ORIGINAL ARTICLE

# Phosphohistone H3 labelling for histoprognostic grading of breast adenocarcinomas and computer-assisted determination of mitotic index

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**Background:** Microscopic evaluation of mitotic figures is a routine procedure in the assessment of the histoprognostic grade of tumours. Nevertheless, their count may be fraught with difficulties. As histone H3 phosphorylation at serine 10 is closely linked to chromosomal condensation, a new monoclonal antibody directed to phosphorylated histone H3 (PPH3) was recently proposed to detect mitotic cells.
**Aim:** To test the reliability of this antibody in detecting and counting mitotic figures in sections of breast adenocarcinomas, because of the importance of mitotic count in histoprognostic grading.
**Methods:** The pattern of PPH3 staining in formalin-fixed paraffin wax-embedded tissues, including normal tissues and a series of 39 breast adenocarcinomas, was examined. A new computer-assisted method was also developed for determining the mitotic index.
**Results and conclusions:** In all tissues tested, PPH3-labelled mitotic figures were easily detected, allowing a rapid identification of the area of highest mitotic activity. In breast carcinomas, a strong correlation was observed between PPH3-stained and haematoxylin and eosin-stained mitotic counts ($r = 0.86$, $p < 0.0001$). Counting of prophase nuclei that coexpress cyclin B1, a marker of the G2/M phase, was possible by PPH3 staining; its accuracy led us to reconsider the tumour grade in three cases. Finally, an automatic computer-assisted method was designed for assessing mitotic index with confocal microscopy and image-analysis software.

Microscopic evaluation of mitotic activity is a routine procedure in assessing the grade of malignancy in tumours[1] such as soft tissue sarcomas,[2] meningiomas[3] or breast adenocarcinomas.[4] In addition, recent evidence showed that the entry into the M phase of the cell cycle sensitises tumour cells to new anti-cancer strategies.[5] Nevertheless, the method of counting is subjective and time consuming and is associated with problems of reproducibility in differentiating mitotic figures from apoptotic or necrotic cells, requiring the experience of trained histopathologists.[6 7] Therefore, immunostaining of proliferating cell nuclear antigen (PCNA) or Ki-67 has been proposed to enhance the reliability of the determination of proliferation activity.[8 9] However, as Ki-67 is expressed by cells throughout the cell cycle from the late G1 phase,[10] the count of Ki-67-positive nuclei is higher than the haematoxylin and eosin (H&E)-based mitotic count. It is thus uncertain whether mitotic count has the same relevance as the count of Ki-67-positive or PCNA-positive cells.

Recent studies have documented a tight correlation between histone H3 phosphorylation and mitotic chromatin condensation, with an antibody selective for the Ser-10 phosphorylated histone H3 (PPH3) amino-terminus.[11–14] Accordingly, PPH3 may be used to detect mitotic figures. However, there is only limited experience on this marker with regard to the histoprognostic grading of human tumours,[15] a key issue in the diagnostic surgical pathology of some epithelial and non-epithelial tumours.

Therefore, the goal of this study is to assess the reliability of this antibody in detecting and counting mitotic figures by immunohistochemistry in breast adenocarcinomas, for which the mitotic count is part of the histoprognostic grading. We also propose a new computer-assisted method for determining the mitotic index with confocal microscopy and image-analysis software.

## PATIENTS AND METHODS

### Tissue samples

Formalin-fixed paraffin wax-embedded human tissues were selected from the archives of the Department of Pathology, University Hospital, Nantes, France, and were processed according to the guidelines of the French Ethics Committee for Research on human tissues.

### Normal control tissues

As controls to validate the immunoperoxidase and double-immunofluorescence staining, we chose tissues characterised by a spatially restricted proliferation compartment: normal colonic mucosa displaying mitotic figures only at the base of the crypts, hyperplastic lymph nodes and tonsils containing many mitotic figures in their germinal centres.

### Breast adenocarcinomas

Thirty nine cases of breast adenocarcinomas diagnosed between 2001 and 2003 were selected (grade I ($n = 13$), grade II ($n = 13$) and grade III ($n = 13$) according to the Scarff–Bloom–Richardson histological grading system modified by Elston and Ellis[4]). For each tumour, the paraffin-wax-embedded section with the highest mitotic activity on H&E staining was selected for the immunohistochemical study.

**Abbreviations:** H&E, haematoxylin and eosin; PCNA, proliferating cell nuclear antigen; PPH3, phosphorylated histone H3; TDM, texture different moment

### Immunoperoxidase staining of paraffin-wax-embedded sections

For each sample, a section was stained by an indirect immunoperoxidase method with a rabbit polyclonal anti-PPH3 antibody (Upstate Biotechnology, Lake Placid, New York, USA; 1:300, 1 h incubation). Briefly, 3-μm-thick sections were stained by a standard three-step streptavidin–biotin peroxidase method with 3,3′-diaminobenzidine as a chromogen (ChemMate Streptavidine Peroxydase Kit, Dakocytomation, Trappes, France) after appropriate antigen retrieval for 30 min in a boiling sodium-citrate buffer (pH 6).

### Quantification of immunohistochemical staining

For tumours, the maximal mitotic count was obtained by summing up the number of mitotic figures counted on H&E-stained sections in 10 consecutive fields (×400 magnification) in the area of highest mitotic activity, usually at the periphery of the tumour. We then counted PPH3-positive nuclei with morphological features of prophase, metaphase, anaphase and telophase, characterised by strong and dense staining of chromatin clumps. PPH3-positive nuclei with fine granular staining and intact nuclear membranes were, however, excluded from the quantification, as they corresponded to cells in interphase (see Results).

### Double-immunofluorescence staining and confocal microscopy

To examine whether anti-PPH3 antibody was restricted to mitotic figures, double-staining experiments with cyclin B1, a cyclin specifically associated with the G2/M transition,[16] and PPH3 were performed in both normal (colonic mucosa) and tumour tissues. In fact, cyclin B1 accumulates in the cytoplasm of cells in the late G2 phase and translocates to the nucleus in the prophase phase of mitosis.[16] Three cases of breast adenocarcinomas were examined: grade II (n = 1) and grade III (n = 2). A mixture of the primary antibodies (anti-PPH3, 1:150 and anti-cyclin B1, 1:30, Dakocytomation) was applied on paraffin-wax-embedded sections for 1 h, after appropriate antigen retrieval, as mentioned earlier. After washing, sections were incubated for 30 min with alexa fluor 488-conjugated goat anti-rabbit and alexa fluor 568-conjugated goat anti-mouse antibodies (1:200, Molecular Probes, Eugene, Oregon, USA). Nuclear staining was carried out with TO-PRO-3 (1 μM, Molecular Probes).[17] Sections were then mounted using Prolong antifade medium (Molecular Probes). Imaging was carried out on a Leica TCS-SP confocal laser-scanning microscope (Leica, Heidelberg, Germany) with an ×63/1.4 oil objective lens. Image processing was performed using the TCS-NT software (Leica).

### Computer-assisted count of mitotic figures by confocal microscopy

Paraffin-wax-embedded tissue sections of breast adenocarcinomas (n = 3), labelled with PPH3 (green) and TO-PRO-3 (red), were analysed with the image-processing software Metamorph (V.4.6.5; Universal Imaging Corporation, Downingtown, Pennsylvania, USA) to assess an automatic mitotic index. Briefly, for each field analysed, a series of sections was taken (step: 0.5 μm) and projected onto a single image. The projected green and red images were then analysed separately. The number of nuclei per field was automatically counted throughout the red images. This morphometric analysis first measured the standard nucleus size to use this as a parameter for counting. Then, the number of PPH3-positive mitotic figures per field was inferred from a morphometric analysis of the green images and restricted to objects that meet two criteria: the standard nucleus size and the mitotic cell texture different moment (TDM). Only the PPH3-positive cells with TDM from 0 to 5 were counted, as those that had a TDM>5 were unwanted cells.

### Statistical analysis

Statistical analysis was carried out with Statview F-4.5 (Abacus Concepts, Berkeley, California, USA). Wilcoxon's paired test was carried out to compare (1) the mitotic figure count assessed with H&E staining and PPH3 immunoperoxidase labelling in 10 consecutive fields (×400 magnification) of the 39 breast carcinomas and (2) the mitotic index assessed with PPH3 immunoperoxidase staining and PPH3 immunofluorescence, followed by the computer-assisted method, in three breast carcinomas. Spearman's correlation was used to assess the relationship between variables. For all analyses, a probability value (p) of less than 0.05 was considered to be significant.

## RESULTS

### Mitotic cell staining with anti-PPH3 antibody in normal tissues

All mitotic figures assessed by H&E staining present at the base of colonic mucosa crypts (fig 1A) and in the hyperplasic germinal centres of lymph nodes and tonsils (data not shown) were strongly stained with the anti-PPH3 antibody. Furthermore, as expected, double-immunofluorescence staining with anti-PPH3 and anti-cyclin B1 antibodies showed that all prophase figures coexpressed PPH3 and cyclin B1 in the nucleus (fig 1B,C).

In addition, non-mitotic (interphasic) sparse nuclei exhibited a finely granular staining, preferentially lining the nuclear membrane. This staining pattern was observed at the base of some colonic crypts (fig 1A), but not in the surface epithelium, and in a few lymphocytes scattered in the interfollicular areas of the lymphoid tissues. These non-mitotic cells could be divided into two classes according to double-immunofluorescence staining: cells displaying a cytoplasmic cyclin B1 staining corresponding to the late G2 phase (fig 1D,E) and cells scoring negative with cyclin B1 both in the cytoplasm and the nucleus, thus indicating that they were out of the G2 phase.

### Pattern of PPH3 staining in breast adenocarcinomas

All mitotic figures assessed by standard histology were strongly labelled with the anti-PPH3 antibody in every sample, thus allowing a rapid identification of the area of highest mitotic activity for subsequent counting (fig 2A,B). Furthermore, we observed many prophase nuclei, characterised by strong and dense staining of chromatin clumps, and some interphase nuclei (fig 2A). On the basis of double-immunofluorescence staining and confocal microscopy analysis, all mitotic figures coexpressed PPH3 and cyclin B1, particularly prophase figures for which a colocalisation was observed in the nucleus, and interphase nuclei could be classified either in the late G2 phase (cytoplasmic cyclin B1 positive) or out of the G2 phase (cyclin B1 negative; data not shown). Furthermore, we confirmed that cells displaying morphological features of apoptosis or necrosis were PPH3 negative (fig 2A), showing that mitotic count based on immunohistochemistry was more accurate than that determined on H&E staining.

### Comparison between PPH3-based and standard mitotic counts in breast adenocarcinomas

We next compared mitotic counts in PPH3-stained and H&E-stained paraffin-wax-embedded sections according to the Scarff–Bloom–Richardson histoprognostic grading modified by Elston and Ellis,[4] in 39 breast adenocarcinomas. A strong correlation was found between the two methods in all tumour grades ($r = 0.86$, $p<0.0001$). As shown in table 1, no

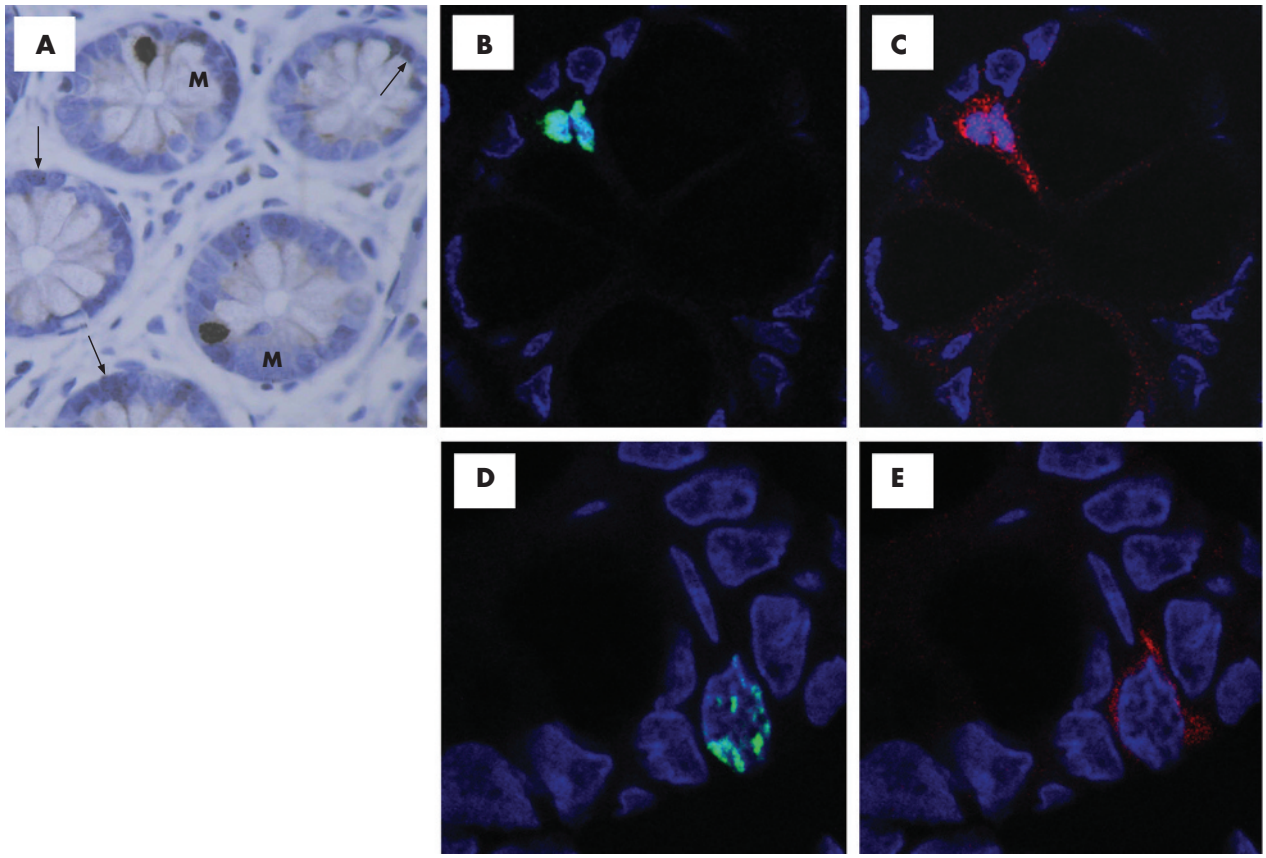


**Figure 1** Normal colonic mucosa. (A) PPH3 immunoperoxidase staining. PPH3-positive mitotic figures (M) are observed at the base of some crypts. Sparse PPH3-positive non-mitotic (interphasic) nuclei are also observed (arrows). Original magnification ×400. PPH3 (B and D, green) and cyclin B1 (C and E, red) double staining followed by confocal microscopy. Cyclin B1 and PPH3 are coexpressed in the nucleus of prophase figures (B, C). In a PPH3-positive non-mitotic (interphasic) cell, cyclin B1 staining is cytoplasmic, corresponding to cells in the late G2 phase (D, E). Original magnification ×630.

significant difference was found between these two methods, whatever the tumour grade. Interestingly, detection of mitotic figures with the PPH3 antibody was more sensitive than H&E staining in all tumour grades, especially in grades II and III. This difference in sensitivity is explained by the fact that prophase figures, which are not taken into account on H&E staining, can be detected in PPH3-stained sections. PPH3 staining was particularly useful in detecting mitotic cells in high-grade tumours with dense cellularity and numerous apoptotic or necrotic cells. In all, 5 of the 13 grade I (38.4%), 7 of the 13 grade II (53.8%) and 5 of the 13 grade III (38.5%) carcinomas showed a higher number of PPH3-positive mitotic cells compared with H&E staining. Furthermore, when the global histoprognostic score was

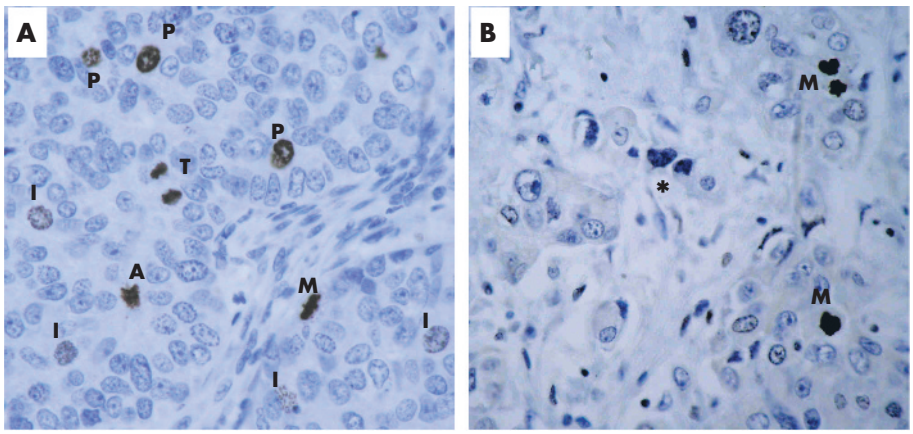


**Figure 2** Grade III breast adenocarcinoma. PPH3-immunoperoxidase staining (A, B) allows a rapid identification of the area of highest mitotic activity. It permits identification of mitotic figures (P, prophase; M, metaphase; A, early anaphase; T, telophase), and non-labelled apoptotic nuclei (asterisk) are easily distinguished. Some non-mitotic (interphasic) nuclei are also PPH3 positive (I). Original magnification ×400.

**Table 1** Comparison between mitotic figure counts on haematoxylin and eosin (H&E) and phosphorylated histone H3 (PPH3)-stained sections in 39 breast adenocarcinomas

| SBR grade | Cases | H&E (MFs per 10 HPF), mean (SE; range) | PPH3 (MFs per 10 HPF), mean (SE; range) | p Value |
|---|---|---|---|---|
| I | 13 | 3 (0.4; 1–6) | 4 (1; 1–15) | NS* |
| II | 13 | 9 (2; 2–26) | 13 (3; 1–42) | NS* |
| III | 13 | 32 (4; 12–57) | 36 (4; 15–74) | NS* |

MF, mitotic figures; NS, not significant; SBR, Scarff–Bloom–Richardson. HPF magnification ×400.
*No statistical difference was found between these two methods (Wilcoxon's non-parametric paired test).

**Table 2** Comparison between determination of mitotic index (MI) on phosphorylated histone H3 (PPH3)-stained breast adenocarcinomas by visual and automatic computer-assisted methods

| Cases | Visual MI | Automatic MI | p Value |
|---|---|---|---|
| 1 | 0.52 | 0.51 | NS* |
| 2 | 0.52 | 0.48 | NS* |
| 3 | 0.82 | 0.86 | NS* |

NS, not significant.
Results are expressed as the mean of MI assessed after PPH3 staining by visual and automatic computer-assisted methods in 10–14 HPF per case.
*No statistical difference was found between these two methods (Wilcoxon's non-parametric paired test).

re-evaluated on the basis of PPH3 staining in breast adenocarcinomas, it was shifted from grade I to grade II in one case, and from grade II to grade III in two cases.

### Confocal microscopy-based and automatic computer-assisted counts of PPH3-positive mitotic figures in breast adenocarcinomas

We compared the mitotic index in PPH3-stained paraffin-wax-embedded sections assessed visually with those assessed by a computer-assisted method in three cases of breast adenocarcinomas. Visual and automatic mitotic indices were assessed as the ratio of the number of PPH3-labelled mitotic figures to the total number of nuclei in 10–14 consecutive fields (4000–6500 nuclei counted). In each case, a good correlation between these two methods was found, and there was no statistically significant difference (table 2).

## DISCUSSION

Although previous studies have dealt with PPH3 staining of colorectal adenocarcinomas and ovarian serous adenocarcinomas,[18 19] we are aware of only one study that focused on a tumour type, meningiomas, which requires a histoprognostic grading.[15] This is why we focused on a tumour type in which the mitotic count is relevant to the prognosis—that is, breast adenocarcinomas. Our study showed a very good correlation between the mitotic figure count assessed with PPH3 labelling and that assessed by standard H&E sections. PPH3 mitotic figure count has several advantages. Firstly, it allows clear and unambiguous identification of mitotic figures, especially of prophase nuclei, a finding supported by the nuclear coexpression of PPH3 and cyclin B1, and consistent with previous results.[19] As a consequence, PPH3 labelling improves the mitotic count. Accordingly, the histoprognostic grade of breast adenocarcinomas was shifted from grade I to grade II in one case, and from grade II to grade III in two cases. In addition, PPH3 staining is also a useful marker in tumours in which a high degree of apoptosis and necrosis can be misinterpreted in H&E staining. Furthermore, it allows a rapid detection of the area of highest mitotic activity, even at low-power view. Interestingly, it may be useful to detect and count mitotic figures more precisely on small biopsy specimens that are sometimes crushed or contain a dense distorted tumour infiltrate.

We also observed some non-mitotic (interphasic) cells labelled with anti-PPH3 antibody, which displayed a finely granular staining. Juan *et al*[12 13] found the same weak and punctate expression of PPH3 in interphasic leukaemic cells. Wolffe and Hayes[20] also described interphase cells with a low level of histone H3 phosphorylation, which was interpreted as chromatin modification related to gene transcription. On the basis of cyclin-B1 staining, these non-mitotic (interphasic) cells could be divided into two classes: cells in the late G2 phase of the cell cycle (cytoplasmic cyclin-B1 positive) and cells out of the G2 phase (cyclin-B1 negative). Whatever the explanation of this immunolabelling of interphase nuclei, the important point is that these nuclei can be easily distinguished because of their specific staining pattern. In this context, further work is needed to explore the biological significance of this histone H3 phosphorylation out of the M phase.

Only a few reports described an automatic counting of immunostaining signals. For example, counting of fluorescence in situ hybridisation signals in tissue sections showed a strong correlation between visual and automatic counting after removal of artefacts and noise with image-processing techniques.[21] In the present study, the good correlation between visual and computer-assisted counts of PPH3-positive mitotic figures with confocal microscopy suggests that this new automatic method is rapid, objective, reproducible and less time consuming for determining the mitotic index in tumours. This method should be promising in the future, and may be useful in grading other tumour types in which the mitotic index has also been shown to have prognostic significance. For a more precise automatic mitotic index determination, however, a specific method for differentiating nuclei of tumour cells from those of stromal cells needs to be developed.

### Take-home messages

- PPH3 staining allows clear identification of mitotic figures, especially prophase nuclei.
- In some tumours, PPH3 staining is useful to distinguish mitotic figures from morphological mimics, for example, nuclei of cells undergoing apoptosis or necrosis.
- PPH3 staining allows a rapid detection of the area of highest mitotic activity, even at low-power magnification.
- This study demonstrates the feasibility of a computer-assisted determination of the mitotic index using PPH3 staining and image-analysis software.

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