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A New Rabbit Monoclonal phospho-histone H3 (pHH3) Hybridoma: An Immunohistochemical Comparison Study with a Rabbit Polyclonal pHH3

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Background

Microscopic evaluation of mitotic figures by Hematoxylin and Eosin (H&E) staining is a routine procedure in the assessment of the prognostic grade of the tumors.[1] Nevertheless, counting mitotic figures is a manual and time consuming process, with assorted difficulties and intra-observer assessment variability.[2] Histone H3 (Ser10) is phosphorylated in association with mitotic chromatin condensation in late G2 and M phase of the cell cycle, and thus phospho-histone H3 (pHH3) can distinguish mitoses from apoptotic nuclei.[3] The immunohistochemical (IHC) staining of Serine-10 (Ser10) pHH3 has been reported to be comparable to mitotic figure staining in the H&E section.[4-6] Most IHC staining publications of pHH3 have used a commercially available rabbit polyclonal pHH3 (P) antibody. However, a monoclonal pHH3 would offer an advantage of a specific epitope and would eliminate the pitfalls of batch to batch variation.

Materials and Methods

A rabbit monoclonal (RM) hybridoma Ser10 pHH3 [BC37] (Biocare Medical) was developed and confirmed by Western blot and ELISA. Three cases of normal tonsil and of melanoma were selected for IHC evaluation. To confirm that the rabbit monoclonal antibody and the rabbit polyclonal antibody both bind to pHH3 at Ser10 specifically, a blocking experiment using two histone 3 sequences which included Ser10 was developed. Peptide Block #1 is Ser10 non-phosphorylated histone 3 and Peptide Block #2 is Ser10 phosphorylated histone 3. Both antibodies were incubated with Peptide Block #1 and Peptide Block #2 for 30 minutes before performing IHC staining. A control group without peptide was used by adding equal volumes of antibody dilution buffer.

Immunohistochemistry

Whole tissue samples of tonsil and melanoma were selected, deparaffinized and hydrated down to water. Slides containing tissues cut at 4µm were placed in a modified citrate buffer solution and heated to 110 °C for 15 minutes in a pressure cooker. Slides were cooled and placed on an automated stainer. The pHH3 rabbit monoclonal and rabbit polyclonal antibodies were incubated for 30 minutes, followed by a rabbit HRP-polymer detection system and visualized with DAB.

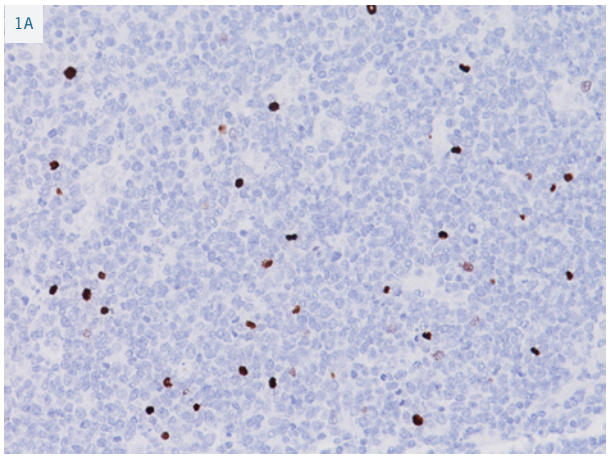
Quantification of pHH3 positive cells

For each case tested, pHH3 staining was recorded by counting five fields of view with a 20x objective. Quantitation of the pHH3 mitotic figures was calculated using a computer assisted imaging system (Applied Imaging Systems) to count unlabeled cells and mitotic figures in prophase, metaphase, anaphase and telophase (without counting interphase granulated nuclei). It was noted that interphase granulated nuclei were seen at a much higher frequency with the pHH3 (P) compared to the pHH3 (RM). This specificity was achieved by adjusting the calculator program to score cells in interphase at 1+ intensity and all other cells in mitosis at 2+ and 3+ staining. Tabulation was achieved with an H-scoring method that counted the total mitotic count at 2+ and 3+ staining intensity and subtracted 1+ staining from the total count.

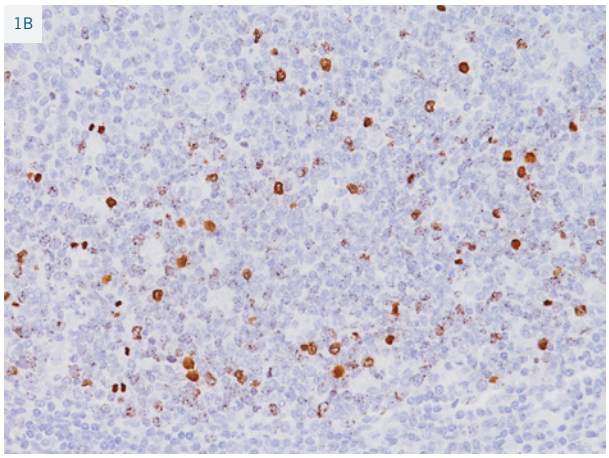
Results

The percentage of mitotic figure (MF) counts in normal tonsil stained with pHH3 (P) was 2.5% vs. 1.4% when stained with pHH3 (RM) (Table 1). However, in tonsil, pHH3 (RM) displayed stronger staining intensity in MF without granular staining in interphase nuclei, unlike the polyclonal pHH3 which demonstrated a much higher expression of granular staining in interphase nuclei (Figures 1A, 1B, respectively). Peptide Blocking Experiment #1 (Ser10 non-phosphorylated histone 3) showed a reduction of MF with pHH3 (RM) from 1.4% to 1.2% vs. with pHH3 (P) which displayed a drop from 2.5% to 1.7% (Figures 1C, 1D, respectively). Peptide Blocking Experiment #2 (Ser10 phosphorylated histone 3) showed no staining expression with either antibody. In melanoma, pHH3 (RM) displayed a MF count of 0.9% vs. pHH3 (P) which displayed 1.0% (Table 2, Figures 2A, 2B).

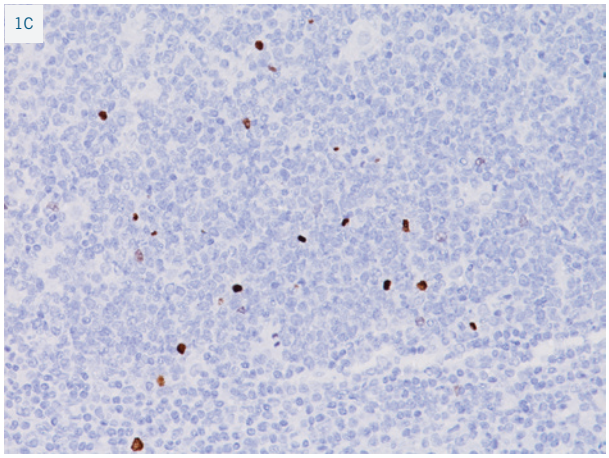
Figure 1



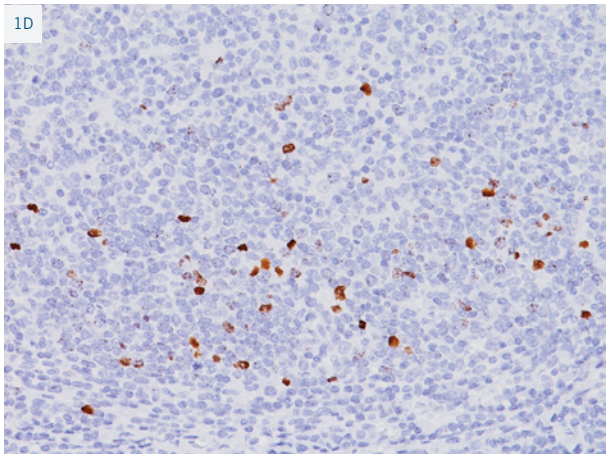
pHH3 (RM) No Treatment on Tonsil



pHH3 (P) No Treatment on Tonsil

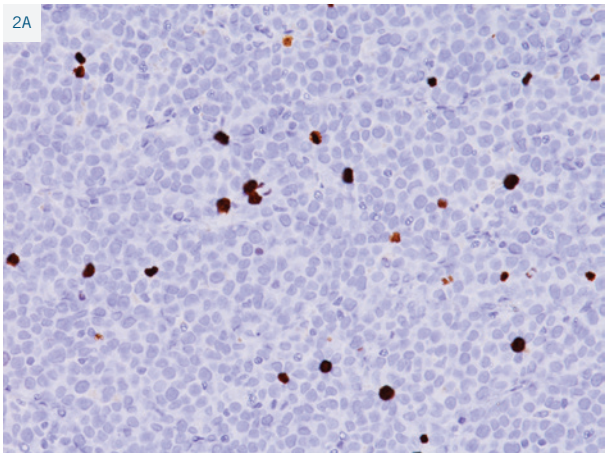


pHH3 (RM) Peptide Block #1 on Tonsil

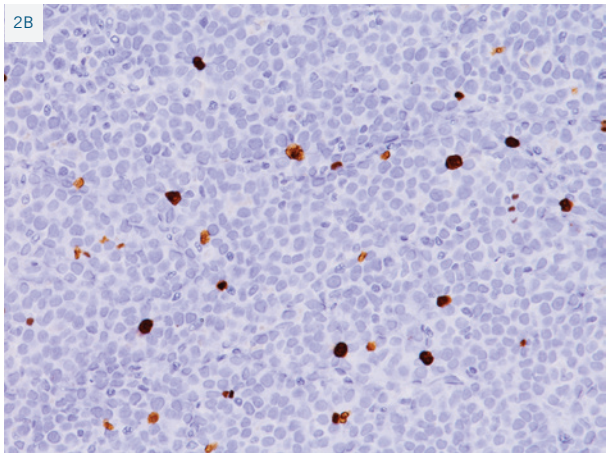


pHH3 (P) Peptide Block #1 on Tonsil

Figure 2

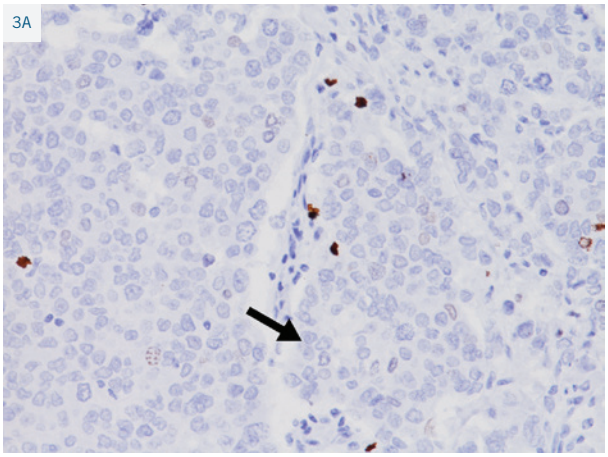


pHH3 (RM) Staining Melanoma

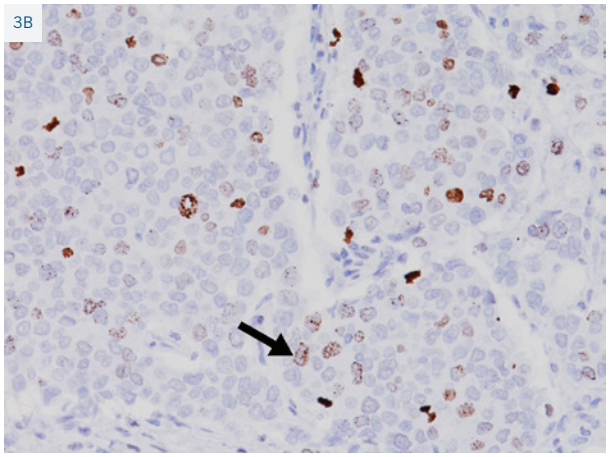


pHH3 (P) Staining Melanoma

Figure 3



pHH3 (RM) No Granular Staining on Melanoma



pHH3 (P) with Granular Staining on Melanoma

Table 1

Tonsil	Cells Counted	Mitotic Figures	% Stained
pHH3 (RM)	15639	219	1.4
pHH3 (P)	15510	381	2.5
pHH3 (RM) with Peptide Block #1	16431	197	1.2
pHH3 (P) with Peptide Block #1	15909	270	1.7

Table 2

Melanoma	Cells Counted	Mitotic Figures	% Stained
pHH3 (RM)	16479	148	0.9
pHH3 (P)	17517	180	1

Discussion

This study presents the first monoclonal pHH3 developed for IHC and compares it with a rabbit polyclonal pHH3 that has been utilized in previous publications. The rabbit monoclonal pHH3 (RM) vs. rabbit polyclonal pHH3 (P) appears to be more specific in the analysis of mitotic figures in late G2 and M-phase in the cell cycle. Peptide Block #1 was developed to block-out non-phosphorylated histone 3 and to document if pHH3 antibodies cross-react with these binding sites. In certain melanoma cases, granulated nuclei at interphase were more prominent with pHH3 (P) compared to pHH3 (RM) (Figures 3A, 3B). This could be due to fixation artifacts. Clearly the data demonstrates that pHH3 (P) stains a higher number of nuclei at interphase when compared to pHH3 (RM). This could be interpreted that pHH3 (P) is not completely phospho-specific and/or pHH3 (P) cross-reacts with more nuclei at interphase compared to pHH3 (RM). The use of an image analysis system is a clear advantage for tabulating MF vs. the manual counting of MF on an H&E stain.

Conclusion

The rabbit monoclonal pHH3 demonstrated strong and specific staining for mitotic figures. Its monospecificity to targeted epitopes at mitosis is a clear advantage to its polyclonal counterpart which demonstrated cross-reactivity probably due to recognizing multiple epitopes. The use of an image analysis system made recording pHH3-stained mitotic figures much easier and less time consuming. This method should offer superior reproducibility over the standard H&E method, which is more dependent on the observer's experience. Finally, rabbit polyclonal antibodies have been shown to have potential staining inconsistencies due to their batch to batch variations, and thus monoclonal antibodies are generally preferred.

References

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