

Featured Article

Selective Tyrosine Hyperphosphorylation of Cytoskeletal and Stress Proteins in Primary Human Breast Cancers: Implications for Adjuvant Use of Kinase-Inhibitory Drugs

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ABSTRACT

Purpose: Small-molecule growth factor receptor inhibitors block cell growth *in vitro* and downstream signaling *in vivo*, but controlled trials in patients with advanced solid tumors have yielded disappointing response rates. To clarify this discrepancy, we compared the patterns of tyrosine phosphoprotein expression in human cancer cells and primary tumors.

Experimental Design: Immunoaffinity chromatography, two-dimensional electrophoresis, and antiphosphotyrosine immunoblotting were combined with mass spectrometry to determine the phosphoproteomic signatures of 40 matched normal and malignant tissues from patients with breast or liver cancer. The identities and abundance of the detected tyrosine phosphoproteins were compared with those of ligand-responsive A431 cells.

Results: Patterns of tyrosine-phosphorylated proteins are similar among normal tissues of the same origin but vary markedly between different tissues. Primary breast tumors exhibit a strikingly homogeneous tyrosine phosphorylation profile, whereas liver cancers display greater phosphoproteomic diversity. The main breast-tumor-specific tyrosine phosphoproteins are cytoskeletal molecules (actin, tubulin, and vimentin) and molecular chaperones (Hsp70, Hsc71, and Grp75). In contrast, control studies in ligand-stimulated A431 human cancer cells revealed an additional phosphorylated subset of prometogenic phosphoproteins (Grb2, Shc, Jnk2, phospholipase C- γ , and phosphatidylinositol 3'-kinase).

Conclusions: Identification of cytoskeletal and stress proteins as the most abundant tyrosine phosphoproteins in

breast tumors implicates these molecules, rather than prometogenic effectors, as the prime stoichiometric substrates for kinase-inhibitory anticancer drugs *in vivo*. Because phosphorylated cytoskeletal proteins and chaperones mediate cell motility and apoptotic resistance, respectively, these data raise the intriguing possibility that small-molecule tyrosine kinase inhibitors may be of greatest value either as adjuvant antimetastatic/invasive drugs or as chemo-/radio-sensitizers.

INTRODUCTION

Maximum dose escalation of conventional DNA-damaging drugs fails to improve overall survival in chemosensitive malignancies such as breast cancer (1), suggesting by default that evolving anticancer strategies should focus on more specific treatments with higher toxic:therapeutic ratios (2, 3). Consistent with this, recent progress has derived from the use of target-specific drugs such as imatinib, a tyrosine kinase inhibitor useful in both *Bcr/Abl*-expressing leukemias and *c-Kit*-expressing stromal tumors (4), and trastuzumab, a humanized monoclonal antibody that inhibits ErbB2-overexpressing human breast cancer (5). Like other differentiation inducers (6, 7), binding of trastuzumab to ErbB2 causes receptor down-regulation (8–10) and thus induces inhibition of antiapoptotic Akt signaling (11) partly via prevention of receptor activation by extracellular proteases (12).

These historical breakthroughs have galvanized expert opinion over the long-term prospects for treating cancer with small-molecule growth factor receptor inhibitors (13, 14). In contrast to the groundbreaking success of trastuzumab, however, the orally bioavailable catalytic tyrosine kinase inhibitor gefitinib (ZD-1839) has had a problematic therapeutic debut (15, 16). The clinical efficacy of this epidermal growth factor receptor (EGFR) inhibitor does not correlate with EGFR expression levels (17, 18), whereas the response rates to the drug in unselected populations appear low and unassociated with cytotoxic synergy (19). Moreover, consistent with inhibition of EGFR kinase activity (20), overall EGFR expression levels rise severalfold (rather than declining as occurs after antibody therapies) in human cells and tumors treated with gefitinib (21, 22). This latter observation is of concern given that the ErbB3 protein, a constitutively kinase-inactive (23) and down-regulation-deficient molecule (24), functions *in vitro* as a prometogenic oncoprotein (25) via its affinity for dimerizing with heterologous ligand-activated receptors. Rather than acting as a pure tumor antagonist then, gefitinib may convert active EGFR into an overexpressed kinase-inactive (ErbB3-like) cell-surface receptor that sequesters EGFR-specific [epidermal growth factor (EGF) and transforming growth factor- α] and other type I receptor ligands (*e.g.*, betacellulin and heparin-binding-EGF; Ref. 26), thus triggering secondary dimerization (27) associated with prolonged activation of other recep-

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tors (28). These complexities caution against framing clinical predictions on the basis of simplistic mechanistic assumptions.

To improve our understanding of this interactive molecular circuitry, we recently developed a phosphoproteomic approach to the analysis of receptor tyrosine kinase signaling *in vitro* and have shown that five molecular subclasses (mitogenic effectors, adhesion proteins, adaptors, cytoskeletal proteins, and chaperones) are comparably phosphorylated in response to growth factors (29). Here we seek to clarify the therapeutic potential of catalytic tyrosine kinase inhibitors by extending this approach to the comparative phosphoproteomic profiling of primary human tumors and adjacent normal tissue.

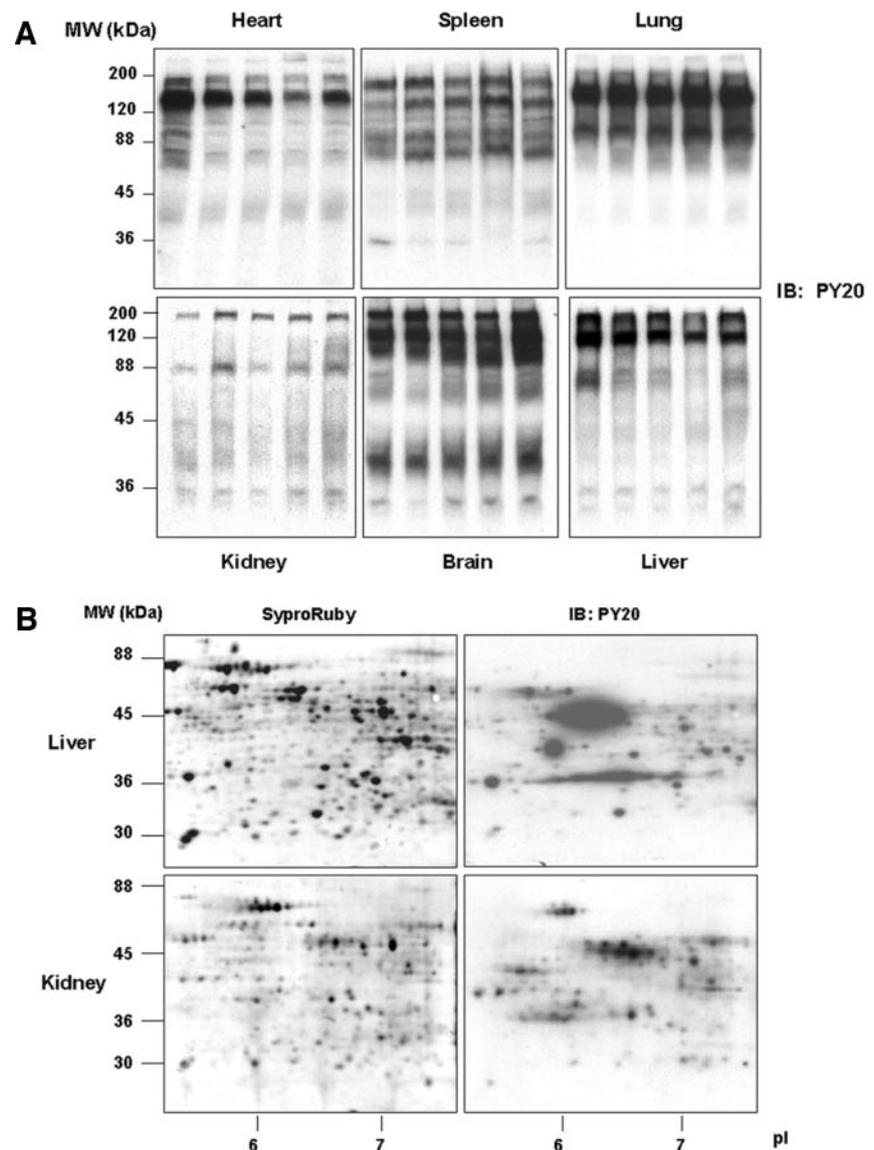
MATERIALS AND METHODS

PY20 peroxidase-conjugated phosphotyrosine antibody for immunoblotting was purchased from Transduction (Lexington,

KY), and 4G10 phosphotyrosine antibody (30) for immunoaffinity column purification was prepared as described previously (29). Protein lysis, chromatography, two-dimensional PAGE, and mass spectrometry were performed according to standard protocols (29). Gels were scanned with a Typhoon 8600 laser imager; spot detection was carried out using Image Master 2D Elite software, and spot excision was performed by an automated spot picker (Amersham Biosciences). Mass spectrometric data were used to interrogate either the National Center for Biotechnology Information database by use of the MASCOT search engine or to interrogate the SWISS-PROT database by use of the ProteinLynx package.

For animal studies, male Balb/C mice were sacrificed by cervical dislocation and handled according to animal guidelines. For tissue collection and analysis, approvals were obtained from the Tissue Repository and Ethics Committees of the National Cancer Centre. Forty matched malignant and adjacent normal

Fig. 1 Characterization of organ-specific tyrosine phosphoproteomes in normal tissues. **A**, comparative tyrosine phosphorylation profiles from six normal murine tissues, as labeled, assessed by one-dimensional antiphosphotyrosine immunoblotting (IB: PY20). Molecular markers in kilodaltons are shown at the left. **B**, two-dimensional separation of proteins and tyrosine phosphoproteins from murine liver and kidney. *Panels at left* show total proteomes imaged by use of SyproRuby protein stain, whereas *panels on the right* show the corresponding positioning of immunoreactive phosphotyrosine as detected by antiphosphotyrosine immunoblotting (IB: PY20).



breast and liver tissues removed from cancer patients during routine surgery were snap-frozen. Tissues were subsequently washed in ice-cold PBS, dissected into small pieces, transferred to lysis buffer, and homogenized with an Ultra-Turrax T25 (Jenke and Kunkel, Staufenh, Germany) by pulsing at -4°C four times for 15 s each time. Lysates were centrifuged, passed through a $0.45\ \mu\text{m}$ filter, clarified by centrifugation, stored at -70°C , and then analyzed in batches.

RESULTS

To assess the sensitivity and utility of phosphoproteomic profiling *in vivo*, we first examined normal mouse tissues. These analyses indicated that each tissue expresses its own characteristic profile of tyrosine-phosphorylated species, as determined by the pattern of differentially sized phosphoproteins; in contrast to the variations among tissue types, close similarity was apparent between tissue phosphoprotein profiles of the same organ type (Fig. 1A). A two-dimensional comparative analysis of the proteomic and phosphoproteomic differences between such normal tissues is presented in Fig. 1B (*left and right panels*, respectively). Patterns of tyrosine phosphoprotein immunoreactivity differed substantially between hepatic and renal tissues, suggesting that the functional differences between these tissue types are reflected in their phosphoproteomic profiles.

Having established the practicability of the above approach, we proceeded to analyze 40 matched primary tumor and adjacent normal (B) or liver (L) tissues from cancer patients. Ten μg of protein from the dissected tissue lysates

were first resolved by use of one-dimensional antiphosphotyrosine immunoblotting, and a duplicate gel was stained for proteins. As shown in Fig. 2A, breast tumor tissues displayed a higher level of tyrosine phosphorylation than normal tissues. Five of these 10 tumor specimens (samples B1, B4, B5, B8, and B10) were characterized by immunodetectable phosphorylation of a 185-kDa protein (Fig. 2A, *arrow on right*) corresponding to the molecular weight of ErbB2, as corroborated by immunohistochemical studies performed for clinical staging (data not shown). As is apparent in Fig. 2A, several other tyrosine-phosphorylated bands were detectable in most tumors; for example, a prominent tumor-specific 44-kDa tyrosine phosphoprotein band was subsequently identified as actin (see below).

Primary liver tumors appeared to be more heterogeneously tyrosine-phosphorylated than breast tumors; furthermore, unlike normal breast tissues, adjacent liver tissues were at least as heavily tyrosine-phosphorylated as liver tumors (samples L1, L3, L4, L6, L8, L9, and L10) if not more so (Fig. 2B). Although unexpected, we propose that this could be consistent with the presence of a preneoplastic field defect induced by chronic viral inflammation (31).

Cancer-specific differences in breast tumor tyrosine phosphorylation were further assessed by use of two-dimensional PAGE analyses of three primary breast tumors (B8, B9, and B10) previously characterized as having representative one-dimensional tyrosine phosphorylation profiles. As shown in the two-dimensional phosphoproteomic comparison of a typical breast (B10) and liver (L4) tumor in Fig. 3, differences in

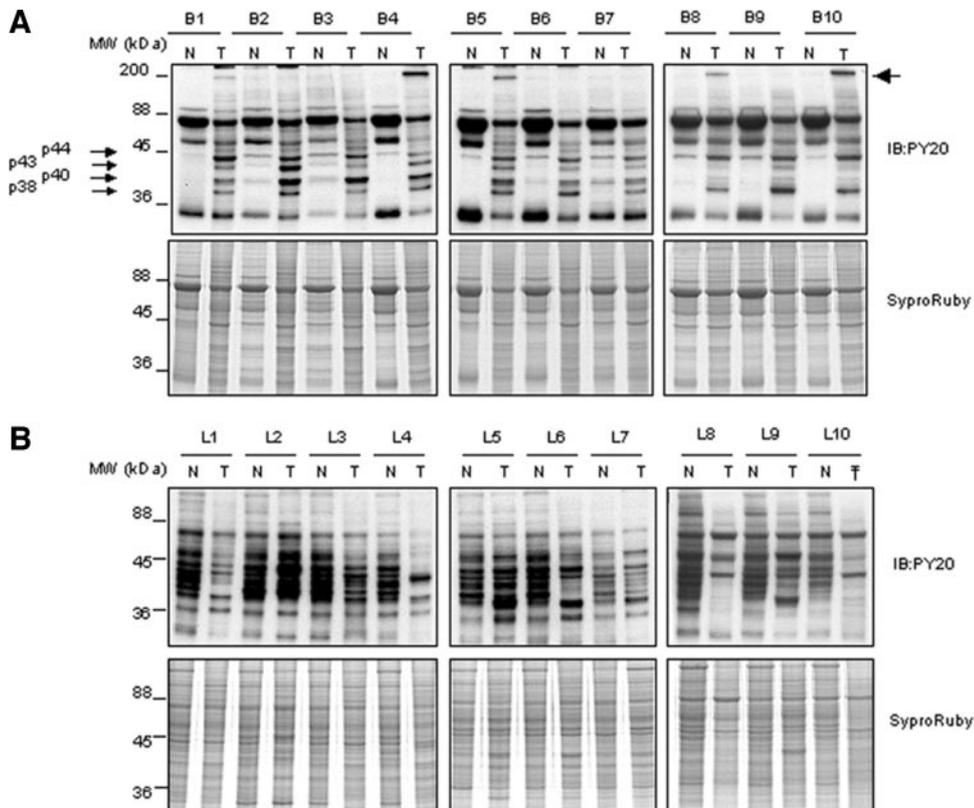


Fig. 2 One-dimensional antiphosphotyrosine immunoblotting of adjacent normal (N) and tumor (T) tissues from breast (Lane pairs B1–B10 in A) and liver cancer (Lane pairs L1–L10 in B) patients. One set of samples was immunoblotted with PY20H (*top gels*) and a further set was stained for total protein content with SyproRuby (*bottom gels*). Arrow at right indicates the position of ErbB2.

protein tyrosine phosphorylation patterns are evident not only between normal (Fig. 3, *upper left*) and malignant tissues (Fig. 3, *upper right*) of the same organ, but also between malignant tissues of breast (Fig. 3, *top right*) and liver (Fig. 3, *second from bottom, right*) origin, and between normal tissues of breast (Fig. 3, *top left*) and liver (Fig. 3, *second from bottom, left*) origin. Of note, these different phosphoprotein profiles were similarly reproduced on examination of other samples.

To confirm whether these pattern differences were indeed phosphorylation-specific, tumor tissues were lysed in the presence or absence of the tyrosine phosphatase inhibitor sodium orthovanadate. Spots that were rapidly dephosphorylated in the absence of vanadate (Fig. 4) were interpreted as being phosphorylated, whereas spots that persisted despite vanadate omission were deemed nonphosphospecific. Accordingly, only the former subset of spots were processed for identification as tyrosine phosphoproteins.

The tyrosine phosphoproteome from a larger breast tumor (sample B9) was then analyzed by mass spectrometry. This procedure confirmed that two main subclasses of tyrosine phosphoproteins were differentially detectable in tumor compared with adjacent normal tissue: cytoskeletal proteins (actin, vimentin, and tubulin- β) and molecular chaperones (Hsc71, Hsp70, and Grp75). We then compared this (presumably constitutive) pattern of tyrosine phosphorylation with that inducible *in vitro* by EGF, which is well known to activate a critical tumorigenic signaling pathway in human breast cancer (32). This comparison revealed a similar overall spectrum of phosphorylated cytoskeletal and chaperone proteins in both EGF-stimulated A431 cells and breast cancer; in contrast, however, the tumor phosphoproteome was characterized by more intense cytoskeletal protein phosphorylation and by little detectable phosphorylation of the

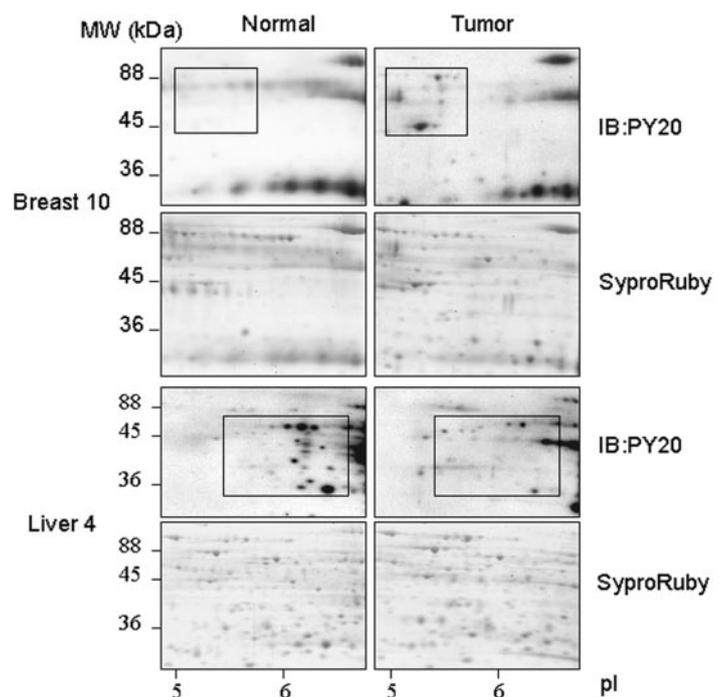
mitogenic effector molecules that were readily detectable in the ligand-primed cell line (Table 1).

DISCUSSION

Conventional two-dimensional proteomic analyses are hampered by interpretational difficulties relating to quantification of the complex data sets involved (33). Strategies to optimize the clinical utility of proteomic-based technologies are thus keenly sought. A popular approach has been to simplify the data by focusing on a molecular subclass of diagnostic and/or therapeutic interest, such as membrane proteins (34), enzymes (35), or circulating biomarkers (36). More recently, functional protein subsets have been defined by use of phosphorylation-specific antibodies to identify downstream substrates of receptor or nonreceptor tyrosine kinases (37, 38). This strategy seems well suited to the characterization of human cancers (*a*) because tyrosine kinases are critical effectors of human carcinogenesis (39), (*b*) because the phosphoprotein composition of such cancers correlates with tissue-specific patterns of cytotoxic sensitivity (40), and (*c*) because growth factor-dependent tyrosine kinases have recently become a prime target of anticancer drug development (41).

We previously analyzed oncoprotein function by creating antibodies specific for either phosphotyrosine (42) or site-specific phosphorylated protein isoforms (43). More recently we have extended this signal-profiling strategy to incorporate all tyrosine-phosphorylated signaling proteins and substrates via combination of immunoaffinity chromatography, two-dimensional PAGE, and mass spectrometry (29). In applying this approach to the *in vivo* setting, the present study has generated two new insights. The first of these is that human breast cancers

Fig. 3 Two-dimensional phosphoproteomic analysis of primary human breast (*Breast 10*) and liver (*Liver 4*) tumors and their respective adjacent normal tissues. The boxed areas highlight the key differences in tyrosine phosphorylation patterns between normal and tumor tissues and show the positions of the breast tumor tyrosine phosphoproteins (*top right*) subsequently identified by mass spectrometry. IB:PY20, antiphosphotyrosine immunoblotting.



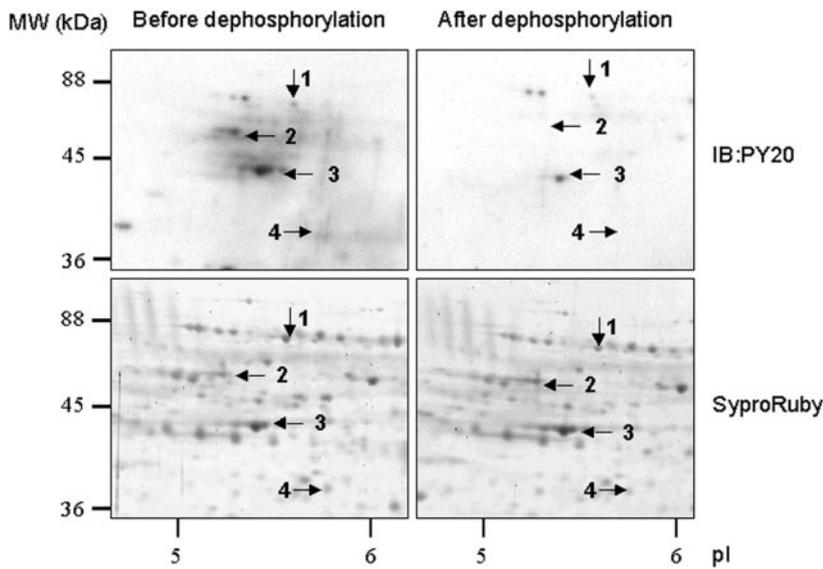


Fig. 4 *In vitro* dephosphorylation of breast cancer (specimen B10)-associated putative tyrosine phosphoproteins. To facilitate dephosphorylation, sodium orthovanadate was omitted from the lysis buffer. Arrows show the positions of protein spots thus identified as dephosphorylation sensitive and, hence, unequivocally phosphorylated. IB:PY20, antiphosphotyrosine immunoblotting.

exhibit a distinctive phosphoproteomic signature, suggesting a commonality of signaling pathways involved in tumor progression; this observation augurs well for the eventual utility of pathway-specific tyrosine kinase antagonists in this disease. The second novel finding is that the breast-cancer-specific tyrosine phosphoproteome predominantly comprises cytoskeletal and chaperone phosphoproteins. This latter profile differs from the phosphorylation pattern documented in ligand-stimulated human cancer cells, in which context promitogenic effector phosphoproteins are as readily detectable as cytoskeletal and stress

phosphoproteins. Because gefitinib triggers the dephosphorylation of all such substrates *in vitro* (Ref. 29; see Table 1), we submit that the main pharmacological effect of tyrosine kinase antagonists in breast cancer may be to reverse the tumor-specific phosphorylation of these latter (cytoskeletal and chaperone) phosphoprotein subclasses.

Previous studies have confirmed the ability of tyrosine kinases to phosphorylate actin (44), vimentin (45), and the Hsc71 (46)/Hsp70 heatshock protein family (47). In functional terms, cell tolerance of stress due to starvation or heat is in-

Table 1 Comparison of tyrosine phosphorylation patterns in cancerous and adjacent normal breast tissues (columns 3 and 2, respectively) and in A431 cancer cells (columns 4, 5, and 6)

Protein ID ^a	Protein class	Tyrosine phosphorylation pattern ^b				
		Human breast tissue		Human A431 cells		
		Adjacent normal breast	Primary breast cancer	EGF-activated cells ^c	Ligand-starved cells ^d	Gefitinib/EGF-treated cells ^e
Actin	Cytoskeletal	+	+++	++	+	-
Vimentin	Cytoskeletal	+	+++	-	-	-
Tubulin- α	Cytoskeletal	-	-	++	+	-
Tubulin- β 1	Cytoskeletal	-	+	++	+	-
Ezrin	Cytoskeletal	-	-	+	-	-
γ -Catenin	Cytoskeletal	-	-	+	-	-
Hsc71	Chaperone	-	+	+++	+	-
Hsp70	Chaperone	-	+	++	+	-
Hsp90	Chaperone	-	-	++	+	-
Grp78/(BiP)	Chaperone	+	+	++	+	-
Grp75	Chaperone	-	+	++	+	-
PLC- γ	Mitogenic	-	-	+	-	-
PI3K/p85	Mitogenic	-	-	+	-	-
Shc	Mitogenic	-	-	+	-	-
Grb2	Mitogenic	-	-	+++	-	-
Jnk2	Mitogenic	-	-	+	-	-

^aID, identification; EGF, epidermal growth factor; PLC, phospholipase C; PI3K, phosphatidylinositol 3'-kinase.

^b+, detectable phosphorylation; ++, moderate phosphorylation; +++, strong phosphorylation; -, no phosphorylation.

^cCells stimulated with 50 ng/ml EGF for 15 min.

^dQuiescent cells.

^eCells coexposed to EGF and the epithelial growth factor receptor inhibitor gefitinib.

creased by tyrosine phosphorylation of actin (48) and Hsp70-family proteins (49), respectively, whereas more generalized tyrosine phosphorylation of focal adhesion proteins induces endothelial destabilization (50). The latter events are potentially induced by upstream phosphotyrosine-dependent cascades involving proinvasive/-metastatic effectors such as Src (51, 52) and phosphatidylinositol 3'-kinase (53). Hence, because most cancer-specific phenotypes imply clonal selection via the acquisition of a growth advantage, our data showing tumor hyperphosphorylation of cytoskeletal and stress proteins suggest a hitherto unrecognized pathogenetic role for these tyrosine kinase substrates in tumor progression. Given the importance of actin and vimentin in governing cell motility (54) and invasion (55) and the evidence for heatshock (56) and other stress proteins (57) in modulating tumor cell survival and anticancer drug resistance, these data raise the possibility of a direct role for tyrosine phosphorylation in modulating the prometastatic and antiapoptotic phenotypes typical of cancer progression (58). Of potential clinical relevance to these respective possibilities, animal models of gefitinib action have confirmed both organ-specific inhibition of metastatic growth (59) and potentiation of cytotoxic cell kill (60).

As is true of most tissue-based research, the interpretability of our findings is limited by several considerations. First, the relatively small number of matched adjacent normal and malignant tissue specimens restricts the power of our conclusions, making it essential that any such correlations be reassessed in larger studies. Second, our tissue-handling protocol did not incorporate laser microdissection, a qualitative gold standard for minimizing sampling error (61); the main reason for this experimental compromise relates to the much larger amount of tissue needed for phosphoprotein (as opposed to mRNA) identification. However, the apparent cancer-specific homogeneity of the one-dimensional tyrosine phosphorylation profiles provides reassurance as to the tumor selectivity of macrodissection in these experiments, whereas previous studies indicated that tissue/tumor heterogeneity may not weaken either the predictivity (62) or sensitivity (63) of such analyses. A third concern relates to the use of the EGFR-overexpressing A431 cell system as a comparator for the breast tissue phosphoproteome. Although the cell lineage is not identical, this system was chosen for technical and logistic reasons: (a) the high level of EGFR expression in this cell line greatly facilitates phosphoprotein identification by two-dimensional PAGE and mass spectrometry, compared with other EGF-responsive cell lines (e.g., Swiss 3T3 cells);⁶ and (b) EGFR expression is common in more aggressive breast cancers (64) and may thus be relevant to anticancer tyrosine kinase inhibition (21).

Finally, it should be noted that our observations by no means exclude the presence of tyrosine-phosphorylated mitogenic effector proteins in primary human breast cancers, nor do they exclude the efficient dephosphorylation of such proteins *in vivo* by kinase inhibitors; for example, the comparison with ligand-stimulated A431 cells is rendered less useful by the likelihood that inducible signaling pathways are down-regulated

in vivo by constitutive autocrine or paracrine loops. Nonetheless, our findings clearly indicate that the most abundant steady-state tumor-specific intracellular targets for kinase-inhibitory drugs are the catalytically inactive terminal substrates implicated in motility and resistance rather than the "headline" mitogenic effector proteins. The importance of this finding lies in its potential implications for the strategic use of kinase-inactivating drugs.

In conclusion, phosphoproteomic fingerprinting of human breast tumors has selectively identified phosphoproteins relevant to metastasis and resistance as stoichiometrically important therapeutic targets of kinase-inhibitory anticancer drugs. Empirical studies will be needed to determine whether prophylactic use of tyrosine kinase inhibitors may inhibit these malignant phenotypes and, by so doing, improve survival outcomes in patients with breast cancer and other common solid tumors. The testable hypothesis that such benefits may be obtainable in the adjuvant setting by use of pathway-specific drugs lacking major cytotoxic activity in the palliative setting should not be rejected out of hand because of the traditional reliance of clinical researchers on early-phase metastatic response rates.

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⁶ Y. P. Lim, unpublished data.

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