Brief fixation enables same-day breast cancer diagnosis with reliable assessment of hormone receptors, E-cadherin and HER2/Neu

Altuna Halilovic,1 Joris Bulte,2 Yvonne Jacobs,1 Hanneke Braam,1 Patricia van Cleef,1 Margrethe Schlooz-Vries,2 Annelies Werner,2 Oliver Boelens,3 Iris Nagtegaal,1 Hans de Wilt,2 Peter Bult1

ABSTRACT

Aims Preoperative core needle biopsy (CNB) is commonly used to confirm the diagnosis of breast cancer. For treatment purposes and for determining histological type, especially in case of neoadjuvant therapy, oestrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor 2 (HER2) status and E-cadherin assessments are crucial. Considering the increasing demand for same-day diagnosis of breast lesions, an accelerated method of CNB processing was developed, in which the tissue fixation time is radically reduced.

Methods To determine whether short fixation time frustrates assessment of ER, PR and E-cadherin immunohistochemistry (IHC) and HER2 fluorescence in situ hybridisation (FISH), 69 consecutive patients with 70 invasive breast carcinomas were included through the same-day diagnostics programme of breast lesions of the Radboud university medical center and the hospital Pantein. IHC for ER, PR and E-cadherin and HER2 FISH were compared between CNBs fixed for approximately 60–90 min and traditionally fixed resection specimens.

Results Overall agreement between CNBs and resection specimens was 98.6% for ER (p<0.001; κ=0.93), 90.0% for PR (p<0.001; κ=0.75), 100% for E-cadherin (p<0.001; κ=1.00) and 98.6% (p<0.001; κ=0.94) for HER2 FISH. Positive and negative predictive values were respectively 98.4% and 100% for ER, 95.9% and 76.2% for PR, 100% and 100% for E-cadherin and 90% and 100% for HER2 FISH.

Conclusions Hormone receptors and E-cadherin IHC and HER2 FISH are highly comparable between briefly fixed CNBs and the corresponding traditionally fixed resection specimens, and can therefore reliably be used in the daily clinical practice of same-day diagnostics of breast cancer.

INTRODUCTION

Preoperative core needle biopsy (CNB) is commonly used to confirm the diagnosis of breast cancer. Particularly in the case of neoadjuvant therapy, status assessment of oestrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) on CNB is crucial.1 2 Also, E-cadherin assessment may have added value next to morphological examination of the H&E slide to diagnose invasive lobular carcinoma,3–6 which may influence additional imaging and subsequent therapy. Therefore, status assessment of ER, PR, E-cadherin and HER2 is standardly performed on all cases of invasive breast cancer diagnosed in our hospitals. In our hospital, HER2 status assessment is determined by double probe fluorescence in situ hybridisation (FISH), which is in line with the American Society of Clinical Oncology/College of American Pathologists (ASCO/CAP) guideline.2

Over the years, there has been an increasing demand for same-day diagnosis of breast lesions, because it improves patient satisfaction and decreases patient anxiety.7–9 In our hospitals, this has led to an accelerated method of CNB processing (aCNB) in which the tissue fixation time is radically reduced.10 Preanalytical factors such as fixation time, however, can influence above-mentioned test results.1 2 11 12 The ASCO/CAP guideline recommends a minimum fixation time of 6 hours in 10% neutral buffered formalin (NBF).1 2 This is substantially longer than for aCNBs, which in our hospitals is <2 hours. There is only some published data, suggesting that shorter fixation time does not hinder assessment of the ER, PR and HER2 status.13–19 No studies have been published studying the effect of short fixation time on E-cadherin assessment.

The aim of this study is therefore to evaluate the effect of brief fixation of CNBs on ER, PR and E-cadherin immunohistochemistry (IHC) and HER2 FISH in invasive breast cancer.

MATERIALS AND METHODS

Patients

The Radboud university medical center (Radboudumc) has employed a same-day diagnostics programme for breast lesions using aCNB since January 2008,10 and the affiliated hospital Pantein in Boxmeer, since February 2010. From these programmes, a representative cohort was included for the current study, including all consecutive patients (69) with invasive breast cancer between September 2012 and July 2013, if both the CNB and resection were obtained in one of two hospitals and the cold ischaemia time of the resection specimens was <2 hours, excluding patients receiving neoadjuvant therapy. No ethical approval was required according to current Dutch legislation, as we used leftover coded material.20

Tissue processing

Fresh CNBs, extracted using 14–18 G needles on a true cut CNB gun (Bard Biopsy Systems), were immediately placed in Unifix (Klinipath BV Duiven,
The Netherlands). Fixation time prior to processing was estimated at approximately 30–60 min. CNBs were placed in a rapid microwave histoprocessor (Pathos, Milestone Srl, Sorisole, Italy) which processes the specimens in approximately 1 hour and 26 min. The detailed protocol of the microwave histoprocessor is described elsewhere. Total fixation time for aCNBs varied from approximately 60 up to 90 min. Finally, CNBs were routinely embedded in paraffin blocks. Excision specimens were sliced in approximately 4–5 mm thick slices and were fixed overnight in accordance with the current ASCO/CAP guideline recommendations (≥6 hours).2, 21

**Immunohistochemistry**

An extensive description of the immunohistochemical process was previously published.21 Antibody-specific variances are specified: heat-induced antigen retrieval for ER and E-cadherin staining was conducted in 10 mM EDTA (pH 9.0) in a pretreatment module (Thermo Scientific, Thermo Fisher Scientific, Waltham, Massachusetts, USA) and for PR staining in 10 mM sodium citrate (pH 6.0) in a high-pressure cooker (2100 Retriever, Aptum, Bio-Connect, Huissen, the Netherlands). Slides were incubated with either rabbit monoclonal antibody to E-cadherin (clone SP1, dilution 1:80, Thermo Scientific), mouse monoclonal antibody against PR (clone PgR636, dilution 1:250, Dako, Glostrup, Denmark) or mouse monoclonal antibody against E-cadherin (clone SPM471, dilution 1:300, Thermo Scientific) at room temperature (60 min).

**Immunohistochemical scoring**

All specimens were scored independently by two blinded observers (PB and AH), including an experienced breast pathologist. In case of interobserver discrepancies, consensus was reached after discussion at a two-headed microscope. Scoring of ER-positive and PR-positive nuclei was performed according to the current ASCO/CAP guideline.2 The positive and negative predictive value for PR was 95.9% and 76.2%, respectively. Seven tumours were PR discordant.

**HER2 FISH**

Sections of 4 μm were cut, mounted and subsequently dried at 56°C (for at least 45 min). They were deparaffinised in xylene and brought to tap water through graded alcohols. Pretreatment with 10 mM sodium citrate (pH=6.0) at 96°C (10 min) followed. After cooling down and rinsing in demineralised water (demin), slides were treated with 0.01 M HCl (5 min) followed by pepsin digestion at 37°C (7 min for CNBs and 15 min for resection specimens). Following rinsing in 0.01 M HCl and subsequently phosphate buffered saline (PBS), slides were fixed in 1% formaldehyde/PBS (5 min). The slides were finally dehydrated in increasing ethanol series and air-dried. For the in situ hybridisation (ISH) process, 10 μL custom-made probe ERBB2-HER2/Neu 17Q12/SE-17 (KB-00007 Kreatech, Leica, Rijswijk, The Netherlands) was applied to the pretreated slides, the area cover-slipped and sealed with photo glue. The slides were subsequently placed in a hybridiser (DAKO) for denaturation at 80°C (10 min) and hybridisation overnight at 37°C. Following rinsing successively in 2×saline sodium citrate (SCC) (5 min at 45°C), 2×SCC/0.3% nonyl phenoxypolyethoxethanol (NP-40) (3 min at 73°C), 2×SCC and demin, slides were again dehydrated in increasing ethanol solutions and air-dried. Finally, the slides were mounted with a solution containing both 4,6-diamidino-2-phenylindole (DAPI) and Vectashield (Vector, Burlingeham, Amsterdam, The Netherlands). HER2 and CEP17 signals were scored using a Leica DMRBE (Leitz) fluorescence microscope as previously described.22 Scoring was performed by three experienced technicians (PVG, YJ and HB), and in case of doubt, an experienced breast pathologist (PB) assisted the technicians. HER2 amplification was defined as an HER2/ CEP17 ratio of 2 or more, which is in concordance with the current ASCO/CAP guideline.2

**Statistical analysis**

Data were analysed using IBM SPSS Statistics for Windows (V20.0, IBM, Armonk, New York, USA). For ER, PR, E-cadherin and HER2 status assessments, level of agreement was calculated and Cohen’s κ statistics were assessed to estimate the level of agreement between CNB and resection specimen results beyond chance. κ values were judged as previously described.23 Positive and negative predictive values were calculated in order to determine the accuracy of CNB IHC and FISH in predicting positive or negative receptor expression in the resection specimen. Quantitative correlation between ER and PR percentages in CNBs and resection specimens was calculated by means of Spearman’s correlation coefficient, after subdividing results into categorised data (0%–9%, 10%–30%, 31%–70% and 71%–100%).

**RESULTS**

**Patients**

In total, 69 consecutive patients with 70 invasive breast cancer underwent aCNB, with subsequent resection of the tumour. One patient had two primary breast carcinomas, both on the right side. Further patient and clinicopathological characteristics are shown in table 1. The H&E staining showed no differences between CNB and resection specimen regarding morphological features such as nuclear appearance, growth pattern and mitoses.

**Hormone receptor expression**

The correlation and according overall agreement between ER and PR percentages in CNBs and resection specimens are shown in tables 2 (for ER) and 3 (for PR). The positive and negative predictive value for ER expression was 98.4% and 100%, respectively. Only one tumour was ER-positive in the CNB and negative in the resection specimen. This case was PR-negative in both the CNB and resection specimen.

The positive and negative predictive value for PR was 95.9% and 76.2%, respectively. Seven tumours were PR discordant.

---

**Table 1 Patient and clinicopathological characteristics**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>N=69 patients/70 tumours (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (year, mean (range))</td>
<td>61.2 (35–87)</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>69 (100)</td>
</tr>
<tr>
<td>Male</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Histological subtype</td>
<td></td>
</tr>
<tr>
<td>Invasive ductal carcinoma</td>
<td>57 (81.4)</td>
</tr>
<tr>
<td>Invasive lobular carcinoma</td>
<td>7 (10)</td>
</tr>
<tr>
<td>Tubular carcinoma</td>
<td>2 (2.9)</td>
</tr>
<tr>
<td>Mucinous carcinoma</td>
<td>2 (2.9)</td>
</tr>
<tr>
<td>Micropapillary invasive carcinoma</td>
<td>1 (1.4)</td>
</tr>
<tr>
<td>Apocrine carcinoma</td>
<td>1 (1.4)</td>
</tr>
<tr>
<td>Bloom-Richardson grading</td>
<td></td>
</tr>
<tr>
<td>Grade I</td>
<td>13 (18.6)</td>
</tr>
<tr>
<td>Grade II</td>
<td>26 (37.1)</td>
</tr>
<tr>
<td>Grade III</td>
<td>31 (44.3)</td>
</tr>
</tbody>
</table>
E-cadherin expression

E-cadherin was positive in 62/70 (88.6%) CNBs and in the same number of resection specimens. Overall agreement for E-cadherin expression was 100% ($\kappa=1.00$; $p<0.001$), as were the positive and negative predictive values.

HER2/Neu amplification

HER2 was amplified in 10/70 (14.3%) CNBs and in 9/70 (12.9%) resection specimens. Overall agreement for HER2 FISH was 98.6% ($p<0.001$; $\kappa=0.94$). The positive and negative predictive value was 90% and 100%, respectively. There was only one discordant case in the present study, in which the HER2 status was positive in the CNB and negative in the resection specimen. This case showed an original HER2/CEP17 ratio of 58/27 (=2.15) in 20 nuclei for the CNB and 38/33 (=1.15) in 20 nuclei for the resection specimen. Upon re-evaluation, the HER2/CEP17 ratio was 177/87 (=2.03) in 60 nuclei for the CNB and 130/59 (=2.20) in 40 nuclei for the resection specimen.

Discussion

In this study, we have shown that brief fixation (60–90 min) of CNBs does not frustrate assessment of ER, PR and E-cadherin IHC and HER2 FISH. Overall concordance between CNB and resection specimen was 98.7% for ER ($\kappa=0.93$), 90.0% for PR ($\kappa=0.75$), 100% for E-cadherin ($\kappa=1.00$) and 98.6% for HER2 FISH ($\kappa=0.94$). Previously, we have already shown, in a two-centre study, that Ki67 analysis in breast cancer CNBs is not hampered by a very short fixation time.21

When comparing our results with traditionally fixed CNBs and resection specimens, similar to higher levels of agreement were seen in the present study. A large meta-analysis including ER status assessment of 2450 patients showed an overall agreement between traditionally fixed CNBs and resection specimens of 92.8% ($\kappa=0.78$).24 Furthermore, data available on the effect of briefly fixed CNB on ER expression showed results analogous to ours, although most of these studies had a small sample size, with only highly expressing tumours included.13–17 Only one study,23 stated that the minimum formalin fixation time should be at least 6–8 hours in order to maintain reliable results for ER expression, regardless of type or size of the specimen. This study however included a small sample size, had an extra processing step in which samples were temporarily stored in 100% ethanol after fixation and there was no precise account of the cold ischaemia time. Most importantly, they compared ER staining using the Q-score method which values intensity and distribution of staining equally.26 This method therefore emphasises both the intensity of staining as the percentage of positive cells, whereas we focused primarily on true discordance (negative vs positive receptor status).

There was only one discordant case for ER expression in the present study (table 2 and figure 1). This case showed positive (41%–50%) expression in the CNB and negative (0%–9%) expression in the resection specimen. Upon re-evaluation, the CNB showed moderately to strongly stained nuclei which were quite uniformly distributed. The resection specimen displayed positive staining of normal breast tissue and several foci of positive invasive cells, in total <10%, staining weakly and located at the generative cells, in total <10%, staining weakly and located at the resection specimen. Upon review, the CNB showed moderately to strongly stained nuclei which were quite uniformly distributed. The resection specimen displayed positive staining of normal breast tissue and several foci of positive invasive cells, in total <10%, staining weakly and located at the

---

Table 2  Correlation of oestrogen receptor percentages between accelerated core needle biopsies and conventionally fixated resection specimens in 70 invasive breast carcinomas

<table>
<thead>
<tr>
<th>Resection specimen</th>
<th>0%–9%</th>
<th>10%–30%</th>
<th>31%–70%</th>
<th>71%–100%</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>CNB 0%–9%</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td>10%–30%</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>31%–70%</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>71%–100%</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>57</td>
<td>59</td>
</tr>
<tr>
<td>Total</td>
<td>9</td>
<td>0</td>
<td>4</td>
<td>57</td>
<td>70</td>
</tr>
</tbody>
</table>

Overall agreement of 98.6% between CNBs and resection specimens ($\kappa=0.93$, $p<0.001$; $\tau=0.85$, $p<0.001$).

CNB, core needle biopsy.
periphery of the tumour. The ASCO/CAP guideline recommends tumours with >1% expression to be scored positive; however, Dutch guidelines maintain a threshold of positivity of ≥10%. Remarkably, there were several resection specimens which stained stronger at the periphery of the tumour compared with the centre, suggesting an (fixation) artefact. Others have indeed suggested that inadequate or delayed fixation of the centre of the tumour can lead to false-negative ER expression.

26,28 However, this seems less likely for our cases, because as a general policy all included resection specimens were sliced in approximately 4.5 mm thick slices and then fixed in NBF with cold ischaemia time <2 hours. Other reasons for the discordance could be tumour heterogeneity and sampling error. CNBs are usually taken from the periphery of the tumour to avoid a necrotic or fibrotic centre. If some tumours especially stain at the periphery of the tumour, this might explain the discordance.

For PR expression, there were seven discordant cases (table 3 and figure 2). Two tumours were PR-positive in the CNB and negative in the corresponding resection specimen. Both resection specimens showed a positive internal control, also with dispersed nuclear staining in <10% of invasive nuclei, mostly located at the periphery of the tumour. CNBs displayed a strong yet heterogeneous staining in 10%–20% and 21%–30% of invasive nuclei, respectively. The same arguments which could explain the discrepant results apply here as for the ER discordant case. Five tumours were positive in the CNB and positive in the resection specimens. These discordant cases showed a highly heterogeneous expression of PR-positive invasive nuclei in the resection specimens. The PR expression percentage was lowest in 3/5 tumours showing 10%–20% expression, 1/5 with 20%–30% expression and 1/5 with 50%–60% expression. Heterogeneous and low receptor expression, together with sampling error, is therefore likely to blame for the discordance. Observer variation is an unlikely explanation for the discordance, since we tackled this confounder by scoring all specimens independently by two blinded observers. In case of discrepancies between the observers, consensus was reached after reviewing the slides together. There was an excellent interobserver agreement for PR (κ=0.89 for CNBs and κ=0.90 for resection specimens) and for ER (κ=1.00 for both CNBs and resection specimens) and E-cadherin (κ=1.00 for both CNBs and resection specimens).

Data comparing PR expression between traditionally fixed CNBs and resection specimens of 2448 patients showed similar levels of agreement (85.2%; κ=0.66) as our briefly fixed CNBs. Furthermore, data comparing the effect of briefly fixed CNB on PR expression showed comparable results. Finally, because corresponding ER expression was positive (and concordant) in all discordant PR cases, this discordance has no direct therapeutical consequences, when hormonal therapy is based on the CNB.

E-cadherin expression showed 100% concordance between briefly fixed CNBs and resection specimens. To our knowledge, this is the first study to compare E-cadherin expression in briefly fixed CNB and resection specimens. No published data were found comparing E-cadherin expression in conventionally fixed CNBs and resection specimens.

Overall agreement between CNBs and resection specimens for HER2 FISH was 98.6%. Papers evaluating HER2 FISH assessment in traditionally fixed CNBs and resection specimens show a comparable level of accordance. Also, studies, yet with very small sample sizes, comparing briefly fixed CNBs (with quite varying fixation times) show similar results. There was only one discordant case in the present study, which showed a positive HER2 status in the CNB and a negative one in the resection specimen. After re-evaluation of the score, in

<table>
<thead>
<tr>
<th>Table 3</th>
<th>Correlation of progesterone receptor percentages between accelerated core needle biopsies and conventionally fixated resection specimens in 70 invasive breast carcinomas</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Resection specimen</td>
</tr>
<tr>
<td></td>
<td>0%–9%</td>
</tr>
<tr>
<td>CNB</td>
<td></td>
</tr>
<tr>
<td>0%–9%</td>
<td>16</td>
</tr>
<tr>
<td>10%–30%</td>
<td>2</td>
</tr>
<tr>
<td>31%–70%</td>
<td>0</td>
</tr>
<tr>
<td>71%–100%</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>18</td>
</tr>
</tbody>
</table>

Overall agreement of 90.0% between CNBs and resection specimens (κ=0.75, p<0.001; r_s=0.89, p<0.001).

CNB, core needle biopsy.

Figure 2  Examples of progesterone receptor (PR) expression (200×).

PR concordant case with high (91%–100%) expression in both the core needle biopsy (CNB) (A) and resection specimen (B); PR discordant case with positive (10%–20%) expression in the CNB (C) and negative (0%–9%) expression in the resection specimen (D); PR discordant case with negative (0%–9%) expression in the CNB (E) and positive expression (10%–20%) in the resection specimen (F).
fact both the CNB and the resection specimen should be assessed as HER2-positive. In the vicinity of the tumour in the resection specimen, we observed a dense lymphocytic infiltrate. We believe these cells may have been included in the initial assessment of the HER2/CEP17 ratio and that this accounts for the observed discrepancy. Similar rates of within-laboratory discrepancies have been previously observed in HER2 FISH testing. For HER2 IHC, a moderate level of agreement cannot precisely pinpoint the preanalytical variations. With the vital use of essential biomarkers. Our testing. For HER2 IHC, a moderate level of agreement cannot precisely pinpoint the preanalytical variations.

We believe these cells may have been included in the initial assessment of the HER2/CEP17 ratio and that this accounts for the observed discrepancy. Similar rates of within-laboratory discrepancies have been previously observed in HER2 FISH testing. For HER2 IHC, a moderate level of agreement cannot precisely pinpoint the preanalytical variations. With the vital use of essential biomarkers. Our testing.

CONCLUSIONS

We have shown that brief fixation of CNBs does not frustrate expression of ER, PR and E-cadherin IHC and HER2 FISH, further supporting same-day diagnostics of breast cancer lesions with the vital use of essential biomarkers. Our findings provide evidence to modify current practice guidelines to accommodate rapid fixation (60–90 min) for ER, PR and E-cadherin IHC and HER2 FISH.

Take home messages

- There is an increasing demand for same-day diagnosis of breast lesions, because it improves patient satisfaction and decreases patient anxiety.
- We have shown that brief fixation of core needle biopsies does not frustrate expression of oestrogen receptor, progesterone receptor and E-cadherin immunohistochemistry and human epidermal growth factor receptor 2 fluorescence in situ hybridisation, which are crucial factors in the diagnosis and treatment of breast cancer.
- This further supports same-day diagnostics of breast cancer lesions with the vital use of essential biomarkers.
- Our findings, together with previously published data, suggest a change of American Society of Clinical Oncology/College of American Pathologists guideline on tissue fixation time.

Handling editor Runjan Chetty

Contributors All authors contributed extensively to the work presented in this article. PB and JHWdW conceived the study and jointly designed the experiments with AH and JPB. Experiments were performed by AH, YNAI, HB, PHoC and PB, AH, JPB, PB, IDN, MS-V, JEMW and OBB performed data collection and analysis. AH, JPB and PB wrote the first concept of the manuscript. All authors discussed the results and implications, and commented on the manuscript at all stages.

Competing interests IN received a speaker’s fee from Sakura Finetek Europe BV.

Provenance and peer review Not commissioned; externally peer reviewed.

REFERENCES

5 Goldstein NS. Does the level of E-cadherin expression correlate with the primary breast carcinoma infiltration pattern and type of systemic metastases? Am J Clin Pathol 2002;118:425–34.


Brief fixation enables same-day breast cancer diagnosis with reliable assessment of hormone receptors, E-cadherin and HER2/Neu

Altuna Halilovic, Joris Bulte, Yvonne Jacobs, Hanneke Braam, Patricia van Cleef, Margrethe Schlooz-Vries, Annelies Werner, Oliver Boelens, Iris Nagtegaal, Hans de Wilt and Peter Bult

J Clin Pathol  published online March 21, 2017

Updated information and services can be found at:
http://jcp.bmj.com/content/early/2017/03/21/jclinpath-2017-204362

These include:

References
This article cites 34 articles, 18 of which you can access for free at:
http://jcp.bmj.com/content/early/2017/03/21/jclinpath-2017-204362
#BIBL

Email alerting service
Receive free email alerts when new articles cite this article. Sign up in the box at the top right corner of the online article.

Notes

To request permissions go to:
http://group.bmj.com/group/rights-licensing/permissions

To order reprints go to:
http://journals.bmj.com/cgi/reprintform

To subscribe to BMJ go to:
http://group.bmj.com/subscribe/