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Original Contribution

Ki67 index and mitotic count: Correlation and variables affecting the accuracy of the quantification in endocrine/neuroendocrine tumors

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ABSTRACT

Quantification of Ki67 and mitosis is time consuming and subject to inter-observer variabilities. Limited studies explored the impact of those variables on the results and the correlation between mitotic count and Ki67 index in endocrine/neuroendocrine tumors, particularly so since the advent of PHH3 antibody and digital pathology. Using Ki67 and mitosis as examples, this study is intended to reveal variables affecting accurate quantification of biomarkers, and to explore the relationship of Ki67 index and mitotic count/index in endocrine/neuroendocrine tumors.

Using both manual and pathologist supervised digital image analysis (PSDIA) methods, we examined the impact of post-analytical variables on the quantification of mitosis and Ki67 index and studied the correlation between them in 41 cases of endocrine/neuroendocrine tumors of variable histological grades/proliferating rates.

We found that the selection of hotspots, field size and especially threshold affected the outcome of quantification of mitosis and Ki67 index; that mitotic count/index strongly ($p < 0.05$) correlated with Ki67 index only in the tumors with peak Ki67 index less than 30% and the correlation was more monotonic (positive, non-linear) than linear. In the hotspots of these tumors, the ratio of mitotic count to proliferating cells defined by Ki67 detection averaged 0.04. We also found that the PHH3 antibody could markedly increase the efficiency and accuracy of mitotic quantification.

A consensus among pathologists is needed for the selection of hotspots, field size and threshold for quantification of mitosis and Ki67 index.

1. Introduction

Eukaryotic cell cycle usually consists of four sequential phases: G1 (gap phase 1), S (DNA synthesis), G2 (gap phase 2) and M (mitosis). G1, S and G2 together are called interphase. In a typical human cell proliferating in culture, interphase might occupy 23 h of a 24-hour cycle, with 1 h for M phase. S phase lasts 10–12 h and occupies about half of the cell cycle time in a typical mammalian cell (Fig. 1). M phase is traditionally divided into five stages – prophase, prometaphase, metaphase, anaphase and telophase (Fig. 2), defined primarily on the basis of chromosome behavior as seen under a microscope [1].

A mouse monoclonal antibody, Ki-67 was first produced and described by Gerdes et al. [2]. Studies showed that the Ki-67 antibody recognized a nuclear antigen – a chromatin protein located on chromosome 10, present in all phases (G1, S, G2 and M) of proliferating cells, but absent in resting cells (G0) [2-5]. Recent studies revealed that Ki67 acts as a biological surfactant to prevent mitotic chromosomes from coalescing [6,7]. Ki67 antigen expression increases in S phase and reaches its highest level in M phase [8].

Theoretically, any tumor cell with detectable Ki67 is in the proliferating phase, so, Ki67 has become a very sensitive marker of proliferation. Studies on Ki67 have proven it as a valuable biomarker for cancer diagnosis, grading and prognosis stratification [9-12].

Mitotic activity has been used as a marker for tumor proliferation for many decades. However there are many variables and subjective interpretations associated with evaluation of mitotic activity [13]. Phosphorylation of histone H3 (PHH3) has been used to measure mitotic activity since 1990 [14]. Since it occurs almost exclusively during mitosis (M phase) and is low in interphase (G1, S and G2) cells, PHH3 is considered as a marker for cell mitotic activity [15].

The current quantitative method for mitotic count in most practices is based on morphology (H&E slides). Not only it is a time-consuming

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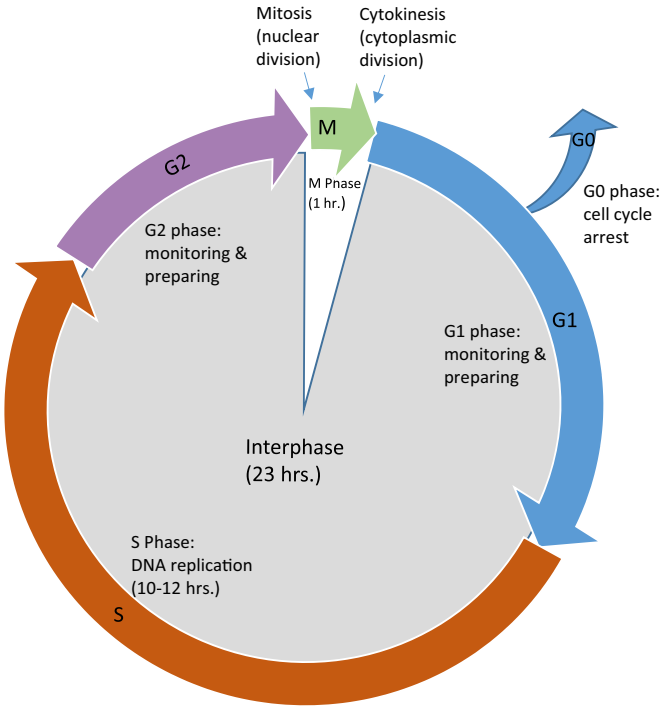

Fig. 1. A typical mammalian cell 24-hour cycle in culture.

process but also the accuracy is often compromised by morphologic imitators, such as degenerating/pyknotic cells (Fig. 3). The value of PHH3 antibodies in mitigating the problems associated with mitosis counting has been recognized recently [16-19].

The commonly used methods for Ki67 index quantification include manual estimation with glass slide and microscope (ME), manual quantification with printed digital images (MQPI) or manual quantification with glass slide and grids under the microscope. Studies have shown pathologist supervised digital image analysis (PSDIA) is more objective and accurate compared to the manual methods for biomarker quantification [11,12,20].

Two studies published in 1990s showed mixed results on the correlation between mitotic count and Ki67 index in breast cancer [21] and mixed cases of colorectal adenocarcinoma, mammary carcinoma, squamous cell carcinoma, non-small cell lung cancer and small cell lung cancer [22] Presently, limited data are available regarding how variables such as the selection of hotspots, field size and threshold could affect the quantification of Ki67 index. Limited data is available regarding the correlation between mitosis and Ki67 index in endocrine/neuroendocrine tumors, especially since the advent of PHH3 antibody and digital pathology.

This study was intended to explore the impact of post-analytical variables on biomarker quantification and the correlation of Ki67 index with mitotic count/index in endocrine/neuroendocrine tumors.

2. Materials and methods

2.1. Cases

Forty-two cases of endocrine/neuroendocrine tumors were included in this study. Among the 42 cases, 1 case was an adrenal cortical carcinoma (ACC) from the pathology archive at the University of Wisconsin-Madison. This case allowed us to explore the impact of different sizes of fields, hotspots, thresholds on the quantification. 41 cases were commercial endocrine/neuroendocrine tumor tissue microarray samples (TMA) (EN801a, BioMax) of variable histological grades/proliferating rates, which we used to study the correlation of Ki67 index and mitotic count/index. Out of the 41 commercial cases of endocrine/neuroendocrine tumors, there were 11 adrenal cortical adenocarcinomas, 4 pancreatic islet cell carcinomas, 2 each of esophageal and cardia neuroendocrine carcinomas, 3 gastric carcinoid tumors, 1 each of colon neuroendocrine carcinoma, small intestine carcinoid and rectal carcinoid tumors, 12 lung atypical carcinoid and 4 lung carcinoid tumors. Each case in the TMA had duplicate cores of 1.5 mm in diameters.

2.2. Immunohistochemistry

PHH3 antibody (rabbit monoclonal, s28, Abcam: ab32388) was used to detect mitoses. Ki67 antibody (mouse monoclonal, Cell Signal: 9449) was used to detect proliferating cells. The experiments were run on the Roche Ventana Medical System's Discovery XT Automated platform, and all reagents were Roche-Ventana proprietary reagents except for the antibody diluent (Biocare Medical) and Harris Modified hematoxylin (ThermoFisher).

2.3. Quantification

2.3.1. Manual methods

To explore the interobserver variability and efficiency, the quantification of mitosis and Ki67 index were performed for the in-house adrenal cortical carcinoma case by three pathologists (RH, RL and WH) using ME method (glass slides) and MQPI (printed image). The results and time spent by each pathologist were recorded.

2.3.2. PSDIA method

To explore the impact of the selection of hotspots, field size and threshold, the quantification of mitotic count/index and Ki67 index were also performed for the in-house adrenal cortical carcinoma case using PSDIA. Whole section IHC slides were scanned with Aperio CS2 (ImageScope, Aperio, Leica). The quantification of mitotic count (PHH3+) and index, and Ki67 index were performed with Aperio nuclear algorithm (Leica) in 4 hotspots using the respective whole section IHC digital slides (by CN and WH). The nuclear algorithms were optimized by pathologist (WH) for the identification of positively stained mitoses and proliferating cells (Ki67 positive cells), respectively. Quantifications were performed in the fields of different sizes (0.05 mm^2, 0.1 mm^2, 0.2 mm^2 and 0.4 mm^2) by scaling up and down in the same hotspots (Fig. 4). A 0.2 mm^2-field has an average of 1046 (935–1150) cells (Table 2). Intense PHH3 staining signal (3+) was used for identifying mitoses (Fig. 5). For Ki67 index quantification, different thresholds (1+, 2+ and 3+) were used.

2.3.3. TMA analysis

The TMA IHC slides (EN801a TMAs) were also scanned with Aperio CS2 (Leica). Three of the 41 cases (EN801a) had no detectable Ki67 staining, therefore were excluded for analysis. The fields for analysis were carefully selected to avoid necrosis and lymphocyte-rich, non-tumor tissue (Fig. 6). A 0.2 mm^2-field is equivalent to one 40× high power field (HPF) for the Olympus BX41. Due to limited size of each core (1.5 mm in diameter), three 0.2 mm^2-fields from each core of the TMA slides, a total of 228 HPFs were selected for quantification. For mitosis quantification, intense PHH3 staining (3+) was considered positive; for the quantification of Ki67 index, 1+ was used as threshold by using Aperio ImageScope and nuclear algorithms. Mean values and the peak values from the three fields of each case were used for analysis. Using Excel, line charts were plotted to show the relationship between the mitotic count/index and Ki67 index. The ratio of mitotic count (PHH3+) to Ki67 positive cells (Ki67+) for each field was calculated. The relationship between the mitotic count/index and Ki67 index were analyzed.

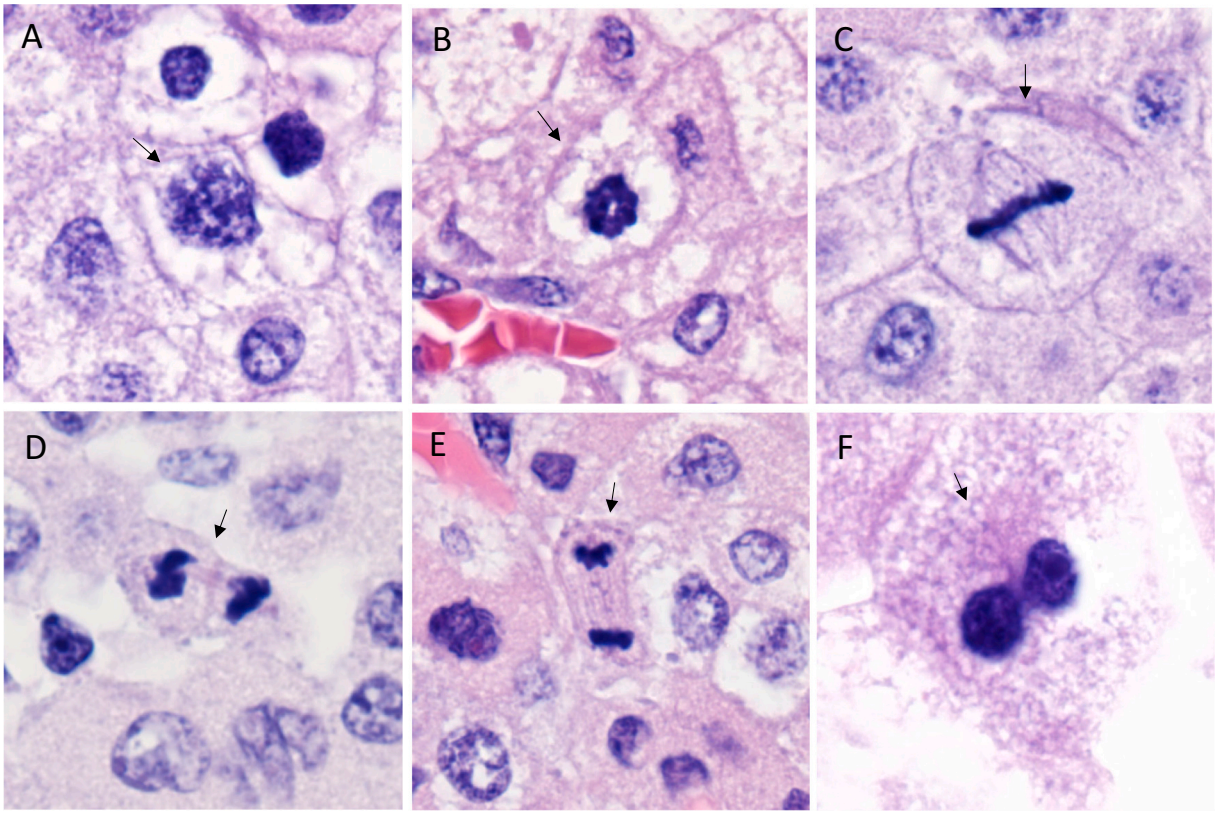

Fig. 2. H&E section. Five stages of mitosis and cytokinesis found in our adrenal cortical carcinoma case: A. Prophase B. Prometaphase C. Metaphase D. Anaphase and E. Telophase F. Cytokinesis. Magnification: 10 × 100.

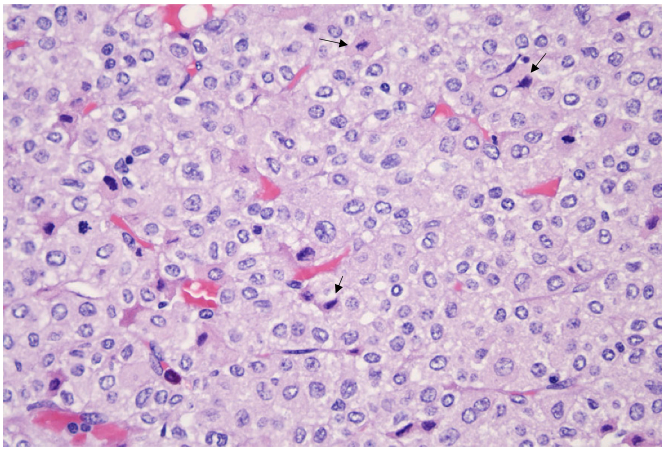

Fig. 3. H&E section. Morphological mimickers of mitosis (arrows). Magnification: 10 × 40.

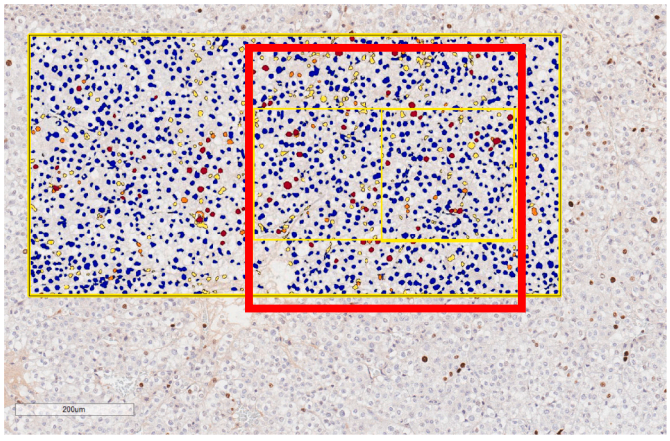

Fig. 4. Quantifications were performed in fields of different size (0.05 mm^2, 0.1 mm^2, 0.2 mm^2 and 0.4 mm^2) by scaling up and down in the same hotspot using Aperio ImageScope software. Red square = 0.2 mm^2. PHH3 staining intensity is color-coded: brown = 3+, orange = 2+, yellow = 1+. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2.4. Statistical analysis

IBM SPSS Statistics 25 was used to perform the statistical analysis. Pearson's correlation was performed to evaluate the linear (two variables change in proportion) degree of correlation and Spearman's correlation was performed to evaluate the monotonic (positive, non-linear) degree of correlation between mitotic count/index and Ki67 index using peak and mean values from the TMA samples, respectively.

3. Results

Using both ME and MQPI methods, there was obvious inter-observer variability for mitotic count and Ki67 index among the 3 pathologists. MQPI was found to be more time-consuming. PHH3 IHC staining enabled the three pathologists to identify more mitoses within a shorter time (Table 1).

We found that even in the same sized fields (hotspots), mitotic count and Ki67 index varied due to tumor heterogeneity, with a difference of Ki67 index of up to 2% between the hotspots. Using the PSDIA method, true hotspots were reliably identified for quantification by sampling a few candidate areas quickly (Table 2, bold numbers).

We also found that field size affected the quantification outcome for both mitotic count and Ki67 index: the smaller the field size, the higher

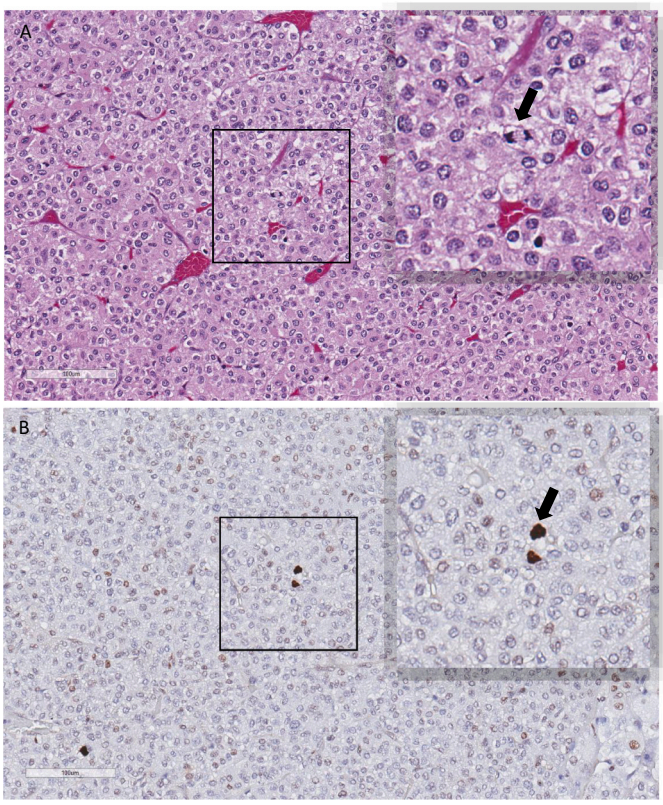

Fig. 5. Mitosis (arrowed) in H&E section (A) and PHH3 stained section (B).

the number (Table 2). The selection of the threshold for identifying Ki67 positive cells (Ki67+) markedly affected the Ki67 index; the difference between thresholds varied from 3% to up to 13% (Table 3).

We found that the PSDIA method could reduce interobserver variability by being able to precisely select the true hotspot in a given slide (Table 2, bold numbers).

We found that mitotic count/index correlated positively with Ki67 index overall ($p < 0.01$), and with r_S (Spearman) greater than r_P (Pearson), indicating there was more of a monotonic (positive but non-linear) than a linear relationship between them (Fig. 7, Table 4). The chart shows that the degree of correlation between peak Ki67 index and mitotic count/index starts to decrease drastically from the 30% point (Fig. 7, blue arrow). To take a closer look at the correlation, we then stratified the cases into three groups based on their peak Ki67+ index as follows: Group 1: peak Ki67 index < 3%, group 2: peak Ki67 index = 3–29%; group 3: peak Ki67 index ≥30%. The mean and peak PHH3+/Ki67+ ratios were 0.10 ± 0.28 and 0.07 ± 0.10 in group 1, 0.05 ± 0.04 and 0.04 ± 0.03 in group 2, and 0.03 ± 0.03 and 0.03 ± 0.02 in group 3, respectively (Table 5). We also found that the tumors in group 1 and 2 had strong correlation between the mitotic count/index and Ki67 index ($p < 0.01$). The correlation was also more monotonic (positive, non-linear) than linear, with $r_S > r_P$. The tumors in group 3 showed positive but weak correlations ($p > 0.05$) between the mitotic count/index and Ki67 index. The mitotic count (n) appeared to correlate with Ki67 index slightly better than mitotic index (%) (Table 6).

4. Discussion

We are aware of the limitations of this study. We would like to

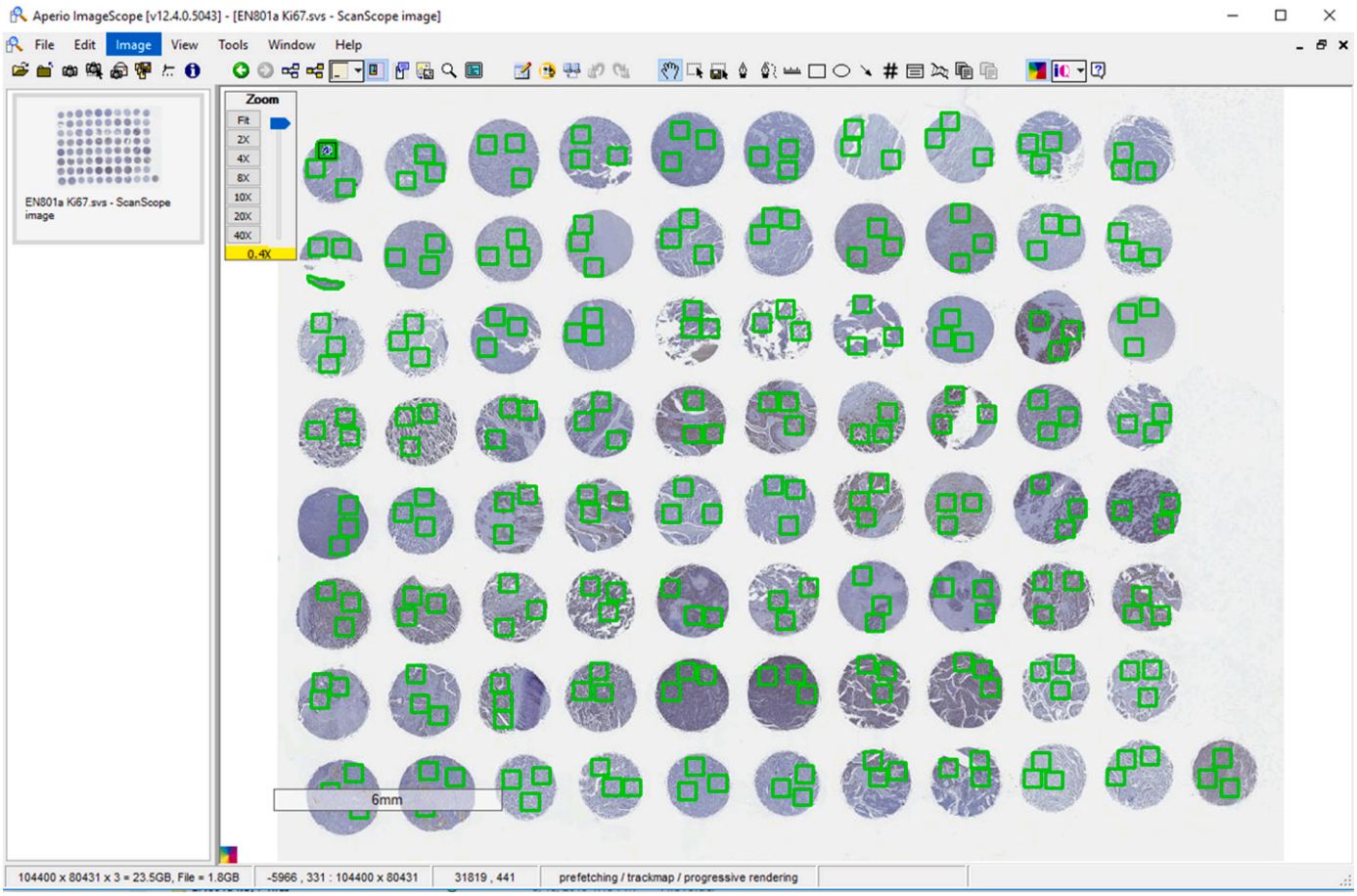

Fig. 6. Three 0.2 mm^2-fields were carefully selected from each core to avoid necrotic and lymphocyte-rich areas and non-tumor tissue.

Table 1
Manual quantification of mitosis and Ki67 index from an adrenal cortical carcinoma by 3 pathologists.

Pathologist	Manual mitotic count				Manual Ki67 index quantification			
	Using H&E slide		Using PHH3-stained slide		ME method (HPF, 0.2 μm^2)		MQPI (0.1 μm^2, 525 cells)	
	n/10 HPF	Time spent (min)	n/10 HPF	Time spent (min)	Ki67+ (%)	Time spent (min)	Ki67+ (%)	Time spent (min)
1	8	5	12	1	4	6	5.7	4
2	7	1.8	18	1	10	2	6.3	12
3	2	1	10	0.3	7	0.5	10	4

HPF = high power field, Min = minutes, ME = manual estimate under microscope, MQPI = manual quantification with printed image.

emphasize that this study was intended to explore the impact of post-analytical variables on the quantification and to study the correlation of Ki67 index with mitotic count/index in endocrine/neuroendocrine tumors, not intended to study the correlation of Ki67/mitosis with the tumor outcome or the correlation of ME with PSDIA. We used one case of in-house adrenal cortical carcinoma to demonstrate interobserver variability using ME and MQPI methods, to explore the impact of the selection of hotspot, field size and threshold on biomarker quantification; and used a TMA of endocrine/neuroendocrine tumors with variable histological grades/proliferating rates to study the correlation of Ki67 index with mitotic count/index.

For the quantification of Ki67 index it is considered acceptable to count between 500 and 1000 tumor cells in the highest labeled area (hotspot) [20,23]. There is a plethora of data published counting a range of 500 to > 2000 tumor cells for Ki67 quantification [11,12,24,25]. Our study and other recent studies [26] showed that field size affected Ki67 quantification and revealed the flaw of this practice of counting an inconsistent number of cells for Ki67 quantification among pathologists.

Mitotic counts have been interpreted as the number per high-powered field (HPF) within the tumor. It is known that different combinations of microscopes and lenses result in HPFs of variable size. In 2018, the International Agency for Research on Cancer (IARC) and World Health Organization (WHO) expert consensus proposed to express the mitotic count per mm^2 and the Ki67 labeling index performed on regions of most intense labeling ("hotspots of at least 0.4 mm^2) [27]. Yet, this proposal did not address a definitive threshold for positive Ki67 labeling.

Table 3
Digital quantification of average Ki67 index with different thresholds in fields of different size from 4 hotspots of an adrenal cortical carcinoma.

Field size (mm^2)	HPF of Olympus BX41 (n)	Total cells (n)	3+ (%)	3+2+ (%)	3 + 2 + 1+ (%)
0.05	0.25	276	5.17	8.305.17	19.06
0.1	0.5	534	4.67	7.63	17.26
0.2	1	1046	4.50	7.41	16.86
0.4	2	2074	3.82	6.76	16.69

Since Ki67 is detectable in all phases of the cell cycle, with increased expression in S phase and peak expression in M phase, theoretically, all cells with detectable Ki67 expression should be considered as proliferative cells (positive cells) [23]. However, it is a common practice that only stronger stained cells (2+ and 3+, or 3+ only) are counted as positive and weakly stained (1+) cells are often disregarded. This inconsistency obviously contributes to prevalent inter-observer variability.

Inter-observer variability is the inherent problem for both ME and MQPI methods. Some studies reported that the overall reproducibility using the MQPI method was better than using the ME method for the quantification of Ki67 index, when all participants were given the same printed images in a study setting [11,20]. But in practice, the MQPI is still plagued with inter-observer variability due to subjective selection of hotspots, field size and threshold.

Table 2
Digital image analysis of mitoses (PHH3+ cells) and Ki67 index in fields of different sizes from 4 hotspots of an adrenal cortical carcinoma.

Field	PHH3 slide					Ki67 slide			
	# Cells	Area (mm^2)	HPF[a] (n)	PHH3+ (n)	PHH3+ (%)	# Cells	Area (mm^2)	HPF (n)	Ki67+[b] (%)
1	237	0.05	0.25	2	0.84	304	0.05	0.25	7.56
2	250	0.05	0.25	3	1.20	278	0.05	0.25	7.56
3	247	0.05	0.25	4	**1.62**	253	0.05	0.25	**9.09**
4	225	0.05	0.25	2	0.89	267	0.05	0.25	8.99
Mean	239.75	0.05	0.25	2.75	1.15	275.5	0.05	0.25	8.3
1	467	0.1	0.5	3	0.64	600	0.1	0.5	8.5
2	489	0.1	0.5	4	0.82	525	0.1	0.5	6.67
3	493	0.1	0.5	5	**1.01**	485	0.1	0.5	**8.87**
4	435	0.1	0.5	3	0.69	527	0.1	0.5	6.46
Mean	471	0.1	0.5	3.75	0.80	534.25	0.1	0.5	7.63
1	915	0.2	1	4	0.44	1150	0.2	1	8.44
2	970	0.2	1	4	0.41	1035	0.2	1	6.48
3	956	0.2	1	7	**0.73**	935	0.2	1	**8.45**
4	867	0.2	1	5	0.58	1067	0.2	1	6.28
Mean	927	0.2	1	5	0.54	1046.75	0.2	1	7.41
1	1801	0.4	2	5	0.28	2248	0.4	2	**7.21**
2	1902	0.4	2	6	0.32	2017	0.4	2	6.65
3	1798	0.4	2	7	0.39	1867	0.4	2	7.02
4	1723	0.4	2	7	**0.41**	2166	0.4	2	6.14
Mean	1806	0.4	2	6.25	0.35	2074.5	0.4	2	6.76

[a] For an Olympus BX41 microscope, 1 HPF (high power field) = 1 40× field = 0.2 mm^2.
[b] Ki67+: 2+ was used as positive threshold; Aperio nuclear algorithm was used for quantification.

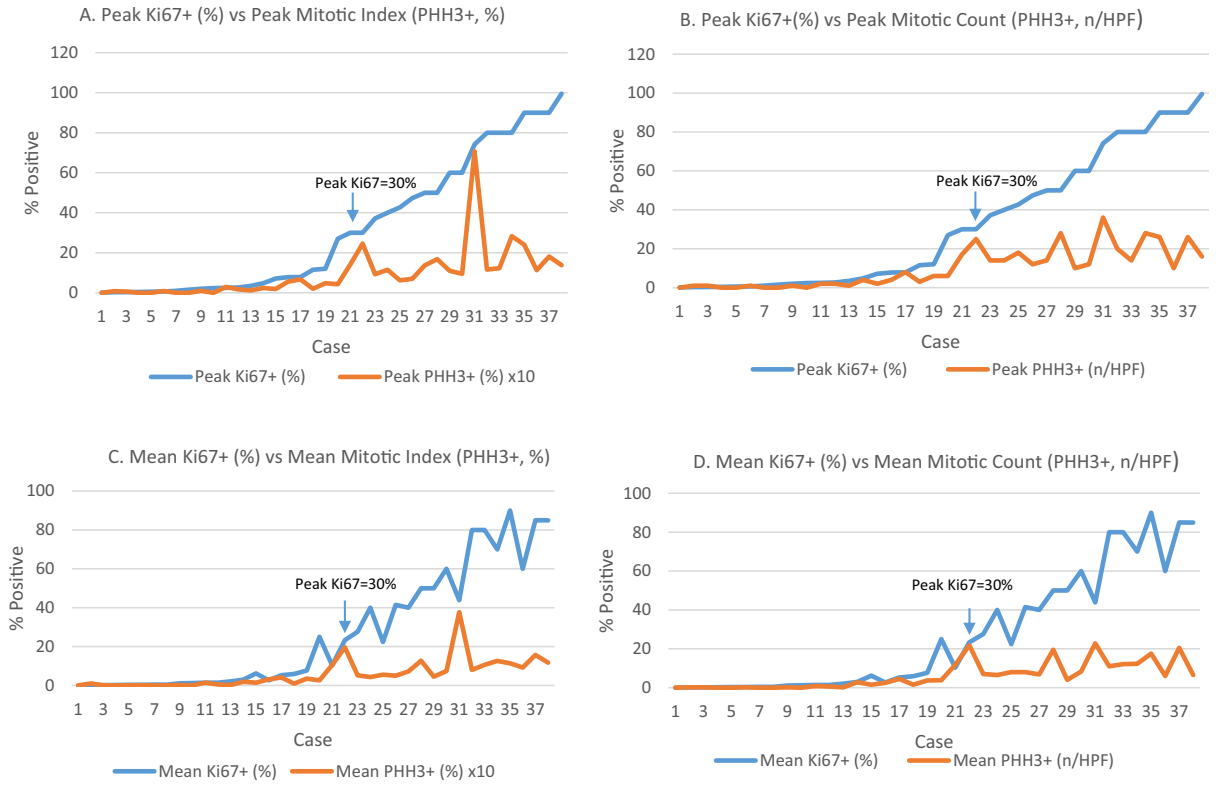

Fig. 7. Line charts illustrate the relationship between the mitotic count/index and Ki67 index from the 38 cases (228 fields) of endocrine/neuroendocrine tumors. Panel A & B show "peak" data correlation, panel C & D show "mean" data correlation. (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.)

Table 4
Overall correlation study between mitotic count and Ki67 index from 38 (228 HPFs) endocrine/neuroendocrine tumors.

Correlations	Peak PHH3 + (%) vs Ki67 + (%)		Peak PHH3 + (n) vs Ki67 + (%)		Mean PHH3 + (%) vs Ki67 + (%)		Mean PHH3 + (n) vs Ki67 + (%)	
	r	*p*-Value	r	*p*-Value	r	*p*-Value	r	*p*-Value
Pearson	0.646	< 0.01	0.814	< 0.01	0.614	< 0.01	0.715	< 0.01
Spearman	0.910	< 0.01	0.898	< 0.01	0.901	< 0.01	0.889	< 0.01

PHH3 + = mitosis, n = mitotic count. Aperio nuclear algorithm was used for quantification. IBM SPSS Statistics 25 was used for analysis.

Table 5
Digital quantification of mitotic count (PHH3 +) and Ki67 index from 38 cases (228 HPFs) of endocrine/neuroendocrine tumors with different proliferating rates (groups).

Group	Case/HPF (n/n)	Mean Ki67 + (%)	Peak Ki67 + (%)	Mean PHH3 + (%)	Peak PHH3 + (%)	Mean PHH3 + (n/HPF)	Peak PHH3 + (n/HPF)	PHH3 + /Ki67 + (ratio)	
								Mean	Peak
1	12/72	0.59 ± 0.53	1.20 ± 0.96	0.03 ± 0.05	0.07 ± 0.09	0.17 ± 0.26	0.67 ± 0.78	0.10 ± 0.28	0.07 ± 0.10
2	8/48	7.21 ± 7.45	10.20 ± 7.39	0.22 ± 0.13	0.36 ± 0.20	2.56 ± 0.26	4.25 ± 0.78	0.05 ± 0.04	0.04 ± 0.03
3	18/108	53.27 ± 24.39	62.83 ± 22.88	1.10 ± 0.78	1.74 ± 1.46	11.69 ± 6.08	18.89 ± 7.51	0.03 ± 0.03	0.03 ± 0.02

Group 1: peak Ki67 < 3%, group 2: peak Ki67 = 3–29%; group 3: peak Ki67 ≥ 30%; HPF, high power field; HPF = a 40× field of Olympus BX41 microscope = 0.2 mm^2.

PSDIA is objective, precise and robust and is considered as the gold standard for biomarker quantification by many [20,28,29]; yet biased data can also be produced if not done correctly [11,30,31]. Unless there is a consensus for the selection of hotspots, field size and threshold, inter-observer variability will persist regardless of methodology used.

With all those powerful software tools, PSDIA clearly has commanding advantages for optimal quantification of Ki67 index and all other biomarkers. It can choose a true hotspot (with highest Ki67 index) by analyzing multiple candidate fields with the click of a button and by setting the field size and threshold precisely within an institution. Ideally, this should be done in a centralized fashion: IHC, algorithm development and quantification should be done by trained personnel under the pathologist's supervision. The final reporting would be done by the supervising pathologist or by individual pathologist who orders the test. This can minimize subjectivity associated with manual quantification at least within an institution. Broadly, guidelines for the selection of field size, hotspot(s) and threshold could be developed by experts and reinforced with a proficiency test program to standardize the practice among institutions. However, PSDIA requires more capital investment in scanner and software, and dedicated technical personnel,

Table 6
Correlations between mitotic count and Ki67 index in the 38 (228 HPFs) endocrine/neuroendocrine neoplasms with different proliferating rates.

Correlations	Group 1 & 2 (20 cases, 120 HPFs)								Group 3 (18 cases, 108 HPFs)							
	Peak PHH3+ (%) vs Ki67+ (%)		Peak PHH3+ (n) vs Ki67+ (%)		Mean PHH3+ (%) vs Ki67+ (%)		Mean PHH3+ (n) vs Ki67+ (%)		Peak PHH3+ (%) vs Ki67+ (%)		Peak PHH3+ (n) vs Ki67+ (%)		Mean PHH3+ (%) vs Ki67+ (%)		Mean PHH3+ (n) vs Ki67+ (%)	
	r	*p*-Value	r	*p*-Value	r	*p*-Value	r	*p*-Value	r	*p*-Value	r	*p*-Value	r	*p*-Value	r	*p*-Value
Pearson	0.646	< 0.01	0.736	< 0.01	0.534	< 0.05	0.686	< 0.01	0.211	0.4	0.184	0.465	0.044	0.862	0.107	0.672
Spearman	0.830	< 0.01	0.834	< 0.01	0.750	< 0.01	0.815	< 0.01	0.265	0.289	0.114	0.653	0.341	0.166	0.137	0.587

Group 1&2 are tumors with peak Ki67 index < 30%, group 3 are tumors with peak Ki67 index ≥30%; PHH3+ (%) = mitotic index, PHH3+ (n) = mitotic count.

which are currently the major predicaments for many institutions.

The PHH3 antibody would be a great addition to the antibody menu. It can help pathologists differentiate mitosis from its mimickers, such as degenerating/pyknotic cells, and detect mitosis quickly, especially in low grade endocrine/neuroendocrine tumors.

While the data from Weinder's group generated from breast carcinoma in 1994 showed strong correlation between Ki67 index and mitotic index ($r_p = 0.76$, $p < 0.0001$)/mitotic count ($r_p = 0.78$, $p < 0.0001$) [21], the data from Rudolph's group generated from mixed cases of colorectal adenocarcinoma, mammary carcinoma, squamous cell carcinoma, non-small cell lung cancer and small cell lung cancer in 1998 showed mixed results in different cancer group [22]. Both the aforementioned work was done manually, and mitosis was counted based solely on morphology.

Using a mitosis-specific antibody PHH3 and digital pathology, our data from endocrine/neuroendocrine tumors showed that mitotic count ($r_p = 0.65$, $p < 0.01$) and mitotic index ($r_p = 0.81$, $p < 0.01$) were positively correlated with Ki67 index overall; their relationship was more monotonic (positive, non-linear) than linear (change in proportion) ($r_s > r_p$, see Table 4). Strong correlation between these two was seen only in tumors with peak Ki67 index < 30% (in group 1&2). (30% was chosen as a cutoff for analysis because it was the starting point from where the degree of correlation between Ki67 index and mitotic count/index drastically decreased in the line charts (Fig. 7)). Interestingly, the ratio of mitosis to Ki67+ cells (the proliferative tumor cells, using 1+ as the threshold) in tumor group 2 (peak Ki67 index in the range of 3–29%) was 0.04, which coincides with the time allocation for M-phase in a typical human cell proliferating in culture of a 24-hour cycle: 1 h for M-phase and 23 h for interphase (1:24 = 0.042) [1].

Tumors with peak Ki67 index of 3–29% typically encompass benign, atypical to frank malignant tumors. Accurate quantification of mitosis and Ki67 index in this group is critical for establishing a diagnosis and for grading. Perhaps one could also use the ratio of mitosis/Ki67+ cells to gauge the accuracy of mitotic count (index) with Ki67 index or vice versa for endocrine/neuroendocrine tumors with low to moderate rate of proliferation. It would be interesting to see if the relationship between mitosis and Ki67+ cells found in our study is universal and could be found in tumors of other types. Future studies using a larger sample size and including different tumor types are needed.

We acknowledge that the 38 cases of endocrine/neuroendocrine tumors included in this study represent a relatively small sample size. Since each case has duplicate cores of 1.5 mm in diameter, from which three 0.2 mm^2 fields (a 0.2 mm^2 field is equivalent to one 40× HPF of Olympus BX41) from each core, a total of 228 fields were included for analysis. We believe the abundance of fields included for analysis made up for the relatively small case number and served the purpose of this study.

In summary, we show in this study that the selection of hotspots, field size and especially threshold are important variables and could affect accurate quantification of mitosis and especially Ki67 index regardless of methodology used. PSDIA is more objective, precise and robust in nature. It can minimize the problems associated manual quantification in the selection of hotspots, field size and threshold. If done correctly, PSDIA could provide more efficient and accurate quantification of Ki67 index and possibly other biomarkers.

Regardless of the methodology, a consensus among pathologists is needed for the selection of hotspots, field size and threshold for the quantification of mitosis and Ki67 index.

Mitotic count/index correlates positively with Ki67 index, but the correlation is strong only in endocrine/neuroendocrine tumors with a mild to moderately proliferating rate (peak Ki67 index < 30%).

CRediT authorship contribution statement

Wei Huang designed the experiment, did the quantification of Ki67 index and mitosis in the endocrine/neuroendocrine tumors in the TMA sections and all the data analysis, and drafted the manuscript.

Christian Nebiolo did the quantification of Ki67 index and mitosis in the adrenocortical carcinoma on the whole sections, and prepared images for Fig. 2.

Karla Esbona did IHC staining of all the sections including optimization of the antibodies.

Rong Hu took part in manual counting mitosis and quantifying Ki67 index on the whole sections and printed images of adrenocortical carcinoma and provided valuable suggestions in the preparation of this manuscript.

Ricardo Lloyd contributed critical input for the design of this study, took part in manual counting mitosis and quantifying Ki67 index on the whole sections and printed images of adrenocortical carcinoma and provided valuable critiques in the preparation of this manuscript.

Declaration of competing interest

No conflict of interest to disclose.

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