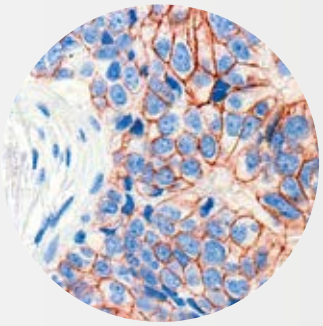
Immunohistochemical detection of phospho-HER-3 in human breast cancer:

Establishing reliable protocols for immunohistochemistry requires a defined evaluation of antibody specificity



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Keywords:
- Target Specificity
- Antigen Retrieval
- Subcellular Localization

Immunohistochemistry (IHC) is one of the most valuable tools to analyze molecular processes in healthy and diseased tissues. Especially the detection of marker proteins in tumor tissue gives important insights into the progression of cancer and its response to drugs. Nevertheless, the implementation of IHC also entails potentially serious pitfalls. Through the handling of specimens after resection, artificial molecular alterations can occur in the tissue. Therefore, a careful and standardized collection of samples is necessary (see Indivumed Standard of Biobanking and www.indivumed.com). Furthermore, unspecific binding of antibodies, which is likely the biggest risk of IHC, reflects an inaccurate molecular reality of the tissue. To prevent a misleading outcome of IHC, Indivumed implements a standardized procedure to establish high-quality staining protocols. This procedure includes a thorough selection as well as the careful evaluation of antibodies using different approaches (immunoblotting, usage of positive and negative controls, evaluation of target distribution in tissue).

The example described here applies Indivumed's procedure to the detection of phosphorylated human epidermal growth factor receptor 3 (HER-3) in human breast cancer tissue.

Material and Methods

Target specificity of antibodies is the most critical aspect for ensuring the reliability of IHC results. Therefore, a standardized procedure to establish IHC protocols has been implemented. To illustrate this procedure, we describe here all steps for the implementation of anti-phospho-HER-3 IHC on formalin-fixed, paraffin-embedded (FFPE) material.

Precise selection of antibody: Attributes such as immunogenicity, purification status and clonality give important hints for the predicted target specificity of antibodies. The applied immunogens predetermine the specificity of the generated antibody. Purification statuses are crucial especially for the specificity of polyclonal antibodies. In contrast to immunogen purified antibodies, purified IgG fractions or whole sera often contain cross reactive or unspecific contaminants. Concerning clonality, monoclonal antibodies often have advantages over polyclonal antibodies. In most cases monoclonal antibodies show high epitope specificity and are well pre-characterized due to a careful hybridoma selection. To implement anti-phospho-HER-3 IHC a monoclonal antibody (clone: 21D3, #4791, Cell Signaling Technology), which was produced against a synthetic peptide, was selected.

Immunoblotting: Immunoblotting serves as a useful tool to determine cross reactivity of antibodies with other proteins. Matched antibody reactivity with protein bands of the predicted molecular weight indicates target specificity. On the basis of literature and expression data bases, BT474 and SKBR3 cells have been selected as positive controls for immunoblotting of phospho-HER-3.

Implementation of IHC on positive controls: IHC is first implemented on positive controls, which either could be cell culture material or tissue. In the case of the anti-phospho-HER-3 antibody, FFPE sections of BT474 and SKBR3 cells were used as positive controls.

Adjustment of antigen retrieval: The inaccessibility of antigens disturbs the reactivity of many antibodies with FFPE tissue. Therefore, antigen retrieval methods such as boiling in EDTA or citrate buffer as well as pre-incubation with proteases improves IHC staining results. To detect phospho-HER-3 in FFPE tissue, boiling in EDTA buffer was selected as the most applicable method here.

Accuracy testing: To assure target specificity of antibodies, the distribution and subcellular localization of IHC staining in positive and negative controls are compared to the results from current literature. Therefore, BT474 cells, SKBR3 cells and human breast cancer tissue were used for an evaluation of the anti-phospho-HER-3 antibody. Since MCF-7 cells do not express phosphorylated HER-3, they were used as negative controls. Additionally, HER-2 negative breast cancer tissue was pre-incubated with alkaline phosphatase (calf intestinal phosphatase) to exclude cross reactivity of the antibody with non-phosphorylated HER-3.

Determination of antibody dilution: To determine the highest possible antibody concentration (lowest dilution) and at the same time the lowest background signal, a titration series is performed.

Approval: The IHC protocol is approved if the target specificity of the relevant antibody was assured in all establishment steps.

Results

The establishment of an IHC protocol is illustrated here using a monoclonal anti-phospho-HER-3 antibody. For this procedure target specificity was first tested by immunoblotting of BT474 and SKBR3 cells (Figure 1). In immunoblots the antibody reacted with a protein band at approximately 185 kDa in both cell lines, which matches the predicted molecular weight of HER-3. No cross reactivity with other proteins has been detected using the anti-phospho-HER-3 antibody. Immunohistochemical staining of anti-phospho-HER-3, a transmembrane receptor, was implemented first on BT474, SKBR3 and MCF-7 cells (Figure 2). Positive reactivity of the plasma membrane in BT474 and SKBR3 cells indicated the specificity of the applied antibody. In contrast, MCF-7 cells were negatively stained, confirming the absence of phospho-HER-3 in this cell line. Considering that MCF-7 cells express non-phosphorylated HER-3, the specificity of the tested antibody to phosphorylated HER-3 can be confirmed as well.

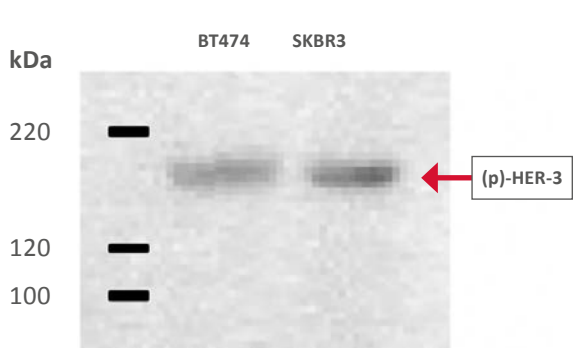

Figure 1: Anti-phospho-HER-3 immunoblot of cell lysates from cultured BT474 and SKBR3 cells.

The target specificity was further evaluated on FFPE tissue samples (Figure 3). In human breast cancer cases, which were negatively tested for HER-2, phospho-HER-3 was localized in

plasma membranes of tumor cells, which is consistent with the predicted distribution. Furthermore, the abolishment of positively stained tumor cells resulting from pre-incubation with alkaline phosphates assured the specific reaction of the antibody with phosphorylated HER-3.

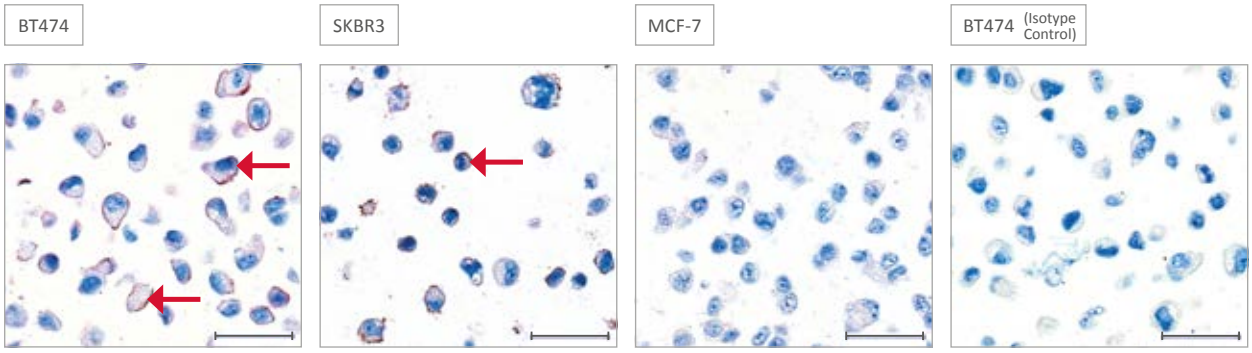

Figure 2: Anti-phospho-HER-3 immunohistochemistry of FFPE cell cultures. A positive staining can be seen at the plasma membrane of BT474 and SKBR3 cells, whereas MCF-7 cells were negative. Scale bars: 50 µm.

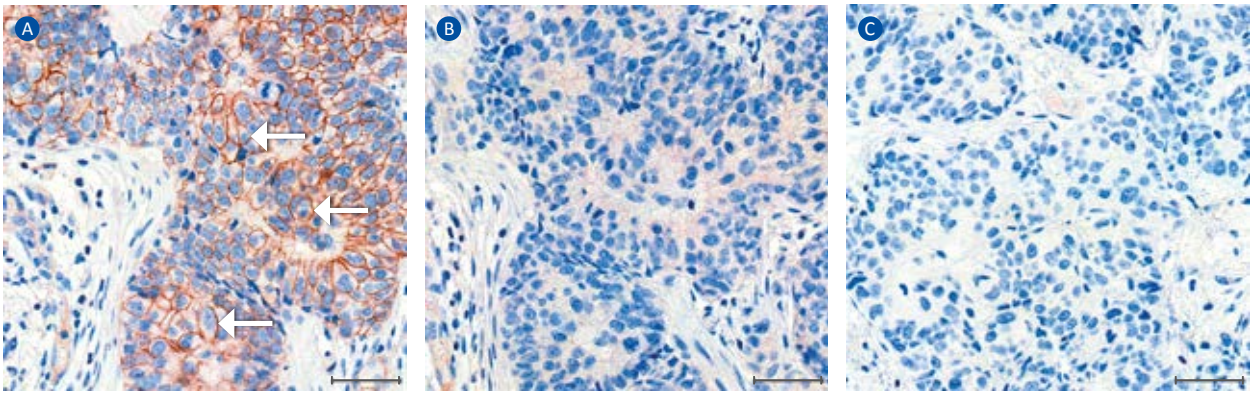

Figure 3: Anti-phospho-HER-3 immunohistochemistry of human breast cancer tissue (HER-2 negative). Positively stained plasma membranes can be seen in human breast cancer cells (A) After pre-incubation with alkaline phosphatase, breast cancer cells are negative (B) Isotype control, no alkaline phosphatse treatment (C) Scale bars: 50 µm.

Conclusion

The target specificity of the anti-phospho-HER-3 antibody has been confirmed with respect to electrophoretic migration properties, IHC of controls (cells and tissue) as well as the evaluation of subcellular localization. This exemplary procedure illustrates all details of the evaluation steps necessary for a reliable IHC protocol establishment. The IHC protocol reported here can now routinely be used for the detection of phospho-HER-3 in FFPE tissue with high precision.

Additional Information

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