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Zwitterionic polymer-coated immunobeads for blood-based cancer diagnostics

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ABSTRACT

Both total plasma and tumor-derived microvesicle (TMV)-associated miRNAs have been proposed as potential blood-based biomarkers for cancer diagnosis. However, there has been no comparison of the two types of miRNAs for biomarker discovery because of technological challenges of isolating TMVs from human plasma. The effective isolation of TMVs can be hardly achieved with conventional immunobead-based methods due to the high content of plasma proteins. In the current study, zwitterionic sulfobetaine-conjugated immunobeads are prepared using cluster of differentiation 83 (CD83) as a candidate protein marker for breast cancer-derived microvesicles. The zwitterionic immunobeads are more than 10-fold efficient for isolating TMVs from clinical plasma samples by suppressing nonspecific protein binding than conventional immunobeads. Early-stage breast cancer can be distinguished from benign breast disease by using the sulfobetaine-modified immunobeads, whereas conventional immunobeads show poor discriminatory performance. Further, we demonstrate that miRNAs in the form of TMVs offer a major improvement over total plasma miRNAs for early cancer detection. The analyses of miRNA expression levels show that in total, 6 miRNAs are significantly upregulated in the CD83-positive microvesicles of breast cancer patients, whereas differential miRNA expression is not detected on using total plasma RNA. The results indicate that our zwitterionic immunobead platform may constitute a powerful tool to identify circulating biomarkers and open a new avenue for highly sensitive blood-based cancer diagnostics.

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1. Introduction

Minimally invasive cancer biomarkers are greatly required for routine clinical practice, and expression signatures of circulating miRNAs are emerging as novel biomarkers. The high stability of miRNAs in body fluids holds great advantages in a clinical setting and has been attributed to the association of miRNAs with microvesicles, Argonaute 2 (Ago2), or high-density lipoprotein (HDL).

Although a close correlation between circulating miRNAs and tumor cells has been suggested, it has been reported that these miRNAs may also be released from various types of normal cells, including blood cells. Pritchard et al. [1] reported that the expression of circulating miRNAs was highly correlated with the miRNA signatures of blood cells, which implies that differentiation of cancer-derived miRNAs from a mixed population of miRNAs is crucial for the discovery of circulating miRNA biomarkers. Therefore, we believe that analyses of tumor-derived microvesicles (TMVs) containing miRNAs offer advantages over miRNA tests of whole unfractionated body fluids.

Microvesicles were first observed in cancer patients in the late 1970s [2]. Thirty years later, several research groups reported that TMVs had distinct miRNA and protein signatures depending on the cancer type [3–5] and that these unique biosignatures made TMVs an ideal candidate as a circulating cancer biomarker. However, TMV isolation is not efficient because TMVs represent a relatively low fraction of microvesicles in body fluids in which other proteins and normal cell-derived microvesicles are present. Moreover, there are no well-defined marker proteins that allow the separation of TMVs from normal vesicles. Mitchell et al. [6] reported difficulty in isolating TMVs from other microvesicles in prostate cancer patients. In a pilot-scale study, Li et al. [7] demonstrated that plasma microvesicles expressing claudin-4, which is often elevated in ovarian tumor cells, were detected in only 51% of ovarian cancer patients. For the same patient samples, CA125 tests demonstrated a

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sensitivity of 71%. Therefore, there is a critical need for efficient capture platforms that would allow the detection of low-abundance TMVs and could be utilized for the discovery of microvesicular protein markers.

There has been a plethora of studies to develop surfaces that are resistant to nonspecific protein adsorption. In recent years, zwitterionic polymers have emerged as superior antifouling materials [8–10]. However, most studies demonstrated the antifouling functionalities of polymer-coated surfaces using serum or single protein solutions, which are much less fouling than plasma; furthermore, none of the studies validated the antifouling properties with undiluted clinical samples.

In this study, we aim to use immunobeads coated with sulfobetaine zwitterionic moieties for isolating TMVs from the plasma of early-stage breast cancer patients. The amounts and miRNA expression signatures of TMVs captured by zwitterionic immunobeads were evaluated to answer circulating miRNAs in the form of TMVs could offer any improvement over total plasma miRNAs as potential diagnostic biomarkers.

2. Materials and methods

2.1. Clinical specimens

Plasma specimens were obtained before initiation of chemotherapy from patients diagnosed with breast disease between February 2011 and June 2012 at Yonsei University College of Medicine. Authorization for the use of the plasma samples for research was obtained from the Institutional Review Board of Severance Hospital, Yonsei University College of Medicine. Clinical and molecular-pathologic information of these samples is provided in Table 1 (details regarding the clinical data are presented in Table S1).

2.2. Plasma preparation

For plasma preparation, peripheral blood (10 mL) was drawn into a K2E Vacutainer tube (BD 367525, Becton Dickinson, Franklin Lakes, NJ). Within 4 h, plasma was obtained by centrifugation at 1300 ×*g* in an Eppendorf-5810R centrifuge with an A-4-81 rotor (Motor Parkway Hauppauge, NY) for 10 min at 4 °C and kept at −80 °C. Before use, frozen plasma was thawed on ice and centrifuged again at 3000 ×*g* for 5 min at 4 °C to remove floating debris.

2.3. Microvesicle isolation by using sucrose gradient ultracentrifugation

Flotation and isolation of microvesicles on a sucrose gradient was performed as previously described [11]. Plasma from 10 patients (5 mL each; patient age, 38–63 years, Table 1 and Table S1) was mixed together, and the mixed plasma (50 mL) was then diluted into 150 mL of PBS. To remove large membrane fragments and other debris, the diluted plasma was centrifuged at 2000 ×*g* for 30 min at 4 °C and subsequently at 14,000 ×*g* for 30 min at 4 °C. The supernatant was then filtered through a 0.22-μm membrane (cat. 162-0020, Thermo Scientific) and ultracentrifuged at 110,000 ×*g* for 120 min at 4 °C in a Sorvall MTX 150 micro-ultracentrifuge (Thermo Scientific) with an S-50A rotor. Pellets collected by differential centrifugation were fractionated by flotation on a continuous sucrose gradient (0.25–2 M in 20 mM HEPES, pH 7.4) at 210,000 ×*g* for 16 h at 4 °C; an SW41 Ti rotor (Beckman) was used. After centrifugation, 12 fractions were collected from the top of the tube, and the density of each fraction was measured using a refractometer (PAL-RI, Atago). For western blot and DLS analyses, all fractions were washed with PBS and concentrated up to 100 μL by using a centrifugation filter with a cutoff of 100 kDa (UFC910024, Millipore).

2.4. Bead preparation

The preparation of SAIT beads was conducted using sulfobetaine moieties [12]. Dynabeads® M-270 amine solution (100 μL, Life Technologies) was placed into an Eppendorf tube and washed with MES buffer containing NaCl (pH 6.0). A PAA solution was prepared in MES buffer, and EDC and NHS were added to the solution as coupling agents. The beads were then reacted in a rotator at RT. After incubation, the beads were washed with MES buffer (pH 6.0) and resuspended in MES buffer containing EDC and NHS. After the activation step, the beads were washed with MES buffer and reacted with protein G solution in MES buffer. After reaction, sulfobetaine (1.2 mg) was added to the solution and incubated. After incubation, the beads were washed with PBST and PBS. Anti-CD83 stock solution was prepared in PBS solution (pH 7.4) and diluted with NaOAc (pH 5.0). The beads that bound protein G were incubated with anti-CD83 solution (80 μg). The beads were then washed with sodium borate buffer (pH 9.3), and the antibody was crosslinked to protein G using DMP (dimethyl pimelimidate dihydrochloride) dissolved in sodium borate buffer (pH 9.3). The reaction was terminated by washing and incubating the beads with ethanolamine. After the quenching step, the beads were washed with PBST and PBS. The prepared beads were washed with PBS and stored in PBS at 4 °C until use.

Table 1
Summarized clinical-pathological features of patients with benign breast disease and breast cancer.

Charateristics samples (n)		Age (year)		Histologic subtype			ER status[a]		PR status[a]		HER2 status[b]		Ki67 status (<14%)	
		Mean	Median (range)	IDC	ILC	Others[c]	Positive	Negative	Positive	Negative	Positive	Negative	Positive	Negative
Overall	T (32)	51.6	50 (35–78)	28	1	3	32	0	18	14	9	23	2	30
	B (30)	44.9	44 (21–59)	–	–	–	–	–	–	–	–	–	–	–
	Gradient ultracentrifugation set													
	T (10)	50.6	50 (38–63)	10			10	0	4	6	2	8	0	10
	B (0)	–												
Study using total plasma	miRNA analysis set													
	T (29)	50.2	49 (35–78)	25	1	3	29	0	17	12	8	21	2	27
	B (25)	43.9	44 (21–59)	–	–	–	–	–	–	–	–	–	–	–
	Bead comparison set													
	T (15)	54.3	54 (37–78)	14	1		15	0	9	6	6	9	1	14
	B (15)	41.5	43 (21–56)	–	–	–	–	–	–	–	–	–	–	–
Study using exosome presenting CD83	Protein analysis set													
	T (30)	51.1	49 (35–78)	26	1	3	30	0	18	12	8	22	2	28
	B (30)	44.9	44 (21–59)	–	–	–	–	–	–	–	–	–	–	–
	miRNA analysis set													
	T (29)	50.2	49 (35–78)	25	1	3	29	0	17	12	8	21	2	27
	B (29)	45	44 (21–59)	–	–	–	–	–	–	–	–	–	–	–

[a] Positive for ER or PR if finding of ≥1% of tumor cell nuclei are immunoreactive.
[b] HER2 immunohistochemistry score (IHC) <3 was defined as HER2 negative and 3 as definitely positive. An IHC score equal to 2 was further analyzed by FISH/CISH and deemed positive if HER2 was amplified.
[c] Multifocal IDC, multiple IDC and mucinous cancer were included.

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