Concordance and Discordance of Mitotic Count, Ki-67 Proliferative Index and Phosphohistone H3 Count in Grading Neuroendocrine Tumors of Gastrointenstinal Tract

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ABSTRACT

Objective: To determine the grades of gastrointestinal neuroendocrine tumours using World Health Organization criteria and Phosphohistone H3 mitotic index and to determine the concordance between the two methods.

Study Design: Cross-sectional study.

Place and Duration of Study: Department of Histopathology, Armed Forces Institute of Pathology, Rawalpindi Pakistan, from Mar to Oct 2019.

Methodology: Forty-two (n=42) patients of either gender between the ages of 18-70 years were enrolled. All the enrolled patients were diagnosed with primary gastrointestinal neuroendocrine tumours and underwent either surgical or endoscopic resection. Tumour grades were determined using World Health Organization criteria and Phosphohistone H3mitotic index. The results obtained with both methods were compared, and the concordance rate was calculated.

Results: When mitotic counts were determined through PHH3, MI resulted in a change of grade of 12 (28.6%) tumours, which were graded by the current WHO system. 11 (26.2%) were changed from grade II to grade I and 1 (2.4%) from grade I to grade I1. The agreement (concordance rate) between the two systems was moderate and statistically significant (71.4%, n=30/42, κ= 0.51, *p*= 0.001)

Conclusion: In the present study, we observed a moderate agreement between the Ki-67 labelling index and the PPH3 mitotic index, and both correlate well with the mitotic counts. The PPH3 mitotic index demonstrated a better correlation with mitotic counts when compared with Ki-67 LI. Hence, the inference can be drawn that the mitotic index with Phosphohistone H3is associated more closely with mitosis in gastrointestinal neuroendocrine tumours.

Keywords: Ki-67, Neuroendocrine tumours, Phosphohistone H3.

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INTRODUCTION

The term neuroendocrine neoplasms (NENs) generally refers to various tumours that arise from the epithelial organs like the pancreas, gastrointestinal tract (GIT), and lungs. These neoplasms comprise 0.5% of all newly diagnosed neoplasms, and in the USA, their annual incidence is reported as about 3.56/per 100,000 individuals.[1,2] Females are more likely to be affected than males (2.5:1), and the GIT is the most frequently involved primary site (approx. 65%), followed by the lung (approx. 25%). Approximately 15-20% of patients have metastases on presentation. In the gastrointestinal tract, the most frequent documented location is the colon and rectum (approx. 65%), followed by the small intestine (35%) and oesophagus/ stomach /appendix (<10%).[3] The existing system of classification (2018), which is also adopted by the World Health Organization (WHO), stratifies these neoplasms based on prognosis. In the gastrointestinal tract, the current classification of neuroendocrine tumours (NETs) is grade 1 (G1), grade 2 (G2), and grade 3 (G3).[4] The WHO classification for gastrointestinal NETs relies entirely on proliferative rate to separate low-grade, intermediate-grade, and highgrade tumours.[5] The proliferative rate can be assessed using mitotic counts or Ki-67 (a cellular marker of proliferation) labelling index. The higher of these two indices is used to define the final grade in cases where the mitotic rate and Ki-67 index are discordant. The optimal cut off values for the Ki-67 labelling index to distinguish low, intermediate and highgrade gastrointestinal NENs have not been conclusively established and may vary depending upon the primary site of the neoplasm. However, the European Neuroendocrine Tumour Society (ENETS), the American Joint Committee on Cancer (AJCC), and both the 2010 and 2017 WHO classifications include a uniform Ki-67 labelling cutoff of <3 percent to define low-grade, 3-20% for intermediate-grade, and >20 percent for high-grade NENs of the tubular gastrointestinal tract (at all sites) and pancreas.[6,7] Another protein called Phos-phohistone H3 (PHH3) is

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also considered a specific marker during mitoses and can be utilized in counting the mitotic figures and grading.[8] Several studies found this marker useful in predicting prognosis in patients with several types of gastrointestinal neoplasm.[9] None the less, its ability to grade NETs of GIT is not completely assessed, particularly when differentiating between G1 and G2 well-differentiated NETs. In a recent study, Kim *et al,* reported that when mitotic counts were determined through PHH3, MI resulted in a change of grade of 25.5% tumours graded by the current WHO system.[10] A total of 21.3% were changed from grade 1 to grade 2 and 4.3% from grade 2 to grade.[1] They further reported that agreement (concordance rate) between the two systems was 75.9%, and the association between these modified grades and the WHO grades was moderate (κ=0.428) but statistically significant (p<0.001).

Several studies reported that histological grading is associated with long term prognosis in patients with NETs. An accurate method is the obvious choice of the reporting pathologist. The present study was planned to determine the grades of gastrointestinal neuroendocrine tumours using WHO criteria and PHH3 mitotic index and determine the concordance rate between the two methods. The better of the two methods would be adopted in future for accurate grading of gastrointestinal NETs in our settings.

METHODOLOGY

The present study was conducted at the Armed Forces Institute of Pathology, Rawalpindi. The study design was comparative cross-sectional, and it was carried out from March 2019 to October 2019. The study was approved by the Institutional Review Board and Ethical Committee (FC-HSP18-6/READ-IRB/19/439, Dated: 12-07-2019). WHO calculator was used for sample size estimation taking 95% confidence level, the anticipated concordance rate of 75.9%[10] with 0.13 absolute precision.[11]

Inclusion Criteria: Patients of either gender between the age of 18-70 years, diagnosed cases of primary gastrointestinal NETs were selected and underwent either surgical or endoscopic resection.

Exclusion Criteria: All the patients who had received chemotherapy or another form of targeted therapy were excluded from the study.

All the specimens obtained were prepared, and formalin-fixed, paraffin-embedded (FFPE) tumour tissue blocks were made for further analysis. An FFPE slide with sections of tumour thickness of 4µm was used for staining by immunohistochemistry. The primary antibodies were directed against PHH3 and Ki-67. Mitotic counts on both H&E and PHH3 stained slides were counted in 50 highpowered fields. The PHH3 MI was estimated from the mean mitotic count and the mean numbers of PHH3-positive nuclei/10 HPFs. Grades of H&E and anti-PHH3 stained sections were determined independently.

Tumors were classified as G1 (<2 mitoses/10 HPFs and/or Ki-67 LI <3%), G2 (2–20 mitoses/ 10HPFs or/and Ki-67 LI 3-20%) and G3 (>20 mitoses/ HPF or Ki-67 LI>20). The results obtained with both the methods were compared, and the concordance rate (number of similar results obtained from both the methods divided by the total number of cases) was calculated. Statistical Package for Social Sciences (SPSS) version 23.0 was used for the data analysis. The kappa (κ) statistic was estimated to measure the degree of agreement between two different grading methods. Kappa values were regarded as highlighting slight (κ≤0.2) agreement, fair (κ=0.21–0.4), (κ=0.41–0.6), substantial (κ=0.61–0.8) or perfect (κ>0.8) agreement. The agreement was considered significant if p-value was calculated as ≤0.05.

RESULTS

The mean age of the study participants was 45.3 ± 18.1 years. A total of 23 (54.8%) were males, and 19 (45.2%) were females (Table-I). Tumour grades determined with the WHO system and through PHH3 MI are represented in Table-II. When mitotic counts were determined through PHH3, MI resulted in a change of grade of 12 (28.6%) tumours, which were graded by the current WHO system.

Table-I: Gender and age distribution of the study participants.

Gender	Frequency	Percent	Mean Age (Years)
Males	23	54.8	50.1 ± 18.2
Females	19	45.2	39.6 ± 16.6

Table-II: Tumor grades with both Ki-67 and PPH3 techniques.

Technique	Grade	Frequency	Percentage
Ki-67	Grade I	20	47.6
	Grade II	13	31.0
	Grade III	9	21.4
	Total	42	100.0
Pph3	Grade I	30	71.4
	Grade II	3	7.1
	Grade III	9	21.4
	Total	42	100.0

A total of 11 (26.2%) were changed from grade II to grade I and 1 (2.4%) from grade I to grade II (Figure).

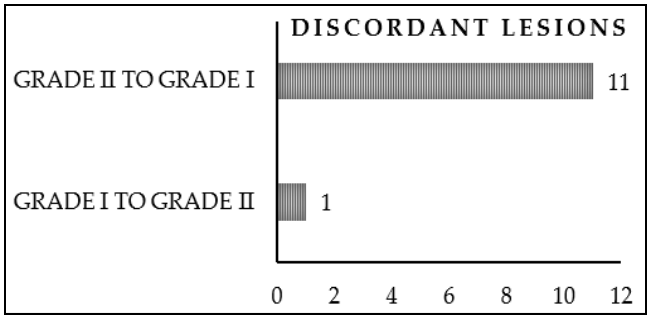

Figure: Distribution of discordant lesions in the study sample.

The agreement (concordance rate) between the two systems was 30 (71.4%), and the agreement between the modified and conventional WHO grades was found to be moderate (κ=0.51) and statistically significant (*p*=0.001), as shown in the Table-III.

Table-III: Agreement between two techniques Ki-67 and PPH3 techniques.

Grades On ki-67	Grades on PPH3			K-value	*p*-value
	Grade I	Grade II	Grade-III		
Grade-I	19 (45.2%)	1 (2.4%)	-	0.517	0.001
Grade-II	11 (26.2%)	2 (4.8%)	-		
Grade-III	0	0	9 (21.4%)		
Overall Concordance Rate Between Two Techniques				71.4% (n=30/42)	

DISCUSSION

The best method to establish the proliferative rate of gastrointestinal NETs is not established. The WHO/AJCC classification system provides criteria for mitotic rate counting and Ki-67 labelling to assess the proliferative rate to separate low-grade, intermediategrade, and high-grade tumours without a preference for one method. Grade assignment based upon these Ki-67 cut offs correlates with patient survival in both primary and metastatic gastrointestinal and pancreatic NETs. However, evaluating mitoses in patients with gastrointestinal NETs using the conventional WHO grading system may not be accurate. This is because darkly stained shrunken or irregular nuclei and apoptotic bodies may mimic mitoses and falsepositive results. PHH3 is another proliferative marker that does not express during interphase or programmed cell death. It is specifically expressed during mitosis making it a more specific mitosis marker.[11,12] In the present study, we determined the grades of gastrointestinal NETs using WHO criteria and PHH3 mitotic index and determined the concordance between the two methods. Our results showed that when mitotic counts were determined through PHH3 MI, a change of grade in 28.6% (n=12/42) tumours were observed, graded by the current WHO system. 26.2% (n=11/42) were changed from grade II to grade I, and 2.4% (n=1/ 42) from grade I to grade II.

There are several possible explanations for a change in tumour grades in 28.6% (n=12/42) patients.[13] Firstly, due to the narrow cut-off of mitotic counts in the current WHO system, it is difficult to distinguish between grade 1 and 2 tumours. The Ki-67 labelling index generally correlates with the mitotic count,[14,15] however, there may be discrepancies. The Ki-67 protein has a short half-life, and its amount and localization change with the cell cycle, which may explain these discrepancies. In all cases with discordance, we used the higher grade as recommended by the WHO classification. Another reason for these changes in grades using the PPH3 index is the lack of optimal cutoff values to standardize grading using Ki-67. The choice of a cut off may be influenced by the patient out come measures (dead versus alive, recurrence versus no recurrence, disease-specific survival, disease-free survival, etc.) and the investigator's interpretation of its clinical significance. A range of values, instead of a single value, may provide similar prognostic significance. Although various investigators have proposed different Ki-67 cut off values for grading, it is increasingly recognized that both Ki-67 and mitotic rate are continuous variables, at least within the low-and intermediate-grade ranges, so it may not be practical to define the absolute values that separate grades. Instead, proliferative rates can be used to define prognosis based upon the absolute Ki-67 and mitotic rate values, with increasing values predicting increasingly aggressive clinical behaviour. This underscores the importance of recording the actual proliferation values in pathology reports rather than simply reporting the grade.[16,17] It is also increasingly recognized that the intent to grade all gastrointestinal NETs using a single system may obscure the inherent biological variability among various primary sites. Heterogeneity of the proliferative rate within a tumour (or among different disease sites if metastases are present) is a common finding in NETs.

Even on a single slide, there may be significant variability in the mitotic count and Ki-67 labelling index. Within the ENETS/WHO classification system, it is recommended that at least 40 HPFs should be counted for mitoses and that areas of highest labelling ("hot spots") should be used to determine the Ki-67 labelling index. If Ki-67 staining is performed on a large specimen, such as a resection, it is relatively simple to scan the tumour at low power to identify the

hot spots of greatest labelling.[18,19] However, within a limited specimen (e.g., core needle biopsy), the Ki-67 index may not be representative. Further more, few data are addressing whether the proliferative rate with in hot spot areas more accurately predicts prognosis than, for example, the average proliferative rate in the entire tumour. These data support counting hot spots to assess the Ki-67 index in heterogeneous tumours. In such cases, a low grade based upon a small, randomly directed biopsy may not represent the true grade of the tumour. In order to better predict patient outcomes, multiple biopsies would be needed, although the optimal number has not been deter-mined.[20,21]

In the present study, we observed a moderate agreement between the Ki-67 labelling and PPH3 mitotic index (concordance rate if 71.4%, κ= 0.51) and both correlate well with the mitotic counts. The PPH3 mitotic index demonstrated a better correlation with mitotic counts when compared with Ki-67 LI. Hence, the inference can be drawn that the mitotic index with PPH3 is associated more closely with mitosis in gastrointestinal NETs. This is due to the fact thatPHH3 stains the cells only in the G2 and M phase of mitosis while Ki-67 expresses itself through out the cell cycle except during the G0 phase. This leads to staining of fewer cells by PPH3 than Ki-67, which results in lower MI values with PPH3. Similar findings have been reported in a recent study by Kim *et al.*[10] Their results demonstrated that mitotic counts determined through PHH3 MI resulted in a grade change of 25.5% tumours, which were graded by the WHO system. A total of 21.3% were changed from grade 1 to grade 2 and 4.3% from grade 2 to grade 1. Kim *et al,* reported that the concordance rate between the two systems was 75.9% in their study, with significant moderate concordance between modified grades and the WHO grades (κ=0.428, p<0.001).[10] Several other studies showed similar findings with PPH3. They reported that PHH3 MI was comparable to the current grading system of WHO and is superior to Ki-67 in the prediction of disease-free survival.[8,22,23] However, in the present study, we did not estimate the survival figures.

Some prognostic parameters used in the Capella and earlier WHO classifications, such as tumour size and the presence of metastasis, are now regarded as part of the staging parameters in the AJCC staging system and are not included in the current WHO classifications. Tumour necrosis is one of the criteria for the intermediate grade in the WHO classification of lung and thymic carcinoids. It was used in the Armed Forces Institute of Pathology, USA classifications of pancreatic NETs. However, necrosis is not a component of the most recent WHO classifications for GEP NETs. Similarly, lymphovascular invasion and perineural invasion are not part of the grading criteria, although they should be recorded as a prognostic factor. Several other markers have been reported to have prognostic value in NETs. Emerging data suggest that programmed cell death ligand 1 (PD-L1) may be a biomarker for high-grade GEP NENs, but confirmatory studies are needed.[24,25]

In summary, the cumulative evidence in the literature and present study results under score the fact that PHH3 appears easier to interpret and more accurate than the prognostic marker (Ki-67) currently in use for grading gastrointestinal NETs. The present study has a few limitations. Firstly, we did not aim toward identifying the cutoff value of PPH3 that could give optimum results.

Secondly, we did not estimate the prognostic significance of the change in grades observed in the present study by measuring long term survival figures. Nonetheless, the prospective nature of the study is its main strength. We recommend further studies to validate our results and to determine the optimal cutoff value for PPH3 for more accurately grading gastrointestinal NETs.

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CONCLUSION

In the present study, we observed a moderate agreement between the Ki-67 labelling index and the PPH3 mitotic index, and both correlate well with the mitotic counts. The PPH3 mitotic index demonstrated a better correlation with mitotic counts when compared with Ki-67 LI. Hence, the inference can be drawn that the mitotic index with PPH3 is associated more closely with mitosis in gastrointestinal NETs.

Conflict of Interest: None.

Authors' Contribution

SH: Data collection, analysis, script, MA: Concept, intellectual and data, MTK: Final approval, HUD: Intellectual contribution, FA:, FR: Data analysis.

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