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NET G3 vs NEC: p53 and Rb1 Immunolabeling in High-grade Gastrointestinal Neuroendocrine Neoplasms - Is It Enough for the Differential Diagnosis?

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ABSTRACT

Background & Aims: High-grade gastrointestinal neuroendocrine neoplasms (GI-NENs) are divided into well-differentiated G3 neuroendocrine tumors (NETs G3) and neuroendocrine carcinomas (NECs), having identical cut-offs of proliferation, but different biomolecular origins. This translates in distinct treatment choices. Our aim was to establish if p53/Rb1 immunohistochemical status in GI-NENs with Ki67 index >20% can predict the histopathological diagnosis.

Methods: p53/Rb1 immunolabelling was performed on 42 cases of high-grade GI-NENs, diagnosed as NET G3, NEC and mixed neuroendocrine-non-neuroendocrine neoplasms (MiNEN) with NEC component. Immunolabeled slides were digitally scanned, with automatic quantification of p53 and Rb1, blind to the diagnosis.

Results: The p53 positive percentage was stratified; two cut-offs were selected, naming the intervals as N (null, <1%), T (tumor, 1%-20%) and C (carcinoma, >20%). The Rb1 expression loss in >90% of neoplastic cells was considered mutational. NETs G3 mainly showed the T status (14/16, 87.5%), followed by N (1/16, 6.25%) and C (1/16, 6.25%); NECs and NEC components in MiNENs predominantly expressed the C status (19/26, 73.08%), followed by N (5/26, 19.23%) and T (2/26, 7.69%) ($p<0.001$, $\chi^2=27.017$). NET G3s showed positive expression for Rb1; 73.08% of NECs expressed negative Rb1 ($p<0.001$, $\chi^2=21.351$). NECs and NEC components in MiNENs showed Rb1 mutational status in 13 C cases (13/19, 68.42%), 4 N cases (4/5, 80%) and in both the T cases ($p=0.002$, $\chi^2=11.187$).

Conclusions: Our results highlight the correlations between the p53/Rb1 immunostainings and the histopathological diagnosis of high-grade GI-NENs. NECs and NEC components in MiNENs showed a p53 mutational status (0% or 21-100%) and predominantly negative Rb1 expression. NETs G3 showed a p53 wild-type status (1-20%) and retained Rb1 expression. These findings suggest that the differential diagnosis of high-grade GI-NENs may benefit from p53/Rb1 immunohistochemical tests in everyday practice.

Key words: high-grade neuroendocrine neoplasms – gastrointestinal localization – NET G3 – NEC – MiNEN – differential diagnosis – p53/Rb1 immunostaining.

Abbreviations: CAPTEM: capecitabine and temozolomide treatment; DAB: 3,3'-Diaminobenzidine; EDTA: ethylenediaminetetraacetic acid; GI-NEN: gastrointestinal neuroendocrine neoplasm; GOF: gain of function; H&E: Hematoxylin & Eosin; HIER: heat induced epitope retrieval; HRP: horseradish peroxidase; IHC: immunohistochemistry; LOF: loss of function; MiNEN: mixed neuroendocrine-non-neuroendocrine neoplasm; NEC: neuroendocrine carcinoma; NEN: neuroendocrine neoplasm; NET: neuroendocrine tumor; PanNEN: pancreatic neuroendocrine neoplasm; WHO: World Health Organization.

INTRODUCTION

High-grade gastrointestinal neuroendocrine neoplasms (NENs) are divided into well-differentiated grade 3 neuroendocrine tumors (NETs G3) and neuroendocrine carcinomas (NECs - conventionally considered G3) [1, 2]. This classification is based on the demonstrated discrepancies between the biomolecular, clinical, epidemiological and prognostic aspects of the two types of tumors, that led to the WHO 2019 reclassification of these neoplasia [1, 2]. These discrepancies stop, unfortunately, at the histopathological level [1, 3-6].

Mixed neuroendocrine-non-neuroendocrine neoplasms (MiNENs) are tumors showcasing both components

(neuroendocrine and non-neuroendocrine); it is recommended that the diagnosis of MiNENs neoplasms to be limited to tumors that have both components in a percentage of at least 30% [1, 7]. The wide availability of immunohistochemical (IHC) testing has led to the conclusion that mixed neoplasms composed of neuroendocrine and non-neuroendocrine components are not as rare as they have been considered in the past [7].

The differentiation of GI-NENs with Ki67 index >20% is sometimes difficult even for the most experienced pathologists [6]. It has been proven that conventional treatment applied for NEC cases obtained a limited response in patients with NETs, making differential diagnosis even more important [8, 9].

No recurrent mutations for G3 GI-NETs were found, so far, regardless of the involved digestive segment [10-13]. In contrast, NECs show TP53 and Rb1 mutations virtually in all possible locations [1, 10, 14, 15]. Due to the frequent identification of TP53 mutations in NECs and MiNENs with NEC component, the two were considered a single category, for statistical analysis purpose [16, 17].

Biomolecular testing is not often accessible or time efficient for the diagnostic process, so immunolabeling intends to become a supportive pillar of the differentiation of high-grade NENs. Thus, in our study, we replicated the testing of the cited IHC panel (TP53 and Rb1) in NETs G3, NECs and MiNENs with NEC component, in order to highlight the possibility of differentiating these entities.

With this research, we aim to establish if p53 and Rb1 IHC positivity percentage in GI-NENs with Ki67 index >20% can predict the histopathological diagnosis of NET G3 or NEC, thus trying to bring its contribution to the collective effort worldwide in the difficult differentiation of these neoplasms.

METHODS

Patients and NEN Initial Diagnosis

The present study includes a number of 42 high-grade gastrointestinal NENs, of which 16 NETs G3, 10 NECs G3 and 16 high-grade MiNENs with NEC component, diagnosed by histopathological and IHC assessment in the Clinical Service of Pathology of Sf. Apostol Andrei Emergency County Hospital, Constanta, Romania, from 2002 to 2021 and subsequently immunolabeled for p53 and Rb1 at the Center for Research and Development of the Morphological and Genetic studies of Malignant Pathology, Constanta, Romania. Patients were retrospectively selected by searching keywords such as "neuroendocrine tumor", "neuroendocrine neoplasm", "neuroendocrine carcinoma" in the hospital's database. The NEN diagnosis was based on the assessment of characteristic morphological features and on IHC testing in order to confirm the neuroendocrine differentiation and to establish the tumor's grading (at least two neuroendocrine markers – chromogranin and synaptophysin, and Ki67) and to exclude certain tumors such as epithelial or lymphoid malignant tumors and small blue cell tumors.

Patients with previously untreated primary NENs of the gastrointestinal tract, with a Ki67 index of >20% in the original diagnosis, were included in our study (NETs G3, NECs and MiNENs with NEC component). We excluded patients with IHC tests performed in other health facilities or solely diagnosed by biopsy, without surgical excision.

Cases diagnosed before 2019 were reevaluated and diagnosed according to the new WHO classification [1, 2]. Because the >20% Ki67 index is often significantly exceeded in NECs and only ocasionally limitated to 20-50% in those which underwent chemotherapy, we considered two thresholds for statistical analysis: >20% to 50% and >50% [1, 18].

We recorded the following clinical and pathological aspects: gender, age, primary site, tumor size, TNM stage and vascular and perineural invasion. After inclusion-exclusion criteria we obtained a group of 42 representative cases, localized in the gastrointestinal tract as follows: 14 cases originating in the stomach, 2 in the small intestine and 26 in the colorectum. Informed consent was obtained from all subjects involved in the study. The study was conducted according to the guidelines of the declaration of Helsinki, and approved by the institutional review board of the Ethics Committee of Sf. Apostol Andrei Emergency County Hospital Constanta, Romania, following European and local regulations (protocol code 36, date of approval 17.12.2020)

p53 and Rb1 Immunohistochemistry

Tissue blocks provided by the Clinical Service of Pathology of the Sf. Apostol Andrei Emergency County Hospital, Constanta were cut into 4 µm sections and applied on a slide. The staining protocol followed the producer's recommendations, as follows: for deparaffinizing we used xylene and decreasing grades of alcohol. The Master Diagnostica protocol for p53 immunostaining included heat induced epitope retrieval (HIER) in ethylenediaminetetraacetic acid (EDTA) pH 8.0 buffer and incubation for 10 minutes at room temperature in ready-to-use rabbit anti-human p53 monoclonal antibody (clone SP5). Detection was performed using Master Polymer Plus Detection System horseradish peroxidase (HRP) (3,3'-Diaminobenzidine (DAB) included; ref. MAD-000237QK). The Novus Biologicals protocol for Rb1 immunostaining included HIER in Tris based pH 9.0 buffer and incubation over night at 4C° with a 1-2 µg/ml dilution of the Rb1 antibody (Rb1/1754). The detection stage required a polymer detection kit that included peroxidase, 2.5% normal horse serum, HRP and DAB reagent – Vector Laboratories, USA and Master Polymer. In the final stage we counterstained with hematoxylin and mounted the glass cover slides.

The resulting slides were afterwards evaluated by two consulting pathologists, blind to the hematoxylin-eosin (H&E) NEN type original diagnosis. Immunolabeled slides with p53 and Rb1 antibodies were scanned using the Huron-TissueScopeTM LE120 scanner, on a 40x platform, with a pixel size of 0.4537x0.4537µm, obtaining whole slide digital images. The pathologists performed an automatic quantification of p53 and Rb1 antibodies using QuPath softwar [19]. For a cell to be considered positive, it had to present nuclear staining. For p53, normal epithelium, mesenchymal cells and lymphocytes were considered internal control; for Rb1 normal epithelium, mesenchymal and endothelial cells staining status were considered internal control. The p53 immunostain was considered as a mutational pattern when complete absent or when moderate to strong nuclear positivity was assessed in

more than 20% of the neoplastic cells [20], and as wild-type pattern when in the 1-20% interval [14]. We stratified the percentage of p53 positive cells in the 42 studied cases by an interval of 20%, and comparing the intervals with the H&E diagnosis we selected two cut-offs (1% and 20%), naming the corresponding positive cells percentage as N (null, <1%), T (tumor, 1%-20%) and C (carcinoma, >20%) (Fig. 1). Concerning Rb1, the loss of nuclear expression in more than 90% of the neoplastic cells was considered to reflect the presence of a mutation [20].

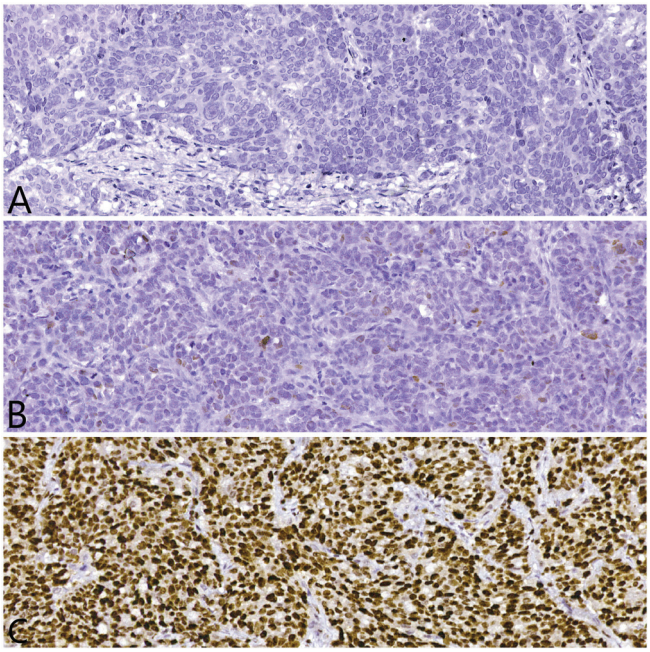

Fig. 1. Different positive cells percentages of p53 immunostaining in GI-NENs: (A) Completely absent p53 nuclear staining in tumor cells (null - N) (original magnification x200); (B) p53 nuclear staining in 1%-20% of tumor cells (tumor - T) (original magnification x200); (C) p53 nuclear staining in >20% of tumor cells (carcinoma - C) (original magnification x200).

Statistical Analysis

Statistical analysis was performed with IBM SPSS 26.0 software, Armonk, NY, USA, 2019; descriptive statistics was performed providing mean and standard deviation (SD) for continuous variable and frequency (percentage) for categorical variables. The association between p53/Rb1 IHC expression and the morphological parameters, including H&E/IHC original diagnosis (according to WHO 2019), was assessed using the Chi Square test/Fisher's exact test and Pearson correlation analysis. A two-tailed p value <0.05 was considered significant.

RESULTS

The clinical characteristics of the 42 included cases are summarized in Table I. Lymph node metastasis was found in 33 cases (33/42, 78,57%) (Table II). Sixteen cases (16/42, 38.1%) were diagnosed as NET G3 (Fig 2B), 10 cases as NEC (10/42, 23.8%) (Fig. 2A) and 16 as high-grade MiNEN with NEC component (16/42, 38.1%). NECs and NEC components in MiNENs featured aggressive behavior: all cases showed perineural invasion (100%); 92.30% showed vascular invasion and 88.46% featured lymph node metastases. On the other hand, NETs G3 featured perineural invasion, vascular invasion, and lymph node metastases in 62.5% of cases (Table II).

Table I. Clinical features

Clinical features	NET G3	NEC	MiNEN with NEC component
Gender, n (%)			
Male	6 (37.5)	6 (60)	8 (50)
Age at diagnosis			
Range	43-81	43-79	43-76
Median	56.5	73	62
Tumor location, n (%)			
Stomach	6 (37.5)	2 (20)	6 (37.5)
Small intestine	2 (12.5)	0 (0)	0 (0)
Colorectum	8 (50)	8 (80)	10 (62.5)
Tumor size, n (%)			
≤5 cm	12 (75)	0 (0)	3 (18.75)
>5 cm	4 (25)	10 (100)	13 (81.25)

*SD: standard deviation; NET: neuroendocrine tumor; NEC: neuroendocrine carcinoma; MiNEN: mixed neuroendocrine-non-neuroendocrine neoplasm.

Table II. Pathological features and Ki67 index

Pathological features	NET G3	NEC	MiNEN with NEC component
TNM stage, n (%)			
I	4 (25)	0 (0)	0 (0)
II	3 (18.75)	2 (20)	4 (25)
III	9 (56.25)	8 (80)	12 (75)
IV	0 (0)	0 (0)	0 (0)
Invasion, n (%)			
Vascular invasion	10 (62.5)	10 (100)	14 (87.5)
Perineural invasion	10 (62.5)	10 (100)	16 (100)
Lymph node metastasis, n (%)			
Positive	10 (62.5)	10 (100)	13 (81.25)
Negative	6 (37.5)	0 (0)	3 (18.75)
Ki67 index, n (%)			
21-50%	12 (75)	4 (40)	2 (12.5)
51-100%	4 (25)	6 (60)	14 (87.5)

For abbreviations see Table I.

NECs and the NEC components in MiNENs showed a high proliferative rate in the original diagnosis, according to the data we collected (mean labeling index of 68.07%). NETs G3, on the other hand, showed a lower index (38.62%) (Table II).

We evaluated the positivity status of p53 (with percentage stratification) and Rb1 for NETs G3 versus NECs and NEC components in the selected MiNENs. After the stratification of p53 positivity percentages, mutational immunostaining patterns (N and C) were detected in 24/26 cases of NECs and NEC components of MiNENs (92.30%) and in only 2/16 cases of NETs G3 (12.5%) (Table III). We found the C pattern to be the most common (20/42, 47.62%). T pattern was evaluated in 16 cases (16/42, 38.1%) and N pattern in 6 cases (6/42, 14.28%) (Table IV). Immunolabeling for Rb1 (Fig. 3) was mutational in 19 cases of NEC and NEC component in MiNENs (19/26, 73.08%). All NETs showed Rb1 positive immunolabeling (Tables III, IV).

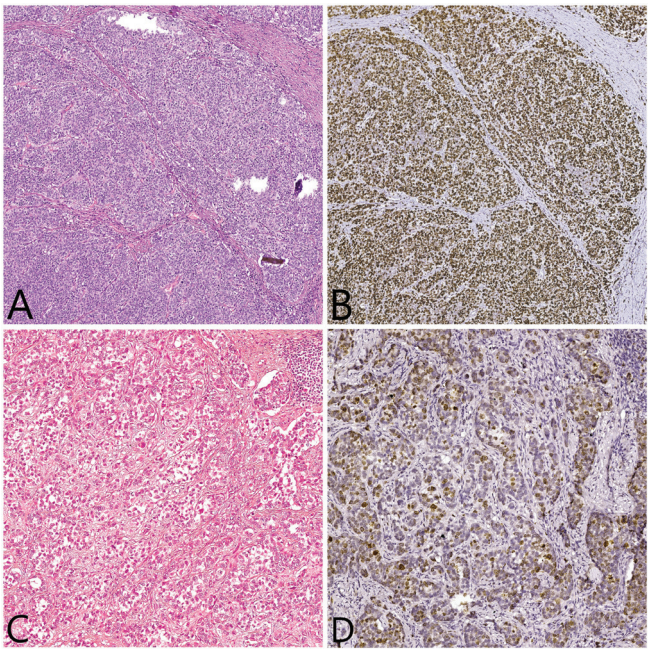

Fig. 2. Histological features of high-grade GI-NENs with corresponding Ki67 immunostaining: (A) Large cell neuroendocrine carcinoma – Malignant epithelial proliferation growing in sheets of relatively large cells with nuclear hyperchromasia and pleomorphism, visible nucleoli and mitotic activity (H&E staining, original magnification x100), and its corresponding Ki67 index of 100%; (B) (original magnification x100); (C) Neuroendocrine tumor G3 – Organoid pattern of growth (H&E staining, original magnification x200) and (D) its corresponding Ki67 index of 60% (original magnification x200).

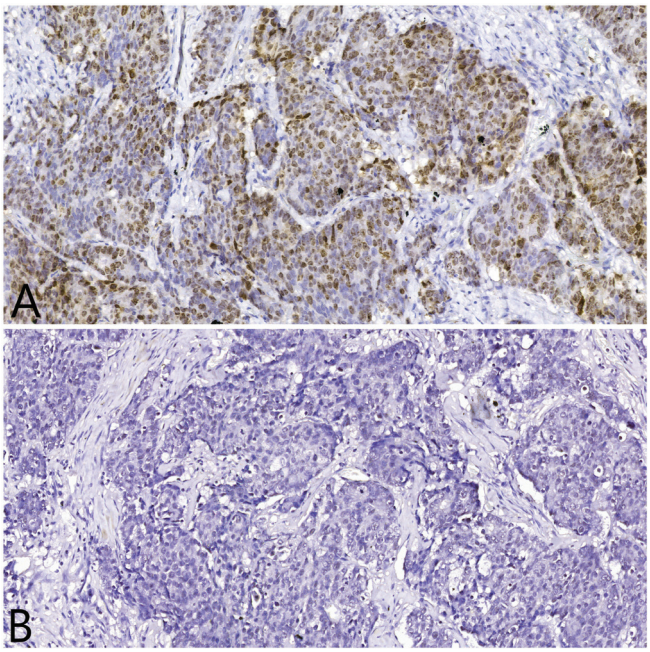

Fig. 3. Rb1 immunostaining status in GI-NENs: (A) Rb1 positive status – nuclear staining in >10% of tumor cells (original magnification x200); (B) Rb1 negative status – loss of nuclear staining in >90% of tumor cells (original magnification x200).

The majority of NET G3 showed a T positivity percentage status (14/16, 87.5%), followed by N (1/16, 6.25%) and C (1/16, 6.25%). NECs and NEC components of MiNENs expressed mainly the C positivity percentage status (19/26, 73.08%), followed by N (5/26, 19.23%) and T (2/26, 7.69%) (Table IV), with a p value <0.001, χ^2=27.017. In contrast, as stated above, all the NET G3 tumors showed positive expression for Rb1 and 73.08% of the NECs present Rb1 negative expression (p<0.001, χ^2=21351, Pearson correlation index r=0.713, p<0.001). NECs and NEC components in MiNENs showed Rb1 mutational immunolabeling in 13 of the C cases (13/19, 68.42%), 4 of the N cases (4/5, 80%) and in both the T cases (Table V) (p=0.002, χ^2=11.187).

85.18% of the tumors with a maximum diameter >5cm showed a p53 mutational pattern (p<0.001, χ^2=17.374). Rb1 showed a slight negative correlation with the tumor size (r=-0.601, p<0.001). 93.33% of the tumors with a ≤5cm diameter showed a positive expression for Rb1 (p<0.001, χ^2=14.013).

Also, 72.22% of cases associating perineural invasion (PNI) showed mutational expression of p53 (p=0.002, χ^2=11.375). 52.77% of the neuroendocrine neoplasms with perineural invasion presented negative expression for Rb1 (p=0.029, χ^2=5783, Pearson correlation index r=-0.371, p=0.0016).

Table III. Comparison between Rb positivity status, p53 positive cells percentage and NEN diagnosis

	Rb status		p53 positive cells percentage (%)					
	+	-	0	1-20	21-40	41-60	61-80	81-100
NET G3	16	-	1	14	1	-	-	-
NEC	2	8	3	2	0	1	1	3
MiNEN with NEC component	5	11	2	-	1	1	5	7

For abbreviations see Table I.

Table IV. Comparison between the Rb IHC status, the percentage of p53 positive cells status and the NEN diagnosis

	Rb IHC status		p	χ^2	p53 positivity percentage status			p	χ^2
	+	-			N	T	C		
NET G3	16	0	<0.001	21.351	1	14	1	<0.001	27.017
NECs and NEC component in MiNEN's	7	19			5	2	19		

For abbreviations see Table I.

Table V. Comparison between the p53 IHC positivity percentage status and Rb1 IHC status in NECs and NEC components in MiNENs

Rb1 IHC status	p53 IHC positive cells percentage status in NECs and NEC components in MiNENs			p	χ^2
	N	T	C	0.002	11.187
+	1	0	6		
-	4	2	13		

There is a strong correlation between the Ki67 and p53 expression, 91.66% of the cases with Ki67 index higher that 50% showing a p53 mutational pattern ($p<0.001$, $\chi^2=21.034$).

DISCUSSION

Due to the NEN G3 heterogeneity, prior to the reclassification of GI-NENs in 2019, the necessity for some sort of division among these neoplasia was enhanced by many publications [21-23].

The WHO 2019 GI-NEN classification is primarily based on the genetic differences between NETs and NECs, TP53 and Rb1 mutations being ones of the main causes of differentiation [1, 2]. Although NETs are characterized by an organoid pattern of growth, compared to the NECs that grow in sheets and frequently show tumor necrosis and a Ki67 proliferation index >75%, the high grade NEN's classification's identical cut-offs of cell proliferation (Ki67 index >20%) raise diagnostic problems that represent a real struggle, regardless of the observer's experience, even more so in health systems where overspecialization is not a common practice or it is impossible/unlikely to achieve [1, 3-6].

The GI-NENs diagnosis frequency in the general population with inconsistent communicated data, generates limitations concerning validating studies and determines the need of replication with data sharing [14, 24].

Gastrointestinal NETs, so far, have not shown recurrent mutations, regardless of their localization, in contrast with well-differentiated pancreatic neuroendocrine tumors (PanNETs), including NET G3, where previous studies have identified the loss of IHC expressions of ATRX and DAXX, which are preserved in NEC [10-13]. TP53 and Rb1 mutations were more extensively studied for pancreatic neuroendocrine neoplasms (PanNENs), but not exclusively [15, 20, 25, 26]. Neuroendocrine carcinomas, in contrast, seem to retain TP53 and RB1 mutations, regardless of their localization [1, 10, 14, 15]. Considering that other studies showed TP53 mutations to be present in NECs and MiNENs with NEC component, and their close genetic relationship with adenocarcinoma, NECs and MiNENs with NEC components were analyzed as a single category in our study [16, 17].

TP53 mutations are commonly found in a wide range of tumors of varying locations. Because the detection of mutations is a big consumer of money, time and effort, it has been frequently attempted to correlate the TP53 status of a tumor with its p53 immunostaining characteristics, because IHC is a common practice in the vast majority of health systems, being relatively affordable and faster to obtain [27]. The correlation between the TP53 mutational status and p53 IHC started to be explored, offering a high potential. A study conducted at Peking Union Medical College Hospital demonstrated that p53 IHC patterns are associated with TP53 mutations type and status in GI-NENs [14]. Based on the above-mentioned studies, we tried to find a statistically relevant correlation between the GI-NENs with >20% Ki67 index histopathological diagnosis and the p53 and Rb1 IHC status. Concerning the p53 abnormal protein expression, mutations are proved to be translated immunohistochemically through a total absence or overexpression [13, 28]; furthermore, some studies correlated the above with the mutation type - loss of function (LOF) and gain of function (GOF) [13, 28, 29]. In our study, 24/26 (92.30%) of NECs and NEC components of MiNENs showed an "all or nothing" p53 IHC positivity percentage (0% or >20%), thus suggesting the involvement of a non-wild-type p53 protein. Only 2/16 NETs G3 showed such p53 positivity percentage. Rapid degradation occurs normally for the wild-type p53 protein, but the GOF mutational forms of p53 are more stable and thus accumulate in tumor cells [30, 31]. Previous studies showed that the prediction of LOF p53 mutations through IHC has a lower sensitivity [32].

The co-inactivation of p53 and Rb1/p16 pathways appears to be a main genetic feature of NECs [15, 33]. Loss of Rb1 was uncommonly reported in pancreatic and extrapancreatic NETs [20, 34]. It has been reported, however, in pancreatic and extrapancreatic NECs of varying locations, such as the gastrointestinal tract, gallbladder and lungs [35-38]. Most importantly, NEC cases showing Rb1 positivity should be subsequently immunolabeled for p16, because the preservation of Rb1 protein is mutually exclusive with p16 preservation, according to studies conducted on PanNENs and lung NENs [15, 34, 35], mirroring the Rb1/p16 relationship involved in tumor suppression [39]. In our study, all NETs G3 preserved Rb1 IHC expression in over 10% of the tumor's cells. 73.07% of NECs and NEC components of MiNENs showed the loss of Rb1 protein in >90% of neoplastic cells.

The differentiation of these malignant neoplasia is essential for the appropriate therapy choice, because the standard treatment with platinum-based chemotherapy applied to neuroendocrine carcinomas obtains a limited response in the case of well-differentiated G3 neuroendocrine tumors [8, 9]. For example, the Nordic NEC trial reported a lower response rate to platinum-based chemotherapy in NEN's with Ki67 < 55% [4].

Gastrointestinal NEC's standard treatment is considered cisplatin accompanied by etoposide; substituting cisplatin with carboplatin or etoposide with irinotecan is considered an alternative [8].

NET G3 prognosis is considered intermediate between NET G2 and NEC [8, 40, 41]. Therapeutic strategies for NET G3 are not yet established, and may include somatostatin analogs and chemotherapy [8, 40]. For neoplasms with Ki67 <55%, capecitabine and temozolomide (CAPTEM) treatment seems promising, with further validation on the way [9].

Although not yet demonstrated to be efficient, as in the case of breast cancer or melanoma [42,43], immunotherapy is considered for future studies, due to the correlation between PDL-1 expression and G3 grading in NENs [8,44].

All of the above highlight the importance of the differential diagnosis in high-grade GI-NENs, for which p53 and Rb1 immunolabeling could be substantially helpful. NET G3 specific trials are greatly necessary to bring clarity in therapeutic strategies.

One of our study's strengths is the automatic quantification performed on digitally scanned immunolabeled slides, thus providing enhanced accuracy and being more time-efficient in data collecting for the statistical analysis. The research's subject, proposed IHC markers to evaluate and objectives were selected after an intensive study of the latest specialized literature in the field of neuroendocrine neoplasms and the results contribute to the international effort to shed light on the differentiation of high-grade neuroendocrine neoplasms, in order to support future NET G3 specific clinical trials in establishing therapeutic strategies for this type of neoplasia.

Our sample size mirrors the GI-NENs diagnosis frequency in the general population and represents a limitation; based on our findings, we highly recommend that such studies should be widely carried out, where possible with DNA sequencing, for further validation.

Neuroendocrine carcinoma is a highly aggressive neoplasm and our study suggests that this entity is genetically distinct from NETs G3; IHC p53 and Rb1 labeling may be a practical method to differentiate the two, thus ensuring the proper therapeutic choice for the patient.

CONCLUSIONS

Our study highlights correlations between p53 and Rb1 immunostaining and the histopathological diagnosis of high-grade gastrointestinal NEN. NECs and NEC components in MiNENs showed an "all or nothing" positivity pattern for p53 (0% or 21-100%) and, for the majority of cases, the loss of Rb1 expression. NETs G3, on the other hand, showed a wild-type p53 pattern (1-20%) and retained Rb1 expression. Concerning the morphological aspects, tumors bigger than 5 cm showed mainly mutational p53 patters; tumors with a ≤5cm diameter retained Rb1 expression, with only one exception. Tumors with perineural invasion showed predominantly a mutational p53 pattern and over 50% of them lost their Rb1 expression. 91.66% of cases expressing a Ki67 index >50% showed a p53 mutational pattern.

Conflicts of interest: None to declare.

Authors' contribution: A.D. conceived and designed the original article, collected the data, and drafted its manuscript. M.A. coordinated the study. C.N.G, I.E.I. provided the clinical data and performed the surgical intervention. A.D. performed the histopathological review for case selection. A.A.N and C.I.O. performed the immunostaining. M.D. and M.A. validated the results. G.I.B. and A.A.N. performed the immunolabeled slides scanning and automatic QuPath quantification. A.A.N. performed the statistical analysis. G.C.C and A.F.M. provided genetics consulting in the correlations of our results with literature review. A.D., A.A.N., G.I.B. were responsible for the final manuscript editing. All authors critically revised the manuscript, approved the final version and agree to be accountable for all aspects of the work.

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