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Original Research

Mitotic Activity in Gastrointestinal Stromal Tumors: Can we use Phosphohistone H3 Immunohistochemistry Instead of Hematoxylin and Eosin for Mitotic Count?

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Abstract

Objectives: In gastrointestinal stromal tumors (GIST), malignancy potential is determined by the prognostic disease risk stratification based on mitosis, tumor size, and location. Phosphohistone H3 (PHH3) is an immunohistochemical marker showing mitotic activity in cells. In this study, we aimed to evaluate mitosis in GIST with PHH3, compare the results with hematoxylin and eosin (HE) stained slides, and examine its relationship with other prognostic data.

Methods: Clinicopathological findings and survival were determined in GIST cases diagnosed between 2006 and 2017. The prognostic risk score was calculated according HE- and PHH3-based mitosis. The cases were classified as Group I: HE + and PHH3 + and Group II: HE + and PHH3–. They were also grouped as those diagnosed before and after 2012 and the staining results of HE and PHH3 were re-analyzed.

Results: Ninety-eight cases were included in the study. Mitosis was detected with both HE and PHH3 in 63.3% of the cases (62/98 cases) (Group I) while in 36.7% of cases, it was detected with HE but not with PHH3 (Group II). In only two cases, the risk score changed with PHH3 (very low → intermedier grade). The ratio of HE + and PHH3 + cases in 2012 and after was significantly higher than HE + and PHH3 – cases. A statistically significant relation was found between HE- and PHH3-based risk scores ($p<0.05$). There was a significant difference between HE-based risk score groups in terms of survival ($p<0.05$), while no difference was observed between the PHH3-based risk score groups ($p>0.05$).

Conclusion: In GIST cases, PHH3 can be used to determine mitosis in more recent blocks, taking into account the technical conditions of the laboratory, but it does not seem to be superior to mitosis detected by HE. Research should continue on new survival determinants for GIST.

Keywords: Gastrointestinal stromal tumor, risk score, mitosis, phosphohistone H3

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Gastrointestinal stromal tumors (GIST) are mesenchymal tumors originating from interstitial Cajal cells known as "pacemaker" cells of the gastrointestinal tract or their stem cell-like precursors.[1] They develop as a result of activating mutations in KIT or PDGFRA, the transmembrane growth factor receptor and express KIT protein (CD117).[2] They are the most common mesenchymal tumors in the gastrointestinal tract and have an incidence of 0.1–3%.[3] They are most common in the stomach and secondly in the small intestine. They can also develop in the esophagus, colon, rectum and even omentum, and mesenteric adipose tissue.

Local tumor recurrences can be found in the abdomen even when the tumor is totally removed and when there is no microscopic tumor at the surgical margins.[4,5] Therefore, all GISTs are considered to have a malignancy potential. Fletcher et al. proposed and approach for defining aggressive behavior in GISTs (very low, low, intermediate, and high risk) by using the National Institutes of Health criteria, tumor size, and mitotic count.[6] Later, these criteria are modified by adding tumor rupture and primary tumor location.[7] Today, progressive (prognostic) disease risk classification, which is determined according to Miettinen and Lasota – Armed Forces Institute of Pathology (AFIP) data, is used.[1,4] According to this classification, the risk of progression can be determined by mitotic rate, tumor diameter and location. In routine daily practice, mitosis evaluated in hematoxylin and eosin (HE) slides is counted in 50 consecutive high power fields (HPF) or in a 5 mm^2 area. During evaluation, crushing and staining artifacts or technical factors such as apoptosis and karyorrhexis observed in necrotic areas as well as experience of the observer are important in determining the number of mitosis (especially ≤5 and >5).[8,9]

Phosphohistone H3 (PHH3) is a mitosis-specific antibody that is an immunohistochemical marker that shows cell nuclei in mitotic activity and thus facilitates counting. In studies, it is emphasized that in tumors, where mitosis has a diagnostic and prognostic value, such as adrenocortical carcinoma, breast carcinoma, melanoma, neuroendocrine tumor, GIST, and leiomyosarcoma, PHH3 can be a reliable determinant.[8-12]

In this study, it was aimed to evaluate the number of mitosis in GISTs immunohistochemically using PHH3, to compare the data obtained with this method with the number of mitosis counted in the HE slides, and to examine the relationship with other prognostic data.

Methods

Archival GIST cases which were all resection specimens and which were diagnosed with morphological and immunohistochemical methods in the pathology departments between January 01, 2006, and May 1, 2017 were included in this two-centered and retrospective study. Demographic features (age and gender), tumor location, tumor size, (mitotic counts based on HE-stained slides, and Ki-67 proliferation index), progressive disease risk (mitosis based on HE, AFIP prognostic risk classification),[1,4] and follow-up durations (in months, for accessible cases) were included in archival data.

Ethical approval for this study was obtained from the Ethics Committee with the number of 10.10.2017/729.

Immunohistochemical Method

For each case, immunohistochemical staining was applied to the paraffin block, in which mitosis was counted on a HE-stained slide. Sections of three micron thickness from selected paraffin blocks were taken into poly-L-lysine coated slides, deparaffinized in a 60°C oven, and then rehydrated by passing through a series of alcohol. It was boiled in the microwave oven 3 times for 5 min, and then, hydrogen peroxide was added to block endogenous peroxide activity. Sections were incubated with rabbit polyclonal anti-PHH3 antibody (Cell Marque, dil/1:100, USA) for 30 min. Afterward, 3'5' diaminobenzidine (DAB) mixture (Leica, Novacastra RE7140-K) was applied. Contrast-stained slides were covered and evaluated.[13] In each case, 50 HPF were counted in HE and PHH3 stained slides. In PHH3 stained slides, positive staining was considered as a mitotic figure. In each stained case, AFIP prognostic risk score was determined according to HE and PHH3 data and categorized (none, very low, low, intermediate, and high). Subsequently, the cases were divided into two groups according to staining compatibility between HE and PHH3: Group I: Cases with HE positive and PHH3 positive staining, and Group II: HE positive and PHH3 negative staining. In addition, the cases were divided into two groups (those diagnosed before 2012 and those diagnosed at 2012 and afterward) and staining compatibility between HE and PHH3 was re-analyzed. Year 2012 was chosen as threshold since it corresponded to the middle of the study period (2006–2017).

In the evaluation of Ki-67 (SP6, Cell Marque, dil/200, USA), nuclear staining was considered positive. The percentage of Ki-67 positive cells in the total number of evaluated cells was calculated. For each case, the tumors were classified into those with <5% positive cells and those with 5% or more positive cells.[14]

Statistical Analysis

Statistical analysis was carried out on data from 98 subjects by IBM SPSS Statistics 23 program. Central tendency measures (mean, and standard deviation) for numerical variables and frequency distributions (number, and percent) for categorical variables were given. Any difference between more than two groups was tested with "one-way analysis of variance" (ANOVA). The results were controlled first by the Levene test for variance homogeneity, and then by the "multiple comparison test" (Bonferonni or Tamhane's T2) to find from which group or groups the difference had originated. Bonferonni was used to examine the difference between groups in the variables that provide variance homogeneity, and Tamhane's T2 test was used to examine the difference between groups in variables that did not provide variance homogeneity. The Chi-square test was used to examine the relationship between two categorical variables while the kappa coefficient, to examine the consistency of the two measurement values, and Kaplan–Meier analysis, for the survival analysis.

Results

Ninety-eight cases were included in the study, 44 (44.9%) of which were female and 54 (55.1%) were male. The mean age was 59.20 ± 12.477, and the majority of patients were under the age of 65 (62.2%). Fifty percent (49 cases) of the cases were located in the stomach, followed by small bowel (34.7%) and colon (8.2%), while 7.1% of the cases (7 cases) were located outside the gastrointestinal tract (retroperitoneum and intraabdominal). Microscopically, 52% of the cases had spindle cells, 41.8% had mixed cellular patterns, and most of the cases (82.7%) showed cellularity. Half of the cases (51%) had fascicular pattern, followed by the storiform pattern (35.7%) and tumor growth was generally expansive (80.6%). Cellular atypia was mostly mild (70.4%). Mitosis counted in HE-stained sections was ≤5 in 50 HPF in 81.6% of cases and >5 in 18.4%. The number of cases with Ki-67 proliferative index <5 and ≥5 was close to each other (48.9% and 51.1%, respectively). When the subjects were evaluated in terms of HE-based risk score, 43.9% of them were found to be very low or low risk, while 16.3% were intermediate and 33.7% were high risk. Of the patients, 62.2% were alive (Table 1).

In 63.3% (62/98 cases) of cases, mitosis was observed with both HE and PHH3 (Group I) (Fig. 1), while in 36.7% of cases, mitoses detected by HE could not be observed with PHH3 (Group II) (Fig. 2). In 26 cases of Group I, mitosis numbers detected by HE and PHH3 were similar. In ten cases, mitosis was less detected by PHH3 than HE, whereas, in 26 cases, mitosis was more detected by PHH3 than HE. In six of these

Table 1. The distribution of cases in terms of age, location, mitosis, Ki-67 index, risk score, survival, morphological features, atypia, and growth pattern

	n	%
Gender		
Female	44	44.9
Male	54	55.1
Age (Mean±SD)	59.20±12.477	
<65	61	62.2
≥65	37	37.8
Location		
Stomach	49	50.0
Small bowel	34	34.7
Colon	8	8.2
Non-GIS	7	7.1
Mitosis (HE-based)		
≤5	80	81.6
>5	18	18.4
Ki-67		
<5	48	48.9
≥5	50	51.1
Risk score (HE-based)		
None	6	6.1
Very low	19	19.4
Low	24	24.5
İntermediate	16	16.3
High	33	33.7
Survival		
Alive	61	62.2
Exitus	37	37.8
Cell type		
Spindle	51	52.0
Epithelioid	6	6.1
Mixed	41	41.8
Cellularity		
Present	81	82.7
Absent	17	17.3
Pattern		
Storiform	35	35.7
Fascicular	50	51.0
Schwannian	9	9.2
Fascicular+storiform	4	4.1
Atypia		
Mild	69	70.4
Intermediate	15	15.3
Severe	14	14.3
Growth pattern		
Expansive	79	80.6
Infiltrative	19	19.4

GIS: Gastrointestinal system; HE: Hematoxylin and eosin.

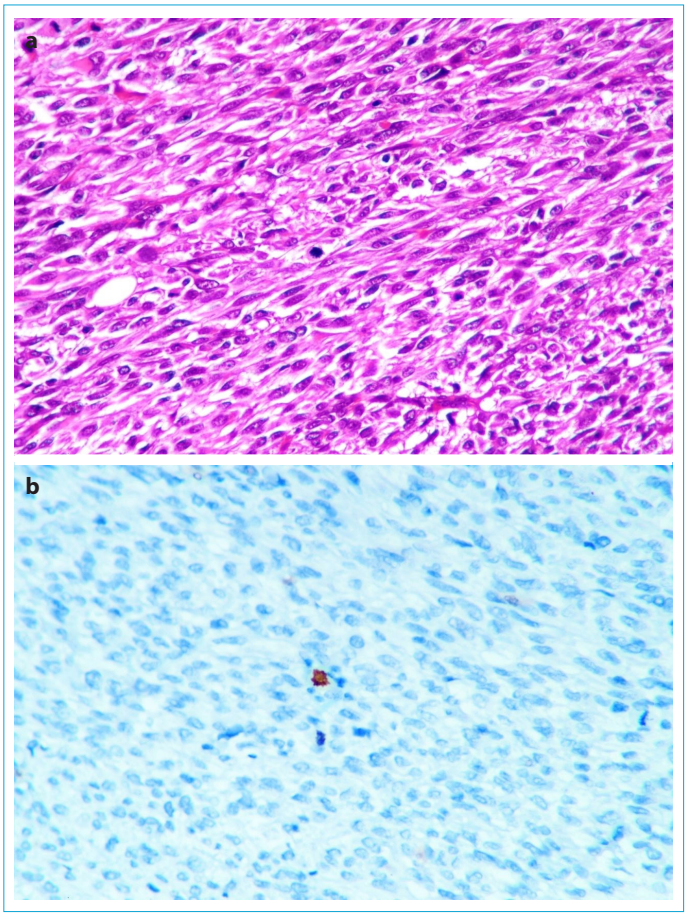

Figure 1. Mitosis (×1000). Detected in a HE-stained tumor cells **(a)**, and detected in by PHH3-immunostain **(b)**.

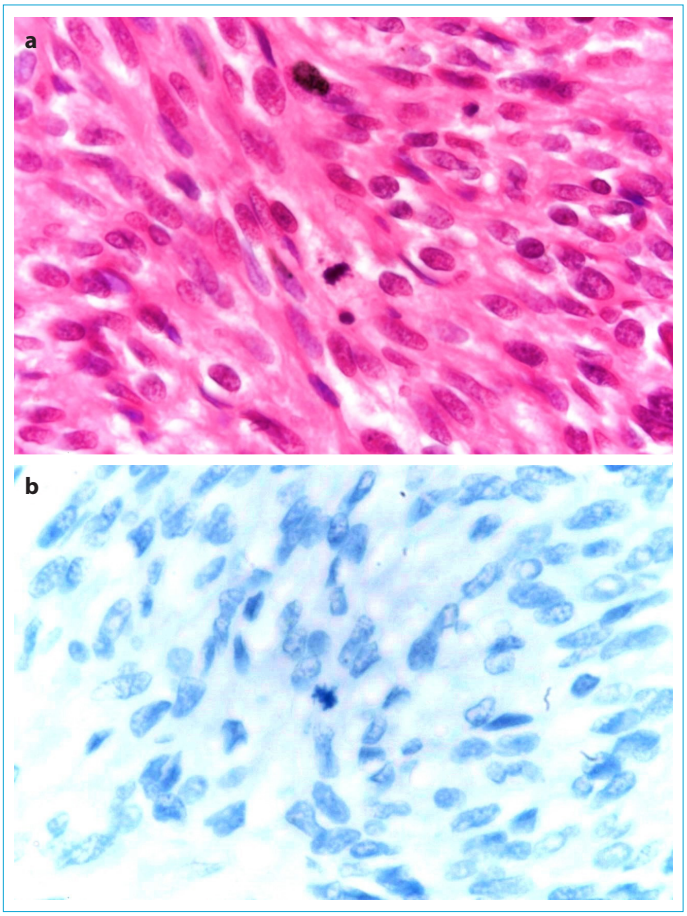

Figure 2. Mitosis (×400). Detected in a HE-stained tumor cells **(a)**, not be observed with PHH3-immunostain **(b)**.

26 cases which had ≤5 mitosis by HE, we detected ≥6 mitosis by PHH3. However, in only two of these cases additional mitosis altered the risk score and consequently, it increased from low-to-intermediate grade.

When the cases were divided into two groups according to the years of diagnosis (before 2012, n=43, 43.9%; 2012 and afterward, n=55, 56.1%), 44.2% (19/43) of the cases diagnosed before 2012, contrary to 78.2% (43/55) diagnosed at 2012 and afterward, showed mitosis in both HE and PHH3. However, mitosis were detected with HE, but not with PHH3-staining in 55.8% (24/43) of pre-2012 cases and in 21.8% (12/55) of cases diagnosed at 2012 and afterward. As a result of Chi-square test, a statistically significant relationship was detected between the groups and the years, and in the group diagnosed at 2012 and afterwards, the rate of mitosis detected in Group I was significantly higher than Group II (Table 2).

As a result of the kappa test, a statistically significant relationship was found between the risk scores based on HE and PHH3 ($p<0.05$, Kappa value 0.856) (Table 3). As a result of the One-way ANOVA test, a statistically significant difference was found between HE-based risk groups in terms of mean Ki-67

Table 2. Mitosis staining with HE and PHH3

	Group I HE(+) and PHH3(+) n (%)	Group II HE(+) and PHH3(-) n (%)	Total n (%)	Chi square/P-value
Before 2012	19 (30.6)	24 (66.7)	43 (43.9)	12.001/0.001
2012 and afterward	43 (69.4)	12 (33.3)	55 (56.1)	
Total	62 (100.0)	36 (100.0)	98 (100.0)	

HE: Hemotoxylin and eosin; PHH3: Phosphohistone H3.

Table 3. Examination of the relationship between risk scores

	Risk Score (HE-based)					Total	Kappa Value	p
	None	Very low	Low	Intermediate	High			
Risk Score (PHH3- based)								
None								
n	5	0	0	0	1	6	0.856	0.000*
	83.3	0.0	0.0	0.0	16.7	100.0		
Very low								
n	1	10	0	1	0	12		
	8.3	83.3	0.0	8.3	0.0	100.0		
Low								
n	0	0	15	1	0	16		
	0.0	0.0	93.8	6.3	0.0	100.0		
Intermediate								
n	0	2	0	10	0	12		
	0.0	16.7	0.0	83.3	0.0	100.0		
High								
n	0	0	0	1	15	16		
	0.0	0.0	0.0	6.3	93.8	100.0		
Total								
n	6	12	15	13	16	62		
%	9.7	19.4	24.2	21.0	25.8	100.0		

*P<0.001. HE: Hemotoxylin and eosin; PHH3: Phosphohistone H3.

values (p<0.05). Accordingly, the Ki-67 average of those with high HE-based risk group was significantly higher than those with low or very low risk. However, there was no statistically significant difference between PHH3-based risk groups in terms of mean Ki-67 values (p>0.05) (Table 4).

Table 4. Comparison of HE- and PHH3-based risk groups and Ki-67 index values

	n	Ki-67		F	p
		Mean	S.D.		
HE-based					
None	6	3.17	2.639	4.827	0.001*
Very low	19	5.37	5.520		
Low	24	4.46	3.611		
İntermediate	16	6.81	6.123		
High	33	10.85	8.519		
PHH3-based					
None	6	3.50	2.881	2.398	0.061
Very low	12	5.00	2.697		
Low	16	5.50	4.775		
İntermediate	12	3.58	1.832		
High	16	7.75	5.170		

*P<0.001. HE: Hemotoxylin and eosin; PHH3: Phosphohistone H3.

As a result of the Kaplan–Meier analysis, a statistically significant difference was found between the HE-based risk score groups in terms of their survival (p<0.05). Accordingly, those who had a low or very low HE-based risk score lived longer than those with a high-risk score. There was no difference between the PHH3-based risk score groups in terms of their survival (p>0.05) (Table 5). According to the Kaplan–Meier analysis, there was no statistically significant difference between the Ki-67 index groups (<5 and ≥5) in terms of survival (p>0.05).

Discussion

The most important prognostic factor in GISTs is the number of mitosis. Mitosis is one of three parameters (tumor diameter, tumor location, and mitosis) considered in the AFIP risk assessment. Accordingly, the detection of ≤5 mitosis in 50 HPF puts the case in a prognosis group between 1 and 3b and the detection of >5 mitosis, in 4–6b.[1,4] However, counting mitosis takes time, is a subjective procedure with very high inter-observer variability, and can be confused with apoptosis.[8,9] PHH3, which is a specific marker of mitosis, has been used as a reliable and easy method to detect mitosis in many different neoplasia such as meningioma, neuroblastoma, adrenocortical tumors, breast, lung, soft tissue, and gastrointestinal tumors.[8-12,15,16] With

Table 5. Comparison of HE- and PHH3-based risk scores and survival values

	Mean				p
	Estimate	Std. Error	95% CI		
			Lower Bound	Upper Bound	
Risk score (HE-based)					
None	85.7	14.9	56.4	114.9	(2<5) 0.036*
Very low	118.8	13.1	93.1	144.5	(3<5) 0.025*
Low	108.9	8.7	91.8	125.9	
İntermediate	75.3	9.0	57.7	92.9	
High	72.9	9.4	54.4	91.3	
Risk score (PHH3-based)					
None	68.8	19.2	31.3	106.4	-
Very low	86.2	7.5	71.5	100.9	
Low	119.9	9.4	101.5	138.2	
Intermediate	71.2	9.6	52.5	89.9	
High	82.5	12.5	57.9	107.1	
Overall	**98.2**	**6.2**	**85.9**	**110.4**	

*P<0.05. HE: Hemotoxylin and eosin, PHH3: Phosphohistone H3.

PHH3 immunostaining, cells in mitosis and areas with high proliferation become evident in brown color and are easily recognized.[12] In the meta-analysis of 19 studies reported between 2011 and 2017, which included different organ tumors, it was stated that PHH3 facilitated counting mitosis and thus, determining grade and it had an important prognostic value.[17]

Relationship between HE and PHH3-Based Mitotic İndex and Risk Score in GIST Cases

In GIST cases, PHH3-based mitotic index was examined in several studies. In a study involving 77 cases, it was reported that the correlation between mitosis number and PHH3, as well as the intraobserver compliance, was high.[12] In the 50-case series of Alkhasawneh et al., with PHH3, 38% of cases received a higher grade than HE-based examination.[18] They concluded that PHH3 was a more sensitive and specific method than HE in determining mitotic figures and found PHH3 useful in risk stratification in GISTs.

In our series, a statistically significant correlation was found between HE-based and PHH3-based mitotic index and hence, risk scores (p<0.05). Only in two cases risk scores had changed and as a result of PHH3-based mitosis values, a higher grade was reported (very low → intermediate grade). However, it was not possible to detect mitosis with PHH3 in about one of three cases. In our study, different results were obtained in the mitosis analysis made from the blocks prepared before and after 2012. When the cases diagnosed before or after 2012 were separately examined, compatibility between HE and PHH3-based mitotic indices was higher in the second group.

Shin et al. mentioned that the factors causing incompatibility between HE- and PHH3-based mitosis depended on high lymphocyte count or tumor cell degeneration on a slide, as well as fixation problems.[12] It is stated that detection of mitosis with PHH3 is superior to counting HE-based mitosis in cases with insufficient fixation.[12] However, it is also known that PHH3 will be positive in the nuclei and mitotic lymphocytes in the interphase, as well as mitotic tumor cells, and that the pathologist may erroneously give a higher mitosis value with PHH3 in lymphocyte-rich cases.[12]

In a recently published study, the PHH3 mitotic index determined manually and the PHH3 index determined by computer-assisted image analysis (comp-PHH3) were compared and comp-PHH3 was found to be a valid alternative.[19]

The incompatibility between HE and PHH3 in our cases diagnosed before 2012 might be due to laboratory techniques and long archival duration of the paraffin blocks, and we believe that this is an issue to be analyzed and clarified. In the literature, there are publications regarding the importance of factors such as fixation time, duration, and fixative selection of tissue for histochemical, immunohistochemical, and molecular studies, and it has been shown in studies that the tissues embedded in long-term paraffin blocks show antigenicity loss over time.[20] Molecular, proteomic and morphological changes may occur

in formalin-fixed paraffin-embedded specimens prepared under suboptimal conditions.[21] In our series, when our cases were classified as before 2012 and 2012 and afterward, there was a significant difference between HE- and PHH3-mitosis staining and therefore, risk scores. According to our findings, reliability of PHH3 has decreased in tissues that have been stored in archive for more than 6 year. Contrary to our expectation, PHH3 immunostaining did not yield a superior result than the mitotic index, we obtained with HE. Only two cases had a grade increase in risk scoring. However, when the risk scores of the cases were compared, the risk scores based on HE and PHH3 were statistically related.

Relationship between HE, PHH3, and Ki-67 Index

In our study, the correlation of Ki-67, an important and widely used proliferative marker, with HE- and PHH3-based mitotic index were investigated. Ki-67 index shows cells in all phases except the G0 phase, while PHH3, only in the late G2 and M phase. According to the literature, the correlation between Ki-67 and HE-based mitotic activity is superior to the correlation with PHH3.[22] The fact that all Ki-67 positive cells do not go to mitosis may explain the incompatibilities between the expression of the two markers.[12] In our study, there was a significant difference in terms of Ki-67 values between risk groups determined according to mitosis detected with HE, in accordance with the literature. High Ki-67 index correlated with high HE staining. However, there was no correlation between PHH3 risk groups and Ki-67 index values. Since $p=0.063$ value determined in this group was statistically very close to $p<0.05$ significance value, this result might be related to our sample size.

Relationship between HE, PHH3, and Survival

A pooled results of a recent meta-analyses showed that high expression levels of PHH3 had a relation with poor overall-survival (HR=2.66), disease-free survival (HR=3.40), and recurrent free survival (HR=2.80).[17] Li et al., in a current study on 98 cases, found a correlation between high PHH positive index and poor prognosis.[23] However, in our study, there was no relationship between PHH3-based risk score and survival. For HE-based risk score, the survival of "very low or low" risk score was found to be longer than those with a high-risk score, while no significant relationship was found between survival with "none" risk score. This might be due to small number of patients in this group and relatively shorter follow-up time. In our study, no significant difference was found between the Ki-67 groups (<5 and ≥5) in terms of survival, and Ki-67 was not found useful in determining the prognosis in GISTs.

Conclusion

In our study, in GIST cases, a correlation was found between HE-based and PHH3-based mitoses. However, in sections prepared from blocks before 2012, PHH3 positivity was less, which might be due to technical issues. There was no relationship between survival and PHH3-based risk score. In the HE-based risk score, there was a relationship between "very low or low" risk score and survival, and the total survival was longer than those with a high-risk score. In terms of mean Ki-67 index, no significant relationship was observed between PHH3-based risk groups, while a significant relationship was observed between HE-based risk groups. Accordingly, mean Ki-67 of those with high HE-based risk group was significantly higher than those with low or very low risk.

As a conclusion, in GIST cases, PHH3 can be used to determine mitosis in newer blocks, considering the technical conditions of the laboratory, but it does not seem superior to mitosis detected by HE. Computer-assisted mitosis counting and artificial intelligence applications developed for PHH3 may be an auxiliary tool that will enable larger areas to be counted in an easier and shorter time in the near future. In addition, research on new survival determinants for GIST should go on.

Disclosures

Ethics Committee Approval: Ethical approval for this study was obtained from the Ethics Committee with the number of 10.10.2017/729.

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Conflict of Interest: None declared.

Authorship Contributions: Concept – S.S.E., S.S., S.H.K., A.E.G.; Design – S.S.E., S.S., S.H.K.; Supervision – S.S.E., S.S., S.H.K.; Materials – S.S.E., S.H.K., E.K., A.E.G., G.A.D., A.S.; Data collection &/or processing – S.S.E., S.H.K., E.K., A.E.G., G.A.D., A.S.; Analysis and/or interpretation – S.S.E., S.S., S.H.K., A.E.G., A.S.; Literature search – S.S.E., S.S., S.H.K., E.K., G.A.D.; Writing – S.S.E., S.S., S.H.K.; Critical review – S.S.E., S.S., S.H.K., A.E.G., A.S.

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