**Advances in Brief**

**Expression Profile of Tyrosine Kinases in Breast Cancer**

Funda Meric, Wei-Ping Lee, Aysegul Sahin, Haixia Zhang, Hsing-Jien Kung, and Mien-Chie Hung

Departments of Surgical Oncology [F. M., H. Z., M-C. H.], Molecular and Cellular Oncology [W-P. L., M-C. H.], and Pathology [A. S.], The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030, and Department of Biological Chemistry, University of California at Davis Cancer Center, Sacramento, California 95817 [H-J. K.]

**Abstract**

The tyrosine kinase (TK) family includes many growth factor receptors, cell cycle regulators, and oncoproteins. Moreover, the receptor TKs HER2/neu and epidermal growth factor receptor are overexpressed in a subgroup of breast tumors and correlate with more aggressive behavior. Thus, TKs are being actively pursued as therapeutic targets. The purpose of this study was to determine the expression pattern of TKs in breast cancer. Reverse transcription-PCR was performed with degenerate primers based on conserved motifs of the catalytic domains of TKs, and the identities of the reverse transcription-PCR products were determined by digestion with a panel of restriction enzymes. Using a TK display assay, we studied the TK profiles of 13 breast cancer cell lines and two normal immortalized breast epithelial cell lines. The TK display assay reproducibly demonstrated known differences in HER-2/neu expression between cell lines. Several TKs, including receptor TKs Axl, Cak, fibroblast growth factor receptor 4, HEK8, HER2/neu, c-MET, RET, and nonreceptor TKs ARG, BRK, Janus kinase 1, Rak, and YES were detected in breast cancer cells. Several kinases were differentially expressed among the cell lines. Similar TK profiles were found using RNA from human breast tumors. We conclude that there is significant variability in the TK expression pattern of breast cancers. This variability should be considered when selecting TK inhibitors to treat patients.

**Introduction**

TKs are regulatory proteins that play an important role in the cell growth and differentiation of normal cells. The TKs represent a major class of proto-oncogenes and may be involved in the progression and metastasis of cancer cells. Some TKs also affect the sensitivity of tumors to radiation- and chemotherapy-induced apoptosis. As a result, TKs are being actively studied as targets for therapeutic intervention. The best studied are the receptor TKs HER2/neu and EGFR. Overexpression of HER2/neu occurs in 20% of invasive breast cancers and has been found to predict a poorer prognosis than does normal expression (1). Several additional TKs have been found to be expressed or overexpressed in breast cancer (2) including BRK (3), c-Src (4), and FGFR (5). In contrast, loss of normal expression of other TKs such as Syk (6) and c-KIT (7) have been reported in breast cancer and been proposed to play a role in carcinogenesis.

Trastuzumab, a monoclonal antibody against HER2/neu, is now in clinical use both as a single agent and in combination with chemotherapeutic agents. TK inhibitors targeting HER2/neu and EGFR are also being tested in clinical trials. Understanding the TK expression patterns in breast cancer is essential for selecting particular TK inhibitors for clinical use and for identifying new therapeutic targets.

We hypothesized that breast cancers have significant variability in their TK expression profiles, and that there may also be novel TKs that are differentially expressed in breast cancer and affect cancer biology. We used a differential display assay based on the conserved sequences in TKs to study their expression profiles in normal breast and breast cancer cell lines.

**Materials and Methods**

**Cell Lines, Cell Cultures, and Breast Specimens.**

Breast cancer cell lines BT474, MCF7, MDA-MB-231, MDA-MB-361, MDA-MB-453, MDA-MB-468, BT20, BT483, BT549, SBKBR3, T47D, ZR75-1, MDA-MB-330, and MDA-MB-435, immortalized breast epithelial cell lines MCF10A and MCF12A, and human rhabdomyosarcoma cell line A204 were obtained from American Type Culture Collection (Manassas, VA) and maintained according to the manufacturer’s specifications. Cells were cultured in a humidified atmosphere of 5% CO₂ and at 37°C. Cells were harvested for RNA and protein analysis at 70–80% confluence. The breast specimens were obtained from the University of Texas M. D. Anderson Tumor Bank.

**TK Display Assay.**

Total RNA was isolated from cell lines and breast tissue with the TRIZOL reagent (Life Technologies, Inc., Carlsbad, CA), and the TKDA was carried out by a

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2 To whom requests for reprints should be addressed, at Department of Molecular and Cellular Oncology, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Box 108, Houston, TX 77030. Phone: (713) 792-3668; Fax: (713) 794-0209; E-mail: mhung@mdacc.org.

3 The abbreviations used are: TK, tyrosine kinase; TKDA, TK display assay; EGFR, epidermal growth factor receptor; FGFR fibroblast growth factor receptor; RT-PCR, reverse transcription-PCR; PDGFR, platelet-derived growth factor receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; JAK, Janus kinase.
modification of the method described by Robinson and colleagues (8, 9). Total RNA was reverse transcribed after annealing with the antisense degenerate primer Primer 3, 5'-CACAG-GTTACCRHAIGMCCAIACRTC-3' /H11032, using the Superscript preamplification system (Life Technologies, Inc.). RT-PCR was performed using degenerate primers; the sense primers were Primer 1, 5'-CAGGTCACCAARRTIDCNGAYTTYGG-3' /H11032, and Primer 2, 5'-CCAGGTCACCAARRTTDCNGAYTTYGG-3' /H11032, and the antisense primer was Primer 3. The mixed bases were defined as follows: N/A/C/T/G, D/A/T/G, H/A/T/C, R/A/G, Y/C/T, M/A/C, and I/deoxyinosine. Alignment of the amino acid sequences corresponding to the primers and the TK consensus sequences are presented in Fig. 1A. RT-PCR was performed with DNA polymerase (FB-6000–60; Fisher Scientific, Pittsburgh, PA) in the presence of 25 mM MgCl₂. Amplification was carried out at an annealing temperature of 44°C for 5 cycles and then at 55°C for 25 cycles. Amplified products were analyzed by gel electrophoresis on an 8% polyacrylamide gel (Fig. 1B). The primer sets yielded RT-PCR products that were consistently 154–170 bp because of the relatively constant spacing of the conserved motifs from which the primers were derived. A DNA was stained with 1 g/ml ethidium bromide. The 154–170-bp RT-PCR products were excised from the gel and eluted. Equal amounts of radioactive DNA (10⁵ cpm) were digested with a panel of restriction enzymes: AciI, AluI, CfoI, DdeI, HaeIII, Hinfl, MnlI, Sau3AI, Sau96I, ScrI, Tru9I, and Rsal. Digestion products and uncut control DNA were resolved on a 6% acrylamide DNA sequencing gel and autoradiographed (Fig. 1B). A sequencing reaction was used as a size marker. The uncut kinases remained as tightly clustered 154–170-bp nucleotide bands (Fig. 2). Up to 10 kinases were cleaved with a given restriction enzyme, and different kinases were represented by bands of different sizes displayed in a DNA sequencing gel (Fig. 2).

Cloning and Sequencing. PCR-amplified products were subcloned into the pCR2.1 vector (Invitrogen, Carlsbad, CA). The recombinant plasmids were sequenced using the T7 Sequence version 2.0 DNA sequencing system (USB Corp., Cleveland, OH). The sequences obtained were compared with GenBank database sequences from the National Center for Biotechnology Information using the BLAST algorithm.

RT-PCR for Specific Kinases. RT-PCR was performed at an annealing temperature of 55°C using primers 5'-GGTG-GCTGTGAAGACGATGA-3' /H11032 and 5'-CTCAGATCTCCTG-3' /H11032 for Axl, 5'-TTGTCGCGACAGGCG-3' and 5'-GATGATGGCCTGCTCCAG-3' /H11032 for Syk, and 5'-AGTAGATGGAGGATGAG-3' and 5'-AGTTTGACAGGTTCCA-
Amplification of GAPDH was performed as a control using primers 5′-AAGGTGAAGGTCGGAGTTCAAC-3′ and 5′-CATGAGTCCTTCCACGATACC-3′. PCR products were separated by 1.5% agarose gel electrophoresis.

**Western Blot Analysis.** Whole-cell extracts were lysed using buffer A [20 mM Tris (pH 7.5), 10% glycerol, 1% Triton X-100, 0.15 mM NaCl, 1 mM β-mercaptoethanol, 1 mM Na3VO4, 1 mM aprotinin, and 1 mM phenylmethylsulfonyl fluoride]. Samples were then centrifuged, and the supernatants were heated to 100°C for 5 min and separated by 8% SDS-PAGE. The membranes were blocked with 5% milk, incubated with antibodies against Axl (Santa Cruz Biotechnology, Santa Cruz, CA), and then stripped and incubated with an antibody against actin (Boehringer Mannheim, Indianapolis, IN). Immunocomplexes were detected with an ECL chemiluminescence system (Amersham Corp., Arlington Heights, IL).

**Northern Blot Analysis.** Total RNA was extracted from cell lines using TRIZOL reagent as described previously. Ten µg of total RNA from each cell line was resolved on a formaldehyde gel and transferred onto nylon membranes (GeneScreen; New England Nuclear Life Science Products, Boston, MA). A 303-bp Axl fragment was randomly labeled with [α-32P]dCTP using the random primer DNA labeling system (Life Technologies, Inc.). The membranes were then stripped and incubated with an antibody against actin (Boehringer Mannheim, Indianapolis, IN). Immunocomplexes were detected with an ECL chemiluminescence system (Amersham Corp., Arlington Heights, IL).

**Results**

**TKDA.** We used TKDA to profile TKs expressed in breast cancer cell lines. The catalytic domains of TKs have conserved motifs that have been commonly used for amplification of TKs using degenerate primers (2). We used primer sets derived from the conserved amino acid motifs DFG and DVW of the subdomains VII and IX in the activation loop of TKs (Fig. 1A). TK profiles of normal breast cell lines MCF10A and MCF12A and breast cancer cell lines BT474, MCF7, MDA-MB-231, MDA-MB-361, MDA-MB-453, MDA-MB-468, BT20, BT483, BT549, SBKB3, T47D, ZR75-1, MDA-MB-330, and MDA-MB-435 were performed. TKDA was performed by loading equal amounts of radioactive DNA (i.e., equal amounts of RT-PCR products) for each cell line onto the sequencing gel. Thus, the assays were standardized using the total TK content rather than the total RNA content as a reference. Although results of this PCR-based assay were not quantitative, we found that the HER2/neu expression detected by TKDA in MCF10A cells, in low HER2/neu-expressing MCF7 cells, and in high HER2/neu-expressing MDA-MB-361 cells was consistent with the expected expression profile (Fig. 2A). Furthermore, when RT-PCR was performed with two primer sets (Primers 1 and 3 versus Primers 2 and 3), similar results were obtained, demonstrating that the results of TKDA were reproducible. Fig. 2B shows one TKDA after restriction enzyme digestion with CfoI. HER2/neu was detected with the expected expression pattern. The expression patterns of Arg, Axl, BRK, Cak, HEK8, JAK1, MET, Rak, and YES were confirmed by subcloning and sequencing of RT-PCR products.

Seven RT-PCR products, the digestion patterns of which did
not correspond to known kinases, were also identified; we hypothesized that these represented either novel kinases or RT-PCR products of nonspecific annealing of the primers. Fig. 3B demonstrates the expression patterns of three of the most abundant potential novel kinases. Two nonkinase products, 18S rRNA and mitochondrial cytochrome c oxidase subunit II, were also amplified, most likely because of primer sequence homology to them.

The TK profiles were compared using cDNAs generated from RT-PCR of cell lines using antisense degenerate Primer 1, an oligo dT primer, or random hexamers. The TK profiles were reproducible regardless of the method used to produce the cDNA. However, optimal results were obtained using the antisense degenerate primer, possibly because that method was the least vulnerable to RNA degradation.

We additionally studied the TK profiles of five normal breast specimens and seven invasive carcinomas (six ductal and one lobular) with TKDA. Overall, the TK profile of the breast tissues was remarkably similar to that of the breast cell lines. All TKs detected in breast cell lines were detected in breast tissue. An additional TK, Tie, was detected only in the tissue; this kinase may be endothelial in origin.

**RT-PCR Analysis of Specific Kinases.** TKDA revealed differential expression of Axl in breast cancer cell lines; therefore, we further evaluated Axl expression at the RNA and

![Table and Figure](image-url)
protein level. RT-PCR using Axl-specific primers detected Axl mRNA in normal MCF10A and MCF12A breast cell lines and in breast cancer cell lines BT549 and MDA-MB-231 (Fig. 4A). However, Northern blot analysis demonstrated that the mean expression level of Axl was higher in BT549 cells and 12-fold higher in MDA-MB-231 cells than in normal breast cells MCF10A and MCF12A, MDA-MB-435, and MDA-MB-453, yielding a 303-bp product (top panel). RT-PCR was performed with primers for GAPDH as a control (lower panel). B, expression of Axl protein. Cell extracts were obtained from normal breast cell line MCF10A and breast cancer cell lines MCF7, BT20, BT549, MDA-MB-231, MDA-MB-435, and MDA-MB-453. Western blot analysis was performed using anti-Axl antibodies (top panel) and anti-actin antibodies as a loading control (bottom panel). C, detection of Axl mRNA by Northern blot analysis. Ten μg of total RNA from MCF10A, MCF12A, BT549, and MDA231 cell lines were analyzed. Hybridization was carried out with an Axl probe (top panel) or a GAPDH probe (bottom panel).
expressed in breast cancer are proposed to play a role in breast cancer biology. For example, EGFR and HER2/neu are overexpressed in 35–50% and 20–30% of invasive breast cancers, respectively, and predict a poor prognosis (1, 12, 13). BRK, a TK cloned from a metastatic breast tumor (3), has been reported to be overexpressed in two thirds of breast tumors (14), the c-Met receptor has been found to be expressed at high levels in poorly differentiated breast cancer cells (5), and c-Src has also been reported to be overexpressed in breast cancer (4). In addition, McLeskey et al. (5) showed that several breast cancer cell lines express high levels of FGFR; 10% of primary breast tumor samples have been found to have amplification of the FGFR4 gene (15), and 9% amplification of FGFR1 (16). In contrast, c-kit has been found to be expressed significantly less in breast cancer than in normal breast tissue (7, 17). Furthermore, Syk has been reported to be lost in invasive breast cancer and is proposed to be a potential tumor suppressor in human breast carcinomas (6). Thus, several TKs are expressed in breast cancer, potentially exerting some opposing effects on cell growth and proliferation.

In the present study, we found that the receptor TK Axl was overexpressed at the mRNA and protein level in some breast cancer cell lines. Axl was initially identified as a protein encoded by a transforming gene from primary human myeloid leukemia cells (18). Growth arrest-specific gene 6, which is the ligand for Axl (19), mediates mitogenic activity in Axl-overexpressing cells (20). Overexpression of Axl has been reported in thyroid cancer (21) and hepatocellular cancer (22). The expression of Axl has also been found to be higher in a metastatic prostate cell line than in a less aggressive prostate carcinoma cell line or in normal prostate cells (23). Axl has also been reported to be expressed 10-fold more in colon cancer metastases than in other normal and malignant tissues (24). Our study suggests that Axl is overexpressed in a subgroup of breast cancers. We have reported recently that adenovirus type 5 E1A down-regulates the expression of Axl at a transcriptional level, and that down-regulation of Axl is involved in E1A-mediated growth suppression and E1A-induced apoptosis (25). Further study is needed to determine whether Axl will be useful as a therapeutic target in a subgroup of breast tumors.

Craven et al. (24) and Cance et al. (26) have previously attempted to systematically identify TKs expressed in breast cancer cell line 600PEI and in a primary tumor library by performing RT-PCR with a different set of degenerate primers and flanking a short RNA sequence (120 bp without primers). The advantages of TKDA are that it allows for simultaneous profiling of up to 40–50 TKs (8), as well as provide a potential for novel gene discovery. It may be less sensitive to RNA degradation than are other RNA-based assays, because the primers chosen for TKDA are close to the 3’ end of the RNA and flank a short RNA sequence (120 bp without primers). The disadvantages of TKDA are that it, similar to other PCR-based assays, has a contamination potential, and there is also a potential for nonspecific priming with degenerate primers, as we observed when they amplified with 18S rRNA and mitochondrial cytochrome c oxidase subunit II; however, >90% of clones obtained with degenerate primers are protein kinases (8). There is also a potential for misidentification of TKs with the assay, because identification is dependent on restriction digest pattern; thus, attention must be paid to ensure that all digestions actually work, and consideration must be given to performing confirmatory double digests if needed. Another limitation of the assay is that it is not quantitative: it is more useful for determining the presence or absence of TK expression than for determining relative expression levels. TKDA is fairly labor intensive and requires large amounts of RNA to produce reproducible results. Therefore, it is unlikely to be used routinely in clinical practice; however, it is a valuable research tool. Overall, the kinase profiles found were fairly reproducible in cell lines. However, the expression of four protein kinases (RET, Axl, GCK, and one potential novel kinase) were found to be more variable. The expression of these kinases may be sensitive to culture conditions, such as cell confluence, pH, and relative serum starvation, or may represent a variation in assay conditions.

In summary, we used a TKDA to profile the TK expression pattern in breast cancer. Several TKs were found to be differentially expressed in commonly used breast cancer cell lines. This heterogeneity should be taken into consideration when studying breast cancer biology. Tailoring TK inhibitors to the expression profile of each tumor may enhance their therapeutic efficacy.

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References


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