Brief Fixation Does Not Affect Assessment of Hormone Receptor Expression in Invasive Breast Carcinoma Biopsies

Paving the Road for Same-day Tissue Diagnostics

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Abstract: In patients with invasive breast carcinoma, estrogen receptor α (ERα) and progesterone receptor (PR) expressions need to be assessed in core-needle biopsies (CNBs) before the start of neoadjuvant systemic treatment. Current guidelines recommend a minimum formalin fixation time of 6 hours. Considering the increasing demand for same-day diagnostics in oncology, more rapid tissue processing with shorter fixation times is required. To identify whether brief fixation (< 6 h) of CNBs compared with conventionally fixed resection specimens provides for reliable immunohistochemical assessment of ERα and PR expression, 78 consecutive patients diagnosed with invasive breast carcinoma were included through the same-day diagnostics programme of the UMC Utrecht. Paraffin-embedded CNBs fixed for approximately 45 minutes were retrieved. Immunohistochemistry for ERα and PR was compared between the briefly fixed CNBs and conventionally fixed resection specimens. All slides were reviewed by means of consensus scoring by 2 blinded observers. Overall agreement between CNB and resections was 73/74 (98.6%) for ERα (κ = 0.85; 95% confidence interval [CI] = 0.56-1.00) and 69/75 (92.0%) for PR (κ = 0.81; 95% CI = 0.66-0.96). For ERα, positive and negative predictive values were 98.6% (95% CI = 0.91-0.99) and 100.0% (95% CI = 0.31-1.00), respectively. For PR, positive and negative predictive values were 100.0% (95% CI = 0.91-1.00) and 76.0% (95% CI = 0.54-0.90). In conclusion, analysis of hormone receptor expression in briefly fixed CNB seems comparable to results from conventionally fixed resection specimens of the same tumor.  

Key Words: fixation time, estrogen receptor, progesterone receptor, immunohistochemistry, breast carcinoma

Accurate evaluation of estrogen receptor α (ERα) and progesterone receptor (PR) is of great clinical importance in invasive breast carcinoma. Results of immunohistochemical analysis determine the type of systemic treatment. Interpretation of receptor expression may be influenced by a variety of preanalytic factors, such as cold ischemia time, type of fixative, and duration of fixation. The American Society of Clinical Oncology and the College of American Pathologists (ASCO/CAP) Guideline Recommendations advise a minimum fixation time of 6 hours, on the basis of previous research showing erroneous results for ERα expression in breast carcinoma samples fixed under 6 hours. However, an increasing demand for rapid diagnostics in oncology is observed, assuming that providing patients with a definitive diagnosis at the day of presentation reduces anxiety. Patterns of highly abnormal cortisol secretion were found in women who had not yet heard their diagnosis on the fifth day after large-core breast biopsy. Negative effects of uncertainty may be reduced with more rapid communication of biopsy results. To provide a conclusive, histologically proven diagnosis within 1 day, ultrafast processing of histologic samples is required. In case of neoadjuvant chemotherapy or locally ablative therapy, ERα and PR expressions then need to be assessed on briefly fixed core-needle biopsies (CNB). Evidence in the literature is scarce, but there are data available implying that brief fixation of a surgical breast carcinoma specimen does not compromise assessment of ERα and PR expression. The aim of this study was to thoroughly evaluate the effect of brief fixation of CNB on ERα and PR staining.

MATERIALS AND METHODS

Patients

A same-day diagnostics program for breast lesions was introduced in our hospital in November 2011. In this program, patients with suspected malignancy are referred for CNB with the aim to inform patients about their diagnosis and fitted treatment regimen on the same day as the biopsy.
Within our same-day diagnostics database, all admissances between November 2011 and December 2012 were systematically searched. Patients were consecutively included when both the CNB and the subsequent resection specimen contained invasive breast carcinoma. Patients undergoing neoadjuvant therapy, patients with multifocal disease, and cases in which no marker studies were conducted in the excision specimen were excluded from the study.

**Ethics Statement**

As archival pathology material was used and the included patients are not affected by the study, no ethical approval is required according to Dutch legislation. Anonymous or coded use of redundant tissue for research purposes is part of the standard treatment agreement with patients in our hospitals, and informed consent was therefore not required.

**Tissue Processing**

The CNBs were immediately placed in 10% neutral buffered formalin and sent to the pathology laboratory to be registered (estimated fixation time up to now 15 min). Then, specimens were registered and immediately placed in an automated tissue processor (Peloris; Leica, Valkenswaard, The Netherlands). In short, the Peloris, which runs with the xylene replacement isopropyl alcohol (IPA), started with a 30-minute formaldehyde fixation step under vacuum, followed by dehydrating ethanol 70% (37°C for 1 min and 60°C for 5 min) and 80/20 ethanol/IPA baths (60°C for 16 min), IPA 100% (60°C for 12 min) and paraffin saturation (85°C for 30 min and 65°C for 5 min). Hereafter, tissue samples were routinely embedded in paraffin. Assuming a preprocessing formaldehyde exposure window (including transportation to the pathology laboratory and administration) of 15 minutes, total formaldehyde fixation time before and during processing thereby was approximately 45 minutes. Excision specimens were fixed overnight in compliance with the current ASCO/CAP guideline recommendations (6 to 72 h). Cold ischemia time in all surgical specimens was <2 hours.

**Immunohistochemistry**

Paraffin blocks were cut into sections of 4 μm thickness and mounted. Slides were baked at 37°C and immunohistochemically stained for ERα or PR by means of the Bond Max autostainer (Leica Biosystems, Nussloch, Germany). Incubation was performed using mouse monoclonal antibodies from Dako (Glostrup, Denmark) against ERα, clone ID5 (dilution 1:50), and PR, clone 636 (dilution 1:100). Slides from the excised specimens had been stained before for ERα and PR shortly after surgery for diagnostic purposes according to the same staining protocol. Percentages of ERα-positive and PR-positive nuclei were estimated by consensus of 2 blinded observers including an experienced breast pathologist. ERα and PR percentages ≥10% stained cells were considered positive. Staining in the normal breast parenchyma (when present) was noted. Surgical specimen slides were reviewed microscopically or digitally.

**Statistical Analysis**

Statistic analyses were performed using IBM SPSS Statistics for Windows (version 20.0, IBM Corp., Armonk, NY). Contingency tables were made and overall agreement between the CNB and resection specimens were calculated for ERα and PR. k statistics were calculated to estimate the level of agreement beyond chance. Values of k > 0.6 were correlated with good agreement, values between 0.4 and 0.6 were considered as moderate agreement, values between 0.2 and 0.4 as fair, and values < 0.2 as slight agreement. To assess the accuracy of immunohistochemical tests on briefly fixed specimens in predicting either positive or negative receptor expression, we calculated positive and negative predictive values. To illustrate the range of difference among concordant and discordant cases, contingency tables were made for categorized data.

**RESULTS**

**Patients**

Between November 2011 and December 2012, 650 patients underwent biopsy at our same-day diagnosis breast clinic. Confirmed invasive breast carcinoma was found in 113/650 patients (17.4%) in a consecutive series. A total of 72/113 (63.7%) cases were eligible for analyses. Reasons for exclusion were the administration of neoadjuvant therapy before surgery (n = 24), multifocal disease (n = 13), no marker studies performed in the excision specimen (n = 2), and absence of remaining diagnostic material (n = 2). Mean age of the patients was 60.5 years (range, 39 to 89 y). Mean time between biopsy and surgery was 27.5 days (range, 7 to 51 d). A total of 44/72 patients (61.1%) had invasive ductal carcinoma, 9/72 (12.5%) invasive lobular carcinoma, 18/72 (25.0%) invasive ductulolobular carcinoma, and 1/72 (1.4%) tubular carcinoma.

In 6 patients, 2 biopsies of the same tumor had been performed. Therefore, 78 biopsies were compared with 72 corresponding surgical specimens.

**Comparison of Receptor Expression in CNB and Resection Specimens**

Two cases were excluded for both ER and PR evaluation, as biopsy material had been washed off during immunohistochemistry. For the ER study, 2 more cases were excluded because immunohistochemistry slides from resection specimens were missing, and digital images were not available. For the PR study, 1 case was excluded for the same reason. Morphology of all CNBs was unremarkable in the hematoxylin and eosin–stained sections, and normal breast parenchyma, when present in the CNB, always showed the expected staining pattern for ERα and PR.

ERα status was positive in 64/68 (94.1%) resection specimens. ERα status was positive in 71/74 (95.9%)
biopsies. Mean percentage of ERα-positive tumor cells was 87% in the resection specimen group and 91% in the biopsy group. In 1 patient, the ERα status was positive in the initial biopsy specimen but ERα negative in the subsequent excision specimen. This case had usual ERα staining in the normal breast parenchyma of the CNB, and PR was positive. Overall agreement for ERα was thereby 98.6% (κ = 0.85; 95% confidence interval [CI] = 0.56-1.00) (Table 1). The positive and negative predictive values for biopsy ERα status were 98.6% (95% CI = 0.91-0.99) and 100.0% (95% CI = 0.31-1.00), respectively. For categorized data we found that 60/74 (81.1%) had a biopsy score identical to the categorized score in the resection specimen (Table 2).

PR status was positive in 52/69 (75.4%) resection specimens. PR status was scored positive in 50/75 (66.7%) biopsies. Mean percentage of PR-positive tumor cells was 58% in the resection specimen group and 44% in the biopsy group. All 6 discordant cases were negative at biopsy and positive in the excision specimen. All these cases displayed the expected PR staining in the normal breast parenchyma, when present, in the CNB. Overall agreement for PR between biopsies and excision specimens was thereby 92.0% (κ = 0.81; 95% CI = 0.66-0.96) (Table 3). For PR discordant cases, mean difference in the scored PR-positive percentage between CNB and resection specimen was 35.5% (Table 4). The positive and negative predictive values for PR status were 100.0% (95% CI = 0.91-1.00) and 76.0% (95% CI = 0.54-0.90), respectively. All PR discordant cases had concordant, positive ERα scores.

**TABLE 1. Agreement of ERα Expression Between Ultrashort Fixed CNB and Conventionally Fixed Resection Specimens of Invasive Breast Carcinoma Patients (98.6%, κ = 0.85; 95% CI = 0.56-1.00)**

<table>
<thead>
<tr>
<th>Resection Specimen</th>
<th>ERα−</th>
<th>ERα+</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>CNB</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ERα−</td>
<td>3</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>ERα+</td>
<td>1</td>
<td>70</td>
<td>71</td>
</tr>
<tr>
<td>Total</td>
<td>4</td>
<td>70</td>
<td>74</td>
</tr>
</tbody>
</table>

**TABLE 2. Contingency Table for Categorized ERα Scores in Ultrashort Fixed CNB and Conventionally Fixed Resection Specimens of Invasive Breast Carcinoma Patients**

<table>
<thead>
<tr>
<th>Resection Specimen</th>
<th>0%-5%</th>
<th>10%-35%</th>
<th>50%-75%</th>
<th>90%-100%</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>CNB</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0%-5%</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>10%-35%</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>50%-75%</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>90%-100%</td>
<td>1</td>
<td>1</td>
<td>6</td>
<td>56</td>
<td>64</td>
</tr>
<tr>
<td>Total</td>
<td>4</td>
<td>1</td>
<td>7</td>
<td>62</td>
<td>74</td>
</tr>
</tbody>
</table>

**TABLE 3. Agreement of PR Expression Between Ultrashort Fixed CNB and Conventionally Fixed Resection Specimens of Invasive Breast Carcinoma Patients (92.0%, κ = 0.81; 95% CI = 0.66-0.96)**

<table>
<thead>
<tr>
<th>Resection Specimen</th>
<th>PR−</th>
<th>PR+</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>CNB</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PR−</td>
<td>19</td>
<td>6</td>
<td>25</td>
</tr>
<tr>
<td>PR+</td>
<td>0</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Total</td>
<td>19</td>
<td>16</td>
<td>35</td>
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</tbody>
</table>

**DISCUSSION**

In this study, we compared the excision specimens of 78 patients with invasive breast carcinoma with the preceding CNB from the same tumor to evaluate whether brief fixation (approximately 45 min) compromises assessment of ERα and PR expression in breast carcinoma biopsies. Agreement between CNB and resection specimen was 98.6% for ERα (κ = 0.85) and 92.0% for PR (κ = 0.81).

Discordant results for ER expression were observed in 1 case. Review of the ERα discordant case showed sparse (weak) staining at the periphery of the resection specimen (Fig. 1). Therefore, inadequate fixation of the resection specimen may have accounted for the large difference in receptor expression between the 2 samples. We also observed that CNB consistently showed well-circumscribed uniformly stained nuclei of high intensity, whereas whole sections often showed areas of weaker intensity (Fig. 2). Although weaker intensity is not taken into account during the scoring process, these findings suggest that larger specimens perhaps rely more on longer fixation time than smaller CNB for ERα analysis.

Discordant results for PR expression were observed in 6 cases (Fig. 3). In PR discordant cases, mean difference in receptor expression between CNB and resection specimen was 35.5%. The question here is whether these discrepancies were due to brief fixation or rather caused by observer variation or tumor heterogeneity. Although observer variation cannot be completely excluded as an additional source of error, we tried to minimize this through consensus scoring of all specimens by 2 observers. Rather, the observed discrepancies seem to be due to sampling from a heterogenous tumor. Normal breast...
parenchyma in the CNB, when present, always showed the expected staining pattern with dispersed strongly positive cells, indicating that antigenicity was well preserved despite the ultrashort fixation (Fig. 2).

Further, our concordance rates are completely in line with those from a meta-analysis of 21 studies including conventionally fixed tissues of 2450 patients demonstrating an overall agreement between CNB and

FIGURE 1. Positive ER staining in a briefly fixed biopsy (A and B) but negative ER staining in vast majority of corresponding resection specimen (C and D); positive ER staining in small portion of the peripheral zone of corresponding resection specimen (E and F).
Resection specimens of 92.8% for ERα ($\kappa = 0.78$) and 85.2% for PR ($\kappa = 0.66$). Moreover, in the present study, no patient had discordance between both ERα and PR between CNB and resection specimens, thereby having no consequences for the indication for hormonal therapy when based on the CNB. These data suggest that...
brief formalin fixation for as little as 1 hour does not compromise assessment of ERα and PR expression. According to the laws that govern the diffusion of fixatives into tissues, the penetrated depth in millimetres is equal to the square root of the fixation time in hours, multiplied by a coefficient of diffusibility ($K$) for that fixative. For 10% neutral buffered formalin (an aqueous solution of 4% formaldehyde), varying $K$-values have been reported.

**FIGURE 3.** Two cases of negative PR staining in briefly fixed biopsies (A and C) but positive staining in corresponding resection specimens (B and D); a concordant case with positive progesterone staining in both biopsy and resection specimen (E and F).
as methods varied greatly among studies. In the literature, a frequently cited publication reported $K = 0.78$, implying that a specimen measuring 1.6 mm in size will be fully penetrated at 1 hour formalin immersion. According to the same equation, after 2 hours immersion, formalin will have diffused to the core of a 2.2-mm-sized specimen. This knowledge implies that smaller CNB will be fixed more quickly than their larger, surgical counterparts. However, in the Consensus Recommendations on ER Testing in Breast Cancer by Immunohistochemical Analysis, it is argued that, although the penetration rate of smaller samples is faster, actual fixation is a chemical reaction that takes an additional amount of time. The cross-links that are established during the actual fixation process are thought to affect epitope recognition by antibodies. In recommendation 4 of the Consensus Recommendations, it is specified that a minimum of 25 hours fixation time in formalin is required to secure chemical fixation of a 4-mm-thick tissue block. This recommendation is based on studies that determined the amount of bound $^{14}$C-formaldehyde in animal tissues. Nevertheless, evidence is scarce, and assumed antigenicity changes remain poorly understood.

To our knowledge, this is the largest study to date to describe the effect of brief fixation on ER$\alpha$ and PR expression in invasive breast carcinoma biopsies. One previous study compared immunohistochemistry results for both ER$\alpha$ and PR expression, and 2 studies compared results for ER$\alpha$ in briefly fixed breast carcinoma specimens. In the first, ER$\alpha$ and PR results were described for different fixation times in samples from the surgical specimen of 1 patient with highly ER$\alpha$-positive and PR-positive breast carcinoma. From this specimen, 16 CNB-sized pieces were fixed in 10% formalin for different time periods (ranging from 1 to 168 h) before staining. No differences in both ER$\alpha$ and PR expression were observed between samples fixed during different time periods. The first of the other 2 studies on solely ER$\alpha$ expression was performed in a similar manner, cutting 12 small pieces per mastectomy of 10 patients with (highly ER$\alpha$-positive) invasive breast carcinoma. No significant staining differences were observed between samples from the same tumor that had been fixed for different time periods (1, 3, 6, and 9 to 10 h). Here, the authors suggest that the total time of formalin immersion of a tissue sample may be more critical for immunohistochemistry results than the theoretical time to complete the chemical fixation. Findings from our study support this latter suggestion. However, the ASCO/CAP guideline recommendation for minimum fixation time is based on the second study, which demonstrated that a minimum of 6 to 8 hours is necessary for reliable ER$\alpha$ results (n = 24). Limitations of these studies are that they were conducted in small series of tumor excision specimens with high receptor levels. In contrast, our study was conducted among a significantly larger number of patients, although there were still few negative cases in the ER$\alpha$ group. This implies that the positive predictive value can be accurately estimated, but the negative predictive value is less irrefutable.

Ideally, we would have fixed an extra set of CNBs for at least 6 hours to compare results with those of the briefly fixed portion. However, performing extra core biopsies is a burden to patients, and dividing the standard number of core biopsies between brief and conventional fixation would not have led to a final diagnosis on the same day for those patients with noninvasive lesions in the briefly fixed biopsies. Therefore, we completely switched to brief fixation when the same-day diagnostics program was initiated. The fixation duration can be quite adequately, although not completely accurately, established at 45 minutes for all biopsies. According to protocol, biopsies are immediately transported to the pathology laboratory where they are directly placed in the automated tissue processor. We are not able to comment on the effects of fixation under 45 minutes or of other preanalytical variables such as heating the fixative or agitation.

In conclusion, our findings demonstrate that brief formalin fixation of CNB for as little as 45 minutes does not seem to compromise assessment of ER$\alpha$ and PR expression by immunohistochemistry. This may have important implications for current practice as the time to final diagnosis can be significantly reduced, and reliable same-day histologic diagnosis of breast lesions becomes feasible. Further, current guidelines may have to be updated in the light of this new evidence.

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REFERENCES


