Activated Mitogen-activated Protein Kinase Expression during Human Breast Tumorigenesis and Breast Cancer Progression

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Abstract

Purpose: The purpose of this study is to address the hypothesis that activated mitogen-activated protein kinase (MAPK; extracellular signal-regulated kinases 1 and 2) has a role in breast tumorigenesis, breast cancer progression, and the development of tamoxifen resistance.

Experimental Design: H-score analysis and a specific antibody for the immunohistochemical detection of activated MAPK in formalin-fixed, paraffin-embedded tissue sections were used to compare expression in: (a) human breast tumors and their matched adjacent normal breast tissue; (b) primary human breast tumors and their matched lymph node metastases; and (c) primary breast tumors from patients who later proved to be sensitive or resistant to tamoxifen treatment.

Results: Active MAPK expression was detected in 48% of primary human breast tumors and was significantly increased in tumors compared with adjacent normal breast (Wilcoxon test, \(P = 0.027\)). A significant positive association \((\chi^2, P = 0.02; n = 55)\) was obtained between active MAPK and the presence of lymph node metastases. Moreover, increased active MAPK (Wilcoxon test, \(P = 0.0098\)) was found in concurrent lymph node metastases compared with primary breast tumors. No significant difference in active MAPK was found in primary tumors of patients who later responded to tamoxifen or did not respond to tamoxifen.

Conclusions: These data suggest that active MAPK is a marker of breast cancer metastasis and has a role in the metastatic process. However, active MAPK is unlikely to be a marker of endocrine sensitivity or involved in de novo tamoxifen resistance.

Introduction

Ligand-independent activation of ERs\(^3\) has been extensively documented in experimental models (1). Consequently, it has been speculated that such a mechanism could, in part, underlie estrogen-independent activation of ERs and, therefore, may be associated with altered ER activity that is thought to underlie the altered estrogen action that occurs during human breast tumorigenesis (2) and/or breast cancer progression, in particular, the development of antiestrogen resistance (1). Previously, we had developed an estrogen-independent (as defined by loss of growth responsiveness to estradiol) ER+ human breast cancer cell line (T5-PRF) by long-term growth in estrogen-depleted media (3). Among other changes (3, 4), these estrogen-independent cells contained a significant increase in activated MAPK (5), as well as an increased apparently ligand-independent activity of the endogenous ER (4). Recently, increased activated MAPK was found in another cell line model of apparently estrogen-independent proliferation (6). MAPK has been implicated in the ligand-independent activation of ERs because it can directly phosphorylate ERs on serine 118, leading to ligand-independent ER activation and the loss of tamoxifen inhibition of ER-mediated transcriptional activation (7). Furthermore, treatment of cells with EGF or IGF-I that activates the Ras/Raf/MAPK pathway also activates the ERs in a ligand-independent fashion, and this is accompanied by serine 118 phosphorylation of ERs (7). These data suggest the possibility that increased activated MAPK in estrogen target tissues in vivo could effect estrogen and antiestrogen responsiveness. Interestingly, an increased expression and activity of MAPK in human breast tumors compared with normal breast tissues were reported (8), although only one breast tumor case was matched to its own adjacent normal breast tissue, all others were independent samples. If confirmed, this observation suggests that increased MAPK during human breast tumorigenesis, especially in ER+ breast tumorigenesis, could also contribute to the altered estrogen action that occurs during this process (9, 10). However, the relationship of activated MAPK to steroid receptor status to other known prognostic variables in breast cancer and to breast cancer progression, in particular antiestrogen sensitivity and resistance, has not been documented. In this study, we have investigated the expression of activated MAPK in human breast tissues directly in vivo using immunohistochem-

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1 The abbreviations used are: ER, estrogen receptor; MAPK, mitogen-activated protein kinase; EGF, epidermal growth factor; IGF, insulin-like growth factor; PR, progesterone receptor; ERK, extracellular signal-regulated kinase; TBS, Tris-buffered saline; NEB, New England Biolabs.
istry and assessed the relationship of activated MAPK expression with known prognostic variables and progression in human breast cancer.

Materials and Methods

Human Breast Tissues. All breast samples used for this study were selected from the National Cancer Institute of Canada/Manitoba Breast Tumor Bank (Winnipeg, Manitoba, Canada). As was previously described (11), tissues are given to the Bank from cases at multiple centers within Manitoba, rapidly collected and processed to create matched formalin-fixed, paraffin-embedded and frozen tissue blocks for each case with the mirror image surfaces oriented by colored inks. The histology of every sample in the Bank is uniformly interpreted by a pathologist in H&E-stained sections from the face of the paraffin tissue block. This information is available in a computerized database along with relevant pathological and clinical information and was used as a guide for selection of specific paraffin and frozen blocks from cases for this study. For each case, interpretation included an estimate of the cellular composition (including the percentage of invasive epithelial tumor cells and stroma), tumor type, and tumor grade (Nottingham score). Steroid receptor status was determined for all primary tumor samples by a ligand-binding assay performed on an adjacent portion of tumor tissue. Tumors with ER levels ranging from 0.8 to 83 fmol/mg of total protein were considered ER+, and tumors with PR levels ranging from 2.7 to 323 fmol/mg protein. Seventeen tumors were ER+ and 4 were ER−. PR levels determined by ligand-binding assays ranged from 9.5 to 146 fmol/mg protein, and the PR levels ranged from 9.5 to 216 fmol/mg protein. One of these tumors were PR− and the rest were PR+. The ER levels for the tamoxifen-resistant cases ranged from 4.6 to 107 fmol/mg protein, and the PR levels ranged from 8.8 to 143 fmol/mg protein. One of these tumors was PR− and the rest were PR+. There were no significant differences between the two groups with respect to ER levels or grade scores, however, there was a significantly statistical difference between the groups with respect to PR levels. The tamoxifen-sensitive group had a significantly (P = 0.0064, Mann-Whitney test, two-tailed) higher median PR level (40.5 fmol/mg protein) than the tamoxifen-resistant group (14.8 fmol/mg protein).

Cohort 1. Twenty-six primary human breast tumor biopsies were selected. For each case, matched adjacent normal and tumor tissue blocks were available. The quality of each block and the relative cellular composition were determined by the histopathological assessment of sections from adjacent mirror image paraffin-embedded tissue blocks as described previously (11). The presence of normal ducts and lobules as well as the absence of any atypical lesion was confirmed in all normal tissue specimens. In three cases, the normal tissue sections did not contain normal glands and, therefore, were excluded from the normal versus tumor analysis, leaving 23 cases. The ER levels ranged from 0.8 to 83 fmol/mg protein. Five tumors were ER− and 18 were ER+. The PR levels ranged from 2.2 to 112 fmol/mg protein. Fifteen tumors were PR+ and 8 tumors were PR−. The tumors spanned a range of grades (grade scores 5–9) as determined by the Nottingham grading system.

Cohort 2. To identify cases that responded divergently to tamoxifen, a review of 1000 consecutive cases identified 490 cases that were ER+ and node negative. Among these cases, 196 were identified that had been treated with adjuvant tamoxifen after surgery ± local radiation. From these cases, a subset of 15 was selected that had shown progression of disease (either dead or alive with recurrent disease, referred to as tamoxifen-resistant cases). A similar control subset (n = 14) was specifically selected to comprise cases with similar lengths of follow-up (resistant, 34 months versus sensitive, 39 months), ER status, tumor grade, and tumor histology but that showed no progression of disease (referred to as tamoxifen-sensitive cases). The ER levels for the tamoxifen-sensitive cases ranged from 4.4 to 146 fmol/mg protein, and the PR levels ranged from 9.5 to 216 fmol/mg protein. One of these tumors were PR− and the rest were PR+. The ER levels for the tamoxifen-resistant cases ranged from 4.6 to 107 fmol/mg protein, and the PR levels ranged from 8.8 to 143 fmol/mg protein. One of these tumors was PR− and the rest were PR+. There were no significant differences between the two groups with respect to ER levels or grade scores, however, there was a significantly statistical difference between the groups with respect to PR levels. The tamoxifen-sensitive group had a significantly (P = 0.0064, Mann-Whitney test, two-tailed) higher median PR level (40.5 fmol/mg protein) than the tamoxifen-resistant group (14.8 fmol/mg protein).

Cohort 3. Sections from 21 primary human breast tumor samples and their matched lymph node metastases were selected. For the primary tumor samples, the ER levels, determined by ligand-binding assays, ranged from 0 to 298 fmol/mg protein. Seventeen tumors were ER+ and 4 were ER−. PR levels determined by ligand-binding assays ranged from 2.7 to 323 fmol/mg protein. Fourteen tumors were PR+ and 7 were PR−.

Antibodies. The following antibodies specific for dually phosphorylated (active) forms of the MAPK isoforms, ERK1 and ERK2 (p44/42), were used in this study: (a) phospho-p44/42 MAPK (Thr202/Tyr204) rabbit polyclonal antibody (9101S; New England Biolabs, Beverly, MA); (b) phospho-p44/42 MAPK (Thr202/Tyr204) E10 monoclonal antibody (9106L; New England Biolabs); and (c) antiactive MAPK rabbit polyclonal antibody (V8031; Promega, Madison, WI). The antibodies used for immunohistochemistry were validated by the following method. Estrogen-depleted MCF-7 breast cancer cells were treated for 3 h with 50 μM of the MAPK kinase 1 inhibitor, PD98059 (Calbiochem, La Jolla, CA), or vehicle (DMSO) alone. Half of the cells from each group was extracted and analyzed by Western blotting. The remainder was embedded in 3% agar, formalin fixed, paraffin embedded, and processed for immunohistochemistry (12). Western blot analysis (Fig. 1) showed a significant decrease in the M44,000/M42,000 ERK1/2 MAPK bands of the PD98059-treated cell extracts compared with the vehicle alone-treated cells using an antibody that recognized only the dually phosphorylated (active) MAPK isoforms ERK1 and ERK2. No change in total MAPK levels was seen when the blot was stripped and reprobed with an antibody recognizing total MAPK (ERK1(C-16) catalog no. sc93-G; Santa Cruz Biotechnology), supporting the conclusion that inhibition of the MAPK kinase 1 that activates ERK1 and ERK2 led to decreased detection of active MAPK with no effect on total MAPK levels, which were equivalent between the two treatment groups. Immunohistochemistry, using two different antibodies (polyclonal NEB 9101S and monoclonal NEB 9106L antibodies) to active MAPK, showed the presence of nuclear and some cytoplasmic staining in some but not all cells. The cell pellet sections were assessed by semiquantitative scoring using an H-score system, as described previously (13). Importantly, the intensity and the percentage of cell staining were significantly reduced in the PD98059-treated cells compared with the vehicle alone-treated cells (Fig. 2). The immunohistochemistry results were therefore consistent with the Western blot analysis. The polyclonal antibody (Fig. 2, B and E) gave a better signal immunohistochemically and was used on randomly selected human breast tumor sections. The results showed little if any
Western blot analysis of T-5 human breast cancer cells with and without PD98059 treatment. T-5 human breast cancer cells were exposed to 50 nm PD98059 for 3 h and proteins extracted (14). Proteins were resolved by 10% PAGE/SDS as described in the “Materials and Methods” section. A, expression of active MAPK visualized using antiactive MAPK rabbit polyclonal antibody (V8031; Promega) and chemiluminescence. B, blots from A were stripped and reprobed with goat anti-ERK1 (total MAPK; Santa Cruz Biotechnology) and visualized via chemiluminescence.

Fig. 1 Western blot analysis of T-5 human breast cancer cells with and without PD98059 treatment. T-5 human breast cancer cells were exposed to 50 nm PD98059 for 3 h and proteins extracted (14). Proteins were resolved by 10% PAGE/SDS as described in the “Materials and Methods” section. A, expression of active MAPK visualized using antiactive MAPK rabbit polyclonal antibody (V8031; Promega) and chemiluminescence. B, blots from A were stripped and reprobed with goat anti-ERK1 (total MAPK; Santa Cruz Biotechnology) and visualized via chemiluminescence.

activated MAPK expression is increased in breast tumors compared with normal breast tissue, we used a

then stripped (0.2 M glycine, 0.1% SDS, 1% Tween-20 (pH 2.2)) for 1 h at room temperature and blocked for 1 h at room temperature with 0.2% I-block in TBS. The membrane was then probed with 1/1000 dilution of goat anti-ERK1 (total MAPK; Santa Cruz Biotechnology) in 0.2% I-block overnight at 4°C, followed by incubation with 1/5000 donkey antigoat antibody conjugated to horseradish peroxidase for 1 h at room temperature. Detection was with the enhanced chemiluminescence detection system as described above.

Immunohistochemistry. In all cases, tissue samples had been fixed for 18–24 h in 10% buffered formalin before routine embedding in paraffin wax. Five-μm thick sections were cut, mounted on Superfrost/Plus slides (Fisherbrand), dried overnight at 37°C, dewaxed in xylene (4 min), and rehydrated in graded alcohol. DAKO EnVision System peroxidase (DAKO Diagnostics Canada, Inc.) was used for immunohistochemistry staining. After an initial pilot study using both the monoclonal (NEB 9106L) and polyclonal (NEB 9101S) antibodies, subsequent staining of human breast tissues was done using the polyclonal antibody to active MAPK. Blocking steps included peroxidase blocking reagent (0.03% hydrogen peroxide containing sodium azide) for 5 min to block endogenous peroxidase and Universal Blocker (DAKO Diagnostics Canada, Inc.) for 15 min to prevent nonspecific staining with antibody from both mouse and rabbit. Tissue sections were incubated overnight at 4°C with the primary antibody (1:250 dilution in antibody diluting buffer; DAKO Diagnostics Canada, Inc.) after an initial incubation with the same antibody at 37°C for 30 min. After the overnight incubation, slides were treated with labeled polymer (goat antirabbit and goat antimouse immunoglobulin in Tris-HCI buffer containing carrier protein and antimicrobial agent) for 30 min at room temperature. Finally, slides were incubated for 10 min with substrate 3-amino-9-ethylcarbazole. Each incubation step was followed by a 2-min TBS wash twice. The slides were counterstained with hematoxylin, immersed in a bath of ammonia water, rinsed in distilled water, and a coverslip applied using an aqueous mounting medium.

Levels of expression were scored semiquantitatively under the light microscope by assessing the average signal intensity (on a scale of 0–3) and the proportion of cells showing a positive signal (0, none; 0.1, less than one tenth; 0.5, less than one half; and 1.0 greater than one half). The intensity and proportion scores were then multiplied to give an H-score (13).

Statistical Analysis. Differences between normal samples and their matched tumors were tested using the Wilcoxon matched pairs test, two-tailed. Correlation between activated MAPK expression and tumor characteristics was tested by calculation of the Spearman coefficient r. A χ2 test was used to determine statistical significance of the association between active MAPK and nodal status. Fisher’s exact test was used to test for differences in frequency of detection of active MAPK between groups.

Results

Activated MAPK Expression Is Increased during Human Breast Tumorigenesis. To confirm and extend previous data suggesting that activated MAPK expression is increased in breast tumors compared with normal breast tissue, we used a

background staining in these tissue sections, and positive nuclear staining was seen in some of the epithelial tumor cells (Fig. 2. A and D). Therefore, this antibody was used for additional immunohistochemical analysis of activated MAPK in formalin-fixed, paraffin-embedded sections of human breast tissues.

Western Blot Analysis. Estrogen-depleted MCF-7 cells that had been treated or not treated with PD98059, as described above, were extracted using Buffer J [0.5 M Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, and 0.2 M DTT] containing 1 tablet/10 ml complete protease inhibitor mixture tablets (Roche, Mannheim, Germany) that were optimized to inhibit proteolysis and phosphatases, as described previously (14). Aliquots of the extracts were analyzed using 10% SDS-PAGE with a 4% stacking gel at 200V for 45 min at room temperature according to the Laemml method (15). Gels were transferred to nitrocellulose using 3-(cyclohexylamino)propanesulfonic acid transfer buffer [10 mM 3-(cyclohexylamino)propanesulfonic acid (pH 11.0) and 20% methanol] and transferred for 1 h at 120V at 4°C. Blots were blocked overnight at 4°C in 0.2% (w/v) I-block (Tropix, Foster City, CA) in TBS. Blots were incubated with rabbit antiactive-MAPK antibody (Promega, 1/1000 in 0.2% I-block in TBS containing 0.5% Tween-20) overnight at 4°C, followed by goat antirabbit antibody conjugated to horseradish peroxidase (Bio-Rad, 1/5000) for 1 h at room temperature. Detection was carried out using the enhanced chemiluminescence detection system according to the manufacturer’s instructions (Amer sham, Buckinghamshire, United Kingdom). The membrane was
polyclonal antibody recognizing only active MAPKs ERK1/ERK2 as described and validated above to compare active MAPK expression in 23 breast tumor samples with their matched adjacent normal breast tissues containing normal ductal epithelium. Interestingly, of the 23 samples studied, only 11 (48%) had detectable staining in the tumor epithelium. There did not appear to be any relationship of frequency of detection of activated MAPK expression and ER status, although in this cohort only 6 of the tumors were ER−. The tissue sections were subjected to semiquantitative H-score analysis using sections obtained from agar-embedded cell pellets of PD98059-treated and -untreated cell lines as controls (Fig. 2, B and E). An example of activated MAPK staining in a human breast tumor and its matched adjacent normal breast tissue is shown in Fig. 2, A and D, respectively. The data were analyzed by a Wilcoxon matched pairs statistical test. The expression of active MAPK seen in breast tumors (median for all 23 tumors, 0.1; median for the 12 tumors with detectable staining only, 0.6) was significantly increased \( P < 0.027, n = 23; P = 0.027, n = 12 \) compared with their adjacent matched normal breast tissue (median for all 23 normal tissues, 0.0; median for the 12 normal samples whose corresponding tumor had detectable staining, 0.0).

Table 1 Characteristics of the primary tumors of patients who relapsed (tamoxifen resistant) or remained disease free (tamoxifen sensitive) after tamoxifen adjuvant therapy

<table>
<thead>
<tr>
<th></th>
<th>ER (fmol/mg protein)</th>
<th>PR (fmol/mg protein)</th>
<th>Grade(^a)</th>
<th>Age (yr)</th>
<th>Follow-up (mo)</th>
</tr>
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<tbody>
<tr>
<td>Tamoxifen sensitive</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>30</td>
<td>40.5(^b)</td>
<td>6</td>
<td>72.5</td>
<td>30</td>
</tr>
<tr>
<td>Max(^c)</td>
<td>146</td>
<td>216</td>
<td>9</td>
<td>87</td>
<td>76</td>
</tr>
<tr>
<td>Min(^d)</td>
<td>4.4</td>
<td>9.5</td>
<td>4</td>
<td>47</td>
<td>13</td>
</tr>
<tr>
<td>Tamoxifen resistant</td>
<td>14.2</td>
<td>14.8(^b)</td>
<td>6</td>
<td>67</td>
<td>32</td>
</tr>
<tr>
<td>Median</td>
<td>107</td>
<td>143</td>
<td>9</td>
<td>89</td>
<td>63</td>
</tr>
<tr>
<td>Max</td>
<td>4.6</td>
<td>8.8</td>
<td>5</td>
<td>49</td>
<td>9</td>
</tr>
</tbody>
</table>

\(^a\) Nottingham score.

\(^b\) Significant difference, \( P = 0.0064 \) (Mann-Whitney rank sum test, two-sided), between the two subgroups for this parameter.

\(^c\) Maximum value observed in cohort.

\(^d\) Minimum value observed in cohort.


Activated MAPK Expression Is Not Altered between Breast Tumors That Are Tamoxifen Sensitive or Tamoxifen Resistant. Ligand-independent activation of ERs is thought to be, at least in part, a possible mechanism associated with tamoxifen resistance in ER+ breast tumors. To determine whether activated MAPK expression could be a predictor of tamoxifen sensitivity in primary breast cancers, tumors described under cohort 2 were examined immunohistochemically as described above for activated MAPK expression. The tumors were all ER+ and node negative and were the primary tumors obtained from patients who were later treated with tamoxifen (as described above) and remained disease free (tamoxifen-sensitive cases, \( n = 14 \)) or relapsed (tamoxifen-resistance cases, \( n = 15 \)). Tumor characteristics are detailed in Table 1. It should be noted that there were no statistically significant differences between the sensitive and resistant groups with respect to ER levels, tumor grade, age, or time of follow-up. However, a statistically
significant difference was observed between the level of PRs in the primary tumors of the sensitive and resistant cases (Mann-Whitney rank sum test, \( P = 0.0064 \)). Higher levels of PRs were observed in the primary tumors of tamoxifen-sensitive cases (median, 40.5; \( n = 14 \)) versus tamoxifen-resistant cases (median, 14.8; \( n = 15 \)).

Ten of 29 tumors had detectable active MAPK staining (7 of 14 sensitive and 3 of 15 resistant); using Fisher’s exact test, there was no statistically significant difference (\( P = 0.128 \)) between the two groups in terms of the frequency of detection of active MAPK. When active MAPK was quantified using H-score analysis, there was no statistically significant difference in active MAPK expression between the two groups (sensitive median, 0.05 versus resistant median, 0.0; Mann-Whitney rank sum test, \( P = 0.1 \)). It was concluded that activated MAPK as measured in primary breast tumors before beginning tamoxifen treatment was unlikely to be a marker of endocrine sensitivity.

**Activated MAPK Expression Is Associated with Lymph Node Metastases.** Correlation between activated MAPK expression and tumor characteristics was tested by calculation of the Spearman coefficient \( r \) using active MAPK H-scores obtained from all tumors in both cohorts 1 and 2. No statistically significant correlations were found with grade, cellular composition, ER level, or PR level. However, a significant positive association was obtained between active MAPK expression and the presence of lymph node metastases (\( \chi^2 \), \( P = 0.02 \)). This suggested that active MAPK may be a marker of metastases and could be associated with progression in human breast cancer.

To investigate this further, an additional cohort of 21 primary human breast tumor samples and their matched lymph node metastases (cohort 3) were provided by the National Cancer Institute of Canada/Manitoba Breast Tumor Bank. Nineteen of 21 primary tumors (90%) had detectable active MAPK expression, and 19 of 21 lymph node metastases (90%) had detectable active MAPK expression. This increased frequency of detection of active MAPK expression as compared with the original cohort 1 (48%), where there was a mixture of node positive and node negative primary breast tumors, is consistent with the statistically significant association of active MAPK expression and lymph node involvement identified in cohort 1. This difference in frequency of detection between the two cohorts is significant (\( P = 0.0034 \), Fisher’s exact test). Also, the pattern of active MAPK detection seemed to be conserved between each primary and its matched lymph node metastasis, i.e., if the primary tumors (19 of 21) had detectable active MAPK, so did its matched lymph node metastases (19 of 21); if the primary tumor did not have detectable active MAPK (2 of 21), then its matched lymph node metastasis was also negative (2 of 19).

**Activated MAPK Expression Is Increased in Lymph Node Metastases Compared with the Primary Breast Tumor.** When the level of active MAPK expression was semiquantified using H-score analysis as described in the “Materials and Methods” section, a statistically significant increase (Wilcoxon matched pairs test, \( P = 0.0098 \)) in active MAPK expression was found in the lymph node metastases (median score, 1.0; \( n = 21 \)) versus the primary breast tumor (median score, 0.2; \( n = 21 \)). Examples of increased expression of active MAPK in lymph node metastases compared with their matched primary breast tumor are shown in Fig. 3. These data suggest that not only is active MAPK expression a potential marker of metastasis but that it is also increased during breast cancer progression.
Discussion

There are several studies in vitro using breast cancer cell lines that suggest a role of activated MAPK in human breast cancer and possibly altered estrogen/antiestrogen responsiveness (5, 6, 16–18). The data presented here confirm and extend previous data that suggested increased expression of active MAPKs ERK1 and ERK2 occurs during human breast tumorigenesis in vivo (8). Using multiple samples of human breast tumors and their matched adjacent normal breast tissues together with immunohistochemical detection of dually phosphorylated (active) MAPK, our data provide unequivocal evidence that increased active MAPK expression occurs in ~50% of primary breast tumors compared with their adjacent normal breast tissues. Conclusions reached from previously published data were derived from comparisons between breast tumors and independent cases of normal breast tissue and benign breast lesions. In only one case was the sample matched from the same patient. The combined data suggest that increased expression of active MAPK frequently occurs during human breast tumorigenesis and, in part, may play a role in this process. The reasons for increased active MAPK expression are unknown, although it may, in part, be because of increased total expression of MAPK in addition to increased activity of growth factor receptor-induced cell proliferation pathways (19, 20).

In examining the relationship of active MAPK expression in primary human breast tumors with known prognostic variables, increased active MAPK expression was unrelated to tumor type, grade, or steroid receptor status. However, a positive association of active MAPK detection with the presence of lymph node metastases was observed. This suggests that active MAPK may be a marker of nodal metastases and poor prognosis. Furthermore, the level of active MAPK in lymph node metastases was significantly increased above that found in their matched primary tumors, suggesting that the increased active MAPK may have a mechanistic role in the metastatic progression of breast tumors. Receptor tyrosine kinase activation of signal transduction pathways often includes activation of the ERK 1/2 MAPKs. Several growth factor receptor pathways are known to be deregulated in breast tumors; in particular, the c-erbB2 receptor is amplified, and associated with a poor prognosis in ~30% of human breast cancers (21). Both EGF and IGF-I receptor pathways are often increased in human breast tumors, and increased EGF receptor activity is also associated with a poor prognosis (22). In contrast, increased IGF receptor levels are usually associated with a good prognosis (23). Alterations in the extracellular environment (e.g., extracellular matrix components), which occurs during tumorigenesis and metastasis, can also affect MAPK activity (19). Therefore, it is possible that the increased active MAPK seen in lymph node metastases may be due, in part, to increased and/or deregulated activity of such receptor tyrosine kinase pathways, altered extracellular environment, as well as an increased level of expression of total MAPK (8). Therefore, multiple mechanisms are likely to be responsible for the increased active MAPK seen during breast tumorigenesis and breast cancer progression. Irrespective of the mechanism(s) by which active MAPK is increased, our data suggest that it may be an excellent marker for predicting micrometastases and identifying a subgroup of node negative breast cancers with a poor prognosis. However, this requires additional investigation.

The data presented here suggest that active MAPK is increased in breast tumors compared with their matched adjacent normal breast tissues, suggesting that active MAPK is increased during breast tumorigenesis and may have a role in breast tumorigenesis. A more detailed study of preneoplastic breast lesions would be necessary to support this hypothesis and identify the stage at which this increase occurs. However, in ER+ breast tumorigenesis, such an increase in activated MAPK may have a role in the deregulated and altered action of estrogen thought to occur during this process (2). Although our data suggest that increased active MAPK expression was unrelated to steroid hormone receptor status in primary breast tumors, we have found a proportion (41%) of ER+ tumors that can contain increased active MAPK expression. Because active MAPK is able to directly phosphorylate ERα (7) and apparently induce ligand-independent activation of the receptor, the hypothesis has been suggested that this may underlie, in part, the development of tamoxifen resistance. To address this issue, we compared the expression of active MAPK in primary tumors of patients that later were classified as tamoxifen sensitive or resistant. These breast tumors were all ER+ and node negative. We found no significant differences in either the frequency of detection or the level of active MAPK expression between the primary tumors from patients that subsequently were shown to be tamoxifen sensitive and the primary tumors from patients that subsequently were shown to be tamoxifen resistant. These data suggest that active MAPK expression in ER+ primary human breast tumors is not a marker of endocrine responsiveness and is unlikely to be involved in de novo tamoxifen resistance. We cannot, however, exclude the possibility that altered active MAPK expression may be involved in acquired tamoxifen resistance. In this study, we have only measured active MAPK in the primary tumors of ER+ patients as yet unexposed to any form of adjuvant treatment. Previous data and our current data show that not all ER+ breast cancers will respond to tamoxifen treatment despite never having seen tamoxifen before. This type of resistance is referred to as de novo resistance. To study acquired tamoxifen resistance, it would be necessary in future studies to acquire biopsy material from breast cancer metastases that develop in patients whose original breast cancer responded to tamoxifen and then disease recurrence occurred during tamoxifen treatment and/or use xenograft mouse model systems of acquired tamoxifen-resistant human breast cancer cells (16).

In summary, we have investigated the expression of the active MAPKs ERK1 and ERK2 during human breast tumorigenesis and breast cancer progression. Significantly increased active MAPK was found in primary breast tumors compared with their adjacent matched normal breast tissues, was correlated with nodal metastasis when detected in primary breast tumors, and was significantly increased in lymph node metastases compared with their matched primary breast tumors. These data suggest that not only is active MAPK a marker of progression in human breast cancer but may also have a role in both breast cancer progression as well as breast tumorigenesis.
References

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