Assessment of HER2 status in breast cancer biopsies is not affected by accelerated tissue processing

Joris P Bulte,1 Altuna Halilovic,2 Shona Kalkman,3 Patricia H J van Cleef,2 Paul J van Diest,3 Luc J A Strobbe,4 Johannes H W de Wilt1 & Peter Bult2

1Department of General Surgery, Radboud University Medical Center, 2Department of Pathology, Radboud University Medical Center, Nijmegen, 3Department of Pathology, University Medical Center Utrecht, Utrecht, and 4Department of Surgical Oncology, Canisius-Wilhelmina Hospital, Nijmegen, the Netherlands

Aims: To establish whether core needle biopsy (CNB) specimens processed with an accelerated processing method with short fixation time can be used to determine accurately the human epidermal growth factor receptor 2 (HER2) status of breast cancer.

Methods and results: A consecutive case–series from two high-volume breast clinics was created. We compared routine HER2 immunohistochemistry (IHC) assessment between accelerated processing CNB specimens and routinely processed postoperative excision specimens. Additional amplification-based testing was performed in cases with equivocal results. The formalin fixation time was less than 2 h and between 6 and 72 h, respectively. Fluorescence in-situ hybridisation and multiplex ligation-dependent probe amplification were used for amplification testing. One hundred and forty-four cases were included, 15 of which were HER2-positive on the routinely processed excision specimens. On the CNB specimens, 44 were equivocal on IHC and required an amplification-based test. Correlation between the CNB specimens and the corresponding excision specimens was high for final HER2 status, with an accuracy of 97% and a kappa of 0.85.

Conclusions: HER2 status can be determined reliably on CNB specimens with accelerated processing time using standard clinical testing methods. Using this accelerated technology the minimum 6 h of formalin fixation, which current guidelines consider necessary, can be decreased safely. This allows for a complete and expedited histology-based diagnosis of breast lesions in the setting of a one-stop-shop, same-day breast clinic.

Keywords: accelerated processing, breast cancer, core needle biopsy, diagnosis, formalin fixation time, HER2 status, histology, one-stop-shop

Introduction

For breast cancer diagnosis, a pre-operative histological confirmation is mandatory, including determination of oestrogen and progesterone receptor (ER and PR) and human epidermal growth factor receptor 2 (HER2) status for treatment decisions, such as indication for and selection of neo-adjuvant systemic therapy. Due to increasing emphasis on speed in the diagnostic process, an accelerated processing method for core needle biopsy (aCNB) for breast lesions was developed in our breast clinics.1,2 With the use of microwave-based rapid histoprocessors the fixation time for certain histology specimens, such as CNBs, can be reduced to less than 1 h. The American Society of Clinical Oncology and the College of

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American Pathologists (ASCO/CAP) guideline recommends a minimum fixation time of 6 h in neutral-buffered formalin (NBF), precluding the use of accelerated processing techniques. Although there is ample literature supporting a maximum fixation time, there are actually few data to support the recommended minimum fixation time of 6 h. The previous version of the ASCO/CAP guideline cites only one pilot study in support of a minimum fixation time, which was, however, omitted in the 2013 version of the guidelines. The authors of a recent systematic review found six studies regarding short fixation time for receptor status in general, only two of which addressed HER2 immunohistochemistry (IHC) testing. Both were small-scale pre-clinical pilot studies comparing samples from 10 and one HER2-positive tumours, respectively, testing fixation times varying between 1 and 168 h. The authors concluded that data on the reliability of hormone receptor status or HER2 status after a short fixation time are scarce, but the status of highly expresser breast cancers does not seem to be altered. The reliability of hormone receptor status and fluorescence in-situ hybridisation (FISH) determination of HER2 status is supported further by a more recent publication.

As aCNB is a relatively new technique, there is little information available on the reliability of hormone receptor and HER2 determination on aCNB specimens. The Dutch 2012 breast cancer guideline suggests using either IHC or FISH as a first-line test and using more advanced techniques, such as multiplex ligation-dependent probe amplification (MLPA) to establish a definitive HER2 status if the first result is equivocal. A recent preclinical study of 40 tissue fragments cut to the size of 17 g biopsy specimens using much shorter fixation times (1–24 h) suggests that HER2 testing using both IHC and FISH is reliable. However, no studies have been published regarding actual CNB specimens. Most clinics perform HER2 status only for malignant cases and report this as an addendum to the pathology report the next day. To use pre-operative determination of HER2 status for making adequate treatment decisions in routine breast cancer care, it is crucial to know whether it can be determined reliably on aCNB specimens. We investigated the reliability of HER2 status assessment in CNB specimens that underwent accelerated processing. Herein, we compared IHC results of aCNB with routinely processed resection specimens using a consecutive patient series from two high-volume breast cancer units that have adopted the aCNB method for routine care.

Materials and methods

Patients

Between November 2011 and January 2012 we included consecutive cases from the University Medical Centre Utrecht (UMC Utrecht) breast clinic, and between September 2012 and July 2013 we included consecutive cases from Radboud University Medical Center (Radboud UMC). 144 cases in total. All samples were from patients who underwent aCNB and subsequent resection of the corresponding lesion. Neo-adjuvant systemic therapy and multifocal disease were exclusion criteria. Excision specimens were used as controls. These were fixed overnight in compliance with the current ASCO/CAP guideline recommendations (≥6 h fixation time). Cold ischaemia time in all surgical specimens was less than 2 h; the total fixation time was approximately 6–72 h, with three specimens fixed for a maximum 95 h because of fixation during a holiday weekend.

Tissue processing

In Radboud UMC, all aCNB specimens were transported in 3.7–4% formaldehyde solution containing zinc sulphate, acetate buffered at pH 5.6–5.8 (Unifix; Klinipath BV, Duiven, the Netherlands). Fixation time prior to registration at the pathology department was approximately 30 min. We included an extra 30-min fixation step with formaldehyde if material was registered within 30 min of biopsy acquisition. Thereafter, specimens were processed using a rapid microwave histoprocessor (Pathos; Milestone Srl, Sorisole, Italy). The processing programme started with a 20-min formaldehyde fixation step, leading to a total fixation time of 50–80 min. The full programme takes 1 h 26 min. Details of this process have been described previously.

In UMC Utrecht, all aCNB specimens were transported in 10% neutral buffered formalin (NBF) and sent to the pathology laboratory to be registered (estimated fixation time approximately 15 min). Specimens were placed subsequently in an automated tissue processor (Peloris™; Leica, Valkenswaard, the Netherlands). The processing programme started with a 30-min formaldehyde fixation step under vacuum, leading to a total fixation time of approximately 45 min. The full programme, which has been described previously in greater detail, takes 1 h 39 min.

HER2 IHC staining

In Radboud UMC, HER2 IHC staining was performed manually using the Dako HercepTest™ Kit (Code © 2018 The Authors. Histopathology Published by John Wiley & Sons Ltd, Histopathology, 73, 81–89.
K5204: Dako, Glostrup, Denmark), largely according to the protocol in the manufacturer’s guide. In short, 4-μm sections of all paraffin blocks were cut and mounted. Slides were incubated at 56°C, deparaffinised in xylene and brought to denim water through graded alcohols. Heat-induced (121°C) antigen retrieval with sodium citrate (pH 6.7) in a pressure-cooker followed (30 min). Following washing in wash buffer, peroxidase-blocking reagent was applied for 5 min. Next, the primary antibody (prediluted rabbit anti-human HER-2 protein) was applied (30 min). Afterwards, slides were incubated with visualisation reagent for 30 min. Following rinsing in washing buffer, visualisation was achieved with bright diaminobenzidine (dilution 1:25, Immunologic, Duiwen, the Netherlands). Slides were counterstained with Mayer’s haematoxylin (1 min). Some slides were evaluated previously for diagnostic purposes, in which case the slides were retrieved from the pathology archive. Most slides were stained for the purpose of this study.

In UMC Utrecht, paraffin-embedded biopsy blocks were cut into 4-μm sections and mounted. Slides were baked at 37°C and stained immunohistochemically for HER2 by means of the Bond Max autostainer (Leica Biosystems, Nussloch, Germany). Incubation was performed using rabbit monoclonal antibodies from Thermo Fisher Scientific (Waltham, MA, USA) against HER2, clone SP3 (dilution 1:100). Slides from the excised tumour specimens had been IHC-stained previously for HER2 shortly following surgery for diagnostic purposes according to the same staining protocol. Appropriate controls were used throughout.

HER2 IHC SCORING

In accordance with ASCO/CAP guidelines, HER2 IHC was scored using the Dako scoring system as 0, 1+, 2+ or 3+; 0 and 1+ were considered a negative result, 2+ equivocal and 3+ a positive result.1 In accordance with these guidelines we defined a score of 0 as fewer than 10% of tumour cells staining positive, a score of 1+ as more than 10% of tumour cells staining positive, but no circumferential staining was present, a score of 2+ as more than 10% of tumour cells showing weak or moderate circumferential staining and a score of 3+ as more than 10% of tumour cells showing a strong circumferential staining.2

In Radboud UMC, IHC was scored and reported independently by two blinded investigators (A.H. and P.B.). When initial assessment was not identical, a consensus was reached. In UMC Utrecht, stained slides were reviewed by means of consensus scoring by two blinded observers (S.K. and P.D.), including an experienced breast pathologist. Surgical slides stained previously for diagnostic purposes were reassessed.

Similar to ASCO/CAP guidelines, the Dutch 2012 breast cancer guideline mandates an additional amplification-based test for equivocal cases (IHC scores 2+). According to routine care, this test was thus performed in all cases with HER2 IHC scores of 2+.10

AMPLIFICATION-BASED TESTS

Radboud UMC uses a double-probe FISH test, UMC Utrecht an MLPA test. This additional determination was taken into account to create a dichotomous variable HER2-positive or -negative.

HER2 FISH was performed as described elsewhere.9 For the ISH process, a custom-made probe ERBB2-HER2/neu 17Q12/SE-17 (KB-00007 Kreatech: Leica, Rijswijk, the Netherlands) was used. Scoring of HER2 FISH was conducted as described by the latest ASCO/CAP guideline for HER2 status assessment.1

MLPA was performed as described elsewhere.13 Approximately 1 cm² of invasive tumour was identified on haematoxylin and eosin (H&E)-stained 4-μ thick sections and isolated. The tissue was incubated with proteinase K, then centrifuged and analysed with MLPA using the P004 HER-2 kit (MRC Holland, Amsterdam, the Netherlands).

DATA ANALYSIS

Data were analysed using IBM srsS Statistics for Windows version 20.0 (IBM, Armonk, NY, USA) and a kappa statistic was calculated to assess the correlation beyond chance of the aCNB specimen HER2 IHC score and the excision HER2 IHC score. Furthermore, we analysed the result of combining a 0 and 1+ score, as the difference has no clinical significance (both are considered negative). Lastly, we analysed HER2 status as a dichotomous variable considering the result of both IHC and additional testing.

ETHICAL STANDARDS

According to Dutch legislation, no approval from a research ethics committee was required for this study, as coded tissue obtained from routine diagnostic workflow was used and the included patients are not affected by the study. Anonymous or coded use of redundant tissue for research purposes is part of the standard treatment agreement with patients in our
hospitals, to which patients may opt out. None of the included patients submitted an objection against use of residual materials.

### Results

A total of 144 cases were eligible for analysis: 75 from UMC Utrecht and 69 from Radboud UMC. This included 102 invasive ductal carcinomas, 20 invasive ductolobular carcinomas, 16 invasive lobular carcinomas and six invasive carcinomas of other subtypes. Eighty-seven patients were treated by lumpectomy, 57 by mastectomy. All cases were female, aged between 35 and 89 years (median = 60). The results of HER2 IHC analysis are displayed in Table 1. - 15 cases (10%) were HER2-positive on excision. The concordance of HER2 status assessment between aCNB and resection specimens was 50% (κ = 0.33). Immunohistochemistry on the aCNB specimens was more often equivocal (44 of 144 versus 31 of 144 cases) compared to the resection specimens, but this was not statistically significant (P = 0.081, χ² test).

Data are also shown for Radboud UMC and UMC Utrecht separately, in italics and underscored, respectively.

Table 2 shows correlation of aCNB and resection when 0 and 1+ scores are combined in a single category. Accuracy was increased by 28 cases to 100 of 144 cases (69%, κ = 0.39), which was statistically significant compared to Table 1 (P = 0.0008). The accuracy for results of immunohistochemistry combined with additional testing was 97% (κ = 0.85), as displayed in Table 3. The data set contains only four discrepant cases. Sensitivity of combined IHC and amplification-based testing was 87%, specificity 98%.

When these analyses were performed for the two centres separately, the raw IHC concordance for Radboud UMC and UMC Utrecht were 54% (κ = 0.35) and 47% (κ = 0.13), respectively. When additional testing was taken into account, Radboud UMC (using FISH) had an accuracy of 96% (κ = 0.84) and UMC Utrecht (using MLPA) of 99% (κ = 0.85).

The complete data set with all IHC results, including all available amplification-based results, is provided as online additional material in Supporting information, Table S1.

### Discussion

In this study, we investigated the reliability of HER2 status assessment using immunohistochemistry as the first test on breast cancer core needle biopsies that underwent accelerated processing (aCNB). The current ASCO/CAP guidelines preclude this determination, as they require a formalin fixation time of at least 6 h.³ As only one study is known to have assessed the reliability of HER2
assessment with fixation times of less than 6 h, it seems reasonable to dissuade pathology laboratories from using shorter fixation techniques. However, efforts to reduce time to final diagnosis and treatment have become more pressing during recent years, given its impact on stress reduction in patients. This effort not only affects patients who are diagnosed with cancer but, even more so, patients diagnosed with benign disease. Therefore, the recommendation for at least 6 h of formalin fixation warrants re-examination based on robust scientific evidence.

We used patient cohorts from two centres to investigate whether reliable HER2 status assessment can be performed in conjunction with aCNB. Our dual-centre study of 144 consecutive cases showed a strong correlation between HER2 status determined on aCNB specimens and HER2 status on routinely processed resection specimens. Although at first glance the accuracy of immunohistochemistry (Figure 1) was 50%, most of the disparities were not clinically relevant, as equivocal results in routine practice always require additional assessment by means of amplification-based testing such as FISH (Figure 2) or MLPA. The clinically relevant result ‘HER2-positive’ or ‘HER2-negative’ (Figure 3) takes the results of these additional tests into account, and this correlation between aCNB specimens and resection specimens was excellent in the present study, with a 97% accuracy and a kappa of 0.85 (considered to be a strong correlation).

MLPA is a low-cost and fast PCR-based test for HER2 amplification. It quantitatively tests amplification in small quantities of DNA extracted from paraffin blocks, and has been shown to have a high concordance with the results of ISH-based techniques. MLPA is therefore considered a good alternative for FISH.

For hormone receptors, the concordance between regularly processed core needle biopsies (rCNB) and the whole tumours after resection has been fairly well established. Techniques for receptor status determination have been standardised since publication of the ASCO/CAP guidelines. A recent meta-analysis concluded a pooled concordance rate of 94% for ER status. Concordance is slightly lower for PR status, due possibly to more intrinsic tumour heterogeneity. A recent meta-analysis concluded a pooled concordance rate of 85% for PR status.

Until recently, HER2 status concordance varied considerably between different laboratories. However, since the introduction of a consensus statement on the definition of HER2 positivity by ASCO/CAP, reported variation has decreased dramatically in the literature. A recent meta-analysis, considering only studies using the ASCO/CAP protocol, found a concordance rate between rCNB and resections of 98%. Our present results with aCNB and corresponding resections are in line with these published results.

Table 3. Results of immunohistochemistry combined with amplification-based testing for equivocal results

|            | aCNB | Excision |
|------------|------|----------|
|            | HER2 status | Negative | Positive | Total |
|            |     |          |          |       |
| Excision   |     |          |          |       |
| Negative   | 127 | 2         | 129      |       |
| Positive   | 2   | 13        | 15       |       |
| Total      | 129 | 15        | 144      |       |

Bold type, all cases (kappa 0.85); italic type, Radboud UMC cases only (kappa 0.84); underscored: UMC Utrecht cases only (kappa 0.85).

HER2, human epidermal growth factor receptor 2; aCNB, accelerated processing method for core needle biopsy.

When comparing immunohistochemistry of the aCNB specimens to the excision specimens in the present study, only IHC score 3+ correlated closely. On more thorough inspection it became clear that IHC classification of the biopsy result was often only one point off from the resection specimen. In fact, this was the case in 62 of the 72 disparate cases. This disparity has a large influence on the kappa statistic of the IHC data. However, it is important to realise that in clinical practice these differences are often irrelevant, as IHC scores 0 and 1+ are both considered HER2-negative. When scores 0 and 1+ were combined, accuracy increased to 69% (κ = 0.39). Furthermore, all cases with IHC 2+ scores were subjected to further testing. Therefore, the correlation observed in our cohort was similar to the previously reported correlation between rCNB specimens and excision specimens. We therefore propose that HER2 status can be established reliably on aCNB specimens.

One could argue that the use of two different amplification-based testing methods is a weakness of
our study. However, we believe it can actually be considered a strength, as it proves that both techniques can be used in aCNB specimens. The correlation between aCNB specimens and resection specimens was equally good for both techniques, considering both the separate and the combined data set. Moreover, both techniques are considered validated methods for routine diagnostic testing, so no meaningful differences in results ought to occur. Discrepancies follow from the practical reality that there is (tolerable) variability in the different diagnostic testing methods used in practice, not from the fact that one method is superior to the other. The relatively small sample size of the data set leaves some room for error, but the present one is still among the biggest series available. As experience proceeds, more data will become available. Meanwhile, both techniques have their own advantages and disadvantages.

FISH was used in most of the initial trials for adjuvant HER2-targeted therapy, and therefore relates the results directly to trial results. Furthermore, it provides a direct morphological control between the

Figure 1. Examples of immunohistochemistry of HER2 in core needle biopsies (A–D) and excision specimens (E–H). A,E, Score 0 (in E some cytoplasmic staining); B,F, score 1+; C,G, score 2+; D,H, score 3+. © 2018 The Authors. Histopathology Published by John Wiley & Sons Ltd, Histopathology, 73, 81–89.
HER2 test and the diagnostic tumour slides. Conversely, MLPA is fast, cheap, easy to perform and more quantitative than IHC.13

Adaptation of the aCNB technique depends upon two factors. First, reliable logistics, as stringent workflow in outpatient clinic, radiology and pathology...
departments, is a prerequisite for a same-day diagnostics programme and, secondly, is solid evidence that the technique allows reliable interpretation of the results. The use of this technique for basic histology was established previously.1 The reliability for hormone receptor status, E-cadherin, Ki-67 and HER2 FISH has also been described.9,12

In conclusion, this study shows that HER2 immunohistochemistry can be determined reliably using aCNB, clearing another important hurdle to wide acceptance and implementation of the aCNB technique in daily clinical practice.

Conflicts of interest
The authors declare that they have no conflicts of interest.

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**Supporting Information**

Additional Supporting Information may be found in the online version of this article:

**Table S1.** Complete caselist.