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Comparative profiling of breast cancer tissue membrane proteome by use of SDS-PAGE based cICAT method

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Breast cancer is the most common cancer among women worldwide. There is an emerging interest in protein expression profiling with the aim of identifying novel diagnostic markers and therapeutic targets in breast cancer. We analyzed breast cancer tissues by using the cICAT (cleavable isotope-coded affinity tag) labeling approach and tandem mass spectrometry. Breast cancer and matched normal tissues were fractionated by ultracentrifugation to enrich membrane proteins, and cICAT labeled proteins were separated by SDS-PAGE. A total of 364 proteins were identified and quantified. Among the proteins that showed >1.5 fold difference between normal and cancer (36.6%), five (RPN1, ERp29, Trop2, PrP and PIGR) were selected for confirmation. Western blot on 10 pairs of normal and cancer tissues revealed that the expression of RPN1 and ERp29 were increased in breast cancer tissue, while Trop2, PrP and PIGR were decreased. Those Western blot results were highly consistent with cICAT results. Higher level of RPN1 and ERp29 and lower level of Trop2 in breast cancer cell lines compared to normal breast cell lines were observed. The result supports reliability of our SDS-PAGE based ICAT method in quantifying cancer tissue proteome.

INTRODUCTION

In recent years the discovery of cancer biomarkers has become a major focus of cancer research. An ideal cancer marker is a protein or protein fragment that can be easily detected in the patient's blood or urine et al, but not detected in a healthy person. Today, the most common use of cancer biomarkers is for detection of early disease and recurrent disease. In the future, better tests that can predict tumor outcome on advancer and predict the response of individual tumors to particular therapeutic drugs may be developed (Chatterjee and Zetter, 2005). Worldwide, breast cancer is one of the most frequent and deadly cancers. It is estimated that more than 200,000 patients are diagnosed with breast cancer annually (Siegel et al., 2014). Breast cancer is a complex disease. Accumulation of numerous and often unknown molecular alterations causes cell proliferation, genetic instability, and acquisition of an increasingly invasive and resistant phenotype. The combinatorial origin, the heterogeneity of malignant cells and the variability of the host background create molecularly distinct subgroups of tumors endowed with different phenotypes and clinical outcomes (Bagshaw et al., 2005; Mathelin et al., 2006; Weigel and Dowsett, 2010).

Proteomic analyses make the global comparison of proteins from almost any biological sample, thus enabling the identification of multiple proteins of interest in a single experiment. Differential protein expression between different conditions, e.g. diseased and normal, may be examined by applying proteomic tools within a well-defined hypothesis (Zhao et al., 2009). Current biomarker discoveries by use of isotopically labeled protein digests of samples such as tissues and blood, followed by liquid chromatographic separation and mass spectrometric (LC-MS) analyses have produced putative cancer markers. The isotope-coded affinity tags (ICAT) strategy is a leading technology for relative protein quantification, relying on post-harvested, stable isotope labeling (Gygi et al., 1999). In this method, cysteine residues are specifically derivatized with reagent containing either $^{12}C_9$ or $^{13}C_9$ as well as a biotin group for subsequent affinity purification. Typically, one sample in pair-wise comparison is labeled with light version of the reagent, while the other sample is labeled with heavy version. The two samples are then digested with trypsin, fractionated by ion exchange chromatography, affinity purified through biotin tag, and then analyzed by LC-MS/MS (Kang et al., 2012; Shin et al., 2014). We, however, opted against ion exchange chromatography and used SDS-PAGE instead. Since gel-separated proteins are highly denatured, it will be more amenable to membrane proteins (F et al., 2010; Li et al., 2003). Membrane proteins are involved in a multitude of cellular processes such as signal transduction, cellular adhesion, ion transport, and drug resistance. Their identification

provides clues to the understanding of cellular functions and mechanisms. Proteins bearing multiple transmembrane helices, which include many transporters and receptors, are very hydrophobic. Because such proteins are difficult to solubilize and are therefore often poorly represented as part of the total protein pool, the identification and quantification of membrane proteins remain important challenges. We tried to tackle the problem by employing SDS-PAGE based ICAT method which had previously been developed by Ramus et al (Ramus et al., 2006).

Here we report the proteomic analysis of breast cancer proteome by SDS-PAGE based cICAT. The analysis was performed on membraneous proteins which were enriched by ultracentrifugation. We have successfully applied cICAT method to identify 364 proteins among which 135 were differentially expressed by more than 1.5 fold between normal and tumor tissues. Several candidates of differentially expressed proteins (DEPs) were further confirmed using Western blot on tissue samples and various breast cell lines. The differences in expression levels of RPN1 (dolichyl-diphosphooligosaccharide-protein glycosyltransferase), ERp29 (Endoplasmic Reticulum protein 29), Trop2 (tumor-associated calcium signal transducer 2), PrP (Major prion protein), and PIGR (polymeric-immunoglobulin receptor) were consistent with the cICAT data. The result supports reliability of our SDS-PAGE based cICAT method in quantifying cancer tissue proteome.

RESULTS AND DISCUSSION

Sub-proteome analysis of breast cancer tissue by fractionation

We have compared protein expression profiles between tumor and matched non-tumor tissues from breast cancer patients. The non-tumor tissues were obtained at the sites more than 5 cm away from the tumor margin of the cancer core. We focused on membrane proteins. Thus, we enriched membrane fraction by use of differential centrifugation and adopted SDS-PAGE based cICAT as a quantitation method since SDS-PAGE is very powerful in solubilizing hydrophobic proteins. Another benefit of using membrane fraction is that it is relatively free of blood contamination. The non-tumor tissue was relatively richer in circulating blood than the tumor tissue (Kim et al., 2009). We reasoned that if we focused on more clearly fractionated parts without blood proteins of our target sample, interference originated from contamination would be diminished.

Membrane proteins are often difficult to extract from their native environment without high concentrations of detergents, such as SDS. However, in such conditions, the labeling of cysteine residues present on hydrophobic proteins with cICAT reagents requires adaptation of the standard protocol that is not compatible with the presence of high concentrations of SDS (Smolka et al., 2001). The major difference between the classical cICAT standard protocol and SDS-PAGE based cICAT was the use of SDS-PAGE instead of cation exchange chromatography

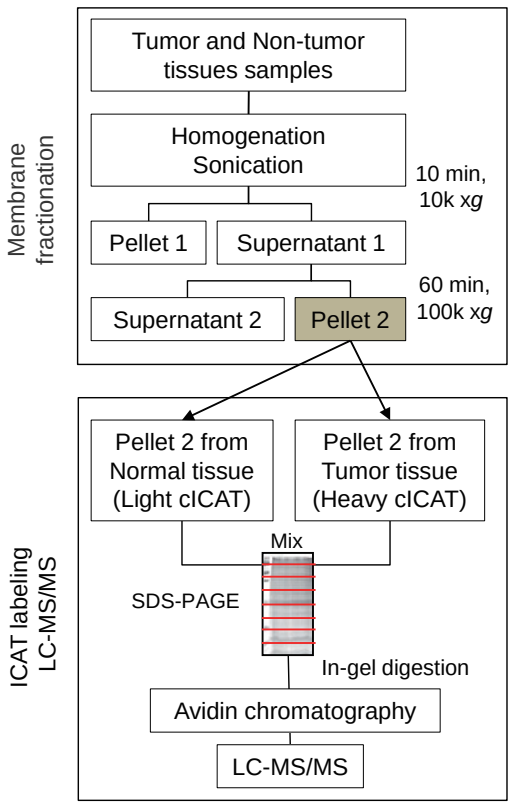

FIGURE 1 | Flow chart for quantitative analysis of breast cancer tissue membrane proteome by SDS-PAGE-based cICAT.

(Figure 1). The cICAT-labeled proteins were pre-fractionated by SDS-PAGE into 8 partitions according to molecular weight range. Such pre-fractionation before in-gel digestion would reduce complexity of the sample (Li et al., 2003).

Identification of differentially expressed proteins (DEPs) from membrane fraction

To determine the number of protein identified from all the 8 gel bands, we incorporated the search results of the eight LC-MS/MS runs and performed TPP analysis. Finally, a total of 364 proteins were identified (Supplementary Table S1). Among these, 45 proteins were up-regulated (>1.5 fold) and 90 proteins were down-regulated (<1.5 fold) in the cancer tissues. The identified proteins could be categorized into six main groups by cellular location (Figure 2A): membrane or membrane interacted proteins (43%), cytoplasm proteins (16%), cytoskelectal nucleus (17%), extracellular proteins (8%), mitochondria endoplasmic reticulum proteins (4%), secreted proteins (5%). Given that global genomic analysis predicts that 20-30% of all open reading frames encode membrane proteins (Wallin and von Heijne, 1998), 43% being membrane proteins among all of the identified proteins in our results indicate that the membrane proteins were moderately enriched by our membrane fractionation method. It appears

TABLE 1 | Characteristics of breast tissues and cell lines used in this experiment

Experiment	Age/Cell line	Histology	T	N	M	Stage	ER/PR	Her2
			Tissue					
cICAT/WB	45	Infiltrating duct carcinoma	2	1	0	IIB	-/+	2+
	45	Infiltrating duct carcinoma	2	0	0	IIA	-/-	2+
	30	Infiltrating duct carcinoma	2	0	0	IIA	-/-	0
	30	Infiltrating duct carcinoma	3	3	0	IIIC	-/-	0
WB	40	Micropapillary carcinoma	2	2	0	IIIA	-/-	3+
	40	Infiltrating duct carcinoma	2	0	0	IIA	-/-	3+
	51	Invasive apocrine carcinoma	1	0	0	I	-/-	1+
	60	Infiltrating duct carcinoma	2	0	0	IIA	-/-	3+
			Cell line					
WB	Hs 578Bst	Normal					-/-	-
	Hs 578T	Primary ductal carcinoma					+/-	-
	MDA-MB-231	Adenocarcinoma					-/-	-
	MCF 7	Adenocarcinoma					+/-	-
	SKBR-3	Adenocarcinoma					-/-	-
	ZR-75-1	Adenocarcinoma					+/-	+

Three parameters of TNM classification for each breast tumor are defined; T, Size or direct extent of the tumor; N, degree of spread to trigonal lymph nodes; M, presence of metasis.

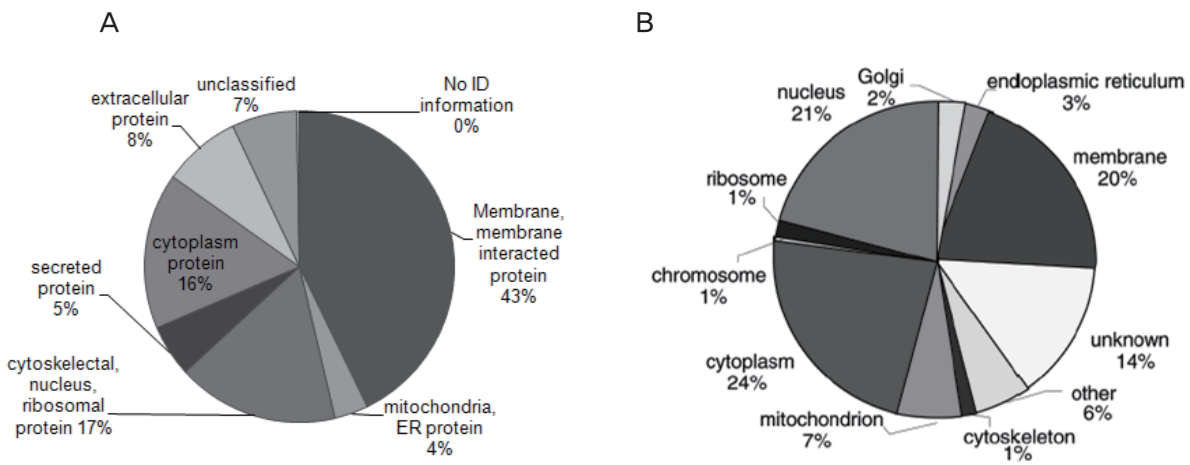

FIGURE 2 | **Classification of identified proteins based on their cellular location.** (A) This study. (B) Data from Gong et al (Gong et al., 2008).

clearer when our data are compared to a report by Gong et al., in which breast cancer tissues were analyzed by LC-ESI-MS/MS combined with sequential protein precipitation and solubilization: the largest breast cancer whole tissue proteome reported so far (Gong et al., 2008). As seen in Figure 2B, the larger categories comprised proteins from the cytoplasm (24%), nucleus (21%), and membrane (20%) in total breast tissue proteins. Details of peptide identification for all the DEPs in our study are summarized in Supplementary Table S1.

Verification of differential expression by Western blot

Among the 135 DEPs, only 21 have antibodies commercially available and not all of the antibodies were suitable for Western blotting. After careful evaluation of the specificity of the antibodies for Western blotting we chose five to validate. The five target proteins were RPN1, ERp29, Trop2, PrP and PIGR. Their

TABLE 2 | List of proteins tested by Western blot

IPI accession	UniProt accession	Protein Name	cICAT ratio (N/C)[a]	Location	Peptide used for quantification[b]
IPI00024911	P30040	Endoplasmic reticulum protein ERp29	0.42	ER lumen	GQGVYLGMPGC*LPVYDALAGEFIR
IPI00025874	P04843	Dolichyl-diphosphooligosaccharide--protein glycosyltransferase (ribophorin, RPN1)	0.63	ER membrane	VAC^ITEQVLTLVNKR
IPI00293898	P01833	Polymeric-immunoglobulin receptor (PIGR)	1.53	Plasma membrane	GSVTFHC^ALGPEVANVAK
IPI00297910	P09758	Tumor-associated calcium signal transducer 2 (Trop2)	1.77	Plasma membrane	ALGSGMAVDC*STLTSK
IPI00646733	P04156	Major prion protein (PrP)	1.8	Plasma membrane	VVEQMC^ITQYER

[a]Note that the ratio represents normal/cancer. [b]C*: Cysteine residue labeled with heavy cICAT, C^: Cysteine residue labeled with light cICAT

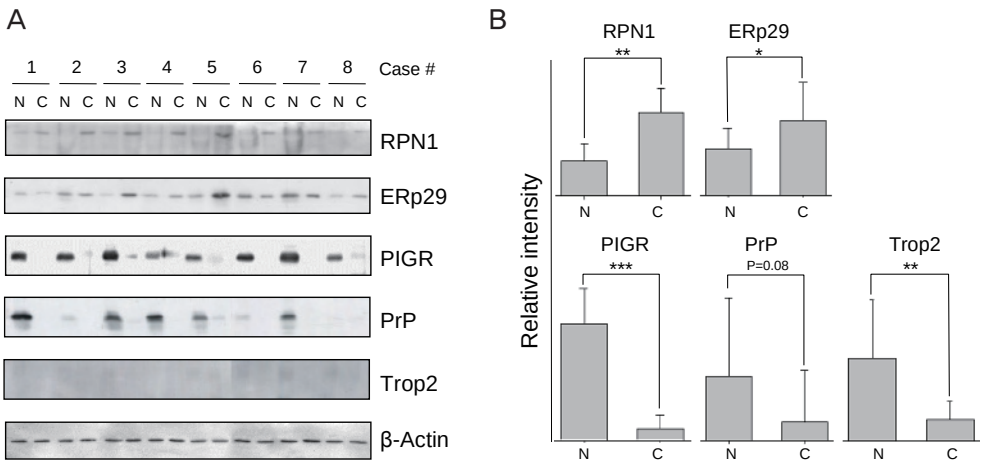

FIGURE 3 | Western blot analysis of five representative DEPs in breast cancer tissues. (A) Western blot analysis of RPN1, ERp29, PIGR, PrP, and Trop2 was performed on eight pairs of breast cancer tissues and matched normal tissues. β-Actin was used as a control. Samples #1~#4 are those used in quantitative proteomic analysis (B) Box-plots for the expression level of five DEPs. Western blot images were quantified by densitometric scanning. P-values: $p<0.05$ (*); $p<0.01$ (**); $p<0.001$ (***). C: cancer tissue, N: matched normal tissue.

cICAT quantitation result is summarized in Table 2. We tested the expression of RPN1, ERp29, Trop2, PrP and PIGR by Western blot on eight pairs of normal and cancer tissues (Figure 3). As compared to normal tissue, the expression of RPN1 and ERp29 were increased in breast cancer tissue (RPN1: 2.4-fold, p=0.001; ERp29: 1.6-fold, p=0.03). In contrast, Trop2, PrP and PIGR showed decreased expression in cancer tissue (Trop2: 3.8-fold, p=0.001; PrP: 1.9-fold, p=0.08; PIGR: 1.6-fold, p=0.0002). The Western blot results were highly consistent with the cICAT result (Table 2 and supplementary Figure S1).

Ribophorins are integral membrane glycoproteins that are exclusively localized to the rough endoplasmic reticulum (Bagshaw et al., 2005). ERp29 protein, which is located to the ER lumen, plays a role in the processing of protein secretion, possibly by participation in the folding of proteins. ERp29 has been found highly expressed in a variety of tumors and cancer cell lines, but an apparent paucity in other cases leaves both the nature and generality of its involvement in tumorigenesis unclear (Shnyder et al., 2008). Participation of ERp29 in the production of secreted and endomembrane proteins are supported by several lines of evidence (Hubbard, 2002). ERp29 induces breast cancer cell growth arrest and survival through modulation of activation of p38 and upregulation of proteins associated with ER (Gao et al., 2012). Also, CK19 expression results in ER stress and negatively regulates ERp29 expression by up-regulating p38/XBP-1 signaling. The attenuation of ERp29 under ER stress is probably a novel indicator and/or modulator in defining tumor cell dormancy and/or metastasis (Bambang et al., 2009). In our

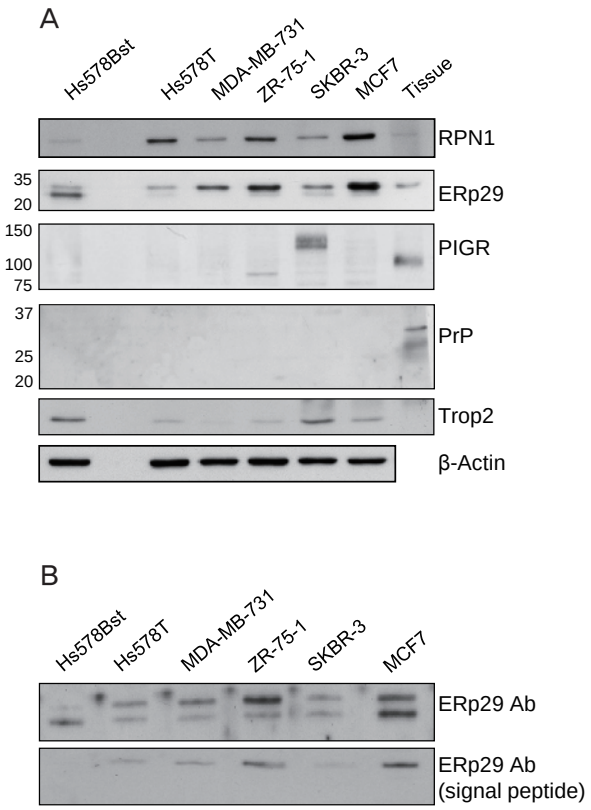

FIGURE 4 | Western blot analysis of five representative DEPs in breast cell lines. (A) Western blot analysis of RPN1, ERp29, PIGR, PrP, and Trop2 was performed on cancer cell lines (Hs578T, MDA-MB-231, SK-BR-3, ZR-75-1 and MCF7) and a normal breast cell line (Hs578Bst). β-Actin was used as a control. (B) Western blot analysis of ERp29 was performed with antibodies targeting its main chain (upper image) or signal peptide (lower image).

study, RPN1 and ERp29 protein expressions were increased in breast cancer tissue.

Among 5 proteins, PIGR, Trop2 and PrP protein expressions were decreased in cancer tissue. PIGR is a single-pass type I membrane protein and expressed on several glandular epithelia including those of liver and breast. The receptor binds polymeric immunoglobulin molecules at the basolateral surface of epithelial cells (Berntsson et al., 2014). PrP protein is a glycosylphosphatidylinositol (GPI)-anchored cell surface glycoprotein that is expressed in various tissues with most abundant expression in the nervous systems (Prusiner, 1998). Several groups have reported PrP expression in various human cancers, suggesting a contributory role of PrP in cancer biology (Li et al., 2009; Pan et al., 2006; Sollazzo et al., 2012). Trop2 is encoded by a gene on chromosome 1p32. It is a cell surface glycoprotein that is not or scarcely expressed in normal adult tissues but that is overexpressed in a significant subset of colorectal carcinomas. Trop2 was shown to form an oncogenic fusion protein with cyclin D1 (Huang et al., 2005). But, in our study, Trop2 showed decreased level in breast cancer tissue suggesting that Trop2 expression is differentially regulated depending on cancer type.

Confirmation of protein expression in breast cancer cell lines

The expression of five target proteins was tested in breast cancer cell lines (Hs578T, MDA-MB-231, SK-BR-3, ZR-75-1 and MCF7) and a normal breast cell line (Hs578Bst). RPN1 protein showed high expression levels in Hs578T, ZR-75-1 and MCF7. ERp29 protein showed high expression levels in MDA-MB-231, ZR-75-1, SKBR-3 and MCF7. In contrast, lower level of Trop2 was detected in cancer cell lines. PIGR and PrP protein were hardly detected in any of the cell lines tested.

Interestingly, ERp29 showed two bands at the molecular size of 29 kDa (increased in cancer cell line) and 26 kDa (decreased in cancer cell line). We suspected that 29 kDa band might represent phosphorylated ERp29 or precursor form of ERp29 with signal peptide uncleaved. Band pattern of ERp29 in ZR-75-1, SKBR-3 and MCF7 did not change upon treatment with alkaline phosphatase prior to electrophoresis, suggesting that band splitting was not due to phosphorylation (data not shown). We next raised a polyclonal antibody by using signal peptide of ERp29 (sequence position: 1-32, MAAAV PRAAF LSPLL PLLLG FLLLS APHGG SG). The antibody recognized only the upper band (Figure 4B). This suggests that a great portion of ERp29 exists in precursor form in cancer cell lines while in Hs578Bst the majority exists in cleaved form. Judging by the molecular weight, the ERp29 detected in breast cancer tissues corresponded to precursor form (Figure 3).

CONCLUDING REMARKS

In this study, we exploited SDS-PAGE based cICAT to quantify breast cancer tissues. Breast cancer and matched normal tissues were fractionated by ultracentrifugation to enrich membrane proteins, and cICAT labeled proteins were separated by SDS-PAGE. SDS facilitated solubilization of membrane proteins while it did not exert any harmful effect on cICAT labeling on cystein residue. A total of 364 proteins were identified and quantified, 43% of which were classified as membrane or membrane interacted proteins. It reveals that SDS-based cICAT is suitable for quantitation of membrane proteins. The quantitation result was further supported by Western blot on five representative proteins. The result supports reliability of our SDS-PAGE based cICAT method in quantifying cancer tissue proteome.

METHODS

Tissues and cell lines

Tissues were collected at the Breast Care Center of Seoul National Hospital in Korea. Authorization for the use of these tissues for research purposes was obtained from the Institutional Review Board of Seoul National University Hospital (Kim et al., 2009). Specimens of primary breast cancer and control normal tissues were matched according to TNM classification. The diagnosis was performed by experienced pathologists

(Table 1). Tissue specimens were stored in a liquid nitrogen tank. For cICAT analysis, we selected four pairs of breast cancer tissues and matched normal tissues, and combined each type of tissue separately. For validation by immunoblotting, we used 4 more tissue pairs. All of the patients had the diagnosis of an infiltrating ductal carcinoma. Normal tissues were sampled and were obtained from sites more than 5 cm away from the tumor margin of the cancer. All of the tissues were obtained and stored with the consent of each patient (Lee et al., 2005). For proteomic analysis, we chopped up tissues with scissors and pulverized them using liquid nitrogen in a mortar. After suspension in phosphate-buffer saline (PBS), the pulverized tissues were washed gently by swirling for 10 seconds and centrifuged shortly at 10,000×g. The supernatant was discarded and the clear pellet was stored at -80°C until needed.

Five human breast cancer cell lines (Hs578T, MCF7, SKBR-3, MDA-MB-231, and ZR-75-1) and one normal breast cell line (Hs578Bst) were obtained from American Type Culture Collection (ATCC) (Manassa, VA, USA) or Korean Cell Line Bank (Seoul, Korea). SKBR-3 and ZR-75-1 were grown in RPMI 1640 (GIBCO, Cat. 11875-119, USA) supplemented with 10% v/v FBS (GIBCO, Cat. 16000-044, USA), 1% penicillin and streptomycin (GIBCO, Cat. 10378-016, USA). MDA-MB-231 cell line was grown in DMEM (GIBCO, Cat. 11995-065, USA) with the same supplements, and Hs578T and MCF7 were cultured in the same media to which 0.01 mg/mL insulin (Sigma-Aldrich, Cat. I3536, USA) was added. Hs578Bst was cultured in the same medium for Hs578T to which 0.01 mg/mL EGF (Sigma-Aldrich, Cat. E9644, USA) was added. All the cell lines were cultured at 37°C in a humidified incubator with a mixture of 95% air and 5% CO_2

Sample preparation and isotope labeling

Prepared breast tissue pellet was resuspended in 50 mM Tris-HCl buffer (pH 8.3) containing 5 mM EDTA, 0.3% (w/v) β-mercaptoethanol, 0.1 M NaF, 1 mM PMSF, 0.0125% digitonin and a protease inhibitor cocktail and disrupted by sonication. The resulting tissue suspension was first submitted to a short centrifugation at 10,000×g at 4°C to eliminate nuclei and intact cells. The clear supernatant was transferred to a clean ultracentrifuge tube and ultracentrifuged for 1 h at 100,000×g at 4°C (Beckman, 90 Ti rotor, USA). The supernatant was then discarded and the pellet was resuspended in the same buffer and centrifuged again for 1 h at 100,000×g at 4°C. Pellets, containing the insoluble proteins, was finally resuspended in 50 mM Tris-Cl buffer (pH 8.3) containing 5 mM EDTA, 8 M urea and 4% (w/v) SDS. After protein concentration determination by Bradford assay, equal amounts of each sample (breast normal tissue and cancer tissue) were labeled with cleavable ICAT (cICAT) reagent (Applied Biosystems, Cat. 4339035, USA) according to the manufacturer's protocol

First, protein disulfide bonds were reduced with 250 mM tris(2-carboxyethyl)phosphine (TCEP) (Sigma-Aldrich, Cat. 646547, USA) for 30 min at 37°C. Cysteine residues were then alkylated with 350 nmol of cICAT reagent for 2 h in the dark at 37°C (normal breast tissue: light cICAT; cancer tissue: heavy cICAT). The alkylation reaction was quenched with 1.75 μmol of DTT for 5 min. The two samples were mixed and loaded on a 12% polyacrylamide gel and submitted to electrophoresis. The gel was stained with Coomassie Brilliant Blue.

In-gel digestion, affinity chromatography, and LC-MS/MS

The whole gel lane was excised into 8 bands of equal areas with a surgical blade. Each band was then cut into small pieces and placed into 1.5 ml microtube. Gel pieces were destained with a solution of 25 mM ammonium bicarbonate in 50% acetonitrile at 25°C for 10 min and dried in the air. Gel pieces were rehydrated with a solution of sequencing-grade trypsin (12.5 ng/μL; Promega, Cat. V5111, USA) in 25 mM ammonium bicarbonate, after which digestion was carried out for 16 h at 37°C. The reaction was quenched with 0.5% phosphoric acid. Tryptic peptides recovered from the gel pieces were subjected to affinity chromatography using the avidin cartridge (Applied Biosystems, Cat. 4326694, USA). That is, the tryptic peptides were suspended in PBS, loaded on an avidin-catridge, and then washed with PBS followed by 50 mM ammonium bicarbonate in 20% methanol, pH 8.3. cICAT-labeled peptides were eluted with 0.4% TFA in 30% acetonitrile, dried *in vacuo*, redissolved in 90 μl of 95% TFA, incubated at 37°C for 2 h to cleave off the biotin moiety from the cICAT-labeled peptide, and finally dried again.

Samples were reconstituted in 0.4% acetic acid and an aliquot (~1 μg) was injected into a reversed-phase capillary column (15 cm x 75 μm) packed in house with C_{18} resin (Magic C_{18} AQ 200Å; Michrom BioResources, Cat. H252, USA) using a helium pressure cell. An Agilent nanoflow 1200 series HPLC system (Agilent technology, USA) was used at a flow rate of 400 nl/min. The column was equilibrated with 95% solvent A (0.1% formic acid in H_2O) + 5% solvent B (0.1% formic acid in acetonitrile) prior to use. The cICAT labeled peptides were eluted with a linear gradient of 10 to 30% solvent B over 85 min. The HPLC system was coupled to an LTQ XL mass spectrometer (Thermo Scientific, USA) equipped with an in-house built micro-spray device. The spray voltage was set to 1.9 kV, and the temperature of the heated capillary was set to 250°C. The MS survey was scanned from 300 to 2000 m/z, and followed by three data-dependent MS/MS scans with the following options: isolation width, 1.5 *m/z*; normalized collision energy, 25%; dynamic exclusion duration, 180 sec.

Analysis of mass spectrometric data

Peak lists were generated using Extract-msn program (v3) in Bioworks package v3.2 (Thermo Scientific, USA) with the following parameters: minimum ion count threshold, 15; minimum intensity, 100. The peak lists were compared against the human International Protein Index database including known contaminants (IPI, versions 3.24, European Bioinformatics Institute, www.ebi.ac.uk/IPI) using the SEQUEST (TurboSequest version 27, revision 12) allowing two missed cleavages (trypsin) and ±0.5 and ±3 Da mass tolerance for MS/MS and MS respectively. cICAT option (+227.26 Da fixed modification plus +9 Da variable modification) on cysteine residue was used and a variable modification of methionine oxidation (+16 Da) was allowed. Peptide assignment and quantification were performed with the Trans-Proteomic Pipeline provided by Institute for Systems Biology (TPP, version 4.0, http://www.proteomecenter.org). The SEQUEST search output was used as an input for Peptide-Prophet module and peptides with probabilities greater than 0.05 were included in the following Protein-Prophet. Proteins with probabilities greater than 0.5 were put into manual inspection to evaluate MS/MS spectral quality (Kang et al., 2010). False discovery rate was 10% at the cut-off value of 0.5 before manual inspection. From a list of 519 proteins, 62 proteins were removed due to lack of quantification information and 93 proteins due to their unreliable mass spectra during manual inspection. The number of removed protein (93 ea) by manual inspection exceeded the number of estimated false positives (~ 52 ea) and as a result the false discovery rate for the final data set will be almost zero.

Western blot

For Western blotting, 16 clinical specimens, each from controlled normal and cancer tissues procured by HSA/IgG depletion, were analyzed. HSA/IgG depletion was performed by using a Vivapure Anti-HSA/IgG kit according to the manufacturer's protocol (Sartorious group, Cat. VS-SP08HAIGG, Germany). Six kinds of breast cancer cell line were performed Western blot for confirmation. The proteins (20 μg) from each sample were individually separated by 8~12% SDS-PAGE and electro-transferred onto PVDF membranes using the Mini Trans-Blot® cell (Bio-Rad, USA). The membranes were serially probed with one of the following primary antibodies: anti-RPN1 (1:200, Santa Cruz Biotechnology, Cat. sc-98270, USA), anti-PIGR (1:200, Santa Cruz, Cat. sc-374343), anti-ERp29 (1:1000, Novus Biologicals, Cat. NBP1-33076, USA), anti-PrP (1:200, Santa Cruz, Cat. sc-15312), and anti-TROP-2 (1:400, R&D systems, Cat. AF650, USA). ERp29 antibody recognizing signal peptide (sequence

position: 1-32, MAAAV PRAAF LSPLL PLLLG FLLLS APHGG SG) was produced by Abfrontier (Abfrontier Co. Ltd, Korea).

Donkey anti-rabbit IgG horseradish peroxidase (1:4000, GE Healthcare, Cat. NA934, England) or sheep anti-mouse IgG horseradish peroxidase (1:4000, GE Healthcare, Cat. NA931) or donkey anti-goat IgG horseradish peroxidase (1:4000, Santa Cruz, Cat. sc-2020) in TBS-T buffer were used as secondary antibodies. The chemiluminescent signals were detected using ECL™ Western blotting Detection (GE Healthcare, Cat. RPN2232SK). Dephosphorylation assay was treated by 10 units of shrimp alkaline phosphatase (Promega, Cat. M9910, USA) for 30 min at 30°C and immunoblotted. Band intensity of Western blot image was quantified using ImageQuant version 5.3. (GE Healthcare, Sweden)

CONFLICTS OF INTEREST

The authors declare no potential conflicts of interest.

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SUPPLEMENTARY INFORMATION

Supplementary Figure S1 and Table S1 are available in the website (http://www.bdjn.org/)

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