-sized (30-150 nm) extracellular vesicles that are secreted from various cells and may carry proteins, lipids, miRNAs and mRNAs within them (1, 2). In recent years, exosomes have been highlighted as an important biomarker for various diseases and mediators for cell-to-cell communication during progression, invasion, metastasis, and recurrence in many cancers (3-5). Exosomes are found in various body fluids such as plasma, serum, urea, saliva, and tears (6-9). Therefore, exosomes hold potential for being biomarkers for early detection of diseases and their prognosis. Among all body fluids, tears are the most suitable source for exosome isolation because they have less protein and debris when compared to blood and can be isolated non-invasively.

Previously, tear exosomes have been used for the diagnosis of eye diseases (10). The present report is the first to characterize tear exosomes from cancer patients. Previously there was one report that characterized tear exosomes in multiple sclerosis (11). Due to the ease of tear collection and its non-invasive nature, we explored the potential of tears for breast cancer. We isolated tear exosomes from metastatic breast cancer patients and normal volunteers and analyzed oncogenic miRNAs, such as miR-21, 200c, 1246, 17-5p and 373, that have previously been detected in serum exosomes of breast cancer patients (Table I). Our results showed that exosomes from tears of breast cancer patients can be a potential source of cancer biomarkers.

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Materials and Methods

Study population. We recruited 5 patients with metastatic breast cancer (mean age=58.6 years; age range=44-73 years) and 8 healthy volunteers (mean age=27 years; age range=21-38 years) (Table II) and collected their tear samples. Four out of five patients had distant metastases after surgery, while one patient had progressive unresectable cancer. All the patients were undergoing chemotherapy

Table I. *miRNAs proposed for early detection and prognosis of breast cancer prognosis.*

micro RNA	Year published	miRNA-samples from breast cancer	Dysregulation	Reference
miR-21	2016	Plasma-exosome	Up-regulation	(15)
	2018	-	Up-regulation	(20)
	2018	Plasma	Up-regulation	(18)
	2018	Plasma	Up-regulation	(21)
	2019	Plasma	Up-regulation	(22)
	2006	Tissue	Up-regulation	(23)
miR-200c	2017	Plasma	Up-regulation	(19)
	2019	Plasma	Up-regulation	(22)
miR-17-5p	2017	Serum-exosome	Up-regulation	(14)
	2006	Tissue	Up-regulation	(23)
	2017	Tissue, cell line	Up-regulation	(24)
	2019	Tissue	Up-regulation	(25)
miR-373	2014	Serum	Up-regulation	(13)
		Serum-exosome		
	2013	Serum	Up-regulation	(26)
	2008	Tissue	Up-regulation	(27)
miR-1246	2016	Plasma-exosome	Up-regulation	(15)
	2016	Serum	Up-regulation	(28)
	2017	Tissue	Up-regulation	(29)

or endocrine therapy. All the patients were estrogen receptor-positive and human epidermal growth factor receptor 2 (HER2)-negative. Three patients had invasive ductal carcinoma, while one had invasive lobular carcinoma. One of the healthy volunteers provided both tears and blood. All patients and healthy volunteers had signed an informed consent to participate in the study which was approved by the Ethics and Scientific Committee of Kobe University, Japan.

Tear fluid collection. Tears were collected from both eyes by placing a Schirmer tear test strip (Eagle Vision TN, USA) on each eye, for 5 min or more. Subsequently, the Schirmer strips were transferred to 1-5 ml of phosphate-buffered saline (PBS) (Nacalai Tesque, Kyoto, Japan) and kept overnight at 4°C (10).

Exosome purification from tear fluid. To remove debris and the other large impurities, samples were centrifuged at $2,000 \times g$ for 15 min at 4°C. Subsequently, the exosomes were precipitated by ultracentrifugation at $100,000 \times g$ for 90 min at 4°C. Finally, exosomes were washed once with PBS and re-precipitated by ultracentrifugation at $100,000 \times g$ for 90 min at 4°C (Figure 1A). The resultant exosomes were measured for their protein content using Qubit Protein Assay Kits with a Qubit3.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA).

Western blot. Equal amounts of exosomes were lysed in RIPA buffer and western blot analyses were performed as described previously (12). The following antibodies were used to detect the exosomes: anti-CD9 antibody (sc-59140; Santa Cruz Biotechnology, Dallas, TX, USA), anti-CD63 antibody (557305; BD, Franklin Lakes, NJ, USA).

Table II. *Clinical characteristics of breast cancer patients and healthy volunteers in the study.*

	Patients	Healthy volunteer
Total	5	8
Age	44-75	21-38
Operation	4	-
Metastatic	5	-
Estrogen receptor status		
Positive	5	-
Negative	0	-
HER2 status		
Positive	0	-
Negative	5	-

Extraction of micro RNAs and qRT-PCR. Micro RNAs were extracted from tear exosomes using QIAzol and an miRNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The RNAs was quantified using Qubit microRNA Assay Kits with a Qubit 3.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) and an Agilent Bioanalyzer 2100 with an RNA6000 Pico kit (Agilent Technologies, Inc., Santa Clara, CA, USA).

For qRT-PCR analysis, cDNAs were generated from total RNA using a TaqMan® MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific Inc.) according to the manufacturer's protocol. The cDNA samples were stored at -20°C until further use. Real-time PCR was performed in triplicate with diluted cDNAs using a Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA). Based on published literature on their constitutive expression, miR-16-5p and syn-cel-39, were used for data normalization. The relative expression of each miRNA was calculated using the $2^{-\Delta\Delta C_t}$ method (13, 14).

Statistical analyses. The Mann-Whitney *U*-test was used to determine the differential expression of miRNAs between the two groups. Statistical analyses were performed using Statcel 4 Software (OMS Publishing Inc., Tokyo, Japan).

Results

Exosome isolation from tears. Tear exosomes from healthy volunteers were detected with anti-CD9 and anti-CD63 antibodies with western blotting. Tear exosomes had a significantly higher expression of exosome markers (CD9, CD63) than serum exosomes (Figure 1B).

Micro RNA isolation from tear-derived exosomes. Total RNA extracted from tear exosomes was analyzed using the Agilent RNA6000 Pico kit (Figure 1C). The majority of exosome RNA content comprised of small RNAs (<200 nt), indicating that tear exosomes are enriched in short RNAs. Ribosomal RNA was not detected in tear exosomes.

Figure 1. Analysis of protein and miRNA from the tear exosomes. (A) Experimental design for the isolation of tear exosomes (Tear-Exo). (B) Amount of exosomal markers, CD9 and CD63, in tear and serum exosomes (Serum-Exo) fractions. (C) Analysis of miRNA from tear exosomes with a Bioanalyzer 2100.

Figure 2. Relative levels of miRNA levels in tear exosomes of patients and healthy volunteers. Levels of miR-21, 200c, 17-5p, 373 and 1246 in tear exosomes were determined evaluated in Tear-Exo by qRT-PCR and assessed by the $2^{-\Delta\Delta C_t}$ method. The data were statistically analyzed using the Mann-Whitney test and the results displayed as box plots. Horizontal lines depict median value. The height of the boxes is the interquartile range represent the values between the 75th and 25th percentiles of individual fold changes.

Comparison of miRNAs from tear exosomes from patients with metastatic breast cancer and healthy volunteers. To determine whether breast cancer-specific microRNAs could be detected from tear exosomes, we analyzed the amount of miR-21, 200c, 1246, 17-5p, and 373 from tear exosomes from patients with metastatic breast cancer and healthy controls. The relative amount of miR-21 and 200c from breast-cancer patient tear exosomes was significantly higher than that of healthy controls. The relative amount of miR-1246, 17-5p and 373 did not show a statistically significant difference between patients and healthy controls (Figure 2).

Discussion

The present study is the first to analyze tear exosomes from cancer patients. Tears contain little sugar and have a different protein composition compared to serum (30). In the present report we observed that tear exosomes had a significantly higher content of exosome markers (CD9, CD63) than serum exosomes. In the future we plan to further characterize tear exosomes and compare them with serum exosomes from healthy volunteers and breast cancer patients. Many factors make tears suitable for diagnostic purposes; they include ease of collection, non-invasive

procedures, and feasibility to obtain sufficient quantity of the sample. However, thus far, there has been no report on the use of tears from cancer patients for diagnostic or prognostic purposes. Although tear proteins have been analyzed in cancer patients (31), the present study is the first to analyze tear exosomes in cancer-patients. We expect in the near future tear exosomes will be used more often to identify biomarkers for cancer patients. Our results showed that the amount of miR-21, and miR-200c were significantly higher in tear exosomes isolated from patients with metastatic breast-cancer than those of healthy controls. These findings establish tears as a useful material for identifying potentially diagnostic and prognostic biomarkers for metastatic breast cancer. It has previously been reported that the human plasma samples from breast cancer patients carry a higher load of miR-21 than healthy controls (15). Further, a large number of studies have examined the function of miR-21 as a biomarker for cancer detections (16-18). MiR-200c has been shown to be differentially expressed in metastatic breast cancer (19). In our study, we observed that significantly higher level of miR-200c in tear exosomes obtained from breast cancer patients than from healthy controls. Another advantage of using tears for diagnostic purposes is that less purification is required. Since tears contain comparatively less protein and cellular debris than other body fluids, they have a shorter sample-processing time. For instance, in the present study, despite having a low sample volume, we needed just three steps before the samples could be used for qRT-PCR.

Although tears have certain advantages over other types of samples, they may be difficult to collect from elderly patients and patients with dry-eye syndrome. In such patients, eye-drop assisted tears might be useful, but need experimental validation. Although the results from the present study are very interesting and hold promise, their validity is limited by the small number of samples used in this study. In the future, we will analyze a larger number of tear exosomes at each stage of breast cancer to better determine diagnostic and prognostic markers and combinations. In the future, we will also perform electron microscopy of tear exosomes.

Conflicts of Interest

The Authors declare no competing financial interests.

Authors' Contributions

S.I., and R.S designed the study, performed the experiments, collected and analyzed the data and wrote the paper; S.M., M.B., T.K. and H.T. collected tear samples from patients; H.K., Y.K., T.T., and R.M.H gave technical support and conceptual advice.

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