The importance of tissue handling of surgically removed breast cancer for an accurate assessment of the Ki-67 index

Nobuyuki Arima,1 Reiki Nishimura,2 Tomofumi Osako,2 Yasuyuki Nishiyama,2 Mamiko Fujisue,2 Yasuhiro Okumura,3 Masahiro Nakano,3 Rumiko Tashima,4 Yasuo Toyozumi5

ABSTRACT

Aim Insufficient attention for the Ki-67 immunohistochemistry has been given to the importance of tissue handling for surgical breast cancer specimens. We sought to investigate the effect of fixation status on the Ki-67.

Methods We examined the effect of fixation, time to and duration of fixation using surgical specimens, and finally, compared the paired Ki-67 index in the tumor between core needle and surgical specimen.

Results The Ki-67 was significantly higher when 10% neutral buffered formalin was used (p=0.0276). Insufficient fixation caused a drastic reduction in the Ki-67 index (p=0.0177), but not significant in oestrogen receptor (ER) and human epidermal growth factor receptor 2 (HER2). Sixteen hours delayed time to fixation also caused a reduction of the Ki-67 (p=0.0284), but not significant in ER. Prolonged fixation significantly led to a gradual reduction in the Ki-67 in a time-dependent manner, but not in both ER and HER2. Finally, cutting the tumor before fixation improved fixation status and consequently caused an increased level of the Ki-67 index (r=0.8595).

Conclusions Tissue handling of surgical specimen is critical for assessing the Ki-67 compared with ER and HER2. We should pay more attention to tissue fixation status for the standard assessment of the Ki-67 index.

INTRODUCTION

The Ki-67 is a proliferative cell marker that is expressed in all phases of the cell cycle except the G0 stage.1 It has generally been used as a way to determine the malignant potential of a tumour and as a prognostic marker in patients with malignant neoplasms (brain tumours, gastrointestinal and pancreatic neuroendocrine tumours, lymphomas and breast cancers).2–6 Patients with human epidermal growth factor receptor 2 (HER2)–negative luminal-type breast cancer with higher cell proliferation demonstrated more unfavourable prognosis compared with those with lower cell proliferation.7–9 Therefore, the 2011 and 2013 St. Gallen international consensus meeting recommended chemotherapy in addition to hormone therapy for HER2–negative luminal-type breast cancer with higher Ki-67 index.10–12 However, a standard assessment of the Ki-67 index has not yet been established, and the most recent studies have only focused on interobserver variability in the interpretation of these values.13–15

The American Society of Clinical Oncology (ASCO)/the College of American Pathologists (CAP) guidelines recommend proper tissue handling for hormone receptors (oestrogen receptor [ER] and PgR) and HER2.16–18 but insufficient attention has been given to the importance of tissue handling for the Ki-67 index. The only mention to this effect was presented in a review article by Dowsett et al, who suggested that the pre-analytical setting is a potential factor that might affect the Ki-67 immunohistochemistry (IHC).3

Formerly, we evaluated the Ki-67 index of surgically removed cancers using slides prepared at several different institutes. The analysis revealed that there were many cases with extremely low or with a diminished level of the Ki-67 protein even in tumour cells with a high grade. This led to the hypothesis that postoperative tissue handling of surgically removed breast cancer might strongly affect the Ki-67 index. Postoperative tissue handling of surgical specimens that might affect IHC includes the size of the specimen, time to fixation from tumour removal, type of fixative and duration of fixation, and the effects of these factors on several biomarkers have been studied.19–27 However, rigorous analysis on the importance of tissue handling for the Ki-67 protein has not yet been performed. Therefore, we thoroughly examined the various fixation conditions of surgically removed breast cancer tissue and their effects on the Ki-67 index.

MATERIALS AND METHODS

Materials, histological examination and IHC

Surgically removed breast cancer tissue in cases with either conservative resection or mastectomy at Kumamoto City Hospital were used in this study. Histopathological examination was routinely performed using formalin fixed paraffin embedded (FFPE) tissue. The antibody used for IHC was Ki-67 (clone MIB-1, mouse monoclonal, Dako, Glostrup, Denmark), HER2 (clone 4B5, rabbit monoclonal, Roche Diagnostics, Tokyo, Japan) and ER (clone SP1, rabbit monoclonal, Roche Diagnostics). IHC was performed using BENCHMARK XT (Ventana, Tucson, USA), and the details for this procedure can be found in the two articles published by Nishimura et al in 2010.6–9 The Ki-67 index was determined by

To cite: Arima N, Nishimura R, Osako T, et al. J Clin Pathol Published Online First: [please include Day Month Year] doi:10.1136/jclinpath-2015-203174
calculating the proportion of positive-nuclear staining cells in the hot spot (at least 500 cells). ER expression was calculated as the percentage of positive cancer cells. The staining pattern of HER2 (invasive cancer cells) was divided into the following four groups: 3+ (strong and diffuse), 2+ (moderate and diffuse), 1+ (focal and weak) and 0 (negative). Assessment of IHC was performed by two specialised pathologists.

Type of fixative
The surgically removed breast cancer tissue of 1310 patients from June 2009 to June 2013 was used in this study. Cancer tissues in the first group (n=655) were fixed with 10% neutral buffered formalin (NBF) for the last two years, while those in the second group (n=655) were fixed with 15% unbuffered formalin for the first two years. The Ki-67 index between the two groups was then evaluated and compared. Time to fixation from tumour removal, duration of fixation and the preparation of the FFPE and IHC methods were identical for the two groups.

Time of fixation
Time to fixation from tumour removal
Samples of fresh tumour sliced from surgically removed cancer tissue were prepared and stored for a duration period of 1–16 h before formalin fixation. These tissues were wrapped with polyethylene film at room temperature to avoid dehydration. Biomarker expression was evaluated by using IHC slides when the sliced tumour tissue reached the designated cold ischaemia time.

Time of fixation
Insufficient fixation
Samples of fresh tumour sliced from a surgically removed breast cancer were prepared and fixed with formalin followed by the preparation of the FFPE tissue. A comparison of the biomarker expression was then made between tumours with an insufficient fixation period (3 h) and those with a full fixation period (48 h).

Prolonged fixation
Samples of fresh tumour derived from the same removed breast cancer were prepared and fixed with formalin for a designated duration time (from 48 h to 90 days), followed by the preparation of the FFPE tissues. Expression of biomarkers was then analysed using IHC. The same procedure was used for each of the samples other than duration of fixation.

Effect of cutting the centre of a fresh tumour just before fixation
The surgically removed breast cancer tissue of 1190 patients from March 2009 to November 2011 was used. For the latter half of this period, the central portion of the surgically removed breast cancer was cut just before fixation in the first group (n=594) in order to promote formalin penetration into the tumour tissue more quickly. The tumours in the second group (control group, n=594) were not pretreated in the first half of this period. The Ki-67 index between the two groups was then evaluated and compared.

Comparison between core needle biopsy and surgically removed cancer tissue
The cancer tissue of 136 patients in which core needle biopsy was performed before surgical resection was used. Neoadjuvant cases were excluded. Core needle tissues were fixed for 6–48 h and the surgically removed tissues were fixed for 48–96 h after cutting the centre of the tumour just before formalin fixation.

The Ki-67 index in each of the 136 pairs of core needle and surgically removed cancer tissue was analysed.

Statistical analysis
Unpaired t test was used to compare the mean Ki-67 index for type of fixative (Welch’s t test) and the effect of tumour cutting assays (Student’s t test). Paired t test was used to compare the mean Ki-67 index and the mean percentage of ER-positive cancer cells for time to fixation, insufficient fixation and prolonged fixation assays. The Pearson’s correlation coefficient was used to analyse the relationship between the Ki-67 index in the core needle tissue and the surgically removed breast tissue.

RESULTS
Type of fixative
The mean Ki-67 index in cancers fixed with 10% NBF was 30.5±22.9%, and the mean Ki-67 index in cancers fixed with 15% unbuffered formalin was 27.8±20.9%. The results revealed that there was a significant difference (p=0.0276) in the Ki-67 index between the two different types of fixatives (table 1).

Time to fixation
Sixteen hours delayed time to fixation caused a significant reduction of the Ki-67 index (p=0.0284, table 2), but not in ER expression (p=0.3129, table 3), although <10 h delayed time to fixation did not reveal a recognisable influence on the Ki-67 and ER expression (data not shown).

Time of fixation
Insufficient fixation
Insufficient fixation caused a significant reduction of the Ki-67 index (p=0.0177) and the percentage of ER-positive cells (p=0.0364), although the reduction of the Ki-67 index was more drastic compared with ER (tables 2 and 3). In an HER2-positive breast cancer, a serious reduction in the Ki-67 index (from 33% to 5%) was observed in the insufficient fixative (W elch’s t test) and the effect of tumour cutting caused a significant reduction of the Ki-67 index (p=0.0276) in the Ki-67 index between the two different types of fixatives (table 1).

Table 1 Effect of type of fixative on Ki-67 immunohistochemistry

<table>
<thead>
<tr>
<th>Type of fixative</th>
<th>No. of samples</th>
<th>Ki-67 index (mean±SD: %)</th>
<th>p Value (Welch’s t test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10% NBF</td>
<td>655</td>
<td>30.5±22.9</td>
<td>0.0276</td>
</tr>
<tr>
<td>15% F</td>
<td>655</td>
<td>27.8±20.9</td>
<td></td>
</tr>
</tbody>
</table>

10% NBF, 10% neutral buffered formalin; 15% F, 15% unbuffered formalin.

Table 2 Effect of time of fixation on Ki-67 immunohistochemistry

<table>
<thead>
<tr>
<th>Conditions</th>
<th>No. of samples</th>
<th>Ki-67 index (mean±SD: %)</th>
<th>p Value (paired t test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time to fixation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Immediate</td>
<td>10</td>
<td>43.1±11.5</td>
<td>0.0284</td>
</tr>
<tr>
<td>16 h</td>
<td></td>
<td>36.7±18.4</td>
<td></td>
</tr>
<tr>
<td>Insufficient fixation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>48 h</td>
<td>8</td>
<td>21.8±10.8</td>
<td>0.0177</td>
</tr>
<tr>
<td>3 h</td>
<td></td>
<td>8.8±9.4</td>
<td></td>
</tr>
<tr>
<td>Prolonged fixation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>48 h</td>
<td>10</td>
<td>36.3±13.6</td>
<td>vs 48 h</td>
</tr>
<tr>
<td>14 days</td>
<td></td>
<td>26.6±15.8</td>
<td>0.0414</td>
</tr>
<tr>
<td>28 days</td>
<td></td>
<td>22.4±11.2</td>
<td>0.0042</td>
</tr>
<tr>
<td>56 days</td>
<td></td>
<td>14.8±6.5</td>
<td>0.0003</td>
</tr>
</tbody>
</table>
fixation setting (figure 1A, B), but HER2 overexpression remained the same (figure 1C, D).

Prolonged fixation
The Ki-67 index gradually decreased in a time-dependent manner in ER-positive cancers, and this reduction was significant when tumours with a 2-day-long fixation period was regarded as controls (table 2), while a reduction of ER labelling was not significant (table 3). Figure 2A demonstrated that prolonged fixation caused a gradual reduction of the Ki-67 index in ER-positive cancer cells from 41% to 17%, but there was no recognisable change in ER labelling in the same tumour even in a 60-day-long fixation period. Similarly, the Ki-67 index visually decreased in a time-dependent manner from 74% to 13% in HER2-positive cancer cells, but only a slight decrease in HER2 expression in the same tumour was observed even in a 84-day-long fixation period (figure 2B).

Effect of cutting the centre of the surgically removed breast tumour just before fixation on the Ki-67 index
There was a significant difference between the average Ki-67 index (26.2%±19.8%) in tumours in which the centre of fresh tumours had been cut just before fixation and the control group (23.5%±18.6%) in which the centre of fresh tumours had not been cut (p=0.0181) as shown in table 4.

Comparison of the Ki-67 index between core needle and surgical specimen
There was a strong correlation (Pearson’s correlation coefficient=0.8595) between the Ki-67 index of core needle and the Ki-67 index of surgical specimen in the same tumour (figure 3).

DISCUSSION
The results in this study clearly demonstrated that proper tissue handling of surgically removed breast cancer tissue was extremely important for a standard assessment of the Ki-67 index in contrast to ER or HER2.

Out of several factors related to tissue handling that might influence IHC, insufficient fixation caused a most serious negative effect on the Ki-67 index in proliferating tumour cells, although a mild but significant reduction of the percentage of ER-positive tumour cells was also observed in the same condition. On the other hand, overexpression of HER2 protein (score 3+) was remained unchanged even in this condition. These results suggest that the effect of fixation status on IHC differs in varying degrees depending on the type of biomarker antibody used. In contrast to core needle biopsy, insufficient fixation is more likely to occur in surgically removed tissues when they are inadequately handled after removal. Breast tissue is mainly composed of fat and connective tissue and is often hard to fix with formalin because formalin penetrates tissue at an average rate of 1 mm/h.19 The result that the Ki-67 index in cases where the centre of the tumour was cut before fixation

### Table 3

<table>
<thead>
<tr>
<th>Conditions</th>
<th>No. of samples</th>
<th>ER (mean±SD: %)</th>
<th>p Value (paired t test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time to fixation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Immediate</td>
<td>4</td>
<td>96.8±4.50</td>
<td>0.3129</td>
</tr>
<tr>
<td>16 h</td>
<td></td>
<td>89.5±16.4</td>
<td></td>
</tr>
<tr>
<td>Insufficient fixation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>48 h</td>
<td>8</td>
<td>87.1±25.3</td>
<td>0.0364</td>
</tr>
<tr>
<td>3 h</td>
<td></td>
<td>66.1±34.6</td>
<td></td>
</tr>
<tr>
<td>Prolonged fixation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>48 h</td>
<td>10</td>
<td>95.8±4.3</td>
<td>vs 48 h</td>
</tr>
<tr>
<td>14 days</td>
<td></td>
<td>88.3±12.6</td>
<td>0.2152</td>
</tr>
<tr>
<td>28 days</td>
<td></td>
<td>84.5±18.6</td>
<td>0.2665</td>
</tr>
<tr>
<td>56 days</td>
<td></td>
<td>85.8±15.4</td>
<td>0.2010</td>
</tr>
</tbody>
</table>

### Table 4

<table>
<thead>
<tr>
<th>Cutting the tumour</th>
<th>No. of samples</th>
<th>Ki-67 index (mean±SD: %)</th>
<th>p Value (Student’s t test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>With cut</td>
<td>594</td>
<td>26.2±19.8</td>
<td>0.0181</td>
</tr>
<tr>
<td>Without cut</td>
<td>594</td>
<td>23.5±18.6</td>
<td></td>
</tr>
</tbody>
</table>
Vals after appropriate gross inspection and margins designa-

enzymatic degradation. In our results, although several hours
of activity of these molecules can occur after the surgical inter-
ruption of blood

cut the fresh tumour or inject formalin around a tumour

During both warm ischaemia time (time from the interruption
of the blood supply to the tumour by the surgeon to the exci-
ton of the tissue specimen) and cold ischaemia time (time from
excision to the initiation of tissue fixation), the progressive loss
of activity of these molecules can occur after the surgical inter-
ruption of blood flow, leading to tissue ischaemia, acidosis and
enzymatic degradation. In our results, although several hours
to <10 h delayed time to fixation from tumour removal did not
cause a recognisable effect on the Ki-67 index as well as the per-
centage of ER-positive tumour cells, 16 h delayed time to fix-
ation caused a significant reduction of the Ki-67 index but not
the percentage of ER-positive cells. Portier et al. demonstrated
that cold ischaemia time (up to 3 h) had no deleterious effect on
the detection of HER2 via in situ hybridisation (ISH) or IHC.
On the other hand, Khoury et al. indicated that the HER2
fluorescence ISH (FISH) test was particularly vulnerable to cold
ischaemia in contrast to ER, PgR and HER2 IHC, and they
recommended not to delay formalin fixation for >1 h. Pinhel
et al. also showed that delayed fixation did not cause a signifi-
cant reduction in the Ki-67, ER, PgR and HER2 proteins but
had an effect on the phosphorylated proteins (p-Akt and
p-Erk1/2). Thus, cold ischaemia for several hours is not likely to

have a serious effect on ER, PgR, HER2 and Ki-67 IHC but has
a negative effect on the HER2 FISH and the some proteins
related to signal transduction. Therefore, we should follow
the ASCO/CAP guidelines for hormone receptors and HER2, which
recommended that removed breast specimens should be fixed as
quickly as possible.

Prolonged fixation (overfixation) is likely to be less problem-
atic than insufficient fixation (underfixation) but potentially
could also lead to false-negative results caused by excessive
protein cross-linking by formaldehyde. In addition, the pro-
longed exposure of tissue to formalin inhibits the recovery of
nucleic acids. In this study, we clearly demonstrated that
prolonged fixation caused a significant reduction in the
Ki-67-positive cells but not in ER and HER2-positive cells,
although Goldstein et al. and Tong et al. already indicated that
prolonged fixation does not affect ER, PgR and HER2
expression in IHC. Overfixed cancer tissues lead to weak Ki-67
nuclear labelling in a time-dependent manner, which resulted in
a disturbance in the detection of Ki-67-positive cells. Further-
more, Selvarajan et al. showed that HER2 gene amplifi-
cation detected by FISH is affected by prolonged fixation as
well. Thus, prolonged fixation as well as insufficient fixation
should be avoided in order to get an accurate Ki-67 index.

The ASCO/CAP guideline for hormone receptors and HER2
recommended 10% NBF. Formalin-lacking buffer has a
limited shelf life and degrades rapidly and the degradation of
formalin is believed to contribute to the poor quality of nucleic
acids obtained from FFPE tissue. On the other hand, NBF has a
longer shelf life and the tissue fixed with NBF yields a consist-
ently better quality RNA. We showed that cases fixed with
10% NBF had a significantly higher Ki-67 index than those
fixed with 15% unbuffered formalin, which indicates that NBF
fixation maintain the better quality of not only nucleic acids but
also proteins.

Standard fixation times are a minimum of 5 h for needle and
endoscopic biopsy specimens and ≥12 from larger specimens.
Because it is usually harder to affect the fixation status in core
needle specimens, it may be the optimal standard for IHC. In
contrast, handling surgically removed breast tissue samples is
more problematic. In order to prevent insufficient fixation, we
should cut the fresh tumour or inject formalin around a tumour
using a syringe before fixation. Additionally, comparing the
Ki-67 index between core needle biopsy and surgical resection
tissue derived from the same tumour seems to be important to
evaluate whether or not the surgically removed tissue was

![Figure 3](image-url) Comparison of the Ki-67 index between core needle and surgical specimen in the same tumour. The Ki-67 index in each of the 136 pairs of core needle and surgical specimen originating from the same tumour were analysed. The Ki-67 index independently scored were strongly correlated (Pearson’s correlation coefficient = 0.8595).

Take home messages

- Sixteen hours delayed time to fixation caused a significant
  reduction of the Ki-67 index, but oestrogen receptor (ER)
  expression was not significantly affected.
- Insufficient fixation caused a drastic and a mild reduction
  of the Ki-67 index and ER expression, respectively, while
  human epidermal growth factor receptor 2 (HER2)
  expression was not affected.
- Prolonged fixation caused a significant reduction of the
  Ki-67 index, but either ER or HER2 expression was not
  significantly affected.
- Tissue handling of surgical specimen is more critical for a
  standard assessment of the Ki-67 compared with ER and
  HER2.
suitably handled. Our result in this study demonstrated that the Ki-67 index between these tissues was closely concordant, which indicates that the surgical breast samples were properly handled.

In summary, proper tissue handling is extremely important for an assessment of the Ki-67 index. The first step to standardise the Ki-67 index between laboratories and institutes is to make sure whether the surgical specimens are properly handled.

Handling editor Cheok Soon Lee

Contributors Each of the authors contributed to the manuscript. NA wrote the manuscript. RN, TO, YN, MF, YO, MN, RT and YT made corrections in the manuscript. All authors are responsible for the overall content of the manuscript.

Competing interests None declared.

Patient consent Obtained.

Ethics approval The Ethics Committee of Kumamoto City Hospital.

Provenance and peer review Not commissioned; externally peer reviewed.

Data sharing statement All data in this study are shared by authors, and we do not have additional unpublished data.

REFERENCES