Enzyme-Linked Immunosorbent Assay of c-erbB-2 Oncoprotein in Breast Cancer

Avril Nugent, Enda McDermott, Karen Duffy, Niall O'Higgins, James J. Fennelly, and Michael J. Duffy

Amplification or increased expression of the c-erbB-2 gene has previously been reported to be a prognostic marker for breast cancer. Gene amplification is usually measured by Southern blotting, whereas increased protein expression is usually detected by immunochemistry. We measured c-erbB-2 protein with an enzyme-linked immunosorbent assay (ELISA). High concentrations of oncoprotein were found in 25 of 161 (16%) primary breast cancers and in 3 of 6 (50%) breast cancer metastases. High concentrations were not found in normal breast tissue or benign breast tumors. In the primary cancers, high concentrations of c-erbB-2 protein were found more frequently (a) in estrogen receptor-negative tumors than in estrogen receptor-positive tumors, (b) in progesterone receptor-negative tumors than in progesterone-positive tumors, and (c) in axillary node-positive cancers than in node-negative cancers. Patients with tumors containing high amounts of the c-erbB-2 protein had a significantly shorter (P < 0.001) disease-free interval and overall survival rate than did patients with low amounts. We conclude that assay of c-erbB-2 protein by ELISA is simple, rapid, and quantitative and offers important prognostic information in breast cancer.

Additional Keyphrases: oncogene · estrogen and progesterone receptors

The c-erbB-2 gene was first identified as the transforming gene in chemically induced rat neuroblastomas, where it was given the name neu (1). Subsequently the human homolog of this gene was cloned and given the name c-erbB-2 (2) or HER-2 (3). The c-erbB-2 gene was later found to encode a protein very similar in structure to the epidermal growth factor receptor; i.e., it contained an extracellular ligand-binding domain, a transmembrane domain, and an intracellular domain with tyrosine kinase activity (for review, see reference 4). This type of structure suggests that the c-erbB-2 gene codes for a growth factor receptor. Recently, Lupu et al. (5) described a putative ligand for the c-erbB-2 protein.

The c-erbB-2 gene is amplified in 10-30% of most types of human adenocarcinomas, including breast cancers (4, 6, 7). In 1987, Slamon et al. (8) reported that amplification of c-erbB-2 correlated significantly with...
poor prognosis in 86 patients with axillary node-positive breast cancer. Since this original report by Slamon et al., >30 publications have appeared describing the presence of either the c-erbB-2 gene or its protein product in human breast cancer (reviewed in references 6 and 7). Although most of these reports confirm the original findings of Slamon et al.—i.e., most find a significant relationship between either c-erbB-2 gene amplification or overexpression and poor prognosis in breast cancer (9–12)—a few find no such relationship (13, 14).

Measurement of c-erbB-2 gene amplification is usually performed by Southern blotting and the use of radiolabeled probes. This approach is labor intensive, expensive, and difficult to standardize. The c-erbB-2 oncoprotein is usually assayed by immunocytochemistry of formalin-fixed and paraffin-embedded tumor sections. This technique is simpler than Southern blotting, but is only semiquantitative. Furthermore, fixation of tissue in formalin can lead to loss or underestimation of the c-erbB-2 protein (9). Here, we describe the use of an enzyme-linked immunosorbent assay (ELISA) to measure c-erbB-2 protein in breast cancer extracts.³

Materials and Methods

Breast tumors were rapidly frozen in liquid nitrogen and then transferred to a −70 °C freezer. Tumors were homogenized with a Micro Braun Dismembrator (Braun, Melsungen AG, FRG). c-ErbB-2 protein was then assayed by using ELISA kits from Applied Biotechnology Inc. (Cambridge, MA), according to the protocol recommended by the supplier. We used an arbitrary cutoff point of 10 U/µg protein to indicate "normal" or high concentrations. Estrogen (ER) and progesterone (PR) receptors were also assayed by using ELISA kits from Abbott Diagnostics, (Abbott Park, IL). The cutoff point for both ER and PR was 200 fmol/g (wet weight) of tumor.

Results

The distribution of c-erbB-2 protein in different types of breast tumors is shown in Figure 1. Above-normal (>10 U/µg total protein) concentrations of the oncoprotein were found in 25 of 161 (16%) primary breast carcinomas and in 3 of 6 (50%) breast cancer metastases. No high concentrations were found in any of 10 specimens of normal breast tissue or in any of 14 benign breast tumors.

The relationship between c-erbB-2 protein concentrations in primary breast cancers and other established prognostic markers is shown in Table 1. High concentrations of c-erbB-2 protein were found more frequently in both ER- and PR-negative tumors than in tumors positive for these receptors: for ER-negative vs ER-positive samples, chi-square = 4.63, P <0.05; for PR-negative vs PR-positive samples, chi-square = 8.65, P <0.005. High concentrations of c-erbB-2 proteins were also found more frequently in breast cancers with metastasis to axillary nodes than in tumors without nodal metastasis (chi-square = 5.11, P <0.025). However, c-erbB-2 protein concentrations did not correlate significantly with tumor size.

The relationship between c-erbB-2 status of the primary cancer and both the disease-free interval and the overall survival of patients is shown in Figure 2. Patients with tumors having c-erbB-2 concentration >10 U/µg had a significantly shorter disease-free interval (chi-square = 11.3, P <0.001) and shorter overall survival (chi-square = 10.9, P <0.001) than did patients with tumors containing <10 U/µg protein. A similar relationship is seen if the cutoff point for c-erbB-2 is 5 U/µg protein: for disease-free interval, chi-square = 9.46, P <0.005; for overall survival, chi-square = 9.9, P <0.05.

Discussion

This is one of the first reports describing an ELISA to measure c-erbB-2 oncoprotein in human tumors. The ELISA is considerably simpler and faster than Southern blotting and should be easier to standardize. Further-

### Table 1. Relationship between c-ErbB-2 Protein Concentrations and Various Markers of Primary Breast Cancers

<table>
<thead>
<tr>
<th>Marker</th>
<th>n</th>
<th>Above-normal c-erbB-2 conc.*</th>
<th>Chi-square</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>ER status</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>99</td>
<td>10 (10.1)</td>
<td>4.63</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Negative</td>
<td>62</td>
<td>15 (24.2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PR status</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>57</td>
<td>4 (7.0)</td>
<td>8.65</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td>Negative</td>
<td>64</td>
<td>19 (27.9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Node status</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>63</td>
<td>4 (6.4)</td>
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</tr>
<tr>
<td>Positive</td>
<td>80</td>
<td>17 (21.3)</td>
<td>5.11</td>
<td>&lt;0.025</td>
</tr>
<tr>
<td>Size</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1</td>
<td>23</td>
<td>1 (4.4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T2</td>
<td>100</td>
<td>18 (18.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T3</td>
<td>23</td>
<td>4 (17.4)</td>
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<td></td>
</tr>
<tr>
<td>T4</td>
<td>3</td>
<td>0 (0)</td>
<td></td>
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</tr>
</tbody>
</table>

* >10 U/µg protein.

N.S., not significant.
more, unlike immunocytochemistry, an ELISA yields quantitative results.

Using a cutoff point of 10 U/μg protein, we found that 16% of primary breast cancers had above-normal concentrations of c-erbB-2 oncoprotein. This frequency of c-erbB-2 gene activation in breast cancer is similar to that previously described in studies involving immunocytochemistry and Southern blotting (6, 7). The inverse relationship between c-erbB-2 gene amplification and both ER and PR has also been reported previously (15–17). However, some investigators failed to find a significant relationship between c-erbB-2 protein concentrations (by immunocytochemistry) and ER status (11, 13). Similarly, our finding of a greater frequency of oncoprotein in primary tumors from node-positive patients than in node-negative patients has also been previously described (18). However, as with the relationship between ER and c-erbB-2 concentrations, reports on the association between c-erbB-2 protein and nodal status are conflicting (11, 13).

Of the six metastatic lesions we examined, the primary tumor was available for only one. In that case, both the primary and metastatic tumors contained high concentrations of c-erbB-2—22.8 U/μg protein in the primary tumor and 40.0 U/μg protein in the metastasis.

Our results showing the relationship between high concentrations of c-erbB-2 protein, as determined by ELISA, and poor prognosis in breast cancer agree with the majority of reports performed with other methods to detect activation of this gene (reviewed in references 6 and 7).

The tissues used in this study were samples taken without conscious bias from a tumor bank. This sample may thus contain a disproportionate number of larger specimens, because only excess material remaining after ER and PR assays could be stored in the tumor bank. This type of bias is common when multiple biochemical assays are carried out on the same breast tumor and may contribute to the conflicting results seen with c-erbB-2 protein and other new prognostic markers. For example, the original report by Slamon et al. (8), showing a relationship between c-erbB-2 gene amplification and poor prognosis in breast cancer, dealt with only axillary node-positive patients. Our data on the c-erbB-2 ELISA should thus be designated as results of a pilot study (18). These results need to be confirmed with larger numbers of patients and more representative samples.

In conclusion, the ELISA for c-erbB-2 protein is simple, quick, and quantitative. If the results in this preliminary study are confirmed, assay of c-erbB-2 oncoprotein by ELISA should permit the introduction of this analyte into routine clinical laboratories.

References
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Fig. 2. Relationship between c-erbB-2 protein in primary breast cancers and (top) disease-free interval and (bottom) overall survival. Cutoff point for normal values was 10 U/μg protein.
Comparison of Plastic vs. Glass Evacuated Serum-Separator (SST™) Blood-Drawing Tubes for Common Clinical Chemistry Determinations

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We evaluated a plastic evacuated blood-drawing tube containing an integral serum-separating barrier gel, by direct comparison with a glass counterpart. The plastic tube demonstrated no differences when compared for common clinical chemistry analytes with multiple types of instruments and systems. A total of 260 such different combinations were studied with emphasis on tests sensitive to drawing and handling indexes such as lactate dehydrogenase and potassium. A total of six separate blood drawings were tested with no significant differences noted in these tests. The total study included subjective evaluations of the plastic tube's use as a blood-drawing device and objective studies based on quantitative test results from normal and hospitalized patients and use of the primary sampling tubes (both plastic and glass) for 48-h storage.

Glass evacuated blood-drawing tubes have been the standard device for obtaining blood from patients for clinical laboratory testing since the 1960s. Major developments in the past 40 years include the introduction of an integral gel polymer into the evacuated tube, which, on centrifugation, converts the primary tube to a closed-container serum separator. Other improvements of note are the choice of "soft" vs. "hard" glass, different polymer-separator formulations, and the inclusion of anticoagulants to produce plasma rather than serum. Each of these developments generated appropriate evaluation studies to ensure that consistent analytical equivalence to the original products existed. Laessig et al. (1) tested the initial serum-separator tube for 81 analytes and methods, primarily using serum from apparently healthy individuals. Laessig et al. (2) also compared hard with soft glass tubes for 34 analytes. Plasma-separator tubes were evaluated by Chan et al. (3) for routine chemistry, drug, and thyroid-function assays with samples from hospitalized patients and healthy volunteers and by Doumas et al. (4) for 25 chemistry analytes with samples from apparently healthy adults.

Existing studies of polymer-containing tubes for routine blood collection seem to conclude that (a) barrier-gel tubes are not appropriate for all samples for therapeutic drug monitoring (5), especially for tricyclic antidepressant drugs (6); (b) silicon-based polymer-gel tubes give slightly higher results for ionized calcium in serum (7); and (c) the evacuated tubes alone may cause a certain amount of hemolysis (8). More recently, concern for the safety of laboratory employees and for the humane disposal of laboratory waste have led to the development of plastic serum-separator tubes. Plastic blood-drawing tubes have potential advantages relating to (a) breakage: plastic tubes will not shatter when inadvertently dropped on a hard surface; (b) safety: breakage of a plastic tube, i.e., because of crushing or centrifugation, does not result in shards that increase the risk of puncture wounds and blood exchange; (c) disposal: plastic may be incinerated to a very small mass, whereas glass generally requires disposal in a landfill; (d) weight: plastic tubes generally weigh 30% less than their glass counterparts; and (e) robotics: the slight flexibility of plastic tubes makes them amenable to such processing.

Plastic blood-drawing devices have been used by clinical laboratories for several years, especially as disposable syringes for obtaining arterial specimens. Plastic, however, is generally perceived to be less inert than glass as a blood-container material and, therefore, might be expected to lead to interferences with common clinical laboratory procedures. Studies comparing plastic with glass, other than for blood-gas analytes, are rare. Falch (9) compared polypropylene Microtainers™ (Becton Dickinson Labware, Lincoln Park, NJ) with glass Vacutainers™ (Becton Dickinson) for 16 chemical constituents, but the Microtainers collected capillary blood whereas the Vacutainers collected venous blood. Falch attributed small differences observed in sodium and calcium results to interference from the plastic Microtainers. A large difference found for thyroid-stimulating hormone was later determined to be due to the