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Identification of ganglioside GM2 activator playing a role in cancer cell migration through proteomic analysis of breast cancer secretomes

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Key words

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Cancer cell secretomes are considered a potential source for the discovery of cancer markers. In this study, the secretomes of four breast cancer (BC) cell lines (Hs578T, MCF-7, MDA-MB-231, and SK-BR-3) were profiled with liquid chromatography–tandem mass spectrometry analysis. A total of 1410 proteins were identified with less than 1% false discovery rate, of which approximately 55% (796 proteins) were predicted to be secreted from cells. To find BC-specific proteins among the secreted proteins, data of immunohistochemical staining compiled in the Human Protein Atlas were investigated by comparing the data of BC tissues with those of normal tissues. By applying various criteria, including higher expression level in BC tissues, higher predicted potential of secretion, and sufficient number of tandem mass spectra, 12 biomarker candidate proteins including ganglioside GM2 activator (GM2A) were selected for confirmation. Western blot analysis and ELISA for plasma samples of healthy controls and BC patients revealed elevation of GM2A in BC patients, especially those who were estrogen receptor-negative. Additionally, siRNA-mediated knockdown of GM2A in BC cells decreased migration *in vitro*, whereas the overexpression of GM2A led to an increase in cell migration. Although GM2A as a diagnostic and prognostic marker in BC should be carefully verified further, this study has established the potential role of GM2A in BC progression.

Breast cancer is a major common cancer in women worldwide. In 2013, approximately 232 340 new cases and 39 620 deaths from BC were estimated in the USA.[1] The high mortality related to BC is thought to be because of the advanced stage of disease at presentation. Tumor progression toward increasing metastatic potential is a complex, multistep cascade of events that occurs when cancer cells reduce their adhesion, increase their motility, and therefore develop an ability to invade local tissues.[2] Therefore, discovery of metastasis-promoting or migration-related proteins for prognosis and monitoring is beneficial in the fight against BC and enormous efforts have been made to characterize useful biomarkers for BC.[3,4]

Recently, the development of proteomic techniques has led an expansion of the search for new biomarkers.[5] The most useful biomarkers can be assayed in non-invasively obtained body fluids such as plasma or serum, and be linked to BC by a defined mechanism involving cancer proliferation, migration, and metastasis.[6] However, the discovery of biomarkers using plasma samples is challenging due to high complexity and wide dynamic range of proteomes. The cancer secretome, which is the whole collection of proteins released by cancer cells or tissues, has been proposed as an alternative source of tumor markers. The rationale supporting this strategy is that secretomes, being much closer to tumor cells than plasma, may be enriched with secreted proteins relevant to the disease and also be more likely to be present in the blood. Therefore, they may play an important role in many vital biological and physiological processes related to cancers and become appropriate targets for non-invasive early diagnosis or monitoring of tumor progression.[7–9]

In this study, secretomes derived from four BC cell lines were first analyzed by LC-MS/MS in order to search for tumor markers that could also be found in blood plasma. Differentially expressed proteins between normal and cancer breast tissues were then selected by using a public database of immunohistochemical images. By applying various criteria, including higher expression level in BC, higher predicted potential of secretion, and sufficient number of tandem mass spectra, 12 biomarker candidate proteins, including GM2A, were selected for confirmation. The expression of these candidate proteins were confirmed through Western blot analysis and ELISA on plasma of healthy control and BC patients. It was found that GM2A existed at a higher level in BC patient plasmas. Knockdown of GM2A in BC cells impaired cell

migration. Our results suggest a promoting effect of GM2A in BC progression.

Materials and Methods

Preparation of secretomes and cell lysates. Hs578T, MCF-7, MDA-MB-231, and SK-BR3 cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin (Gibco, Rockville, MD, USA) at 37°C with 5% CO_2 incubator. In the case of Hs578T, 0.01 mg/mL insulin (Sigma-Aldrich, St. Louis, MO, USA) was further supplemented. We prepared, trypsin-digested, and fractionated cell secretomes and lysates as described in our previous study.[10]

Liquid chromatography–tandem mass spectrometry. Tryptic digests were separated using a reversed phase Magic C18 column (75 μm) on an Agilent 1200 HPLC system, (Agilent Technologies, Santa Clara, CA) with a linear gradient of 10–40% in acetonitrile containing 0.1% formic acid for 90 min (400 nL/min). The HPLC system was coupled to an LTQ-XL mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA). The electrospray ionization source was operated in the positive ion mode (300–2000 m/z) with spray voltage set at 1.9 kV, capillary voltage at 30 V, and the heated capillary temperature at 250°C. Each scan cycle consisted of one full MS scan in profile mode followed by six data-dependent MS/MS scans with the following options: isolation width, 1.5 m/z; normalized collision energy, 25%; and dynamic exclusion duration, 180 s.

Analysis of mass spectrometric data. All tandem mass spectral data were searched by the SEQUEST algorithm (TurboSequest version 27, revision 12) against the human UniProtKB database (released in March 2012) supplemented with 199 experimentally validated FBS contaminant sequences.[11] Two trypsin missed cleavages, fixed modification of carbamidomethylation at cysteine (+57.02 Da), and variable modification of oxidation at methionine (+15.99 Da) were allowed. Mass tolerances for MS/MS and MS were set to ±0.5 and ±2 Da, respectively. Peptide and protein assignment and validation (false discovery rate <1%) were carried out using the Trans Proteomic Pipeline version 4.5 (http://www.proteomecenter.org).

Bioinformatic analysis. We used *in silico* tools (http://www.cbs.dtu.dk/services/) to predict various secretion pathways such as SignalP (version 4.0),[12] SecretomeP (version 2.0),[13] and TMHMM (version 2.0).[14] Ingenuity Pathway Analysis (http://www.ingenuity.com) was used to determine the subcellular localization and biological function of proteins. The HPA version 9.0 (http://www.proteinatlas.org) is a public database with millions of immunohistochemical images and was used to compare protein expressions between normal and BC tissues. All of the secreted proteins were further analyzed to ascertain whether they had been reported in the PPD (http://www.plasmaproteomedatabase.org). Oncomine version 4.4.4.4 (https://www.oncomine.org), a cancer microarray database and Web-based data-mining platform, was used to evaluate gene expression levels in BC tissues. Microarray data related to BC were analyzed and integrated through the data-mining platform.[15,16]

Transfection and real-time PCR. To inhibit the expression of GM2A, 26–39 nM GM2A siRNA duplex and scrambled siRNA as a control (Integrated DNA Technologies, Coralville, IA, USA) were transfected to cells using TransIT-TKO transfection reagent (Mirus, Madison, WI, USA). To overexpress GM2A in cell lines, 4 μg GM2A human cDNA (Origene, Rockville, MD, USA) and porcine cytomegalovirus as a control were transfected into the cells using X-tremeGENE HP DNA transfection reagent (Roche, Mannheim, Germany). After incubation for 48 h, the expression of GM2A was measured by quantitative RT-PCR (gene expression, $2^{-\Delta\Delta CT}$ methods) using the StepOnePlus Real-Time PCR system (Applied Biosystems, Framingham, MA, USA) and Western blot analysis (protein expression).

Migration assay. Cell migration was analyzed using the Oris Cell Migration Assay Kit (Platypus Technologies, Madison, WI, USA) following the manufacturer's instructions. Briefly, cells were allowed to migrate for 30 h and were stained with 5 μM calcein AM (Molecular Probes, Eugene, OR, USA). The fluorescence was then recorded using a fluorescence filter set (excitation, 485 nm; emission, 528 nm).

Human plasma. Plasma samples were collected from 104 BC patients (stage 0, 6 patients; stage I, 24; stage II, 61; stage III, 12; and stage IV, 1) and 40 healthy controls who did not show any observable diseases at the time of collection. Detailed sample information is provided in Table S1. The plasma was prepared as suggested by the HUPO Plasma Proteome Project.[17] Biospecimens for this study were provided by the Asan Medical Center (Seoul, Korea) and Ajou Human Bio-Resource Bank (Suwon, Korea), members of the National Biobank of Korea supported by the Korean Ministry of Health and Welfare. All samples were obtained with informed consent under Institutional Review Board-approved protocols (IRB No. 2013-0761).

Western blot analysis. After separation by SDS-PAGE, proteins were transferred to PVDF membranes (20 × 15 cm). All membranes were blocked with 5% skim milk in TBS-T buffer for 1 h at 25°C, and incubated with primary antibodies overnight at 4°C. Membranes were then incubated with secondary antibodies for 1 h at 25°C, washed, and visualized with the ECL primer (GE Healthcare, Waukesha, WI, USA). The primary antibodies used in this study were directed against the following proteins: GM2A, ATP6AP2 (Atlas Antibodies, Stockholm, Sweden), FBLN1, and IGFBP5 (Abnova, Taiwan, China).

Enzyme-linked immunosorbent assay. The concentration of GM2A in human plasma was measured by using commercialized ELISA kits (MyBioSource, San Diego, CA, USA) and calculated from a six-point standard curve (0–800 ng/mL GM2A). A quality control sample prepared by plasma samples pooled from 54 BC patients was included to monitor within-batch and batch-to-batch variations.

Statistical analysis. Differences between controls and cancer patients in the plasma levels of GM2A were analyzed using a non-parametric Mann–Whitney *U*-test[18] and a receiver–operating characteristic curve.[19] Statistical analyses were carried out using MedCalc version 11.5.1.0 (MedCalc Software, Mariakerke, Belgium).

Results

Secretomes of four breast cancer cell lines. The overall process of searching potential BC markers is shown in Figure 1(a). From the MS data for secretomes of four BC cell lines (MCF-7, MDA-MB-231, SK-BR-3, and Hs578T), 936, 603, 585, and 475 human proteins were identified, respectively (Fig. 1b, Table S2). To check for contamination by intracellular proteins, α-tubulin was measured by Western blot to assess the release of intracellular proteins due to cell death. α-Tubulin was scarcely detected in the secretomes, but it was clearly detected in cell lysates (Fig. 1c).

The 1410 non-redundant human proteins were further analyzed using bioinformatic programs for prediction of protein

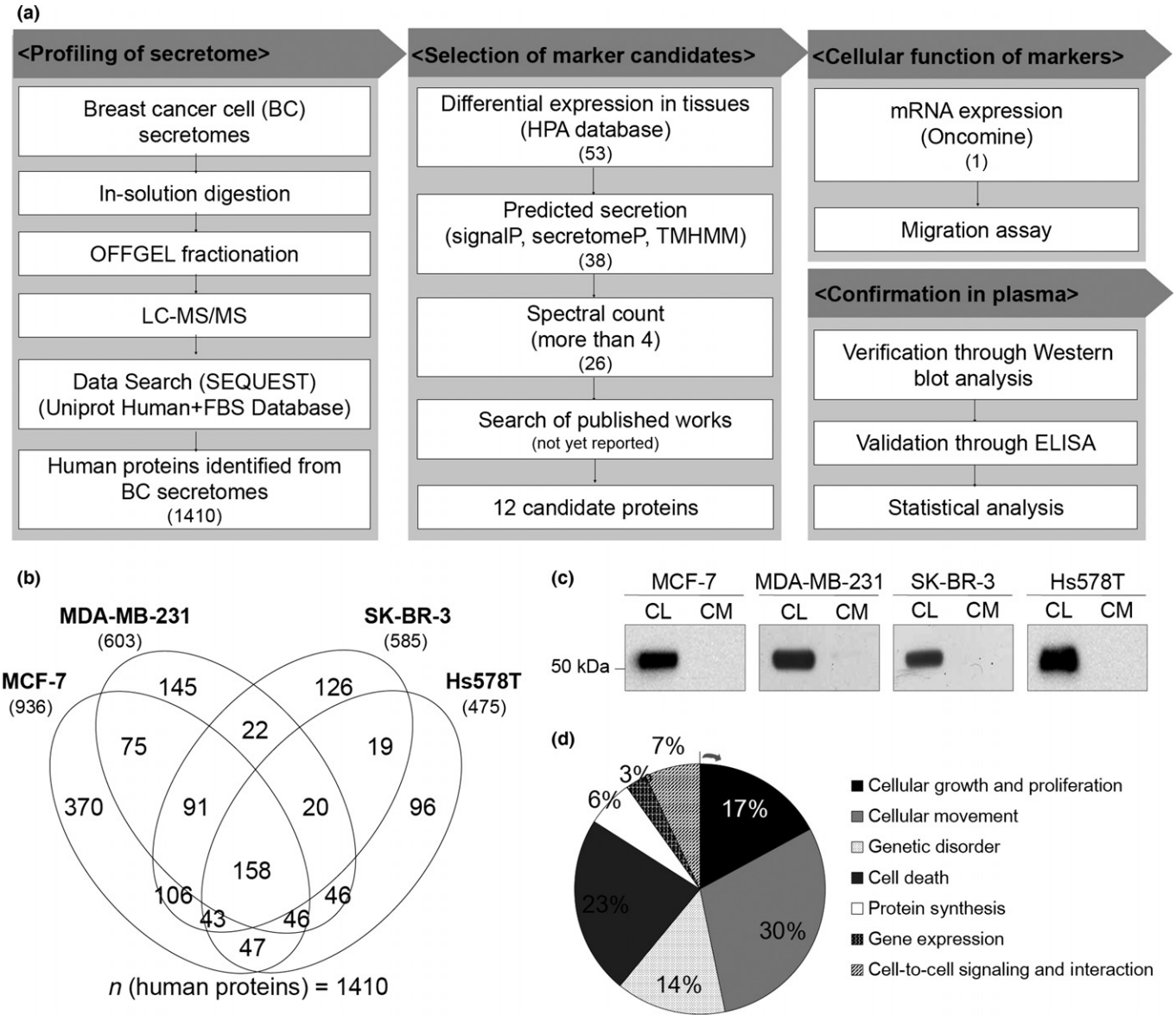

Fig. 1. Schematic workflow and proteomic analysis of secretomes from breast cancer cell lines. (a) Experimental workflow for discovery breast cancer markers. (b) Venn diagrams and the number of identified human proteins in secretome of four breast cancer cell lines (Hs578T, MCF-7, MDA-MB-231, and SK-BR3). (c) Proteins (10 μg) in cell lysates (CL) and conditioned media (CM) were analyzed by Western blotting using an anti-α-tubulin antibody as a quality control. (d) Classification of proteins according to biological function. HPA, Human Protein Atlas; IHC, immunohistochemistry; MS/MS, tandem mass spectrometry.

secretion. Using the SignalP program, 349 proteins were found to be secreted through the classical secretory pathway (cut-off values for SignalP, no TM networks > 0.45, TM networks > 0.5). The SecretomeP program predicted that 425 proteins were released through the non-classical secretory pathway (SignalP signal peptide = No and SecretomeP score > 0.5). An additional 22 proteins were determined as integral membrane proteins through TMHMM. Collectively, these analyses predicted that 55% (796/1410) of the identified proteins were released into the conditioned media of cultured cancer cells through various secretory pathways (Table S3). In addition, 85% (1192/1410) of the identified proteins were previously reported as plasma proteins in PPD. This indicates that many proteins identified in the cell secretomes actually get into blood by various secretion pathways. The major biological functions of secretomes, as shown in Figure 1(d), were related to cellular movement (30%), cell death (23%), cellular growth and proliferation (17%), genetic disorder (14%), cell–cell signaling and interaction (7%), protein synthesis (6%), and gene expression (3%).

Selection of marker candidates for breast cancer. In the HPA database, 53 of 1410 proteins were found to be more strongly stained in BC tissues than in normal tissues. Of these 53 proteins, 38 were predicted to be secreted proteins based on the above-mentioned *in silico* programs. We then excluded proteins that had less than four tandem mass spectra for protein identification and that had already been previously reported in relation to BC. In the final outcome (see Fig. 1a for the strategy for selecting candidate proteins), 12 proteins met all the criteria (listed in Table 1).

Detection of candidate proteins in breast cancer secretome and plasma by Western blot analysis. Western blot analyses of the

Table 1. Twelve proteins selected as marker candidates for breast cancer

Accession no.	Protein name	Gene name	MS/MS spectral count				HPA database					
			MCF-7	MDA-MB-231	SK-BR-3	Hs578T	HPA Ab†	Average IHC score		Percent of location		
								Normal	Breast cancer	n/c/m	c/m	n
Q99538	Legumain	*LGMN*	7	24	13	1	HPA001426	1	2	0	100	0
O75787	Renin receptor	*ATP6AP2*	0	7	3	0	HPA003156	1	1.77	0	100	0
P24593	Insulin-like growth factor-binding protein 5	*IBP5*	0	14	0	5	CAB009216	1	1.58	0	100	0
P17900	Ganglioside GM2 activator	*GM2A*	1	4	5	2	HPA008063	0	2.5	0	100	0
P21741	Midkine	*MK*	0	11	19	0	CAB010055	0	1.8	9	82	0
P51884	Lumican	*LUM*	31	0	0	0	HPA001522	0	1.32	17	50	0
P53004	Biliverdin reductase A	*BLVRA*	0	5	0	5	HPA042865	0	1.08	18	73	0
Q08629	Testican-1 (protein SPOCK)	*SPOCK1*	112	0	0	0	HPA007450	0	1.58	0	100	0
Q12907	Lectin mannose-binding 2	*LMAN2*	2	9	2	0	HPA003927	0	1.64	0	82	0
Q96AG4	Leucine-rich repeat-containing protein 59	*LRRC59*	1	4	0	0	HPA030827	0	1.77	0	100	0
P24821	Tenascin	*TENA*	0	0	22	0	CAB004592	0	1.59	0	100	0
P23142	Fibulin-1	*FBLN1*	6	0	0	13	HPA001612	0	1.75	0	100	0

†Accession number of Human Protein Atlas (HPA) antibody. c, cytoplasmic; IHC, immunohistochemistry; m, membranous; MS/MS, tandem mass spectrometry; n, nuclear.

12 proteins were first pursued using the secretomes and we were able to find that all of the candidate proteins were clearly present in the conditioned media of BC cell lines (data not shown). Then we used Western blot analysis in order to examine whether the 12 proteins could be detected in plasma samples that were pooled separately from 20 healthy controls and 54 BC patients. Eleven of the 12 candidate proteins were already reported in PPD, but only four proteins, FBLN1, ATP6AP2, GM2A, and IGFBP5, were clearly detected in the plasma (Fig. S1).

mRNA expression of four candidate proteins. The Oncomine database was used to examine how the mRNA expressions of four proteins detected in plasma were expressed in various datasets of BC. The expression levels of three proteins, GM2A, FBLN1, and IGFBP5, in BC tissues ($n = 53$) were higher than those of normal breast tissues ($n = 6$) ($P < 0.001$; Fig. 2a, S2).[20–22] Additionally, *GM2A* gene expression was 2.42-fold higher in ER-negative BC specimens compared to ER-positive BC specimens (Fig. 2b). In contrast, ATP6AP2 showed a negative correlation between gene expression and immunohistochemical data (Fig. S2), and was excluded from further validation. In a previous study of RAF1/MAP2K-transfected MCF-7 BC cell line,[22] GM2A expression was activated by RAF1 and MAP2K, both known to play a key role in cell migration, invasion, and metastasis (Fig. 2c). However, IGFBP5 expression was inhibited by either RAF1 or MAP2K and no change was reported for FBLN4 under the same conditions (Fig. S2). Therefore, we selected GM2A as a BC marker candidate and extracted a list of genes that were coexpressed with GM2A with a correlation coefficient >0.9. We subjected the genes to Ingenuity Pathway Analysis to decipher a common role. As seen in Figure 2(d), all of the genes were relevant to epithelial cancer in their biological functions. Finally, we attempted to test the role of GM2A in cell motility.

Correlation with GM2A expression and motility of breast cancer cells. In order to study the role of GM2A in BC progression, we controlled GM2A expression levels in the four BC cell lines and examined its effect on cell migration. The results applied to GM2A siRNA showed that the expression of GM2A was significantly reduced at both mRNA (Fig. 3a) and protein levels (Fig. 3b) when compared to the levels in the control siRNA. As seen in Figure 3(c), GM2A inhibition decreased the migration of BC cells compared to the control cells, particularly in MDA-MB-231 and SK-BR-3 cell lines.

Cell migration assays were also carried out after overexpression of GM2A. Transfection of the *GM2A* gene into BC cells resulted in an increase of GM2 at the protein level (Fig. 3d), which led to a significant increase in the migration rate (Fig. 3e). Knockdown or overexpression of GM2A showed little effect on cell proliferation (Fig. S3). Thus, these findings from the cell migration assays indicated that GM2A could play a role in progression of BC.

Validation of GM2A in BC patients' plasma samples. To evaluate the diagnostic usefulness as a marker, as an initial test, Western blot analyses were carried out using the plasma samples of 54 BC patients and 20 healthy controls. Although the expression levels of GM2A for all of the BC plasma samples were not higher than those in healthy control samples, ER-negative (Fig. 4a, black circles) compared to ER-positive samples showed a much higher level of GM2A. The levels of the other three candidate proteins did not change significantly in the plasma of cancer patients (data not shown). We then used a commercialized ELISA kit to further evaluate GM2A using samples from 38 healthy controls and 104 BC patients, which included the samples used in Western blot analyses (Table S1). Intra-assay coefficient of variation was less than 10%. Compared to the plasma of healthy controls, the plasma level of GM2A was 1.25-fold higher in patients with BC (median values, 5.91 μg/mL *versus* 4.74 μg/mL, $P = 0.0576$; Fig. 4b). The difference was more significant in ER-negative BCs ($P = 0.0276$ for ER-negative *vs* healthy control; $P = 0.2277$ for ER-positive *vs* healthy control). The area under the curve value was 0.604 for all BC patients *versus* healthy

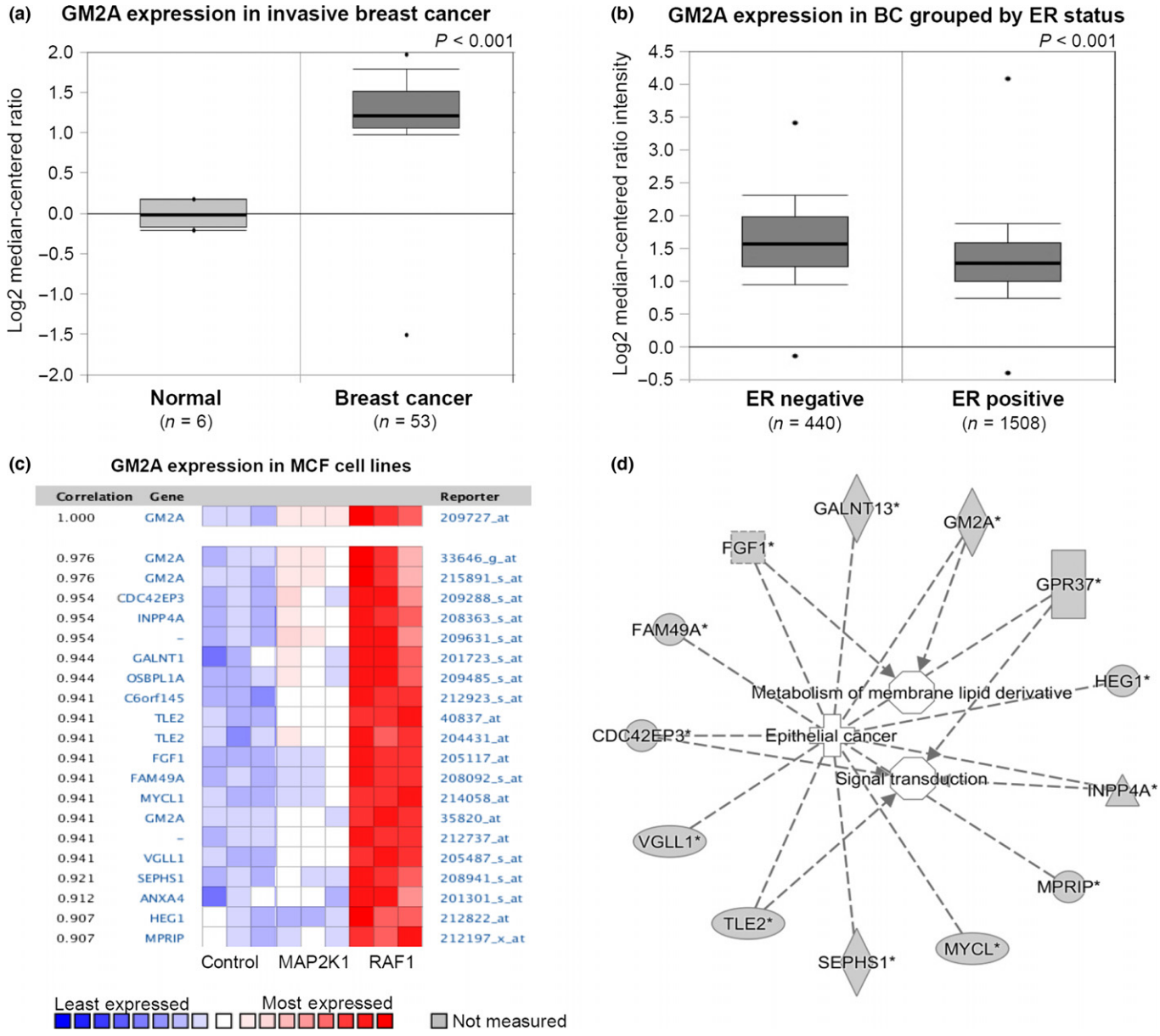

Fig. 2. mRNA expression of ganglioside GM2 activator (GM2A). (a) Gene expression levels obtained from the Oncomine database were compared between breast cancer (BC; $n = 53$) and normal breast tissues ($n = 6$).[20] (b) GM2A expression levels in BC tissues were compared between estrogen receptor (ER)-negative ($n = 440$) and ER-positive samples ($n = 1508$).[21] (c) Gene expression level of GM2A and GM2A-correlated genes (correlation coefficient > 0.9) in MAP2K- and RAF1-transfected MCF-7 breast cancer cell lines is represented as a heat-map. (d) Interaction networks between GM2A and GM2A-correlated genes by Ingenuity Pathway Analysis. The asterisks (*) indicate multiple identifiers in the dataset file map to a single gene in the molecular network.

controls ($P = 0.048$) (Fig. 4c) and 0.641 for BC patients with ER-negative *versus* healthy controls ($P = 0.022$) (Fig. 4d).

Discussion

As numerous proteins have been found to be differentially expressed in BC tissues, recently many researchers have made efforts to discover biomarker candidates, focusing on those with a higher chance of detection in bodily fluids such as serum, plasma, milk, and urine.[23–25] Due to the high complexity and dynamic range in serum and plasma proteomes, proteomic analysis of the secretomes derived from cultured cancer cells could be an alternative source to find serological markers. In this study, 85% (1192/1410) of the identified proteins from four BC cell lines were reported in PPD. Thus, proteomic profiling of secretomes by shotgun proteomics is an effective method to further the discovery of serological diagnostic markers. Additionally, to increase the identification of true-positive human secreted proteins using LC-MS/MS, tandem mass spectral data were searched against a composite database that included experimentally validated FBS contaminant sequences as well as human sequences.[11]

As shown in Figure 1(a), our own strategy for the selection of marker candidates was designed. Using this strategy, 1410 human secreted proteins from four BC cell lines were identified (Fig. 1b), and combined with the quantitative information of immunohistochemistry images from the HPA public database. We hypothesized that if any protein found in the BC cell

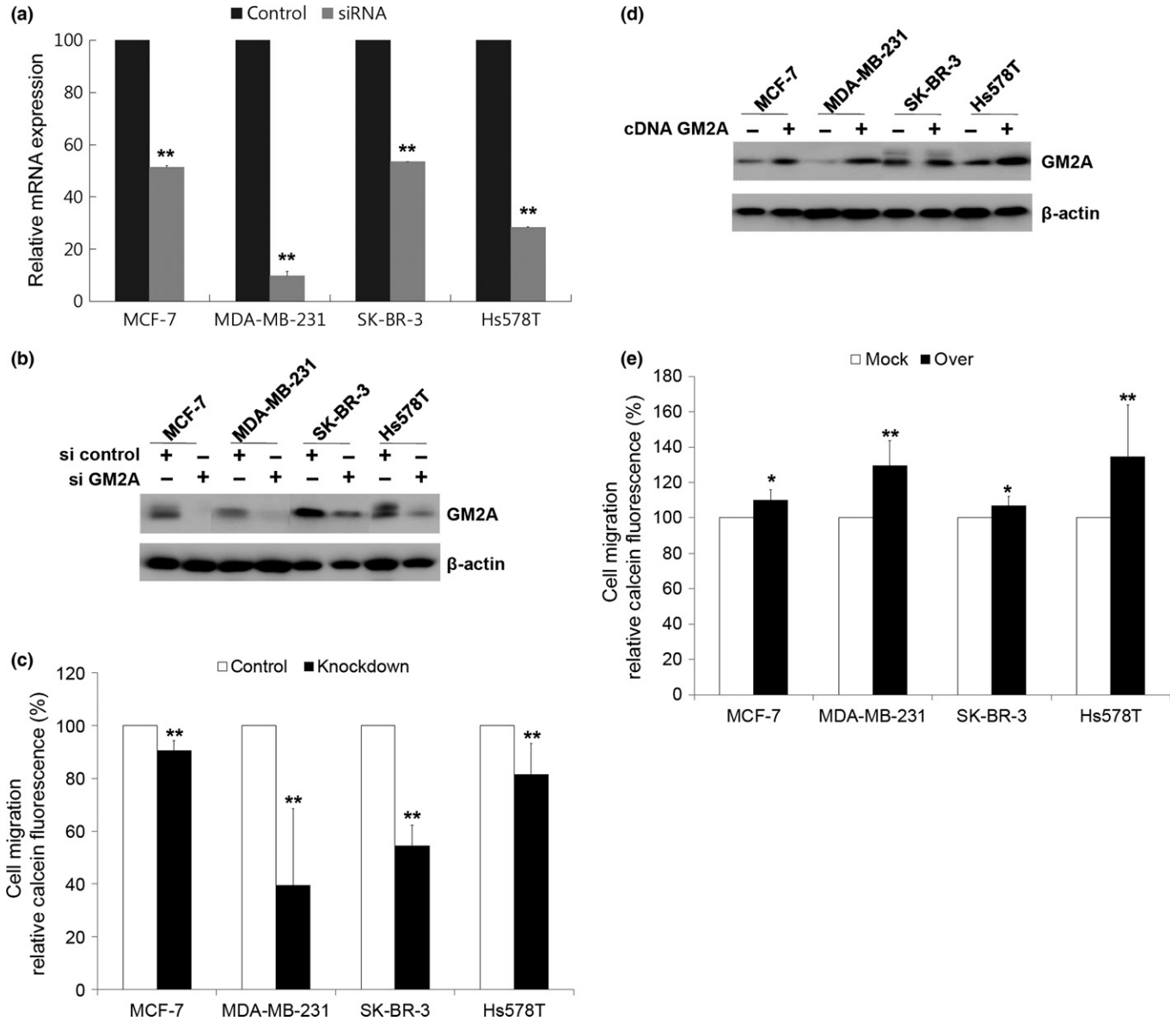

Fig. 3. Effect of ganglioside GM2 activator (GM2A) alteration in breast cancer cells on cell motility/migration. (a) mRNA expression of GM2A in siRNA-treated breast cancer (BC) cells was measured by quantitative real-time PCR. Error bars represent SD of quadruplicated measurements. (b) Protein level of GM2A in siRNA-treated BC cells was measured by Western blot. (c) Cell migration was analyzed for siRNA-treated BC cells (triplicates). (d) Immunoblot analysis of GM2A overexpression in BC cells. (e) Cell migration was analyzed in GM2A-overexpressing (Over) BC cells (triplicates). $*P < 0.01$; $**P < 0.001$.

secretomes showed quantitative changes between the normal and BC tissues, they could stand a better chance of becoming serological markers. Using the HPA database, we were able to compare protein expressions between breast tumor and normal breast tissues without undertaking complex experiments.[26] Such valuable reference datasets have facilitated the discovery of markers like GM2A in this study. The analysis of secretomes using *in silico* programs revealed that approximately 55% of the proteins were predicted as secretory proteins (Table S3). This is reasonable as most studies on cell secretomes have reported that approximately 45–60% of the identified proteins were secretory proteins.[27] In our previous research, the proteins not predicted as secretory proteins by *in silico* programs, unlike the ones predicted as such, were not frequently detected in plasma,[10] therefore, they were not subjected to further analysis. The public gene expression database Oncomine further provided us with valuable information regarding cancer specificity of the target proteins. The gene expression level of GM2A was much higher in breast tumor tissues than in normal tissues (Fig. 2a); in particular, the level was higher in patients with ER-negative disease compared to those with ER-positive disease (Fig. 2b). The underlying mechanism of GM2A regulation by ER signaling is not well known and remains to be established. The protein level of GM2A was also higher in plasma samples of BC patients, mostly in ER-negative cases (Fig. 4b). Although protein abundance is not a simple function of DNA copies and mRNA levels, recently published research has revealed that genes that are dysregulated at the level of DNA or RNA are also mostly dysregulated at the level of protein.[28]

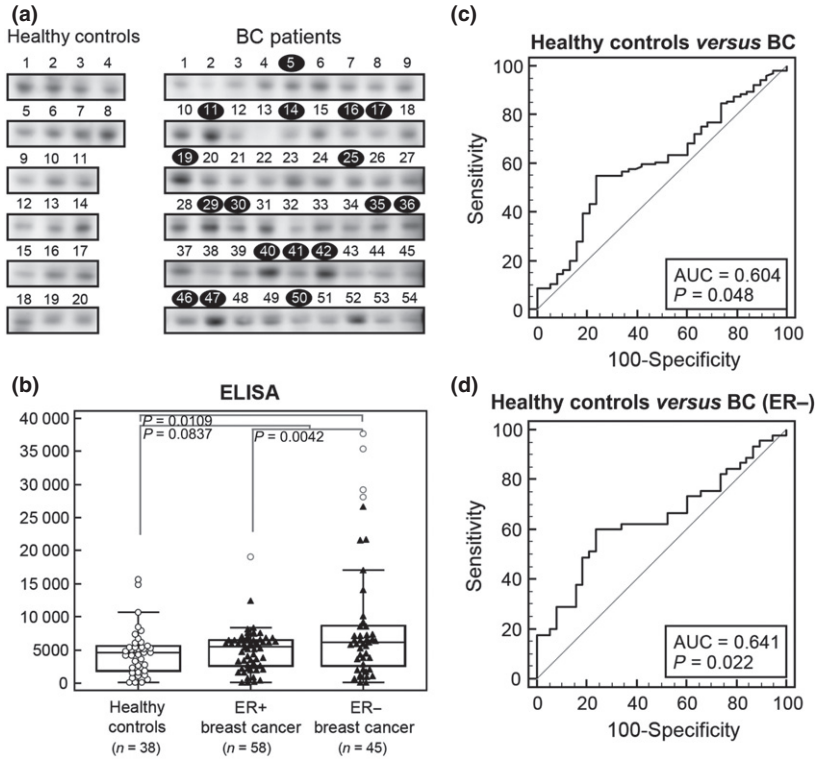

Fig. 4. Ganglioside GM2 activator (GM2A) levels in plasma samples of breast cancer (BC) patients. (a) Immunoblot of GM2A in plasma samples (10 μg) from healthy controls and BC patients. Black circles indicate estrogen receptor (ER)-negative samples. (b) ELISA analysis of GM2A in plasma samples of BC patients and healthy controls. (c) Receiver–operating characteristic (ROC) curve of GM2A for discriminating BC from healthy controls. (d) ROC curve of GM2A for discriminating ER-negative BC from healthy controls. AUC, area under the ROC curve.

Ganglioside GM2 activator encodes a small glycolipid transport protein. This protein binds gangliosides and catalyzes the degradation of the ganglioside GM2.[29] Gangliosides are expressed as lipid-bound sialic acids. Gangliosides have been shown to inhibit cell motility by downregulating epidermal growth factor receptor activity and the phosphatidylinositol 3-kinase–protein kinase B signaling pathway.[30] Another study reported that the GM2/GM3 complex strongly inhibited cell motility through the CD82/cMet-mediated pathway.[31] Therefore, our results showing higher expression of GM2A, which activates degradation of ganglioside GM2, in BC also support those previous findings and provide a connecting link for GM2A activity, cell motility, and cancer progression. That is, overexpression of GM2A increased the motility of BC cells, while knockdown decreased the cell motility. Although we do not have conclusive evidence because we tested only four BC cell lines, such an effect was more prominent in ER-negative cells from the BC cell migration assay (for example, MDA-MB-231 *vs* MCF-7). These results may provide a clue for the ELISA results in which GM2A was found more frequently in ER-negative patients than ER-positive patients (Figs. 2b,4). The ELISA data do not provide strong evidence that GM2A is useful as a screening marker for BC. However, both specificity and sensitivity increased for ER-negative patients. In order to exploit GM2A as a diagnostic marker, further study is required. It may include stratification of BC patients through measurement of GM2A in a larger sample set followed by statistical analysis in relation to various clinical information.

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Disclosure Statement

The authors have no conflict of interest.

Abbreviations

ATP6AP2	renin receptor
BC	breast cancer
ER	estrogen receptor
FBLN1	fibulin-1
GM2A	ganglioside GM2 activator
HPA	Human Protein Atlas
IGFBP5	insulin-like growth factor-binding protein 5
LC	liquid chromatography
MS	mass spectrometry
MS/MS	tandem mass spectrometry
PPD	Plasma Proteome Database

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Fig. S1. Western blot analysis of candidate proteins in breast cancer cell lines and blood plasma samples.

Fig. S2. RNA expression and immunohistochemistry images of candidate proteins from the Human Protein Atlas database.

Fig. S3. Images of cells transfected with ganglioside GM2 activator (GM2A) siRNAs or GM2A.

Table S1. Plasma samples used in this study.

Table S2. Identification of secretomes from four breast cancer cell lines (MCF-7, MDA-MB-231, SK-BR-3 and Hs578T) by liquid chromatography–tandem mass spectrometry.

Table S3. Analysis of secreted proteins and predicted secretion pathways.