Proteomic Study Reveals That Proteins Involved in Metabolic and Detoxification Pathways Are Highly Expressed in HER-2/neu-positive Breast Cancer*

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The receptor tyrosine kinase ErbB2 (HER-2/neu) is over-expressed in up to 30% of breast cancers and is associated with poor prognosis and an increased likelihood of metastasis especially in node-positive tumors. In this proteomic study, to identify the proteins that are associated with the aggressive phenotype of HER-2/neu-positive breast cancer, tumor cells from both HER-2/neu-positive and -negative tumors were procured by laser capture microdissection. Differentially expressed proteins in the two subsets of tumors were identified by two-dimensional electrophoresis and MALDI-TOF/TOF MS/MS. We found differential expression of several key cell cycle modulators, which were linked with increased proliferation of the HER-2/neu-overexpressing cells. Nine proteins involved in glycolysis (triose-phosphate isomerase (TPI), phosphoglycerate kinase 1 (PGK1), and enolase 1 (ENO1)), lipid synthesis (fatty acid synthase (FASN)), stress-mediated chaperonage (heat shock protein 27 (Hsp27)), and antioxidant and detoxification pathways (haptoglobin, aldo-keto reductase (AKR), glyoxalase I (GLO), and prolyl-4-hydroxylase β-isoform (P4HB)) were found to be up-regulated in HER-2/neu-positive breast tumors. HER-2/neu-dependent differential expression of PGK1, FASN, Hsp27, and GLO was further validated in four breast cancer cell lines and 12 breast tumors by immunoblotting and confirmed by partially switching off the HER-2/neu signaling in the high HER-2/neu-expressing SKBr3 cell line with Herceptin treatment. Statistical correlations of these protein expressions with HER-2/neu status were further verified by immunohistochemistry on a tissue microarray comprising 97 breast tumors. Our findings suggest that HER-2/neu signaling may result, directly or indirectly, in enhanced activation of various metabolic, stress-responsive, antioxidative, and detoxification processes within the breast tumor microenvironment. We hypothesize that these identified changes in the cellular proteome are likely to drive cell proliferation and tissue invasion and that the key cell cycle modulators involved, when uncovered by future research, would serve as naturally useful targets for the development of therapeutic strategies to negate the metastatic potential of HER-2/neu-positive breast tumors. Molecular & Cellular Proteomics 4:1686–1696, 2005.

Traditional cancer chemotherapy agents designed to block cell division are toxic to healthy cells as well as to cancer cells. Targeting specific metabolic pathways to stop cancer growth is potentially less toxic to normal cells and can improve tolerability considerably. Thus, anticancer drug discovery has shifted from the traditional empiric random screening approach to a more rational and mechanistic, target-based approach whereby specific abnormalities in cell functioning are modulated in a classical drug (ligand)-receptor fashion. The HER/ErbB family of transmembrane receptors is one of the most exciting targets currently under evaluation.

This ErbB family of receptor tyrosine kinases includes four closely related members: HER-1/ErbB1 (also known as the epidermal growth factor receptor), HER-2/ErbB2 (also known as HER-2/neu),1 HER-3/ErbB3, and HER-4/ErbB4. These receptors initiate signals by forming ligand-induced combinations of homo- and heterodimers (1) and play a critical role in the pathogenesis of breast cancer. HER-2/neu, one of the most well characterized breast cancer oncogenes, is amplified in about 20–30% of all human breast cancers (2, 3) and is also overexpressed in a variety of other human tumors, including ovarian, lung, gastric, and oral cancers. It appears...
not to bind to any known ligand but is the preferred heterodimerization partner for the other family members (4). The ErbB2/3 heterodimer efficiently activates the phosphatidylinositol 3-kinase (PI3K)/AKT/PTEN pathway and the Ras/Raf/mitogen-activated protein kinase (MAPK) pathway, which are essential in cellular survival, by phosphorylating and inactivating growth-inhibitory and proapoptotic proteins. aberrant expression of ErbB receptors triggers the activation of multiple downstream signal transduction pathways and plays a key role in inducing increased cell proliferation and differentiation, decreasing apoptosis, and enhancing tumor cell motility and angiogenesis. Clinically accumulated evidence has shown that HER-2/neu overexpression in tumors is associated with a poor prognosis and a more aggressive phenotype. In particular, tumors with HER-2/neu overexpression are known to be refractory to various types of chemo- and endocrine therapy and to be associated with shortened overall survival (5, 6).

The genes/proteins regulated by HER-2/neu-mediated signal transduction pathways have been investigated recently (7–10). Transcriptome analysis revealed that a large number of these differentially regulated genes were involved in cell-matrix interactions, cell proliferation, and transformation (8). Many interferon-inducible genes were found to be downregulated, consistent with increased proliferation of the ErbB2-overexpressing cells, whereas the key cell cycle modulators, including cyclin D2, were up-regulated in the breast cell lines exposed to hergulin β1, an ErbB-specific growth factor (9). Kumar-Sinha et al. (10) reported that HER-2/neu regulated fatty acid synthase (FASN) expression through the PI3K pathway, whereas Menendez et al. (11) showed that FASN modulated HER-2/neu transcription and suggested HER-2/neu could function as a sensor of energy imbalance in the FASN-overexpressing tumor cells. Several other targets of the ErbB2/PI3K signal transduction cascade have been implicated in tumor angiogenesis and metastasis as well (12, 13), leading to stimulated expression of vascular endothelial growth factor (12) and enhanced cytoskeletal reorganization and tumor cell motility (14). The prometastatic gene S100A4 is another gene shown to be up-regulated by the HER-2/neu signaling network that includes the PI3K, AKT1, and extracellular signal-regulated kinase 1/2 pathways (13). HER-2/neu itself is strongly inhibited by a tumor suppressor protein, ARF, an alternative reading frame protein, which inhibits HER-2/neu-mediated oncogenic growth by negating its effect in blocking cancer cells from undergoing programmed cell death or apoptosis (15).

Recent advances in proteomic technology have provided powerful analytical tools to identify the differentially expressed and/or post-translationally modified proteins as potential biomarkers in tumors (16, 17) and blood (18, 19). Two-dimensional electrophoresis (2-DE), as the principal tool in proteomics, is able to resolve thousands of proteins in one experiment and provide the highest resolution in protein separation. The aggregate protein information attainable from such proteomic analyses would provide a vast resource for a better understanding of the translated protein milieu and dynamic protein-protein interactions that occur within the tumor microenvironment during oncogenesis and pathogenesis. Deciphering the changes in the proteome in the tumor cells compared with the normal untransformed counterparts also provides a basis for the identification of potential targets for early disease detection and for the rational designs of diagnostic and therapeutic methods. A few studies on HER-2/neu-induced changes in protein expression have been reported using breast cancer cell lines (20), but none, to our knowledge, have been performed on HER-2/neu-positive and -negative clinical breast tumor tissues to discover the deregulated proteins and/or phosphoproteins involved in the HER-2/neu signal transduction pathways in the tumor microenvironment.

The development of tumor-specific biomarkers and discovery of novel target-based drugs have been challenged by the heterogeneous populations of cells present in human breast tumor tissues. To overcome this problem, we used laser capture microdissection (LCM) to procure relatively homogeneous cell populations from both the HER-2/neu-positive and -negative breast tumors to generate well defined differential protein profiles and to identify the deregulated proteins associated with the HER-2/neu oncogene. In a previous study (21), we identified seven of the 21 spots with at least 5-fold changes in spot volume in HER-2/neu-positive tumors using the Ettan MALDI-TOF mass spectrometer. In this study, the remaining spots were analyzed with the MALDI-TOF/TOF MS/MS tandem mass spectrometer, and nine more proteins with high Mascot scores were identified. Four differentially expressed proteins were further validated using a combination of immunoblotting, monoclonal antibody inhibition of HER-2/neu signaling, and immunohistochemistry, the latter performed on 97 breast tumors compiled into a breast tumor tissue microarray (TMA). We present data to suggest that these proteins may represent some of the key enzymes or non-enzyme mediators in the signal transduction cascade that are involved in the underlying mechanisms that collectively confer the poor prognosis associated with HER-2/neu overexpression in breast tumors.

**EXPERIMENTAL PROCEDURES**

**Cell Culture, Clinical Tissue Selection, and Laser Capture Microdissection**

**Cell Lines—**Four commercially available breast carcinoma cell lines, MDA-MB-231, MCF-10A, BT474, and SKBr3 (kindly given by Dr. Q. C. Lau, Oncology Research Institute, National University of Singapore), were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, and 100 mg/ml streptomycin. Two of these cell lines, BT474 and SKBr3, were known to be high expressers of HER-2/neu oncoprotein, whereas the MDA-MB-231 and MCF-10A cell systems had not demonstrated any HER-2/neu expression. The adherent cells in culture were harvested by trypsinization before reaching confluence; the cells were treated at 37 °C for 5–10 min with trypsin-EDTA solution (Sigma) and washed with PBS buffer. The proteins were extracted using the Mammalian
Protein Extraction Reagent (M-PER, Pierce). To determine the influence or effect of HER-2/neu signaling on the protein expression of various differentially expressed proteins, the HER-2/neu-overexpressing SKBr3 cell line was treated with Herceptin at a final concentration of 200 μg/ml for 48 h at 37 °C (10) to inhibit HER-2/neu expression. These analyses were done in triplicates. Total proteins were extracted and used for Western blotting.

Clinical Tumor Samples—Stringent selection of the tumor tissue samples and the procurement of homogeneous populations of tumor cells using the PixCell II laser capture system for proteomic analyses were as described previously (21). Briefly 10 HER-2/neu-positive and 15 HER-2/neu-negative frozen tumor tissues and their matched normal tissues were obtained from the Tissue Repository of the Singapore National University Hospital. The tumors were obtained following regulations set by our Institutional Review Board for tissue usage, including informed patient consent and anonymization prior to release for research use. For the proteomic studies, which were carried out in triplicates to ensure reproducibility of results, we carefully selected six tumors, controlling for histological type or grade (all of Grade 3), nodal status (all node-negative), and estrogen receptor (all estrogen receptor-negative), thus ensuring that HER-2/neu status remains the major discerning variable. Three of the selected tumors were HER-2/neu-positive, and the remaining three were HER-2/neu-negative. The decision to work with proteins pooled from a small number of well matched tumors was made to minimize the misinterpretation of protein profiles arising from random differences in gene expression of different tumors and to obtain sufficient materials for downstream analyses. Multiple 5-μm-thick sections were cut from the frozen tissues fixed in 70% ethanol and embedded in paraffin, further stained in hematoxylin in the presence of protease inhibitor mixture tablets (Complete Mini, Roche Applied Science), and dehydrated in xylene. Cells were microdissected using the PixCell II LCM system (Arcturus Engineering Inc., Mountain View, CA) with 10,000,000 cells, corresponding to 50,000–70,000 cells, within the set maximum time period of 30 min per slide. The cells were immediately lysed in the appropriate protein extraction buffer.

Sample Preparation

Protein sample preparation and separation by 2-DE were performed as stated in our previous work (21). Generally the microdissected cells were lysed in a 40 mM Tris-HCl buffer containing 7 M urea, 2 M thiourea, 4% CHAPS, 1% Mega-10, 0.5% Triton X-100, 50 mM dithiothreitol, 1% IPG buffer (pH 3–10 nonlinear), and 2 M tributylphosphine. The protein concentrations were determined using the PlusOne 2-D Quantitation kit (Amersham Biosciences) with a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE). The protein molecular weight (M) or pl and species and allowed for carboxymethylation of cysteine and partial oxidation of methionine residues. Up to one missed tryptic cleavage was considered for all tryptic mass searches. Protein scores greater than 75 were considered as significant (p < 0.05). Further confirmation of protein identifications was obtained by duplicating the protein identification using the MS-Fit software (prospector.ucsf.edu).

Western Blotting and 2-D Immunodetection

For 2-D immunodetection, the same protein samples used for the protein profile analysis were tested. The pooled proteins were first separated using IPG DryStrips (7 cm, pH 3–10) and further separated by 10% SDS-PAGE. For Western blotting, two HER-2/neu-overexpressing cell lines, BT474 and SKBr3, and two HER-2/neu-negative cell lines, MDA-MB-231 and MCF10A, as well as 12 clinical specimens, six each from HER-2/neu-positive and HER-2/neu-negative tumor cells procured by LCM, were analyzed. The protein mixture from each case was individually separated by 10% SDS-PAGE and electrotransferred onto PVDF membranes using the SemiDry apparatus (Bio-Rad). The membranes were serially probed with one of the following primary antibodies: anti-FASN (1:500, BD Biosciences), anti-Hsp27 (1:1000, Santa Cruz Biotechnology, Inc.), anti-PGK1 (1:500, Santa Cruz Biotechnology, Inc.), anti-GLO (1:500, kindly given by Dr. K. D. Tew from Institute of Cancer Research, University of London), and anti-β-actin (1:5000, Sigma). Goat anti-rabbit IgG horseradish peroxidase (1:10,000, Zymed Laboratories Inc.) or goat anti-mouse IgG horseradish peroxidase (1:5000, Molecular Probes) in TBS-Tween 20 buffer were used as secondary antibodies. The chemiluminescent signals were detected using SuperSignal® West Pico Chemiluminescent Substrate (Pierce). Signals were captured with the MULTIscan™ plate reader.
FASN Is Highly Expressed in HER-2/neu-positive Tissues and Cell Lines—FASN shows two immunoreactive protein spots on the 2-D immunoblot (Fig. 2). The relative intensities of spot 1 showed a 2.3-fold increase in HER-2/neu-positive tumors compared with HER-2/neu-negative tumors, whereas spot 2 had a 4.5-fold higher intensity in HER-2/neu-positive tumors than in the -negative tumors. Based on the estimated $M_r$ values of the two spots on 2-DE, we concluded that spot 2 ($\sim$48 kDa) is the Fab B (3-oxoacyl-acyl-carrier-protein synthase) fragment and spot 1 ($\sim$85 kDa) is the Fab B + Fab D (acyl-carrier-protein)-S-malonyl transferase) fragment of FASN. The enhanced expression of FASN was further verified by Western blotting. As shown in Fig. 3A, two bands of FASN were immunodetected. Interestingly, band 2 (Fab B, $\sim$48-kDa molecular mass) gave a more pronounced quantitation variance between HER-2/neu-positive and -negative tumors with five of six cases of the former showing higher expression of this fragment. Fig. 3B shows the FASN expression in the four breast cancer cell lines. FASN was strongly immunodetected in the SKBr3 cell line and weakly detected in the BT474 cell line. In SKBr3 cells that were treated with the monoclonal antibody Herceptin for 48 h, the expression of FASN was reduced by more than 2-fold, indicating the role of HER-2/neu signaling in FASN expression (Fig. 4). Immunohistochemical analysis performed on the breast cancer TMA section re-

**Immunohistochemistry on Tissue Microarrays**

Two breast cancer TMAs consisting of 97 tumors and corresponding matched normal tissues, respectively, were constructed as reported previously (22). The DAKO Envision system was used for the immunostaining of the TMA sections. Brief sections were deparaffinized in xylene and rehydrated in graded alcohols. Antigen unmasking was undertaken using DAKO® Target Retrieval Solution in a microwave oven. Endogenous peroxidases were removed using 3% hydrogen peroxide in methanol. Sections were incubated for 1 h with anti-FASN (1:500), anti-Hsp27 (1:500), anti-GLO (1:200), and anti-PGK1 (1:200) at room temperature followed by detection with labeled dextran polymer conjugated with peroxidase and DAB -substrate chromogen solution. Nuclei were lightly stained with Mayer’s hematoxylin. Each antibody was applied to three sections. The staining level was scored as negative (0), weak (1), moderate (2), and strong (3) by a pathologist and a research scientist independently, according to the staining intensity of the tumor cells. Cases with discrepant scores were rescored by the same or additional scorers to obtain a consensus score, failing which the scores were not included in the data analysis. All the sections were examined without prior knowledge of the clinical information for any of the cases.

**Statistical Analysis**

A Fisher’s exact test was used to compare the expression of FASN, Hsp27, PGK1, and GLO between HER-2/neu-positive and -negative tumors. A p value (two-sided) of 0.05 was considered statistically significant.

**RESULTS**

**Identification of Differentially Expressed Proteins**—To identify the proteins whose expressions were strongly associated with HER-2/neu status in breast cancer, we compared the protein profiles between pooled HER-2/neu-positive and -negative tumor cells. A representative 2-DE gel image of a protein profile from LCM-procured HER-2/neu-positive tumor cells is shown in Fig. 1A. Using the 2-D Elite software (Amer-
revealed the cytoplasmic staining of FASN with high FASN expression in the HER-2/neu-positive tumors \((p < 0.002)\) (Table II). Representative examples of positive and negative immunostaining are shown in Fig. 5. Taken together, our collective observations suggest that HER-2/neu signaling regulates FASN expression in breast cancer.

**Heat Shock Chaperone Hsp27 Is Highly Expressed in HER-2/neu-positive Tissues and Cell Lines**—We showed that Hsp27, a predictor of poor outcome and response to therapy in breast cancer \((23, 24)\), was differentially expressed in HER-2/neu-positive and -negative breast tumors. The 2-D immunoblot analysis of Hsp27 (Fig. 2) showed that only one spot was detected in tumor tissues with significantly enhanced expression in the HER-2/neu-positive tumors. No other isoforms were identified. Fig. 3, A and B, summarizes the Western blot analyses of Hsp27 expression levels, normalized to the expression level of \(\beta\)-actin, in 12 clinical tissue samples and four breast cancer cell lines, respectively. The overall

**Table I**

Summary of overexpressed proteins in HER-2/neu-positive breast tumors identified by MALDI-TOF/TOF MS/MS

| Proteins (spot no.) (molecular mass/pl) | Accession no. | Sequence coverage \(b\) | -Fold increase \(c\) | No. of matched peptides | MS/MS peptide sequence (ion scores) | Mascot score | Function |
|---------------------------------------|--------------|--------------------------|-----------------|------------------------|----------------------------------|-------------|----------|
| Hsp27 (spot 4) \((22.8 \text{ kDa/5.98})\) | NP_001531 | 47 | 5.52 (0.86) | 7 | \(1^{12}\)GPSWDPLLFR \((40)\) | 381 | Chaperone protection |
| TPI (spot 5) \((26.6 \text{ kDa/6.4})\) | NP_000356 | 74 | 5.43 (0.87) | 15 | \(2^{28}\)LDQQAFGLPR \((66)\) | \(3.4^{9}\)VSLDNHFAFAPDLTVK \((82)\) | | |
| GLO (spot 6) \((21.0 \text{ kDa/5.12})\) | NP_006699 | 28 | 27.3 (1.08) | 6 | \(1.3^{34}\)VPADTFVCAFTYIDFAR \((65)\) | \(6^{66}\)DCGATWWVLGHSER \((32)\) | | Glycolysis |
| ENO1 (spot 7) \((47.5 \text{ kDa/7.01})\) | NP_001419 | 39 | 7.01 (1.04) | 7 | | | | |
| PGK1 (spot 8) \((45.0 \text{ kDa/8.3})\) | NP_000282 | 23 | 5.20 (0.76) | 5 | | | | |
| FASN (spot 9) \((276 \text{ kDa/6.0})\) | NP_004095 | 6 | 10.86 (1.75) | 12 | \(1^{102}\)GHTGVWGVSGSETSELR \((26)\) | \(1^{214}\)AFDTAGNYCRR \((55)\) | | |
| | | | | | \(2^{258}\)EQGTVFSGDQEQLR \((87)\) | \(2^{296}\)VDKPQELNGTR \((27)\) | | |
| Haptoglobin (spot 10) \((45.9 \text{ kDa/6.13})\) | NP_005134 | 22 | 7.22 (1.06) | 7 | \(1^{102}\)GHTGVWGVSGSETSELR | \(2^{214}\)AFDTAGNYCRR | | Antioxidant protection |
| P4HB (spot 11) \((57.5 \text{ kDa/4.76})\) | NP_000909 | 26 | 5.03 (0.64) | 8 | \(1^{217}\)VGYSGWGR | \(2^{237}\)YMLPVADQDCQR | | Detoxification protection |
| AKR (spot 13) \((36 \text{ kDa/7.7})\) | NP_001345 | 32 | 5.21 (1.03) | 5 | \(1^{327}\)YKPESELTAE | | | Detoxification protection |

\(a\) Reference for the protein identification.

\(b\) Sequence coverage of the matched peptides in protein.

\(c\) -Fold increase was the average (±S.D.) of triplicate 2-DE gels.
expression of Hsp27 was >2-fold higher in HER-2/neu-positive tumors compared with that in HER-2/neu-negative tumors. Only the cell line SKBr3 expresses a high level of Hsp27 followed by much weaker expression in the BT474, MDA-MB-231, and MCF-10A cell lines (Fig. 3B). We also observed that reducing the HER-2/neu expression in the SKBr3 cell line by Herceptin treatment resulted in lower expression of Hsp27 in the treated SKBr3 (Fig. 4).

In situ immunohistochemical staining of Hsp27 provided the tumor-dependent expression variances. Fig. 5 shows examples of cases with strong and weak cytoplasmic staining for Hsp27. Statistically overexpression of Hsp27 was mostly observed in HER-2/neu-positive tumors (p = 0.011), confirming the positive correlation of Hsp27 expression with HER-2/neu status in breast cancer (Table II).

**PGK1 Is Overexpressed in HER-2/neu-positive Tissues and Cell Lines**—We found that three enzymes involved in the glycolysis pathway, TPI, PGK1, and ENO1, were more highly expressed in the HER-2/neu-positive tumors. At least five isoforms of PGK1 were detected by 2-D immunoblotting (Fig. 2) with all five spots registering increased intensities (~1.5-4-fold increase) in HER-2/neu-positive tumor tissues. Western blot analysis of cell lines and tumor tissues also showed that PGK1 was, overall, more highly expressed in HER-2/neu-positive tumors (Fig. 3A). PGK1 is highly expressed in the HER-2/neu-overexpressing cell line SKBr3, whereas HER-2/neu-negative cell lines MDA-MB-231 and MCF-10A showed relatively lower expression (Fig. 3B). In addition, we observed that PGK1 expression was affected (decreased) by partially switching off HER-2/neu signaling by blocking it with Hercep-
tin treatment as shown in Fig. 4. Immunostaining performed on the breast tumor TMA sections also showed a differential staining pattern of PGK1 (Fig. 5) with strong staining of PGK1 mostly observed in HER-2/neu-positive cases ($p = 0.027$) (Table II). Taken together, we conclude that PGK1 expression is regulated by HER-2/neu status in breast cancer.

**Table II**

| Staining scores $^a$ | HER-2/ neu-negative $(n = 66)$ | HER-2/ neu-positive $(n = 2)$ | $p$ value $^b$ |
|----------------------|-------------------------------|-------------------------------|----------------|
| **FASN**             |                               |                               |                |
| 0–1                  | 20 (30%)                      | 4 (19%)                       |                |
| 2                    | 35 (53%)                      | 5 (24%)                       |                |
| 3                    | 11 (17%)                      | 12 (87%)                      | 0.002          |
| **Hsp27**            |                               |                               |                |
| 0–1                  | 14 (21%)                      | 3 (14%)                       |                |
| 2                    | 40 (61%)                      | 7 (33%)                       | 0.011          |
| 3                    | 12 (18%)                      | 11 (53%)                      |                |
| **PGK1**             |                               |                               | 0.027          |
| 0–1                  | 20 (30%)                      | 3 (14%)                       |                |
| 2                    | 36 (55%)                      | 9 (43%)                       |                |
| 3                    | 10 (15%)                      | 9 (43%)                       |                |
| **GLO**              |                               |                               | 0.023          |
| 0–1                  | 18 (27%)                      | 2 (10%)                       |                |
| 2                    | 38 (58%)                      | 10 (48%)                      |                |
| 3                    | 10 (15%)                      | 9 (43%)                       |                |

$^a$ Staining scores: 0–1, negative to weak; 2, moderate; 3, strong.

$^b$ Fisher’s exact test.

**Fig. 4.** Regulation of FASN, Hsp27, PGK, and GLO by HER-2: representative immunoblot analysis of SKBr3 cells with or without Herceptin treatment. SKBr3 cells were treated with Herceptin (final concentration, 200 μg/ml) for 48 h, and the expression levels of proteins were analyzed by Western blot. Signals were detected as described in Fig. 2. β-Actin levels served as a loading control. Results are the average of three independent experiments.

**Fig. 5.** Representative immunohistochemical staining of FASN, Hsp27, PGK1, and GLO on tissue microarrays. A–D show strong staining, and E–H show weak staining. Sections were deparaffinized in xylene and rehydrated in graded alcohols. After unmasking antigen and removing endogenous peroxidases, sections were incubated for 1 h with antibodies against FASN, Hsp27, GLO, and PGK1, respectively, at room temperature followed by detection with labeled dextran polymer conjugated with peroxidase and DAB $^1$-substrate chromagen solution. Nuclei were lightly stained with Mayer’s hematoxylin. The extent of staining was scored as negative (0), weak (1), moderate (2), and strong (3) according to the staining intensity of the tumor cells.

**GLO Is Overexpressed in HER-2/neu-positive Tissues and Cell Lines**—We identified several proteins with antioxidant and detoxification functions (haptoglobin, GLO, P4HB, and AKR) that are all potentially associated with HER-2/neu signaling. We first validated the GLO expression in both cell lines and clinical specimens. As shown in the 2-D immunoblot, the intensity of the immunodetected GLO spot was about 5-fold higher in HER-2/neu-positive tumors (Fig. 2). The high GLO expression detected in the HER-2/neu-positive breast tissues (Fig. 3A) and cell lines (Fig. 3B) was in keeping with the Western blot results. The significant reduction in GLO expression in SKBr3 cells treated with Herceptin further confirmed the association of GLO expression with HER-2/neu signaling (Fig. 4). In addition, the differential expression of GLO was also verified as statistically significant by the immunohistochemical analysis carried out on the TMA sections. None of the normal tissues showed any staining for GLO, but many of the tumor tissues on the TMA sections showed variable but positive staining by the GLO-specific antibody (Fig. 5), correlating well with HER-2/neu overexpression ($p = 0.023$, Table II). These findings indicate that HER-2/neu mediates the stimulation of GLO expression and thus enhances the self-protection of cells by detoxification.
DISCUSSION

Amplification/overexpression of the HER-2/neu oncogene correlates with a poor clinical prognosis and also with the adverse response of the tumor to systemic therapy. Identification of genes/proteins that are differentially expressed in HER-2/neu-dependent breast tumors is essential in elucidating the mechanistic basis of their increased metastatic potential. Although a number of genes and pathways have been identified by cDNA microarray analysis to be up- or downstream targets of HER-2/neu signaling (7, 8), the deregulation of genes found in cell lines may not always be consistent with that in tumors (7) mainly due to the interaction between stromal and tumor cells in the tumor microenvironment. In addition, there is evidence indicating that mRNA levels may not necessarily predict the translated protein levels (9). Proteomics, as a complementary tool to genomic research, is thus increasingly being used to examine cancer-related changes in protein expression with the aim of identifying the markers or potential therapeutic targets and/or increasing the understanding of the pathogenesis of disease. Our findings in this proteomic study indicate that certain specific proteins involved in glycolysis, fatty acid synthesis, heat shock protein chaperonage, and detoxification are more highly expressed in HER-2/neu-positive breast tumors compared with the HER-2/neu-negative breast tumors.

FASN—The association of FASN with HER-2/neu overexpression, first reported by cDNA microarray analysis (10), has been confirmed by our results. FASN is the major enzyme required for the anabolic conversion of dietary carbohydrates to fatty acid. The fatty acids synthesized, or their derivatives, are essential components of biological membranes, serve as metabolic fuel. Significantly higher levels of FASN have been found in many tumors relative to their normal counterparts and were evident in the early stages of tumor development in prostate (25) and breast cancer (26). In our study, the enhanced levels of FASN in HER-2/neu-positive tumors were confirmed by immunoblotting and TMA immunohistochemical analysis (p = 0.002). Reducing the HER-2/neu expression by Herceptin treatment significantly decreased FASN levels in SKBr3 cell lines. The breast cancer-associated FASN hyperactivity can potentially be exploited as a target for the development of new therapeutic antimetabolites. Data from studies on the regulation of FASN and HER-2/neu appear inconsistent with some claiming that HER-2/neu regulates FASN expression via the PI3K pathway (10) and others counterclaiming that FASN inhibits HER-2/neu transcription via up-regulation of PEA3, a transcription repressor of HER-2/neu (11). However, the synergistic cytotoxicity to HER-2/neu overexpressors of both chemical FASN inhibitors and the antibody trastuzumab against p185-HER-2 provides a molecular rationale for novel therapeutic strategy to treat the more aggressive HER-2/neu-positive breast cancer (11, 29).

Hsp27—The chaperone protein Hsp27 is involved in various cellular stress responses, apoptosis, and actin reorganization. Overexpression of Hsp27 could protect malignantly transformed cells from apoptotic cell death and foster resistance to chemotherapeutic agents and irradiation (30). Hsp27 is overexpressed in numerous human cancers, including breast invasive ductal carcinoma and cell lines (8, 20) and other carcinomas (31, 32), and recently was reported to play a cytoprotective role in hormone-refractory prostate cancer (33). It interacts with the cytochrome c-Apaf-1-dATP complex in the procaspase-9 pathway and also binds to F-actin, preventing the subsequent cytochrome c release (34). Using proteomic analyses, we established that there is differential expression of Hsp27 in breast cancers with/without HER-2/neu overexpression. However, based on the 2-D immunoblot of breast tumors, we did not find any other isoforms as were reported to be present in renal cell carcinoma (32). The failure to detect phosphorylated Hsp27 isoforms in our cases is probably due to the sample treatment, which may have caused the dephosphorylation of Hsp27, or perhaps the level of the other isoforms may be too low to be detected in our experiments. Our immunohistochemistry analysis showed that Hsp27 was expressed in the cytoplasm, and the staining scores were closely associated with the HER-2/neu status. Partially switching off the HER-2/neu signaling reduced the expression of Hsp27 in the SKBr3 cell line. The enhanced level of Hsp27 in HER-2/neu-positive tumors may be one of the factors contributing to its aggressive tumor behavior and poor prognosis. However, concrete evidence awaits the revelation of the molecular mechanisms directly stimulating the overexpression of Hsp27 by activation of HER-2/neu signaling.

Glycolytic Enzymes TPI, PGK1, and ENO1—Three enzymes, TPI, PGK1, and ENO1, involved in the glycolytic pathway were also found to be significantly overexpressed in the HER-2/neu-positive breast tumors. Overexpression of these enzymes may well relate to the increased requirements of both energy and protein synthesis/degradation pathways in the rapidly growing tumors. PGK1 functions as an enzyme in glycolytic metabolism and as a secreted protein by tumor cells to participate in the angiogenesis process as a disulfide reductase (35) and is transcriptionally activated by hypoxia-inducible factor-1 (36). PGK1 also influences DNA replication and repair in mammalian cell nuclei (37) and induces a multidrug resistance (MDR) through an MDR-1-independent mechanism (38). Recently PGK1 has been found in a variety of other cancers, such as renal cancer (39) and squamous cell carcinoma (16), and is considered an appropriate target molecule for specific immunotherapy of HLA-A2+ colon cancer patients (40). In our study, high expression of PGK1 in HER-2/neu-positive breast cancers was further confirmed by both Western blotting and immunohistochemistry with significant correlations (p = 0.027) to the HER-2/neu oncogene. A significantly reduced PGK1 level in SKBr3 cells treated with
Herceptin provided evidence that HER-2/neu signaling regulates PGK1 expression in breast cancer. We showed that PGK1 was post-translationally modified by 2-D immunoblotting but failed to observe this phenomenon in the 2-DE silver-stained gel. This may be because we only selected spots with ~5-fold change in volume, thus excluding spots such as those of PGK1 with lower ratios. It is also possible that the amounts of the other PGK1 isoforms are too low to be detected by silver staining. The isoform (spot 4/5), with its relatively high intensity, was most probably the spot that was picked from the 2-D silver-stained gel for MS/MS analysis. Therefore, the various isoforms of PGK1 may be specifically correlated with HER-2/neu status and associated with the more aggressive behavior of breast cancer. Confirmation of this requires further investigation.

Although the mechanism(s) by which HER-2/neu signaling regulates PGK1 expression is yet unclear, it is recognized that enhanced glycolytic metabolism will provide more energy (ATP) for cell proliferation and growth and will increase the concentration of metabolites such as lactate and pyruvate to regulate hypoxia-inducible gene expression, including that of vascular endothelial growth factor. The cross-talk between HER-2/neu-mediated signals and the sink of energy and/or fuel produced from related metabolic pathways remain to be better defined.

Antioxidant and Detoxification Proteins—We demonstrated a high expression of four proteins, haptoglobin, P4HB, GLO, and AKR, that are involved in antioxidative and detoxification pathways in HER-2/neu-positive tumors and the sink of energy and/or fuel produced from related metabolic pathways remain to be better defined. The reduced GLO level found in Herceptin-treated SKBr3 cells. GLO is an essential component in pathways leading to the detoxification of methylglyoxal, a side product of glycolysis. Accumulation of methylglyoxal causes DNA modification and protein cross-links and thus initiates the activation of apoptosis. GLO was reported to be highly expressed in apoptosis-resistant leukemia cells (41), solid tumors (42), and Alzheimer disease (43). Overexpression of this protein may serve to protect cells from toxic events and induce a prosurvival response to chemotherapy or environmental carcinogens. Recently, the potential of GLO as an apoptosis resistance factor and a target in cancer chemotherapy was recognized (44), but its regulation in tumor cells and its involvement in apoptosis and drug resistance have not been fully elucidated. Our findings may prompt renewed interest in the interaction between the regulation of GLO and HER-2/neu-mediated signaling and in the development of GLO inhibitors as novel anticancer drugs. Further study of regulation of the other three proteins, haptoglobin, P4HB, and AKR, by HER-2/neu signaling may reveal novel self-protective mechanisms in the aggressive breast tumors.

In summary, we have identified HER-2/neu-associated proteins that are related to cellular metabolic and detoxification activities in the tumor microenvironment. Taken together with the decreased expression of the four proteins by inhibition of HER-2/neu expression in the SKBr3 cell line, the proteins that we have identified in this study appear to be involved in multiple diverse pathways and have established roles in cellular intermediary metabolism. Our hypothesis is that these diverse pathways are interlinked as part of the signal transduction cascade initiated by HER-2/neu activation as depicted in Fig. 6. Our proposal is substantiated by a number of experimental and clinical observations.

FASN has been reported to be regulated by HER-2/neu signaling via the PI3K pathway (10). The chaperone activity of
Hsp27 is regulated by heat-induced changes in phosphorylation and oligomerization. Phosphorylation of Hsp27 is catalyzed by MAPK-activated protein kinase-2, a target of the p38 MAPK, which is also regulated by HER-2/neu signaling, and acts upstream of the apoptosome by preventing cytochrome c release. In HER-2/neu-positive tumors, the increased energy requirement for cell proliferation could be partly supported by the up-regulation of the glycolytic enzymes, such as TPI, PGK1, and ENO1. The HER-2/neu-dependent differential expressions of these proteins suggest cross-talk, directly or indirectly, between HER-2/neu signaling and this central intermediary metabolic pathway. Besides the energy and intermediary metabolites used for biosynthesis and metabolism, the glycolytic pathway also produces by-products that are toxic to cell survival, such as methylglyoxal, which increases and initiates the potentiality of cell death. Potential cytotoxicity can be regulated by initiating detoxification processes, which can be stimulated, for example, by enhancing the expression and activity of GLO. Although the regulation and involvement of GLO in tumorigenesis and drug resistance have yet to be fully elucidated, our proteomic study suggests the probable intricate interactions between HER-2/neu signaling and detoxification (Fig. 6).

The up-regulated proteins/enzymes may yet be potential therapeutic targets in developing drugs against the highly aggressive HER-2/neu-positive breast tumors. Future work will need to address how HER-2/neu signaling regulates the expression of these identified proteins and how the HER-2/neu oncoprotein senses and modulates the burden of energy and the metabolites produced in tumor cells to enhance their own survival.

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