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ORIGINAL ARTICLE

Phosphohistone H3 (PHH3) as a surrogate of mitotic figure count for grading in meningiomas: a comparison of PHH3 (S10) versus PHH3 (S28) antibodies

Napaporn Puripat[1] · Kongsak Loharamtaweethong[1]

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Abstract

Mitotic figure (MF) counting is important in the evaluation of meningioma grading. Nevertheless, mitosis assessment on hematoxylin and eosin (H&E)-stained slides may be problematic because of technical factors and pathologist's experience. Phosphohistone H3 (PHH3) is a mitosis-specific antibody that has proven to facilitate mitotic count in various tumors. However, the antibody performance between PHH3 serine10 (S10) and serine28 (S28) has never been compared in these tumors before. In this study, 48 cases of meningioma (28 grade I, 14 grade II, 6 grade III) were evaluated using immunohistochemical stains for four commercially available PHH3 (S10) and S28 antibodies to identify MFs and validate PHH3 intra- and interobserver reproducibility and agreement. Two pathologists counted MFs on both H&E- and PHH3-stained slides. H&E and PHH3 MFs were highly correlated (Spearman's rho = 0.96 for PHH3 (S10)-Biocare, 0.96 for PHH3 (S10)-CST, 0.91 for PHH3 (S28)-Abcam, and 0.89 for PHH3 (S28)-Santa Cruz. The mean difference between an H&E and PHH3 mitotic count is 0.81 for PHH3 (S10)-Biocare, 0.95 for PHH3 (S10)-CST, − 0.97 for PHH3 (S28)-Abcam, and − 0.97 for PHH3 (S28)-Santa Cruz. For comparison among four PHH3 antibodies, PHH3 mitotic counts had both a good intra- and interobserver reproducibility ($p > 0.05$). Regarding to World Health Organization (WHO) grade, there was not a significant discrepancy in the stratification of tumor grades for all four PHH3 antibodies in terms of interobserver agreement. The Cohen's kappa coefficient (K) was 0.93 for PHH3 (S10)-Biocare, 0.82 for PHH3 (S10)-CST, 0.76 for PHH3 (S28)-Abcam, and 0.80 for PHH3 (S28)-Santa Cruz. Considering survival analyses, all five proliferation indices were univariately associated with recurrences. Increased PHH3 mitotic indices (MIs) were significantly associated with recurrence-free survival in univariate Cox proportional hazards regression analysis ($p < 0.001$) and remained an independent predictor in multivariate analysis ($p < 0.05$). The appropriate prognostic cutoff values for recurrence prediction were 5 or more per 10 high-power fields (HPFs) for PHH3 (S10) and 3 or more per 10 HPFs for PHH3 (S28).

Keywords Phosphohistone H3 · Serine 10 · Serine 28 · Mitosis · Meningioma grading · Comparison

Introduction

Meningiomas account for approximately one third of primary intracranial neoplasms and represent the largest group of benign tumors. They are most frequently encountered in middle-aged and elderly patients. Most of them are benign and slow-growing and are usually curable after complete resection. Nonetheless, they have an intrinsic trend to recur, and recurrence depends strongly on tumor grade, proliferative activity, tumor subtype, brain invasion, and extent of resection [1, 2].

The 2016 revision of the World Health Organization (WHO) classification of meningiomas stratifies these tumors into three prognostically significant grades based on the number of mitotic figures (MFs) per 10 consecutive high-power fields (HPFs; 0.16 mm^2) in the area of highest mitotic activity as follows: benign (WHO grade I) with < 4 mitoses/10 HPFs, atypical (WHO grade II) with 4–19 mitoses/10 HPFs, and anaplastic (WHO grade III) with ≥ 20 mitoses/10 HPFs [2].

✉ Kongsak Loharamtaweethong
kongsakloharamtaweethong@hotmail.com

[1] Department of Anatomical Pathology, Faculty of Medicine, Vajira Hospital, Navamindradhiraj University, 681 Samsen Road, Dusit, Bangkok 10300, Thailand

Consequently, correct count for MF is essential to this diagnostic purpose. However, the quantitation of MF can be difficult due to some factors, for example, the heterogeneity of mitotic activity in different areas of the tumor and subjectivity in discriminating MF on H&E slides from nuclear changes occurring in apoptotic cells, crushed cells, pyknosis, or necrosis [3].

PHH3 is a core histone protein, which together with other histones forms the major protein constituents of the chromatin in eukaryotic cells. In mammalian cells, PHH3 is negligible during interphase but reaches a maximum for chromatin condensation during mitosis [4]. Immunohistochemically, PHH3 specifically detected the core protein histone H3 only when phosphorylated at serine 10 (S10) or serine 28 (S28). They stain mitotic figures from early prophase through metaphase, anaphase, and telophase [5]. PHH3 can serve as a mitotic marker to distinguish MFs from apoptotic bodies and some problematic situations, such as crushed artifact, distorted cells, necrosis, and karyorrhectic debris. Although PHH3 S10 and S28 antibodies were observed in several tumors [5], such as breast [6, 7], melanoma [8, 9], glioma [10], and also in meningioma [11, 12], they lacked of the comparative performance between such antibodies.

Therefore, the present study was performed to assess the diagnostic role of four commercially available PHH3 antibodies (S10 and S28) as a mitotic marker in comparison with standard H&E-stained slides. In addition, we also evaluated the results of PHH3 staining for determination of the PHH3 (S10) and (S28) mitotic indices (MIs) in the correlation between PHH3 MI and recurrence-free survival which compared the results with those for H&E MI. All parameters of proliferation activity were also evaluated for prognostic significance. Based on these findings, we provided the optimal cut-off values for PHH3-specific mitotic thresholds with prognostic and grading implications.

Materials and methods

Meningioma tissue samples

Institutional Review Board approval at the Navamindradhiraj University was obtained for this study. Between 2012 and 2017, 48 patients with meningiomas were selected from the archives of the Department of Anatomical Pathology. All available formalin-fixed paraffin-embedded (FFPE) tissue specimens were reviewed. Only diagnostic criterion for mitotic count was included in this study for meningioma classification according to current WHO 2016 criteria. The slide containing the highest mitotic activity on H&E was selected from each tumor. Of each tumor, a representative FFPE sample was selected, containing morphologically well-preserved tumor tissue, and tissue sections were stained with H&E and immunostained for four commercially available anti-PHH3 antibodies.

Meningioma cases with clinical data and follow-up

For statistical analyses of recurrence-free survival, only cases followed up until recurrence or death or for at least 3 years were included. Clinical data recorded included patient's sex, age, tumor locations, date of surgery for primary and recurrent tumor resections, extent of resection, and date of recurrence as detected radiologically (computed tomography or magnetic resonance imaging). The extent of resection was documented along the guidelines proposed by Simpson [13]. Complete surgical eradication of the tumor (gross total resection [GTR]) was designated as Simpson grade I or II, corresponding to macroscopically determined complete tumor resection with bipolar coagulation of the dural insertion. Anything less than GTR was designated as subtotal resection (STR).

Immunohistochemistry

Immunohistochemistry was performed on the Leica Bondmax platform (Leica Micro-systems, Buffalo Grove, IL, USA) on 2-μm-thick consecutive sections with procedures performed according to the manufacturer's instructions. Rabbit monoclonal anti-PHH3 (S10) [1:150, Biocare #ACI3130A], rabbit polyclonal anti-PHH3 (S10) [1:100 Cell Signaling System (CST) #9701], rabbit monoclonal anti-PHH3 (S28) [1:1000, Abcam #ab32388], and mouse monoclonal anti-PHH3 (S28) [1:500 Santa Cruz #SC-374669] were used as primary antibodies. Human vermiform appendix was used as a positive control in all immunohistochemical reactions.

Slide scoring

In this study, 2-μm H&E-stained FFPE slide MFs were counted in 10 consecutive HPFs, which allowed calculation of the mitotic rate per 0.16 mm^2 surface area. PHH3-positive cells were counted in the same way as MFs in H&E sections in 10 HPFs and expressed per 0.16 mm^2 surface area as well. Cells with brown staining and the following features were considered as positively stained MFs: loss of nuclear membrane, presence of chromosome condensation, and arranged either along a plane or separated (Fig. 1a–f). Nuclei with intact, smooth nuclear membranes and absence of striking chromosome condensation were considered to be nonspecific and were disregarded (Fig. 1g–i).

For H&E-stained slides, two raters counted MFs simultaneously via multi-head microscope and the final counting were accomplished by rater consensus (gold standard). For PHH3-stained slides, two raters counted PHH3-positive MFs

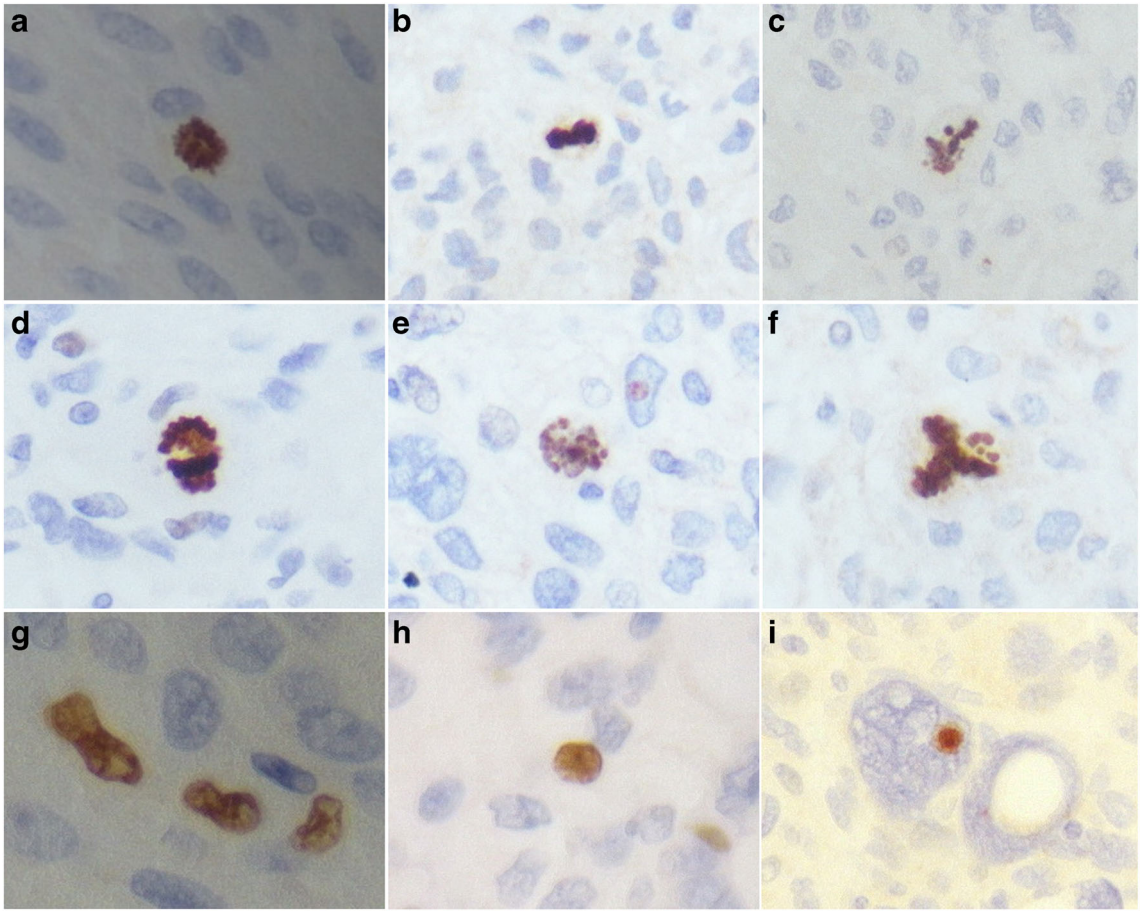

Fig. 1 **a**–**f** Examples of PHH3-positive mitotic figures: absence of nuclear membrane and presence of chromosome condensation (**a**); condensed chromosomes arranged along a plane (**b**); chromosome separation (**c**–**e**); and tripolar mitosis (**f**). **g**–**i** Non-specific PHH3 staining. Nuclei with positive staining, with intact, smooth nuclear membranes and without striking chromosome condensation are not counted

individually via multi-head microscope. Each slide was read twice by two raters. The second reading was made 2 weeks after the first without knowledge of first reading results. Each microscopic field was located and recorded by a vernier scale in both *x*- and *y*-axes. As a result, the second reading was accomplished by the previous recorded localization to evaluate the same microscopic field.

Statistical analysis

Statistical analysis was made using Stata Statistical Software, College Station, TX: StataCorp LP (Stata Software; http:// www.stata.com). The test is significant when p value is < 0.05.

For different PHH3 antibodies, intrarater reproducibility was computed using paired t tests to compare results between the first and the second readings for each case and each rater. To assess interrater reproducibility, Student's test was performed to compare first reading results for each slide between the two raters. The relationships between H&E and PHH3 were studied using Spearman's rho correlation. Linear regression analysis was performed to evaluate the correlation between H&E and different PHH3 antibodies. Bland-Altman plots were used to evaluate the mean difference between H&E mitotic count and PHH3-based mitotic index for the first rater. Agreement between the H&E-based grade and PHH3-based grade was evaluated by calculating the Cohen's kappa coefficient. Receiver operator characteristics (ROC) were used for determination of the appropriate cutoff levels (based on maximization of the Youden index) for each proliferation index regarding discrimination between recurrent and non-recurrent meningiomas. Recurrence-free survival times were computed and distributions estimated by using the Kaplan-Meier plots. The log-rank test was used for univariate survival analyses. Adjusted for patients' age and extent of tumor resection, Cox proportional hazards modeling techniques were used for multivariate analyses.

Table 1 Comparisons between readings and raters with different PHH3 antibodies

PHH3 mitotic	Rater 1 Mean ± SD	*p* value[a]	Rater 2 Mean ± SD	*p* value[a]	*p* value[b]
Biocare (1st reading)	6.25 ± 8.45	0.785	6.27 ± 8.44	0.569	0.990
Biocare (2nd reading)	6.23 ± 8.53		6.23 ± 8.54		1.000
CST (1st reading)	6.40 ± 8.36	0.038	6.40 ± 8.40	0.060	1.000
CST (2nd reading)	6.58 ± 8.68		6.58 ± 8.67		1.000
Abcam (1st reading)	4.46 ± 7.43	0.086	4.58 ± 7.59	0.204	0.935
Abcam (2nd reading)	4.25 ± 7.52		4.46 ± 7.80		0.894
Santa Cruz (1st reading)	4.46 ± 7.69	0.799	4.60 ± 7.88	0.622	0.927
Santa Cruz (2nd reading)	4.44 ± 7.61		4.65 ± 8.03		0.897

[a] Paired *t* test for comparison between the two readings

[b] Student's test for comparison between the two raters

Results

MF counting on H&E-stained slides

Twenty-eight benign WHO grade I; a mean value of 0.89 MFs/10 HPFs (range, 0–3), 14 atypical WHO grade II; 6.12 MFs/10 HPFs (range, 4–10), and 6 anaplastic, WHO grade III; 24.83 MFs/10 HPFs (range, 21–28) meningiomas were evaluated.

Intra- and interrater reproducibility of PHH3 mitotic counts

Statistical analysis revealed no significant difference for both raters for the first and second readings for PHH3 (S10)-Biocare, PHH3 (S10)-Abcam, and PHH3 (S28)-Santa Cruz but a significant difference appeared for PHH3 (S10)-CST for rater 1 ($p = 0.038$). Regarding the PHH3 results for

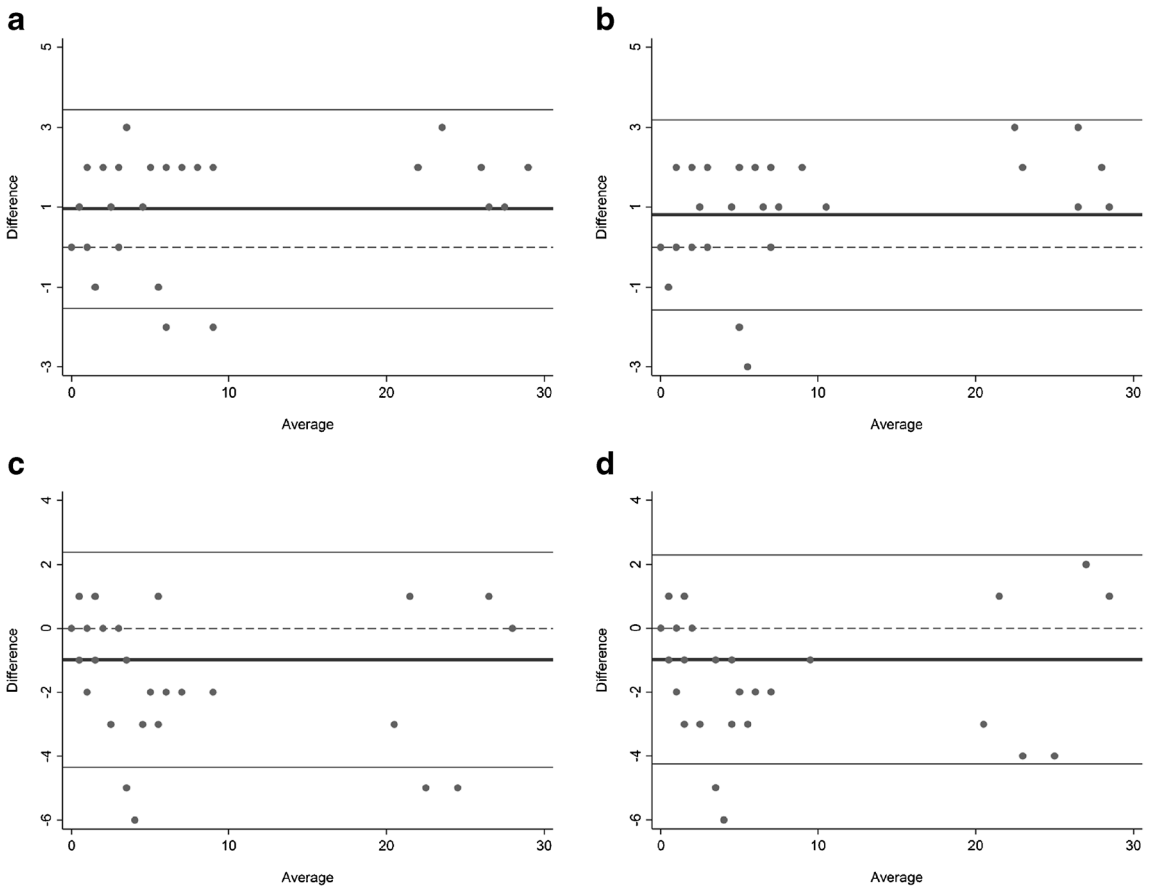

Fig. 2 Correlation between the first H&E-based and PHH3-based mitotic count for the rater 1. The Bland-Altman plots demonstrate the mean difference between these two counts for each PHH3 antibody. **a** H&E versus PHH3 (S10)-Biocare. **b** H&E versus PHH3 (S10)-CST. **c** H&E versus PHH3 (S28)-Abcam. **d** H&E versus PHH3 (S28)-Santa Cruz

Table 2 Percentage of agreement between two pathologists evaluated by Cohen's kappa coefficient for stratifying meningiomas according to the WHO grade

PHH3-based grade	H&E-based grade						Agreement (%)	*K*	*p* value
	Grade I		Grade II		Grade III				
PHH3 (S10)-Biocare									
Grade I	26	(54.2)	0	(0.0)	0	(0.0)	95.8	0.93	< 0.001
Grade II	2	(4.2)	14	(29.2)	0	(0.0)			
Grade III	0	(0.0)	0	(0.0)	6	(12.5)			
PHH3 (S10)-CST									
Grade I	23	(47.9)	0	(0.0)	0	(0.0)	89.6	0.82	< 0.001
Grade II	5	(10.4)	14	(29.2)	0	(0.0)			
Grade III	0	(0.0)	0	(0.0)	6	(12.5)			
PHH3 (S28)-Abcam									
Grade I	28	(58.3)	6	(12.5)	0	(0.0)	87.5	0.76	< 0.001
Grade II	0	(0.0)	8	(16.7)	0	(0.0)			
Grade III	0	(0.0)	0	(0.0)	6	(12.5)			
PHH3 (S28)-Santa Cruz									
Grade I	28	(58.3)	5	(10.4)	0	(0.0)	89.6	0.80	< 0.001
Grade II	0	(0.0)	9	(18.8)	0	(0.0)			
Grade III	0	(0.0)	0	(0.0)	6	(12.5)			

Data are presented as *n* (%)

K Cohen's kappa coefficient

between two raters, the differences between two readings were not significant in all four PHH3 antibodies ($p > 0.05$). The tests are summarized in Table 1.

Correlation between H&E and PHH3 results

The PHH3 count was comparable with H&E count by performing with all four PHH3 antibodies. H&E and PHH3 mitotic rates were highly correlated (Spearman's rho = 0.96 PHH3 (S10)-Biocare, 0.96 PHH3 (S10)-CST, 0.91 PHH3 (S28)-Abcam, and 0.89 PHH3 (S28)-Santa Cruz. Linear regression established a strong significant correlation between the HES and all four PHH3 methods used ($p < 0.001$). The Bland-Altman plots show that the mean difference between an HES and PHH3 mitotic count is 0.81 for PHH3 (S10)-Biocare, 0.95 for PHH3 (S10)-CST, − 0.97 for PHH3 (S28)-Abcam, and − 0.97 for PHH3 (S28)-Santa Cruz. The results are shown in Fig. 2.

Interobserver agreement on H&E-based grade and PHH3-based grade

According to the WHO meningioma grading, the percentage of agreement between the two pathologists, about the H&E-based grade and PHH3-based grade in our study, was relatively high in all primary antibodies, usually over 85%. Table 2 shows the Cohen's kappa coefficients for agreement between

Table 3 Results of receiver operating characteristics (ROC) curve analyses for optimal cutoff values

Classification variable	Cutoff*	Sensitivity		Specificity		AUC	
		%	(95% CI)	%	(95% CI)	AUC	(95% CI)
H&E MI	≥ 4/10 HPFs	88.9	(51.8–99.7)	76.5	(58.8–89.3)	0.83	(0.70–0.96)
PHH3 MI							
Biocare	≥ 5/10 HPFs	88.9	(51.8–99.7)	82.4	(65.5–93.2)	0.86	(0.73–0.98)
CST	≥ 5/10 HPFs	100.0	(66.4–100)	70.6	(52.5–84.9)	0.85	(0.78–0.93)
Abcam	≥ 3/10 HPFs	77.8	(40.0–97.2)	79.4	(62.1–91.3)	0.79	(0.63–0.95)
Santa Cruz	≥ 3/10 HPFs	77.8	(40.0–97.2)	82.4	(65.5–93.2)	0.80	(0.64–0.96)

H&E MI mitotic index assessed on H&E-stained slides, *PHH3 MI* mitotic index assessed in anti-phosphohistone H3-immunolabeled specimens, *CI* confidence interval

*Most appropriate cutoff point with the highest discriminatory power for distinguishing between recurrent and non-recurrent meningiomas

Table 4 Univariate Cox proportional hazards regression survival analyses of proliferation assessment methods

Variables	Recurrence rate (%)	Hazard ratio (95% confidence interval)	Median recurrence-free survival time (month)	*p* value*
H&E MI, ≥ 4/10 HPFs	50	18.44 (2.29–148.43)	48	< 0.001
PHH3 MI				
Biocare, ≥ 5/10 HPFs	57	24.46 (3.02–197.8)	45	< 0.001
CST, ≥ 5/10 HPFs	54	11.93 (2.45–58.15)	45	< 0.001
Abcam, ≥ 3/10 HPFs	50	10.25 (2.11–49.80)	45	< 0.001
Santa Cruz, ≥ 3/10 HPFs	54	11.93 (2.45–58.15)	45	< 0.001

*Log-rank test

the diagnostic methods. The best agreement ($K = 0.81–1.00$) was PHH3 (S10)-Biocare (0.93) and PHH3 (S10)-CST (0.82).

Meningioma cases with clinical data and follow-up

Among these patients, 43 with complete clinical data and followed up were included for analyses. The mean age of patients at initial diagnosis was 49 years (range, 36–62 years) and 67% were female. A GTR was achieved in 32 cases, whereas 11 patients underwent only STR. The median follow-up for all patients studied was 58 months (range, 36–68 months). All cases were followed up until recurrence or a minimum of 3 years were included in survival analyses. Initial diagnoses were WHO grade I meningioma in 27 of these patients, WHO grade II meningioma in 13 patients, and WHO grade III meningioma in 3 patients. The overall recurrence rate was 20.9% ($n = 9$), and the recurrence rate (RR) was significantly higher for meningiomas treated by STR (5/11; RR = 45.5%) than for those treated by GTR (4/32, RR = 12.5%). The recurrence rate in WHO grade I meningiomas was 9% in the STR subset (1/11) and 3.7% (1/27) in the entire cohort (GTR and STR subsets). Recurrences were documented in 46.2% (6/13) of WHO grade II meningiomas and 66.7% (2/3) of anaplastic meningiomas.

Recurrence-free survival analyses

Results of ROC analysis for H&E MI, PHH3 (S10) MI, and PHH3(S28) MI are shown in Table 3. ROC analysis displayed the following cutoff values with maximization of Youden index for each MI as the most appropriate: H&E MI, 4 or more per 10 HPFs; PHH3 (S10)-Biocare MI, 5 or more per 10 HPFs; PHH3 (S10)-CST MI, 5 or more per 10 HPFs; PHH3 (S28)-Abcam MI, 3 or more per 10 HPFs; and PHH3 (S28)-Santa Cruz MI, 3 or more per 10 HPFs. Using cutoff value as 5 or more per 10 HPFs for PHH3 (S10) and 3 or more per 10 HPFs for PHH3 (S28) significantly demonstrated cases at risk of recurrence.

In addition, PHH3 (S10) and PHH3 (S28) MIs were comparable to the standard H&E MI in the discrimination between recurrent and non-recurrent cases, as shown by ROC analyses. The highest discriminatory power among the proliferation indices is suggested for the PHH3 (S10) and PHH3 (S28) MIs, which exhibit the greatest areas under the curve. The results of the log-rank test for H&E MI, PHH3 (S10)-Biocare MI, PHH3 (S10)-CST MI, PHH3 (S28)-Abcam MI, and PHH3 (S28)-Santa Cruz MI are summarized in Table 4, and the Kaplan-Meier curves are illustrated in Fig. 3. Univariate analyses revealed a significantly higher risk of recurrence for higher proliferation, as measured by all 5 methods. The hazard ratios were 18.44 for a H&E MI of 4 or more per 10 HPFs, 24.46 for a PHH3 (S10)-Biocare MI of 5 or more per 10 HPFs, 11.93 for a PHH3 (S10)-CST MI of 5 or more per 10 HPFs, 10.25 for a PHH3(S28)-Abcam MI of 3 or more per 10 HPFs, and 11.93 for a PHH3 (S28)-Santa Cruz MI of 3 or more per 10 HPFs. Results of multivariate Cox hazards modeling are summarized in Table 5. All 5 proliferation indices could be identified as independent predictors of worse outcome (recurrence risk), adjusted for age and extent of tumor resection.

Discussion

Accurate assessment of mitotic activity in meningiomas is essential to predict the biological behavior of meningioma. Meningiomas are graded based on mitotic count into grade I (< 4 mitoses/10 HPFs), grade II (4–19 mitoses/10 HPFs), and grade III (≥ 20 mitoses/10 HPFs) on standard H&E-stained slides, which might be subject to intra- and interobserver variability. This may result in errors and inconsistencies in assessing mitotic count, especially when the scoring is close to four. Consequently, accurate histologic grading is needed for proper medical treatment decisions.

Although Ki-67 is thought to correlate with behavior of meningiomas, it is not routinely used for the grading of meningiomas, and there is no agreed-on cutoff value. Based on

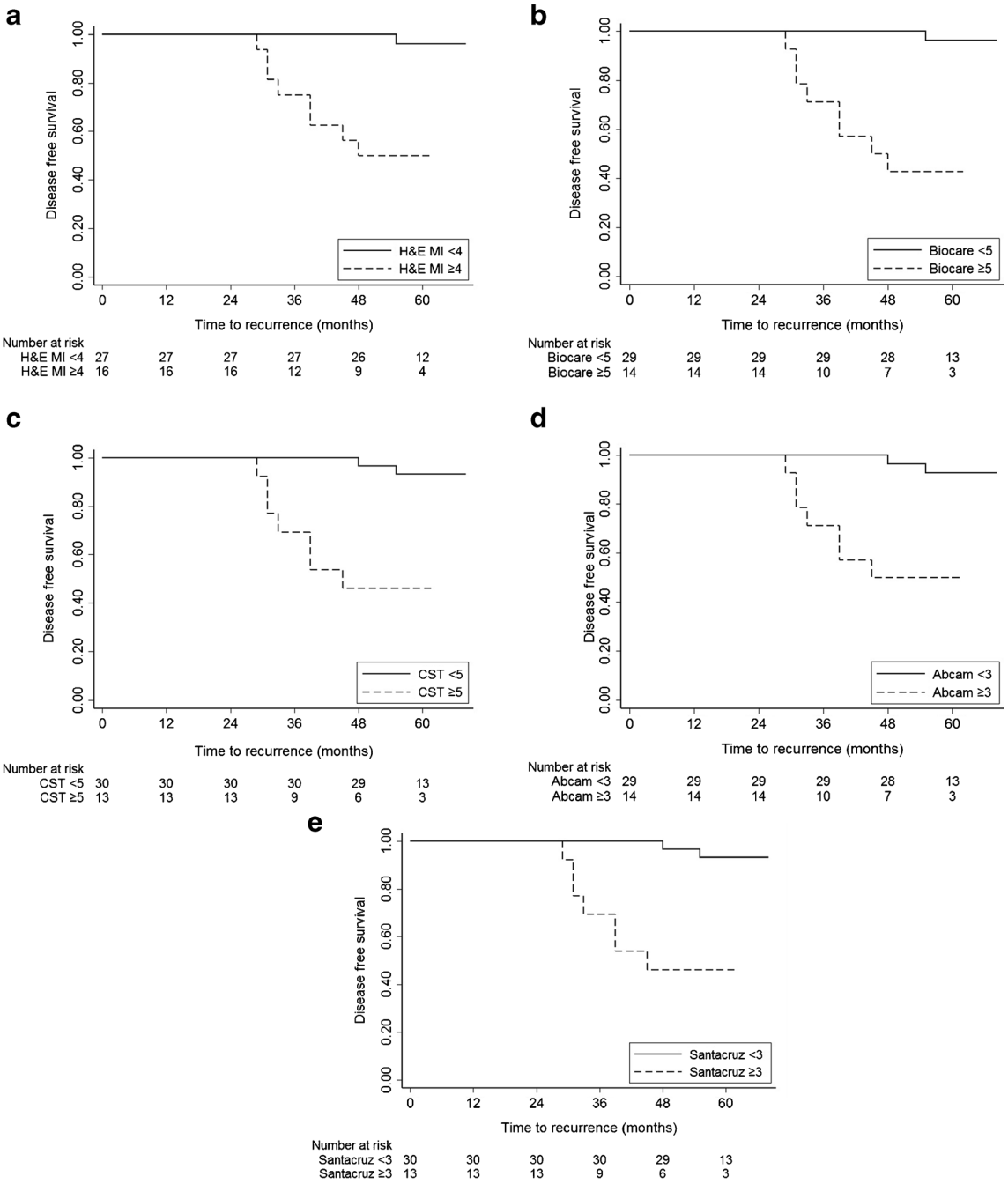

Fig. 3 Relationship of mitotic indices to recurrence-free survival in 43 patients followed up until recurrence for at least 3 years. The Kaplan-Meier curves for the H&E mitotic index (MI) (**a**), phosphohistone H3 (PHH3)-Biocare MI (**b**), PHH3-CST MI (**c**), PHH3-Abcam MI (**d**), and PHH3-Santa Cruz MI (**e**) demonstrate the significantly worse outcome for meningiomas with higher mitotic activity

the largest study, Winther et al. [12] reported their analyses regarding Ki-67 and PHH3 labeling indices (LI) in human meningiomas and compared the parallelity with the standard H&E MI. They found that all three proliferation parameters were significantly correlated with WHO grade. The optimal cutoff values for recurrence prediction were 3% for the Ki-67 LI and 0.5% for the PHH3 MI. However, PHH3 MI was significantly associated with recurrence-free survival in univariate Cox proportional hazards regression analysis ($p = 0.011$) and remained an independent predictor in multivariate analysis ($p = 0.005$), while traditional H&E MI and Ki-67 LI did not reach statistical significance. As a result, Ki-67 LI showed to have less powerful prognostic value than those with PHH3 MI parameter. In addition, Ki-67 LI tends to overestimate the proliferative activity of the tumor because it is able to stain cells that are in several phases of the cell cycle (non-G0 phase), including M, G1, G2, and S phases [14, 15]. In addition, tumor-infiltrating immune cells can also be stained with

Table 5 Multivariate recurrence-free survival analysis by Cox hazard regression in 43 meningioma cases

	Univariate analysis		Multivariate analysis	
	HR (95% CI)	*p* value	HR_{adj}[a] (95% CI)	*p* value
H&E MI, ≥ 4/10 HPFs	18.44 (2.29–148.43)	0.006	22.7 (2.57–200.68)	0.005
PHH3 MI				
Biocare, ≥ 5/10 HPFs	24.46 (3.02–197.8)	0.003	27.76 (3.14–245.24)	0.003
CST, ≥ 5/10 HPFs	11.93 (2.45–58.15)	0.002	17.12 (3.08–95.13)	0.001
Abcam, ≥ 3/10 HPFs	10.25 (2.11–49.80)	0.004	10.31 (2.06–51.65)	0.005
Santa Cruz, ≥ 3/10 HPFs	11.93 (2.45–58.15)	0.002	17.12 (3.08–95.13)	0.001

HR hazard ratio, HR_{adj} adjusted hazard ratio, *CI* confidence interval

[a] Adjusted hazard ratio estimated by Cox hazard regression adjusted for age and extent of resection

Ki-67, leading to an overestimation of the proliferative activity of a given tumor. As a result, PHH3 has the advantage of being mitosis specific, staining cells in the G2 to M transition.

Theoretically, PHH3 specifically detected the core protein histone H3 only when phosphorylated at S10 or S28. As a consequence, anti-PHH3 (S10) and S28 antibodies are widely used in studying in mitotic counting. However, most studies investigated on PHH3 (S10) which showed good correlation with H&E-stained mitotic count. Consequently, the data on antibody performance between them has still limited. Regarding to PHH3 (S28), Sun et al. [16] demonstrated that pHH3 S10 was superior to S28. However, the sample size was small in this study, including 10 cases of melanoma and 10 cases of unclassified subtypes of ovarian tumor. In addition, scoring of PHH3 was performed as a percentage of immunopositive cells, rather than being expressed as the routine visual scoring system, i.e., the number of positively stained cells per 10 HPFs. Hence, it is difficult to determine the antibody performance. In contrast to the previous study, our study showed that the difference between them is relatively small and did not reach statistical significance.

Overall, our results reveal that PHH3 (S10) and S28 staining within a given tumor can be useful, it allows for rapid screening of mitoses in the tumor hot spot at low power, and is less time-consuming than counting mitoses on routine H&E-stained slides. Our study also supported the opinion of previous studies that PHH3 (S10) immunostaining is a valuable diagnostic tool by providing a more efficient mitotic count assessment with little background staining. Similar to previous studies in meningiomas, our results demonstrate that H&E mitotic counts strongly and significantly correlate with PHH3 mitotic counts both PHH3 (S10) and S28 antibodies. Regarding the performances of PHH3 and H&E staining, our results are consistent with those of others [17–25]. PHH3 staining facilitates the identification of mitoses, providing more efficient, more sensitive, and more specific assessment, with little background staining. In addition, there was excellent intra- and interobserver agreement among observers in our study. Although PHH3 scores are slightly higher with PHH3 (S10) or lower with PHH3 (S28) than traditional mitosis counts obtained with standard H&E-stained slides, the difference did not reach statistical significance and the results still show a similar trend for tumor grade.

Considering tumor grade, there was a different histologic grade assigned using PHH3 immunostains compared with standard H&E. Our study demonstrated that 4.2% and 10.4% of cases being upgraded when using Biocare and CST, whereas 12.5% and 10.4% of cases being downgraded when using Abcam and Santa Cruz, respectively. However, the results are still comparable with standard H&E count. This may be explained by the sensitivity and specificity of PHH3 for staining mitotic figures and highlighting nuclear detail. Cases that had higher mitotic counts on H&E compared with PHH3 may have resulted from miscounting of crushed cells, apoptotic bodies, or karyorrhectic debris as actual mitotic figures or from random sampling error in a given mitotic hot spot. We also occasionally observed PHH3 immunostaining in crushed nuclei but were not able to differentiate whether this was true positive or nonspecific staining (data not shown). Additionally, it is important for the method of PHH3 counting to be standardized in order to allow comparisons between the findings of various studies. The definition of an HPF and the number of fields required often vary between studies and are highly dependent on the specifications of the microscopes being used. Besides the staining procedure used, several additional factors may have a significant impact on the final result such as prolonged cold ischemia time [26], decalcified samples, application of a standardized counting procedure, *heterogeneity* of *mitotic activity* in different areas of the tumor [27], the experience of the observer, and MFs in non-tumoral cells should be considered as well.

More importantly, owing to different sensitivity of the PHH3 (S10) and (S28) mitosis counting methods may occur, the mitotic thresholds proposed by the WHO should be reconsidered if PHH3 MIs are applied to tumor grading.

Perry and coworkers [28] defined the threshold value of 4 or more mitoses per 10 consecutive HPFs based on the results of multivariate survival analyses on H&E-stained tumor specimens. In the present study, multivariate Cox hazards modeling technique demonstrated the PHH3 (S10) and PHH3 (S28) MIs as an independent predictor of recurrence-free survival with the high sensitivity and specificity in the detection of recurrent cases. By using discriminant analyses, prognostic PHH3-specific threshold values could be defined, which permits discrimination between each risk group or, rather, tumor grade. However, as it holds for other immunohistochemical markers of proliferation, there is inconsistency among laboratories in terms of staining results and interpretation of PHH3 immunohistochemical reactions. Hence, it is difficult to determine agreed-on cutoff value for PHH3 MIs and the thresholds suggested herein may not be extrapolatable to other laboratories.

In our study, 5 or more PHH3 (S10)-positive mitoses per 10 HPFs and 3 or more PHH3 (S28)-positive mitoses per 10 HPFs demonstrate meningiomas with significantly higher risk of recurrence within 3 years after primary surgery, independent of patient age or extent of tumor resection. These findings emphasize the reliability and advantages of the PHH3 mitosis counting method in the appropriate risk stratification of patients with meningioma. However, owing to the small sample size of anaplastic meningiomas in our study, it is difficult to identify a cutoff point in these cases. Further studies in larger series are needed to determine the optimal cutoff value in this subgroup.

In conclusion, our study demonstrated that PHH3 is a rapid, easy, and sensitive method for the assessment of mitotic activity. Both PHH3 (S10) and S28 are correlated well with the mitotic count on H&E. Based on survival analyses, mitotic thresholds of PHH3 (S10) MI of 5 or more per 10 HPFs and PHH3 (S28) MI of 3 or more per 10 HPFs were proposed for the most appropriate prognostic cutoff values for the prediction of recurrence-free survival.

Authors' contribution All authors of the manuscript made substantial contributions to the conception or design of the work; the acquisition, analysis, or interpretation of data for the work; drafting the work and/or revising it critically for important intellectual content; final approval of the version submitted for publication; and agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

More specifically, authors' contribution is listed as follows:

NP: concept, development of the methods, case selection, supervision, preparation of the manuscript, finalization, and approval of the manuscript

KL: case selection, application and refinement of the methods, analysis of data, preparation of the manuscript, finalization, approval of the manuscript, and acts as corresponding author

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Compliance with ethical standards

This retrospective study was approved by the institutional ethical committee of the Navamindradhiraj University. The study did not include animals; therefore, issues relating to animal welfare do not apply.

Conflict of interest The authors declare that they have no conflicts of interest.

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