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Proteomic Analysis of Breast Cancer Tissues to Identify Biomarker Candidates by Gel-Assisted Digestion and Label-Free Quantification Methods Using LC-MS/MS

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This study presents a proteomic method that differentiates between matched normal and breast tumor tissues from ductal carcinoma *in situ* (DCIS) and invasive carcinoma from Korean women, to identify biomarker candidates and to understand pathogenesis of breast cancer in protein level. Proteins from tissues obtained by biopsy were extracted by RIPA buffer, digested by the gel-assisted method, and analyzed by nano-UPLC-MS/MS. From proteomic analysis based on label-free quantitation strategy, a non-redundant list of 298 proteins was identified from the normal and tumor tissues, and 244 proteins were quantified using IDEAL-Q software. Hierarchical clustering analysis showed two patterns classified as two groups, invasive carcinoma and DCIS, suggesting a difference between two carcinoma at the protein expression level as expected. Differentially expressed proteins in tumor tissues compared to the corresponding normal tissues were related to three biological pathways: antigen-processing and presentation, glycolysis/gluconeogenesis, and complement and coagulation cascades. Among them, the up-regulation of calreticulin (CRT) and protein disulfide isomerase A3 (PDIA3) was confirmed by Western blot analysis. In conclusion, this study showed the possibility of identifying biomarker candidates for breast cancer using tissues and might help to understand the pathophysiology of this cancer at the protein level.

Key words: Breast cancer, Ductal carcinoma *in situ*, Biomarker candidates, Proteomics, LC-MS/MS

INTRODUCTION

Breast cancer is the most common cause of death in women worldwide (Parkin et al., 2005). In Korea, the incidence of cancer has increased rapidly since 2001, even in the face of decreasing mortality rates reported in many other developed countries. The westernization of the Korean lifestyle, a lower rate of breastfeeding, lower birth rates, and an increase in the number of check-ups for breast cancer could partially explain this trend (Yoo et al., 2002). However, risk factors for cancer such as age, socioeconomic status, reproductive events, family history, and lifestyle explain only a small part of the cases because the etiology of breast cancer is known to be so complex (Son et al., 2006). Although previous studies have tried to demonstrate possible risk factors of breast cancer by molecular techniques using DNA or RNA from tumor cell lines or tumor tissues, it would be difficult to show the relationship to the expression of the targeted proteins (Truong et al., 1999; Kenny et al., 2007). Since breast cancer is known to progress from benign to invasive lesions (Beckmann et al., 1997; Russo and Russo, 1999; Polyak, 2001), it is possible to hypothesize that there might be differences in the microenvironments and

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tumor-related proteins among the various stages of breast tumors, such as adenoma, ductal carcinoma *in situ* (DCIS), and invasive breast cancer. Therefore, analysis of differentially expressed tumor-related proteins might be helpful to identify candidate biomarkers for tumors in the development of breast cancer.

Recently, various proteomic approaches have been regarded as successful methods in identifying tumor-related protein markers and in understanding the molecular mechanism of breast cancer metastasis (Bouchal et al., 2009). For these studies, 2-D polyacrylamide gel electrophoresis (PAGE) has usually been used. However, it has been reported that this method has some disadvantages including limited separation based on *pI* and molecular weight, and poor detection of membrane and low-abundance proteins (Sutton et al., 1995). To overcome the disadvantages of 2-D PAGE, nano-LC-MS/MS technology is introduced in this study.

Furthermore, pretreatment of proteomes from tissue or cell culture samples is very important for LC-MS/MS analysis because high concentrations of detergent are incompatible with subsequent trypsin digestion, reducing the potential to successful identification of proteins and resulting in inaccurate quantification of each protein. Recently, the gel-assisted digestion method exhibited a greater degree of compatibility with high concentrations of detergents allowing for the efficient removal of detergents and solvents (Han et al., 2008).

In this study, LC-MS/MS analysis with gel-assisted digestion method was used to increase the number of proteins identified from normal and tumor tissues. To quantify the proteins, IDEAL-Q software (Tsou et al., 2010), a newly reported and reliable tool, was used for label-free quantification of the proteins from the matched tissues (normal and tumor parts of the breast tissue from each patient) of 3 patients. From the 298 proteins identified and 244 proteins quantified, we selected three biological pathways through a hierarchical tree algorithm and confirmed two proteins by western blot analysis. This study is the first in-depth proteomic approach for matched normal and tumor breast tissues from Korean patients, and the strategy used in this study is a good approach to identify biomarker candidates for other diseases.

MATERIALS AND METHODS

Patient characteristics

The study protocol was approved by the Institutional review board of Kyungpook National University Hospital. Informed consent was confirmed by the IRB. Tumor samples of one DCIS and two invasive ductal carcinomas, and the matched normal samples were provided from three postmenopausal women. Both tumor and normal samples originated from the same breast in each case. Tumor size ranged between 0.5 and 2.5 cm. Tissue pieces were weighed, shock-frozen in liquid nitrogen, and kept at −80°C until use.

Gel-assisted digestion

To extract proteins from each tissue (1~2 g/tissue), 100 μL of RIPA buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) was added into each tissue and homogenized by plastic tip homogenizer. The tissue debris was pelleted by centrifugation at 1,200 g for 15 min at 4°C. The supernatant contained 350~500 μg per each sample. For gel-assisted digestion, 50 μg of this extracted protein was resuspended in 50 μL of 6 M urea, 5 mM EDTA, and 2% (w/v) SDS in 0.1 M triethylammonium bicarbonate (TEABC) and incubated at 37°C for 30 min for complete dissolution. Proteins were chemically reduced by adding 3 μL of 10 mM Dithiothreitol (DTT) and alkylated by adding 3 μL of 50 mM iodoacetaminde (IAA) (in a dark reaction) at room temperature for 20 min. To incorporate the proteins in the gel directly into the Eppendorf vial, 18.5 μL of acrylamide/bisacrylamide solution, 2.5 μL of 10% Ammonium persulfate (APS), and 1 μL of 100% TEMED was then applied to the protein solution. The gel was cut into small pieces and washed several times with 1 mL of TEABC containing 50% acetonitrile (ACN). The gel samples were further dehydrated with 100% ACN and then completely dried by SpeedVac. Proteolytic digestion was then done with trypsin (protein:trypsin = 50:1, w/w) in 25 mM TEABC incubating overnight at 37°C. Peptides were extracted from the gel using sequential extraction with 200 μL of 25 mM TEABC, 200 μL of 0.1% TFA in water, 200 μL of 0.1% TFA in ACN, and 200 μL of 100% ACN. The solutions were combined and concentrated in a SpeedVac.

LC-methods

The nanoscale LC separations of the tryptic peptide mixtures (0.5 μg per each LC run) were done using a nanoAcquity system (Waters Corporation), equipped with a Symmetry C18 (5 μm) 300 μm × 5 mm precolumn and a BEH C18 (1.7 μm) 75 μm × 25 cm analytical reversed phase column (Waters Corporation). Analysis of all samples was performed in triplicate. Mobile phase A was water with 0.1% formic acid and mobile phase B was 0.1% formic acid in ACN. The samples were initially transferred in mobile phase A to the precolumn at a flow rate of 10 μL/min for 5 min. The peptides were separated with mobile phase B over a gradient of 1 to 80% over 95 min at a flow rate of 300 nL/min, followed

by a 20 minute rinse with 80% of mobile phase B (Moon et al., 2011).

Data acquisition and processing

The analysis of tryptic peptides was done using a Q-Tof Premier mass spectrometer (Waters Corporation) (Cho et al., 2012). Accurate mass LC-MS data were collected in a data dependent mode of acquisition. The NanoLockSpray source was used for accurate mass measurement, and the lock mass channel was sampled every 30 sec. The method included a full sequential MS scan (m/z 400-1600, 0.6 sec) and three MS/MS scans (m/z 100-1990, 1.2 sec per scan) on the three most intense ions present in the full-scan mass spectrum. Raw MS/MS data were converted into a peak list using MASCOT Distiller version 2.0 (Matrix Science) with the default parameters. All MS/MS samples were analyzed using MASCOT version 2.2.1 (Matrix Science). Mascot was set up to search the IPI HUMAN 3.70 database. The database search against Mascot was done with a fragment ion mass tolerance of 0.1 Da and a parent ion tolerance of 0.05 Da. Two missed cleavages were allowed for the trypsin digestion. Carbamidomethylation of the cysteines was considered as a fixed modification, whereas oxidation of methionines was considered as variable modifications. To evaluate the false discovery rate (FDR) of protein identification, we repeated the searches using identical search parameters and validation criteria against a randomized decoy database created by Mascot. Peptides identities were assigned if their mascot ion scores for $p < 0.05$. Proteins with more than 2 peptides were identified with confidence. The FDRs with Mascot protein scores >34 ($p < 0.05$) ranged below 2%.

Relative quantification by IDEAL-Q

The quantitative analysis of peptides in our label-free experiments was done by IDEAL-Q software (Tsou et al., 2010). The raw data files acquired from Waters Q-TOF Premier were converted into mzXML formatted files by the program massWolf, and the search results in MASCOT were exported in eXtensive Markup Language data (.XML) format. After the data conversion, the results from the peptide identification with confidence ($p < 0.05$) from each LC-MS/MS run and the corresponding mzXML files were used to perform quantitation analysis by IDEAL-Q (Tsou et al., 2010).

Hierarchical clustering and KEGG pathway analysis

Hierarchical clustering of 59 proteins was done with the Cluster/TreeView analytic package (Eisen, Stanford University). KEGG pathway analysis was done according to the gene ontology (GO) using The Database for Annotation, Visualization, and Integrated Discovery (DAVID, http://david.abcc.ncifcrf.gov/).

Western blot analysis

Normal and tumor tissues were analyzed by western blot to study the expression level of some of the proteins identified by MS. 40 μg of proteins were loaded onto 7% polyacrylamide gels and transferred to nitrocellulose membranes (Whatman). Membranes were blocked for 2 h using 5% skim-milk in TBS containing 0.1% Tween 20 (T-TBS). The primary antibodies were diluted in T-TBS and incubated overnight at 4°C. The primary antibodies used were mouse-monoclonal antibodies to PDI (ERp57, Abcam) and rabbit-polyclonal antibodies to CRT (Abcam). Secondary antibodies consisted of HRP-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology) or goat anti-mouse IgG (Cell signaling), which were detected by the ECL-plus western blotting analysis system (GE Healthcare).

RESULTS AND DISCUSSION

Protein identification using LC-MS/MS

A total of three tumor tissues and three matched normal tissues were collected from three patients with invasive ductal carcinoma (pat. 1 and 2) and ductal carcinoma *in situ* (DCIS; pat.3) for proteomic analysis. The characteristics of the patients and their tumors are presented in Table I. Protein extracts were subjected to gel-assisted digestion with trypsin, which has the advantage to digest proteins in a denatured state without SDS gel separation and eliminates other chemicals included in the denaturation step without desalting (Han et al., 2008). One μg of digested peptide was injected into the LC-MS/MS, and samples were analyzed in triplicate. Fig. 1 shows an outline of the method, which identifies differentially expressed proteins from normal and tumor tissues using nano-UPLC-MS/MS. Through LC-MS/MS, a total of 298 non-redundant proteins were identified, and 244 proteins were quantified using software called IDEAL-Q for label-free quantification (Tsou et al., 2010). This software helped increase the number of proteins quantified by aligning peptide peaks with their elution times as well as signal-to-noise

Table I. Patient information

Patient	Histopathology	Grade	Age	Size of lesion (cm)
Pat 1.	Invasive carcinoma	Low	65	0.5
Pat 2.	Invasive carcinoma	2	58	2.5
Pat 3.	DCIS	2	79	1.7

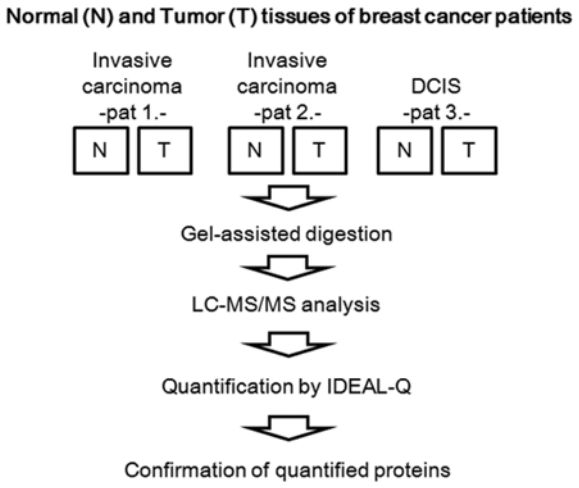

Fig. 1. Experimental strategy for proteomic approaches to identify differentially expressed proteins from breast tumor tissues and normal tissues.

ratio validation on the detected peptide peak clusters for all LC-MS/MS analyses.

Analysis of differentially expressed proteins in breast cancer with hierarchical clustering and pathway analysis

Cluster analysis was done on the identified proteins to evaluate the relationship between differentially regulated proteins and breast tissue samples and to estimate interesting protein expression clusters. By using hierarchical clustering analysis, a clear distinction of the expression patterns was possible by the clustering of these proteins into several characteristic profiles, which split 59 of the proteins into two main clusters: either up-regulated (in red) or down-regulated (in green) (Fig. 2A). Furthermore, these patterns interestingly were classified as two groups, invasive carci-

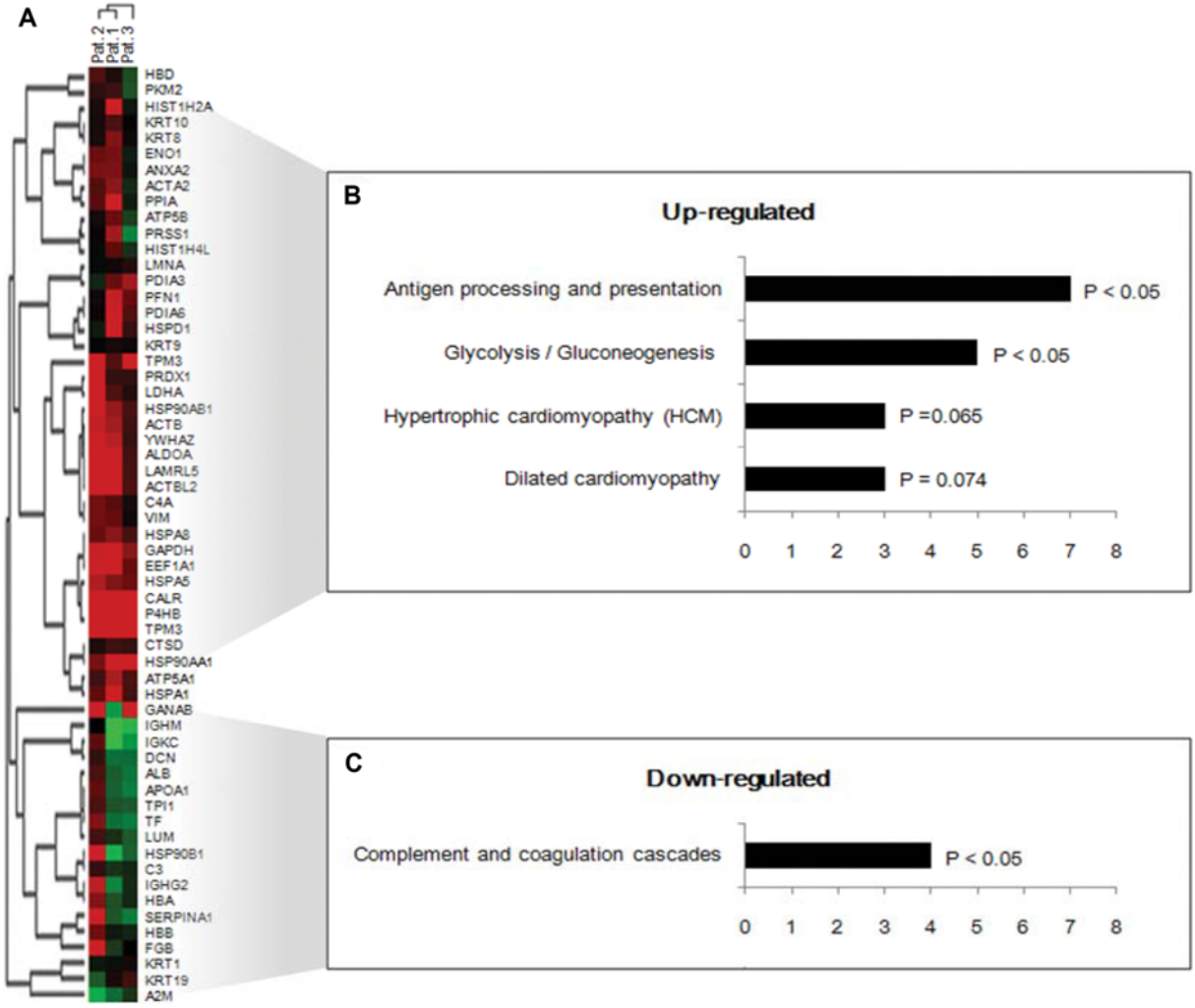

Fig. 2. Clustering analysis of proteins differentially expressed between normal and tumor samples, and KEGG pathways. (**A**) The 59 proteins expressed in the all 3 breast cancer patients were classified into 2 main groups via hierarchical clustering analysis. Up-regulated (**B**) and down-regulated (**C**) proteins were analyzed by KEGG pathway.

noma (patients 1 and 2) and DCIS (patient 3), showing a difference between DCIS and invasive carcinoma at the protein expression level, as expected. Furthermore, to find any pathways which could differentiate the two main clusters, up-regulated and down-regulated clusters were analyzed by the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway (Fig. 2B and 2C). The up-regulated cluster had four pathways: antigen processing and presentation, glycolysis/gluconeogenesis, hypertrophic cardiomyopathy, and dilated cardiomyopathy. Among them, antigen processing and presentation, and glycolysis/gluconeogenesis pathways had meaningful statistics (p-value < 0.05, FDR < 5%). On the other hand, the down-regulated cluster had one pathway, the complement and coagulation cascades (*p*-value < 0.05, FDR < 5%). Therefore, in this study, we focused on these three pathways to identify biomarker candidates, and might understand the pathogenesis of breast cancer at the protein level. The list of proteins related to the three pathways is presented in Table II.

The antigen processing and presentation pathways included three up-regulated proteins, calreticulin (CRT), protein disulfide isomerase A3 (PDIA3), and binding immunoglobulin protein (BiP). These proteins, known as chaperone proteins in the endoplasmic reticulum (ER), are related to antigen processing, which prepares antigens for presentation to special cells from the immune system such as T lymphocytes. Among them, CRT is an ubiquitous protein that was first identified as a Ca^{2+}-binding protein in skeletal muscle sarcoplasmic reticulum (Ostwald and MacLennan, 1974). Later, it was found in nonmuscle endoplasmic reticulum membrane as well as in muscle cells, and cDNA and genes encoding CRT have been isolated from several vertebrates and invertebrates as well as from higher plants (Michalak et al., 1999). Participation of CRT has been implicated in many cellular functions, including Ca^{2+} storage and signaling, lectin-like chaperoning, regulation of gene expression, cell adhesion, and autoimmunity. CRT also has the role of a stress protein, and its production can be induced by a variety of pathophysiological stresses, heat shock, and amino acid deprivation (Conway et al., 1995; Heal and McGivan, 1998). Increased production of CRT has been reported in several cancerous tissues, such as breast, liver, and prostate cancer (Franzen et al., 1996; Bini et al., 1997; Alaiya et al., 2000; Yoon et al., 2000; Yu et al., 2000). Although increases of CRT in tumor cells and prolif-

Table II. The list of proteins related to antigen processing and presentation, glycolysis/gluconeogenesis, and complement and coagulation cascades pathways and ratios of proteins differentially expressed in tumor tissues compared to the corresponding normal tissues in invasive carcinoma (2 patients) and DCIS (1 patient)

Accession	Gene name	Description	Invasive Carcinoma		DCIS
			Pat. 1	Pat. 2	Pat. 3
Antigen processing and presentation (up-regulated)			fold change (T/N)[a]		
IPI00025252	PDIA3	Protein disulfide-isomerase A3	6.0	0.7	3.4
IPI00003362	HSPA5	HSPA5 protein	3.6	7.2	4.4
IPI00003865	HSPA8	Isoform 1 of Heat shock cognate 71 kDa protein	2.4	3.5	4.7
IPI00414676	HSP90AB1	Heat shock protein HSP 90-beta	2.4	T	5.5
IPI00304925	HSPA1	Heat shock 70 kDa protein 1A/1B	2.3	3.1	21.4
IPI00382470	HSP90AA1	Isoform 2 of Heat shock protein HSP 90-alpha	T[b]	3.9	T
IPI00020599	CALR	Calreticulin	T	89.2	T
Glycolysis / Gluconeogenesis (up-regulated)			fold change (T/N)		
IPI00465439	ALDOA	Fructose-bisphosphate aldolase A	2.3	T	19.5
IPI00479186	PKM2	Isoform M2 of Pyruvate kinase isozymes M1/M2	0.4	1.7	2.1
IPI00217966	LDHA	Isoform 1 of L-lactate dehydrogenase A chain	1.5	T	2.6
IPI00465248	ENO1	Isoform alpha-enolase of Alpha-enolase	0.8	3.5	3.9
IPI00219018	GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	4.8	9.5	20.9
Complement and coagulation cascades (down-regulated)			fold change (T/N)		
IPI00298497	FGB	Fibrinogen beta chain	1.0	T	0.5
IPI00478003	A2M	Alpha-2-macroglobulin	0.5	0.1	0.3
IPI00553177	SERPINA1	Isoform 1 of Alpha-1-antitrypsin	0.3	T	0.4
IPI00783987	C3	Complement C3 (Fragment)	0.7	1.9	0.6

[a]Fold change present the ratio of tumor/normal (T/N) tissues. The peptides corresponding to a protein were quantified using IDEAL-Q software. [b]T indicates unique proteins detected twice in tumor group but not in normal group.

erating cells are well known, details on the mechanisms for these increases are as yet undetermined. As previously reported (Ramsamooj et al., 1995), the up-regulation of CRT seems to be the result of cellular stress derived from breast cancer.

The PDIA3 (protein disulfide isomerase A3) and heat shock 70 kDa protein 5 (HSPA5) were up-regulated in tumor tissues. PDIA3 is also a part of the major histocompatibility complex (MHC) class I peptide-loading complex, which is essential for the formation of the final antigen conformation and export from the endoplasmic reticulum to the cell surface (Garbi et al., 2006). This PDIA3 has been detected as up-regulated protein in MCF-7 breast cancer cell line (Bianchi et al., 2005) and this protein may be induced in response to cellular stress in breast cancer state. In humans, HSPA5, known as binding immunoglobulin protein (BiP) (Ting and Lee, 1988; Hendershot et al., 1994), and the 78 kDa glucose-regulated protein (GRP-78) (25) are molecular chaperones located in the lumen of the endoplasmic reticulum (ER) that bind newly-synthesized proteins as they are translocated into the ER and maintain them in a competent state for subsequent folding and oligomerization. BiP is also an essential component of the translocation machinery, as well as plays a role in retrograde transport across the ER membrane of aberrant proteins destined for degradation by the proteasome. BiP is an abundant protein under all growth conditions, but its synthesis is markedly induced under conditions that lead to the accumulation of unfolded polypeptides in the ER. This up-regulated HSPA5 may play a role in suppressing misfolded proteins in breast cancer cells.

Five proteins including fructose-bisphosphate aldolase A, pyruvate kinase isozymes M1/M2, lactate dehydrogenase, alpha-enolase 1, and glyceraldehyde 3-phosphate dehydrogenase were up-regulated in the invasive carcinoma group. Among them, the pyruvate kinase isoenzyme type M2 is expressed in some differentiated tissues, such as lung, fat tissue, retina, and pancreatic islets as well as in cells with a high rate of nucleic acid synthesis such as normal proliferating cells, embryonic cells, and especially tumor cells (Corcoran et al., 1976; Tolle et al., 1976; Reinacher and Eigenbrodt, 1981; Schering et al., 1982; MacDonald and Chang, 1985; Brinck et al., 1994). Lactate dehydrogenase (LDH) is an enzyme present in a wide variety of organisms, including plants and animals. It is used to monitor cancer (especially lymphoma) patients since cancer cells have a high rate of turnover with destroyed cells leading to an elevated LDH activity (Burke et al., 1978). Therefore, up-regulation of these proteins related to glycolysis/gluconeogenesis pathway in breast tumor cells could explain the general concept where rapid growing tumor cells show higher rate of metabolism than in normal cells.

Four proteins including fibrinogen beta chain, alpha-2-macroglobulin, alpha-1-antitrypsin, and complement C3, related to the complement and coagulation cascades pathways were slightly down-regulated in invasive carcinoma. The complement system helps improve the ability of antibodies and phagocytic cells to clear pathogens from an organism (Janeway, 2005). Therefore, in the breast carcinoma stage, these immune functions might be down-regulated to change the environment and so it is better suited for cancer development.

As evidences of the precise identification of proteins by LC-MS/MS, the mass spectrum of each representative peptide from CRT and PDIA3 are shown in Fig. 3C and F. A peptide, EQFLDGDGWTSR, corresponding to the partial amino acid sequence 25-36 of CRT had an ion signal of m/z 705.83. A peptide, TADGIVSHLK corresponding to the partial amino acid sequence 120-129 of PDIA3 had an ion signal of m/z 520.80. The number of peptides used for the identification of CRT and PDIA3 was 3 and 7, respectively.

Validation of differentially expressed proteins in breast cancer patients by western blot

To further validate the results obtained from the relative comparative expression studies using LC-MS/MS, we examined the expression status of several identified proteins using western blot. These representative proteins were selected based on hierarchical clustering and pathway analysis. In cases where antibodies were suitable for western blot, we tested their reactivity with the breast cancer samples as a means of verification. Protein extracts from normal and tumor tissues from 3 patients were separated by SDS/PAGE and blotted onto nitrocellulose membranes. Fig. 3 shows a representative compilation of the western blot analysis for these proteins. These representative proteins included PDIA3 and CRT. The expression level of CRT was significantly increased in the tumor tissues from the invasive carcinoma patients but slightly increased in the tumor tissues from the DCIS patient. In the case of PDIA3, the expression level of the protein was significantly increased in the tumor tissues from both invasive carcinoma and DCIS patients. The results of the western blot analysis with tumor and normal tissues confirmed the LC-MS/MS results ($p < 0.05$). Therefore, this study demonstrates that some of the proteins identified by LC-MS/MS could serve as potential biomarkers in future studies of breast cancer.

The identification of biomarker candidates for breast cancer is so important and many studies using a recent

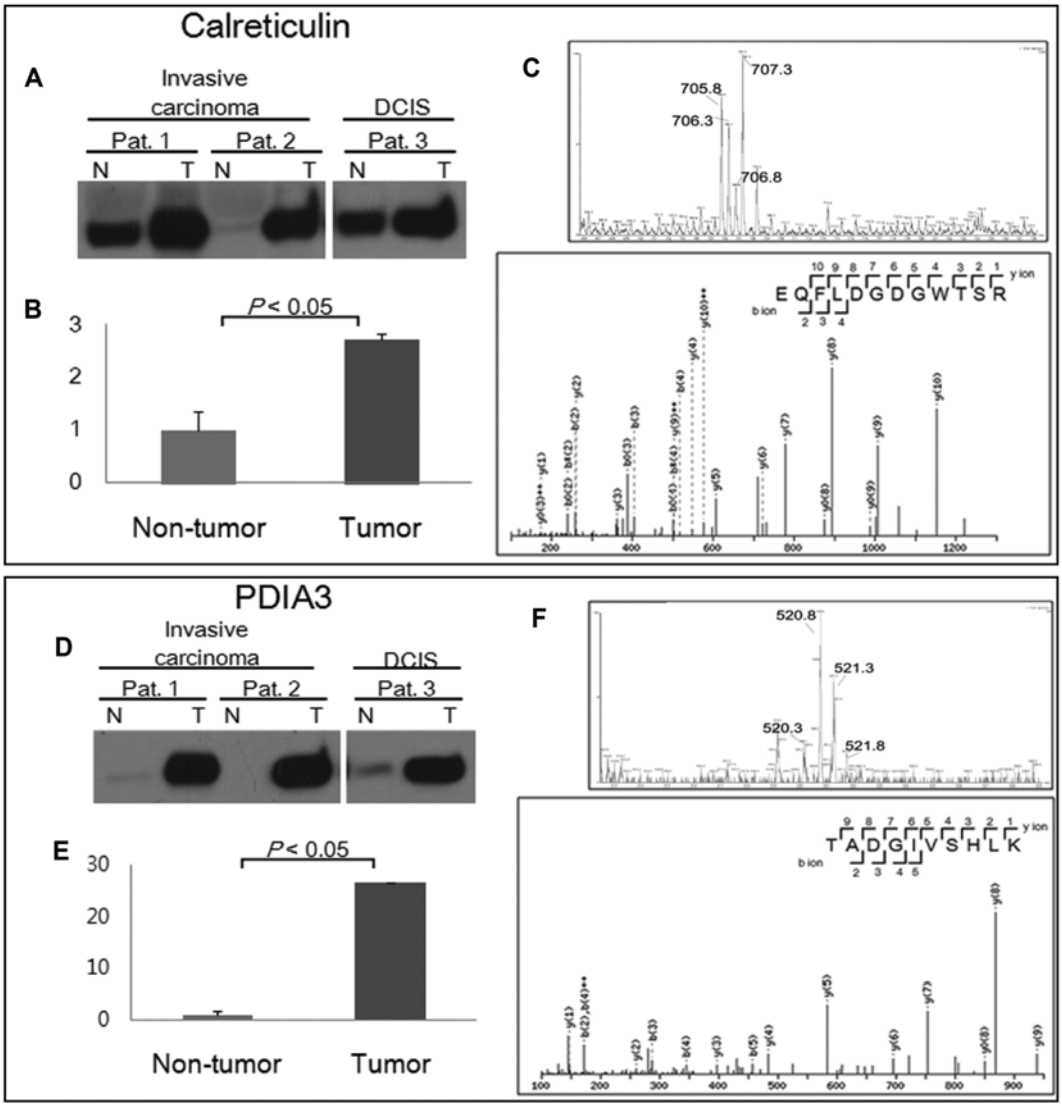

Fig. 3. Expression levels of CRT and PDIA3 in breast cancer samples. In total, three pairs of tumor tissue (T) and matched normal tissues (N) were examined. A, B, and C shows the information for CRT; western blot (**A**), their expression levels (**B**) and MS/MS fragmentation (bottom) of peptide with parent ion spectrum (top) (**C**). D, E, and F shows the information for PDIA3; western blot (**D**), their expression levels (**E**) and MS/MS fragmentation (bottom) of peptide with parent ion spectrum (top) (**F**).

proteomics technology have been reported. There are two factors, a digestion efficiency and a quantification method, which contribute to the quality of proteomic result. To improve these two factors, first, a gel-assisted digestion method has been introduced, which increased the efficiency of digestion of proteins by making proteins denatured and increased the recovery yield of proteins by reducing desalting steps. Second, label-free quantification based on IDEAL-Q software which increased the number of proteins quantified based on several factors explained in result, has been introduced in this study. Finally, 298 proteins were identified from tissues and 244 proteins were quantified with high quality of reliability. Among them, two identified proteins, CRT and PDIA3, related to breast cancer development were confirmed by western blot analysis. Furthermore, this method investigated in this study could be applied to other proteomic research to identify biomarker candidates using clinic tissues.

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