**Abstract**

**Purpose:** Cell signaling pathways include a complex myriad of interconnected factors from the membrane to the nucleus, such as erbB family receptors and the phosphoinositide-3-kinase/Akt/mTOR and Ras-Raf-ERK cascades, which drive proliferative signals, promote survival, and regulate protein synthesis.

**Experimental Design:** To find pivotal factors in these pathways, which provide prognostic information in malignancies, we studied 103 human breast tumors with an immunohistochemical profile, including total and phosphorylated (p) proteins: human epidermal growth factor receptor 2 (HER2), epidermal growth factor receptor, extracellular signal-regulated kinase 1/2, Akt, 4E-binding protein 1 (4EBP1), eukaryotic initiation factor 4E, phosphorylated ribosomal protein S6 kinase 1, phosphorylated ribosomal protein S6, and Ki67. Western blot and reverse lyase protein arrays were also done in a subset of tumors.

**Results:** Significantly, activation of the phosphoinositide-3-kinase/Akt/mTOR cascade was detected in a high proportion of tumors (41.9%). Tumors with HER2 overexpression showed higher p-Akt as compared with negative tumors ($P < 0.001$). Levels of p-Akt correlated with the downstream molecules, p-4EBP1 ($P = 0.001$) and p-p70S6K ($P = 0.05$). Although 81.5% of tumors expressed p-4EBP1, in 16.3% of these tumors, concomitant activation of the upstream factors was not detected. Interestingly, p-4EBP1 was mainly expressed in poorly differentiated tumors ($P < 0.001$) and correlated with tumor size ($P < 0.001$), presence of lymph node metastasis ($P = 0.002$), and locoregional recurrences ($P = 0.002$). Coexpression of p-4EBP1 and p-eIF4G correlated with a high tumor proliferation rate ($P = 0.012$).

**Conclusion:** In this study, p-4EBP1 was the main factor in signaling pathways that associate with prognosis and grade of malignancy in breast tumors. Moreover, p-4EBP1 was detected in both HER2-positive and HER2-negative tumors. This factor seems to be a channelling point at which different upstream oncogenic alterations converge and transmit their proliferative signal, modulating protein translation.

**Human tumors are characterized by their great heterogeneity and histopathologic variability. Currently, >250 malignant tumors and thousands of subtypes and histologic variants have been described. Nevertheless, the classic pathologic criteria, such as tumor size, grade of malignancy, and metastatic dissemination, are generally the most relevant prognostic factors in cancer. In addition to the variability of histopathologic subtypes, molecular study of tumors is even more complex. In all malignant tumors, at least six genetic alterations should affect the main mechanisms of cellular transformation, including growth factor and cell signaling pathways, the cell cycle, apoptosis, and mechanisms implicated in cellular invasiveness, and angiogenesis (1–3). Overall, >350 genes associated with cancer have been identified, representing >1% of the human genome (4). Studies using cDNA expression arrays have detected hundreds of genetic alterations whose true clinical and pathologic significance remains to be described and validated. It is relevant that except in lymphoma, leukemia, small round cell tumors in children, and a large group of sarcomas, which are known to have translocations, the genetic alterations thought to be involved in malignant transformation show considerable redundancy in most carcinomas (4). In breast cancer, one of the main genetic alterations is the amplification of the oncogene human epidermal growth factor receptor 2 (HER2)/neu and the overexpression of its product. Nevertheless, this gene is amplified in only 25% of breast tumors (5–7), a fact suggesting that other factors in these pathways must be activated to maintain the oncogenic signal.**

Epidermal growth factor receptor (EGFR) and HER2/neu, as well as the downstream activation factors extracellular...
HER2/neu amplification or various mutations in EGFR, PTEN, one gene or epigenetic alteration can be present, including inactivated to promote oncogenic proliferation, and more than the cell signaling routes, several factors can be activated or transformation, and conversely, when it is inactivated, a mental studies have shown that 4EBP1 has a central role in cell eIF4E and activation of cell protein synthesis. Recent experiments studies have shown that 4EBP1 is activated in a high percentage of breast tumors, is associated with higher malignant grade, tumor size, and local recurrence regardless of HER2/neu status, and could be proposed as a hallmark in cell signaling.

Materials and Methods

**Tumor cell lines and Western blot assays.** For the current study, we selected the following human cell lines containing different levels of EGFR and HER2/neu from the American Type Culture Collection (Manassas, VA): vulvar squamous carcinoma (A431; ref. 15), colon carcinoma (DiFi; ref. 16), and breast carcinoma (SK-BR-3, MDA-MB-468, T-47D, and BT-474; refs. 17, 18). Cancer cell lines were maintained as monolayers at 37°C and 5% CO2/air in DMEM/Ham’s F-12 supplemented with 10% fetal bovine serum and glutamine (2 mmol/L); in the case of BT-474, insulin at 0.01 mmol/L was added.

Western blot assays were done as previously reported (19, 20) with minor modifications. In brief, tumor cells were grown in 100 mm dishes to subconfluence and exposed to rapamycin analogue mTOR inhibitor for 2 h at doses superior to the IC50 for these cell lines (10 and 100 nmol/L). After removal of the medium, cells were washed twice with ice-cold PBS and scraped into ice-cold lysis buffer [50 mmol/L HEPES (pH 7.0), 10% glycerol, 0.5 mmol/L p-nitrophenylphosphate, 0.5 mmol/L orthovanadate, and 1 mmol/L phenylmethylsulfonyl fluoride]. After removal of cell debris by centrifugation, protein concentration was determined by the Lowry assay (DC Protein Assay; Bio-Rad, Hercules, CA). Lysate samples containing equal amounts of protein were then added to SDS-PAGE loading buffer with 5% phosphoinositide-3-kinase, or Ras, illustrating the redundancy of oncogenic events in tumor cells. This redundancy and the oncogenic alterations in other cellular pathways could explain the low reported response rates to specific biological therapies in cancer (13, 14).

The central aim of the present study is to identify a simplified molecular signature in cell signaling in breast tumors on the basis that a channeling downstream factor or “wild card” might orchestrate the transduction of oncogenic signals to the ribosomes or nucleus through a kind of “bottleneck,” regardless of the individual upstream oncogenic alterations. To investigate this hypothesis, activation of the membrane receptors, EGFR and HER2/neu, two major biochemical pathways, Akt and ERK1/2, and the downstream factors, 4EBP1, p70S6K1, and S6, were studied by immunohistochemistry using phosphorylation-specific antibodies. Expression of these factors was measured in a breast malignant tumor series and correlated with pathologic grade, patient survival, and tumor recurrence to determine which, if any, of the factors fulfilled the concept of a channeling factor or an oncogenic wild card.

This study showed that 4EBP1 is activated in a high percentage of breast tumors, is associated with higher malignant grade, tumor size, and local recurrence regardless of HER2/neu status, and could be proposed as a hallmark in cell signaling.

**Table 1. Characteristics of the breast tumors**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infiltrating carcinomas</td>
<td>103</td>
</tr>
<tr>
<td>Histologic type</td>
<td></td>
</tr>
<tr>
<td>Ductal</td>
<td>97 (94.2)</td>
</tr>
<tr>
<td>Lobular</td>
<td>6 (5.8)</td>
</tr>
<tr>
<td>Histologic grade</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>3 (2.9)</td>
</tr>
<tr>
<td>2</td>
<td>56 (54.4)</td>
</tr>
<tr>
<td>3</td>
<td>44 (42.7)</td>
</tr>
<tr>
<td>pT</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>33 (32.0)</td>
</tr>
<tr>
<td>2</td>
<td>64 (62.2)</td>
</tr>
<tr>
<td>3</td>
<td>6 (5.8)</td>
</tr>
<tr>
<td>pN</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>50 (48.5)</td>
</tr>
<tr>
<td>1</td>
<td>37 (35.9)</td>
</tr>
<tr>
<td>2</td>
<td>6 (5.8)</td>
</tr>
<tr>
<td>3</td>
<td>10 (9.8)</td>
</tr>
<tr>
<td>Hormone receptors</td>
<td></td>
</tr>
<tr>
<td>ER/PR+</td>
<td>75 (72.8)</td>
</tr>
<tr>
<td>HER2/neu</td>
<td></td>
</tr>
<tr>
<td>Overexpression</td>
<td>23 (22.3)</td>
</tr>
<tr>
<td>Amplification</td>
<td>21 (20.4)</td>
</tr>
<tr>
<td>Ki67 (MIB1)</td>
<td></td>
</tr>
<tr>
<td>&gt;20%</td>
<td>29 (28.2)</td>
</tr>
<tr>
<td>≤20%</td>
<td>74 (71.8)</td>
</tr>
</tbody>
</table>

**Fig. 1.** Dose-dependent effects of mTOR inhibition on phosphorylated initiation factor 4EBP1, p70S6K1, and S6 in a panel of cell lines including breast cancer cells (BT-474, SK-BR-3, MDA-468, and T-47D), colon cancer cells (DiFi), and squamous cancer cells (A431). Expression was detected by Western blot.
β-mercaptoethanol and heated for 5 min at 100°C. Electrophoretic transfer to nitrocellulose membranes was followed by immunoblotting with the primary antibodies, as previously described. Finally, membranes were hybridized with the appropriate horseradish peroxidase–conjugated secondary antibody (Amersham Pharmacia Biotech, Little Chalfont, United Kingdom) and detected by chemiluminescence with the SuperSignal West Dura Extended Duration Substrate (Pierce, Rockford, IL).

**Patients.** A total of 103 malignant infiltrating breast tumors (97 ductal and 6 lobular carcinomas) were randomly obtained from the Pathology Department of Vall d’Hebron University Hospital (Barcelona, Spain) during the period from 1998 to 2002. Patient ages ranged from 33 to 75 years, with a mean age of 53. Tumor size, histologic grade, and the number of nodes showing metastasis were determined (Table 1). Histologic grades 1, 2, and 3 were defined according to the criteria of Scarff-Bloom-Richardson, modified by Elston (22). The tumor-node-metastasis stage was defined according to WHO criteria. Hormonal status and the proliferation marker Ki67 were determined by immunohistochemistry. All the patients studied were included in clinical protocols and follow-up was obtained in all cases. The Institutional Review Board Committee on Human Research of the Vall d’Hebron University Hospital approved the collection of tissues and the procedures conducted on these tissues.

**Reagents.** The following primary antibodies were assayed, as described previously (13, 19, 23): mouse monoclonal anti-EGFR clone K1494 (Dako, Carpinteria, CA), mouse monoclonal anti-p-EGFR clone 74 (Chemicon, Temecula, CA), rabbit anti-HER2 Herceptest (Dako), rabbit polyclonal anti-p-HER2 Tyr1248, rabbit polyclonal anti-ERK1/2, rabbit polyclonal anti-Thr202/Tyr204 p-ERK1/2, rabbit polyclonal anti-Akt, rabbit polyclonal anti-Ser473 p-Akt, rabbit polyclonal anti-4EBP1, rabbit polyclonal anti-Thr389 p-4EBP1, rabbit polyclonal anti-Thr70 p-4EBP1, rabbit polyclonal anti-Ser240/244 p-S6, from Cell Signaling in Breast Tumors.
Heat-induced antigen retrieval in a water bath was done using 0.01 mol/L of citrate buffer at pH 6, except for p-EGFR, which required pH 10 buffer, and EGFR, which used proteinase K. The appropriate EnVision detection system (Dako) was used for each antibody. p-EGFR and p-HER2 were detected with the Catalytic System of Amplification from Dako, following the manufacturer’s guidelines.

**Immunohistochemistry evaluation.** To score a tumor cell positive, membrane staining was required for EGFR, membrane or cytoplasmic staining for p-EGFR, strong and complete membrane staining for HER2/neu, membrane staining for p-HER2/neu, nuclear staining for ERK1/2, p-ERK1/2, estrogen receptor, progesterone receptor, and Ki67, and cytoplasmic and nuclear staining for p-Akt. Samples were assessed in a blinded fashion by two investigators (F. Rojo and L. Najera). For the quantitative analysis, a Histo score (Hscore) based on the percentage of stained cells and intensity of staining was calculated evaluating 10 high-power representative fields (>400) on complete tumor sections using optical microscopy (24). The intensity score was defined as follows: 0, no appreciable staining in cells; 1, weak intensity cells, comparable to stromal cells; 2, intermediate intensity of staining; and 3, strong intensity of staining. The score was based on the fraction of positive cells (0-100%) and the Hscore was calculated by multiplying the intensity score and the fraction score, producing a total range of 0 to 300. A tumor was considered positive when at least 1% of stained cells were visualized. This scoring was used for the statistical analysis.

**Fluorescent in situ hybridization for HER2/neu.** Fluorescent in situ hybridization was done according to the PathVysion (Vysis Inc., Downers Grove, IL) guidelines, described in the package insert as approved by the U.S. Food and Drug Administration. In brief, the PathVysion guidelines involve the rehydration of a paraffin-embedded 5-μm-thick section. The section was air-dried, pretreated, and digested with proteinase K before being hybridized with fluorescent-labeled probes for the HER2 gene, and α-satellite DNA for chromosome 17. Nuclei were routinely counterstained with an intercalating fluorescent counterstain, 4,6-diamidino-2-phenylindole. For each tumor, 60 tumor cell nuclei from invasive areas were identified using a Nikon Eclipse E400 fluorescence microscope with a Rhodamine and FITC double filter, and scored for both HER2 and chromosome 17 centromere numbers. HER2 gene amplification was defined as a HER2-to-chromosome 17 ratio of ≥2.0, as required by the guidelines.

**Western blot assays.** From the same patients, a subset of frozen breast tumor samples was analyzed by Western blot, as described previously (25, 26). Several controls and extraction procedures were used to ensure that a degradation artifact was not generated (26).

**Reverse lysate protein arrays.** The previously described tissue processing and lysate preparation (27–29) were done with minor variations. Tissue lysates were loaded onto 96-well plates in serial dilutions (near, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, and 1:128). Dilution series of samples and a reference standard were printed in duplicate onto nitrocellulose-coated glass slides (Schleicher & Schuell Bioniscience, Keene, NH) using a ring-and-pin robotic arrayer (GeneTAC G3 Library Management System; Genomic Solutions, Ann Arbor, MI). The reference standard was prepared from EGF-stimulated MDA-468 and MDA-435 cells. Microarrays were stained as previously described (29) using a biotinyl-linked catalyzed signal amplification system (Dako). Stained arrays were scanned on an HP scanner. Mean pixel intensities

**Table 2. Expression levels of markers in 103 breast tumors**

<table>
<thead>
<tr>
<th></th>
<th>Total levels, N (%)</th>
<th>Hscore [cutoff, 25], N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGFR</td>
<td>35 (33.8)</td>
<td>18 (17.4)</td>
</tr>
<tr>
<td>p-EGFR</td>
<td>18 (17.4)</td>
<td>18 (17.4)</td>
</tr>
<tr>
<td>HER2</td>
<td>23 (22.3)</td>
<td>10 (10.7)</td>
</tr>
<tr>
<td>p-HER2</td>
<td>11 (10.7)</td>
<td>11 (10.7)</td>
</tr>
<tr>
<td>p-ERK1/2</td>
<td>90 (87.3)</td>
<td>48 (46.6)</td>
</tr>
<tr>
<td>p-Akt</td>
<td>61 (59.2)</td>
<td>48 (46.6)</td>
</tr>
<tr>
<td>p-4EBP1</td>
<td>90 (87.3)</td>
<td>61 (59.2)</td>
</tr>
<tr>
<td>pp70S6K1</td>
<td>80 (77.7)</td>
<td>39 (37.8)</td>
</tr>
<tr>
<td>p-S6</td>
<td>80 (77.7)</td>
<td>34 (32.9)</td>
</tr>
</tbody>
</table>

**NOTE:** Total expression was described for all markers. The Hscore cutoff of 25 was considered overexpression, based on median expression in nontumoral breast epithelium.

**Fig. 3.** Expression of biomarkers in various breast tumors. A, strong, diffuse HER2/neu overexpression in an infiltrating ductal breast tumor. B, EGFR expression in low-grade carcinoma with membranous and cytoplasmic staining patterns. Some stromal cells express moderate levels of EGFR. C, nuclear and cytoplasmic staining for p-ERK1/2 in a breast carcinoma. Only nuclear expression due to activated translocation of protein was considered p-ERK1/2 expression. Proteins were detected in stromal elements. D, p-Akt was present in the cytoplasm and nuclei of tumor cells, with a heterogeneous distribution within the tumor. E, p-p70S6K1 was observed in tumor cells with strong nuclear and weak cytoplasmic expression. F, cytoplasmic expression of p-S6 protein. Staining is focal in distribution.
were calculated with background correction using MicroVigene software (v. 2.006, VigeneTech, Inc., North Billerica, MA). For each sample, the slope of the regression line best fitting the linear range of the dilution curve was used to determine the relative protein expression (30). Data were normalized to glyceraldehyde-3-phosphate dehydrogenase as reference standard.

**Statistical analysis.** Statistical analyses were carried out using the SPSS Data Analysis Program, version 10.0 (SPSS, Inc., Chicago, IL). Spearman’s nonparametric correlation test was used to calculate the statistical significance of continuous variables, and the Mann-Whitney U test was used to compare group means. All statistical tests were conducted at a two-sided significance level of 0.05.

**Results**

**Validation of reagents.** To validate antibody specificity, expression of the proteins studied was determined on a panel of cell lines treated with specific erbB receptors and mTOR inhibitors. To show the specificity of p-p70S6K1, p-4EBP1, and p-S6 antibodies, cell lines were treated with a rapamycin-analogue mTOR inhibitor and protein levels were detected by Western blot (Fig. 1). Complete inhibition of p-S6 protein was observed at both doses of mTOR inhibitor in all cell lines. In contrast, p-4EBP1 and p-p70S6K1 levels showed a dose-dependent reduction, as reported by other groups (21). The specificity of the antibodies against membrane receptors, total and phosphorylated ERK1/2, and p-Akt was previously shown (19, 24).

To validate the antibodies in paraffin-embedded tissues, protein levels were compared by Western blot in fresh-frozen tissue samples and in formalin-fixed tissue from the same specimens by immunohistochemistry. Strong significant correlations between the Hscore and relative expression levels by Western blot were shown for EGFR, p-EGFR, HER2/neu, p-HER2/neu, p-ERK1/2, p-Akt, 4EBP1, p-4EBP1, and p-p70S6K1 (Spearman’s correlation test, \( P < 0.05 \)), but not for p-S6 (Fig. 2). This absence of a correlation might be explained by the low and heterogeneous p-S6 expression in tumors.

Finally, to test the Hscore as a reproducible and reliable quantification method for immunohistochemistry, a reverse tissue lysate array approach was done on frozen tissue samples, and results were compared with the Hscore from the same paraffin-embedded specimens. Statistically significant correlations between the Hscore and reverse lysate array results were found for EGFR, p-EGFR, HER2/neu, p-HER2/neu, p-ERK1/2, and p-Akt (Spearman’s correlation test, \( P < 0.05 \); Fig. 3).

**Protein expression profile in breast tumors.** HER2/neu overexpression was detected in 22.3% of breast tumors and gene amplification was confirmed by fluorescent in situ hybridization in 20.4% of cases. EGFR was observed in 33.8% of tumors. Among the signaling factors studied, p-ERK1/2 was detected to some degree in 87.3%, p-Akt in 59.2%, p-4EBP1 in 87.3%, p-p70S6K1 in 77.7%, and p-S6 in 77.7%. Overexpression for these signaling factors was defined by a cutoff of 25 on the Hscore, considering this value as the median of expression in

<table>
<thead>
<tr>
<th></th>
<th>EGFR</th>
<th>HER2</th>
<th>p-ERK1/2</th>
<th>p-Akt</th>
<th>p-4EBP1</th>
<th>p-elF4G</th>
<th>p-p70S6K1</th>
<th>p-S6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor size</td>
<td>0.292</td>
<td>0.078</td>
<td>0.623</td>
<td>0.621</td>
<td>&lt;0.001*</td>
<td>0.057</td>
<td>0.493</td>
<td>0.898</td>
</tr>
<tr>
<td>Histologic grade</td>
<td>0.051</td>
<td>0.557</td>
<td>0.098</td>
<td>0.502</td>
<td>&lt;0.001*</td>
<td>0.039*</td>
<td>0.69</td>
<td>0.649</td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td>0.844</td>
<td>0.026*</td>
<td>0.119</td>
<td>0.015*</td>
<td>0.02*</td>
<td>0.096</td>
<td>0.016*</td>
<td>0.69</td>
</tr>
<tr>
<td>Locoregional recurrence</td>
<td>0.191</td>
<td>0.035*</td>
<td>0.236</td>
<td>0.01*</td>
<td>0.002*</td>
<td>0.114</td>
<td>0.347</td>
<td>0.402</td>
</tr>
</tbody>
</table>

**Table 3.** Statistical significance for biomarkers related to tumor size, histologic grade, lymph node status, and recurrence

*Statistically significant.

To validate the antibodies in paraffin-embedded tissues, protein levels were compared by Western blot in fresh-frozen tissue samples and in formalin-fixed tissue from the same specimens by immunohistochemistry. Strong significant correlations between the Hscore and relative expression levels by Western blot were shown for EGFR, p-EGFR, HER2/neu, p-HER2/neu, p-ERK1/2, p-Akt, 4EBP1, p-4EBP1, and p-p70S6K1 (Spearman’s correlation test, \( P < 0.05 \)), but not for p-S6 (Fig. 2). This absence of a correlation might be explained by the low and heterogeneous p-S6 expression in tumors.

Finally, to test the Hscore as a reproducible and reliable quantification method for immunohistochemistry, a reverse tissue lysate array approach was done on frozen tissue samples, and results were compared with the Hscore from the same paraffin-embedded specimens. Statistically significant correlations between the Hscore and reverse lysate array results were found for EGFR, p-EGFR, HER2/neu, p-HER2/neu, p-ERK1/2, and p-Akt (Spearman’s correlation test, \( P < 0.05 \); Fig. 3).

**Protein expression profile in breast tumors.** HER2/neu overexpression was detected in 22.3% of breast tumors and gene amplification was confirmed by fluorescent in situ hybridization in 20.4% of cases. EGFR was observed in 33.8% of tumors. Among the signaling factors studied, p-ERK1/2 was detected to some degree in 87.3%, p-Akt in 59.2%, p-4EBP1 in 87.3%, p-p70S6K1 in 77.7%, and p-S6 in 77.7%. Overexpression for these signaling factors was defined by a cutoff of 25 on the Hscore, considering this value as the median of expression in...
adjacent normal breast epithelium. Overexpression was detected in <40% of cases for all these markers with the exception of p-4EBP1, which was observed in 59.2% of breast tumors (Table 2; Fig. 4).

**Correlations among the signaling factors.** Tumors with HER2/neu overexpression showed higher expression levels of p-Akt (Mann-Whitney U test, *P* < 0.001), and a significant association was found between EGFR expression and p-ERK1/2 (Spearman’s correlation test, *P* < 0.002). Similarly, there was a correlation between p-Akt expression and both p-4EBP1 and p-p70S6K1 (Spearman’s correlation test, *P* = 0.001 and *P* = 0.05, respectively). Additionally, p-4EBP1 expression levels significantly correlated with p-p70S6K1 and p-eIF4G (Spearman’s correlation test, *P* = 0.002 and *P* = 0.010, respectively) but not with p-S6 (Spearman’s correlation test, *P* = 0.421). Nevertheless, an important subset of these breast tumors (16.39% of cases) had high p-4EBP1 levels, but nonrelevent upstream marker expression, including erbB receptor overexpression and signaling factors, suggesting that 4EBP1 can be phosphorylated by various cellular pathways. Exploring individual factors, HER2 and EGFR were undetectable in 65.57%, p-ERK1/2 in 45.9% and p-Akt in 44.26% of tumors with p-4EBP1 overexpression. This finding was supported by a significant correlation between proliferation marker Ki67 and p-4EBP1 (Spearman’s correlation test, *P* = 0.012). Expression of the total forms was compared with the phosphorylated levels of each protein and significant correlations were observed for EGFR and HER2 (Spearman’s correlation test, *P* < 0.001). In contrast, no correlations were observed for total and phosphorylated protein levels of ERK, Akt, and 4EBP1.

**Correlations between expression profile and clinical and pathologic variables.** Expression levels were correlated with tumor size, histologic grade, presence of lymph node metastasis, and evidence of local recurrence 5 years after diagnosis. Among the markers examined, p-4EBP1 showed the strongest correlations with tumor size (Mann-Whitney U test, *P* < 0.001), histologic grade (*P* < 0.001), and presence of positive lymph nodes (*P* = 0.02; Table 3). Moreover, patients with locoregional disease at 5 years from diagnosis showed higher p-4EBP1 as compared with patients with no recurrence (Mann-Whitney U test, *P* = 0.002; Fig. 5).

**Discussion**

The findings obtained from this study on cell signaling pathways in a series of breast tumors, including growth factor receptors and the ERK1/2 and Akt cascades, suggest that p-4EBP1 is a main factor of these biochemical pathways associated with histologic grade, lymph node metastases, and locoregional recurrences. p-4EBP1 was detected in breast tumors showing HER2/neu overexpression or EGFR expression as well as in a relevant number of tumors without these oncogenic alterations. Phosphorylation of 4EBP1 can be the consequence of several oncogenic events along these biochemical routes, including amplification or mutation of growth factor receptors, loss of function, or mutations in PTEN, phosphoinositide-3-kinase mutations, Ras mutations, and other collateral mechanisms of oncogenic cellular activation (7, 31). These might include phospholipase D activation (32), which could activate the mTOR cascade, the ATM-p53 pathway (33), and possibly other, as yet unknown, kinases or phosphatases. In this scenario of multiple genetic alterations that can trigger the oncogenic pathways, we propose that the phosphorylated form of 4EBP1 might act as a bottleneck or wild card factor through which many upstream oncogenic signals converge, regulating protein synthesis machinery, cell cycle progression, and cell proliferation (Fig. 6). Hence, analysis of p-4EBP1 could provide reliable molecular information on the oncogenic potential mediated by this pathway. Importantly, the results obtained by immunohistochemistry were validated by Western blotting and reverse lysate protein arrays using the same antibodies, underlining the fact that this factor can be studied in paraffin sections.

The results obtained in breast cancer are comparable to those observed in ovarian tumors, in which p-4EBP1 expression was also associated with high histologic grade and poor survival (34). Interestingly, we did not find similar significant correlations with p70S6K1, which was also activated by mTOR. The differences between p-4EBP1 and pp70SK1 expression observed in breast tumors may be an indication that p-4EBP1 could be phosphorylated by other pathways (35) or that mTOR phosphorylates 4EBP1 more actively in breast cancer. It is well known that mTOR-dependent phosphorylation of p70S6K1 is not linearly associated with 4EBP1 phosphorylation (36, 37), and this feature could explain the lack of prognostic value of p-p70S6K1 observed in our study. In our series, p-p70S6K1 was detected by immunohistochemistry in the infiltrating margins of the tumors and not throughout the tumors, as was p-4EBP1. Other reports (38) have shown a

![Fig. 5. Box graphs comparing p-4EBP1 expression for the presence of lymph node involvement (Mann-Whitney U test, *P* = 0.02) and locoregional recurrence (Mann-Whitney U test, *P* = 0.002).](image-url)
prognostic role for p70S6K1 overexpression and amplification only in a subgroup of node-negative premenopausal breast cancer patients with an increased risk of locoregional disease, but this population was not fully represented in our study. In any case, further clinical studies and in vitro analyses are needed to clarify the dissociation between p70S6K and p-4EBP1 in breast tumors.

The results of this study support previous data (6), indicating that 4EBP1 expression can predict poor prognosis in breast tumors, particularly those with HER2/neu overexpression or amplification. In the present study, we found a significant correlation between high p-4EBP1 expression and poor prognosis, not only in tumors showing HER2/neu overexpression or amplification, but also in a group of high-grade breast tumors in which HER2/neu amplification/overexpression was not detected. These findings suggest that p-4EBP1 overexpression in breast cancer indicates poor prognosis regardless of whether there is concomitant HER2/neu overexpression or amplification. In fact, in our series, p-4EBP1 was overexpressed in 80% of breast tumors with negative HER2/neu expression, and >75% of tumors without EGFR expression, and even 16% of tumors were negative for all the upstream marker studies. Activation of the Akt pathway and 4EBP1 may be associated with the response to chemotherapy and radiotherapy because of the activation of cell survival signals (39), and might be a predictive factor of clinical response to specific oncogenic inhibitors. In fact, phospho-4EBP1 was detected in the tumors of patients treated with EGFR inhibitors that showed down-regulation of EGFR protein but no clinical response, indicating that p-4EBP1 was activated by other pathways (13).

We found that total 4EBP1 expression was increased in high-grade tumors as compared with low-grade tumors, and that nearly all the 4EBP1 proteins were phosphorylated. Conversely, in low-grade tumors, there was a clear difference between total 4EBP1 and the phosphorylated protein. 4EBP1 phosphorylation was also regulated at the transcriptional level, whereas 4EBP1 has been reported to be inhibited by p38-MAPK (40) and up-regulated by the forkhead transcriptional factor FOXO (41) as well as the Ras-Raf-ERK signaling pathway (42). Functional 4EBP1 status, i.e., phosphorylated 4EBP1, is essential for eIF4E release. 4EBP1 has eight described phosphorylation sites susceptible to phosphorylation by kinases such as mTOR, phosphoinositide-3-kinase, and ERK1/2. Moreover, mTOR can be activated by serine/threonine kinases and other kinases that can phosphorylate 4EBP1 at Thr 70, the same residue that is recognized by the antibody used in the present study.
the margins of head and neck carcinoma surgical specimens has been related with local recurrence (46). eIF4E and complexes with other members of the family are essential for ribosomal synthesis (47). Moreover, eIF4E can block the exit of cytochrome c from the mitochondria, blocking apoptosis (48). The interrelationship between 4EBP1 and eIF4E is essential for eIF4E control of protein synthesis and translation of key proteins in tumor transformation, such as cyclin D1, Myc, fibroblast growth factor, and vascular endothelial growth factor (49). In fact, in vitro studies have shown that eIF4E is crucial for maintaining malignant breast cell lines (12, 49). Thus, it is clear that an increase in free eIF4E can be critical for the control of apoptosis, protein synthesis, and indirectly, cell proliferation (50). Nevertheless, no significant correlation was found between total eIF4E or p-eIF4G and locoregional recurrence or grade of malignancy in our breast tumor series. Future studies are needed to validate these results with reliable anti-p-eIF4E antibodies in paraffin sections.

In summary, a study of some of the most relevant cell signaling factors revealed that several proliferative cell signaling pathways converge in a few crucial factors. We propose that phosphorylated 4EBP1 may be a wild card or pivotal factor that channels the proliferative oncogenic signal and that activation of this factor could be secondary to several upstream oncogenic alterations of the cell receptors and signaling pathways. The presence of p-4EBP1 can be explained by multiple and redundant oncogenic alterations, such as mutations of PTEN, phosphoinositide-3-kinase, and other, still poorly defined, oncogenic alterations that are activated in malignant cells. Thus, 4EBP1 and the eIF family may become good candidate targets for treatment with specific inhibitors in many tumor types.

Acknowledgments

We thank Celine Cavallo for English language advice and Elisabet Llonch and Sonia Rodriguez for their expert technical work.

References


4E-Binding Protein 1, A Cell Signaling Hallmark in Breast Cancer that Correlates with Pathologic Grade and Prognosis

Federico Rojo, Laura Najera, José Lirola, et al.


Updated version
Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/13/1/81

Cited articles
This article cites 50 articles, 24 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/13/1/81.full.html#ref-list-1

Citing articles
This article has been cited by 32 HighWire-hosted articles. Access the articles at:
/content/13/1/81.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.