Clinical Cancer Research

Identification of Developmental Endothelial Locus-1 on Circulating Extracellular Vesicles as a Novel Biomarker for Early Breast Cancer Detection

Pyong-Gon Moon[1], Jeong-Eun Lee[1], Young-Eun Cho[1], Soo Jung Lee[2], Jin Hyang Jung[3], Yee Soo Chae[2], Han-Ik Bae[4], Young-Bum Kim[5], In-San Kim[6], Ho Yong Park[3], and Moon-Chang Baek[1]

Abstract

Purpose: Currently, there are no molecular biomarkers for the early detection of breast cancer. This study focused on identifying surface proteins found on circulating extracellular vesicles (EVs) for detecting early-stage breast cancer.

Experimental Design: Circulating EVs, isolated from the plasma of 10 patients with breast cancer (stages I and II) and 5 healthy controls, were analyzed using LC-MS/MS. Developmental endothelial locus-1 protein (Del-1) was selected as a candidate biomarker. Two different ELISAs were used to measure Del-1 in plasma samples from healthy controls ($n = 81$), patients with breast cancer ($n = 269$), breast cancer patients after surgical resection ($n = 50$), patients with benign breast tumors ($n = 64$), and patients with noncancerous diseases ($n = 98$) in two cohorts.

Results: Plasma Del-1 levels were significantly higher ($P < 0.0001$) in patients with breast cancer than in all controls and returned to almost normal after tumor removal. The diagnostic accuracy of Del-1 was AUC, 0.961 [95% confidence interval (CI), 0.924–0.983], sensitivity of 94.70%, and specificity of 86.36% in test cohort and 0.968 (0.933–0.988), 92.31%, and 86.62% in validation cohort for early-stage breast cancer by one type of ELISA. Furthermore, Del-1 maintained diagnostic accuracy for patients with early-stage breast cancer using the other type of ELISA [0.946 (0.905–0.972), 90.90%, and 77.14% in the test cohort; 0.943 (0.900–0.971), 89.23%, and 80.99% in the validation cohort].

Conclusions: Del-1 on circulating EVs is a promising marker to improve identification of patients with early-stage breast cancer and distinguish breast cancer from benign breast tumors and noncancerous diseases. *Clin Cancer Res; 22(7); 1757–66. ©2015 AACR.*

Introduction

Breast cancer, the most common type of cancer in women, causes up to 500,000 deaths yearly worldwide (1–3). Although cancer diagnosis and treatment have advanced in recent decades, only some molecular biomarkers, such as autoantibodies, with poor values, for the early detection of breast cancer have been reported (4–6). Identifying a biomarker to detect breast cancer at an early stage will enable the use of less-aggressive treatments and improve clinical outcomes (7). CA 15-3 is the most widely used serum marker in patients with metastatic breast cancer but has a sensitivity of only 60% to 70% (5, 8, 9). Recently, various methods have been reported to detect circulating tumor cells, which are a promising biomarker (10, 11). The FDA has approved the CellSearch system with a sensitivity of about 65% for detecting circulating tumor cells ($\geq$1 cell per 7.5 mL blood) in metastatic breast cancer patients (10, 12). Furthermore, measuring circulating tumor DNA showed superior sensitivity to measuring other circulating biomarkers to monitor metastatic breast cancer and provided the earliest index of treatment response (13, 14).

It is traditionally accepted that metastasis occurs at a late stage of cancer progression (15). However, metastatic dissemination of cancer cells can occur in preinvasive stages of tumor progression in mouse models of breast cancer and in human patients (16–18). This suggests that systemic spread is an early step in breast cancer and implies that unknown systemic factors secreted from the primary tumor may prepare a premetastatic niche and promote early cancerous colonies.

Extracellular vesicles (EVs) are membranous vesicles secreted by various cells. EVs have been classified into several subcategories, including exosomes (50–100 nm in diameter) and microvesicles (100-1,000 nm in diameter; refs. 19, 20). Cancer cells, and neighboring cells in the tumor microenvironment, secrete EVs

[1]Department of Molecular Medicine, Cell and Matrix Research Institute, School of Medicine, Kyungpook National University, Daegu, Republic of Korea. [2]Department of Oncology/Hematology, Kyungpook National University Hospital, Daegu, Republic of Korea. [3]Department of Breast and Thyroid Surgery, Kyungpook National University Hospital, Daegu, Republic of Korea. [4]Department of Pathology, Kyungpook National University Hospital, Daegu, Republic of Korea. [5]Division of Endocrinology, Diabetes and Metabolism, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, Massachusetts. [6]Department of Biochemistry and Cell Biology, School of Medicine, Kyungpook National University, Daegu, Republic of Korea.

Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

P.-G. Moon and J.-E. Lee contributed equally to this article.

Corresponding Author: Moon-Chang Baek, Kyungpook National University, 101 Donging-Dong, Jung-Gu, Daegu 700-422, Republic of Korea. Phone: 82-53-420-4948; Fax: 82-53-426-4944; E-mail: mcbaek@knu.ac.kr

doi: 10.1158/1078-0432.CCR-15-0654

Translational Relevance

Breast cancer is the leading cause of death worldwide and is the most common type of cancer in women. Molecular biomarkers for early detection of breast cancer have not been reported, although survival rates for breast cancer increase with early diagnosis. Using two different ELISAs, the diagnostic performance of Del-1 for breast cancer detection was 84% to 99% for sensitivity and 74% to 86% for specificity. The level of Del-1 on circulating extracellular vesicles (EVs) was significantly elevated at the earliest stage of breast cancer and dropped after surgical resection. The minimally invasive method with high sensitivity and specificity based on the molecular biomarker, Del-1–positive circulating EVs, is promising for the early diagnosis of breast cancer using only one drop of blood.

that play important roles in pro-metastatic signaling, angiogenesis, and immune suppression in an autocrine or paracrine manner (21–24). Cancer cell–derived EVs may have the potential to be used as early biomarkers for various cancers because the membrane vesicles are secreted continuously into body fluids, including blood, from an early stage of the disease (25, 26).

Based on this background information, we hypothesized that EV proteins can be used as early diagnostic biomarkers for breast cancer. We employed a proteomic approach to identify Del-1 as disease-specific proteins on circulating EVs isolated from the plasma of patients with early-stage breast cancer. We then confirmed that the plasma level of Del-1, measured by two different ELISAs, correlated with the presence of breast cancer.

Patients and Methods

Study design and participants

The study design used to identify biomarkers of early-stage breast cancer is illustrated in Fig. 1 and Supplementary Fig. S1. Plasma samples for test set were obtained from the Kyungpook National University Hospital, Daegu, Korea. An independent validation set was obtained from the Chonnam National University Hwasun Hospital, Hwasun, Korea. The histologic cell type and stage of breast cancer of the patients studied in test and validation sets are provided in Table 1. Plasma was obtained from freshly drawn blood using plasma separate tube. Aliquots of each plasma were deep-frozen at storage using liquid nitrogen. All plasma samples were transported to laboratory on dry ice within 24 hours. For analysis, plasma samples were thawed at 4°C. Thawed plasma samples were stored at 4°C for triplicate analysis within 2 weeks. Plasma samples were shaken at 1,500 rpm, 5 minutes right before ELISA. These processes were same for cases and controls.

To identify early biomarker candidates for breast cancer, circulating EVs from 5 patients with stage I breast cancer, 5 with stage II breast cancer, and 5 healthy controls (discovery set, group 1) were isolated, characterized (Supplementary Fig. S2), and analyzed using LC-MS/MS. Del-1 was considered potential biomarker for breast cancer. Plasma samples from 169 patients with breast cancer and 35 healthy controls (test set, group 2) were used to test differential expression of diagnostic marker candidates using two different ELISA methods. We mixed cases and age-matched controls in every plate, and each plate contained standard samples as

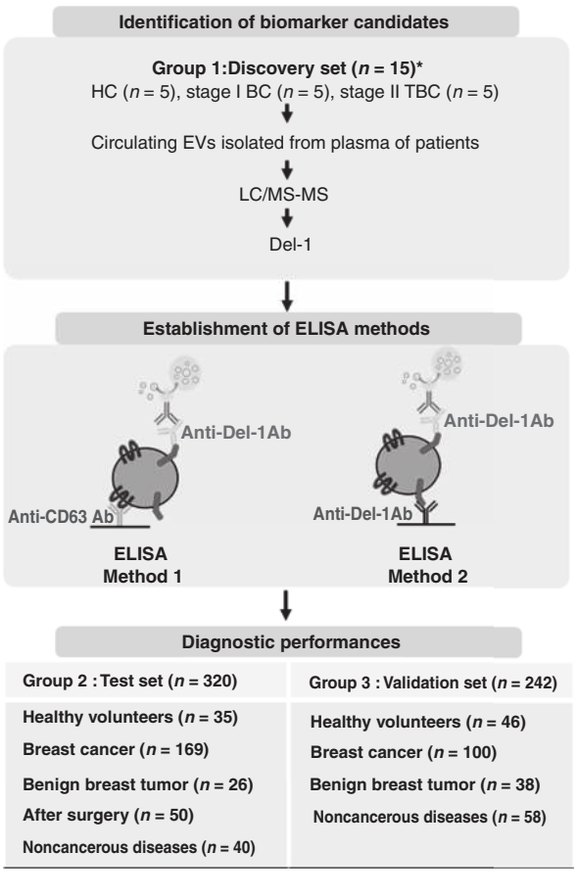

Figure 1.
Overview of the study design. Circulating EVs isolated from plasma of healthy control patients ($n = 5$) and patients with early-stage breast cancer (BC; $n = 10$) were analyzed using LC-MS/MS to identify early biomarker candidates. Biomarker candidates among proteins that were upregulated in breast cancer patients compared with controls were selected based on three filtering criteria (Fig. 2A). Del-1 was evaluated as a novel biomarker for the early detection of breast cancer using plasma (1 μL) from test cohort ($n = 320$) and validation cohort ($n = 242$) by two types of ELISA with different primary capture antibodies. *, Discovery set is included in the test set.

described previously to reduce the variation between plates. Group 2 contained 26 patients with benign breast tumors, 50 breast cancer patients after surgical resection, and 40 patients with noncancerous diseases (thyroiditis, gastritis, hepatitis B, and rheumatoid arthritis). The validation study was conducted with a rigorous predefined statistical analysis plan with prespecified outcome. All validation cohorts were analyzed in a blinded fashion. Unblinding of clinical parameters and corresponding experimental data was performed only after finishing all experiments. To validate biomarker candidates for breast cancer, Del-1 levels were measured using ELISA in 100 patients with breast cancer, 46 healthy controls, 38 patients with benign breast tumors, and 58 patients with noncancerous diseases (validation set, group 3). The TNM Classification of Malignant Tumors (TNM) classification from the 7th edition of the American Joint Committee on Cancer (AJCC) was used to define early-stage breast cancer (0, I, and II; refs. 27, 28). The Reporting Recommendations for Tumor Marker Prognostic Studies (REMARK) criteria, recommended by McShane and colleagues (29), were

Table 1. Characteristics of study population

Characteristic	Total			Test			Validation			*P*[a]
	N (%)	Mean	SD	*N* (%)	Mean	SD	*N* (%)	Mean	SD	
Number of study population	562			320 (56.9)			242 (43.1)			
Age, years					50	10.4		52.3	10.3	0.8090[b]
Breast cancer	269			169 (62.8)			100 (37.2)			
Histologic grade[c]										0.0148[d]
1	49 (18.2)			30 (17.9)			19 (19.0)			
2	136 (50.6)			96 (56.8)			40 (40.0)			
3	84 (31.2)			43 (25.4)			41 (41.0)			
Stage[e]										<0.0001[d]
0	42 (15.6)			37 (21.9)			5 (5.0)			
I	66 (24.5)			48 (28.4)			18 (18.0)			
II	89 (33.1)			47 (27.8)			42 (42.0)			
III	60 (22.3)			25 (14.8)			35 (35.0)			
IV	12 (4.5)			12 (7.1)						
Estrogen receptor (ER)[f]										0.2045[d]
Negative	74 (27.5)			42 (24.9)			32 (32.0)			
Positive	195 (72.5)			127 (75.1)			68 (68.0)			
Progesterone receptor (PgR)[f]										0.7049[d]
Negative	93 (34.6)			57 (33.7)			36 (36.0)			
Positive	176 (65.4)			112 (66.3)			64 (64.0)			
HER2[f]										0.4544[d]
Positive[g]	58 (21.6)			34 (20.1)			24 (24.0)			
Negative	211 (78.4)			135 (79.9)			76 (76.0)			
Healthy control	81			35 (43.2)			46 (56.8)			
Noncancerous diseases	98			40			58			0.4635[d]
Thyroiditis	16 (16.3)			8 (20)			8 (13.8)			
Gastritis	14 (14.3)			7 (17.5)			7 (12.1)			
Hepatitis B	12 (12.2)			6 (15)			6 (10.3)			
Rheumatoid arthritis	56 (57.1)			19 (47.5)			37 (63.8)			
After surgery	50			50						
Benign breast tumor	64			26 (40.6)			38 (59.4)			

[a]*P* value comparing test and validation groups.
[b] *t* test.
[c]modified Scarff–Bloom–Richardson grading system.
[d] χ^2 test.
[e]AJCC staging system.
[f]according to immunohistochemical (IHC) staining of ER, PgR, and HER2.
[g]IHC (3+) or FISH (+).

followed throughout this study. All individuals provided informed consent for blood donation according to a protocol approved by the Institutional Review Board of the Kyungpook National University Hospital.

Proteomic analysis

EVs were purified by differential centrifugation as described previously (30). EVs from plasma of 10 patients with breast cancer (stages I and II) and 5 control (healthy volunteer) patients were analyzed by nano-UPLC and tandem mass spectrometry (Q-Tof Premier; Waters). Data processing, searching, and analyses were performed using Mascot server 2.2 (Matrix Science). Label-free protein quantification was performed using an IDEAL-Q software (31). The analysis was performed three times. EV proteins from MDA-MB-231 cells were analyzed by the same methods. The proteins were considered to be significantly upregulated with a greater than 2-fold difference versus healthy control and set with an adjusted $P < 0.05$.

ELISA

For quantification of Del-1 protein on EVs in plasma using an ELISA method 1, 96-well plates were coated overnight with polyclonal anti-CD63 (ab68418; Abcam) antibody at 100 ng/well in 0.2 mol/L sodium phosphate buffer (pH 6.5). The plates were blocked for 1 hour at 37°C with 200 μL of PBS containing 1% BSA and washed three times with PBS containing 0.05% Tween 20 (PBS-T). Plasma (10 μL) was diluted with blocking buffer (90 μL). The diluted plasma (10 μL) was added to the plates in triplicate and incubated for 1 hour at 37°C. Following three washes with PBS-T, the plates were reacted with monoclonal anti–Del-1 (ab88667; Abcam) antibody, preincubated with peroxidase-conjugated anti-mouse IgG antibody for 30 minutes, and developed with 3,3′,5,5′-tetramethylbenzidine containing hydrogen peroxide. The reaction was stopped with 1 mol/L phosphoric acid, and optical density values were measured at 450 nm on an automated iMark plate reader (BioRad).

For quantification of Del-1 or CD63 proteins in plasma using an ELISA Method 2, the same steps were performed as with Method 1 except for coating the plates with polyclonal anti–Del-1 (ab151308; Abcam) or anti-CD63 (ab68418; Abcam) antibodies to capture each protein. Recombinant Del-1 (6046-ED-050; R&D Systems) or CD63 proteins (H00000967-G01; Abnova) were used to generate standard curves. The levels of CD63 were measured using a monoclonal anti-CD63 (ab8219; Abcam) antibody. The CA 15-3 enzyme immunoassay was performed using an enzyme immunoassay kit (MYM Laboratory & Medical Supply) following the manufacturer's instructions. Each data point is the average of triplicate measurements.

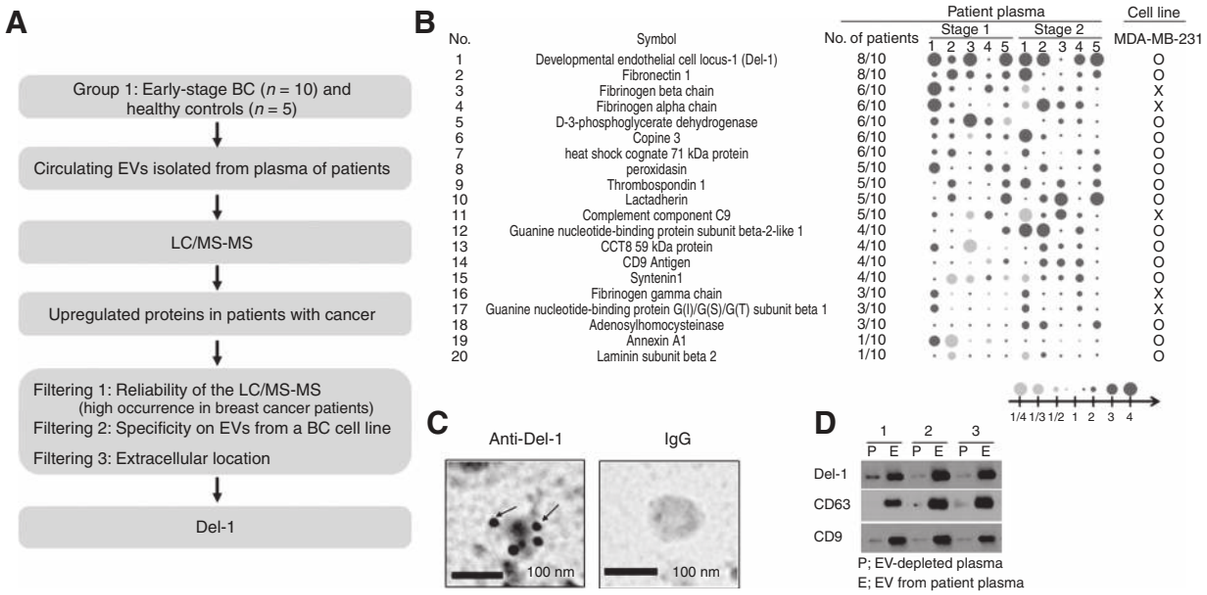

Figure 2.
Identification of Del-1 on EVs as a biomarker candidate for early-stage breast cancer (BC) and characteristics of Del-1 protein. A, an overview of the process for identification of a biomarker of early-stage breast cancer. The EV tryptic peptides (5 μg) from each of 15 samples were analyzed three times by nano-UPLC MS/MS. A total of 846 nonredundant proteins were identified. Among differentially expressed proteins, 49 were upregulated and 74 were downregulated in patients with cancer. Seven upregulated proteins (Del-1, fibronectin 1, fibrinogen beta chain, fibrinogen alpha chain, D-3-phosphoglycerate dehydrogenase, copine 3, and heat shock cognate 71-kDa protein) were selected for further consideration because these proteins were detected with high occurrence in six or more of the 10 breast cancer patients. In addition, EVs derived from MDA-MB-231 cells, a representative breast cancer cell line, were analyzed by LC-MS/MS and compared with EV proteins from plasma. EV proteins that were common in the breast cancer cell line and plasma were determined. Finally, Del-1 proteins that are located extracellularly were selected because they can be easily be detected by appropriate antibodies. B, for identification of differentially expressed proteins in circulating EVs derived from control ($n = 5$) and patients with early-stage breast cancer (stages I and II; $n = 10$), the circulating EVs were analyzed for protein expression by nano-UPLC-MS/MS. A partial list of proteins upregulated in cancer patients is shown. The color and size of each dot indicate the extent of upregulation (red) and downregulation (green) of the listed protein in EVs from cancer patients compared with control patients. The number of patients (out of 10) with proteins upregulated greater than 2-fold is shown. In addition, proteins in EVs isolated from the breast cancer cell line MDA-MB-231 were analyzed by nano-UPLC-MS/MS. The presence of each protein in EVs derived from this cell line is indicated by an O. C, Immunogold labeling of EVs with an anti–Del-1 antibody. EVs were incubated with a primary anti–Del-1 antibody and then incubated with a secondary antibody conjugated to gold particles (9–11 nm). The grid was stained with 0.5% uranyl acetate and examined using transmission electron microscopy. Arrows indicate Del-1 proteins. Scale bar, 100 nm. D, Western blot analysis of EVs isolated from plasma and EV-depleted plasma. Plasma EVs (E) from each patient (n = 3) were isolated by ultracentrifugation then separated from the EV-depleted plasma fraction (P). Each fraction was run on SDS-PAGE and analyzed by immunoblotting using anti–Del-1, anti-CD63, anti-CD9 antibodies. Del-1 protein was mainly detected in the EV fraction, which was verified using the CD63 protein, a representative exosomal marker. The blots shown are representative of three replicates.

Statistical analyses

Descriptive statistics summarized clinical factors; χ^2 and t tests were used to compare the test and validation groups. To estimate the reproducibility and precision of the ELISA results, intra- and interassay coefficients of variation (CV) were calculated, where CV (%) = (SD/mean) × 100. For plasma Del-1, CD63, and CA 15-3 levels, relationships were analyzed using the Kolmogorov–Smirnov test to assess differences between individual variables from two groups. Assessment of the correlation between tumor size and levels of Del-1 or CD63 was performed using a nonparametric Spearman correlation. The diagnostic potential of Del-1 was determined by calculating the receiver operating characteristic (ROC) curve plotted to evaluate the sensitivity and specificity of the biomarker measurements in predicting breast cancer. Discriminative efficacy of Del-1 and optimum diagnostic cutoff were calculated by the area under the ROC curve (AUC) in test set. Using Del-1 threshold values in test set, we calculated diagnostic performance of Del-1 for breast cancer detection in the validation cohort. All validation cohorts were analyzed in a blinded fashion. The Kruskal–Wallis one-way analysis was used to evaluate a significant change of Del-1 in the presence of three types of breast cancer–related receptors. *P* values less than 0.05 indicated statistical significance. Analyses were conducted using MedCalc (MedCalc Software) and Prism (GraphPad Software, Inc.). Data are expressed as mean ± SEs.

Results

Identification of Del-1 protein on circulating EVs in early-stage breast cancer patients versus control

An overview of the process for identification of a biomarker for early-stage breast cancer is illustrated (Fig. 2A).

To identify early biomarker candidates, EVs isolated from the plasma of healthy controls ($n = 5$) and patients with early-stage breast cancer (stages I and II; $n = 10$) were analyzed using proteomics (31). A total of 844 nonredundant proteins were identified (Supplementary Table S1). Among differentially expressed proteins, 49 were upregulated, and 74 were downregulated in patients with cancer (Supplementary Table S1). Seven upregulated proteins were selected for further consideration

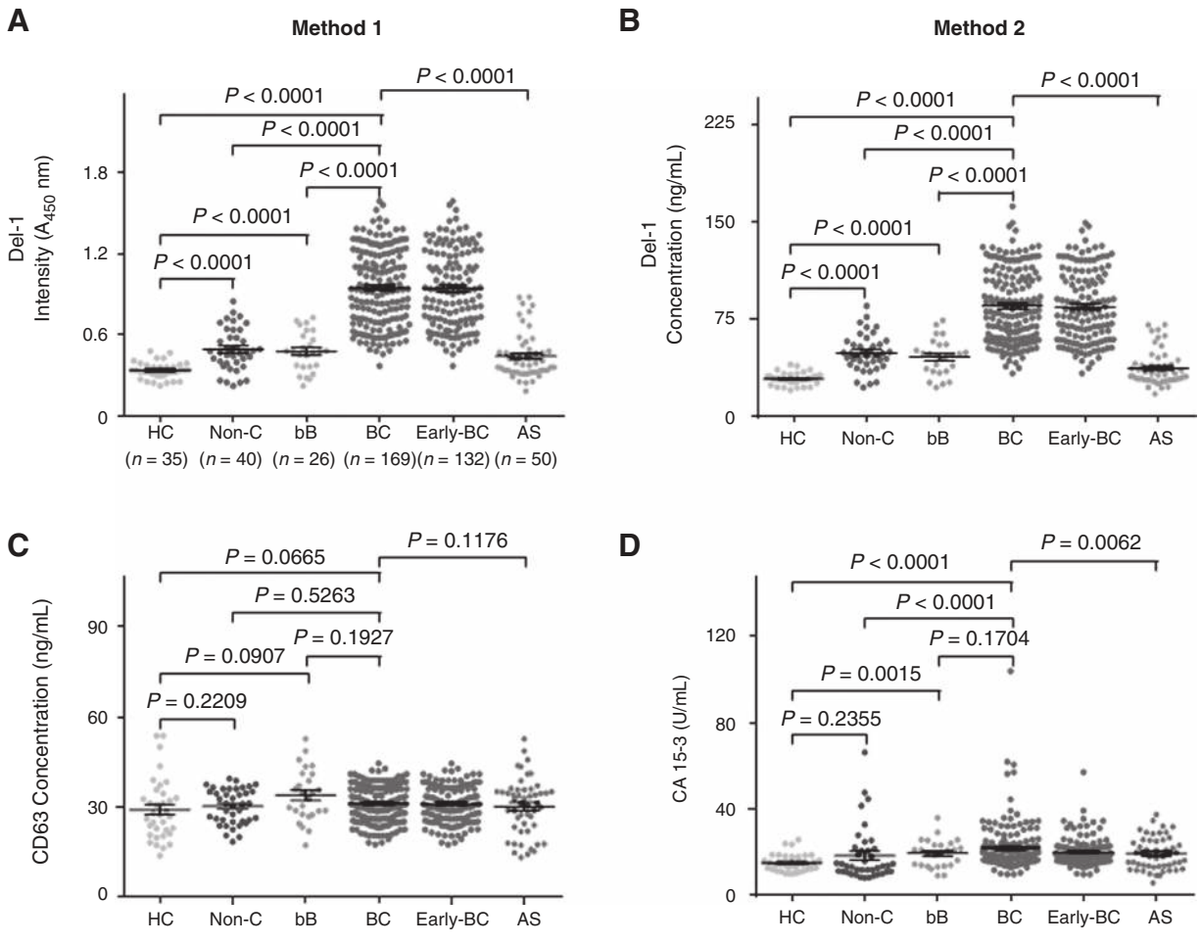

Figure 3.
Del-1 levels in plasma in the test cohort using two types of ELISA. A, Del-1 levels on circulating EVs in plasma using Method 1. B, Del-1 levels in plasma using Method 2. C, CD63 levels in plasma. D, CA 15-3 levels in plasma. All data were obtained using samples from the same subjects. Healthy controls (HC), $n = 35$; noncancerous diseases (Non-C), $n = 40$; benign breast tumors (bB), $n = 26$; breast cancer (BC), $n = 169$; early-stage breast cancer (early-BC), $n = 132$; after surgery (AS), $n = 50$. The black horizontal lines are mean, and error bars are SEs.

because these proteins were detected with high occurrence of six or more among 10 breast cancer patients (Fig. 2B). In addition, exosomes derived from MDA-MB-231 cells, a representative breast cancer cell line, were analyzed by LC-MS/MS and compared with exosomal proteins from plasma (Fig. 2B; Supplementary Table S2). Exosomal proteins that were common in the breast cancer cell line and plasma were determined. Finally, Del-1 that are located extracellularly were selected because they can be easily detected by appropriate antibodies (refs. 32, 33; Fig. 2C). The location of Del-1 on the surface of exosomes was confirmed by immunogold labeling analysis (Fig. 2C). The major location (exosome pellet or exosome-depleted plasma) was determined for Del-1. Del-1 was significantly enriched in the EV fraction ($P < 0.001$ vs. EV-depleted plasma; Fig. 2D).

Levels of Del-1 determined by two different ELISAs in the plasma of breast cancer patients

Del-1 levels in 1 μL of plasma from all patients were assessed by two different ELISAs using different primary capture antibodies (monoclonal anti-CD63 antibody for Method 1 and polyclonal Del-1 antibody for Method 2; Fig. 1). The amount of protein in plasma was quantified based on standard curve generated from recombinant Del-1. Linearity and reproducibility for ELISA Methods 1 and 2 are shown in Supplementary Fig. S3. Total CVs were 7.16% using the ELISA Method 1 and 6.09% using the ELISA Method 2.

We recruited 562 participants: 320 in the test cohort and 242 in the validation cohort (Fig. 1 and Table 1). Del-1 plasma levels were significantly higher in patients with breast cancer in the test cohort [$n = 169$, absorbance at 450 nm (A_{450}) median 0.938, IQR 0.700–1.208; mean 0.950 ± 0.289] than in three control groups [healthy controls, $n = 35$; benign breast tumors, $n = 26$; and noncancerous diseases, $n = 40$; A_{450} median 0.406, IQR 0.330–0.506; mean 0.438 ± 0.146; $P < 0.0001$; Fig. 3A] using the ELISA Method 1. Fifty plasma samples were collected from breast cancer patients after surgical resection. Del-1 levels dropped after surgical resection (0.445 ± 0.174, $P < 0.0001$; Fig. 3A). Using the ELISA Method 2, Del-1 levels were also significantly higher in patients with breast cancer in the test cohort (median, 80.70 ng/mL; IQR, 59.50–108.8; mean, 85.05 ± 28.27) than in three control groups

(median, 39.22; IQR, 28.91–49.58; mean, 41.38 ± 14.61; $P < 0.0001$; Fig. 3B).

To demonstrate the utility of Del-1 as early diagnostic marker for breast cancer relative to other biomarkers, the plasma levels of CA 15-3, a molecular diagnostic biomarker currently used for the detection of metastatic breast cancer (9), was measured for the same subjects (Fig. 3D). In contrast with Del-1, but consistent with previous work (34), CA 15-3 showed no significant change compared with controls at breast cancer stages 0 to III (median, 24.0 U/mL; IQR, 20.6–29.0; $P = 0.444$; Supplementary Fig. S4) but was significantly increased at stage IV (median, 37.8 U/mL; IQR, 22.6–53.0; $P = 0.028$; Supplementary Fig. S4). Although Del-1 levels were higher in breast cancer patients than in controls, the levels of CD63, a representative exosome marker protein, were significantly not changed (Fig. 3C). Similarly, the numbers of circulating EVs per same volume of plasma were significantly not changed between healthy control and breast cancer patients ($P = 0.964$; Supplementary Fig. S5A). These results suggested that the number of EVs is somewhat controlled in human plasma, as shown previously in melanoma patients (21).

The levels of Del-1 did not correlate with the size of the breast tumors (Spearman $r = 0.079$; $P = 0.306$; Supplementary Fig. S5B). Similarly, the levels of CD63 did not correlate with the size of the breast tumors (Spearman $r = 0.055$; $P = 0.447$; Supplementary Fig. S5C). Moreover, Del-1 levels showed no significant correlation with the status of four types of receptors in breast cancers ($ER/PR^{+}Her2^{+}$, $ER/PR^{+}Her2^{-}$, $ER/PR^{-}Her2^{+}$, $ER/PR^{-}Her2^{-}$; $P > 0.05$; Supplementary Fig. S6).

Sensitivities and Specificities of Del-1 for breast cancer diagnosis in test set

ROC curves showed predictive values, and likelihood ratios for Del-1 in the diagnosis of breast cancer are shown in Table 2. The optimum diagnostic cutoff for Del-1 was 0.512 (A_{450}) [AUC, 0.954; 95% confidence interval (CI), 0.923–0.975; sensitivity at 95% specificity, 73.1%] using the ELISA Method 1 (Fig. 4A and Table 2) and that was 53 ng/mL (AUC, 0.937; 95% CI, 0.923–0.975; sensitivity at 95% specificity, 63.9%) using the ELISA Method 2 (Fig. 4A and Table 2) in the test cohort.

A greater proportion of patients with breast cancer in the test cohort were positive for Del-1 using the ELISA Method 1 than the ELISA Method 2 [164 (97.0%) vs. 156 (92.3%) of 169 patients; Fig. 3A]. In the assessment of differential diagnostic accuracy, plasma Del-1 using the ELISA Method 1 (AUC, 0.936; 95% CI 0.899–0.963; sensitivity at 69.6% specificity) had slightly higher AUC values than the ELISA Method 2 (AUC, 0.912; 95% CI 0.871–0.944; sensitivity at 95% specificity, 58.5%) in patients with breast cancer compared with patients with benign breast tumors and noncancerous diseases (Fig. 4B and Table 2).

In the test cohort, 132 (78%) of 169 patients with breast cancer had early-stage disease (stages 0, I, and II). Del-1 plasma levels were significantly higher in early-stage breast cancer than in three control groups ($P < 0.0001$; Fig. 3A and B). Plasma Del-1 using the ELISA Method 1 (AUC, 0.961; 95% CI, 0.924–0.983; sensitivity at 95% specificity, 73.5%) yielded a slightly higher differential diagnosis of early-stage breast cancer from three control groups, compared with the ELISA Method 2 (AUC, 0.946; 95% CI, 0.905–0.972; sensitivity at 95% specificity, 65.0%; Fig. 4C and Table 2). Similarly, plasma Del-1 using the ELISA Method 1 (AUC, 0.939; 95% CI, 0.890–0.971; sensitivity at 95% specificity, 68.8%) yielded a slightly higher differential diagnosis of early-stage breast

Table 2. Results for measurement of plasma Del-1 using ELISA in the diagnosis of breast cancer

	Test set						Validation set					
AUC (95%CI)	**Sensitivity (%)**	**Specificity (%)**	**PPV (%)**	**NPV (%)**	**Positive LR**	**Negative LR**	**AUC (95%CI)**	**Sensitivity (%)**	**Specificity (%)**	**PPV (%)**	**NPV (%)**	**Positive LR**	**Negative LR**
ELISA Method 1													
BC vs. bB, non-C, and HC													
0.954 (0.923–0.975)	95.27	81.19	98.98	51.87	4.67	0.05	0.961 (0.931–0.981)	99.00	86.62	99.34	77.30	6.15	0.01
BC vs. bB and Non-C													
0.936 (0.899–0.963)	88.75	76.24	98.42	33.67	4.27	0.23	0.948 (0.911–0.973)	99.00	81.25	98.85	81.23	4.69	0.01
Early-BC vs. bB, Non-C, and HC													
0.961 (0.924–0.983)	94.70	86.36	99.23	41.35	5.42	0.07	0.968 (0.933–0.988)	92.31	86.62	99.49	100.00	6.44	0.07
Early-BC vs. bB and Non-C													
0.939 (0.890–0.971)	89.39	77.27	99.46	33.26	12.00	0.21	0.957 (0.911–0.983)	92.31	81.25	99.10	100.00	4.92	0.04
ELISA Method 2													
BC vs. bB, Non-C, and HC													
0.937 (0.923–0.975)	93.49	76.24	98.96	45.3	4.71	0.08	0.920 (0.882–0.950)	95.00	80.99	97.04	62.51	4.86	0.06
BC vs. bB and Non-C													
0.912 (0.871–0.944)	85.80	75.24	98.04	33.27	4.82	0.19	0.899 (0.852–0.935)	95.00	74.64	97.68	54.51	3.68	0.07
Early-BC vs. bB, Non-C, and HC													
0.946 (0.905–0.972)	90.90	77.14	99.34	25.17	8.54	0.09	0.943 (0.900–0.971)	89.23	80.99	98.83	100.00	5.12	0.05
Early-BC vs. bB and Non-C													
0.924 (0.874–0.959)	84.85	75.76	98.66	31.64	6.07	0.18	0.925 (0.871–0.962)	89.23	74.64	97.95	100.00	3.88	0.06

Abbreviations: bB, benign breast tumor; BC, breast cancer; HC, healthy controls; LR, likelihood ratio; Non-C, noncancerous diseases; NPV, negative predictive value; PPV, positive predictive value.

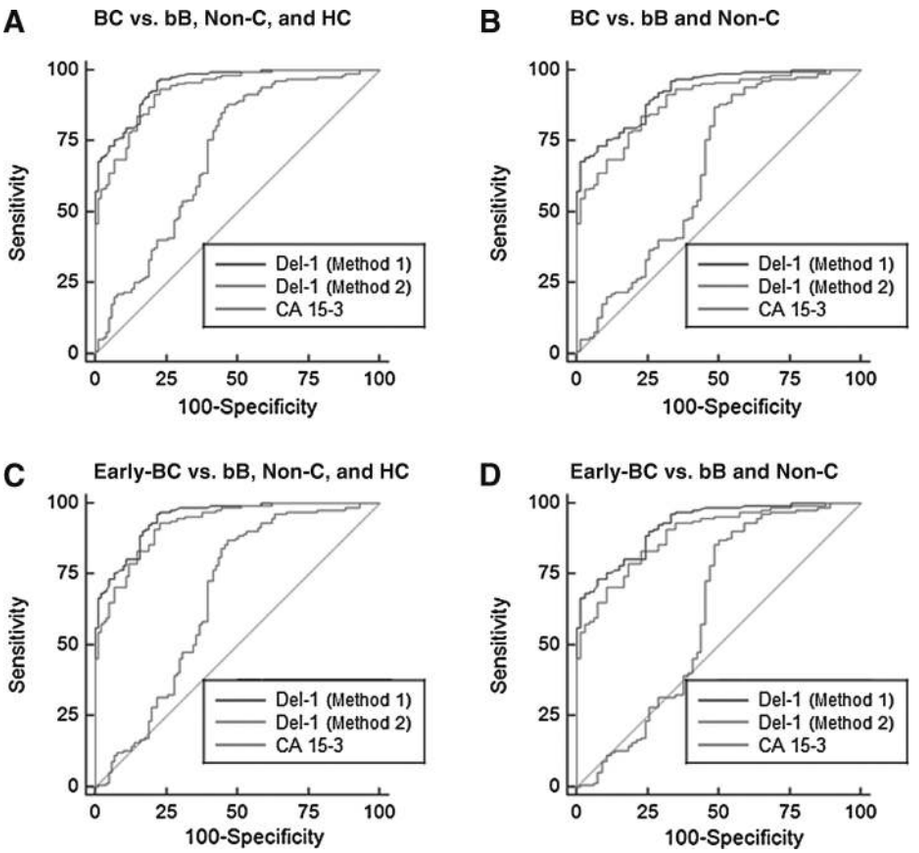

Figure 4.
Diagnostic outcomes for plasma Del-1 in the diagnosis of breast cancer (BC) in the test cohort. A, ROC curves for Del-1 and CA 15-3 for all patients with breast cancer versus three control groups. B, ROC curves for Del-1 and CA 15-3 for all patients with breast cancer versus benign breast tumors and non-cancerous diseases. C, ROC curves for Del-1 and CA 15-3 for patients with early-stage breast cancer versus three control groups. D, ROC curves for Del-1 and CA 15-3 for patients with early-stage breast cancer versus benign breast tumors and non-cancerous diseases. bB, benign breast tumors; Early-BC, early-stage breast cancer; HC, healthy controls; non-C, noncancerous diseases.

cancer from controls with other diseases, compared with the ELISA Method 2 (AUC, 0.924; 95% CI, 0.874–0.959; sensitivity at 95% specificity, 57.7%; Fig. 4D and Table 2). The ROC curves for Del-1 indicated an ability to diagnose early-stage breast cancer.

Diagnostic performance of Del-1 in validation set

Using Del-1 threshold values of 0.512 (A_{450}) for the ELISA Method 1 and 53 ng/mL for the ELISA Method 2, we observed similar results in the validation cohort to those in the test cohort. A patients with breast cancer in the validation cohort were positive for Del-1 using the ELISA Method 1 than the ELISA Method 2 [100 (100.0%) vs. 95 (95.0%) of 100 patients; Fig. 5A]. In the assessment of differential diagnostic accuracy, plasma Del-1 using the ELISA Method 1 (AUC, 0.948; 95% CI 0.911–0.973; sensitivity at 95% specificity, 65.9%) had slightly higher AUC values than the ELISA Method 2 (AUC, 0.899; 95% CI 0.852–0.935; sensitivity at 95% specificity, 56.2%) in patients with breast cancer compared with patients with benign breast tumors and noncancerous diseases (Fig. 5B and Table 2).

The validation cohort confirmed the ability of Del-1 to diagnose breast cancer, especially that with early-stage disease using the two ELISA methods (Fig. 5; Supplementary Fig. S7; and Supplementary Table S2). The abilities of Del-1 to distinguish patients with breast cancer from those with nonmalignant benign breast tumors and noncancerous diseases were also confirmed in the validation cohort (Fig. 5B and D; Supplementary Fig. S7; and Table 2).

Discussion

There is an urgent need to identify novel plasma biomarkers with the ability to more accurately diagnose cancer, especially for detection and screening in early-stage cancer (35). EVs may prove to be useful biomarkers for an improved diagnosis of cancer and other diseases (36, 37), due to several benefits to surpass drawbacks of current clinical biomarkers. EVs contain many disease-associated proteins, giving important information, from origin cells (38). They are stable through protecting their cargo from the attack of nucleases and proteases. Detection of EV-specific proteins reduces the complexity of proteome in body fluids, such as plasma and urine. Therefore, EVs are optimal for multiplexed biomarker analyses that may increase sensitivity and specificity of the diagnostic assay (39). In recent reports, circulating EV miRNAs and EV proteins from biofluids have been shown to be useful biomarkers for the early detection and prognosis of cancer (21, 37, 40). A new role for prostate-specific membrane antigen as a tumor marker for diagnosis and prognostic evaluation of prostate cancer has been suggested (41). In addition, it has been reported that Survivin is released by tumor cells via EVs and hence can serve as a biomarker in patients with early breast cancer (42). In melanoma patients, EV-associated proteins (TYRP-2, VLA-4, HSP70, and HSP90) in the plasma are presented as biomarkers (21). Despite the identification of many potential biomarkers in numerous research papers, only a small number are considered sufficiently reliable to be used in clinical settings. Various

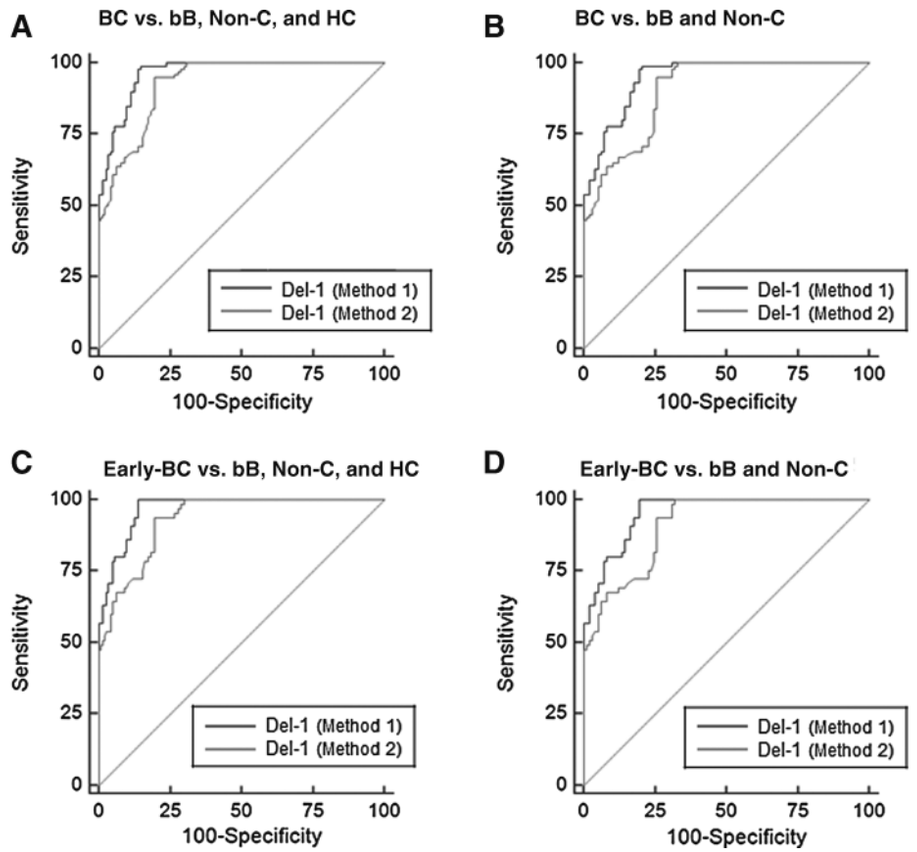

Figure 5.
Diagnostic outcomes for plasma Del-1 in the diagnosis of breast cancer in the validation cohort. A, ROC curves for Del-1 for all patients with breast cancer versus three control groups. B, ROC curves for Del-1 for all patients with breast cancer versus benign breast tumors and noncancerous diseases. C, ROC curves for Del-1 for patients with early-stage breast cancer versus three control groups. D, ROC curves for Del-1 for patients with early-stage breast cancer versus benign breast tumors and noncancerous diseases. bB, benign breast tumors; Early-BC, early-stage breast cancer; HC, healthy controls; non-C, noncancerous diseases.

candidate exosomal proteins have been reported as biomarkers and confirmed by Western blot after EV purification from a small number of clinical samples (21, 43, 44). In the current study, we showed diagnostic accuracy of Del-1 with high sensitivity and specificity in 562 participants from two cohorts using two different ELISAs. This assay is simple, reproducible, quantitative, and minimally invasive using a small amount of plasma (1 μL) without EV purification. The major location (exosome pellet or exosome-depleted plasma) was determined for Del-1, and this protein was significantly enriched in the exosome fraction (Fig. 2D). Exosomal Del-1 using the ELISA Method 1 was a similar diagnostic performance, compared with plasma Del-1 using the ELISA Method 2.

There are some studies for the function of the Del-1 protein in cancer (43) and few relating to breast cancer. Del-1 was first identified as an extracellular matrix protein having 3 N-terminal epidermal growth factor–like domains and the discoidin I–like or factor V C domains, C1 and C2 (45). Del-1 is expressed by endothelial cells during embryonic vascular development (46) and promotes the adhesion of endothelial cells through its interaction with integrin receptors (45). This protein is expressed in other cancers (43, 47, 48) as well as breast cancer (47), suggesting that this protein might have potential as cancer-specific biomarker for various human cancers, including breast cancer.

We found that plasma Del-1 levels had no correlation with the AJCC stage (Fig. 3). This result raised the question as to why there was no relationship between the circulating levels of Del-1 and the tumor burden as one would expect larger tumors to secrete more EVs than smaller tumors. The mechanisms that control the balance between the secretion of EVs and their clearance are not well known in the circulating system. One potential mechanism to regulate EV secretion involves the ability of tumor cells to sense the concentration of EVs in the microenvironment and then alter their secretion (49). These researchers also found that labeled EVs from mammary epithelial cells are internalized into the tumor cells, suggesting a feedback regulatory mechanism for controlling EVs secretion. Another potential mechanism to regulate EV clearance involves special cells, such as endothelial cells, that eliminate circulating EVs through uptake processes. Such scavenging cells might selectively take up EVs by recognizing specific epitopes, such as outer membrane proteins. Recently, a new pathway for microparticle clearance involving Del-1–mediated integrin-dependent endothelial cell uptake from the circulation was reported (32, 50). This strongly supports the possibility that circulating EVs with Del-1 from tumor cells could be selectively eliminated from the circulation by this mechanism. Based on these mechanisms of EV secretion and clearance from the circulation, it is thought that steady-state EV levels in the circulation might be maintained regardless of tumor size.

The patient groups differed to some degree in the diagnostic performance results (Table 2). For example, the positive and negative predictive values of plasma Del-1 for the differential diagnosis of early-stage breast cancer from controls were a bit different because the validation cohort had only 65 patients with early-stage breast cancer, compared with 132 in the test cohort. These findings can be explained by differences in the sample size

between the test and the validation cohorts (Table 1). Despite these differences, the diagnostic capability of plasma Del-1 was generally similar in the two cohorts. The current study is retrospective analysis of individuals with breast cancer. A prospective study will be done in the future to assess whether Del-1 can be validated as *in vivo* marker in patients with breast cancer. The striking decrease in Del-1 concentrations in plasma after surgery suggests that these proteins could be useful surveillance biomarker to assess the response of breast cancer patients to cancer therapies. To further explore this potential role, the long-term follow-up of breast cancer patients who underwent surgery is planned.

To our knowledge, this is the first study to report the diagnostic relevance of Del-1 on plasma EVs as a protein marker for breast cancer in a test cohort and an independent validation cohort. Combining measurement of Del-1 in plasma with imaging information, and other clinicopathologic characteristics, may improve the identification of patients with early-stage breast cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: P.-G. Moon, J.-E. Lee, H. Park, M.-C. Baek
Development of methodology: P.-G. Moon, J.-E. Lee, M.-C. Baek
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): P.-G. Moon, J.-E. Lee, Y.-E. Cho, S. J. Lee, Y. S. Chae, H.-I. Bae, M.-C. Baek
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): P.-G. Moon, Y.-E. Cho, S. J. Lee, Y. S. Chae, I.-S. Kim, M.-C. Baek
Writing, review, and/or revision of the manuscript: P.-G. Moon, J.-E. Lee, Y.-E. Cho, S. J. Lee, Y. S. Chae, Y.-B. Kim, I.-S. Kim, M.-C. Baek
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): P.-G. Moon, Y.-E. Cho, J. H. Jung, H.-I. Bae, M.-C. Baek
Study supervision: M.-C. Baek

Acknowledgments

The authors thank R. Santen and B. Smith for critiquing the article, M. Lee for purification of extracellular vesicles, and S. Lee for helpful comments on the article. They also thank the National Biobank of Korea–Kyungpook National University Hospital and the Chonnam National University Hwasun Hospital for providing plasma samples.

Grant Support

This work was supported by a National Research Foundation of Korea (NRF) grant funded by the Korea Government (NRF-2012R1A2A2A01046512 and 2014R1A5A2009242) and by a grant of the Korean Health Technology R&D project, Ministry of Health & Welfare, Republic of Korea (HI12C0534).

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Received March 17, 2015; revised October 20, 2015; accepted October 21, 2015; published OnlineFirst November 24, 2015.

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