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Differential protein expression in primary breast cancer and matched axillary node metastasis

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Abstract. Axillary lymph node (ALN) metastasis is a key step of tumor progression in breast cancer and is associated with an unfavorable prognosis. However, the mechanisms of this process are not well understood. Proteomic technologies have led to identification of specific protein markers and a better understanding of the cellular processes. To explore this, differential protein expression was analyzed between node-positive breast carcinoma and node-negative breast carcinoma (11 samples) and between primary breast carcinoma and matched metastatic ALN (five pairs) using a combination of 2D-SDS-PAGE and LC-MC/MS. Of the total 678 protein spots, 19 proteins were up-regulated and 3 proteins were down-regulated in node-positive breast carcinomas compared to node-negative breast carcinomas. Four up-regulated proteins were identified, namely Annexin 5, carbonic anhydrase I, peroxiredoxin 6 and proteasome α2 subunit. For proteins altered in metastatic ALN compared to primary tumors, 6 of 14 up-regulated proteins were identified: heat shock 70 kDa protein 5, protein disulfide isomerase, prolyl 4-hydroxylase β subunit precursor, lactate dehydrogenase B, triosephosphate isomerase 1 and β-tubulin and 5 of 23 down-regulated proteins were identified including 90 kDa heat shock protein, chain A apo-human serum transferrin, chain A α1-antitrypsin, enolase 1 and macrophage migration inhibitory factor. Immunohistochemistry showed stronger immunostaining for β-tubulin in metastatic ALN compared to primary breast tumor. All of the identified proteins function in various processes involved in cell survival and growth. Our results suggest that these processes are critical for tumor progression and metastasis and the proteins identified could be candidate markers of clinical usefulness.

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Introduction

Breast cancer is an important health burden worldwide. It is by far the most frequent cancer in women (23% of all cancers), with an estimated 1.15 million new cases in 2002, ranking second overall when both genders are considered together (1). In Thailand, though the incidence is low, it has been continuously increasing throughout the last few decades (2,3). Nowadays, breast cancer has become one of the major focus of public health campaigns for cancer prevention and control in our country.

Several clinicopathological factors have been recognized as prognostic indicators for disease outcome with breast cancer (4,5). Among these, tumor size and axillary lymph node (ALN) metastasis are most important. In one study, patients with a small tumor (<2 cm) and no ALN metastasis had a 5-year survival rate over 95% while those with large tumor (>5 cm) with ALN metastasis, the survival rate was only 45% (4,5). Therefore, detection of breast cancer at the early stage or identification of factors that help predict ALN metastasis is of prime importance.

It is well known that cancer cell transformation and progression are extremely complex events involving mutation or deregulation of a variety of genes controlling cell proliferation, differentiation and cell death (6,7). Different proteins may be up-regulated or down-regulated simultaneously and provoke distinct cell functions. Therefore, the analysis of a single or few proteins is far from the prediction of the clinical outcome. The newly developed proteomics technologies which can identify hundreds of altered proteins in a single experiment, have provided a great opportunity for the identification of novel protein markers (8).

In this study, we attempted to identify proteins that were altered during tumor progression and metastasis of breast cancer. We utilized a proteomics approach using a combination of 2D-SDS-PAGE and mass spectrometry to determine differentially expressed proteins in primary breast cancers with and without ALN metastasis and proteins that altered between primary tumors and their matched metastatic ALN. These results may provide a better understanding of cellular mechanism of tumor progression and metastasis and also help identify candidate markers for further clinical usefulness.

Materials and methods

Tissue samples. Fresh tissue of breast cancer and metastatic ALN were obtained at the time of surgical resection from 11 breast cancer patients of Songklanagarind Hospital, Faculty of Medicine, Prince of Songkla University, Songkhla. The selected tissues were removed from the resection specimens and stored at -70°C until processing. The patients were staged according to the American Joint Committee on Cancer (AJCC) 6th Edition Staging Manual (9). Histopathological characteristics were reviewed and confirmed by a single pathologist before use in the study. The research protocol was approved by the Research Ethics Committee of the faculty.

Protein extraction and two-dimensional gel electrophoresis. To obtain the representative area, each sample was sectioned in 0.5 μm thickness at -20°C, stained with routine H&E and examined under light microscopy. If the tissue sections still contain undesirable areas, the original gross tissues were trimmed, processed for histological procedures, and examined until the specific area was acquired. The representative frozen tissues were ground with lysis buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, 1% DTT and 2% IPG buffer (pH 3-10). The mixture was then frozen with liquid nitrogen, and thawed. The protein mixture was centrifuged at 14,000 rpm for 15 min at 4°C. The supernatant was taken as protein extract for further analysis. Protein concentration was determined using Protein Assay Dye Reagent Concentrate (Bio-Rad) based on Bradford's method using bovine serum albumin as a standard.

The proteins were separated by 13-cm immobilized pH gradient (IPG) strips, pH 3-10 range (linear) followed by SDS-PAGE. Trace contaminants in the protein solution were removed using 2-D Clean-Up Kit (Amersham Biosciences) according to the manufacturer's instruction. Protein extract (75 μg) were subjected to IEF in the first step of protein separation for the total 18.8 kVh at 20°C. The focused IPG strips were then equilibrated with equilibration buffer I containing SDS and 65 mM DTT for 15 min and with equilibration buffer ll containing SDS and 135 mM iodoacetamide for 15 min. The second step of protein separation was performed by 12% SDS-PAGE. The protein spots were visualized by silver staining.

Image analysis. The stained gels were scanned with an Image Scanner™ (Amersham Biosciences) at 300 dpi resolution, and the images were saved as MEL files. The images were further processed by 2D gel analysis software, ImageMaster 2D Platinum (Amersham Biosciences). Cropping of the clusters of spots of all images was performed. Protein spots in the cropped images were detected and matched to a reference gel. The missing spots were added manually to the reference gel and all matched gels. Mismatched spots were edited and then the intensities of protein spots were analysed. To eradicate the error of the different levels of stained protein, normalisation was made by calculating a percentage ratio of individual spot volume to the total volume of protein spots in each gel image.

The comparison of mean intensities of protein spots were compared statistically using t-test for two comparison sets: i) breast cancers from patients with node-negative (Tn^-) and breast cancers from patients with node-positive disease (Tn^+); and ii) primary breast cancers from patients with node-positive disease (Tn^+) and matched metastatic ALN (L) (paired t-test). The level of significance of p-value was set at <0.05.

In-gel digestion with trypsin. Protein spots were washed with 100 μl de-ionized water, 3 times. To destain silver nitrate from the proteins, 30-50 μl of 15 mM $K_3Fe(CN)_6$ and 50 mM $Na_2S_2O_3$ were added and vortexed for 1-2 min. This procedure was repeated until the gel pieces were clear. Afterwards, those gel pieces were washed 3 times with 50 μl of 25 mM NH_4HCO_3, 10 min each time and then the gel was dried completely by SpeedVac. Disruption of disulfide bonds between cysteine residue within polypeptide chains was achieved by adding 50 μl of 0.1 mM NH_4HCO_3, 10 mM DTT, and 1 mM EDTA and incubated at 45°C for 45 min. The solvent was discarded and quickly replaced by a freshly prepared solution of 100 mM of iodoacetamide in 0.1 mM NH_4HCO_3. The solution was then agitated at room temperature in the dark for 30 min. The solution was removed and gel pieces were washed 3 times with 50 μl iodoacetamide washing solution (0.05 mM Tris-HCl, pH 8.5, and 50% acetronitrile), at 10 min each time. The gels were entirely dried. Working trypsin solution (30 μl) (resuspended trypsin in 0.1% acetic acid, 0.05 mM Tris-HCl, 10% acetronitrile, 1 mM $CaCl_2$, pH 8.5) was added and incubated, accompanied with stirring at 37°C overnight. The mixture was removed to a new tube. The gels were soaked in 2% trifluoroacetic acid at 60°C for 30 min and vortexed continuously. The gel pieces were placed in 40 μl of digestive buffer (0.05 mM Tris-HCl, 1 mM $CaCl_2$, pH 8.5) and agitated at 30°C for 10 min. Subsequently, the gels were put in a sonicator for 5 min. Forty microliters of 100% acetronitrile was added and incubated at 30°C for 10 min. The mixture was again placed in sonicator for 5 min. Next, the mixture was pulled on to a new tube and 40 μl of 5% formic acid and 100% acetronitrile (1:1) were added and incubated in the agitator at 30°C for 10 min. The mixture was sonicated for 5 min and pulled on to a new tube. Finally, the SpeedVac was used to dry the samples.

Protein identification. Peptides were analysed by LC-MS/MS (Waters, Micromass Q-Tof micro™). Electrospray ionisation quadrupole-time of flight (Column C18, 300 μm by 15 cm) mass spectrometry was used to analyse the amino acid sequences of the tryptic peptides. The standard peptide (Glu-fiblinopeptide) was injected into the capillary column and afterwards a peptide sample of protein spot was also injected into the same capillary column. Peptides were separated by 2 eluents, including eluent A (3% ACN, 0.1% formic acid in H_2O) and eluent B (0.1% formic acid in 97% ACN). Ninety-three percent of A and 7% of B were used at 0 min, 50% of A and B at 35 min, 20% of A and 80% of B at 45 and 49 min respectively, and 93% of A and 7% of B at 50 and 60 min, respectively.

The system was run until the peptide separation finished. The monoisotopic peptide masses for all samples were measured and then searched into the search engine MASCOT (Matrix Science, London, UK). The data were searched

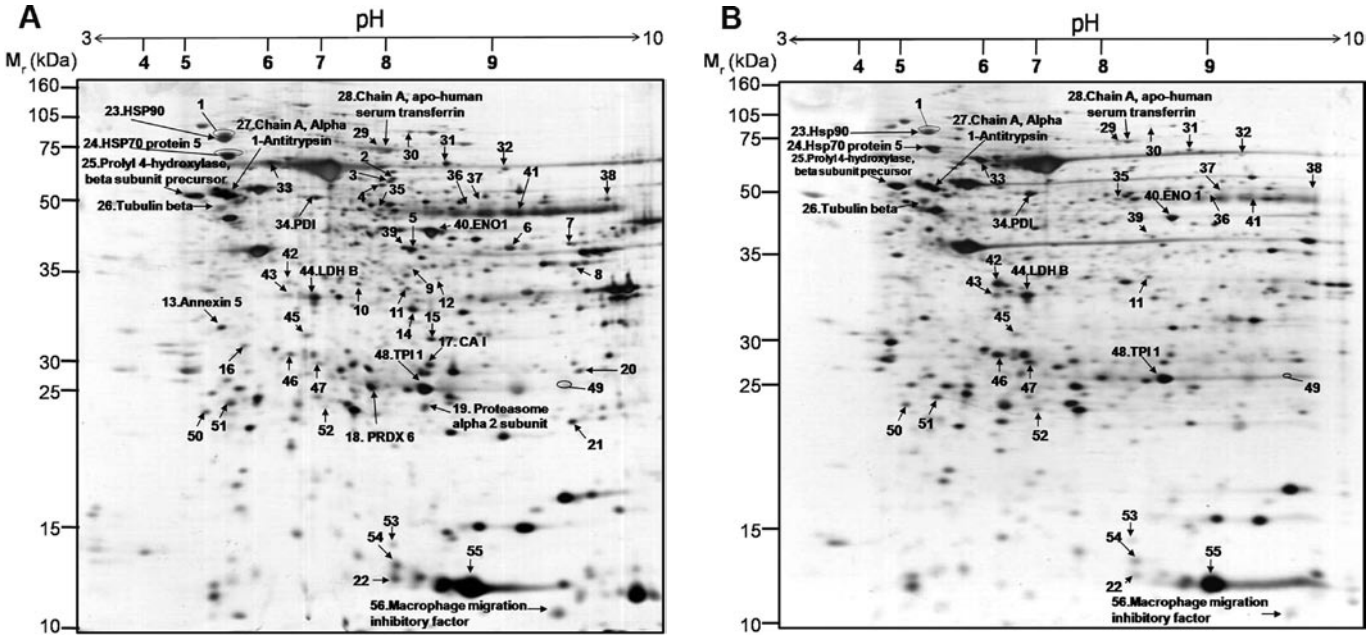

Figure 1. Representative silver-stained 2D-gels of a primary breast tumor (A) and its matched ALN metastasis (B). Differentially expressed proteins are labeled numerically. Spots 1-22 are differentially expressed proteins between node-negative breast tumor and node-positive breast tumor. Spots 1, 11, 22-56 are differentially expressed proteins between primary breast tumor and metastatic ALN. Successfully identified proteins are shown as protein names. CA I, carbonic anhydrase I ; ENO1, enolase 1; LDH B, lactate dehydrogenase B; PRDX 6, peroxiredoxin 6; PDI, protein disulfide isomerase; TPI 1, triosephosphate isomerase 1.

against NCBI non-redundant protein sequence database. The variable modifications (phospho ST), mass values (monoisotopic), protein mass (unrestricted), peptide mass tolerance (± 1.2 Da) and fragment mass tolerance (± 0.2 Da) were included in the search parameters. A maximum of one trypsin missed cleavage was allowed. Protein identifications were considered to be correct when the protein score was greater than based Mowse score ($p<0.05$).

Immnuhistochemistry. Validation study was performed using immunohistochemical staining for β-tubulin on formalin-fixed, paraffin-embedded sections. The three-micrometer sections were incubated at 45°C overnight. The sections were deparaffinized by xylene and rehydrated in graded alcohol. Antigen was retrieved by immersing the sections in Tris EDTA buffer pH 9.0 in a pressure cooker at 95°C for 4 min. Endogenous peroxidase activity was blocked with 3% H_2O_2 for 5 min and the sections were then washed in distilled water and immersed in PBS buffer pH 7.6 for 5 min. To block non-specific proteins, the sections were incubated with 3% normal horse serum in moist chamber for 30 min. The sections were incubated with the antibody against β-tubulin (clone TBN06, NeoMarkers, Fremont, CA, USA) ready to use. The antibody reactions were revealed using EnVision™ system (Dako-Cytomation, Glostrup, Denmark) according to the manufacturer's instruction. Subsequently the sections were incubated in DAB chromogen for 5 min, counterstained with hematoxylin and mounted. Immunoreactivity was evaluated under light microscopy. Sections that incubation with the primary antibody had been omitted were used as a negative control. IHC staining was evaluated under light microscopy and the intensity was scored as 0 (negative), 1+ (weak), 2+ (moderate), and 3+ (strong). Cases were considered positive if 10% or more of the tumor cells showed unequivocal immunoreactivity.

Results

Cases. Eleven cases of breast cancer in women aged 36-76 years were included in the study. The details of the clinico-pathological features are shown in Table I. Five cases were node-negative breast cancers, of which two cases were ductal carcinoma *in situ*. Six cases were node-positive breast disease.

2D-gel electrophoresis and image analysis. The 2D-gel that contained the most abundant protein spots detected by Image master 2D-platinum was used as reference. This reference gel revealed a total of 678 protein spots. The protein intensities of all 678 spots in all gels were estimated. The data were analyzed for two sets of comparisons: i) breast tumor tissue from node-negative disease (Tn^-, n=5 samples) vs. breast tumor tissue from node-positive disease (Tn^+, n=6 samples) using t-test; and ii) breast tumor tissue from node-positive disease (Tn^+, n=5 samples), and their lymph node metastases (L, n=5 samples) using paired t-test.

Twenty-two protein spots were differentially expressed between Tn^+ vs. Tn^- (Fig. 1). Nineteen proteins were up-regulated and three proteins were down-regulated in Tn^+ compared to Tn^-. Thirty-seven protein spots were differentially expressed between L vs. Tn^+. Fourteen proteins were up-regulated and 23 proteins were down-regulated in L compared to their primary breast tumors.

Identification of altered proteins. Protein spots that were statistically different which showed consistent expression

Table I. Characteristics of the cases used in the study.

Case no.	Age	T	N	M	Stage	Grade	Histology
1	52	Tis	0	0	0	-	DCIS
2	47	Tis	0	0	0	-	DCIS
3	72	1	0	0	1	1	IDC
4	46	2	0	0	2A	3	ILC
5	58	1	0	0	1	3	IDC
6	76	2	3	0	3C	2	IDC
7	44	3	2	0	3A	3	IDC
8	36	2	3	0	3C	1	ILC
9	71	2	1	0	3C	3	IDC
10	58	1	1	0	2A	3	IDC
11	52	3	2	0	3A	3	IDC

Three parameters of TNM classification for breast cancer are shown as T, size or direct extent of the tumor; N, degree of spread to axillary lymph nodes; M, presence of metastasis. DCIS, ductal carcinoma *in situ*; IDC, infiltrating ductal carcinoma; ILC, infiltrating lobular carcinoma.

pattern in different cases were subjected to in-gel digestion and identification. Fifty-six protein spots were excised for identification using LC-MS/MS. All proteins were searched against the NCBI database to identify a single protein and its function. Fifteen proteins were successfully identified (Table II). Four up-regulated proteins in the comparison group of Tn^+ and Tn^- were identified including Annexin 5, carbonic anhydrase I, peroxiredoxin 6 and proteasome α2 subunit. Eleven proteins were identified from the comparison group of Tn^+ and L, namely 90-kDa heat shock protein, chain A apo-human serum transferrin, heat shock 70 kDa protein 5, chain A α1-antitrypsin, prolyl 4-hydroxylase β subunit precursor, protein disulfide isomerase, β-tubulin, enolase 1, lactate dehydrogenase B, triosephosphate isomerase 1 and macrophage migration inhibitory factor. These proteins are known to be involved in the processes of stress response, apoptosis, protein synthesis or degradation, homeostasis, cell division and immune defense.

Immunohistochemical staining for β-tubulin. Immunohistochemistry was performed in all 11 cases used in 2D analysis. However, interpretation was not made in 2 cases due to autolysis of the tumor cells. Reactivity for β-tubulin was observed as fine granular cytoplasmic cytoplasm. In all cases, both normal duct epithelium and tumor cells in the breast were stained indifferently with weak (1+) or moderate (2+) intensity (Fig. 2). By contrast, the metastatic tumors in the ALN were stained intensely (3+), which was consistent with the 2D results.

Discussion

Metastasis of cancer cells to axillary lymph node (ALN) is a key step of tumor progression in breast cancer disease and is associated with an unfavorable prognosis. By far, the mechanism of ALN metastasis is not well understood. In the current study, we used a proteomic approach with a combination of 2D-SDS-PAGE and LC-MC/MS to determine and identify differentially expressed proteins between

Table II. List of differentially expressed proteins identified.

Spot ID	MW (Observed value)	pI	F.C.	Expression	Accession no.	Identity score	BMS	Sequence coverage (%)	Protein name
Node-positive breast cancer compared to node-negative breast cancer									
13	31.675	5.39	2.3	Up	gil4502107	527	42	53	Annexin 5
17	28.400	8.66	2.3	Up	gil4502517	212	42	55	Carbonic anhydrase I
18	26.050	7.80	1.7	Up	gil4758638	372	42	72	Peroxiredoxin 6
19	24.275	8.64	1.4	Up	gil4506181	80	42	5	Proteasome α2 subunit
Primary breast cancer compared to matched axillary node									
24	70.250	5.50	2.7	Up	gil16507237	629	44	42	Heat shock 70-kDa protein 5
25	53.875	5.13	1.5	Up	gil20070125	849	42	37	Prolyl 4-hydroxylase, β subunit precursor
34	51.750	6.85	1.6	Up	gil860986	351	42	45	Protein disulfide isomerase
26	49.025	5.39	1.6	Up	gil18088719	782	42	59	β-Tubulin
44	33.350	6.82	2.0	Up	gil4557032	346	42	43	Lactate dehydrogenase B
48	25.700	8.59	1.3	Up	gil17389815	457	42	54	Triosephosphate isomerase 1
23	78.350	5.46	1.6	Down	gil306891	1050	41	32	90-kDa heat shock protein
28	71.250	8.10	1.5	Down	gil110590597	916	41	36	Chain A, apo-human serum transferrin
27	52.750	5.50	1.6	Down	gil157831596	401	57	31	Chain A, α1-Antitrypsin
40	43.325	8.72	1.8	Down	gil4503571	907	42	69	Enolase 1
56	11.050	9.23	2.8	Down	gil4505185	112	42	26	Macrophage migration inhibitory factor

MW, molecular weight; pI, isoelectric point; F.C., fold change of protein expression; BMS, based Mowse score.

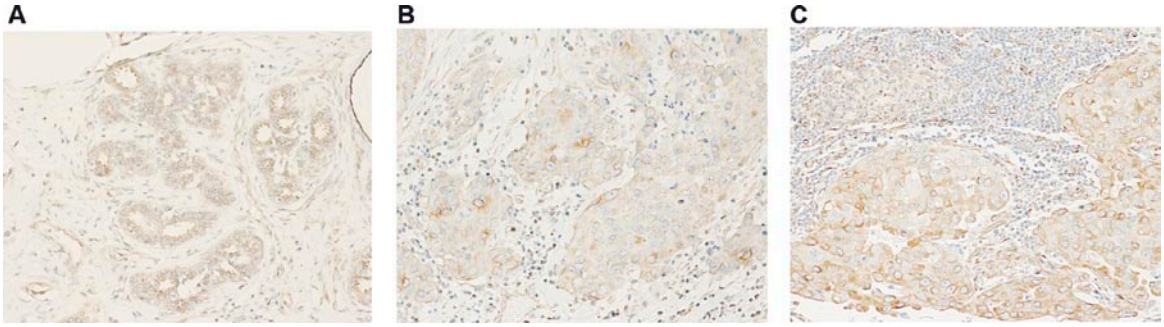

Figure 2. Immunohistochemistry analysis for β-tubulin showing gradual increase in staining intensity from normal breast (A), primary tumor (B) and ALN metastasis (C). Light microscopy, x200.

primary breast tumors and their matched ALN metastasis. We also compared primary tumors between node-negative and node-positive breast cancers. Our results have identified eleven proteins differentially expressed between the comparison groups. These proteins involved stress response, apoptosis, protein synthesis/degradation, homeostasis, cell migration and immune defense. Some of the proteins have been reported in previous study comparing breast tumor and normal tissue (10-12).

The results from 2D-SDS-PAGE showed a very similar protein expression profiles in primary tumor and matched ALN and between tumors with and without ALN metastases. The results are consistent with previous proteomic studies (13-15). These findings are also consistent with genomic profiling studies comparing primary tumors and their matched metastatic ALN (14,15). This suggests that key biological characteristics of primary breast cancer cells are maintained in metastatic cancer cells. However, in spite of the striking overall similarities observed, we detected a few significant altered proteins between the comparison sets. Thirty-seven protein spots among 678 (5.45%) detected spots were differentially expressed between primary tumors and their metastatic ALN and 22 spots among 678 spots (3.24%) between primary tumors with and without ALN metastases. Compared to the study of Vydra *et al* (14), we found a greater number of total protein spots in 2D images (678 vs. 245 spots) and a higher proportion of significant differential proteins (5.45 vs. 1.22%). This may be due to the different samples used in the analyses (clinical samples vs. cultured cells).

Highly proliferative cells and cells exposed to new environments suffer from various cellular stresses. The present study identified at least four stress-response proteins: heat shock 70-kDa protein 5 (HSPA5), 90-kDa heat shock protein (HSP90), protein disulfide isomerase (PDI) and prolyl 4-hydroxylase β subunit precursor. HSPA5 (also known as glucose-regulated protein GRP78) is a major molecular chaperone at the endoplasmic reticulum (ER) (16). Because of its anti-apoptotic properties, it is a key pro-survival component of the unfolded protein response (17). HSPA5 has been reported to be up-regulated in breast cancer compared to normal tissue (10,11,18). In the present study, HSPA5 was up-regulated in metastatic ALN, supporting the theory that HSPA5 plays a role in the protection of metastatic cells in new adverse environments.

HSP90 also has anti-apoptotic properties, and is known to promote cancer cell growth or survival by activating and maintaining the function of major cellular proteins (19). HSP90 was demonstrated as protein and RNA levels to be up-regulated in breast cancer tissue compared to non-cancer tissues (12,20). This protein has been found to be associated with decreased survival in breast cancer (21). However, in the present study, HSP90 was down-regulated in metastatic ALN compared to primary tumors. This finding can not be explained based on current knowledge.

Protein disulfide isomerase (PDI) is an enzyme involved in processing and maturation of secretory proteins in endoplasmic reticulum. The β subunit of Prolyl 4-hydroxylase has been found to be identical to the PDI (22). Schultz-Norton *et al* found that PDI functioned as ER-α chaperones in breast cancer cells (23). It increased the capacity of ER to bind to DNA, enhanced ER-ERE complex formation after exposure to the oxidizing agent. As estrogen binding to an estrogen receptor is critical for breast cancer cell growth, PDI might play a role on survival of metastatic breast cancers in axillary node via an action of ER-α chaperones.

The present study found overexpression of β-tubulin in metastatic tumors compared to their primary tumors. This was validated by immunohistochemical staining in tissue sections. Tubulin is a major component of microtubules, important cellular cytoskeletons which have various cell function including cell growth and division, maintenance of cell shape, transport of intracellular vesicles and organelles and cellular signaling (24). Overexpression of β-tubulin in metastatic tumor suggests that metastasized tumor cells utilize microtubule functions, many of which involve in metastatic cascade. Studies in other cancers also demonstrate invasive and metastatic roles of β-tubulin (25,26).

Since reactive oxygen species (ROS) are usually found in increased numbers in cancer cells, peroxiredoxin (PRDX), an enzyme that detoxifies hydrogen peroxide and various peroxides is expected to be overexpressed. One previous study reported expression of peroxiredoxin isoforms I-VI in 52-94% of breast carcinomas (27). Our findings show that PRDX6 was up-regulated in advanced staged tumors supporting the theory of increased ROS in cancer cells during tumor progression.

In early apoptosis, phosphatidylserine exposes itself on the surface of the dying cells to trigger clearance. Annexin A5 (or Annexin V) binds with high affinity and specificity

to phosphatidylserine, causing a reduction in the clearance of dying cells and consequently interfering with the immunosuppressive effects of apoptotic cells (28). It has also been shown *in vivo* study that impaired clearance of dying tumor cells can lead to tumor rejection (29). In contrast to the evidence of these studies, Annexin A5 was found to be up-regulated in tumors with ALN metastasis in the present study, consistent with report of other cancers (30). Most recently, Annexin A5 has been demonstrated to have possible influence on proliferation and invasion capacity of cancer cells via deregulation of the genes implicated in cell motility (31).

Proteasome α2 subunit, a subunit of proteosome, functions in degradation of damaged proteins by proteolysis (32). Previous studies have reported alteration of proteasome subunit α type 1 in infiltrating breast carcinomas (11). In this study, proteasome α2 subunit was up-regulated, indicating increased damage to proteins in cancer cells.

Previous studies have shown that growing cancer cells increased in both aerobic as well as anaerobic glycolyses through their exposure to physiological stresses (33,34). Supporting this basis, the present study revealed up-regulation of triosephosphate isomerase 1 and lactate dehydrogenase B, enzymes involved in these two processes, in metastatic ALN. These suggest that metastatic cells have higher levels of physiological stress. Up-regulation of carbonic anhydrase I, a zinc-containing metalloenzyme, which maintain pH homeostasis and ion transport also supports the increased metabolism of tumor cells (35). Carbonic anhydrase I was also reported to be up-regulated in breast cancer tissue compared to normal tissue in a proteomic study (12). Many carbonic anhydrase inhibitors have been shown to inhibit tumor cell proliferation *in vitro*, including breast cancer cell lines (36).

Iron is essential for proliferation of normal and neoplastic cells (37). *In vivo* studies have demonstrated that excess iron, with its capability of generating ROS, may contribute to malignant transformation, increased metastasis and invasiveness of various cancers including breast cancer (38,39). Net iron equilibrium requires both apotransferrin and ferrotransferrin (40). The present study found down-regulation of chain A apo-human serum transferrin (chain A apo-hTF), a transferrin without iron. This may disrupt iron equilibrium and promote cell proliferation. However, the exact role of chain A apo-hTF on tumor cell proliferation and metastasis needs more investigation.

Others differentially expressed proteins found in the present study included β-tubulin, chain A α1-antitrypsin, enolase 1, and macrophage migration inhibitory factor (MIF). Up-regulation of β-tubulin, a major protein in mitotic spindles, suggests enhanced cancer cell division. Chain A α1-antitrypsin is a serine proteases inhibitor. Serine proteases are known as an extracellular matrix degrader (41). Down-regulation of chain A α1-antitrypsin found in the present study, may indicate enhanced function of serine proteases to degrade extracellular matrix. Enolase 1 (also known as α-enolase), is a glycolytic enzyme expressed in most tissues. More recent evidence has shown that enolase also functions as a plasminogen receptor (42) and may also play a role in tissue invasion (43). Increased enolase 1 expression has been demonstrated in various cancers including breast cancer (44). In the current study, enolase 1 was found to be down-regulated in metastastic ALN compared to primary tumor, consistent with those previous reports.

Macrophage migration inhibitory factor (MIF) was found to be down-regulated in metastatic ALN. MIF is a pleiotropic cytokine and mediator of acute and chronic inflammatory diseases. Many studies revealed contradictory effects of MIF on tumor biology. Overexpression of MIF was reported in various cancers including breast cancer (45-47). However, Pozzi and Weiser demonstrated that recombinant MIF can activate macrophages which became cytotoxic for tumor cells *in vitro* (48). Most recently, Verjans *et al* have reported a dual role of MIF in human breast cancer depending whether it is intracellular or extracelluar expression (49). Collectively the evidence suggests that expression of MIF in breast cancer is a complex situation and has different effects on tumor biology.

Certain limitations of the present study should be noted. The 2D-PAGE technology requires a large amount of protein extract. If there are only small subpopulations of tumor cells within the primary site with higher metastatic potential, altered proteins may not be detected. Selection bias for protein identification may occur because sharp and sizable protein spots in 2D gel were more likely to be selected for further protein identification and small protein spots were not obtained. And finally, the present study included only a small number of samples and thus some altered proteins may not have been statistically different. Even with these limitations, however, we successfully identified 15 proteins, all of which are associated with tumor cell growth and survival. To our best knowledge, this is the second comparative proteomic study in primary breast tumor vs. matched metastatic ALN. However, our results need further studies to determine whether these proteins are observable on traditional IHC as well as their clinical usefulness.

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