Endocrine Pathology (2021) 32:396–407
https://doi.org/10.1007/s12022-020-09659-6

A Subset of Large Cell Neuroendocrine Carcinomas in the Gastroenteropancreatic Tract May Evolve from Pre-existing Well-Differentiated Neuroendocrine Tumors

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Accepted: 16 December 2020 / Published online: 12 January 2021

Abstract

In the gastro-entero-pancreatic (GEP) tract, neuroendocrine neoplasms (NENs) include well differentiated neuroendocrine tumors (NETs) and high-grade NE carcinomas (NECs), which are thought to make up separate and mutually exclusive tumor entities. Little is known, however, as to whether there may be any pathogenetic link between them. Clustering analysis of a 10-gene panel generated from a previously reported next-generation sequencing analysis on 48 GEP-NENs with clinical annotations was used in the study. Unsupervised cluster analysis showed three histology-independent clusters, namely, C1, C2, and C3, which accounted for 44% of patients but the entire array of mutations. All but two NECs fell into the clusters, yet with different prevalence rates ($p < 0.0001$). A model was devised according to which NETs were likely to evolve into NECs upon progression of C3 into C1 and C2, despite different morphology. The median Ki-67 labeling index was 5% in C3 showing better prognosis and 50% in C1 and C2 experiencing worse prognosis, with an impressive intra-tumor heterogeneity of diversely proliferating tumor areas. This study suggests that a subset of large cell NECs in the gastroenteropancreatic tract may evolve from pre-existing well-differentiated NETs.

Keywords Gastro-entero-pancreatic tract · Neuroendocrine · NET · NEC · Tumor · Carcinoma · Cluster analysis · Transition · Prognosis · Pathogenesis · Mutation

Abbreviations

Genes

APC	APC regulator of WNT signaling pathway
ATM	ATM serine/threonine kinase
ATRX	ATRX Chromatin Remodeler
BRAF	B-Raf proto-oncogene, serine/threonine kinase
CTNNB1	Catenin beta 1
DAXX	Death Domain Associated Protein
IDH1	Isocitrate dehydrogenase [NADP(+)] 1
KRAS	KRAS proto-oncogene, GTPase
PIK3CA	Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha
PTEN	Phosphatase and tensin homolog
RB1	RB transcriptional corepressor 1
TP53	Tumor protein p53

Others

DNA	Deoxyribonucleic acid
GEP	Gastroenteropancreatic
IHC	Immunohistochemistry
LCNEC	Large cell neuroendocrine carcinoma
NE	Neuroendocrine
NEC	Neuroendocrine carcinoma
NEN	Neuroendocrine neoplasm
NET	Neuroendocrine tumor
NGS	Next-generation sequencing
TNM	Tumor node metastasis
WD	Well differentiated
WHO	World Health Organization

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Introduction

In the gastro-entero-pancreatic (GEP) tract, neuroendocrine neoplasms (NENs) include well-differentiated NE tumors (WD-NETs) and NE carcinomas (NECs) [1, 2]. These tumors are graded according to a three-tier scheme based on morphology, mitotic count, and Ki-67 labeling index (henceforth, simply Ki-67), whereby NET-G1 and NET-G2 correspond to WD-NETs and NECs to G3 [3, 4]. However, a new category of NET-G3 has recently been devised in the GEP tract inside the category of NECs, by integrating WD morphology, criteria for G3 (> 20 mitoses per 2 mm^2 and Ki-67 ≥ 20%), upregulation of neuroendocrine markers [5, 6], somatostatin receptors (STTRs) [7, 8] and retinoblastoma [8] along with p53 downregulation [7, 8], and occurrence of Death Domain Associated Protein (*DAXX*) and/or ATRX Chromatin Remodeler (*ATRX*) mutations (at least in the pancreas) along with the lack of the relevant proteins [8–17]. This additional category is deemed to intermediately behave between NET-G2 and NECs [5, 6, 17–20]. Although NETs and NECs are actually considered separate and distinct tumor entities [1, 2, 21], we have recently proposed a new pathogenetic hypothesis according to which NECs in resection specimens of lung and thymus NENs of either small or large cells could reflect secondary development from pre-existing carcinoids [22–25]. Whether such a challenging hypothesis can be supported even in the GEP tract according to the natural history of disease, this is still an unclarified issue.

We herein describe a reappraisal of the mutational profile of a published cohort of 48 GEP-NEN patients [26] aimed to evaluate whether G3 NENs and NETs in the GEP tract may have any developmental relationship. Our findings preliminarily support the hypothesis that a subset of large cell NECs in the gastroenteropancreatic tract may evolve from pre-existing well-differentiated NETs after crucial gene alterations have been acquired for tumor progression.

Materials and Methods

Patients and Tumors

This study deals with a clinically well-annotated cohort of 48 GEP-NENs (25 males and 23 females) from 13 pancreatic and 35 extra-pancreatic digestive sites, which had previously been investigated by means of targeted next-generation sequencing (NGS) analysis [26]. Out of 63 originally reported NE malignancies, 15 tumors developing outside the GEP tract (breast, lung, and head and neck) or presenting as unknown tumor primaries were excluded from the analysis. Anatomical sites other than the pancreas comprised the esophagus (one case), stomach (two cases), small intestine (18 cases), colon-rectum (12 cases), and gallbladder (two cases). Ethnicity was distributed according to 42 Caucasian, five African American, and one Asian patient, whereas smoking habit was present in 22/48 (46%) patients (this information was missing in one patient). Alcohol consumption was documented in 46/48 (96%) patients, with 20 (42%) of whom being active consumers. The patient cohort comprised 12 (25%) G1, 20 (42%) G2, and 16 (33%) G3 tumors, which were classified according to World Health Organization (WHO) classifications [1, 2] and ENETS guidelines (at https://www.enets.org/enets_guidelines.html). In particular, no typical case of NET-G3 could be documented upon morphology in the subgroup of NEC patients, who belonged to the histologic subtype with large cell according to 2017 WHO and 2019 WHO classifications (large cell neuroendocrine carcinoma, LCNEC) [1, 2]. No cases of small cell NECs were present. Twenty-nine out of 48 (60%) tumors had been surgically removed (11 NETs G1, 11 NETs G2, and seven NECs), with no neoadjuvant treatment being administered. Thirty-four (71%) patients were staged IV, with the remaining 14 (29%) being staged I–III according to the current TNM staging system, 8th edition [1, 2]. Eastern Cooperative Oncology Group (ECOG) performance status ranked score 0 in 28 (58%) patients, score 1 in 18 (38%) patients, and score 2 in the remaining two (4%). Paraffin material had been selected for immunohistochemistry (IHC) assessment of Ki-67 and molecular investigations.

Study Design

This is a cancer mutational profile analysis to explore an innovative concept of secondary NECs in the GEP tract evolving from pre-existing WD-NETs through sequential gene mutations, as previously hypothesized in the lung [23, 25, 27] and the thymus [22, 24]. To this purpose, our previous NGS study on GEP-NENs, either primary or metastasis, conducted at Fox Chase Cancer Centre (Philadelphia, USA), was reappraised by clustering analysis [26]. All NENs were sporadic, affected adult patients (≥ 18 years), and included NET-G1, NET-G2, and NEC. All the ten recurrently altered genes from a panel of 50 oncogenes and tumor suppressor genes frequently involved in human cancers by targeted NGS were used as an investigative signature in the study [26]. This 10-gene panel included *APC* (APC regulator of WNT signaling pathway), *ATM* (ATM serine/threonine kinase), *BRAF* (B-Raf proto-oncogene, serine/threonine kinase), *CTNNB1* (catenin beta 1), *IDH1* (isocitrate dehydrogenase [NADP(+)] 1), *KRAS* (KRAS proto-oncogene, GTPase), *PIK3CA* (phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha), *PTEN* (phosphatase and tensin homolog), *RB1* (RB transcriptional corepressor 1), and *TP53* (tumor protein p53), which underwent clustering analysis.

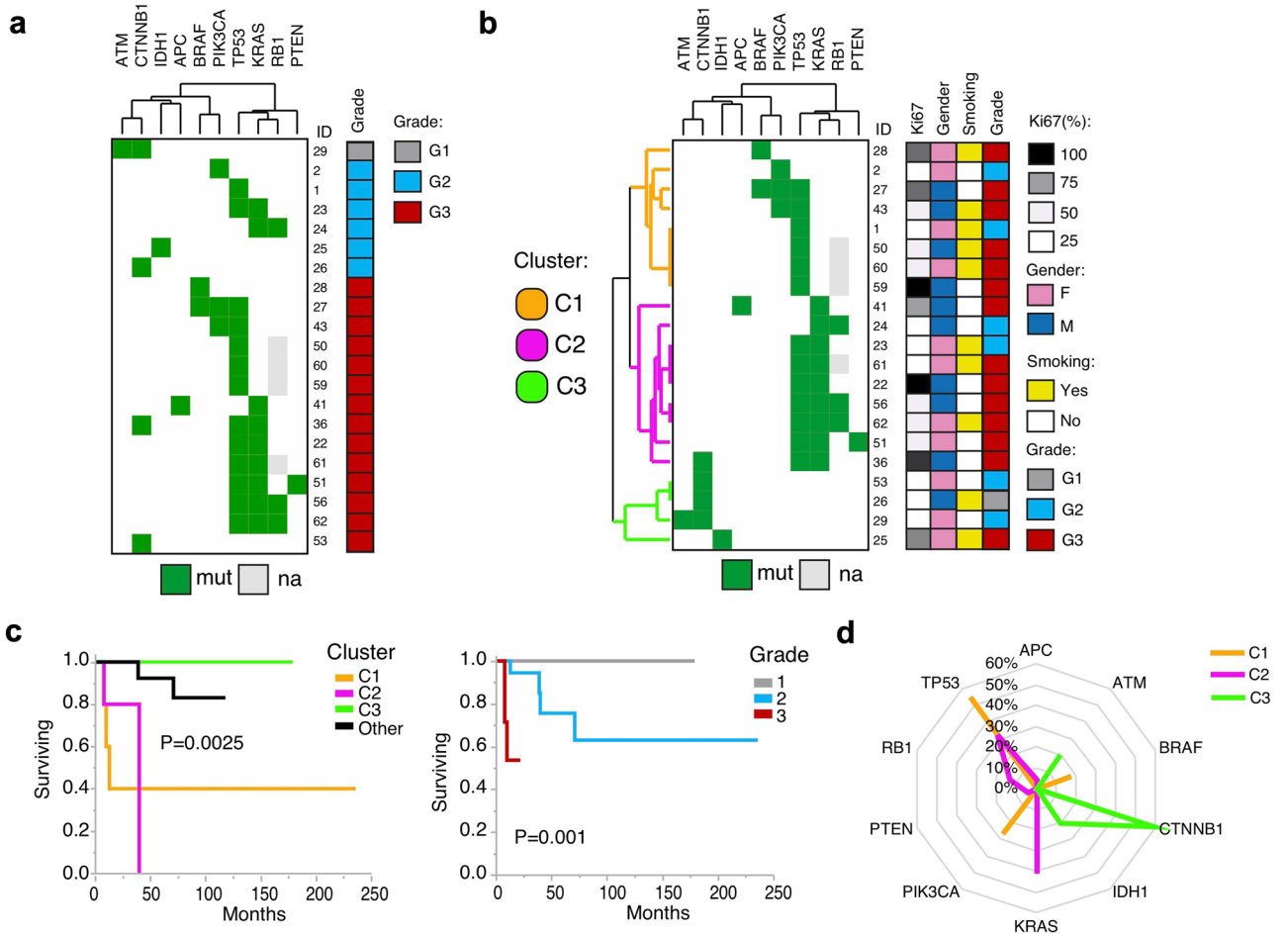

Fig. 1 **a**–**d** Unsupervised and supervised cluster analyses. **a** One-way hierarchical cluster analysis of the 10-gene mutations profile in 21 NEN (with mutations). Samples were ordered based on tumor grade. Colors are as per the legend. **b** Unsupervised two-way hierarchical cluster analysis of the 10-gene mutations profile in 21 NEN. Data on Ki-67, gender, and smoking are presented as well. Colors are as per the legend. In light gray four NECs (three in C1 and one in C2) are reported, where *RB1* assessment was not available. **c** Kaplan-Meier survival plots of the entire cohort of NAN (48 samples) stratified based on cluster identity (on the left) or on tumor grading (on the right). *P* values were calculated by the log-rank test. **d** Radar plot of the prevalence of genetic alterations in the clusters identified: numbers identify the percentages of the relevant gene mutations

Statistical Analysis

Hierarchical clustering analysis was performed as elsewhere detailed [23, 28]. Briefly, we used Spearman rank correlation as similarity metric and Centroid linkage as clustering method in Cluster 3.0 and Java TreeView software environment (http://bonsai.hgc.jp/~mdehoon/software/cluster/software.htm). The three main branches ($N = 3$) of hierarchical clustering were selected to construct clusters. Bar and radar plots were prepared using Excel 2020 (Microsoft Office). Continuous and categorical variables were compared by Kruskal-Wallis test and Fisher's exact test, respectively, using the SAS software, version 9.4 (SAS Institute, Inc., Cary, NC). Kaplan-Meier plots and log-rank test for overall survival were performed using JMP 12 (SAS). Cox univariate and multivariable analyses were performed using the SAS software, version 9.4 (SAS Institute, Inc., Cary, NC). All p values were two-sided, and $p < 0.05$ were considered as significant.

Results

Hierarchical Clustering Analysis Reveals Distinct Groups of Tumors

Supervised cluster analysis of the 10 gene panel in 21 out of 48 (44%) tumor samples with mutations ordered according to tumor grade revealed that mutations in *TP53/KRAS/RB1/PTEN* genes co-occurred more frequently in G2 and G3 tumors (Fig. 1a). Overall, NET-G1 had the lowest mutation

Table 1 Clinicopathologic data according to clustering

	All $N = 48$	Cluster				p value
		C1 $N = 8$ (16.7%)	C2 $N = 9$ (18.8%)	C3 $N = 4$ (8.3%)	"Others" $N = 27$ (56.3%)	
Age at diagnosis [years]						
Median (Q1; Q3)	60 (55.5; 65)	59.5 (57; 64)	58 (51; 68)	64.5 (61.5; 66)	60 (55; 64)	0.66[a]
Min–max	33–84	46–78	48–84	59–67	33–78	
Sex						
Male	25 (52.1%)	4 (50.0%)	5 (55.6%)	1 (25.0%)	15 (55.6%)	0.79[b]
Female	23 (47.9%)	4 (50.0%)	4 (44.4%)	3 (75.0%)	12 (44.4%)	
Ethnicity						
White	42 (87.5%)	6 (75.0%)	9 (100.0%)	4 (100.0%)	23 (85.2%)	0.65[b]
Black	5 (10.4%)	2 (25.0%)	0	0	3 (11.1%)	
Asian	1 (2.1%)	0	0	0	1 (3.7%)	
Smoking status						
Smoker	22 (45.8%)	5 (62.5%)	3 (33.3%)	2 (50.0%)	12 (44.4%)	0.24[b]
Non-smoker	25 (52.1%)	3 (37.5%)	6 (66.7%)	1 (25.0%)	15 (55.6%)	
NA	1 (2.1%)	0	0	1 (25.0%)	0	
Alcohol						
Yes	20 (41.7%)	3 (37.5%)	4 (44.4%)	2 (50.0%)	11 (40.7%)	0.30[b]
No	26 (54.2%)	4 (50.0%)	5 (55.6%)	1 (25.0%)	16 (59.3%)	
NA	2 (4.2%)	1 (12.5%)	0	1 (25.0%)	0	
Site						
Esophagus	1 (2.1%)	1 (12.5%)	0	0	0	0.0018[b]
Stomach	2 (4.2%)	1 (12.5%)	0	0	1 (3.7%)	
Pancreas	13 (27.1%)	1 (12.5%)	3 (33.3%)	2 (50.0%)	7 (25.9%)	
Small intestine	18 (37.5%)	2 (25.0%)	0	1 (25.0%)	15 (55.6%)	
Colon-rectum	12 (25.0%)	2 (25.0%)	6 (66.7%)	0	4 (14.8%)	
Gall bladder	2 (4.2%)	1 (12.5%)	0	1 (25.0%)	0	
Stage						
I–III	14 (29.2%)	3 (37.5%)	1 (11.1%)	1 (25.0%)	9 (33.3%)	0.65[b]
IV	34 (70.8%)	5 (62.5%)	8 (88.9%)	3 (75.0%)	18 (66.7%)	
Grade						
G1	12 (25.0%)	0	0	1 (25.0%)	11 (40.7%)	0.0001[b]
G2	20 (41.7%)	2 (25.0%)	2 (22.2%)	2 (50.0%)	14 (51.9%)	
G3	16 (33.3%)	6 (75.0%)	7 (77.8%)	1 (25.0%)	2 (7.4%)	
Performance status—PS						
0	28 (58.3%)	3 (37.5%)	4 (44.4%)	3 (75.0%)	18 (66.7%)	0.16[b]
1	18 (37.5%)	5 (62.5%)	3 (33.3%)	1 (25.0%)	9 (33.3%)	
2	2 (4.2%)	0	2 (22.2%)	0	0	

Percentages could not add up 100 because of rounding

[a]Kruskal-Wallis test

[b]Fisher's exact test

burden (one out of 12 cases, 8.3%), NET-G2 an intermediate value (six out of 20 cases, 30%), and NECs the highest one (15 out of 16 cases, 93.7%) (Supplemental Table 1). All NECs featured large cell neuroendocrine carcinoma. Expectedly, grade-related survival curves showed the best and the worst prognosis for NET-G1 and NECs, respectively, while G2 run an intermediate clinical course (Fig. 1c). There were no differences in survival between resected and unresected NECs. The whole set of molecular and clinicopathologic data of our cohort of 48 patients is presented in Supplemental Table 1.

Unsupervised cluster analysis by means of the same 10 gene panel pushed three distinct clusters to emerge, namely, C1, C2, and C3 (Fig. 1b). The remaining unclustered tumors (i.e., with no panel-related mutations) were descriptively

labeled as "others" (Table 1). The clusters C1, C2, and C3 comprised eight, nine, and four patients, respectively, and showed different distribution of mutations: the cluster C1 included mutations in *TP53* (six cases), *PIK3CA* (three cases), and *BRAF* (two cases); the cluster C2 mutations in *KRAS* (nine cases), *TP53* (seven cases), *RB1* (three cases), and *APC/CTNNB1/PTEN* (one case for each); and the cluster C3 mutations in *CTNNB1* (three cases) and *ATM/IDH1* (one case for each) (Fig. 1b and d).

To further investigate the relationship existing among C1, C2, and C3, we considered the prevalence of mutations in each cluster (Fig. 2). While no cluster shared all the same mutations, *TP53* were observed in C1 and C2; *CTNNB1* in C2 and C3; and *APC*, *ATM*, *BRAF*, *IDH1*, *KRAS*, *PIK3CA*, *PTEN*, and *RB1* across clusters with different prevalence rates among them (Figs. 2 and 3). Interestingly, the median Ki-67 value ranked 5% (range 1–70%), 50% (range 3.2–95%), and 50% (range 3.2–95%) in clusters C3, C2, and C1, respectively (Fig. 3; Supplemental Table 1).

All but one C2-associated NEC had Ki-67 ranging from 50 to 95% and none of them showed well-differentiated morphology (Supplemental Table 1). In all NEC samples, which belonged to the LCNEC subtype, Ki-67 was heterogeneously distributed inside tumors, showing diversely proliferating tumor areas intermingled with each other (Fig. 4).

Univariate and Multivariable Survival Analyses

Survival curves showed that C3 had the best prognosis, whereas C1 and C2 exhibited the lowest probability of survival with no significant difference between them, in keeping with their tumor composition ($p = 0.69$) (Fig. 1c). As a matter of fact, each cluster remarkably exhibited an unexpected admixture of NETs and NECs, with all but two NECs being distributed among cluster C1 (six out of eight cases, with two NET-G2), cluster C2 (seven out of nine cases, with two NET-G2), and cluster C3 (one out of four cases, with one NET-G1 and two NET-G2) ($p < 0.0001$) (Table 1; Fig. 3). Unmutated tumors according to this 10-gene panel (i.e., the group "others") totaled 27 cases, mostly NETs (25/27 cases, 93%), particularly NET-G2 (14 cases), with only two NECs being on record (Fig. 3). No differences were documented across population as far as age at diagnosis, sex, ethnicity, smoking status, alcohol consumption, tumor stage, and performance status were concerned, although a preferential localization of cluster C2 and the "others" group was seen in the colon-rectum and small intestine, respectively ($p = 0.0018$; Table 1).

Cox univariate analysis showed that tumor site (stomach vs. small intestine), grade, stage IV, and clusters C1 and C2 impaired survival, while cluster C3 did not present any event (Table 2). Multivariable analysis confirmed that only clusters affected survival independently (Table 2).

Discussion

We herein challenge the current pathogenesis of GEP-NENs by favoring the hypothesis that a subset of large cell NECs in the gastroenteropancreatic tract is likely to evolve from pre-existing well-differentiated NETs. In this context, an intra-tumor heterogeneous distribution of Ki-67 as depicted in Fig. 4 could reflect the existence of diversely proliferating cell clones in these secondary NECs as previously indicated in the lung and the thymus [22, 23, 25]. Our study showed that 14 out of 16 (87%) NECs tightly joined NETs (NET-G2 but also NET-G1) to realize three distinct and separate clusters, namely, C1, C2, and C3 (Fig. 1; Table 1), which accounted for 44% of patients according to the relevant 10-gene signature. These clusters revealed genetic lesions which were shared by NETs and NECs (Fig. 3), thus supporting a compelling hypothesis on an evolution of NETs to some NECs. Remarkably, the group of "other" tumors devoid of any mutation with our gene panel mostly included NET-G1 (11 cases) and NET-G2 (14 cases) and predominantly grew in small intestine (15 cases) over the pancreas and colon (Table 1; Fig. 3; Supplemental Table 1). Of note, small intestine is known to harbor a minor fraction of mutational driver events (putative drivers in *CDKN1* and *APC* would be documentable in only 10% of instances or less) [29, 30].

We would like to speculate that according to our hypothesis, NETs clustered in C3, which proliferated by 5% Ki-67, would have a potential to transform into NECs (Table 1). As a matter of fact, cluster C3 harbored *CTNNB1* mutation as dominant alteration, which has been linked to epithelial-mesenchymal transition upon beta-catenin nuclearization and tumor progression in GEP tract [31] NENs. Of the other two genes in the cluster, *ATM* (a master controller of cell cycle checkpoint signaling in response to DNA damage and genome stability) is an independent risk factors for recurrence in pancreatic NENs [32], and *IDH1* (involved in metabolic cytoplasmic NADPH production) has been associated with higher histologic grade, lymphovascular invasion, and recurrence rate in rectal and gastric GEP-NENs [33]. Of note, *ATM* and *IDH1* mutations, which were found in two long surviving metastatic NETs of the pancreas (NET G1 and NET G2, respectively; Supplemental Table 1), could be associated with the occurrence and development of these tumors as documented in low-grade glioma and secondary glioblastoma [34, 35]. Cluster C2, beyond *CTNNB1* mutation, presented with additional master genes repeatedly altered in high-grade NENs at different anatomical sites (including the GEP tract), such as *APC*, *KRAS*, *PTEN*, *RB1*, and *TP53*. In particular, cluster C2 was repository of all *KRAS* mutations [36, 37], which are common to NECs of the lung [23, 27,

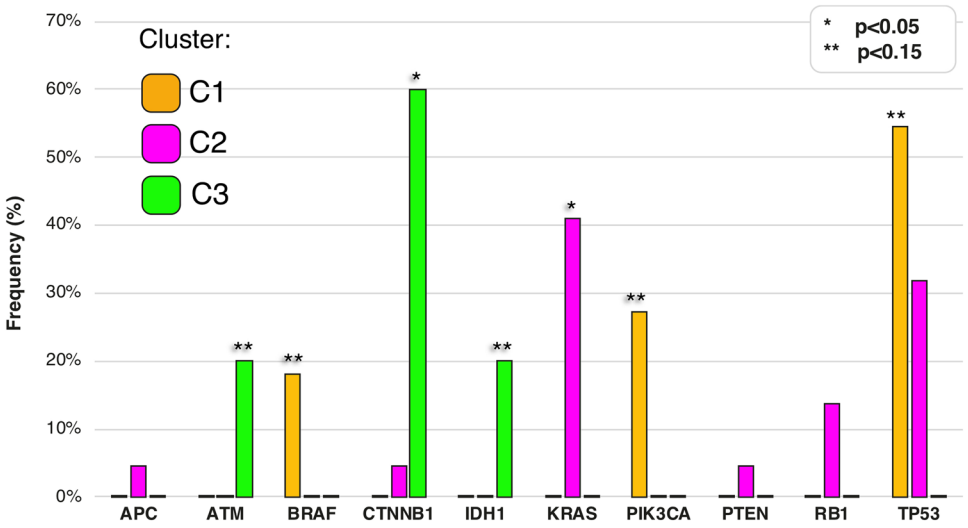

Fig. 2 Distribution of genetic alterations according to clusters (C1→C3). Bar plots indicate frequency (*Y*-axis) of alterations found in each cluster (shown on *X*-axis) highlighted by different colors. Simple asterisks indicate significant *p* values; double asterisks indicate marginal *p* values (calculated by Fisher test)

38–40] and the uterine cervix [41]. Even cluster C1 shared with cluster C2 *TP53* mutations in most instances (75%), along with *PIK3CA* (37%) and *BRAF* (25%) mutations, both of which are frequently documented in gastrointestinal NECs as driver mechanisms [42–44]. Of note, repetitive pathogenic/likely pathogenic *TP53* mutations were found in more aggressive rectal NETs, supporting the hypothesis of a risk evolution [33].

The relevance of clustering to clinics (and hence of gene alterations) even more emerged from the analysis of survival curves, with the best prognosis in C3 and the worst in C1 and C2 (the median Ki-67 ranked 5% in C3 and 50% in C1 and C2) (Fig. 1). These survival trends of clustered NECs were likely to mirror the close relationship among cell differentiation, proliferative activity, and biological aggressiveness in NENs of the GEP tract [16, 17, 45–48]. Noteworthy, cluster C1 (and marginally C2) was also an independent factor of survival on multivariable analysis, thus suggesting a role for gene alteration burden in dictating tumor morphology and clinical behavior.

The heterogeneity in GEP NEC distribution across clusters could reflect major differences in their natural

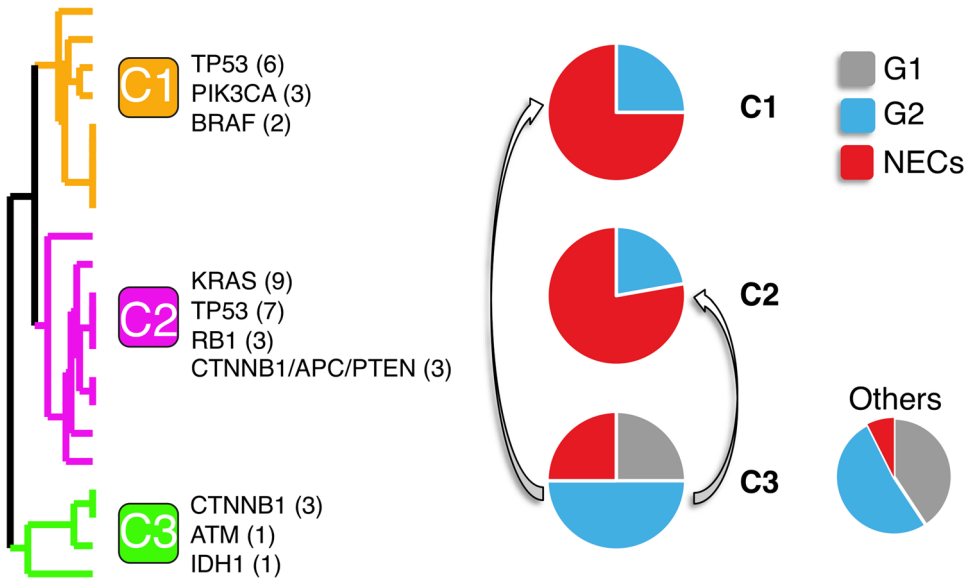

Fig. 3 Relationship between clusters and tumor composition (C1→C3). Pie charts indicate the distribution of different tumor types in the clusters, which are positioned according to the clustering tree shown on the left of picture with the corresponding gene alterations (the numbers in brackets correspond to mutation burden). *APC* (APC regulator of WNT signaling pathway), *ATM* (ATM serine/threonine kinase), *BRAF* (B-Raf proto-oncogene, serine/threonine kinase), *CTNNB1* (catenin beta 1), *IDH1* (isocitrate dehydrogenase [NADP(+)] 1), *KRAS* (KRAS proto-oncogene, GTPase), *PIK3CA* (phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha), *PTEN* (phosphatase and tensin homolog), *RB1* (RB transcriptional corepressor 1), *and TP53* (tumor protein p53)

Table 2 Univariate and multivariable analysis of overall survival

			Univariate an[a]		Multivar.an (stage + grade)		Multivar. an (stage + grade + cluster 4 classes) [b]		Multivar. an (stage + grade + cluster 3 classes) [c]	
	$N = 48$	N deaths = 7	HR (95% CI)	Wald test	HR (95% CI)	Wald test	HR (95% CI)	Wald test	HR (95% CI)	Wald test
≤ 55 years at diagnosis (vs ≥ 65 years)	12	3	1.52 (0.24–9.42)	0.66						
56–64 years at diagnosis (vs ≥ 65 years)	23	2	0.51 (0.07–3.73)	0.51						
Male (vs female)	25	6	4.03 (0.48–33.60)	0.20						
Black/Asian (vs white)	6	1	1.68 (0.20–14.04)	0.63						
Smoker (vs non-smoker)	22	3	0.92 (0.21–4.12)	0.92						
Alcohol yes (vs no)	20	3	1.10 (0.25–4.90)	0.90						
Esophagus (vs small intestine)	1	0	No events							
Stomach (vs small intestine)	2	1	21.60 (1.22–382.33)	0.0361						
Pancreas (vs small intestine)	13	3	4.58 (0.47–44.39)	0.19						
Colon-rectum (vs small intestine)	12	2	5.28 (0.48–58.63)	0.18						
Gall bladder (vs small intestine)	2	0	No events							
Stage IV (vs I–III)	34	7	All events in stage IV patients		All events in stage IV patients		All events in stage IV patients		All events in stage IV patients	
G3 (vs G1-G2)	16	3	18.52 (1.88–182.88)	0.0125	16.53 (1.64–166.77)	0.0174	4.55 (0.30–69.39)	0.28	4.49 (0.30–68.38)	0.28
PS 1–2 (vs 0)	20	3	1.64 (0.36–7.39)	0.52						
Cluster C1 (vs cluster "Others")	8	3	12.08 (1.98–73.89)	0.0070			92.51 (3.41–2509.24)	0.0072		
Cluster C2 (vs cluster "Others")	9	2	10.35 (1.35–79.68)	0.0248			8.41 (0.65–108.88)	0.10		
Cluster C3 (vs cluster "Others")	4	0	No events				No events			
Cluster C1 (vs cluster C3 and "Others")	8	3	13.68 (2.24–83.73)	0.0046					117.98 (4.28–3254.15)	0.0048
Cluster C2 (vs cluster C3 and "Others")	9	2	11.76 (1.53–90.44)	0.0179					10.86 (0.83–141.30)	0.07

[a]Global test: age at diagnosis $p = 0.49$; site $p = 0.22$; cluster 4 classes $p = 0.0440$; cluster 3 classes $p = 0.0114$

[b]Global test for cluster 4 classes: $p = 0.06$; nested likelihood ratio test after adding cluster: $p = 0.0050$

[c]Global test for cluster 3 classes: $p = 0.0188$; nested likelihood ratio test after adding cluster: $p = 0.0076$

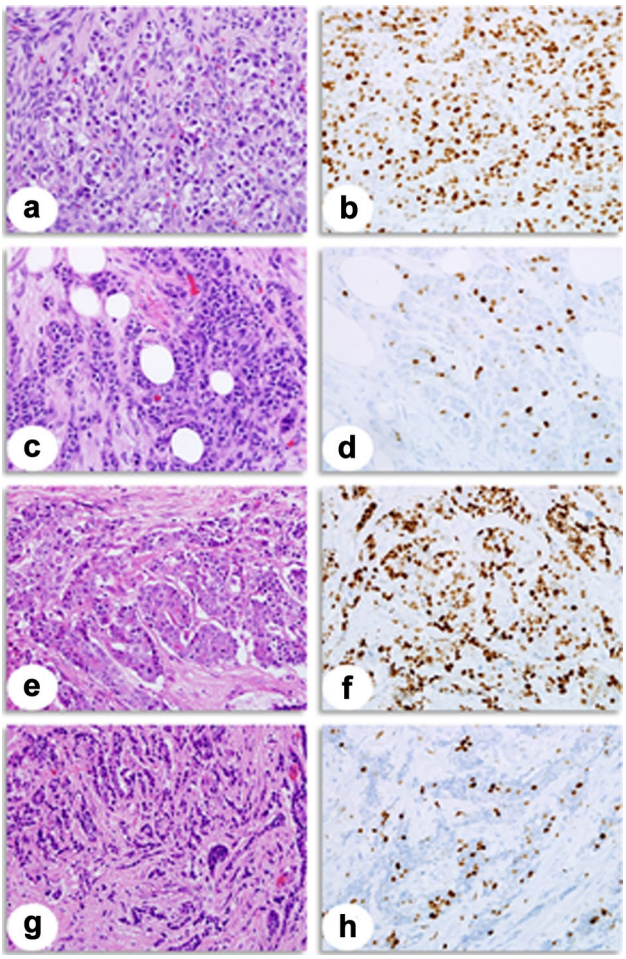

Fig. 4 **a**–**h** Intra-tumor heterogeneity of diversely graded neoplastic components in two different instances of neuroendocrine carcinomas. Trabecular to solid aggregates of cancer cells with poor differentiation featuring LCNEC **a** show high levels of Ki-67 activity **b**, whereas concurrent well differentiated tumor areas in the same lesion **c** exhibit correspondingly lower labeling of Ki-67 consistent with NET G2 **d**. In another case of LCNEC, a high-grade component **e** with an elevated value of Ki-67 **f** contrasts with a well differentiated tumor **g** showing lower activity of Ki-67 consistent with NET G2 **h**. LCNEC stands for large cell neuroendocrine carcinoma; NET stands for neuroendocrine tumor. All pictures were taken at 200X

history [15, 23, 25], which in turn might reflect endogenous and/or exogenous risk factors as a function of the primary anatomical sites (e.g., prior/familial history of non-NE cancer or smoking in the small intestine [49] or urinary bladder [50], smoking in the colon and uterine cervix [51, 52], and non-recent onset diabetes [53] in the pancreas). Indeed, in our hand, *KRAS*-mutated C2 cases developed in the colon and the pancreas. Therefore, endogenous and/or exogenous risk factors might give rise to different genetic/epigenetic alterations at the onset of tumor development according to the different anatomical site (so important for the clinical behavior of GEP NENs), whereby imprinting preclinical phase, clinical outcome, and morphologic appearance [23, 25, 54].

In our series, we did not encounter morphologic NET-G3 and the range of Ki67 in NECs clustering C1 (50–95%) and C2 (30–95%), the survival curves and the prevalence of *TP53* mutations (*TP53* is inactivated in most NECs anywhere [1, 2]) were all in keeping with the diagnosis of NECs. However, less proliferating tumor areas as depicted in Fig. 4 could meet some morphological traits of NET-G3, whose diagnostic recognition may have been blurred at the level of an individual patient's cancer by using morphology or immunohistochemistry as supervised defining criteria [8, 55–57]. Moreover, one case clustering C2, mutated in *TP53* and featuring NEC, fell into the same proliferation category as NET-G3 (mitoses > 20; 20% < Ki-67 < 50%), but was joined two other NETs bearing *TP53* or *RB1* mutation (*RB1* is inactivated in most NECs anywhere) [1, 2] (Supplemental Table 1). In our study, unsupervised clustering analysis (Figs. 1 and 3) revealed a clear admixture of NETs and NECs, which in turn showed admixture of well differentiated NETs with high-grade components (Fig. 4), thus letting us hypothesize an evolution of some NECs with large cell morphology from pre-existing NETs (Fig. 3). This admixture of diversely escalating tumor components in cell proliferation was paralleled by a heterogeneous intra-tumor distribution of Ki-67 (Fig. 4), which could help to recognize these secondarily evolving NECs, as previously observed [6, 22, 23].

Despite limitations of our study principally due to its retrospective character, limited number of tumors, and the small number of genes under evaluation, which precluded an independent validation, a strength point was however represented by unsupervised clustering analysis centered on molecular alterations rather than the more traditional supervised analysis based on tumor categorization that may be challenging especially at the level of an individual patient's cancer.

Conclusions

We herein introduce the concept of secondary large cell NEC in the GEP-NENs which is likely to incorporate an underrecognized perspective mirroring risk factors and the natural history of disease. This is in keeping with other models of NE neoplasms arising elsewhere, in which gene alterations are the best modelers of tumor fate.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s12022-020-09659-6.

Acknowledgments This work is dedicated to the memory of Carlotta, an extraordinarily lively girl who died an untimely death due to cancer

in the prime of her life. The authors are indebted with Ms. Cecilia Pelosi for accurate proofreading of the manuscript.

Authors' Contributors GP conceived and designed the study, drafted and finalized the manuscript, and shared all statistical analyses; FB carried out clustering analysis, supervised all statistical procedures, shared the study design, and contributed to draft and finalize the manuscript; ED performed all statistical analyses; JM, AS, AA, MP, YG, and NV critically revised the manuscript and finalized the manuscript. All authors approved the submitted version.

Data Availability All data and materials being used and presented in this study are available as Supplemental Table 1.

Compliance with Ethical Standards

Disclosures and Declarations F.B is a recipient of grants from the Associazione Italiana Ricerca sul Cancro (AIRC) [IG-22827], the Italian Ministry of Health [GR-2016-02363975 and CLEARLY]. N.V. was supported by Cancer Center Support Grant (CCSG) P30 CA006927 to Fox Chase Cancer Center. All the other authors declare that there are no sources of funding. All authors declare that there are no financial or non-financial interests and that the study does not involve humans and/or animals for which specific approval by the ethics committee and/or informed consent are required. Moreover, the study does not have implications for public health or general welfare.

Conflict of Interest The authors declare that they have no conflict of interest.

Ethics Approval and Consent to Participate As this study dealt with reappraisal of previously generated and authorized molecular data by the Fox Chase Cancer Centre's ethics committee, no further release was necessary. In particular, no new information about patients was collected and no protected health information was used in the current study. The study was performed in keeping with the Declaration of Helsinki and, remarkably, does not contain any individual person's data, but only aggregated information.

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