Handy Manual for Receptor Analyses Using Cytological Specimens from Breast Cancer Metastases

Collaborative Research in National Hospital Organization Network
“Basic Research for the Decision of Drug Therapies Using Cytological Specimens in Breast Cancer Patients” (H26 and H29-NHO Gan-Ippan-02)
Research Group
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Receptor analysis for hormone receptors and human epidermal growth factor receptor 2 (HER2) in breast cancer metastases has been recommended to determine the appropriate therapy, because of potential differences in the receptor expression status between primary and metastatic breast cancer. Cytological analysis is applicable to several types of metastatic lesions as well as to body cavity fluids, and it is a useful approach for patients in a poor clinical condition.

Several studies have reported on assessment of the receptor status using cytological specimens. However, there are some issues in using cytological specimens for receptor analysis in routine practice, most importantly, the difficulty in the ability to consistently prepare multiple good-quality slides. The research representative for this study and her colleagues at the Shikoku Cancer Center recommend the use of the cell block method as a suitable method for preparing cytological specimens for receptor analysis, and have been routinely using this method for receptor analysis at their institution from August 2011.

Herein, we conducted a multi-institutional study to evaluate the usefulness of this method in nine institutions with a high volume of breast cancer patients. The study group consisted of pathologists, surgeons, and medical technologists at these institutions. This method is favored by all the members of the research group, and there was the opinion that the method should be promoted to the public. Therefore, we prepared a handy manual containing a simple description of the method.

We sincerely hope that every pathology laboratory carries this manual and uses it for receptor analysis in cytological specimens of breast cancer. We recommend the best method for evaluation available at this point, because no standardized method has been established until date.

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1. Importance of Receptor Analysis in Breast Cancer Metastases

Receptor analysis of breast cancer metastases has been recommended to enable selection of the appropriate treatment, because of potential differences in the receptor status between primary and metastatic breast cancer.\textsuperscript{1,2} Therefore, receptor analysis of metastatic breast cancers is widely performed in routine practice.

If available, it would be ideal to use tissue samples for the receptor analysis. On the other hand, cytological analysis can be applied to several types of metastatic lesions as well as to body cavity fluids, and it is a useful approach for patients in a poor clinical condition. Therefore, use of cytological specimens for receptor analysis may be a useful alternative to the use of tissue samples.
2. Receptor Analysis Using Cytological Specimens

It is important, for reliable receptor analysis, to have the ability to consistently prepare multiple good-quality slides. There are two methods known to be suitable for receptor analysis in cytological specimens: the liquid-based cytology (LBC) method and the cell block (CB) method (Table). Both methods have been reported analysis of both the hormone receptors3,4 and for HER2 in situ hybridization (ISH).5-7 However, a special equipment is needed for the preparation of cytological specimens using the LBC method, and the consumable reagents required are expensive. On the other hand, CBs can be handled in the same way as tissue blocks. Therefore, the CB method may be more suitable for routine clinical use in hospitals with a pathology laboratory than the LBC method.

In addition, there is a problem in evaluating the HER2 receptor status by the LBC method in routine practice. If the LBC method is used for this analysis, all cases should be examined by HER2-ISH,5 because immunocytochemical detection of HER2 protein overexpression in LBC specimens is unreliable due to unstable staining.8,9 Use of ISH for all cases in routine practice is not practicable, because of the high cost and time-consuming nature of the procedure. Therefore, in practice, often HER2 assessment by immunostaining is performed first, and then ISH is applied only for cases with equivocal results. And for HER2 evaluation performed this way, the CB method should be used.10

Bright-field HER2 dual ISH (DISH) assay is a better choice of method than fluorescence ISH (FISH) assay to assess the HER2 gene amplification status in cytological specimens. The reason is that it is difficult to distinguish scattered tumor cells among the many non-neoplastic cells under the dark-field fluorescence microscope.

Table Comparison between the liquid-based cytology (LBC) and cell block (CB) methods

<table>
<thead>
<tr>
<th></th>
<th>LBC</th>
<th>CB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rate of evaluable specimens1</td>
<td>Almost the same</td>
<td></td>
</tr>
<tr>
<td>Agreement in hormone receptor results with tissue sections1</td>
<td>Both are good</td>
<td></td>
</tr>
<tr>
<td>Agreement in HER2 gene amplification results with tissue sections1</td>
<td>Both are good</td>
<td></td>
</tr>
<tr>
<td>HER2 protein staining result evaluation2</td>
<td>Difficult</td>
<td>Possible</td>
</tr>
<tr>
<td>Necessity to purchase special equipment</td>
<td>Usually necessary</td>
<td>Not necessary</td>
</tr>
<tr>
<td>Price of consumable reagents</td>
<td>Expensive</td>
<td>Reasonable</td>
</tr>
<tr>
<td>Preparing additional slides</td>
<td>Troublesome</td>
<td>Easy</td>
</tr>
<tr>
<td>Preserved condition</td>
<td>Liquid</td>
<td>Blocks</td>
</tr>
<tr>
<td>Preserved duration of cells in the fixative in an immunostainable condition1</td>
<td>Long</td>
<td>Short</td>
</tr>
<tr>
<td>Number of cells on a slide</td>
<td>Usually enough</td>
<td>Less than in LBC</td>
</tr>
</tbody>
</table>

1 According to the results of analysis of histological specimens of primary breast cancer and cytological specimens obtained from the same tumor.3-7
2 Several studies have reported high false-positive rates for HER2 protein staining on LBC slides.8 In a preliminary study conducted at the Shikoku Cancer Center, we were not able to evaluate the staining results for HER2 protein on LBC slides because of strong background staining.
3 The fixative used for the CB preparation is 10% buffered formalin in this setting.
3. Cell Block Method

(1) What is the Cell Block Method
In this method, cell blocks are prepared by embedding cells in paraffin after fixing them with a fixative. CBs can be stored almost permanently, and they can be handled in the same way as tissue blocks. There are several methods available to prepare CBs. We shall introduce the sodium alginate method here, which is used in the hospital that the research representative is working for, and is favored by all members of the study group.

(2) Sodium Alginate Method
Principle: This method is based on the principle that sodium alginate gelates in the presence of calcium ions.
Reagents: 1% sodium alginate, 1 M calcium chloride.
* Each reagent is prepared at a volume of 50 ml, and can be used for 3 months.

Method (Table): The method is shown in the table.11)

(3) Points that Require Special Attention During the Preparation of a Cell Block Making
① It is important to use 10% buffered formalin as the fixative and set the fixation time between 6 and 48 hours to obtain stable staining results.
② It is better to use tubes with a pointed tip so that the cells can be collected in a compact form.
③ It is important to mix the cells well with the solution after the addition of sodium alginate solution if the amount of cells is large. This would help in accommodating all the cells into the block.
④ The gel should be picked out only after it starts to float. Otherwise, if removal/peeling off is attempted while the gel is still at the bottom of the tube, it may result in breakage.
⑤ The gel must be placed in alcohol solution before it is applied to the tissue processor, because it dissolves in formalin solution.

4. Staining methods
Basically, the same method as that for tissue specimens can be used. We used the autostainers at each institution and the same staining protocol as that for tissues. We obtained good staining results in every autostainer.

We confirmed that the Ventana BenchMark (Roche Diagnostics, Basel, Switzerland), DAKO Autostainer (Agilent, Santa Clara, USA) and BOND (Leica Biosystems, Nussloch, Germany) autostainers can be used for hormone receptor staining. For HER2 protein staining, the Ventana BenchMark and DAKO autostainers can be used. The HER2 DISH assay can be conducted using Ventana BenchMark.
**Table  Cell Block Making through Sodium Alginate Method**

1. **Fix cells in 10% buffered formalin for 6 to 48 hours.**
2. **Centrifuge for 5 min at 3000 rpm.**
3. **Cells**
4. **Remove the supernatant.**
5. **Add 0.5 ml of 1% sodium alginate solution and mix.**
6. **The cells should be mixed well with the solution.**
7. **Centrifuge for 5 min at 3000 rpm.**
8. **Put the gel in a cassette.**
9. **It takes approximately 15 min to put the sample into the cassette.**
10. **Keep the gel in alcohol solution if needed, because it dissolves in formalin.**
11. **The paraffin cell block is prepared.**
12. **Put the cassette into a tissue processor.**
13. **Put the gel in a cassette.**

Attention: Change the amount of solution according to the amount of cell sediment.
5. Receptor Status Evaluation Method

(1) Hormone Receptors

The staining results in slides prepared by the CB method can be evaluated in the same way as tissue slides, if the CBs consist mainly of tumor cells. However, such CBs are rare. Therefore, whether the specimen contains positively stained tumor cells or not should be reported first, and then any additional information may be reported according to the reporting format at each institution.

(2) Human Epidermal Growth Factor Receptor Type 2 (HER2)

The staining results of each tumor cell for HER2 protein staining and the HER2 DISH assay on CB slides can be evaluated in the same way as that in tissue slides. However, the percentage of positive cells cannot be evaluated unless the tumor cells exist in a compact form. It is important to use strict scoring criteria for 2+ or 3+ cases in HER2 protein scoring, because of the reported low concordance rate with the results from tissue sections. The HER2 protein 2+ cases should be examined by DISH, because the concordance rate of the results of DISH assay between CB and tissue sections is reported to be high, and we have shown that immunostaining for HER2 protein in breast cancer CBs can be performed in the same way as that in histological specimens, if DISH is used for equivocal cases.
6. Examples of Receptor Status Evaluation

(1) Hormone receptors

Estrogen receptor (ER)
Progestrone receptor (PR)

① Positive

Pleural fluid

H&E
Many tumor cell clusters are seen in an inflammatory background. (Objective, X20)

ER
Stained by Ventana BenchMark.
Almost all the tumor cells are stained. (Objective, X20)

Ascitic fluid

H&E
A few tumor cell clusters are seen among numerous non-neoplastic cells such as inflammatory cells and mesothelial cells. (Objective, X40)

PR
Stained by Ventana BenchMark.
There are a few tumor cells with positively stained nuclei among numerous non-neoplastic cells. (Objective, X40)
H&E
Tumor cell clusters are seen among numerous non-neoplastic cells such as inflammatory cells and mesothelial cells. (Objective, X20)

ER
Stained by Ventana BenchMark.
The tumor cell nuclei show weakly positive staining. (Objective, X20)

Pleural fluid

H&E
Many inflammatory cells and mesothelial cells are seen. No tumor cells are detected. (Objective, X40)

BerEP4
A few atypical cells are stained. (Objective, X40)

ER
Stained by Ventana BenchMark.
The tumor cell nuclei are positively stained. (Objective, X40)

Ascitic fluid

ER
Stained by Ventana BenchMark.
The tumor cell nuclei are positively stained. (Objective, X40)
② Negative

Pleural fluid

H&E
Tumor cell clusters are seen in an inflammatory background. (Objective, X40)

ER
Stained by Ventana BenchMark. All the tumor cells are not stained. (Objective, X40)
(2) HER2

① Positive (Score 3+)

Pleural fluid

H&E
Tumor cell clusters are seen in a hemorrhagic background. (Objective, X40)

HER2 protein
Stained by Dako Autostainer. Strong circumferential membranous staining of the tumor cell membranes is seen. (Objective, X40)

HER2 protein
Stained by Ventana BenchMark. Strong circumferential membranous staining of the tumor cell membranes is seen. (Objective, X40)

HER2 gene by DISH assay
Stained by Ventana BenchMark. HER2 gene amplification is seen in the nuclei of the tumor cells. (HER2/CEP17 signal count ratio, 15.7). (Objective, X40)
② Equivocal (Score 2+)

Pleural fluid

H&E
One tumor cell cluster is seen among many histiocytes. (Objective, X40)

HER2 protein
Stained by Ventana BenchMark. Moderate circumferential staining of the tumor cells is seen. (Objective, X40)

HER2 gene by DISH assay
Stained by Ventana BenchMark. HER2 gene amplification is seen in the nuclei of the tumor cells. (HER2/CEP17 signal count ratio, 3.0). (Objective, X60)
Pleural fluid

H&E
Small clusters of tumor cells are seen in a hemorrhagic background. (Objective, X40)

HER2 protein
Stained by Ventana BenchMark.
Weak circumferential staining of the tumor cells is seen. (Objective, X40)

HER2 gene by DISH assay
Stained by Ventana BenchMark.
HER2 gene amplification is not seen in the nuclei of the tumor cells. (HER2/CEP17 signal count ratio, 1.1). (Objective, X40)
③ Negative (Score 1+)

### Pleural fluid

<table>
<thead>
<tr>
<th>H&amp;E</th>
<th>HER2 protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor cell clusters are seen. (Objective, X40)</td>
<td>Stained by Ventana BenchMark. Weak and incomplete membranous staining of the tumor cells is seen. (Objective, X40)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>H&amp;E</th>
<th>HER2 protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor cell clusters are seen. (Objective, X40)</td>
<td>Stained by Ventana BenchMark. Weak and incomplete membranous staining of a few tumor cells is seen. (Objective, X40)</td>
</tr>
</tbody>
</table>
7. References


