INTRODUCTION

Gastroenteropancreatic neuroendocrine tumors (GEP-NETs) are neuroendocrine tumors characterised with uniform cells and bland cytological features. With the latest updates of WHO Classification of Tumors, NETs are graded as G1, G2 and G3 according to the proliferative index of the tumor evaluated by Ki67 antibody.\(^1\)

Grading of the neuroendocrine tumors depends on their mitotic activity and Ki67 proliferation index.\(^1\) Ki67 proliferation index was strictly graded as <3% grade 1, 3-20% grade 2, >20% grade 3.\(^1\) But the detection of these grades may cause problems, especially in borderline cases. Many detection methods were offered by different authors. The most commonly used technique for determining Ki67 proliferation index is the hotspot counting depending on the advantages of being the cheapest and the fastest method. However, the reproducibility and the interobserver agreement rates can be low.

 evaluation revealed a kappa value of 0.447 indicating moderate agreement between the pathologist and the software.

Conclusion: The total count of the cells both by the analyser and the pathologist were similar. However, improvements are needed to raise the precision for the detection of positive and negative tumoral cells.

Key Words: Neuroendocrine tumor, Ki67, Neuroendocrine tumor grade, Automated detection system.

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Image analysers are another option for counting Ki67 index.\(^2-4\) The use of these methods also changes depending on the technological facilities of the pathology laboratories worldwide; and due to expensive charges, it does not seem possible to set these devices in every laboratory, especially in countries with economical issues.

Depending on all these disadvantages and advantages of these methods, none of them was accepted globally as gold standard. In the study of Reid et al., eye-count on captured/printed images were found as the most cost-effective and reliable method.\(^5\)

Desktop computers or laptops are one of the musts in every pathology laboratory and for every pathologist even in countries with low socioeconomic level. Designing a simple but effective software suitable for every computer would help pathologists define accurate results in many different tumors, grading of tumors or any type of cell calculation.

The aim of this study was to design a simple software which can load captured images right from the microscopic camera application with no additional device, count the immunostained cells and calculate the percentage in addition to compare its results with eye-ball estimation on these captured images.
METHODOLOGY

Ethical approval was taken from Mugla Sitki Koçman University Ethical Committee for Clinical Trials and this study was supported by a project from the Scientific Research Projects Management Unit of Mugla Sitki Koçman University (Grant number: 15/097).

This descriptive study was conducted at Faculties of Medicine and Technology of Mugla Sitki Kocman University between January 2015 to January 2016. Randomly selected 50 GEP-NETs from the archives of the pathology laboratory of the study centre were included in this study. All of these tumors were diagnosed and graded by hotspot counting method. The size of the tumors and the types of the excision procedures were obtained from the pathology reports. For Ki67 immunostaining, 3-4 mm thick sections were cut from the paraffine blocks and the immunostaining procedure was held automatically by Leica Bond-Max using anti-Ki67 antibody (Leica).

Ki67 stained slides were revised, and on x20 objective, the highest nuclear labelled area "hotspot" was selected and microphotograph was captured by using Leica MC120 HD camera attached to DM1000 LED microscope. Images were then loaded to the software designed by Technology Faculty of our instution for Ki67 positive cell counting.

All images were revised by eye-counting percentage of positively stained cells in the hotspot by the same pathologist and the results given by the software and the pathologist was compared for variability.

An algorithm for disassemble positive and negative cells using K-means clusters was developed in this software for detecting Ki67 proliferation index. The images and cell borders were relatively not easily defined in some areas. In such images, Hough transformation was added for increasing the cell detection success. This software, which can be run on a standard office desktop computer/ laptop with a processor of Intel i5 3.20 Ghz and 8 GB video card, can analyse 1360*1024 pixel and .jpeg formatted microscopic images in about 2 seconds and calculate the percentage of cells which gives Ki67 proliferation index.

Two different observers (the pathologist and the software) gave the percentage of Ki67 positive cells and these results were regrouped according to WHO GEP-NET grading scale as <3% = Grade 1, 3-20% = Grade 2 and >20% = Grade 3. Descriptive statistics are reported as the mean ± SD and categorical variables were given as frequencies (percentages). A p-value less than and equal to 0.05 (p ≤ 0.05) was considered as statistically significant. Frequencies and descriptive statistics were run by SPSS v 20.0 and kappa statistics were run by R v 3.2.4 and Cohen's kappa was calculated for interobserver variability.

Figure 1: The screen views of the analyser: (A) Start-up screen; (B) An image loaded in the application (C) result screen.
**RESULTS**

Among 50 GEP-NETs, 27 were males (54%) while 23 were females (46%). The mean age of the patients was 52.3 ±8.80 years. Patients were diagnosed by endoscopic biopsy in 38 cases (76%); and 12 cases (24%) were diagnosed by surgical excision. The average size of the surgically excised tumors was 2.8 ±0.66 cm. Among all tumors, 26 (52%) were located in the stomach, 10 (20%) were appendiceal, 3 (6%) were colorectal, 3 (6%) were ileal, 8 (16%) were pancreatic NETs.

The images were analysed for both the total count of all cells, immunohistochemically stained cells and the percentage of these cells. After counting, the pathologist calculated the percentage. However, the analyser programme gave the percentage automatically. The main screen views of the analyser was given in Figure 1.

Agreement statistic of Cohen's kappa was used while comparing the percentages of the stained and unstained cells so as to grade the tumor. According to Ki67 index calculated by the pathologist with hotspot eye-counting method; 17 cases were grade 1 (34%), 21 cases were grade 2 (42%), and 12 cases were grade 3 (24%). By software, 8 cases were grade 1 (16%), 36 cases were Grade 2 (72%) and 6 cases were grade 3 (12%). Statistical evaluation revealed a kappa value of 0.447 indicating moderate agreement between the pathologist and the software.

<table>
<thead>
<tr>
<th>Analysers grade</th>
<th>Pathologists grade</th>
<th>Total</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>1</td>
<td>8 (16%)</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>9 (18%)</td>
<td>20 (40%)</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>1 (2%)</td>
</tr>
<tr>
<td>Total</td>
<td>17 (34%)</td>
<td>21 (42%)</td>
</tr>
</tbody>
</table>

Among these 50 cases, 33 (66%) were consensus cases in both the pathologist and the software found the same grade. Among consensus cases, 8 were grade 1 (Figure 2, 16%), 20 were grade 2 (40%) and 5 were grade 3 (Figure 3, 10%, Table I).

**DISCUSSION**

Determining Ki67 index in NETs is a common problem in surgical pathology practice. Because of the strict cut-off values of WHO grading of NETs, it is even more important to give distinct results. Different methods were offered in the literature for evaluating accurate Ki67 index because of the low interobserver agreement rates worldwide not just for GEP-NETs but also in various tumor types. Some of these methods were accompanied with automated image analysers. Automated analysers scan the whole slide and trained personnel (technicians or pathologists) choose the "hotspots" and the selected areas were then counted automatically. This method reduces time for evaluation, and is accepted as gold standard but the whole system is expensive to use in every laboratory in every country.

Automated counting by image analysers scan the whole slide, choose the hotspots and count the Ki67 index. In different studies Aperio immunohistochemical nuclear quantifier and Nuclear v9 image analysis algorithm were used for digital image analysis. But in these algorithms, cut off for the size and the shape of the tumor cells were controlled manually because of the need to exclude stromal cells and lymphocytes and this increases the duration of an analysis of an image up to 10 minutes. In automatic counting analysers such as ImageJ, where macro-based markers of different colors are being used, cannot give very accurate results due to fragmented cells.

But these methods need trained technicians who can choose hotspots and this automated analysers are generally used in reference laboratories of Turkey, which have more technical and financial support. In other centres, these analysers are not accepted because of the high cost. Many other methods are still being investigated for detecting exact percentage, which is one of the most important factors for neuroendocrine tumors. Bologna-Molina et al. suggested a method using only a digital camera attached microscope and a computer. In this method, they offered to use a grid in table covering the immunohistochemical image and manually count the cells. However, these methods generally take time to get to the results.
Eye-balling, fastest and the cheapest method, is the most commonly used method for counting Ki67. Although widely used and known as the most practical method, eye-balling has disadvantages such as high inter and intraobserver variability rates and low reproducibility in especially borderline cases. Even though some authors offered this method as reliable, unexperienced pathologists may not overcome the problem of this variability.

In the study by Reid et al., manual Ki67 counting upon camera captured/printed images was offered. The slides of tumors were revised and images from the hotspots were then captured by an attached camera. Ki67 positive and negative cells were counted from the printed images and the percentage was calculated. In this study, it was also captured images from hotspots but transfer the images to the software loaded at the same computer and the software counted and calculated the percentage of Ki67 positive cells. The target of this software was to reduce time loss while counting manually. However a kappa value of 0.447 is not acceptable in this type of study but similar with other studies found in the literature such as Dhall et al. and Tang et al. reported. In the study of Tang et al., a kappa value of 0.24 (moderate agreement) was calculated between the analyser and the observer similar to the kappa value of 0.39 which was reported in the study of Dhall et al. Moderate agreement may be due to the overlapping of the cells in the images on which eye-balling can differ but the software cannot. In addition to that, the heterogeneity of the tumors can also affect the results. For example, high tumoral cellularity may mask the real positive cells that can result undergrading. This phenomenon can also be observed in other types of tumors such as breast cancer samples.

CONCLUSION

Different methods or analysers can be used in detecting positively stained cells, but there should be higher agreement rates when compared with experts.

REFERENCES