The Effect of 96-Hour Formalin Fixation on the Immunohistochemical Evaluation of Estrogen Receptor, Progesterone Receptor, and HER2 Expression in Invasive Breast Carcinoma

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Abstract

We studied the impact of 96 hours of formalin fixation on estrogen receptor (ER), progesterone receptor (PR), and HER2 testing by comparing immunohistochemical results from core biopsy specimens fixed under current American Society of Clinical Oncology (ASCO)/College of American Pathologists (CAP) guidelines with results for corresponding resection samples fixed for 96 hours. Samples enriched with cases showing weak to moderate receptor expression on core biopsy were included in the study. Cases were scored using ASCO/CAP guidelines. Of the 47 cases, only 1 case (2%) showed a qualitative change in result. However, this change was a positive ER result (H score, 1) on the 96-hour fixed resected sample compared with a negative ER result (H score, 0) for the core biopsy. Minimal changes in semiquantitative H scoring were noted for ER and PR that were likely due to tumor heterogeneity and/or intraobserver variability as the variation occurred in both directions. ER, PR, and HER2 immunohistochemical results should be considered valid for cases fixed up to 96 hours.

The assessment of estrogen receptor (ER) status of primary invasive breast cancers is mandatory owing to the considerable benefit that endocrine therapy provides for ER+ tumors and the confirmed absence of benefit for ER–tumors. Accurate determination of biomarkers is essential for the proper management of patients with breast cancer.1-6 The human epidermal growth factor receptor 2 gene (HER2) is overexpressed in approximately 15% to 20% of breast carcinomas.7-9 HER2 positivity is associated with higher rates of recurrence and mortality in patients with newly diagnosed breast cancer, and agents that target HER2 are found to be effective in the metastatic and adjuvant settings, reducing the risk of recurrence and mortality in patients with early-stage disease. This makes HER2 overexpression analysis a standard of care and HER2 another useful marker for therapeutic decision making in breast cancer.10-12

Duration of tissue fixation is one of the key preanalytic variables that must be controlled according to the recent American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) guidelines.13 The current recommendation is to fix breast tissue specimens in 10% neutral buffered formalin (NBF) for 6 to 72 hours for ER and progesterone receptor (PR) testing. Prolonged fixation time is known to reduce the immunoreactivity of many antibodies in paraffin sections.14,15 Overfixation of breast tissue could lead to false-negative hormone receptor results, particularly if accompanied by inadequate antigen retrieval protocols during ER/PR assay performance.13 It is also the statement of ASCO/CAP11 that there are no published data regarding HER2
immunohistochemical testing on tumors fixed for 48 to 72 hours except for 1 study by Ibarra and Rogers.16 Therefore, the current recommendation for fixation intervals for HER2 immunohistochemical testing remained as 6 to 48 hours of fixation for HER2. The ASCO/CAP guidelines recommend that excisional biopsy specimens that have been fixed in formalin for less than 6 hours or longer than 48 hours for HER2 testing should be excluded, or at least any negative result under those circumstances is reported with that information.11

The specific aim of this study was to examine the impact of 96 hours of formalin fixation on biomarker testing of invasive breast cancer samples. The 96-hour period was selected because weekends and holidays juxtaposed to weekends may impose significant staffing demands on a laboratory for proper handling of these specimens according to the ASCO/CAP guidelines.

Materials and Methods

Tumor tissues were obtained prospectively from invasive breast carcinomas resected at our institution within the last 1.5 years. All resection samples were obtained from the operating rooms, and all had recorded cold ischemic times of less than 1 hour. The cases included in this study were not consecutive cases. The tissues were obtained only from cases that had more than sufficient tumor available for diagnostic purposes on gross inspection. An attempt was also made to include a substantial number of weak to moderate hormone receptor–positive cases and equivocal HER2 cases as per previous core biopsy results. Therefore, of the 47 accrued cases, 13% were ER– (6/47; H score, 0), 11% were weak positive (5/47; H score, 1-100), 19% were moderately positive (9/47; H score, 101-200), and the remaining 57% were strongly positive (27/47; H score, >200). With respect to PR, 23% (11/47) were negative, 26% (12/47) were weak positive, 32% (15/47) were moderate, and the remaining 19% (9/47) were strongly positive. With respect to HER2, 9% (4/47) were scored 0, 26% (12/47) were scored 1+, 51% (24/47) were scored 2+, and 15% (7/47) were scored 3+.

The collected tissue was placed in 10% NBF for 96 hours before processing. Thereafter, tissue processing, embedding, sectioning, and staining were identical to the processes used for other routine specimens. In addition to an H&E-stained section, the study tissue was stained for ER, PR, and HER2. ER was assessed using antibody clone SP1 (Ventana, Tucson, AZ) with iVIEW detection on the BenchMark XT (Ventana). PR was assessed using antibody clone 1E2 (Ventana) with iVIEW detection on the BenchMark XT. HER2 protein was analyzed and scored using 4B5 antibody (Ventana) with iVIEW detection on the BenchMark XT. There was no change in our laboratory immunohistochemical protocols for this particular study, ie, pretreatment, antigen retrieval, and the staining platform were identical to those used for breast core biopsy specimens.

The results were qualitatively scored using the ASCO/CAP criteria, ie, 1% of cells with weak staining considered positive for ER and PR, and HER2 scored as negative (immunohistochemical scores 0 and 1+), equivocal (immunohistochemical score, 2+), and positive (immunohistochemical score, 3+). In addition, hormone receptor results were also semiquantitatively scored using an H score method.17,18 The H score is given as the sum of the percentage staining multiplied by an ordinal value corresponding to the intensity level (0, none; 1, weak; 2, moderate; 3, strong). With 4 intensity levels, the resulting score ranges from 0 (no staining in the tumor) to 300 (diffuse intense staining of the tumor). Concurrent with the resected study specimens, the prior diagnostic core biopsy specimens that have been previously stained for ER, PR, and HER2 using identical antibodies were also reviewed and rescored. All cases were scored by 1 pathologist (R.B.).

Qualitative analysis for hormone receptors was performed for the positive-negative scale and the negative, weak, moderate, or strong scale. The McNemar test19 was performed for the dichotomous (2 × 2) categorization (positive-negative). The McNemar test is appropriate for correlated proportions and tests the hypothesis that the marginal probabilities for each outcome are the same. Owing to the small number of discordant pairs, the exact version20 of the McNemar test was performed. The Stuart-Maxwell test,21,22 a generalization of the McNemar test for more than 2 categories, was performed for the negative, weak, moderate, or strong scale. Quantitative analysis was performed on the actual H scores. Spearman correlation coefficients and paired t tests were performed comparing H scores from core biopsy and 96-hour fixed resection samples. In addition, descriptive statistics are reported for the group of samples that were lower at 96 hours and for the group of samples that were higher at 96 hours. Qualitative analysis was performed for HER2 measurement using the Stuart-Maxwell test. All procedures were executed in R (version 2.13.1; The R Foundation for Statistical Computing, Vienna, Austria; http://www.R-project.org).

Results

Of the 47 cases, only 1 (2%) showed a qualitative change in the result for ER. However, this change was a positive ER result (H score, 1) on a 96-hour fixed resected sample compared with a negative ER result (H score, 0) on the core biopsy sample, a difference that was statistically insignificant (P = .999). For different categories (negative, weak, moderate, or strong) of ER results from core biopsy samples, the categorization was similar on 96-hour fixed resected samples in 43
cases (91%), a difference that was statistically insignificant ($P = .317$). For semiquantitative ER results, core biopsy H scores were compared with 96-hour fixed resected sample H scores. The H scores remained the same for 19 cases (40%), were lower than the core sample scores for 17 cases (36%), and were higher than the core sample scores for 11 cases (23%). This subtle variance was observed in all categories of cases (1 negative, 4 weakly, 7 moderately, and 16 strongly ER+ on core biopsy). The mean ER H score for core biopsy samples was 198.7 and for 96-hour fixed resected samples was 192, a difference that was statistically insignificant ($P = .120$). The Spearman correlation coefficient between core biopsy and resection specimen ER H scores was 0.890 ($P < .001$).

For PR, there was no qualitative change in results. For different categories (negative, weak, moderate, or strong) of PR results for core biopsy samples, the categorization was similar on 96-hour fixed resected samples in 37 cases (79%), a difference that was statistically insignificant ($P = .405$). For semiquantitative PR results, core biopsy H scores were compared with 96-hour fixed resected sample H scores. The H scores remained the same for 21 cases (45%), were lower than the core sample scores for 15 cases (32%), and were higher than the core sample scores for 11 cases (23%). Once again, this subtle variance was observed in all categories of cases (6 weakly, 13 moderately, and 6 strongly PR+ on core biopsy). The mean PR H score on core biopsy samples was 110 and for 96-hour fixed resected samples was 103.5, a difference that was statistically insignificant ($P = .247$). The Spearman correlation coefficient between core biopsy and resection specimen PR H scores was 0.939 ($P < .001$).

The semiquantitative ER and PR results for the core biopsy specimens and the 96-hour fixed resected samples are summarized in Table 1 and depicted in scatter plots in Figure 1A and Figure 1B, respectively.

For HER2, the immunohistochemical score remained the same for 46 cases (98%) and changed from 0 (core sample) to 1+ (96-hour fixed sample) in 1 case (2%). The Stuart-Maxwell test failed to show any statistical difference between the core biopsy and 96-hour fixed resected samples in the HER2 immunohistochemical scores ($P = .317$).

### Table 1

<table>
<thead>
<tr>
<th></th>
<th>Lower at 96 h</th>
<th>Mean Difference (SD) for Lower at 96 h [Range]</th>
<th>Same at 96 h</th>
<th>Higher at 96 h</th>
<th>Mean Difference (SD) for Higher at 96 h [Range]</th>
</tr>
</thead>
<tbody>
<tr>
<td>ER H score</td>
<td>17 (36)</td>
<td>34.9 (22.6) [10-80]</td>
<td>19 (40)</td>
<td>11 (23)</td>
<td>-25.5 (21.0) [-70 to -1]</td>
</tr>
<tr>
<td>PR H score</td>
<td>15 (32)</td>
<td>46.7 (33.4) [10-120]</td>
<td>21 (45)</td>
<td>11 (23)</td>
<td>-35.9 (24.4) [-90 to -10]</td>
</tr>
<tr>
<td>HER2 immunohistochemical score</td>
<td>0 (0)</td>
<td>—</td>
<td>46 (98)</td>
<td>1 (2)</td>
<td>—</td>
</tr>
</tbody>
</table>

ER, estrogen receptor; PR, progesterone receptor.

* Data are given as number (percentage) unless otherwise indicated. ER Spearman correlation coefficient, 0.890; PR Spearman correlation coefficient, 0.939; HER2 Spearman correlation coefficient, 0.996; for all, $P < .001$.

#### Figure 1

**H-score scatterplots for estrogen receptor (A) and progesterone receptor (B).** Interpretation of H scores is as follows: 0, negative; 1-100, weak positive; 101-200, moderately positive; >200, strongly positive.
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There were no clinically significant changes observed for 96-hour fixation, defined as a positive category for a core biopsy sample being negative on the 96-hour fixed specimen. Examples of cases showing similar results on core biopsy and 96-hour formalin-fixed resected samples are shown in Image 1 and Image 2.

Discussion

Improving the accuracy of hormone receptor and HER2 testing to increase the usefulness of ER, PR, and HER2 as prognostic and predictive markers in breast cancer is an ongoing challenge. The duration of tissue fixation is one of the crucial preanalytic variables, in addition to cold ischemic time, tissue processing method, and fixative type, that must be controlled and reported for the purposes of standardization and accuracy according to ASCO/CAP guideline recommendations.11,13 Goldstein et al,14 in their study of strongly and diffusely ER+ cases used to evaluate the amount of ER staining lost due to shorter fixation times, reported the minimum formalin fixation time requirement for reliable immunohistochemical ER results as 6 to 8 hours, regardless of the type or size of specimen. Samples for HER2 testing are also recommended to be fixed in 10% NBF for a minimum of 6 hours, regardless of sample size.11

In this study of the impact of prolonged fixation of 96 hours on breast cancer samples, we focused more on low to
moderate ER/PR-expressing and weak to equivocal HER2-expressing tumors because it is these tumors that are likely to be more sensitive to prolonged fixation than high expressing tumors. In the present study, about 77% of our HER2 cases were weak to moderately (equivocal) immunoreactive, while low to moderate ER and PR expression rates were about 30% and 55%, respectively.

A few prior studies have addressed the issue of prolonged fixation. Apple et al.,23 in their study of various preanalytic factors on a mastectomy specimen with a 4-cm, known strong ER+/PR+ (95% of tumor cells positive with an intensity of 3+) and HER2– invasive lobular carcinoma (determined by previous core needle biopsy results), tested different fixation times from 1 to 168 hours and reported that fixation time did not alter the final results of ER and PR staining. Ibarra and Rogers16 examined 10 invasive breast cancer cases exhibiting 3+ expression of HER2 on core biopsy and found its expression to be unaffected by different buffered formalin fixation times from 3 to 120 hours. Tong et al.,24 in their study of 101 invasive breast cancer cases with a prolonged fixation period range of 73 hours, 33 minutes to 102 hours, 30 minutes, concluded that fixation for limited periods beyond 72 hours does not result in a reduction in assay sensitivity in the determination of ER, PR, or HER2 immunohistochemical status.

There also are reports about the effect of extensive overfixation (>96 hours) on breast immunohistochemical results. Oyama et al.25 compared the immunoreactivity for ER between overnight and very long-term (3 weeks) fixation.

Similar results were obtained on the resected sample fixed for 96 hours (E, H&E, ×200; F, ER, ×200; G, PR, ×200; H, HER2, ×200).
Among 10 cases, there were 2 cases in which the Allred score decreased dramatically from 5 through 7 to 0. Therefore, they concluded that there is some degree of influence of prolonged fixation on the immunoreactivity for ER. In addition, Arber examined the effect of formalin fixation on different invasive ductal carcinomas fixed from 7 to 154 days. Of 23 ER+ cases, a significant reduction in immunoreactivity (≥2 grades) was identified in 3 samples, occurring at 57 to 64 days. For 21 PR+ cases, only 1 showed a significant reduction (from 3+ to 1+) at 120 days. Of 9 HER2+ (2+ or 3+) cases, 4 became negative (1+ or 0) at 20, 42, 49, and 99 days, showing that the immunoreactivity of breast prognostic markers is reduced by formalin overfixation only after extensive fixation that may not be clinically relevant.

In agreement with the aforementioned studies, our results show no significant differences in qualitative assessment of ER, PR, and HER2 results due to formalin fixation of 96 hours. The results show a slight difference in quantitative H scores for hormone receptors, but we favor tumor heterogeneity and possibly intraobserver variability as the cause for this slight variation rather than prolonged fixation. This conclusion is supported by an almost equal number of specimens that show higher and lower scores on 96-hour fixed samples compared with the corresponding core biopsy sample fixed in a standardized manner. Although tumors with hormone expression in the low to moderate range are more likely to demonstrate heterogeneous immunostaining between core biopsy and resection samples compared with high-expressing...
tumors, this did not alter the clinically relevant result in this study.

Finally, we chose to examine the 96-hour period because it accounts for long weekends and remote sites, where tissue handling may be a challenge. We conclude that, ER, PR, and HER2 immunohistochemical results should be considered valid for cases fixed in formalin up to 96 hours, specifically for the antibody clones that we validated in this study.

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References


Similar results were obtained on the resected sample fixed for 96 hours (E, H&E, ×200; F, ER, ×200; G, PR, ×200; H, HER2, ×200). The cytoplasmic granular staining (occasionally seen in tumors with apocrine differentiation) with HER2 was observed on the core biopsy and resection specimens.
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