Quantification of HER2 Oncoprotein in Fine-Needle Aspirates of the Breast

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Abstract. Measurements of either HER2 gene overexpression or its gene-coded protein (p185) are clinically useful for predicting prognosis in breast cancer. The measurements are also useful for identifying metastatic breast cancer patients who may benefit from Herceptin treatment. Since fine needle aspiration (FNA) of the breast has become an increasingly popular technique for obtaining tissue specimens, we have developed a sensitive method to quantify p185 in the aspirate. For this procedure, p185 from the cell pellet of FNA is extracted with a buffer containing Triton X-100, and the p185 is measured with an enzyme immunoassay. Most of the malignant breast tumors (N=7) in this study were associated with elevated p185 concentrations (6/7, 319 ± 222 U/mg), compared to the p185 concentrations in normal breast tissue (42.8 ± 35 U/mg, N=47) or benign lesions (43.1 ± 20.2 U/mg, N=22). Quantification of p185 in FNA may improve the assessment of breast cancer patients, revealing whether they are at high risk and may benefit from Herceptin treatment.

Keywords: HER2 oncoprotein, breast carcinoma, fine needle aspirate, p185, Herceptin, ectodomain

Introduction

Overexpression of the HER2 (c-erbB-2/neu) gene has been found to be associated with poor prognosis, short survival time, and recurrence in various carcinomas [1,2]. Measurements of the gene-coded protein have emerged as a useful prognostic tool, especially for breast tumors [2,3]. It has been established that the protein encoded by the HER2 gene is a 185 kDa transmembrane receptor (p185), a glycoprotein having intracellular, transmembrane, and extracellular domains [4,5]. This transmembrane receptor is presumably involved in the regulation of cell growth and cell transformation, while the intracellular or cytosolic domain of the receptor is associated with the tyrosine kinase activity of the protein. The HER2 protein (p185) shows structural and functional homology with epidermal growth factor receptor (EGFR). The most extensive homology between these two molecules is in the tyrosine kinase domain. The extracellular domain or ectodomain of these two receptors is different. Antibody reacting with the extracellular domain of HER2 gene product would not cross-react with EGFR.

Both immunohistochemical and immunoblotting techniques have been employed in the clinical laboratory for measuring the cellular concentration of p185 in tissue or cell membrane extracts [6,7]. However, these approaches are technically involved, laborious, and only semiquantitative. As pointed out by Slamon et al [6], errors could also be derived from the presence of normal cells surrounding the tumor cells, a problem which happens to be more severe in breast cancer where these nonmalignant cells can account for more than 50% of the tumor. Loss of
antigenic immunoreactivity during fixation could be another problem involved with the immunohistochemical procedure.

All of these problems could be avoided if an enzyme immunoassay was used for the quantification of p185, which can be extracted from the cell membrane with a detergent. It has also been reported that overexpression of the HER2 gene may occur without amplification [6]. Conceivably, the quantification of the HER2 oncoprotein or p185 could be clinically more useful than the determination of gene amplification [8]. Another important clinical application of measuring p185 is related to the recent success of treating metastatic breast cancer patients with Herceptin [9,10]. Herceptin, a humanized monoclonal antibody against the ectodomain of HER2 oncoprotein, is most effective in women with metastatic breast cancer who overexpress the HER2 gene in their cancer cells. Consequently, identifying patients who are overexpressing the HER gene is the first step to determining and selecting those who are suitable for Herceptin treatment. Currently the most popular assay for p185 is a semiquantitative immunohistochemical test on breast cancer tissue. A quantitative immunoassay using FNA specimens should benefit patient selection for Herceptin treatment.

Guided fine needle aspiration (FNA) has become increasingly popular in obtaining tissue specimens for the diagnosis of malignant breast diseases. FNA is often regarded as a more accurate tool than imaging or physical examination for the diagnosis of breast lesions [11]. FNA has also been routinely employed to evaluate palpable masses and to distinguish cysts from solid lesions [11]. Evaluation of FNA from primary breast carcinomas for HER2 overexpression by immunocytochemistry has been used as a predictor of response to tamoxifen treatment [12,15]. Replacing tissue biopsies with FNA allows patients to be evaluated every 3-6 months. It has been well established that measurement of the HER2 oncoprotein (p185) in tissue specimens is useful for predicting prognosis for patients with breast cancer based on immunohistochemical procedures or western blot [13]. We anticipated that the replacement of tissue biopsy specimens with smaller FNA specimens and immunohistochemical methods with quantitative immunoassays would allow a more accurate analysis, as well as a more frequent assessment of prognosis, for patients undergoing therapy [14,15].

In this investigation, we made an attempt to develop a procedure to quantify p185 in the FNA specimens. We were interested in knowing whether the commercial assay had sufficient sensitivity and whether there were a sufficient number of cells in the FNA to allow a quantitative determination of p185 to be made. We were also concerned whether the procedure would permit differentiation of malignant tumors from benign and normal breast tissue based on the measurement of p185.

Materials and Methods

The Triton c-erbB-2 (HER2) oncoprotein kit was purchased from Ciba Corning/Triton Diagnostics (Alameda, CA). (Note: this kit is no longer available.) Since the kit uses two monoclonal antibodies, both against the ectodomain of the HER2 oncoprotein, it can measure both the intact transmembrane receptor and the solubilized ectodomain of the HER2 oncoprotein. The BCA kit for protein determination and the DNA assay kit for cells and tissues were obtained from Pierce (Rockford, IL).

Processing FNA specimens.

1. All frozen FNA specimens were thawed at 4°C and centrifuged at 3,500 rpm for 30 min at 4°C (Beckman GPR centrifuge).
2. All cell pellets were washed separately with 1 ml PBS to remove ectodomain molecules and centrifuged at 10,000 rpm for 10 min at 4°C (Eppendorf microcentrifuge 5402). All pellets were suspended separately, after discarding the supernatant, in 200 μl of the extraction buffer (50 mM Tris-Cl, 100 mmol/l NaCl, 0.9 mmol/l EDTA, 0.9 mmol/l PMSF, 9 mmol/l IAA and 9 mmol/l NaN3, 1% Triton X-100, pH 7.5) and rotated overnight at 4°C.
3. Extracts were centrifuged at 10,000 rpm for 10 min (Eppendorf centrifuge). The supernatants were assayed for p185 (50 μl) and protein (10 μl) using the Pierce BCA kit with BSA as calibrator. DNA content was determined fluorometrically using a Pierce DNA assay (50 μl) kit.
4. In order to measure the c-erbB-2 activity of the supernatant with the Triton kit, supernatants were first dialyzed to remove all additives. Following dialysis, supernatants were concentrated by lyophilization and then reconstituted with a 100 µl aliquot of phosphate buffer before an aliquot was withdrawn for the assay.

**Extraction of p185 from breast tissue.**

1. Breast tissue (ca. 0.5 - 0.8 g) was cut into small pieces before it was homogenized in cold 10 mmol/l Tris-HCl buffer containing 1.5 mmol/l EDTA, 0.5 mmol/l dithiothreitol, pH 7.4 with a Polytron homogenizer (0.5 g tissue/ml buffer).
2. The homogenate was centrifuged at 40,000 rpm for 30 min (approximately 100,000 x g) in the Beckman L8-M centrifuge (#50 rotor).
3. p185 was extracted from the pellet by mixing with 200 µl of 50 mmol/l Tris-Cl buffer containing 0.1 mol/l NaCl, 0.9 mmol/l EDTA, 0.9 mmol/l PMSF, 9 mmol/l IAA and 9 mmol/l NaN3, 1 % Triton X-100, pH 7.5 for 30 min at 4°C.
4. p185 and protein concentration of the supernatant, obtained by centrifugation at 10,000 rpm for 10 min (Eppendorf centrifuge), were assayed using the Triton HER2 EIA kit and by the Pierce BCA kit, respectively.

**Superose 12 HR chromatography.** The p185 extracted from FNA pellets was confirmed by chromatography on a FPLC column containing Superose 12. The FPLC automated system and Superose 6 HR 10/30 prepacked column (bed volume 34 ml) were from Pharmacia (Piscataway, NJ). Normally 200 µl of the extract from SK-BR-3 cells was injected directly onto the column after insoluble substances were removed by centrifugation. The buffer used for column equilibration and elution consisted of 0.05 mol/l phosphate containing 0.05 mol/l NaCl at pH 6.8. The flow rate was maintained at 0.5 ml/min and 0.5 ml per tube of eluate was collected. Eluates were used directly for assay of HER2 oncoprotein using the Triton EIA kit without additional treatment.

**Specimens.** All FNA specimens were samples remaining from routine clinical specimens received in the cytopathology laboratory for processing and microscopic examination. Therefore, they did not go through institutional review. They were frozen at -70°C. The breast FNA samples came from a variety of hospital and clinic sites, and needle rinsings were submitted in either saline, ethanol, or other fixatives. The diagnoses were confirmed by a cytopathologist. The normal breast tissues were obtained from breast reduction surgery.

**Results**

**Measurement of HER2 oncoprotein (p185).** P185 is a membrane bound molecule. The molecule was solubilized and released from the cell membrane using a buffer containing detergent, such as Triton X-100. The solubilized p185 in the extracts was then...
Table 1. Concentration of pi85 in cell extracts of fine needle aspirate specimens from normal breast tissue, benign breast lesions, and malignant breast tumors. Data are reported as mean ± SD (median in parentheses).

<table>
<thead>
<tr>
<th></th>
<th>normal breast tissue (N = 50)</th>
<th>benign breast lesions (N = 22)</th>
<th>malignant breast tumors (N = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p185 conc. (U/ml)</td>
<td>14.4 ± 3.5 (13.4)</td>
<td>42.1 ± 50 (14.6)</td>
<td>185 ± 168 (180)*</td>
</tr>
<tr>
<td>p185 conc. (U/mg protein)</td>
<td>37.8 ± 23.8 (17.3)</td>
<td>41.1 ± 18 (41.2)</td>
<td>319 ± 222 (172)*</td>
</tr>
</tbody>
</table>

* P < 0.05 vs normal breast tissue and vs benign breast lesions.

Table 2. Concentration of p185 and its ectodomain in individual fine needle aspirate specimens from the breast.

<table>
<thead>
<tr>
<th>Patient's diagnosis</th>
<th>p185 conc. in cell extract (U/ml)</th>
<th>p185 conc. in cell extract (U/mg protein)</th>
<th>ectodomain conc. in supernatant (U/ml)</th>
<th>ectodomain conc. in supernatant (U/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>cancer</td>
<td>2.6</td>
<td>52</td>
<td>16</td>
<td>11</td>
</tr>
<tr>
<td>cancer</td>
<td>2.5</td>
<td>31</td>
<td>6.1</td>
<td>1.3</td>
</tr>
<tr>
<td>cancer</td>
<td>105.7</td>
<td>423</td>
<td>28</td>
<td>23</td>
</tr>
<tr>
<td>cancer</td>
<td>&gt;240</td>
<td>&gt;100</td>
<td>29</td>
<td>41</td>
</tr>
<tr>
<td>benign cyst</td>
<td>5.2</td>
<td>35</td>
<td>12</td>
<td>18</td>
</tr>
<tr>
<td>benign cyst</td>
<td>10.6</td>
<td>48</td>
<td>8.1</td>
<td>0.1</td>
</tr>
<tr>
<td>benign cyst</td>
<td>5.5</td>
<td>39</td>
<td>4.9</td>
<td>0.2</td>
</tr>
<tr>
<td>benign cyst</td>
<td>2.6</td>
<td>29</td>
<td>6.1</td>
<td>5.5</td>
</tr>
<tr>
<td>benign cyst</td>
<td>3.0</td>
<td>33</td>
<td>4.7</td>
<td>0.7</td>
</tr>
<tr>
<td>SK-BR-3 cells (control)</td>
<td>119</td>
<td>121</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

quantified using the Triton EIA kit. In this study we found that it was possible to measure p185 quantitatively in FNA specimens due to both the presence of sufficient amounts of p185 in the FNA specimens and the sensitivity of the EIA kit. We found that elevated concentrations of p185 were present in a high percentage of extracts of FNA from malignant tumors (Table 1, Fig. 1).

As shown in Fig. 1, based on p185 measured in U/mg, at least 70% (5 out of 7) of the FNA from malignant tumors had elevated p185 concentrations. In the past, only approximately one-third of all breast cancer cases were reported to overexpress HER2 gene [1,2,16]. We need a larger number of specimens from malignant breast tumors to confirm the result of the current study. To compare the concentration of p185 among three different sources we expressed p185 in terms of either total protein concentration of the extract, the volume of the extract, or the DNA content of the cell pellet (Fig. 1). It appears from results plotted in Fig. 1 that expressing p185 concentrations in U/mg was the best, since it provided the best differentiation of malignant tumor from normal or benign breast tissue. Also, as demonstrated in Fig. 1, two FNA specimens showing elevation in U/ml became normal when the expression was changed to U/mg.

The mean concentrations of p185 in FNA from normal breast tissue, benign lesions, and malignant breast tumors are shown in Table 1. When p185 concentration is expressed in U/mg, there is very little difference between normal and benign breast tissue (p = 0.36). However, a more than seven-fold increase of p185 concentration is found in the malignant breast tissue (p = 0.04, levels in benign breast tissue vs malignant breast cancer). These p values were estimated by Student's t-test, assuming samples of unequal variance.
HER2 activity of the supernatant. The HER2 oncprotein activity was also measured in the supernatants, which were separated from cells in the FNA by centrifugation. As shown in Fig. 2, an almost two-fold increase in the mean, expressed as U/ml, or an approximately three-fold increase in the mean, expressed as U/mg, was found in supernatants of FNA from malignant tumors compared to those from normal breast cysts. Although higher oncprotein activity was found in the supernatant of FNA from breast tumor tissue than in that from normal breast tissue, the increase of oncprotein activity was much higher in cell extracts of the breast tumors when the oncprotein activities in individual FNA samples were compared (Table 2). The amount of oncprotein activity in the supernatants did not appear to correlate exactly with the activities in the cell extracts. Therefore, measuring the oncprotein activity in the supernatant appears to be less useful clinically. Furthermore, the procedure of measuring oncprotein activity in supernatants is more time-consuming and technically complex.

Normal p185 concentration. To establish normal reference levels for p185 in FNA, p185 concentrations were measured in 50 FNA specimens from normal breast tissue. The mean was 37.8 ± 23.8 U/mg. The upper normal value (95% confidence level) was 85.2 U/mg. The p185 concentration was also measured in the membrane extract from normal breast tissue (not the FNA). The values we obtained from the normal breast tissue, although slightly higher, were close to those in FNAs of normal breast tissues. The mean ± SD from the normal breast tissue was 41.9 ± 23.5 U/mg. (Fig. 3, Table 3). As shown in Fig. 3, elevated HER2 oncprotein activity was also detected in breast tumor tissues. For reasons not entirely clear to us we found that only approximately 12% of the tumor tissues had elevated oncprotein activity. The mean ± SD was equal to 117 ± 270 U/mg, which was

![Fig. 2. Average concentrations of the ectodomain of HER2 oncprotein in FNAs from malignant breast tumors and normal breast cysts. Concentration of the ectodomain in the supernatant of the FNA, after dialysis and lyophilization, was determined using a Triton EIA kit. The means ± SD for cancer were 19.6 ± 10.7 U/ml and 19.0 ± 17.1 U/mg, whereas those for normal cysts were 7.8 ± 3.1 U/ml and 5.6 ± 8.4 U/ml, respectively.

Table 3. Mean p185 concentrations in cytosols and detergent extracts of specimens of normal breast tissue and malignant breast tumors. (Note, these are macrospecimens, rather than fine needle aspirate specimens.)

<table>
<thead>
<tr>
<th>Type of specimen</th>
<th>Number of specimens</th>
<th>mean p185 conc. (U/ml)</th>
<th>mean p185 conc. (U/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extracts of normal breast tissue</td>
<td>15</td>
<td>11.1</td>
<td>41.9</td>
</tr>
<tr>
<td>Extracts of malignant tumors</td>
<td>106</td>
<td>384</td>
<td>117</td>
</tr>
<tr>
<td>Cytosols of normal breast tissue</td>
<td>14</td>
<td>15.4</td>
<td>2.8</td>
</tr>
<tr>
<td>Cytosols of malignant tumors</td>
<td>123</td>
<td>9.8</td>
<td>3.8</td>
</tr>
</tbody>
</table>
Fig. 3. p185 concentration in the extract of malignant breast tumors and normal breast tissue. Breast tissue (not FNA), after homogenization and removal of cytosol, was extracted with buffer containing 1% Triton X-100. The extract was assayed for p185 determination using a Triton EIA kit. The mean ±SD for normal tissue was 41.9 ± 23.5 U/mg, whereas that for tumors was 117 ± 279 U/mg. substantially less than in the cell extracts of FNAs (319 ± 222 U/mg) from malignant breast tumors.

HER2 Oncoprotein in supernatant and cell extract. In our earlier study, we found that all the oncoprotein activities in the SK-BR-3 cell extract were derived from the intact molecule, the p185, or the HER2 intact transmembrane protein [17]. In contrast, all the oncoprotein activities detected in the cell medium were derived from the ectodomain of the HER2 oncoprotein. Apparently the ectodomain of the intact transmembrane molecule can be cleaved and released into the cell medium. In fact, in serum, only the ectodomain of HER2 oncoprotein could be found [16,17]. Because the Triton kit reacts with both the intact molecule and the ectodomain of the HER2 oncoprotein, the assay by the Triton kit does not determine whether the oncoprotein activity is derived from the intact molecule (p185) or the ectodomain. To find out what type of HER2 oncoprotein was present in the supernatant and the cell extract of FNA, chromatography of both supernatant and cell extract was performed on a prepacked column containing

Fig. 4. Chromatography of supernatant and extract of FNA containing HER2 oncoprotein activity on a Superose 12 column. Supernatant and Triton X-100 extract of FNA were chromatographed on a prepacked Superose 12 column with a Pharmacia FPLC apparatus. Eluates were assayed with a Triton kit that detects both the ectodomain and the intact molecule (p185). Open circles represent p185 extracted from SK-BR-3 cells, which were used as a control.

Superose 12. Superose 12 chromatography separates the ectodomain from the intact receptor, which allows identification of the type of oncoprotein the oncoprotein activity is associated with. As shown in Fig. 4, based on the position of the peak activity on the elution profile, almost all HER2 oncoprotein activity in the cell extract was derived from the intact transmembrane receptor, whereas the oncoprotein activity in the cell supernatant was associated with the ectodomain of p185, exactly the same molecule that was found in the serum.
Discussion

HER2 oncoprotein (p185) is routinely measured in the cytosol of the breast tumor tissue, because along with estrogen and progesterone receptors, it is part of the risk panel used to assist in the selection of proper management for patients with breast cancer [19]. Since FNA is becoming increasingly popular as a diagnostic modality, quantification of p185 in FNA specimens would allow a more frequent and precise assessment of patients [15]. Therefore, our first attempt was to find out whether the p185 assay was sufficiently sensitive to measure p185 immunoreactivity in the FNA. In fact, we were not certain whether a sufficient amount of p185 could be extracted from cells of FNA specimens for a quantitative determination. Developing the technique to extract p185 from the cells of FNA for quantitative determination by immunoassay would provide many advantages over the traditional immunohistochemical procedure.

The importance of measuring HER2 gene amplification and its overexpression is well known. In the past, the immunohistochemical technique has been the most frequently used method for detecting HER2 overexpression. However, this technique is at most semiquantitative and involves some technical difficulties. Recently FNA has become increasingly popular; therefore, we have made an effort to establish a procedure allowing us to quantify HER2 oncoprotein (p185) in FNA specimens to predict survival and response to therapy. The results of our current investigation clearly indicate that there are sufficient amounts of p185 extractable from the cell pellet of FNA specimens, as measured by the commercial EIA kit from Triton. Our data also indicate that a potential differentiation between malignant breast tumor tissue and normal or benign breast tissue is most likely possible.

Nugent et al [18] also found elevated concentrations of c-erbB-2 (HER2) oncoprotein in breast cancer tissue with an enzyme linked immunosorbent assay. Despite using different assay kits and extraction methods, they found elevated oncoprotein concentration more frequently in breast tumors associated with metastases. No elevation was detected by them in normal breast tissue or benign breast tumors. It should be noted that p185 measurement has been reported to be useful in selecting therapy for breast cancer patients. It was found [19] that overexpression of c-erbB-2 was a useful marker to identify the patients who are most likely to benefit from high doses of adjuvant chemotherapy. However, the data may not be sufficient, as stated in the guidelines from ASCO [20], to recommend that c-erbB-2 overexpression be used for the management of breast cancer patients.

Even though oncoprotein activity was also higher in the supernatant from malignant breast tumors, we felt it was more reliable to differentiate malignant from normal or benign breast tissue based on the measurement of oncoprotein activity in cell extracts. This is because the difference in activity was much larger between malignant, versus normal or benign cell extracts, and it was the encoded protein of the HER2 gene (p185) and not the secondary cleaved product of the oncoprotein that was being measured. The oncoprotein activity detected in the supernatant was entirely derived from the ectodomain, the cleaved product of p185. The concentration of the ectodomain in the supernatant appears to be controlled not only by the gene expression but also by a second reaction that controls the cleavage of the intact transmembrane receptor. Until we gain a more complete understanding of what controls the second reaction (most likely a proteolytic reaction catalyzed by an unknown protease [16,17]), we cannot give any clinical interpretations based on the oncogene activity of the supernatant. This could also be the reason that we did not find a perfect correlation between the oncogene activity of supernatant and cell extract.

We hope the results of the present study will stimulate a continued investigation, including a larger number of specimens, especially FNA from malignant breast tumors, in order to confirm that the percentage of malignant breast tumors which overexpress the HER2 gene is truly higher than the 35% that has generally been thought to be the norm [6]. If this is true, we anticipate that more patients with malignant breast tumors would benefit from treatment with Herceptin.

Our current protocol yields 200 µl extract for each FNA specimen, in which only 50 µl was used for the p185 assay and 10 µl for the protein assay. It appears that the remaining 140 µl cell extract may be used for
the determination of additional risk markers. It may be possible to analyze multiple risk factors in FNA simultaneously, for instance by using the cytosol for estrogen and progesterone receptor assays, the detergent extract for HER2 oncoprotein determination, and the nuclear fraction for p53 mutant protein analysis and telomerase measurement. The DNA from the cell nucleus can also be used for mutation detection after PCR amplification.

Perhaps a more accurate risk assessment can be made because a panel of risk markers, rather than a single marker, is measured. We plan to see if this is possible and if more sensitive assays can be developed to meet this challenge. We also plan to broaden our experiments to include FNAs from other tissues.

References


