Immunocytochemical Localization of Estrogen and Progesterone Receptor and Prognosis in Human Primary Breast Cancer

Angelika Reiner,1 Birgitt Neumeister, Jürgen Spona, Georg Reiner, Michael Schemper, and Raimund Jakesz

Institute of Clinical Pathology [A. R., B. N.], First University Clinic of Surgery [G. R., M. S., R. J.], First University Clinic of Gynecology [J. S.], University of Vienna, A-1090 Vienna, Austria.

ABSTRACT

An immunocytochemical assay (ICA) for the measurement of estrogen receptor (ER) and progesterone receptor (PgR) has been evaluated in 426 human primary breast carcinomas. For estrogen receptor determination ER ICA was used. PgR ICA was performed using the monoclonal antibody KD 68. Assay results for progesterone receptor immunocytochemistry were in agreement (P < 0.0001) with those of biochemical determination in 74%. Progesterone receptor positivity determined with a semiquantified approach based on intensity and heterogeneity of immunocytochemical staining correlated significantly with biochemically determined progesterone receptor levels (P = 0.0001). Survival data showed a significantly better overall survival for patients with either ER ICA- or PgR ICA-positive carcinomas (ER ICA, P < 0.00001; PgR ICA, P = 0.004). Patients with both negative ER ICA and PgR ICA showed a poorer prognosis than patients with only one negative receptor. In ER ICA- and PgR ICA-positive carcinomas a trend could be found that patients whose carcinomas contained high numbers of receptor-positive tumor cells had a better survival. This study demonstrates that ER ICA and PgR ICA are strong prognostic indicators and that the proportion of steroid hormone receptor-positive tumor cells seems to be of clinical importance.

INTRODUCTION

Steroid hormone receptor status is known to be an important predictor of prognosis and response to endocrine therapy in breast cancer patients. In the past it was determined by biochemical assay. This assay is associated with certain methodological difficulties. The major limitations are that a certain amount of tissue is required and that the tissue needs to be homogenized for extraction of the receptor prior to the assay. Therefore, the biochemical assay cannot be performed in very small tumors and intratumoral variations of steroid hormone receptor cannot be determined. These problems were overcome by the development of monoclonal antibodies against steroid hormone receptors and immunohistochemical assay (1–3). Although there exist reports from different centers about correlations of immunocytochemical assay of ER2 with biochemical techniques (4–9), and a few reports about the more recently developed immunocytochemical assay for PgR (10–12), only little is known about the clinical significance of the immunocytochemical assays (10, 13, 14).

Therefore, it was the aim of our study to describe how the recently developed PgR ICA works in our hands and more importantly to examine ER ICA and PgR ICA as prognostic indicators.

MATERIALS AND METHODS

Patients

Tumor samples were obtained from 426 consecutive patients with primary breast cancer. Six % of the patients were younger than 40 years, 21% were between 40 and 50 years, and 73% were more than 50 years. Two hundred, thirty-two patients (54%) had a modified radical mastectomy and in 174 cases (41%) a breast-conserving procedure was possible, mainly combined with an axillary dissection. In 15 patients with high operative risk a simple mastectomy was performed. Five patients with large tumors (Tx, T4) had just a tumor biopsy prior to systemic therapy and underwent modified radical mastectomy thereafter. The distribution of receptor-positive and receptor-negative patients was equal within the different operations. All patients with conservative surgery received radiation therapy to the remaining ipsilateral breast and an electron boost or interstitial radiotherapy with 192Ir to the tumor site. The administration of adjuvant systemic treatment depended on eligibility to enter different adjuvant trials on operable breast cancer or on hormone receptor status and nodal status. Thus, 79 patients received adjuvant polychemotherapy, 133 patients had adjuvant endocrine therapy (tamoxifen), 25 had a combination of polychemotherapy and hormonal therapy, and 189 had no adjuvant systemic therapy. In summary, most of the patients with hormone receptor-positive tumors were treated with tamoxifen alone or combined with polychemotherapy. Only 29 patients with ER ICA-positive tumors (10%) and 50 patients with ER ICA-negative tumors (37%) had polychemotherapy alone. Patients without systemic adjuvant treatment (n = 189) had ER ICA-negative tumors (40%) and ER ICA-positive tumors (60%). The clinical follow-up was conducted at 3-month intervals for 36 months and at 6-month intervals thereafter. The patients were followed up to 62 months (median 36 months).

On pathological examination tumors were smaller than 2 cm in 62% and 2 cm or larger in 38% of the cases. Lymph node metastases were found in 48% of patients. Histological tumor types were invasive ductal carcinoma NOS in 71%, invasive lobular carcinoma in 13%, lobular ductal carcinoma in 4%, and mucoid, medullary, and ductal in situ carcinoma in 2% each of the cases. The remaining carcinomas consisted of rare tumor types as tubular, papillary, or comedo carcinoma. Histological tumor grade according to the World Health Organization was classified in 25% as highly differentiated, in 45% as intermediate, and in 30% as poorly differentiated.

Receptor Analysis

Immediately after surgery tumor specimens were trimmed of adjacent fat and connective tissue.

Biochemical Receptor Analysis. For biochemical analysis specimens were quick-frozen in liquid nitrogen and ER and PgR measured using the DCC assay and Scatchard analysis. ER and PgR values >10 fmol/mg protein were considered positive, and values <10 fmol/mg protein were considered negative.

Immunocytochemical Assay. For immunocytochemistry tumor samples were either quick-frozen in liquid nitrogen and stored in an Ultrafreeze unit at −70°C for as many as 4 years or were frozen immediately in a Cryocut unit at −20°C using Tissue-Tek (Miles Laboratories, Inc., Elkhart, IN) and processed thereafter. All tumor samples were sectioned at 5 μm thickness at −20°C, thaw mounted on glass slides, and fixed in 3.7% phosphate-buffered formaldehyde for 10 min. All further processing for ER ICA was performed according to the instructions in the Abbott ER ICA Monoclonal Kit (Abbott...
Laboratories, Diagnostics Division, North Chicago, IL). For PgR ICA pre-diluted rat monoclonal antibody against human PgR (KD 68) was provided by Abbott Laboratories. The immunohistochemical method used was the same as for ER ICA. In summary, it consisted of the sequential application of monoclonal anti-PgR antibody for 30 min, followed by incubations with bridging antibody and peroxidase-antiperoxidase complex for 30 min each. All incubations were performed at room temperature. The sites of immunoprecipitate formation were identified by diaminobenzidine. All reagents used for PgR ICA were exactly the same as provided with the ER ICA kit. Controls consisted of ER- and PgR-positive cells as provided with the kit and treated with either anti-ER or anti-PgR antibody and control antibody, respectively. In addition a series of tumor specimens was treated with the primary and control antibodies. Specimens showing positive results with the primary antibodies failed to show positive reaction with the control antibody. Tumor specimens were classified as ER ICA or PgR ICA positive when positively stained cells could be identified. In cases of positive staining further parameters analyzed were intensity and heterogeneity of staining. The intensity of staining was subjectively evaluated as 1, 2, or 3, with 1 representing the weakest and 3 the most intense staining. When some variability in staining intensity within a tumor was noticed, the average degree of intensity was considered representative. Heterogeneity represented the proportion of positively stained tumor cells. It was evaluated as 1, 2, 3, or 4 by estimation on screening wide areas within each tissue section: 1, <10% stained cells; 2, <30%; 3, <70%; and 4, >70% stained cells, compared with the total of tumor cells. Results of determination of intensity and heterogeneity were combined and evaluated as follows: 2 and 3 points, low; 4 and 5 points, intermediate; and 6 and 7 points, high positivity of ER ICA or PgR ICA. All cases with negative staining were scored as 0.

Statistical Analysis

Overall survival and disease-free survival was described by Kaplan-Meier (15) estimates, using the programs of the BMDP statistic package. Possible prognostic differences of groups were analyzed by the log rank test (16) and the generalized Wilcoxon test (17). The proportional hazards model by Cox (18) was used to investigate the prognostic effect of receptor measurements while simultaneously adjusting for other factors of assumed prognostic importance. The prognostic strength of a receptor is described by an estimate of the relative risk of dying within short time intervals for “negative” versus “positive” patients. The probability that such or greater relative risk estimate differs from 1 only by chance is shown by a two-sided $P$ value.

For DCC dichotomized at 10 fmol/mg, association with PgR ICA (positive versus negative) was tested by standard $\chi^2$ analysis. For DCC continuous, monotone association with PgR ICA ($-, +, ++, +++$) was confined by the test for Kendall’s $t$ (19). The hypothesis of monotone association of heterogeneity and intensity of staining with DCC was examined similarly.

RESULTS

Localization of Progestosterone Receptor in Breast Cancer Specimens.

Positive staining with PgR ICA was localized to the nuclei of breast cancer cells in all histological specimens (Fig. 1). Heterogeneity of staining was observed frequently. No tumor specimen presented with 100% stained cells. PgR ICA was positive in 58% of the carcinomas.

Relationship between PgR ICA and Biochemically Determined PgR Status. A statistically significant correlation ($P < 0.0001$) was observed between PgR ICA and biochemically determined PgR status. Results for both methods agreed in 74% of the cases. In only 4% of the cases PgR ICA was negative, compared with DCC. In 22% of the cases PgR ICA was positive, whereas PgR was negative in DCC (Table 1). The relationship between PgR status as determined by DCC and semi-quantified PgR ICA is shown in Fig. 2. There was a significant correlation of PgR levels as determined by both methods ($P = 0.0001$). Among PgR ICA high tumors 73% had PgR values $>50$ fmol/mg protein. In PgR ICA intermediate and low carcinomas PgR values $>50$ fmol occurred less frequently (64 and 43%, respectively). However, in all groups of PgR ICA-positive carcinomas a few biochemically PgR-negative carcinomas occurred, but the frequency decreased with increasing PgR positivity (48, 30, and 22%). In PgR ICA-negative carcinomas there occurred a few biochemically PgR-positive tumors, but PgR values never exceeded 200 fmol/mg protein.

Relationship between ER ICA and PgR ICA. Eighty-one % of the carcinomas showed concordant positive or negative results in immunocytochemical ER and PgR status ($P < 0.0001$) (Table

![Fig. 1. Immunocytochemical localization of PgR in an infiltrating ductal carcinoma. A, histological specimen treated with PgR ICA (×220). B, negative control treated with control antibody (×220).](cancerres.aacrjournals.org)
2). In 3% carcinomas were observed which were positive in PgR ICA only. The majority of these carcinomas (7 of 10) showed only weak PgR ICA positivity and 8 of 10 carcinomas presented with <30% PgR ICA-positive tumor cells. However, two carcinomas were observed with intermediate and one carcinoma with high PgR ICA positivity.

Association of ER ICA and PgR ICA with Clinical Follow-up. Women with either ER ICA-positive or PgR ICA-positive carcinomas had a more favorable overall survival than women with steroid hormone receptor-negative carcinomas (Fig. 3). Regarding women with steroid hormone receptor-positive carcinomas there seemed to be no clear difference in survival depending on the grade of positivity, although patients with ER ICA high and PgR ICA high carcinomas had the best overall survival (97% for each receptor). Overall, patients with ER ICA-positive tumors are alive in 93% and those with PgR ICA-positive tumors in 91% of the cases. Patients with ER ICA-negative or PgR ICA-negative carcinomas had a significantly worse prognosis (75% for each receptor). Survival data for both steroid hormone receptors in immunohistochemistry are demonstrated in Fig 4. Patients with both negative ER ICA and PgR ICA show a poorer prognosis (only 60% are alive) than patients with only one negative receptor. In contrast patients with both ER ICA- and PgR ICA-positive carcinomas are alive in 90% of the cases. There was no additive effect in survival in patients when both receptors were positive. In Fig. 5 overall survival of patients with receptor-positive carcinomas in relation to the proportion of receptor-positive tumor cells is demonstrated. A trend could be found that patients whose carcinomas contained >30% receptor-positive tumor cells had a better survival.

In Fig. 6 the relationship between survival of patients with receptor-positive carcinomas and staining intensity is demonstrated. Although patients with carcinomas with high staining intensity have the best prognosis, staining intensity alone does not discriminate satisfactory.

Various results on the prognostic effect of receptors obtained from Cox analyses are summarized in Table 3. The results for ER ICA and biochemically determined ER status are based on 304 patients, and those for PgR ICA and biochemically determined PgR status are based on 212 patients. For comparability the regression models and corresponding univariate analysis were based on the same patients. The results from the regression model are all adjusted for the following factors: histological tumor grade, axillary lymph node status, tumor size, conserva-
receptor-positive carcinomas in relation to staining intensity. +, weak; ++, intermediate; ++++, high staining intensity. A, ER ICA; B, PgR ICA.

Table 3 Comparison of prognostic effect of receptors in immunocytochemical and biochemical assays by Cox analysis

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<thead>
<tr>
<th>Receptor</th>
<th>Univariate analysis</th>
<th>Regression model</th>
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<tr>
<td></td>
<td>Relative risk</td>
<td>P</td>
</tr>
<tr>
<td>ER-ICA</td>
<td>3.03</td>
<td>0.0001</td>
</tr>
<tr>
<td>ER-DCC</td>
<td>2.27</td>
<td>0.0002</td>
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<td>PgR-ICA</td>
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<td>0.0001</td>
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<tr>
<td>PgR-DCC</td>
<td>1.56</td>
<td>0.003</td>
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tive surgery or radical mastectomy, and adjuvant therapy being either none, chemoendocrine, or combined chemotherapy and hormone therapy. However, the receptors are not mutually adjusted in accordance with the aims of this project. As it is demonstrated ER ICA and PgR ICA are prognosticators independent of other prognostic factors. Moreover, the P values for ER ICA and PgR ICA are of higher statistical significance than those for the biochemical assay.

DISCUSSION

Monoclonal antibodies to ER and PgR were used to localize steroid hormone receptors in histological breast cancer samples. PgR localization was always limited to the cell nucleus. This is in agreement with other reports on PgR immunocytochemistry (3, 10, 20) and with the concept of intranuclear steroid hormone receptor localization. Intranuclear localization of ER was already reported in many previous studies (1, 2, 4–9). Similar to ER ICA we could demonstrate a significant correlation between PgR ICA and biochemically determined PgR status. Overall agreement of PgR positivity or negativity by both methods was 74%. This result is comparable to the data of others (10). However, there was some disagreement mainly in cases which were PgR ICA positive but biochemically PgR negative. This finding is also similar to ER ICA (9). Partly, this may be due to the fact that in our series of carcinomas only 40% were biochemically PgR positive. Although this percentage of PgR positivity is within the range of different reports (21–23), there exist reports with considerably higher frequencies of PgR-positive carcinomas (10, 24). Other reasons for discrepant results between immunohistochemistry and biochemical receptor determination may be prolonged specimen storage, alterations of the epitope during tissue fixation, and differences between the biochemical and immunocytochemical steroid hormone receptor assays themselves.

As is known for biochemically determined steroid hormone receptors there exists an association between ER and PgR status. Only 19% of the carcinomas were positive for one receptor only. The majority was positive for ER alone and in 3% only PgR positivity was found. This percentage is lower than for biochemical assays in which approximately 10% of the carcinomas are ER negative but PgR positive. The majority of these carcinomas showed only weak PgR ICA positivity. However, there was one case with high PgR ICA positivity. An explanation for this result could be that in rare cases a dissociation of the function of ER and PgR exists in tumor cells. This was suggested for mammary cancer cell lines (25). It is also supported by simultaneous immunocytochemical demonstration of ER and PgR within the same section of breast cancer, where cells exist which coexpress both receptors or only either one receptor (26).

For semiquantification of steroid hormone receptor immunocytochemistry we used a simple score based on heterogeneity and staining intensity. As in a former report for ER ICA (9) we are now able to demonstrate that this score seems to work quite well also for PgR ICA. This score is simple and easy to perform and therefore can be applied by any competent pathologist.

Immunocytochemical analysis of both ER and PgR was found to predict overall survival. This was true for univariate and also for multivariate analysis when receptor immunocytochemistry prognostic power was kept independently of all other important prognostic parameters. A similar result for ER ICA on a limited number of patients is found in the literature (13). In this study ER ICA was related independently to prognosis of lymph node status and treatment. Moreover, in our study the comparison of the prognostic significance of immunocytochemically and biochemically determined steroid hormone receptor status showed that the P values were of higher significance for immunocytochemistry than for biochemical analysis. This suggests that the immunocytochemical assay is of higher discriminating power. This is in agreement with the result in another study (14). In that study, ER ICA predicted survival and disease-free survival for the whole observation time of 10 years, while biochemically determined ER status lost its predictive value after 5 years. Moreover, we found differences in survival for both ER ICA and PgR ICA, depending on the grade of positivity. The differences between the different grades of positivity were not statistically significant. But the observation time for steroid hormone receptor-positive carcinomas in our study was rather short and therefore it seems possible that differences can be demonstrated more clearly at a later date. Therefore, we assume that the semiquantitative evaluation of steroid hormone receptor immunocytochemistry is of clinical importance. This agrees with some reports in the literature in which a correlation between biochemical steroid hormone receptor values and survival was described (27).
Immunocytochemistry also gives information about the heterogeneity of steroid hormone receptor distribution. As our survival data suggest this seems to be of clinical importance, since patients whose carcinomas contain high levels of steroid hormone receptor-positive cells (>30%) show a better overall survival. This agrees with the results that high rates of recurrences occur in patients whose tumors contain high proportions of ER-negative tumor cells (28). On the basis of this it is tempting to speculate that in the future patients with only a few steroid hormone receptor-positive, but a large proportion of negative tumor cells may be selected for adjuvant combined endocrine and chemotherapy.

One can conclude that steroid hormone receptor immunocytochemistry adds important information to the currently used biochemical steroid hormone receptor assay. The most important findings are that both ER ICA and PgR ICA are strong prognosticators for overall survival and that the proportion of steroid hormone receptor-positive tumor cells seems to be of clinical importance. Although our results are encouraging, one has to keep in mind that immunocytochemistry detects the receptors but does not assure that the receptors are functional.

REFERENCES

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