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Comparation of the quantification of the proliferative index KI67 between eyeball and semi-automated digital analysis in gastro-intestinal neuroendrocrine tumors

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Abstract

Background: Neuroendocrine tumors (NETs) constitute tumors widely distributed and with heterogeneous biological behavior. For gastrointestinal neuroendocrine tumors (GI-NETs) the following prognostic factors have been identified: location, production of hormones, size and proliferative grade. The latter must be calculated using proliferation index by the number of mitosis or the proportion of tumor cells positive for Ki67 immunostaining. The objective of this study was to use a quantitative tool to calculate the Ki67 index in GI-NETs.

Material and methods: We reviewed 40 cases of GI-NETs diagnosed at the Department of Pathological Sciences, Santa Casa de Misericórdia de São Paulo Hospital between 2004 and 2014 and compared the Ki67 index by manual count using scanned photomicrographs with semi-automated digital analysis (MC) and eyeball estimation (EE) of the histological slide.
After Ki67 immunostaining, the slides were scanned with 3DHistech Pannoramic Scanners. Hot spots were selected and exported in a high-resolution image format and the Ki67 index was calculated with semi-automated image analysis software (AxioVision 3.0). Ki67 immunoreactivity was expressed as the percentage of tumor cells with nuclear staining (number of positive tumor cells/a minimum of 500 total tumor cells).

Results: We compared the classification of the neuroendocrine tumor by using the two methods in the semi-automated method 26 maintained the same grade, while 14 were re-classified, 4 being upgraded and 10 downgraded.

Conclusion: In the EE method there was a larger estimate of the percentage of positivity for KI67. As the Ki67 values are the criteria for the classification of neuroendocrine tumors, the semi-automated method can have less error.

Keywords: Neoplasms, Neuroendocrine tumors, Digital imaging analysis, Ki-67 antigen, accuracy

Background

Neuroendocrine tumors (NETs) constitute a wide range of tumors derived from neuroendocrine cells (NE) that are widely distributed throughout the human body, with heterogeneous clinical behavior in terms of local growth and the presence of metastasis. The incidence of the disease is 1–2/100.000 inhabitants, and NETs represent 0.5% of all malignant neoplasms. This group of neoplasms predominantly affects females, and the most prevalent age group is above 50 years of age (Modlin et al. 2003; Caldarella et al. 2011; Taal and Visser 2004).

Since 2004, the Ki67 (MIB-1) cell proliferation marker has been used as the grading parameter for prognostic in gastroenteropancreatic neuroendocrine tumors (GEP-NET) as a method of distinguishing low-grade, intermediate-grade and high-grade tumors and motivated the European Neuroendocrine Tumor Society (ENETS) to propose a 3-tiered grading system

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(Strosberg et al. 2009; Rindi et al. 2006; Rindi et al. 2007; Klimstra et al. 2010a). The WHO also recommends using either mitotic rate or the Ki67 labeling index for histological grading of the inherent biologic aggressiveness of gastroenteropancreatic neuroendocrine tumors (GEP-NETs) (Kulke et al. 2010; Klimstra et al. 2010b).

Thus, NETs are divided into 3 grades: G1 or low grade (mitoses < 2/10HPF or 2mm^2, or Ki67-labeling index of < 3%); G2 or intermediate grade (mitoses of 2 to 20/HPF, or Ki67-labeling index of 3 to 20%); and G3 or high grade (mitoses > 20/10HPF or Ki67-labeling index > 20%). The objective of this classification system was to introduce standardized and yet practical prognostic categories that can predict the prognosis of NETs (Bosman et al. 2010). Although the treatment between G1 and G2 is similar, some cases may present unexpected evolution, especially G2 cases with a proliferative index greater than 10% for Ki67 (Nuñez-Valdovinos et al. 2018).

Despite the use of this grading classification, the behavior of neuroendocrine tumors is uncertain; aggressiveness is variable, with low grade tumors presenting aggressive manifestations such as metastasis, and sometimes there is discordance between the mitotic rate and Ki67 index (Basturk et al. 2015).

The literature describes different methods to evaluate Ki67 expression. One of the most practical and most commonly used in pathological anatomy laboratories is semiquantitative counting (eyeball estimation). Although it is low-cost and does not need a lot of time (less than one minute), it has low accuracy. A practical and reliable alternative demonstrated by Reid et al. is a manual count from printed photomicrographs of areas with high positivity for Ki67 (hot spots) (Reid et al. 2015). This method has been assessed by a number of other studies (Tang et al. 2012; Cottenden et al. 2018; Kroneman et al. 2015; Young et al. 2013).

Thus, we propose to use an alternative method to count the number of positive Ki67 cells in gastrointestinal neuroendocrine tumors, where the histological sections will be scanned and the proliferative index will be manually counted using scanned slides with semi-automated digital analysis (MC) and compared with the results of eyeball estimation (EE).

Materials and methods

The study was approved by the local ethics committee, number 006253/2015. This work does not represent a clinical trial and was therefore not registered as such.

We retrospectively identified 74 cases with confirmed diagnoses of gastrointestinal neuroendocrine tumors (GI-NET) between 2004 and 2014 from the computer records of the Department of Pathological Sciences, Santa Casa de Misericórdia de São Paulo Hospital, included well differentiated NETs and poorly differentiated neuroendocrine carcinoma (NECs). A total of 40 of these cases were retrieved with their corresponding paraffin blocks from the files of the Hospital Pathology Division. Unfortunately, 34 cases without paraffin-embedded were not available for review and Ki67 imunohistochemical staining.

Paraffin-embedded tissue blocks with presence of tumor were cut into 4 μm-thick sections for immunohistochemistry. These sections were deparaffinized and subjected to antigen retrieval in a pressure cooker in a sodium citrate buffer (pH 6.0). Endogenous peroxidase was blocked using 5% hydrogen peroxide. Nonspecific staining was blocked in 2% normal swine serum. The slides were incubated with the primary antibodies (Ki67 – Mib-1 clone, 1:160; Dako Corporation, Carpinteria, CA, USA), counterstaining was performed using Mayer's hematoxylin. Positive control tissue (lymph node) was stained in parallel with all the study cases. All cases were ki67 immunostaining at the same time.

Immunohistochemically stained slides were scanned with 3DHistechPannoramic Scanners (3Dhistech, Budapest, Hungry), using a 20x objective. The areas of highest density of Ki67 immunoreactivity (hot spots) were manually selected by an experienced pathologist for quantification in magnification of 400x (Fig. 1). These areas were exported in a high-resolution image format and analyzed through semi-automated digital analysis software (AxioVision 3.0 – ZeissGMb, Germany) (Figs. 2 and 3). To each case selected 2 or 4 fields hot spots. Ki67 immunoreactivity for all assessment methods was expressed as the percentage of tumor cells with nuclear staining, considering the number of positive tumor cells divided by a minimum of 500 total tumor cells (Figs. 2 and 3). Were considered positive cells when a brown color was observed in the nucleus and/or nucleolus. The cells positive were marked and numbered with green colour and the negatives cells with red colour in magnification of 400x until 500 cells in the total

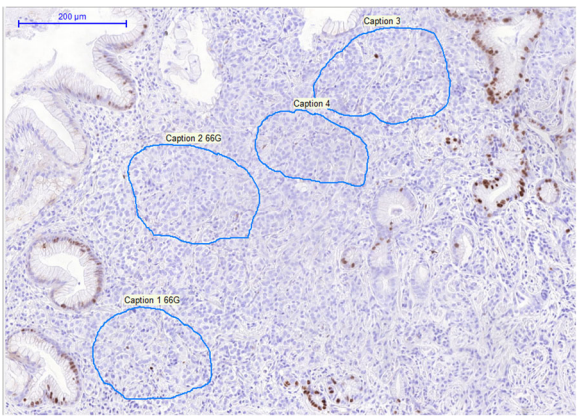

Fig. 1 Image scanning for quantitative immunohistochemical analysis and areas of "hot-spot selected for count

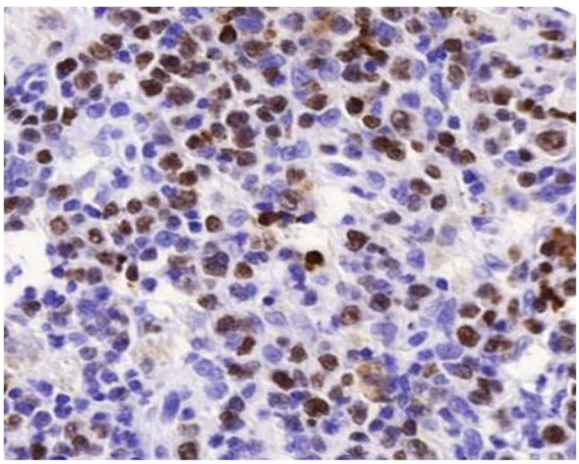

Fig. 2 Ki67 positive nuclear immunoreactivity for the count (brown colour)

(negatives and positives). Thus, the cases were divided into 3 grades: G1 or low grade (Ki67-labeling index of < 3%); G2 or intermediate grade (Ki67-labeling index of 3 to 20%); and G3 or poorly differentiated neuroendocrine carcinomas, (Ki67-labeling index > 20%) (Bosman et al. 2010). Table 2 shows a comparative analysis between the MC and EE methods in respect of identification of the three groups of GI-NET.

The estimation EE by pathologist of the Ki67-labeling index was based in the inspection of the images, without performing formal counting of individual cells.

Table 2 shows a comparative analysis between the MC and EE methods in respect of identification of the three groups of GI-NET.

The mitotic index was not included, due to the limitation of the amount of tumor tissue in 23 of the biopsies,

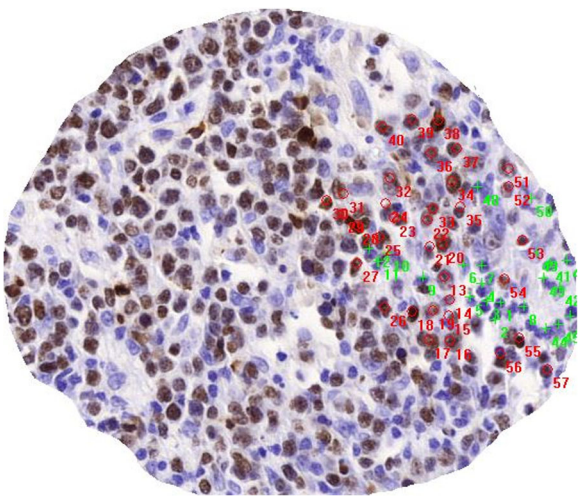

Fig. 3 Manual count of the digital analysis, the red dots are the positive cells for Ki67

making it impossible to count 50 high power fields, as recommended in the literature (Kim and Hong 2016) most cases were 0 mitoses/10HPF.

Results

After selection, we studied 40 cases of GI-NET. The distribution by sex, age, organ and Ki67 are shown in Table 1. The cases included 22 biopsies and 18 surgical resections.

To compare the methods of quantification of the proliferative index, divided in 3 groups according to index proliferative. (Table 2).

When we compared the classification of neuroendocrine tumors based on MC and EE, there was a mismatch in classification in 35% (14/40) of the cases. 10 cases were downgraded and 4 cases upgraded when we used MC and compared with EE.

Discussion

The Ki67 proliferative index has been incorporated into the WHO classification (Bosman et al. 2010) for GI-NET grading and is one of the most reliable prognostic factors. However, Ki67 assessment has limitations due to lack of accuracy, uniformity, reproducibility and consistency in quantification, especially for intermediate-grade tumors with equivocal "gray zones".

Most papers show variabilities in interlaboratory and interobserver reproducibility in interpretation of Ki67 immunoreactivity and differences in the method of counting such as eyeball estimation, manual count with photomicrographs and digital analysis (Reid et al. 2015; Tang et al. 2012; Cottenden et al. 2018; Kroneman et al. 2015; Young et al. 2013). Therefore, there remain several challenges in determining the proliferation index in a given tumor, including the method of counting, tumor heterogeneity, and defining what constitutes a hot spot and what constitutes positive staining. However, Kroneman et al. (Kroneman et al. 2015) showed that the three quantification methods for the Ki-67 index had almost perfect agreement and correlated with patient survival. EE had higher predictive ability for survival and recurrence, although the values were significantly less than those of the other two methods. Other papers have shown that although EE is quick and low-cost it is error prone and produces different estimates depending on the individual observer (Tang et al. 2012).

Manual counting of printed photographs is increasingly considered to be the most accurate approach to determine the Ki67 index and NET grading (Reid et al. 2015). However, since manual counting is more expensive and time consuming it is not adopted in diagnostic routine, even if it is more effective (Cottenden et al. 2018).

Table 1 Distribution of the 40 cases by sex, age (median) and Ki67 (mean and median) in relation to the different organs

	Number	Female	Male	Range Age	Median age	Range Ki67	Mean Ki67	Median Ki67
Stomach	19	11	8	38–86	50.6	0–56	10.5	2.8
Small Intestine	10	6	4	45–87	64	0–3.8	1.4	0.95
Large Intestine	6	4	2	40–85	58	0–69.5	14.7	5
Cecal appendix	5	3	2	15–80	28	0–11.6	2.7	0.7
Total	40	24	16	15–87	54	0–69.5	7.8	1.82

Another form of counting described in literature is automatic digital image analysis (DIA), the time for this type of counting is unclear, however there is a need to standardize the programs for each case, and it is important to photograph areas with the best representation. In addition, it can induce errors such as counting stromal cells or lymphocytes.

We adopted the manual count method using scanned slides and semi-automated digital analysis rather than printing the photograph. First the slides are scanned, which does not need to be done by the pathologist. Then the most representative areas are selected by the pathologist and transferred as image to an analysis program for manually counting using a cell counting tool. These are high-resolution images, so we do not have to depend on a perfect print to visualize the nuclei staining. In addition, we can increase the magnification of the images for better visualization. In our results, there was a tendency for the grades to decrease, mainly from G2 to G1 (9 cases) and from G3 to G2 in (1 case); when we used digital analysis with a manual count our results were similar to Cottenden and et al. (Cottenden et al. 2018). Previous studies have also shown increases in this type of disagreement, mainly G1 to G2 (Tang et al. 2012; Kroneman et al. 2015).

Programs to count semi-automated digital analysis are easy to access and free download, as QuPath, has been developed for research applications at the Centre for Cancer Research & Cell Biology at Queen's University Belfast, as part of research projects funded by Invest Northern Ireland and Cancer Research UK (Bankhead et al. 2017). Unfortunately, scanner equipment have an expensive price to private or public laboratory, but if increase the needs probably in the future this equipment will be more affordable.

Table 2 Distribution by proliferative grade according to eyeball estimation (EE) and manual count (MC)

EE/MC	G1	G2	G3	Total
G1	17	**4**	0	21
G2	**9**	5	0	14
G3	0	**1**	4	5
Total	26	10	4	40

Legends *EE* eyeball estimation, *MC* manual count, *G1* grade 1, Ki67 < 3%; G2 = grade 2, Ki67 = 3 to 20%; G3 = grade 3 Ki67 > 20%
The numbers in boldface means that it changed degree

Eyeball estimation is now strongly discouraged unless a tumor shows an unequivocally low or high index, but if fairly close to the established categorical cutoffs, there may be an error. EE may, therefore, be appropriate in cases with very low or very high Ki-67 indexes such as < 1 or > 20%, but not in cases with a low to intermediate Ki-67 index or with greater heterogenicities.

When we evaluate the percentage of positive cells by the EE, we usually do it by high increase fields and this type of evaluation can vary depending on the microscopic whose size is very variable, so it is important to count a number of tumor cells.

The main objective was to compare two methods to evaluate th Ki67 index in TNE-GI and with results to do grading classification of the GI-TNE. As there were 23 biopsies, these cases should not often show the actual classification, but we compared the results between two methods with same material that may in the future be used in material suitable for analysis.

Conclusion

In view of the differences between the counting methods, it is worthwhile continuing studies aimed at developing a new methodology for the quantification of Ki67. Further studies are needed to identify accurate, clinically appropriate ways of making estimations which will help to assess the evolution of the disease and the prognosis for patients.

Abbreviations

DIA: Automatic digital image analysis; EE: Eyeball estimation; ENETS: European Neuroendocrine Tumor Society; GI-NETs: Gastrointestinal neuroendocrine tumors; HPF: High power fields; Ki67 (MIB-1): Anti-Ki67 antibody; MC: Semi-automated digital analysis; NE: Neuroendocrine cells; NECs: Poorly differentiated neuroendocrine carcinoma; NETs: Neuroendocrine tumors; WHO: World Health Organization

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Authors' contributions

MLB: Participated in the acquisition of clinical and pathological data. FSK: Participated in the acquisition of clinical and pathological data. FDCB: Responsible for the conception and design of the study, participated in the analysis and interpretation of data, and drafted the manuscript. All authors read and approved the final manuscript.

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Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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