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Original Article

Clinicopathologic characteristics of neuroendocrine tumors with assessment by digital image analysis for Ki-67 index with a focus on the gastroenteropancreatic tract: a single-center study

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Abstract: Objectives: Neuroendocrine tumors (NETs) are a heterogeneous group of tumors that arise at various sites throughout the body. The gastroenteropancreatic (GEP) tract is the most common site of NETs. We investigated the clinicopathologic features of patients with GEP-NETs and the utility of digital image analysis, which was compared to eyeball estimation, a conventional method used to determine the Ki-67 labeling index. Methods: The clinicopathologic data of GEP-NET patients at Gangnam Severance Hospital from January 2008 to October 2019 were retrospectively analyzed. Each case was reclassified according to the 2019 World Health Organization classification system, to which the classification of grade 3 was added. Comparisons between eyeball estimation and the digital image analysis method for Ki-67 index assessment were performed by calculating Cohen's kappa (κ) coefficient. Results: In total, 345 patients with GEP-NETs were enrolled. The mean age was 49.3 (range 13-79) years, with more male (61.1%) than female patients. The primary tumor sites were the rectum (70.1%), pancreas (12.5%), stomach (6.7%), and duodenum (5.8%). Overall, 298 (86.4%), 35 (10.1%), 2 (0.6%), and 10 (2.9%) patients exhibited grade 1, 2, and 3 and neuroendocrine carcinoma, respectively. Statistical analysis revealed that age > 50 years, tumor size > 2 cm, and presence of lymphovascular invasion, nodal metastasis, and distant metastasis were significantly associated with short overall survival. Additionally, 283 patients underwent digital image analysis of the Ki-67 index, and substantial agreement was found between the two methods (κ value: 0.765). Conclusions: Eyeball estimation revealed non-inferior results compared with digital image analysis. Further research is needed to evaluate the possibility of using digital image analysis as an alternative analysis method.

Keywords: Neuroendocrine tumor, eyeball estimation, digital image analysis, Ki-67 labeling index

Introduction

Neuroendocrine tumors (NETs) comprise a heterogeneous group of tumors originating from neuroendocrine cells distributed throughout the body [1, 2]. NETs exhibit various clinical and biologic behaviors in relation to the location of the primary tumor, origin of neuroendocrine cells, and pathologic features [3-6]. Most NETs are derived from the gastroenteropancreatic (GEP) system [7]. According to a multicenter study of 4,951 gastroenteropancreatic neuroendocrine tumors (GEP-NETs), the rectum (48.0%) was the most frequent location of GEP-NETs in patients from South Korea, followed by the stomach (14.6%), pancreas (8.7%), and colon (7.9%), whereas the small intestine was the most common location in a report of western patients [3, 8]. In South Korea, the incidence of GEP-NETs has increased over recent decades, primarily due to increased detection of rectal NETs [3]. However, few studies have investigated the biologic nature of GEP-NETs.

In the first World Health Organization (WHO) classification of NETs published in 1980, the term carcinoid was used regardless of the biologic behavior or pathologic features. The subsequent WHO classification published in 2000 divided NETs into well-differentiated endocrine tumors, well-differentiated endocrine carcinomas, and poorly-differentiated endocrine carcinomas according to the degree of differentiation [9]. Well-differentiated NETs are divided

into benign and low-grade malignant tumors according to the tumor size, mitotic rate, Ki-67 index, lymphovascular invasion (LVI), and symptoms. In the 2010 WHO Classification of Tumors of the Digestive System, NETs are graded as grade 1, grade 2, and neuroendocrine carcinoma (NEC) according to their morphology, mitosis, and Ki-67 labeling index, regardless of their location, size, or extent [10]. NECs were defined as having > 20 mitoses per 10 high-power fields or a Ki-67 index > 20% and were subdivided into small-cell NEC and large-cell NEC based on their morphology [10]. However, many studies have demonstrated a better prognosis for NECs exhibiting well-differentiated morphology than for poorly-differentiated NETs, indicating a need for modification of the grading system [11-13]. In the 2017 WHO Classification of Tumors of the Endocrine Organs, a new category of well-differentiated grade 3 NETs was introduced, which was initially validated for the pancreas and then extended to the entire digestive tract in the 2019 WHO Classification of Tumors of the Digestive System [14].

Expression of the Ki-67 protein is a reliable biomarker of tumor cell proliferation and growth [15]. The Ki-67 proliferation index, which is defined as the percentage of tumor cells with Ki-67-positive immunostaining among the total tumor cells [16], has been established as a key prognostic or predictive indicator in various tumor types. Therefore, evaluation of the Ki-67 proliferation index is commonly performed during histopathologic examination [15, 16]. Although the Ki-67 proliferation index is essential for NET grading, the standardized method used to evaluate the index is still controversial. The most widely used method in daily routine practice, which is advocated by some as a reliable method, is eyeball estimation. However, several recent studies have shown that this method is inaccurate and unreliable, especially for 'gray zone' tumors, which are difficult to classify as either low- or high-grade tumors [17, 18]. With the development of new techniques, automated counting using digital image analysis (DIA) has been proposed as an alternative to conventional assessment methods (eyeball estimation, manual counting of printed images).

In this study, we aimed to explore the clinicopathologic features (sex, age, tumor size, tumor location, LVI, distant metastasis, immunohistochemical staining distribution) and biological behavior of GEP-NETs by reviewing previous cases. We utilized the 2019 WHO classification system to re-evaluate previous diagnoses and determine the clinical utility of this new system. Finally, to evaluate automatic counting using DIA as an alternative option to conventional eyeball estimation, we compared the Ki-67 proliferation index values measured by DIA and eyeball estimation.

Materials and methods

Patients

Patients who were diagnosed with GEP-NETs at Gangnam Severance Hospital, Yonsei University College of Medicine between January 2008 and October 2019 were enrolled. Patients with biopsy specimens or Ki-67 immunostained slides that were deemed inadequate for assessment were excluded from this study. Clinical data regarding age, sex, tumor characteristics, treatment, and follow-up period were collected from electronic medical records.

Pathologic diagnosis

The pathologic diagnosis of GEP-NET according to the previous WHO classification was reclassified based on the 2019 WHO Classification of Tumors of the Digestive System. Neuroendocrine neoplasms were classified as grade 1, grade 2, or grade 3, or NEC based on morphologic features and the proliferative activity of the tumor, which was measured using the Ki-67 index and the mitotic rate. Grade 1 indicated a Ki-67 index less than 3% and a mitotic count of less than 2 per 2 mm^2. Grade 2 indicated a Ki-67 index of 3% to 20% or a mitotic count of 2 to 20 per 2 mm^2. Grade 3 was defined as a well-differentiated tumor with a Ki-67 index greater than 20% or a mitotic count greater than 20 per 2 mm^2. NEC was defined as a poorly-differentiated tumor with a Ki-67 index greater than 20% or a mitotic count greater than 20 per 2 mm^2. The grade determined according to the Ki-67 index was usually higher than the grade classified according to the mitotic count, and the higher of the two determined the classification.

Ki-67 immunostaining and counting methodologies

Immunohistochemical staining was performed on 4-um thick tissue sections from formalin-fixed paraffin-embedded (FFPE) tissue. Ki-67 immunostaining was performed on a represen-

tative slide from each patient following manufacturer recommendations using automated slide stainer (Ventana Discovery XT, Ventana Medical System, Tucson, AZ, USA) (clone MIB-1, 1:160; Dako Corporation, Carpinteria, CA, USA). Staining was considered positive when nuclear staining was brown. The Ki-67 proliferation index was defined as the number of positively-stained tumor cells divided by the total number of tumor cells present in tissue, expressed as a percentage. For example, if a tumor sample contained 500 cells and 50 were positively stained for Ki-67, the Ki-67 proliferation index would be 10%. The Ki-67 index was assessed in the area with the highest positivity rate (hot spot). Two methods were applied for comparison: eyeball estimation and DIA.

Eyeball estimation: This is a traditional method for calculating the Ki-67 index, that is widely used and advocated by the European Neuroendocrine Tumor Society and the North American Neuroendocrine Tumor Society [19, 20]. This method consists of estimating the percentage of positive nuclear staining at intermediate power (10× objective) but not actually counting individual cells. To obtain the Ki-67 proliferation index, a pathologist with more than 10 years of experience (SJS) evaluated each slide, avoiding areas with extensive necrosis or high inflammatory cell infiltration as much as possible, and selected hotspots for analysis. The number of positively-stained cells within the selected hotspots was then estimated without manual counting.

Digital image analysis: All available Ki-67 immunostained slides were scanned using PANNORAMIC® 250 Flash III DX scanner (3D Histech, Hungary). The image analysis was performed using 3D Histech QuantCenter (3D Histech) platform, which offers various quantification modules. For this study, NuclearQuant module was utilized to detect and quantify positive nuclei; thereby determining the Ki-67 proliferation index. A pathologist with less than 5 years of experience (JHP) manually annotated hot spots, defined as areas with high Ki-67 labeling. Areas with significant necrosis or inflammatory cell infiltration were avoided. A hot spot area was designated to contain minimum of 1000 cells. In cases where the total number of cells was less than 1000, as many cells as possible were included. NuclearQuant module analyzes scanned digital images to detect and filter nuclei based on their size, color, intensity, and contrast. The detection of nuclei was performed using various parameters, such as size of the object and contrast of intensity at the object's edge. Each detected nucleus was categorized into four levels (0, +1, +2, and +3) based on the intensity of 3,3'-Diaminobenzidine (DAB) staining observed within the object. These parameters were calibrated to achieve the highest accuracy using testing ten randomly selected NET sections that were not included in the cohort. These processes were carried out to exclude non-tumor cells, such as endothelial cells and intratumoral lymphocytes, from the analysis. A score greater than 1+ was considered positive.

Statistical analysis

Statistical significance was established using SPSS statistical software (version 21.0; SPSS Inc., Chicago, IL, USA). Clinicopathologic features and basic variables are presented using descriptive statistics. Cohen's kappa (κ) coefficient was estimated to assess the diagnostic concordance between eyeball estimation and DIA [21]. Higher κ values indicate better consistency. The degree of agreement was determined as follows: 0.81-1.0, 0.61-0.8, 0.41-0.6, 0.21-0.4, and 0-0.2 indicated nearly perfect concordance, substantial agreement, moderate agreement, fair agreement, and poor concordance, respectively [21]. Kaplan-Meier survival analysis was performed using R software ver. 4.2.0 for Windows to analyze the parameters and prognosis of GEP-NETs. The overall survival of patients was calculated from the time of initial diagnosis until death from any cause. Differences between subgroups were assessed using log-rank tests. Significance was set at $P < 0.05$.

Ethics statement

All procedures performed in the current study were approved by the Institutional Review Board of Gangnam Severance Hospital (Approval No. 3-2019-0219), and informed consent was waived.

Results

Clinical information

A total of 345 patients diagnosed with GEP-NETs at the Gangnam Severance Hospital between January 2008 and October 2019 were

Table 1. Clinicopathologic characteristics of patients included in this study

	All (n = 345; 100%)	Grade 1 (n = 298; 86.4%)	Grade 2 (n = 35; 10.1%)	Grade 3 (n = 2; 0.6%)	NEC (n = 10; 2.9%)
Mean age, years (range)	49.3 ± 12.7 (13-79)	48.1 ± 12.4 (13-74)	55.0 ± 12.4 (30-79)	62.5 ± 19.1 (49-76)	61.5 ± 6.6 (50-71)
Age, years					
10 ≤ age < 20	1 (0.3%)	1 (0.3%)	0	0	0
20 ≤ age < 30	14 (4.1%)	14 (4.7%)	0	0	0
30 ≤ age < 40	69 (20%)	65 (21.8%)	4 (11.4%)	0	0
40 ≤ age < 50	96 (27.8%)	90 (30.2%)	5 (14.3%)	1 (50%)	0
50 ≤ age < 60	88 (25.5%)	69 (23.2%)	15 (42.9%)	0	4 (40%)
60 ≤ age < 70	52 (15.1%)	42 (14.1%)	5 (14.3%)	0	5 (50%)
70 ≤ age < 80	25 (7.2%)	17 (5.7%)	6 (17.1%)	1 (50%)	1 (10%)
Tumor size, cm (range)	0.95 ± 1.13 (0.03-8.80)	0.67 ± 0.51 (0.03-3.80)	2.31 ± 1.82 (0.3-7.5)	1.50 ± 0.85 (0.9-2.1)	4.43 ± 2.09 (1.7-8.8)
Tumor location					
Stomach	23 (6.7%)	12 (4%)	4 (11.4%)	1 (50%)	6 (60%)
Duodenum	20 (5.8%)	17 (5.7%)	3 (8.6%)	0	0
Ileum	1 (0.3%)	1 (0.3%)	0	0	0
Colon	6 (1.7%)	5 (1.7%)	0	0	1 (10%)
Rectum	242 (70.1%)	234 (78.5%)	8 (22.9%)	0	0
Appendix	6 (1.7%)	6 (2.0%)	0	0	0
Pancreas	43 (12.5%)	22 (7.4%)	19 (54.3%)	1 (50%)	1 (10%)
Ampulla of Vater	2 (0.6%)	1 (0.3%)	1 (2.9%)	0	0
Common bile duct	2 (0.6%)	0	0		2 (20%)
Lymphovascular invasion					
Present	29 (8.4%)	8 (2.7%)	14 (40%)	1 (50%)	6 (60%)
Absent	316 (91.6%)	290 (97.3%)	21 (60%)	1 (50%)	4 (40%)
Lymph node metastasis					
Present	19 (5.5%)	4 (1.3%)	7 (20%)	1 (50%)	7 (70%)
Absent	326 (94.5%)	294 (98.7%)	28 (80%)	1 (50%)	3 (30%)
Distant metastasis					
Present	7 (2%)	2 (0.7%)	2 (5.7%)	1 (50%)	2 (20%)
Absent	338 (98%)	296 (99.3%)	33 (94.3%)	1 (50%)	8 (80%)
Death record					
Present	5 (1.4%)	2 (0.7%)	0	0	3 (30%)
Absent	340 (98.6%)	296 (99.3%)	35 (100%)	2 (100%)	7 (70%)
Treatment					
Endoscopic resection	266 (77.1%)	255 (85.6%)	11 (31.4%)	0	0
Surgery	69 (20%)	41 (13.8%)	23 (65.7%)	2 (100%)	3 (30%)
Surgery + CTx/RTx	10 (2.9%)	2 (0.7%)	1 (2.9%)	0	7 (70%)

NEC, neuroendocrine carcinoma; CTx, chemotherapy; RTx, radiotherapy.

included in this study. The patients' ages ranged from 13 to 79 years, with a mean age of 49.29 ± 12.68 years. The most commonly diagnosed age group was 40-49 years, accounting for 27.8% of all patients. The frequency and proportions of age groups were as follows: 1 patient aged 10-19 years (0.3%), 14 patients aged 20-29 years (4.1%), 69 patients aged 30-39 years (20%), 96 patients aged 40-49 years (27.8%), 88 patients aged 50-59 years (25.5%), and 25 patients aged 70-79 years (7.2%). The sex ratio (male to female) was 1.78:1 (221/124). The average tumor size, as measured from postoperative resection samples, was 0.95 ± 1.13 cm (range 0.03-8.8 cm) (**Table 1**).

Primary tumor sites

The rectum was the most common site, with the ileum, colon, appendix, ampulla of Vater, and common bile duct accounting for only a small proportion of sites in all patients. The proportions of affected organs in the patient cohort were as follows: 23 in the stomach (6.7%), 20 in the duodenum (5.8%), 1 in the ileum (0.3%), 6 in the colon (1.7%), 242 in the rectum (70.1%), 6 in the appendix (1.7%), 43 in

Table 2. Rate of CD56, synaptophysin, and chromogranin A immunohistochemical positivity by tumor origin

	CD56	Synaptophysin	Chromogranin A
Stomach	15/19 (78.9%)	17 (100%)	9/14 (64.3%)
Duodenum	13/16 (68.8%)	16 (100%)	11/14 (78.5%)
Ileum	-	1 (100%)	-
Colon	4 (100%)	3 (100%)	3 (100%)
Rectum	139/143 (97.2%)	179 (100%)	12/124 (9.7%)
Appendix	3 (100%)	4 (100%)	3/4 (75%)
Pancreas	37/41 (70.7%)	29 (100%)	21/27 (77.7%)
Ampulla of Vater	2 (100%)	1 (100%)	-
Common bile duct	2 (100%)	1/2 (50%)	1/2 (50%)
Total	215/230 (93.5%)	297/298 (99.7%)	83/229 (36.3%)

Table 3. Comparison of Ki67 counts obtained using two different methodologies

Eyeballing \ Automatic	Grade 1	Grade 2	Grade 3	NEC	Total (digital image)
Grade 1	234	15	0	0	249
Grade 2	2	20	0	0	22
Grade 3	0	0	2	0	2
NEC	0	0	0	10	10
Total (eyeballing)	236	35	2	10	283

Cohen's kappa coefficient: 0.765 (p value < 0.001). NEC: neuroendocrine carcinoma.

the pancreas (12.5%), 2 in the ampulla of Vater (0.6%), and 2 in the common bile duct (0.6%) (**Table 1**).

Lymphovascular invasion and metastasis

Twenty-nine patients exhibited LVI (8.4%, 29/345). Of the 43 patients with pancreatic NETs, 16 exhibited LVI (37.2%, 16/43). Six of 23 patients with stomach NETs exhibited LVI (26.1%, 6/23). All patients with common bile duct NETs exhibited LVI (100%, 2/2). In contrast, only five patients with rectal NETs exhibited LVI (2.1%, 5/242). Lymph node metastasis was observed in 19 patients (5.5%, 19/345). Six of 43 patients with pancreatic NETs exhibited node metastasis (14%, 6/43). Four of 23 patients with gastric NETs showed node metastasis (17.4%, 4/23). All patients with common bile duct NETs exhibited node metastasis (100%, 2/2). Distant metastasis was found in seven patients (2%, 7/345). The liver was the only site of distant metastasis. Five of 43 patients with pancreatic NETs exhibited distant metastasis (11.6%, 5/43). The remaining two cases of distant metastasis were observed in patients with rectum and stomach NETs (**Table 1**).

Pathologic features and immunohistochemical profile

Among 345 GEP-NETs, the most common tumor grade was grade 1 (86.4%, 298/345), followed by grade 2 (10.1%, 35/345), NEC (2.9%, 10/345), and then grade 3 (0.6%, 2/345). The positivity rates of immunohistochemical staining for CD56 and synaptophysin were 93.5% (215/230) and 99.7% (297/298), respectively. The positivity rate of immunohistochemical staining for chromogranin A was 36.3% (83/229), which was lower than those of CD56 and synaptophysin. In particular, rectal NETs exhibited only 9.7% (12/124) positivity for chromogranin A (**Table 2**).

Comparison between the eyeball method and digital image analysis

Of the 345 GEP-NETs, only 283 patients underwent DIA to determine the Ki-67 labeling index. The diagnostic concordance between eyeball estimation and automatic counting with DIA revealed substantial agreement (k = 0.765). Among the cases where discrepancies were identified (n = 17), 15 were diagnosed as grade 1 by eyeball estimation but as grade 2 by DIA, while the remaining two cases were diagnosed as grade 2 by eyeball estimation but as grade 1 by DIA. No discrepancy was identified for grade 3 or NEC (**Table 3**).

Survival analysis

Kaplan-Meier survival analysis of clinicopathologic parameters revealed that age > 50 years (P = 0.029), tumor size > 2 cm (P = 0.048), and the presence of LVI (P < 0.001), nodal metastasis (P < 0.001), and distant metastasis (P = 0.028) were significantly associated with short-

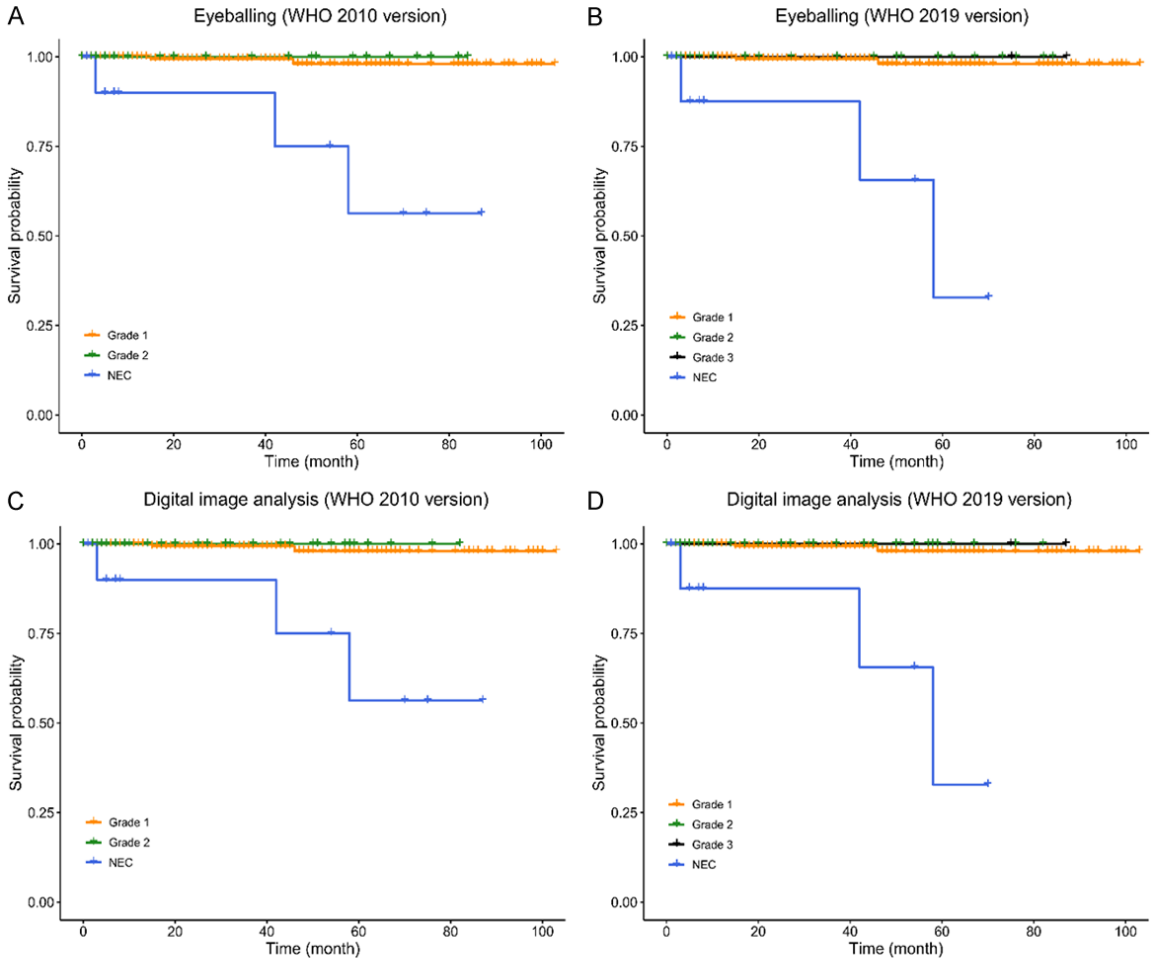

Figure 1. Kaplan-Meier survival analysis by Ki-67 labeling index assessment method and classification criteria. Overall survival by grade using (A) eyeball estimation/the 2010 WHO classification system, (B) eyeball estimation/the 2019 WHO classification system, (C) digital image analysis/the 2010 WHO classification system, and (D) digital image analysis/the 2019 WHO classification system is shown.

er overall survival. Survival analysis was also performed according to tumor grade using two different WHO classification criteria (2010 version and 2019 version) and two distinct Ki-67 counting methods (eyeball estimation method and DIA). Four survival curves were generated based on the combination of these factors. In all four survival analyses, significant differences were observed in overall survival between grade 1 and NEC and between grade 2 and NEC. However, neither the DIA nor eyeball estimation method revealed significant differences in overall survival between grade 3 and NEC. The eyeball estimation method demonstrated non-inferior results compared to DIA regarding overall survival (**Figure 1**).

Discussion

The average age of the GEP-NET patients was 49.3 years, and the largest age group was 40-49 years, followed by 50-59 years. Although many previous studies reported that the age groups most susceptible to NETs were 50-59 years, 60-69 years, or older, the age group in this study was slightly younger [22-24]. Song et al. suggested that this difference may be the result of early detection of asymptomatic patients through advances in detection methods (endoscopic technology, imaging methods) [25]. In addition, the widespread national health checkup system in South Korea may have contributed to the early detection of NETs

through increased endoscopy rates. Cho et al. also suggested that the increase in NET incidence in recent years was largely due to an increase in the detection of rectal NET because of more frequently performed procedures such as endoscopy [3].

In this study, the distribution of GEP-NETs by organ was as follows: rectum (70.1%), pancreas (12.5%), stomach (6.7%), and duodenum (5.8%). Previous studies conducted in South Korea, Japan, Taiwan, and the United States have reported distributions of GEP-NETs by primary organ. In a multicenter study of 4951 patients in South Korea, Cho et al. reported the following distribution of GEP-NETs by primary organ: rectum (48.0%), stomach (14.6%), pancreas (8.7%), colon (7.9%), and small intestine (7.7%) [3]. The rectum exhibited the highest incidence in South Korea in another study as well [26], and other organs exhibited no significant difference in rank. According to a study by Ito et al. in Japan, the top four primary sites of GEP-NETs were the rectum (55.7%), small intestine (18.9%), stomach (15.1%), and colon (2.1%) [27]. In a study by Tsi et al. with 2187 patients in Taiwan, the top four primary sites were the rectum (25.4%), stomach (7.4%), pancreas (6.0%), and colon (5.3%) [23]. A large-scale study of 64,971 patients conducted in the United States determined the frequencies of GEP-NETs in the small intestine (1.05/100,000), rectum (1.04/100,000), and pancreas (0.48/100,000) [22]. These differences in distribution could be attributed to factors such as race, region, and the number of cohorts studied.

In all seven patients exhibiting distant metastasis in our study, the site of metastasis was the liver. This predominance has been confirmed in other studies. For example, in China, the most frequent site of metastasis was the liver (75%) [25]. In a study in the US of 12,501 patients with gastrointestinal tract NETs using the Surveillance, Epidemiology, and End Results database, 65.21% of GEP-NETs metastasized to the liver [28].

The confirmation of NETs requires immunohistochemical detection of neuroendocrine markers. Currently, the most widely used immunohistochemical markers for NETs are synaptophysin and chromogranin A [29]. CD56, also called neural cell adhesion molecule, is often used as a general marker and exhibits high sensitivity. However, its use is discouraged because CD56 is expressed in several non-NETs (lack of specificity). Synaptophysin is generally more sensitive than chromogranin A, which is expressed in almost all well-differentiated tumors [29]. Chromogranin A has limited sensitivity, especially for hindgut origin NETs (from the left transverse colon to the anus), and is expressed in only 20-50% of cases [30]. In this study, NETs originating from the rectum expressed chromogranin A in only 9.7% of the total cases (12/124), which is consistent with previous studies.

The 2010 WHO classification system divided NETs into well-differentiated grade 1 and 2 tumors and poorly-differentiated (grade 3) NEC tumors. At that time, no cases of well-differentiated tumors with a high mitotic count or Ki-67 index were thought to exist among NECs [31]. However, since well-differentiated tumors with a high Ki-67 index or mitotic count were reported to have a better prognoses than other NECs, the need for a new category of such cases was advocated [12, 32]. Thus, in the 2019 WHO classification, NETs were classified into four categories: 1, 2, 3, and NEC [25]. Upon reclassification of previously diagnosed cases using the new grading system in our study, two cases were reclassified as grade 3. Notably, both patients with grade 3 tumors survived. A survival analysis comparing the grade 3 and NEC groups showed no significant difference. However, it should be noted that the number of patients classified as grade 3 was limited, which may have resulted in a lack of power to detect significant differences between the two groups. Further studies with larger sample sizes are necessary to confirm these findings.

Ki-67 is a reliable marker of the proliferative index. It has been used as a prognostic factor in various types of tumors for more than 20 years [33]. Currently, it has been established as an integral factor in determining the GEP-NET grade; however, a consensus on the best method to determine the Ki-67 labeling index has not been reached. Several methods are available to assess the Ki-67 labeling index, each with advantages and disadvantages. The most widely used method in daily practice is eyeball estimation, as advocated by some pathologists and guidelines [19, 20].

Eyeball estimation is also the most commonly used method in actual diagnostic settings at our institution. However, its accuracy and reproducibility have been questioned in recent studies [17, 18, 33]. As an alternative option, the manual counting method is labor-intensive compared with eyeball estimation. This method consists of counting the number of Ki-67-positive cells among at least 500 total tumor cells in a microscopic field or camera-captured/printed image. Another method is a computer-assisted method, which has been attempted since the mid-1990s [34] but has not been widely applied in clinical practice. However, as limitations have been overcome, an increasing number of studies have reported positive results.

We assessed the level of agreement between the eyeball estimation and DIA, and the diagnostic concordance revealed a moderate degree of agreement (Cohen's kappa coefficient, 0.765). Specifically, all discrepancies were between grade 1 and grade 2, and there was no difference between the two methods in the survival analysis. These results suggest that eyeball estimation is a reliable and non-inferior method compared with DIA for evaluating the Ki-67 proliferation index values in NETs. This automatic quantification is expected to be more accurate and less time consuming than previous manual methods, thereby becoming an alternative option for prognosis prediction. Boukhar et al. compared the DIA method with manual counting using the printed image method for GEP-NETs [34], and DIA exhibited non-inferior results compared with manual counting conducted by experts in terms of accuracy and time consumption. Tang et al. demonstrated better outcomes for DIA than for eyeball estimation [18]. Zhong et al. evaluated the degree of agreement between eyeball estimation and DIA in invasive ductal carcinoma of the breast [35]. An excellent degree of agreement was observed in the study cohort, indicating the applicability of DIA in daily practice.

The feasibility of a method for use in daily practice depends not only on its performance but also on its cost-effectiveness. In a study by Reid et al., based on the cost/benefit ratio and reproducibility, the manual counting method using camera-captured/printed images was the most practical one [33]. Eyeball estimation is a quick and easy method, but its accuracy is problematic.

Due to the limitations of DIA, such as lower accuracy, longer turnaround times, and higher costs than those of manual counting methods, it has not yet been widely adopted in clinical practice. However, with the accelerated adoption of digital pathology and the rapid evolution of artificial intelligence in diagnostic settings in recent years, we anticipate that DIA will become increasingly accurate and efficient and will be implemented more widely in routine clinical practice in the near future.

The limitations of this study are its retrospective design and limited sample size. The prognostic utility of the classification of grade 3 NETs in our patient cohort could not be accurately evaluated. Considering the low incidence of GEP-NETs, evaluation with a larger population of tumors is needed.

Regarding the comparison of automated counting and eyeball estimation to determine the Ki-67 labeling index in GEP-NETs, our study found no significant differences in their performance. Given the benefits of automation in terms of objectivity and efficiency, further research is warranted to evaluate the utility of DIA-based approaches.

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Disclosure of conflict of interest

None.

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