Proteomic Analysis of Potential Breast Cancer Biomarkers

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1. Introduction

1.1 Cell signalling and tumorigenesis

In multi-cellular organisms, cells have to communicate with each other in order to control their proliferation, differentiation, survival and to perform diverse physiological functions. Cells release and receive signals to induce these different states of growth either by direct cell-to-cell interaction or via secreted molecules. This communication is elicited through so-called signaling molecules such as transmembrane receptors that are embedded in cell membrane and can activate intracellular signal transduction cascades which ultimately lead to gene activation or repression and a cellular response. According to the specificity, strength and duration of the signal received, the cell will proliferate, differentiate, change shape, migrate, and enter into growth arrest or undergo apoptosis. These complex signaling networks are highly regulated and alterations of the normal intracellular signals can lead to the development of diseases such as cancer. It is now known that a series of genetic mutations are required for the progressive conversion of normal human cells into cancerous cells. Hanahan and Weinberg have proposed a model of tumorigenesis, whereby several physiological conditions are required before cells become tumorigenic (Hanahan and Weinberg, 2011). These include self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of programmed cell death, limitless replicative potential, sustained angiogenesis, tissue invasion and metastasis. In proliferative signaling pathways for example, numerous proto-oncogenes or tumor suppressors have been identified, the mutation of which cause amplification of signaling or loss of negative regulation resulting in over-proliferation and eventual tumor formation. Unlike normal cells, which tightly regulate extracellular ligand levels, receptor expression and secondary signaling molecules, cancer cells often lose the ability to regulate these signaling events. For example, overexpression of receptor tyrosine kinases (RTKs) (Libermann et al., 1985), mutation of RAt Sarcoma (Ras) protein (Marshall, 1996) or the overexpression of PI3K (phosphatidylinositol 3-kinase) (Sulis and Parsons, 2003) are thought to lead to cell transformation.

1.2 Correlation between tumor secreted proteins and cancer

During tumor metastasis, cell-cell interactions are decreased leading to cell dissociation and detachment from the primary tumor. On the other hand, cell-extracellular matrix

interactions are increased facilitating tumor cell migration and metastasis. Thus, during tumorigenesis and metastasis, the secreted proteins in the extracellular space are majorly responsible for growth control, cell adhesion/migration, matrix-degradation, invasion and angiogenesis (Mbeunkui et al., 2006b). Importantly, these tumor cell secreted proteins majorly enter body fluid system such as blood, urine, lymph fluid and can be measured by non-invasive tests. Thus, analysis the tumor secreted proteins is a promising strategy to discover cancer biomarkers.

1.3 Detection of breast cancer markers with high-throughput technologies

Breast cancer is one of the leading causes of death among women around the world. The 5-year survival rate for breast cancer is near 97% when tumors are confined to breast tissue, but decrease dramatically to 23% when tumors have metastasized to other organs at the time of diagnosis (Kulasingam and Diamandis, 2007b; Jemal et al., 2004). Previous studies indicated that the transformation and metastasis of normal breast cells are correlated to altered expression in both transcription and translation levels (Nuyten and van de Vijver, 2008; Morrow, 2007; Lee et al., 2007; Kulasingam and Diamandis, 2007a; Hondermarck et al., 2002). To better understand the molecular mechanisms associated with tumorigenesis and metastasis, it is necessary to identify gene expression signatures and protein expression markers among non-tumorigenic breast cells, non-invasive breast cancer cells, and invasive breast cancer cells. At the transcription level, microarray strategies have been used to classify breast tumors as highly invasive and non-invasive cancer (Sorlie et al., 2003; Nagaraja et al., 2006). At the translation level, proteomic strategies have been used to discern cancer markers from non-invasive and invasive breast cells (Nagaraja et al., 2006; Pawlik et al., 2006; Pucci-Minafra et al., 2002; Varnum et al., 2003). Nagaraja *et al.* compared the proteomic profiling of cell lines corresponding to healthy breast cells, non-invasive breast cancer cells, and invasive breast cancer cells using two-dimensional gel electrophoresis (2-DE). Pucci-Minafra *et al.* compared a ductal infiltrating carcinoma-derived cell line with a non-tumoral mammary epithelial cell line using 2-DE, silver staining, immunodetection, and N-terminal sequencing and identified 58 differentially expressed proteins. In contrast to these cell line based studies, Pawlik *et al.* and Varnum *et al.* analyzed differentially expressed proteins among nipple aspirate fluid samples from tumor-bearing and disease-free breasts. Although these identified proteins are primarily abundant proteins, few of them have been validated as biomarkers.

During tumorigenesis and metastasis, secreted proteins in the extracellular space are major factors in growth control, cell motility, cell invasion, angiogenesis and matrix-degradation (Mbeunkui et al., 2006a). Consequently, the analysis of tumor secreted proteins is a promising strategy for identifying cancer biomarkers. In the past few years, researchers have used proteomic analysis to identify some secreted biomarker candidates for human cancer using 2-dimensional differential in-gel electrophoresis (2D-DIGE) and liquid chromatography-tandem (LC-tandem) mass spectrometry. These markers have been found in lung cancer, liver cancer, pancreatic cancer and colorectal cancer (Xue et al., 2008b). In breast cancer research, Kulasingam and Diamandis used a liquid chromatography-mass spectrometry/mass (LC-MS/MS) strategy to analyze and compare the expression of extracellular and membrane-bound proteins in conditioned media of three breast cell types corresponding to a normal control and cell lines derived from stage 2 and stage 4 patients. Their studies identified numerous marker proteins from conditioned media (Kulasingam and Diamandis, 2007b).

1.4 Utilization of 2D-DIGE / MALDI-TOF MS-based strategies in the global analysis of breast cancer markers

2-DE is currently a key technique in profiling thousands of proteins within biological samples and plays a role complementary to LC/MS-based proteomic analysis (Timms and Cramer, 2008a). However, reliable quantitative comparisons between gels and gel-to-gel variations remain the primary challenge in 2-DE analysis. A significant improvement in the gel-based analysis of protein quantitation and detection was achieved by the introduction of 2D-DIGE, which can co-detect numerous samples in the same 2-DE. This approach minimizes gel-to-gel variations and compares the relative amount of protein features across different gels using an internal fluorescent standard. Moreover, the 2D-DIGE technique has the advantages of a broader dynamic range, higher sensitivity, and greater reproducibility than traditional 2-DE. This innovative technology relies on the pre-labeling of protein samples with fluorescent dyes (Cy2, Cy3 and Cy5) before electrophoresis. Each dye has a distinct fluorescent wavelength, allowing multiple experimental samples with an internal standard to be simultaneously separated in the same gel. The internal standard, which is a pool of an equal amount of the experimental protein samples, helps provide accurate normalization data and increase statistical confidence in relative quantitation among gels (Timms and Cramer, 2008b; Westermeier and Scheibe, 2008; Marouga et al., 2005; Lai et al., 2010; Chou et al., 2010; Huang et al., 2010; Chan et al., 2005; Chan et al., 2009).

Followed the separation of the proteins from biological samples by 2-DE and proteolysis of interested spots by a specific protease, subsequently mass spectrometry is an accurate and sensitive tool to identify these interesting proteins/peptides. Basically, there are two major types of approach used in the identification of proteins and peptides. The first one is matrix-assisted laser-desorption/ionization mass spectrometry (MALDI-TOF-MS) (Karas and Hillenkamp, 1988) and the other one is electrospray ionization mass spectrometry (ESI-MS) (Fenn et al., 1989; Whitehouse et al., 1985). MALDI-TOF-MS relies on ions generated from a solid phase using laser pulses. The sample is usually applied in a matrix solution [eg. 2,5-dihydroxybenzoic acid (DHB) and alpha-cyano-4-hydroxycinnamic acid (CHCA)] that facilitates the ion formation by absorption of photon energy from a laser source. ESI-MS generates ions from a liquid phase. The sample, in a solvent mixture is directly sprayed into the mass spectrometry where an electrostatic field is formed between the capillary and the walls of the mass spectrometer. As the droplets form and travel, they evaporate and the resulting charged particles enter into the gas phase. Each ion is separated in the mass spectrometry according to its mass-to-charge ratio (m/z ratio). Protein identification by mass spectrometry can be carried out by peptide mass mapping using MALDI-TOF-MS or by further peptide fragmentation to generate sequence data using tandem mass spectrometry (MS/MS) (Henzel et al., 1993; James et al., 1993; Mann et al., 1993; Yates, III et al., 1993).

1.5 Application of luminal epithelial cell models with various invasive stages in the discovery of breast cancer markers

A direct comparison of cancer tissue with normal tissue is the best theoretical method of obtaining protein expression signatures during tumor progression. However, a direct comparison of clinical samples increases the amount of false positives due to the heterogeneity of tumor specimens, which interferes with the identification of tumor-specific markers. For this reason, well-characterized model cell lines established from normal and

tumor tissue are recognized as more informative in cancer proteomics research. In the field of breast cancer research, MCF-10A, MCF-7 and MDA-MB-231 are widely used to represent non-tumorigeneic breast luminal epithelial cells, non-invasive breast cancer cells derived from the luminal duct and metastatic breast cancer cells derived from the same tissue, respectively (Singh et al., 2006; Lu et al., 2006). Accordingly, we are introduced a proteomics strategy to discover the putative diagnostic markers and therapeutic targets from this cell model system. To achieve these goals, it is necessary to identify potential biomarkers that reflect the progression of tumorigenesis. Thus, we compared the proteomic profiles of total cellular proteins and secreted proteins of this cell model system using 2D-DIGE to quantitatively identify putative transformation markers in breast cancer.

2. 2D-DIGE and MALDI-TOF MS analysis of secretomes across MCF-10A, MCF-7 and MDA-MB-231 cells

Secreted proteins, plasma membrane bound proteins and extracellular proteins mediate cell attachment, cell motility, cell-cell interactions and cell invasion. These proteins have the highest possibility of being found in the circulation system, including the blood, and thus serve as cancer markers or important markers involved in cancer formation (Xue et al., 2008a). To identify potential proteins that may be involved in tumor formation and metastasis, this study develops a strategy for preparing secreted proteins from normal and cancer cell lines with minimal cytosolic protein contamination. Although these cell lines are generally grown in serum-supplemented media, a serum-free conditioned medium is necessary to prevent serum protein contamination and to allow accurate detection of proteins secreted by cells. A serum-free medium is believed to affect the growth of cells and the production of secreted proteins; however, recent studies indicate that the serum-free condition does not significantly affect the composition of the secreted proteins (Yamaguchi et al., 1990; Inoue et al., 2000). In addition, it is impossible to prevent cell death, and the release of considerable amounts of cytosolic proteins into culture media in either the serum-free condition or the serum-supplemented medium. Accordingly, an intensive wash step was performed prior to incubating these cells in serum-free media to remove both cytosolic proteins and serum proteins. Meanwhile, the incubation time in serum-free media was optimized in advance, minimizing the serum-free induced autolysis of the cells, and enabling the recovery of an adequate amount of secreted proteins for 2D-DIGE analysis. The concentration of secreted proteins in this study was extremely low at approximately 1~2 μg / ml. For this reason, a concentration step was essential to enrich secreted proteins enough for proteomics analysis, and a desalt step was also required for the 2D-DIGE experiment. In this secretomic analysis, MCF-10A, MCF-7 and MDA-MB-231 were grown on cell culture dishes and the confluency of cells was checked prior to incubation in serum-free culture media to ensure that no other exogenous proteins were present. To minimize cell autolysis induced by starvation and to maximize secreted protein concentration in the media, the starvation time of each cell line has to be optimized. Through immunoblotting, the lactate dehydrogenase (LDH) and β-tubulin levels were detected in the 1000-fold concentrated serum-free media starting at 48~60 hrs and at 60~72 hrs, respectively (Figure 1). LDH and β-tubulin are both cytoplasmic proteins and their levels in the media represent the amount of cell death taking place in cell culture. Accordingly, a starvation period of 30 hrs was chosen for further 2D-DIGE based secretomic analysis.
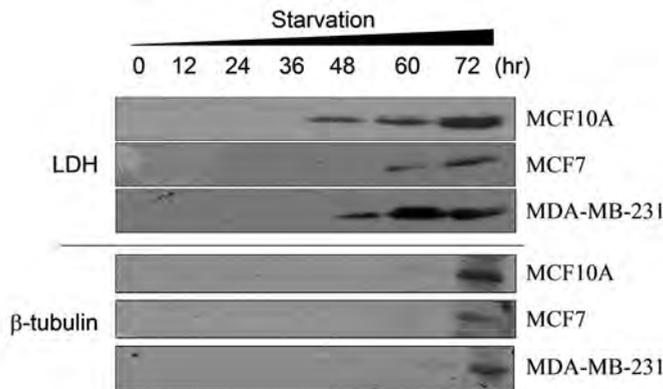
Fig. 1. Optimization of starvation time for secretomic analysis. MCF-10A, MCF-7 and MDA-MB-231 on cell culture dishes were used to check starvation induced cell autolysis by detecting the release of cytoplasmic proteins, LDH and β-tubulin in serum-free media. The serum-free media were harvested and concentrated 1000-fold at indicative starvation periods prior to performing immunoblotting analysis.

Subsequently, proteins secreted from each cell type were enriched from the serum-free medium followed by labeling with CyDyes for 2D-DIGE analysis. The secretomic profiling of MCF-10A, MCF-7 and MDA-MB-231 were visualized using a fluorescence scanner and the images were superimposed using ImageQuant software (Figure. 2). To investigate the potential involvement of secreted proteins in tumorigenesis and metastasis for human breast cancer, biological variation analysis of spots showing greater than 1.5-fold change in expression with a *t*-test score of less than 0.05 were visually checked before confirming the alterations for protein identification. MALDI-TOF MS identification revealed 50 unique differentially expressed proteins across MCF-10A, MCF-7 and MDA-MB-231 (Table 1). Of the proteins identified, 42 were differentially expressed between MCF-7 / MCF-10A, 44 of them were differentially expressed between MDA-MB-231 / MCF-10A, and 37 proteins were differentially expressed between MDA-MB-231 and MCF-7. In the three cell lines investigated, 39% of the total proteins identified were extracellular and plasma membrane-anchored proteins (Figure 3A) indicating that these membrane-associated proteins might be trimmed off the plasma membrane by proteases or might not be completely integrated into the plasma membrane. Most of the identified proteins were involved in signaling transduction, redox-regulation and metabolism (Figure 3B). To our knowledge, 14 out of these identified spots, including tetratricopeptide repeats 3 (IFIT3), have not been reported in any breast cancer related studies. Consequently, these proteins might have the potential to be putative breast cancer markers. As expected, this 2D-DIGE experiment also identified a number of reported breast cancer markers, including Cathepsin D (Zhang et al., 2007) and Insulin-like growth factor-binding protein 4 (IGFBP4) (Mita et al., 2007). These results demonstrate that the proposed approach significantly enriches secreted proteins and membrane proteins in comparison with the previous report that only 2% of the entire mammary epithelial cell proteomes are classified as secreted and membrane proteins (Jacobs et al., 2004). On the other hand, 61% of the total identified proteins in the medium were neither secreted proteins nor membrane-bound proteins. Most of them were sub-located in the cytoplasma, implying that some level of cell necrosis or autolysis was taking place.
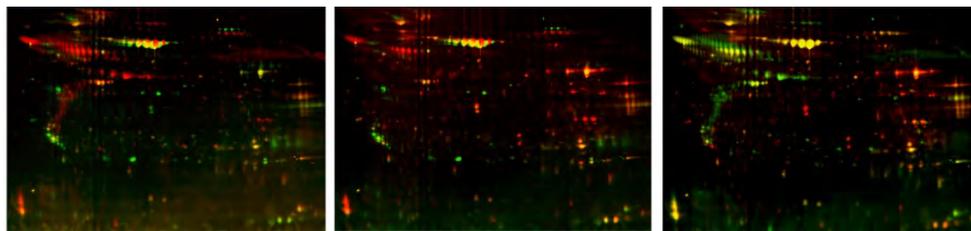
MCF7 (Red) / MCF10A (Green)   MDA231 (Red) / MCF10A (Green)   MDA231 (Red) / MCF7 (Green)

Fig. 2. **Secretomic comparisons across MCF-10A, MCF-7 and MDA-MB-231 cells using 2D-DIGE.** Protein samples (50µg each) enriched from serum-free media were labeled with CyDyes and separated using 24 cm, pH 3-10 non-linear IPG strips. 2D-DIGE images of MCF-10A, MCF-7, and MDA-MB-231 at appropriate excitation and emission wavelengths were pseudo-colored and overlaid with ImageQuant Tool (GE Healthcare).

Secretomic profiles of MCF7 cells compared to MCF10A cells		Secretomic profiles of MDA-MB-231 cells compared to MCF10A cells	
upregulated	*downregulated*	*upregulated*	*downregulated*
Cell cycle	*Biosynthesis*	*Biosynthesis*	*Cell motility /Ca regulation*
E3 ubiquitin-protein ligase CCNB1IP1	Inositol monophosphatase	3'(2'),5'-bisphosphate nucleotidase 1	Plastin-2 / L-plastin
Cell motility	*Cell motility / protease inhibitor*	Inositol monophosphatase	*Immune regulation*
Merlin / Neurofibromin-2	Arfaptin-1	*Cell cycle*	Complement C3
Cytoskeleton	Moesin	Cyclin-dependent kinase inhibitor 1B	*Membrane trafficking*
Actin	*Membrane trafficking*	E3 ubiquitin-protein ligase CCNB1IP1	Rab GTPase-binding effector protein 2
Alpha-actinin-4	Rab GTPase-binding effector protein 2	*Cytoskeleton*	*Metabolism*
Dynein heavy chain 6	*Metabolism*	Dynein heavy chain 6	Carbonic anhydrase 2
Electron transport	Alpha-enolase	*Electron transport*	*Protease Inhibitor*
NADH dehydrogenase iron-sulfur protein 6	Carbonic anhydrase 2	NADH dehydrogenase iron-sulfur protein 6	SERPIN B1
Metabolism	*Protease Inhibitor*	*Metabolism*	SERPIN B5
Aldose reductase	SERPIN B1	Aldose reductase	SERPIN 1
Cytosolic non-specific dipeptidase / Peptidase	SERPIN B5	Cytosolic non-specific dipeptidase / Peptidase A	*Protein folding*
Protease inhibitor	SERPIN 1	Phosphoglycerate mutase 1	Heat shock protein beta-1
SERPIN A3	*Protein folding*	Purine nucleoside phosphorylase	*Redox regulation*
Protein folding	Glutathione S-transferase P	*Signal transduction*	Glutathione S-transferase P
Heat shock protein beta-1	Superoxide dismutase [Mn], mitochondrial	Insulin-like growth factor-binding protein 4	*Signal transduction*
Redox regulation	*Signal transduction*	Rab GDP dissociation inhibitor alpha	14-3-3 protein beta/alpha
Glutathione S-transferase Mu 3	14-3-3 protein beta/alpha	TNFAIP3-interacting protein 2	14-3-3 protein zeta/delta
Peroxiredoxin-1	14-3-3 protein zeta/delta	MAGUK p55 subfamily member 2 / MPP2	IFIT3
Peroxiredoxin-2	IFIT3	*Signal transduction /Ca regulation*	*Transport*
Signal transduction	*Transport*	Annexin A5	Selenium-binding protein 1 / SELENBP1
Insulin-like growth factor-binding protein 4	Plasma membrane calcium-transporting ATPase 2	*Transport*	Plasma membrane calcium-transporting ATPase 2
Rab GDP dissociation inhibitor alpha	*Vascular Transport*	Bestrophin-3	*Vascular Transport*
TNFAIP3-interacting protein 2	Vacuolar protein sorting-associated protein 54	*Unknown*	Vacuolar protein sorting-associated protein 54
MAGUK p55 subfamily member 2 / MPP2		GRAM domain-containing protein 2	
Signal transduction /Ca regulation			
Protein S100-A14			
Transport			
Bestrophin-3			
Unknown			
GRAM domain-containing protein 2			

Table 1. Identification of differentially expressed secreted proteins across MCF-10A, MCF-7, and MDA-MB-231 breast cells obtained after 2D-DIGE coupled with MALDI-TOF mass spectrometry analysis. The functional classes of identified proteins were obtained from the Uniprot website (http://www.uniprot.org/). The average ratio (≥ 2 fold) of differentially expressed proteins across MCF-7 / MCF-10A and MDA-MB-231 / MCF-10A were calculated considering 3 replica gels ($p < 0.05$) and listed in this table.
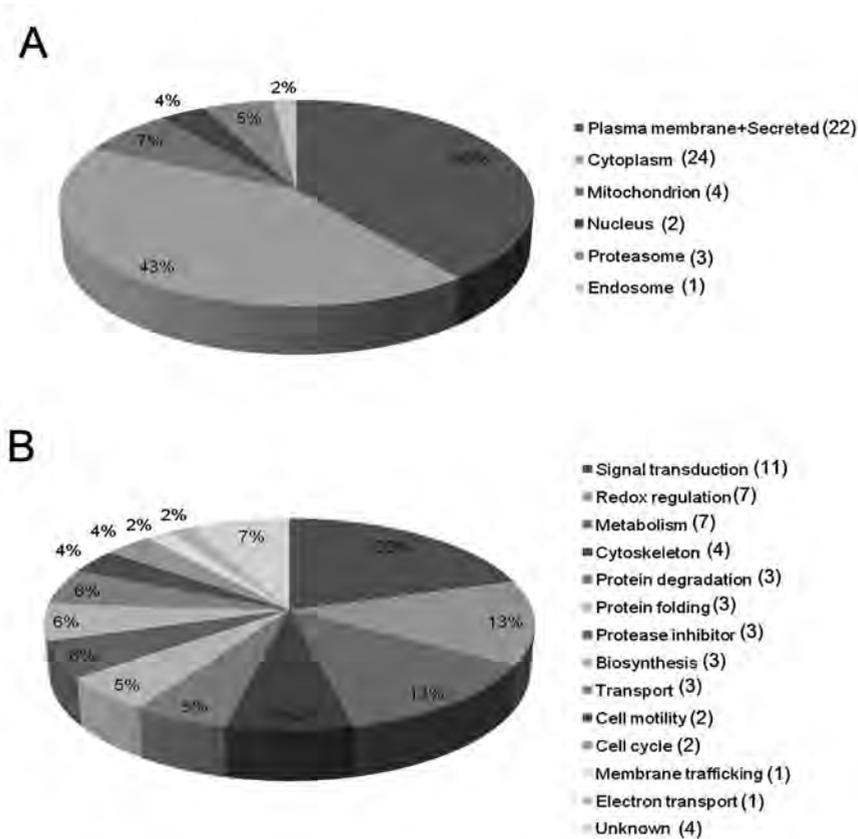
Fig. 3. Percentage of secreted proteins identified from serum-free media by 2D-DIGE / MALDI-TOF MS for MCF-10A, MCF-7 and MDA-MB-231 cells according to their sub-cellular locations (A) and biological functions (B).

3. 2D-DIGE and MALDI-TOF MS analysis of total intracellular proteomes across MCF-10A, MCF-7 and MDA-MB-231 cells

To identify the altered abundances of proteins and relate them to the tumorigenesis of breast cancer, the proteomic profiles of MCF-10A, MCF-7 and MDA-MB-231 were analyzed. Triplicates of the three different cell lysates were compared using 2D-DIGE to obtain an overview of breast cell tumorigenesis. Image analysis using DeCyder v7.0 clearly defined more than 2500 protein spots (Figure 4). To reduce the intrinsic variability derived from protein samples and gel-to-gel variation, only those protein spots that appeared in all of the triplicate gel images were used for statistical analysis. Furthermore, biological variation analysis of spots showing greater than 1.5-fold change in expression with a t-test score of less than 0.05 were visually checked before confirming the alterations for protein identification. MALDI-TOF MS identification revealed 133 unique differentially expressed proteins across MCF-10A, MCF-7, and MDA-MB-231 (Table 2). Of the 133 proteins

Proteomic profiles of MCF7 cells compared to MCF10A cells		Proteomic profiles of MDA-MB-231 cells compared to MCF10A cells	
upregulated	downregulated	upregulated	downregulated
Carbohydrate Metabolism	**Carbohydrate Metabolism**	**Cytoskeleton**	**Cell motility**
Fructose-bisphosphate aldolase A	L-lactate dehydrogenase B chain	Vimentin	Rho-associated protein kinase 2
Glucose-6-phosphate 1-dehydrogenase	Pyruvate kinase isozymes M1/M2	**Gene regulation**	F-actin-capping protein subunit beta
Triosephosphate isomerase	**Cytoskeleton**	Achaete-scute homolog 4	Cofilin-1
Cell motility	Glial fibrillary acidic protein	Serine-threonine kinase receptor-associated protein	**Cytoskeleton**
Rho GTPase-activating protein 5	**Metabolism**	**Growth Regulation**	Glial fibrillary acidic protein
Rho-associated protein kinase 2	Leukotriene A-4 hydrolase	Prohibitin	**Gene regulation**
Gene regulation	Phosphoserine aminotransferase	**Metabolism**	Heterogeneous nuclear ribonucleoproteins A2/B1
Zinc finger protein 433	Carbonic anhydrase 2	Lactoylglutathione lyase	Alpha-1-fetoprotein transcription factor
Nuclear receptor subfamily 5 group A member 2	**Protein metabolism**	Aldose reductase	**Growth Regulation**
Growth Regulation	Aminopeptidase B	Alpha-enolase	**Metabolism**
Protein CASC2, isoform 3	**Protein folding**	**Protein metabolism**	Carbonic anhydrase 2
Metabolism	Protein disulfide-isomerase A3	Elongation factor 1-delta	**Protein metabolism**
Lactoylglutathione lyase	cyclophilin A	Elongation factor 1-gamma	Aminopeptidase B
Protein metabolism	**Redox regulation**	**Protein degradation**	**Protein degradation**
Elongation factor 2	Glutathione S-transferase P	Protein folding	Cathepsin D
Elongation factor 1-gamma	**Signal transduction**	Endoplasmic reticulum protein ERp29	**Protein folding**
Protein degradation	Inhibitor of apoptosis-like protein 2	Nucleophosmin	cyclophilin A
Cathepsin D	**Signal transduction /Ca regulation**	**Redox regulation**	Heat shock protein beta-1 / HSP 27
Protein folding	Calcium-dependent protease small subunit	Flavin reductase	**Redox regulation**
HSP 27	Annexin A2	Glutathione S-transferase Mu3	Peroxiredoxin-6
Endoplasmic reticulum protein ERp29	Annexin A5	**Signal transduction**	Glutathione S-transferase P
FK506-binding protein 4	**Transport**	Growth factor receptor-bound protein 2 (Grb2)	**Signal transduction**
Heat shock protein 75 kDa	Chloride intracellular channel protein 4	**Transport**	ATP synthase subunit beta
Redox regulation	Voltage-dependent anion-selective channel protein 1	Chloride intracellular channel protein 1	PI3-kinase p110 subunit alpha
Flavin reductase	Voltage-dependent anion-selective channel protein 2		Inhibitor of apoptosis-like protein 2
Glutathione S-transferase Mu3	**Cell-cell interaction**		**Signal transduction /Ca regulation**
Peroxiredoxin-4	Galectin-1		Calcium-dependent protease small subunit
Signal transduction	**Protease inhibitor**		Annexin A2
Rab GDP dissociation inhibitor beta	Serpin B5		Annexin A3
PI3-kinase p110 subunit alpha			Annexin A4
Growth factor receptor-bound protein 2			**TCA cycle**
MAGUK p55 subfamily member 2			Malate dehydrogenase
Prostaglandin E synthase 3			**Transport**
Rho-related BTB domain-containing protein 2			Voltage-dependent anion-selective channel protein 1
Signal transduction /Ca regulation			Voltage-dependent anion-selective channel protein 2
Annexin A3			Chloride intracellular channel protein 4
Annexin A6			Cellular retinoic acid-binding protein 2
Transport			**Cell-cell interaction**
Selenium-binding protein 1			Galectin-1
Cellular retinoic acid-binding protein 2			**Protease inhibitor**
ALG-2-interacting protein 1			Serpin B5

Table 2. Identification of differentially expressed total cellular proteomes across MCF-10A, MCF-7, and MDA-MB-231 breast cells obtained after 2D-DIGE coupled with MALDI-TOF mass spectrometry analysis. The functional classes of identified proteins were obtained from the Uniprot website (http://www.uniprot.org/). The average ratio (≥ 2 fold) of differentially expressed proteins across MCF-7 / MCF-10A and MDA-MB-231 / MCF-10A were calculated considering 3 replica gels ($p < 0.05$) and listed in this table.

identified, 107 of them had differential expressions between MCF-7 / MCF-10A, 63 were differentially expressed between MDA-MB-231 / MCF-10A and 96 had differential expressions between MDA-MB-231 and MCF-7. Almost half of the total proteins identified in this breast cell model were cytosolic proteins (Figure 5A), and most of the identified proteins were involved in signaling transduction, metabolism, protein folding, and cell motility (Figure 5B). To our knowledge, 51 of these identified spots, including calumenin, have not been reported in any breast cancer related studies. As such, these proteins might have the potential to be putative breast cancer markers. As expected, some well-known breast cancer markers, such as 14-3-3 proteins (Danes et al., 2008), annexins (Cao et al., 2008a), calmodulin (Gallo et al., 2008), anterior gradient homolog 2 (AGR-2) (Zweitzig et al., 2007; Fritzsche et al., 2006), Galectin-1 (Jung et al., 2007) and Rho-associated protein kinase-2

(ROCK2) (Fu et al., 2008b), were also identified in this 2D-DIGE experiment, lending credence to the reliability of early phase biomarker detection using this experimental strategy.

MCF7 (Red) / MCF10A (Green)   MDA231 (Red) / MCF10A (Green)   MDA231 (Red) / MCF7 (Green)
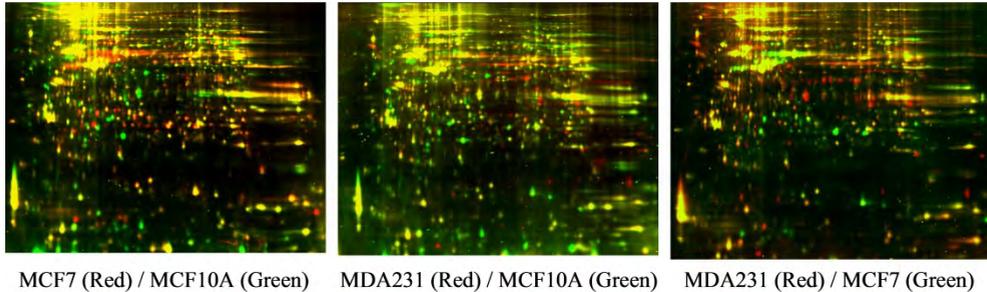
Fig. 4. **Proteomic comparisons across MCF-10A, MCF-7 and MDA-MB-231 cells using 2D-DIGE.** Protein samples (150µg each) purified from total cell lysates were labeled with Cy-dyes and separated using 24 cm, pH 3-10 non-linear IPG strips. 2D-DIGE images of MCF-10A, MCF-7 and MDA-MB-231 at appropriate excitation and emission wavelengths were pseudo-colored and overlaid with ImageQuant Tool (GE Healthcare).

4. Validation of characterized breast cancer markers through immunoblotting and immunofluorescence

The secretomic study indentified some of the well-characterized breast cancer related cytosolic proteins such as Cyclophilin A, 14-3-3delta and peroxiredoxin 2 in culture media (Harding and Handschumacher, 1988; Aitken, 2006; Fujii and Ikeda, 2002). It is essential to validate the levels of these cytosolic proteins in the medium from independent experiments. To this end, the expression level of cyclophilin A, 14-3-3delta and peroxiredoxin 2 from the culture media of MDA-MB-231, MCF-7 and MCF-10A were validated with immunoblotting. The results indicate that both the proteomic and immunoblot analysis showed cyclophilin A and 14-3-3 delta down-regulated in MCF-7 in comparison to the levels in MCF-10A. In contrast, peroxiredoxin 2 showed up-regulation in MCF-7 in comparison to the levels in MCF-10A. Comparing the secreted protein levels between MCF-10A and MDA-MB-231 indicates that the peroxiredoxin 2 and 14-3-3 delta expression levels increased in MDA-MB-231 and MCF-10A, respectively; however, the cyclophilin A level showed no significant change (Figure 6 A~C). This observation confirmed that cyclophilin A, 14-3-3delta and peroxiredoxin 2 were differentially secreted across the breast cells.

Immunoblot and immunofluorescence analysis were carried out to further confirm the differential protein levels observed in total cellular proteins (annexin-2, cathepsin D, profilin, protein disulfide isomerase A1 and Histone deacetylase 1 (HDAC1)) across MDA-MB-231, MCF-7 and MCF-10A (Figure 6 D~H). These proteins have been reported to play important roles in cytoskeleton regulation, proteolysis, calcium regulation, protein disulfide bond rearrangement and chromatin assembly during tumorigenesis (Feldner and Brandt, 2002; Liaudet-Coopman et al., 2006; Sharma and Sharma, 2007; Fu et al., 2008a; Kawai et al., 2003). The results of the immunoblotting indicate that cathepsin D and protein disulfide isomerase (PDI) showed up-regulation in MCF-7 cells but down-regulation in MDA-MB-231 compared to the two protein expressions in MCF-10A. The expression levels of the profilin

and annexin 2 proteins showed down-regulation in MCF-7 but no significant changes in MDA-MB-231 compared to the levels in MCF-10A. These immunoblotting results demonstrate a positive correlation with the 2D-DIGE results (Figure 6 D~G). In addition to immunoblotting, validation was also performed with immunofluorescent analysis. Figure 6H shows that most of the HDAC1 signal was distributed within the nucleus, which is consistent with the subcellular location of HDAC1 in cells. As expected, the fluorescent intensity with the same exposure indicates that HDAC1 showed increased expressions in MCF-7 and MDA-MB-231 compared to its expression in MCF-10A. Altogether, the results from immunoblotting and immunofluorescent agreed with the results from 2D-DIGE data.
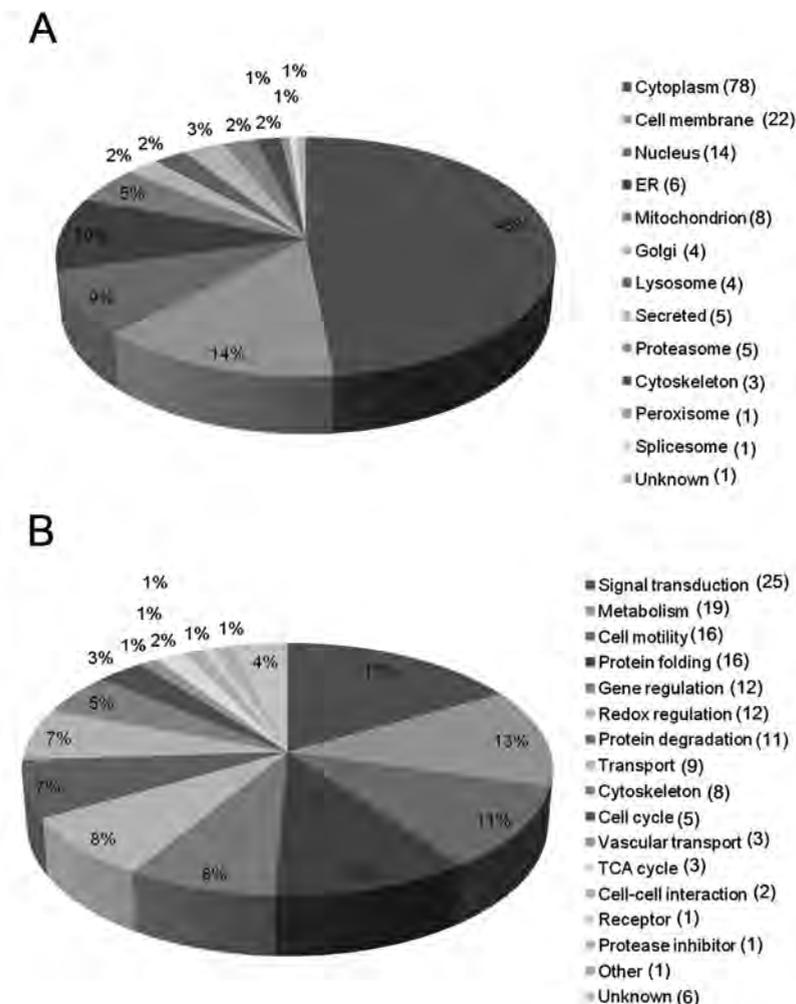
Fig. 5. Percentage of total cellular proteins identified by 2D-DIGE / MALDI-TOF MS for MCF-10A, MCF-7 and MDA-MB-231 cells according to their sub-cellular locations (A) and biological functions (B).
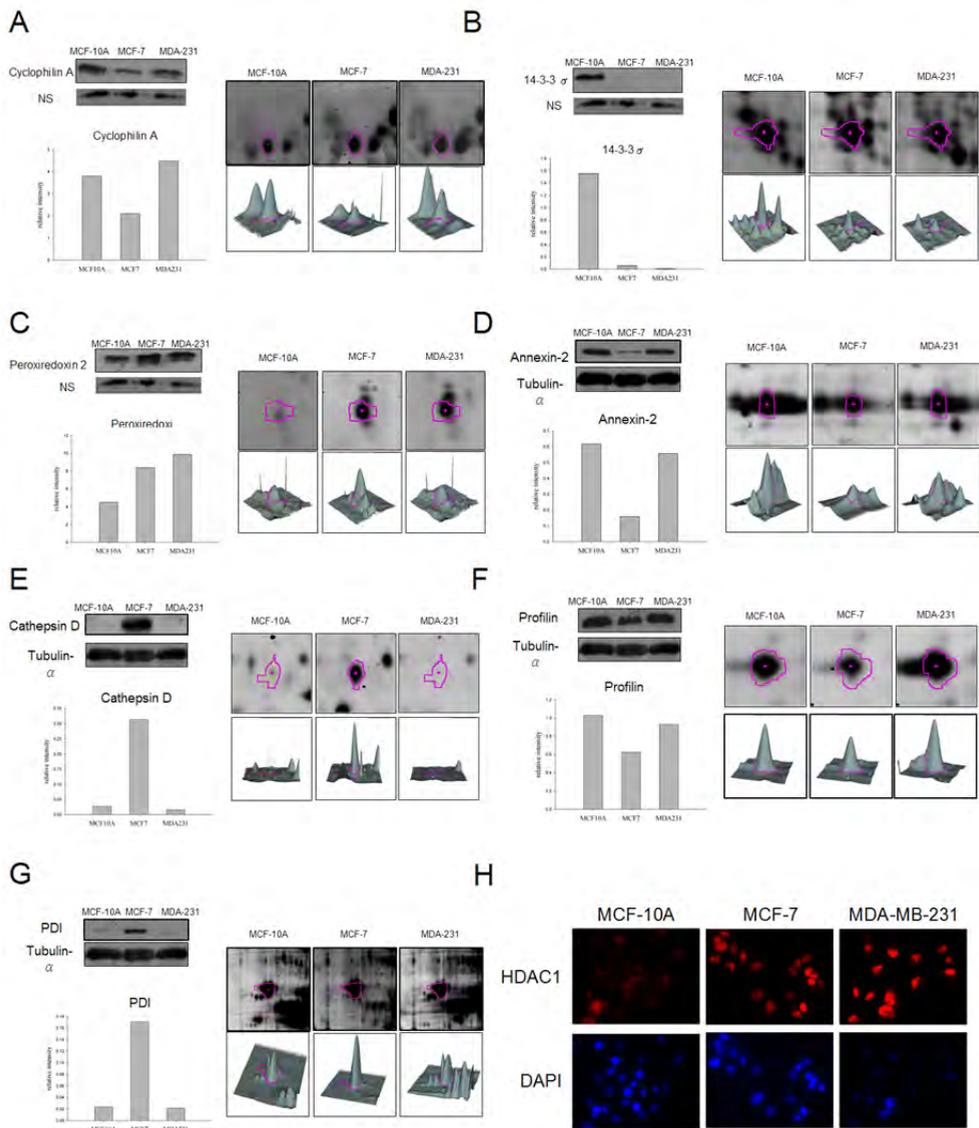
Fig. 6. Representative immunoblotting and immunofluorescent analyses for selected differentially expressed proteins identified by proteomic analysis in MCF-10A, MCF-7 and MDA-MB-231 cells. The levels of identified proteins in serum-free media, (A) Cyclophilin A, (B) 14-3-3 delta and (C) Peroxiredoxin 2 and total cellular proteins, (D) Annexin-2, (E) Cathepsin D, (F) Profilin and (G) Protein disulfide isomerase A1 in MDA-MB-231 and MCF-7 versus MCF-10A confirmed by immunoblot (left top panels), densitometry results with normalized values using nonspecific bands (NS) of secreted proteins and α-tubulin as loading controls (left bottom panels), protein expression map (right top panels) and three-

dimensional spot image (right bottom panels). (H) MCF-10A, MCF-7 and MDA-MB-231 cells were fixed and incubated with anti-HDAC antibody and stained with a Texas Red-conjugated secondary antibody (Red). Nuclei were stained with DAPI (Blue). Each set of three fields was taken using the same exposure, and images are representative of three different fields. Scale bar = 20μm.

5. Validation of unreported identified putative tumorigenic markers through immunoblotting and immunofluorescence

The cellular proteomic and secretomic analyses above reveal that a number of unreported identified proteins may be putative breast cancer markers (Tables 1 and 2). To verify this observation, immunoblotting and immunofluorescence were used to validate these differentially expressed proteins including bestrophin 3, membrane protein, palmitoylated 2 (MPP2), parvalbumin, PDZ and LIM domain protein 1 (PdLIM1), IFIT3 and barrier to autointegration factor 1 (BANF1) as these proteins showed relatively significant changes (> 3 fold) in comparison with most of the unreported identified proteins across MCF-10A, MCF-7 and MDA-MB-231. The immunoblotting analysis of concentrated serum-free media shows that more bestrophin 3 was secreted in the cell lines of MCF-7 and MDA-MB-231 than MCF-10A, while MPP2 was only detected in MDA-MB-231. Notably, the bestrophin 3 blotting result did not completely agree with the 2D-DIGE data, where levels in MCF-7 were higher than MDA-MB-231 (Figure 7A). Using immunofluorescent staining, the robust increase of parvalbumin signal in both the MCF-7 and MDA-MB-231 cells was first confirmed after comparison with the signal in MCF-10A. Parvalbumin was primarily localized in the nucleus, which coincided with the DAPI stained nucleus. Further investigation of parvabumin expression in other breast cancer cell lines indicates that parvabumin was over-expressed in MDA-MB-453, a line of non-invasive breast cancer cells, and slightly up-regulated in MDA-MB-361, an adenocarcinoma with metastatic ability (Figure 7B). These results imply that parvabumin might have the potential to be a breast cancer marker. In contrast, PdLIM1, a cytosolic protein, was down-regulated in all breast cancer lines: MCF-7, MDA-MB-231, MDA-MB-453 and MDA-MB-361 (Figure 7B). In addition, IFIT3, a plasma membrane protein, was down-regulated in transformed cells, especially in MCF-7 and MDA-MB-231, and was consistent with the proteomic data from 2D-DIGE (Figure 7B). Interestingly, BANF1, a major nucleus-located protein, was distributed in the cytoplasma of the MCF-10A cells, but was confined within the nucleus in MCF-7, MDA-MB-231 and MDA-MB-453 cells; in addition, BANF1 was distributed within the cytoplasma and nucleus in MDA-MB-361 (Figure 7B). These results indicate that the BANF1 levels were different between healthy breast cells and breast cancer cells, and that the subcellular locations of the protein may account for tumorigenesis.

Nuclear distribution protein nudE homolog 1 (NDE1), GRAM domain containing 2 (GRAMD2), Parvabumin and bestrophin 3 (Best3), have not been reported in previous breast cancer studies, implying that these proteins need to be further investigated to confirm them as valuable breast cancer markers. In order to examine the expression levels of the newly identified breast cancer markers in clinical specimens, we had used plasma specimens from healthy donors and breast cancer patients to compare the expression levels of the markers including NDE1, GRAMD2, Parvabumin and Best3. The results showed that these identified markers were significantly increased in breast cancer patients rather than in the healthy donors and these increases were observed in both non-metastatic and metastatic breast cancers (Figure 8).
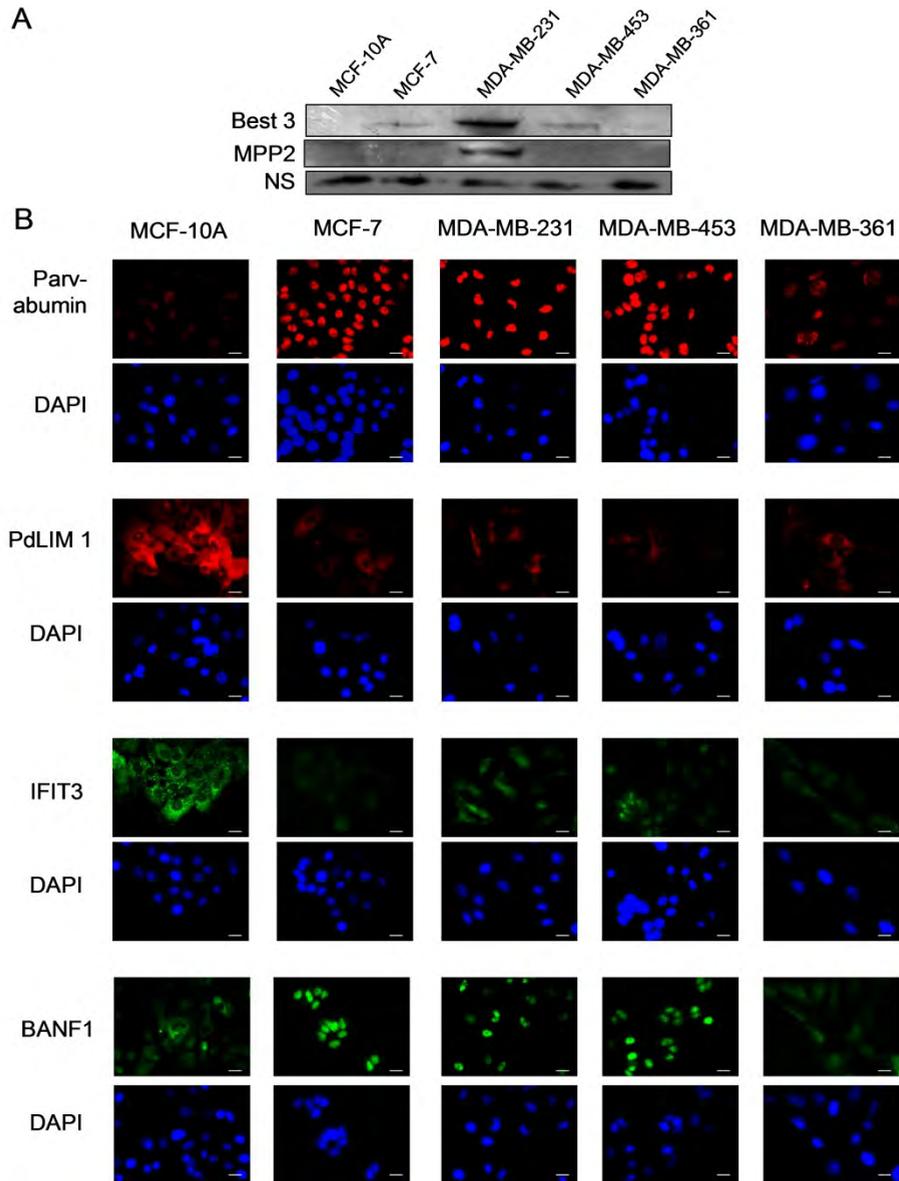
Fig. 7. Immunoblotting and immunofluorescence analyses of the expression and protein localization changes of newly identified putative breast cancer markers across MCF-10A, MCF-7, MDA-MB-231, MDA-MB-453 and MDA-MB-361 cells. (A) The profile of the secreted proteome changes across MCF-10A, MCF-7, MDA-MB-231, MDA-MB-453 and MDA-MB-361 cells. The serum-free media from the cell lines was concentrated and 10μg of the total protein was resolved using SDS-PAGE and immunoblotted for MPP2 and Bestrophin 3. NS represents a nonspecific band used to show equal loading of secreted proteins. (B) 5×10^4

MCF-10A, MCF-7, MDA-MB-231, MDA-MB-453 and MDA-MB-361 cells were seeded on cover slips before fixation and staining for Parvabumin, BANF1, PdLIM1 and IFIT3. Each set of three fields was taken using the same exposure, and images are representative of three different fields. Scale bar = 20μm.
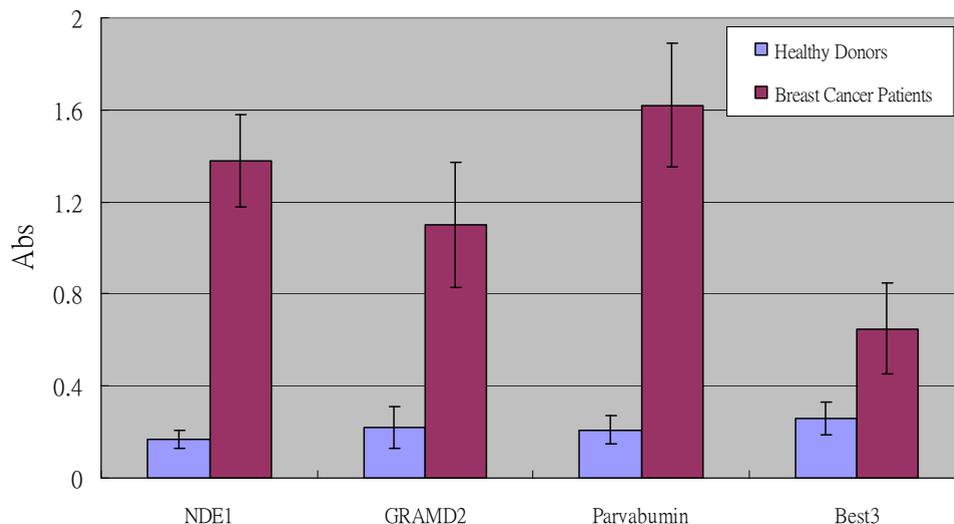
Fig. 8. ELISA analysis of plasma NDE1, GRAMD2, pavabumin and best3 levels in healthy donors, breast cancer patients. Plasma samples were obtained from 30 healthy individuals, 30 breast cancer patients (15 without detectable metastasis and 15 breast cancer patients presenting metastasis) at the time of serum collection. 50 μg of plasma samples were coated onto each well of 96-well plate for ELISA analysis and the absorbance was measured at 450 nm using Stat Fax 2100 microtiterplate reader.

6. Functional classifications of the identified breast cancer markers

With the basis of a Swiss-Prot search and KEGG pathway analysis, numerous potential biological functions of the identified proteins across MCF-10A, MCF-7 and MDA-MB-231 were determined. The information should be useful for studying the mechanisms of breast cancer tumorigenesis and metastasis. Figure 9 compares the expression profiles of the identified differentially expressed proteins in these 3 cell lines. Proteins known to regulate cell cycle are found to be upregulated in both MCF-7 and MDA-MB-231 (Figure 9A), and are associated with the promotion of tumorigenesis (Dictor et al., 1999). In addition, the expression of proteins linked to redox-regulation increased in the MCF-7 cells in comparison to the levels in MCF-10A (Figure 9B). Induced expression of these proteins may be able to account for cancer development and progression. For example, Noh *et al.* showed that peroxiredoxins are greatly over-expressed in most breast cancer tissues (Noh et al., 2001). Proteomic analysis also reveals that proteins involved in carbohydrate metabolism are significantly over-expressed in MCF-7 cells (Figure 9C). This demonstrates that cancer cells rely heavily on glycolysis to obtain ATP for proliferation and tumorigenesis in the presence

of adequate oxygen levels (Lopez-Lazaro, 2008); this mechanism has been implicated in numerous cancer therapies (Gatenby and Gillies, 2007; Rivenzon-Segal et al., 2003). Figures 9D~F show the downregulated profiles of proteins in both MCF-7 and MDA-MB-231 cells. These proteins are involved in calcium regulation, vascular transport and protease inhibition. Calcium-binding proteins, such as annexin-1, whose function is modulated by an estrogen receptor, have been reported to show decreased expression in correlation with breast cancer development and progression(Ang et al., 2009; Cao et al., 2008b; Shen et al., 2006; Shen et al., 2005). The S100 protein family is a family of low molecular weight calcium-binding proteins that is responsible for the regulation of protein phosphorylation, intracellular calcium homostasis, the dynamics of cytoskeleton constituents and cell proliferation (Donato, 2003). The S100 family has become a major interest because of its deregulated expression in human diseases, especially in cancer. According to Ji *et al.* (2004), S100 families exhibit significantly reduced expression in esophageal squamous cell carcinoma (Ji et al., 2004) and are hence recognized as a prognostic esophageal cancer marker. In here, S100A14 was identified as downregulated in MCF-7 and MDA-MB-231, suggesting their potential roles in breast cancer. Interestingly, proteins involved in vascular transport, including Rab GTPase-binding effector protein and vacuolar protein sorting-associated protein 54, were decreased in expression in MCF-7 and MDA-MB-231 (Figure 9F). This may be explained by a previous report indicating that the downregulation of Rab5 GDP/GTP exchange factor enhances receptor tyrosine kinase signaling and promotes the growth factor-directed migration of tumor cells (Hu et al., 2008). However, there are few studies on tumorigenesis regarding the roles of the Rab GTPase-binding effector protein and the vacuolar protein sorting-associated protein 54. Serpin is a group of proteins able to inhibit protease and block the growth, invasion, and metastatic properties of breast tumors. Hence, serpin families function as tumor suppressors in cancer research (Sager et al., 1997). The downregulation of serpin is well-correlated with the progression of breast cancer (Webber et al., 2008) and our own observations in MCF-7 and MDA-MB-231 cells (Figure 9F).

Other differentially expressed proteins of interest across MCF-10A, MCF-7 and MDA-MB-231 include cathepsin D, bestrophin-3 and interferon-induced protein with IFIT3. Cathepsin D, a lysosomal aspartic protease, is over-expressed in estrogen receptor positive breast cancer cells (Rochefort, 1999) and is generally of good prognostic value in comparison with estrogen receptor negative breast cancer in clinical studies (Rochefort, 1998). Our study indicates that cathepsin D is highly expressed in MCF-7, both in total cellular proteins or in secreted fraction. In contrast, cathepsin D is significantly down-regulated in MDA-MB-231 cells compared with MCF-7. Thus, our proteomic results display good correlation with these reports. To our knowledge, bestrophin-3, a cGMP-dependent calcium-activated chloride channel, has not been reported to be associated with cancer and shows upregulation in MCF-7 and MDA-MB-231 in this study. Nevertheless, the related study in bestrophin-1 shows the protein improves intracellular Ca^{2+} signaling and increases cell growth rate in colonic carcinoma cells. The proliferation of the cells is significantly suppressed by bestrophin-1 RNA interference treatment (Spitzner et al., 2008). This indicates bestrophin-3 may be a potential target for breast cancer therapy. IFIT3 plays a key role in the antiproliferative activity of the interferon-related signaling pathway through inducing expression of cell cycle inhibitors, p21 and p27 proteins (Xiao et al., 2006). The 2D-DIGE results in this study show that IFIT3 is downregulated in both MCF-7 and MDA-MB-231 cells, implying that breast cancer cells may maintain a high level of proliferative activity by downregulating the expression of IFIT3.
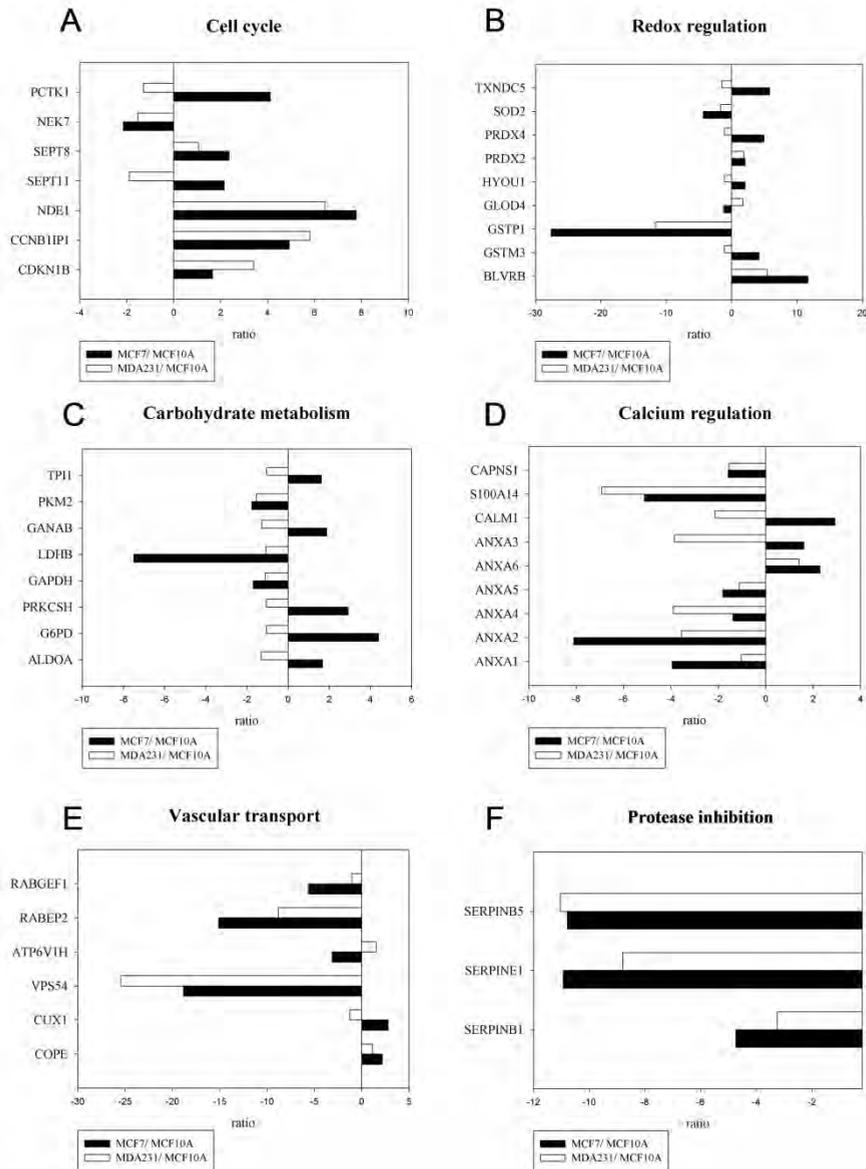
Fig. 9. Expression profiles for proteins potentially contributing to (A) cell cycle (B) redox regulation (C) carbohydrate metabolism (D) calcium regulation (E) vascular transport (F) protease inhibition in comparing MCF-7 and MDA-MB-231 with MCF-10A. White bars represent fold change in protein expression in MDA-MB-231 versus MCF-10A. Black bars represent fold change in protein expression in MCF-7 versus MCF-10A. The vertical axis indicates the identified proteins; the horizontal axis indicates the fold change in protein expression. Additional details for each protein can be found in Table 1 and Table 2.

7. Different proteomic approaches in the study of breast cancer markers

Results of this study include the differentially expressed protein profiles of intracellular proteins and extracellular secreted proteins in non-transformed and transformed breast cell lines. The 2D-DIGE strategy is powerful enough to identify numerous breast cancer signatures and offers a complementary role to LC/MS-based proteomic analysis. Even though the global coverage of protein mixtures identified by LC-MS based analysis is generally higher than that of 2-DE based analysis, 2-DE based analysis offers some distinct advantages, such as direct protein quantification at protein isoform levels instead of peptide levels to reduce analytical variations (Timms and Cramer, 2008a). Using the LC-MS/MS strategy, Kulasingam and Diamandis analyzed and compared the expressions of extracellular and membrane-bound proteins in conditioned media of three breast cells corresponding to the normal control cells and cell lines derived from stage 2 and stage 4 patients, respectively (Kulasingam and Diamandis, 2007b). Their study identified 1062 differentially expressed proteins across these three cell lines. A comparison between Kulasingam's study and our 2D-DIGE secretomic study shows that 25 out of 50 identified differentially expressed secreted proteins coincide with Kulasingam's study, indicating that both LC-MS/MS and 2D-DIGE are potential tools for discovering breast cancer markers with reasonable reproducibility. However, another 25 out of these 50 identified proteins have never been published in Kulasingam's study or any other studies, demonstrating that 2D – DIGE, compared with LC-MS/MS, plays a complementary role in the discovery of biomarkers.

In previous research, Nagaraja *et.al.* used traditional 2-DE with post-stains (silver stain and coomassie blue stain) to reveal 26 differentially expressed proteins among transformed breast cells with different levels of invasiveness and normal cells which were the same cell lines used in the present study (Nagaraja et al., 2006). Their study showed no evidence of visualizing protein spots with sensitive strategies, and protein expression changes were not quantifiable because no broader linear-ranged methods and statistical analysis were employed. Only six out of those 26 proteins coincide with our statistical 2D-DIGE data, which implies that differences might have derived from artificial variations or from results with no statistical analysis.

Mitochondria are key organelles in mammary cells in responsible for several cellular functions including growth, division and energy metabolism. In our recent works, mitochondrial proteins were enriched for proteomics analysis with the state-of-the-art 2D-DIGE and matrix-assistant laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) strategy to compare and identify the mitochondrial protein profiling changes between three breast cell lines with different tumorigenesity and metastatic potential. The mitochondrial proteomics demonstrate more than 1500 protein features from equal amount pooled of mitochondrial proteins of these three breast cancer lines and 125 differentially expressed protein spots were identified by peptide fingerprintings. In which, 33 identified proteins belong to mitochondrial proteins. 18 out of these 33 identified mitochondrial proteins such as short calcium-binding mitochondrial carrier protein-1 (SCaMC-1) have not been reported in breast cancer research in our knowledge. Additionally, mitochondrial protein prohibitin has shown to be differentially distributed in mitochondria and in nucleus for healthy breast cells and breast cancer cell lines, respectively. This approach provides comprehensive studies examining mitochondrial proteins in various stages of breast cancer progression and these identified proteins may be further evaluated as potential breast cancer risk factors for breast cancer initiation and progression.

8. Conclusion

The transformation of a normal cell into a cancer cell has been correlated with alterations in gene regulation and protein expression. To identify altered proteins and link them to the tumorigenesis of breast cancer, non-tumorigenic breast epithelial cells (MCF-10A) were used to distinguish their proteomes from non-invasive breast cancer cells (MCF-7) and invasive breast cancer cells (MDA-MB-231) for the identification of the potential breast cancer markers in transformed breast cells. Using the 2D-DIGE and MALDI-TOF MS techniques, the differentially expressed extracellular secreted proteins and total cellular proteins across MCF-10A, MCF-7 and MDA-MB-231 were quantitatively identified. More than 180 unique differentially expressed secreted and intracellular proteins from these three different cell lines have been identified by proteomic analysis. In which, 14 of the secreted proteins and 51 of the total cellular proteins have not been previously reported in breast cancer research. Some of these unreported proteins have further been verified in other breast cancer cell lines, such as MDA-MB-453 and MDA-MB-361 cells, and clinical specimens. Although breast cell lines have been used widely to study the biological and molecular heterogeneity of breast cancer, it is important to assess their relation to *in vivo* genotypes and phenotypes of breast cancer. According to gene and protein expression profiling, breast cell lines were recently better classified to five major subtypes: luminal-A, luminal-B, ERBB2, basal-like and normal-like, which may not completely correspond to biological reality but have shown a direct correlation with clinical outcomes of this disease (Kao et al., 2009; Charafe-Jauffret et al., 2006; Perou et al., 2000; Chin et al., 2006). It is thus possible to predict the differences in proteins identified among MCF-10A (normal-like), MCF-7 (luminal) and MDAMB-231 (basal-like/post-epithelial mesenchymal transition) are due to the cell lines representing different molecular subtypes of breast cancer in addition to reflect different stages of breast cancer development. Moreover, these three mammary epithelium cells have been commonly selected to compare in many studies as MCF-7 is estrogen receptor positive while MDA-MB-231 is estrogen receptor negative. Therefore, the identified protein signatures in MCF-7 are possible link to estrogen-stimulated progression of non-invasive breast cancer. To sum up, proteomics strategy has offered opportunity to investigate the putative breast cancer markers from various breast cell lines and may aid in developing identified proteins as useful diagnostic and therapeutic candidates in research on cancer and proteomics.

9. Acknowledgments

10. References

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