Ki-67 Index of 55% Distinguishes Two Groups of Bronchopulmonary Pure and Composite Large Cell Neuroendocrine Carcinomas with Distinct Prognosis

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Keywords
Large cell neuroendocrine carcinoma · Ki-67 index · Combined large cell neuroendocrine carcinoma · Napsin A

This work is dedicated to the memory of Laura Salvaterra, a courageous woman who battled against cancer. This is an invitation to fight cancer every day in her name, even after she has left us.

Abstract
Background: Little information is available concerning prognostic factors for bronchopulmonary large cell neuroendocrine carcinomas (BP-LCNECs) and even less is known about combined LCNECs (Co-LCNECs). We investigated whether an integrated morphological, immunohistochemical, and molecular approach could be used for their prognostic evaluation. ***Methods:*** Morphological (including combined features), proliferative (mitotic count/Ki-67 index),

immunohistochemical (napsin A, p40, TTF-1, CD44, OTP, SSTR2A, SSTR5, mASH1, p53, RB1, and MDM2), and genomic (*TP53*, *RB1*, *ATM*, *JAK2*, *KRAS*, and *STK11*) findings were analyzed in BP-LCNECs from 5 Italian centers, and correlated with overall survival (OS). The Ki-67 index was expressed as the percentage of positive cells in hot spots as indicated in the WHO 2019 Digestive System Tumors and, for Co-LCNECs, the Ki-67 index was evaluated only in the LCNEC component. ***Results:*** A total of 111 LCNECs were distinguished into 70 pure LCNECs, 35 Co-LCNECs (27 with adenocarcinoma [ADC] and 8 with squamous cell carcinoma [SqCC]), and 6 LCNECs with only napsin A immunoreactivity. The Ki-67 index cutoff at 55% evaluated in the neuroendocrine component was the most powerful predictor of OS (log-rank $p = 0.0001$) in all LCNECs; 34 cases had a Ki-67 index <55% (LCNEC-A) and 77 had a Ki-67 index ≥55% (LCNEC-B). Statistically significant differences in OS (log-rank $p = 0.0001$) were also observed between pure and Co-LCNECs. A significant difference in OS was found between pure LCNECs-A and Co-LCNECs-A ($p < 0.05$) but not between pure LCNECs-B and Co-LCNECs-B. Co-LCNEC-ADC and LCNEC napsin A+ cases had longer OS than pure LCNEC and Co-LCNEC-SqCC cases (log-rank $p = 0.0001$). On multivariable analysis, tumor location, pure versus combined features, and napsin A, but no single gene mutation, were significantly associated with OS after adjustment for Ki-67 index and study center ($p < 0.05$). ***Conclusions:*** The Ki-67 proliferation index and the morphological characterization of combined features in LCNECs seem to be important tools for predicting clinical outcome in BP-LCNECs.

Introduction

Bronchopulmonary neuroendocrine neoplasms (BP-NENs) represent a special category of lung neoplasms. In the WHO 2015 classification of lung tumors, large cell neuroendocrine carcinoma (LCNEC) was distinguished from large cell carcinoma and regrouped with the typical carcinoid, atypical carcinoid, and small cell neuroendocrine carcinoma (SCNEC) into the NEN category [1]. Owing to their poorly differentiated features, LCNECs and SCNECs were classified as high-grade BP-NENs, distinct from the low-grade typical carcinoid and intermediate-grade atypical carcinoid.

BP-LCNEC represents a rare histological type of lung cancer with an incidence of about 3%, while BP-SCNEC is much more frequent and accounts for 15–20% of all lung cancers [1, 2]. According to the WHO 2015 classification, histological features characteristic of BP-LCNECs are: (1) neuroendocrine pattern (organoid nesting, palisading, and trabeculae); (2) high mitotic rate (>10 per 2 mm^2); (3) necrosis (often extensive); (4) cytology (cells with moderate-to-abundant cytoplasm, vesicular-to-clumped chromatin, and frequent, often prominent nucleoli); and (5) positivity, in >10% of tumor cells, for immunohistochemical neuroendocrine markers (chromogranin A [CgA], synaptophysin [Syn], and CD56 [also known as neural cell adhesion molecule]) [1]. BP-SCNECs share some characteristic features with BP-LCNECs while differing in that they present with smaller cells, a low nuclear-to-cytoplasmic ratio, finely granular chromatin, absent or inconspicuous nucleoli, and nuclear molding [1, 3].

The latest WHO classification of lung NEN reported a variant of BP-LCNEC, the combined LCNEC (Co-LCNEC), characterized by an LCNEC with components of adenocarcinoma (ADC) or squamous cell carcinoma (SqCC), the most frequent combined histotypes [4–6], as well as the rarer giant cell carcinoma or spindle cell carcinoma. If these components are clearly identified, regardless of their proportion, the neoplasm is considered a Co-LCNEC and the component present must be confirmed by immunohistochemistry (IHC) [2]. In particular, napsin A positivity, combined or not with thyroid transcription factor 1 (TTF-1) staining, should be observed in the ADC component and p40 positivity in the SqCC component [1, 7, 8]. Several studies have reported the identification of Co-LCNEC in 30% of 81 [9], 20% of 56 [9], and 12% of 250 [6] studied LCNECs, respectively [10]. Overall survival (OS) and recurrence-free survival did not reveal any statistically significant differences between histologies in any of the studied cohorts.

In addition, a newly introduced subtype of BP-LCNEC, focally expressing the exocrine marker napsin A but lacking a distinct adenocarcinomatous component, has recently been described [11]. Molecularly, this neoplasm reveals mutations typical of lung ADCs (such as *KRAS* and/or *STK11*) and has been designated as LCNEC with ADC-like features [11]. Moreover, high-throughput investigations such as whole-exome, genome, and transcriptome sequencing have demonstrated that a subset of LCNEC shares additional genetic abnormalities with ADC or SqCC (*TTF1* amplification, *CDKN2A* deletion, *STK11* and *KEAP1* mutations, and *FGFR1* amplification), suggesting a common cellular origin [12].

As stated above, different studies have focused on the genetic profile of LCNECs [13, 14]. Interestingly, the most frequently mutated genes are tumor suppressor genes including *TP53* (78%), *RB1* (38%), *STK11* (33%), *KEAP1*

(31%), and genes of the RAS pathway (principally *KRAS* [22%]). On the basis of their genomic profile, BP-LCNECs have been distinguished into two major subtypes and one minor subtype. In detail, the small cell lung cancer (SCLC)-like major subtype is identified by *TP53* mutation and *RB1* co-mutation or loss of expression and *MYCL* amplification along with higher proliferative activity, and the non-SCLC (NSCLC)-like major subtype is hallmarked by lack of co-altered *TP53* and *RB1* and the occurrence of *STK11*, *KRAS*, and *KEAP1* mutations, characteristic of NSCLC. On the other hand, a carcinoid-like minor subgroup showing *MEN1* mutation, with low mutational burden, an overt carcinoid-type morphology, and a Ki-67 labeling index (Ki-67) >20% has been identified.

Overall, molecular data suggest that BP-LCNECs constitute a heterogeneous group of neoplasms sharing genomic characteristics with BP-SCNECs and BP-NSCLCs (mainly ADCs). In addition, a recent study has shown that stratification of BP-LCNECs on the basis of their molecular characteristics may predict chemotherapy outcome. Patients with SCLC-like LCNEC have a shorter OS than those with NSCLC-like LCNEC despite higher response rates to chemotherapy [15]. Particularly BP-LCNECs that carry a wild-type *RB1* gene or express the RB1 protein have a better outcome if treated with NSCLC-type chemotherapy, including platinum plus gemcitabine, or taxane-based treatment rather than with platinum-etoposide chemotherapy [16]. Additional differences between BP-LCNECs with SCLC-like molecular features and NSCLC-like tumors were the higher proliferative activity of the former, as revealed by mitotic count (MC) and Ki-67, and a trend toward a shorter recurrence-free survival for the SCLC-like subset [17].

The role of Ki-67 in BP-NEN has been discussed and still remains to be defined, with implications for a potential definition of tumor grade [18–24]. In the WHO 2015 classification of BP-NENs, Ki-67 is recommended only as an additional diagnostic means of defining diagnosis, particularly in cases of small biopsies or with crush artifacts; the distinction into the four main groups of neoplasms continues to be mainly based on histology, MC, and extent of necrosis [1]. In a recent study, conducted on two BP-NEN cohorts from Japan and Germany, Ki-67, evaluated according to the criteria of the WHO 2019 classification of the digestive system for NENs [25], proved to be prognostically more accurate than MC [24]. The superiority of Ki-67, as a prognostic index, over MC has also been demonstrated in studies on gastrointestinal NEC [26], and on digestive system NEC and mixed adenoneuroendocrine carcinoma (MANEC), in which in particular [27–29] a Ki-67 ≥55% was significantly associated with very poor survival of patients. Moreover, a Ki-67 ≥55% was associated with better response to platinum-based chemotherapy [26].

The aim of this study was to analyze a large series of BP-LCNECs in order to (a) assess proliferative (Ki-67) and mitotic indices to distinguish different prognostic groups of BP-LCNEC; (b) evaluate the prognostic value of morphology in terms of pure and combined histological features; and (c) analyze the relationships between histopathological features and molecular alterations.

Materials and Methods

Case Selection, Tissue Sampling, and Clinicopathological Information

The surgical pathology and clinical databases of 5 Italian institutions (Fondazione IRCCS Istituto Nazionale dei Tumori – INT, Milan; ASST Spedali Civili di Brescia – Brescia; Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico – Milan; Ospedale Policlinico Ospedale San Martino – Genoa; and Humanitas Research Hospital – HRH, Rozzano), covering a period from 1975 to 2016, were retrospectively searched for one of the following histological diagnoses: "large cell neuroendocrine carcinoma," "large cell carcinoma with neuroendocrine differentiation," "neuroendocrine carcinoma with large cell features, Co-LCNEC, mixed neuroendocrine/non-neuroendocrine carcinoma." Excluded were (1) cases with only biopsy material available and (2) cases with small cell features, including Co-LCNEC/SCNEC.

A total of 148 candidate cases were identified, and the study was performed according to the clinical standards of the 1975 and 1983 Declaration of Helsinki and was approved by the Ethics Committee of Fondazione IRCCS INT (No. INT 171/16).

The patients' charts and tumor morphology were centrally and blindly reviewed by two expert pathologists (M. Milione and C. Capella). LCNEC identification and subtype characterization were based on the parallel investigation of at least 4 consecutive sections from representative blocks, stained with H&E (hematoxylin-eosin), Syn, CgA, and Ki-67, respectively. A total of 111 cases met all the criteria of the 2015 WHO classification of lung tumors and were enrolled in the study [1]. The cases excluded from the study were 12 SCNECs, 6 Co-LCNECs/SCNECs, 11 large cell carcinomas, 4 poorly differentiated SqCCs, and 4 ADCs. Among the cases selected there were no tumors with an overt carcinoid-type or a neuroendocrine tumor G3-type morphology.

The morphological analysis (Table 1) considered the following: (a) cytological features such as cell size, nuclei and nucleoli; (b) architectural features such as organoid nesting pattern, peripheral palisading, trabeculae and rosette-like areas [1, 3]; (c) tumoral necrosis (absence vs. presence; focal or punctate vs. diffuse or geographic); (d) MC, evaluated in ≥10 high-power fields (HPF; 10 HPF = 2 mm^2), with a threshold of >10 mitoses per 2 mm^2 [1]; (e) pathological tumor staging according to the Union for International Cancer Control/American Joint Committee on Cancer (UICC/AJCC) 8th edition [30]; (f) lymphovascular invasion (evaluated on H&E- and/or CD31-stained sections); (g) perineural in-

Table 1. Characteristics of the patients with neuroendocrine neoplasms of the lung

	All patients	LCNEC-A Ki-67 <55%	LCNEC-B Ki-67 ≥55%	*p* value[1]
Total	111 (100)	34 (100)	77 (100)	
Age				
<50 years	13 (11.7)	4 (11.8)	9 (11.7)	
50–59 years	19 (17.1)	4 (11.8)	15 (19.5)	
60–69 years	37 (33.3)	13 (38.2)	24 (31.2)	
70+ years	42 (37.8)	13 (38.2)	29 (37.7)	0.78
Sex				
Male	74 (66.7)	23 (67.6)	51 (66.2)	
Female	37 (33.3)	11 (32.4)	26 (33.8)	0.88
Smoking status				
Never	1 (0.9)	0 (0.0)	1 (1.3)	
Former	79 (71.2)	23 (67.6)	56 (72.7)	
Current	31 (27.9)	11 (32.4)	20 (26.0)	0.76
Site				
Superior lobe	84 (75.7)	22 (64.7)	62 (80.5)	
Inferior lobe	16 (14.4)	8 (23.5)	8 (10.4)	
Hilar region	11 (8.9)	4 (11.8)	7 (9.1)	0.13
Location				
Central	13 (11.7)	3 (8.8)	10 (13.0)	
Peripheral	98 (88.3)	31 (91.2)	67 (87.0)	0.75
Combined features				
Co-LCNEC-ADC	27 (24.3)	14 (41.2)	13 (16.9)	
Co-LCNEC-SqCC	8 (7.2)	2 (5.9)	6 (7.8)	
LCNEC napsin A+	6 (5.4)	3 (8.8)	3 (3.9)	
Pure LCNEC	70 (63.1)	15 (44.1)	55 (71.4)	**0.02**
Stage				
I	33 (29.7)	7 (20.6)	26 (33.8)	
II	23 (20.7)	11 (32.3)	12 (15.6)	
III	30 (27.0)	9 (26.5)	21 (27.3)	
IV	25 (22.5)	7 (20.6)	18 (23.4)	0.22
Ki-67 index				
Median (range)	66 (20–95)	41.5 (20–55)	73 (56–95)	–
Mitotic count				
Median (range)	34 (13–78)	28 (13–78)	35 (14–72)	**0.04**
Necrosis				
Absent	16 (14.4)	8 (23.5)	8 (10.4)	
Spot	29 (26.1)	12 (35.3)	17 (22.1)	
Map	66 (59.5)	14 (41.2)	52 (67.5)	**0.03**
Microscopic infiltration				
Air spaces	83 (74.8)	25 (73.5)	58 (75.3)	
Bronchus	13 (11.7)	3 (8.8)	10 (13.0)	
Pleura	2 (1.8)	0 (0.0)	2 (2.6)	
Absent	8 (7.2)	4 (11.8)	4 (5.2)	
Extra-lung	5 (4.5)	2 (5.9)	3 (3.9)	0.61
Morphological pattern				
Solid sheet	109 (99.1)	32 (97.0)	77 (100)	
Trabecular	1 (0.9)	1 (3.0)	0 (0.0)	0.30
Lymphovascular/perineural invasion/lymphocytic infiltration				
Vascular invasion	53 (47.8)	19 (55.9)	34 (44.2)	0.30
Perineural invasion	5 (4.5)	2 (5.9)	3 (3.9)	0.64
Intratumoral lymphocytes	53 (47.8)	18 (52.9)	35 (45.5)	0.54
Peritumoral lymphocytes	74 (66.7)	20 (58.8)	54 (70.1)	0.28
Surgery				
Lobectomy	63 (56.8)	25 (73.5)	38 (49.3)	
Bilobectomy/pneumonectomy	19 (17.1)	5 (14.7)	14 (18.2)	
Partial resection	29 (26.1)	4 (11.8)	25 (32.5)	**0.04**
Residual tumor				
R0	100 (90.1)	30 (88.2)	70 (90.9)	
R1/2	11 (9.9)	4 (11.8)	7 (9.1)	0.73

Values denote *n* (%) unless specified otherwise. Bold type denotes significance. LCNEC, large cell neuroendocrine carcinoma; Co-LCNEC, combined large cell neuroendocrine carcinoma; ADC, adenocarcinoma; SqCC, squamous cell carcinoma; R, residual tumor. [1] *p* value based on the Fisher exact test for categorical variables or the Wilcoxon test for continuous variables.

vasion; (h) intra- and peritumoral lymphocytic infiltration; (i) combination with non-neuroendocrine components such as ADC or SqCC, according to the criteria of the WHO 2015 classification of lung tumors, and confirmed by napsin A or p40 IHC; and (j) spread through air spaces, according to the criteria of Kadota et al. [31].

Immunohistochemistry

The IHC study included detection of the following markers: (a) CgA and Syn (general neuroendocrine markers) in order to confirm the presence and extent of the NEC component; (b) Ki-67 for tumoral cell proliferation assessment; and (c) napsin A, TTF-1, CD44, homeobox protein orthopedia (OTP), somatostatin receptor 2A (SSTR2A), somatostatin receptor 5 (SSTR5), mammalian achaete-scute homolog 1 (mASH1), p53, RB1, and mouse double minute 2 homolog (MDM2), using the antibodies listed in online supplementary Table 1 (for all online suppl. material, see www.karger.com/doi/10.1159/000508376), where the protocols are detailed.

Napsin A (in association with Alcian blue staining) and p40 staining was utilized for identifying either a glandular or a squamous component, respectively. Ki-67 evaluation was made using the MIB antibody and expressed as a percentage of at least 500 cells counted in areas of the strongest nuclear labelling (hot spots), as indicated in the gastroenteropancreatic (GEP) neuroendocrine tumor WHO 2019 guidelines [25, 32]; for Co-LCNECs, Ki-67 was evaluated only in the large cell neuroendocrine component, as suggested by a previous study on MANECs of the digestive system [28].

With the exception of TTF-1, mASH1, p53, RB1, and MDM2, all markers were considered positive regardless of the number of positive cells. To minimize variability in assessment, the IHC results for TTF-1, mASH1, p53, RB1, and MDM2 were assessed adopting a scoring system as absent and present with two possible cutoffs (present: 1–49%; or present: 1–30% and >3%). The immunoreactivity and scores for SSTR2A and SSTR5 were evaluated using a four-tiered system, and scores of 0 and 1 were considered negative and scores of 2 and 3 positive, as suggested by Volante et al. [33]. Pathologist agreement is reported in online supplementary Figure 1.

Next-Generation Sequencing

Targeted next-generation sequencing (NGS) was performed using the Ion AmpliSeq™ Cancer Hotspot Panel (Thermo Fisher Scientific) that amplifies 207 amplicons covering about 2,800 COSMIC mutations from 50 oncogenes and tumor suppressor genes commonly mutated in human cancers. For the details on DNA extraction and library preparation, see online supplementary Materials.

Evaluation of Proliferative Cutoff

Receiver operating characteristic (ROC) analysis was used to obtain a cutoff value for Ki-67 that best identified subjects who died early (within 2 years from surgery). The area under the curve was calculated to determine the diagnostic value of the test. The optimal cutoff that minimized false positive and false negative rates was determined using the Youden index, which reaches its maximum for the point on the ROC curve that is furthest from the diagonal.

Statistical Methods

Associations between demographic characteristics, clinicopathological features, and tumor subtype (LCNEC-A with Ki-67 <55% [20–55%] vs. LCNEC-B with Ki-67 ≥55%), including combined features, were assessed using the Fisher exact test for categorical variables and the nonparametric Wilcoxon test for continuous variables. The association between immunohistochemical, histochemical, and molecular features was assessed using the Mantel-Haenszel test for trend across categories. The primary study endpoint was the correlation of OS with primary tumor site, tumor stage at diagnosis, LCNEC differentiation, the presence and type of non-neuroendocrine component (ADC, SqCC, and napsin A expression), and Ki-67, according to parameters defined in other solid tumors.

OS was defined as the time from the date of surgery to the date of death of any cause, or the date of last contact, whichever occurred first. OS curves were drawn using the Kaplan-Meier method. The log-rank test was used to assess the difference in survival rates between patient groups. Univariable and multivariable Cox proportional regression analyses were used to assess the association between clinicopathological characteristics and death. Data analysis was performed using the SAS software (version 9.4; SAS, Cary, NC, USA). All tests were two-sided and p values <0.05 were considered statistically significant.

Results

Patient Characteristics and Treatment

As shown in Table 1, most of the patients were elderly: 79 patients (71.1%) were ≥60 years old, including 42 patients (37.8%) aged ≥70 years. The vast majority of the patients (99.1%) were current or former smokers. Only a small number of tumors (11.7%; n = 13) originated centrally from a main bronchus, while most neoplasms occurred peripherally in the upper lobes of the lungs (75.7%; n = 84). Fifty-six patients (50.5%) had stage I–II disease, and 55 (49.5%) had stage III–IV disease. All patients underwent cancer-directed surgery, including 29 (26.1%) segmentectomies or wedge resections, 63 (56.8%) lobectomies, and 19 (17.1%) bilobectomies or pneumonectomies.

The 25 patients classified as stage IV at diagnosis had oligometastatic disease and received surgery with palliative intent. Data on adjuvant treatment were available for 65 (58.5%) of the patients: 33 (50.8%) of them received platinum-based chemotherapy (pre- and/or postoperatively), 16 (24.6%) received radiotherapy, 15 (23.1%) received combined chemoradiotherapy, and 16 (24.6%) did not receive any adjuvant treatment at all. No difference in the distribution of adjuvant treatments was observed between the LCNEC-A and LCNEC-B patient subgroups.

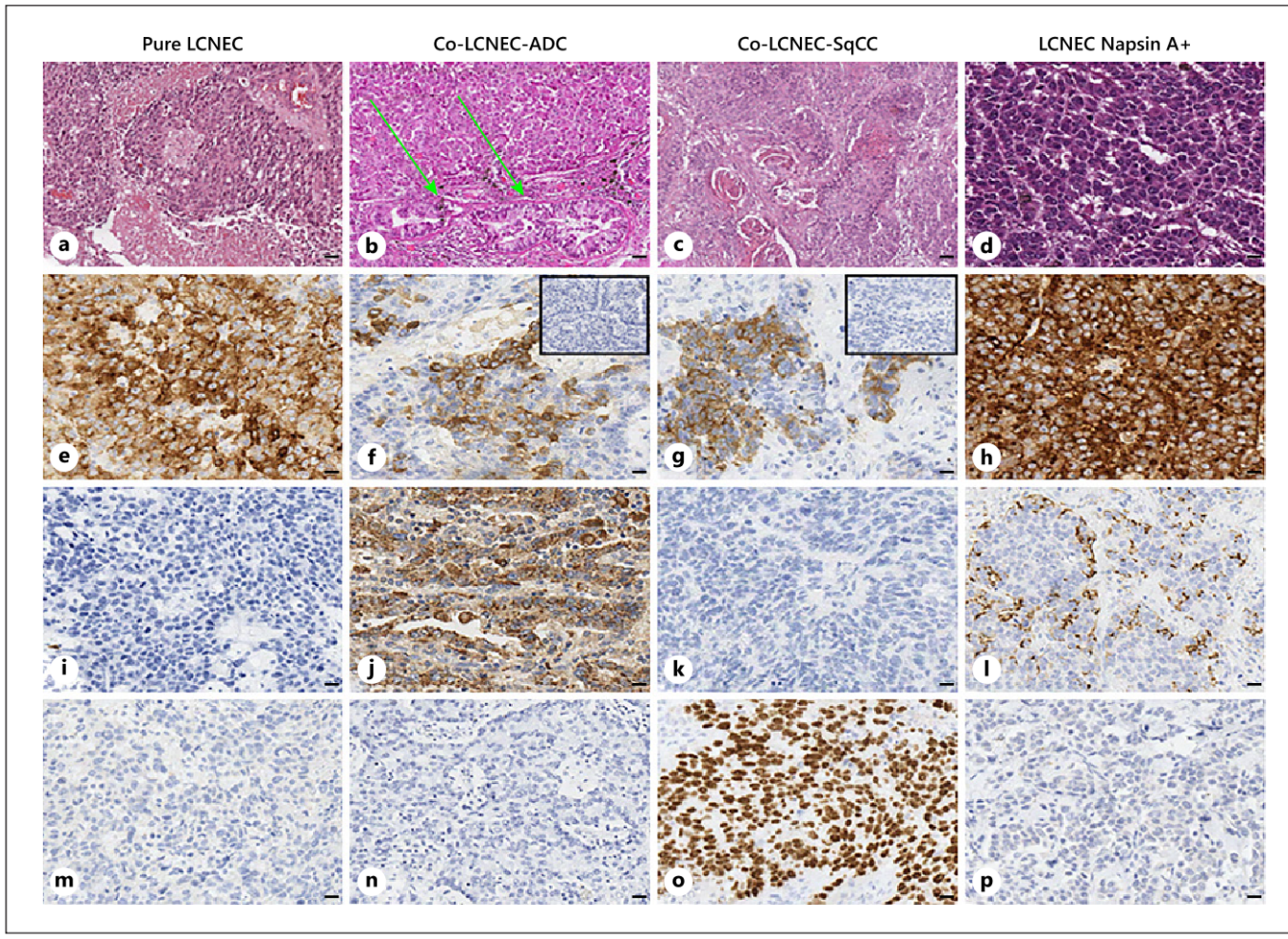


Fig. 1. LCNEC morphological and immunohistochemical spectrum. Pure LCNEC is represented by classic large neuroendocrine cells with abundant cytoplasm and large nuclei with evident nucleoli (**a**) strongly and diffusely staining for Syn (**e**); napsin A (**i**) and p40 (**m**) were not detected in pure LCNEC. Co-LCNEC showed in three combinations: (1) Co-LCNEC-ADC, with well-defined neoplastic glands (**b**, arrows) intermingled with large neoplastic neuroendocrine cells easily detectable only after Syn staining (**f**), and a glandular component negative for Syn (**f**, **inset**). In glandular structures strongly stained by napsin A (**j**), p40 was not detected in either of the aforementioned tumor counterparts (**n**). (2) Co-LCNEC-SqCC, with large neuroendocrine cells, easily detectable by Syn staining (**g**), associated with a squamous component (**c**) not stained by Syn (**g**, **inset**); these neoplastic cells show intense nuclear p40 staining (**o**), but no neoplastic cell shows reactivity against napsin A (**k**). (3) Co-LCNEC napsin A+, where neuroendocrine carcinoma is not distinguishable on morphological study (**d**) from the aforementioned pure LCNEC; its neuroendocrine nature is confirmed by intense and diffuse cytoplasmatic Syn staining (**h**). Of note, in contrast with pure LCNEC, a modest percentage of neoplastic cells shows napsin A immunostaining (**l**). No p40 staining is detected (**p**). Scale bars, 50 μm. ×20. LCNEC, large cell neuroendocrine carcinoma; Co-LCNEC, combined large cell neuroendocrine carcinoma; ADC, adenocarcinoma; SqCC, squamous cell carcinoma; Syn, synaptophysin.

Histopathological Findings

Seventy (63.1%) of the BP-LCNEC tumors were classified as pure LCNECs and 41 (36.9%) as Co-LCNECs (containing at least a 10% ADC or SqCC component). The most frequent sites of microscopic invasion for LCNECs were air spaces (74.8%; $n = 83$), followed by lymphatic and blood vessels (47.8%; $n = 53$), the bronchial wall (11.7%; $n = 13$), and perineural spaces (4.5%; $n = 5$) (Table 1). LCNECs showed focal necrosis in 29 cases (26.1%) and extensive map-like necrosis in 66 cases (59.5%) (Table 1).

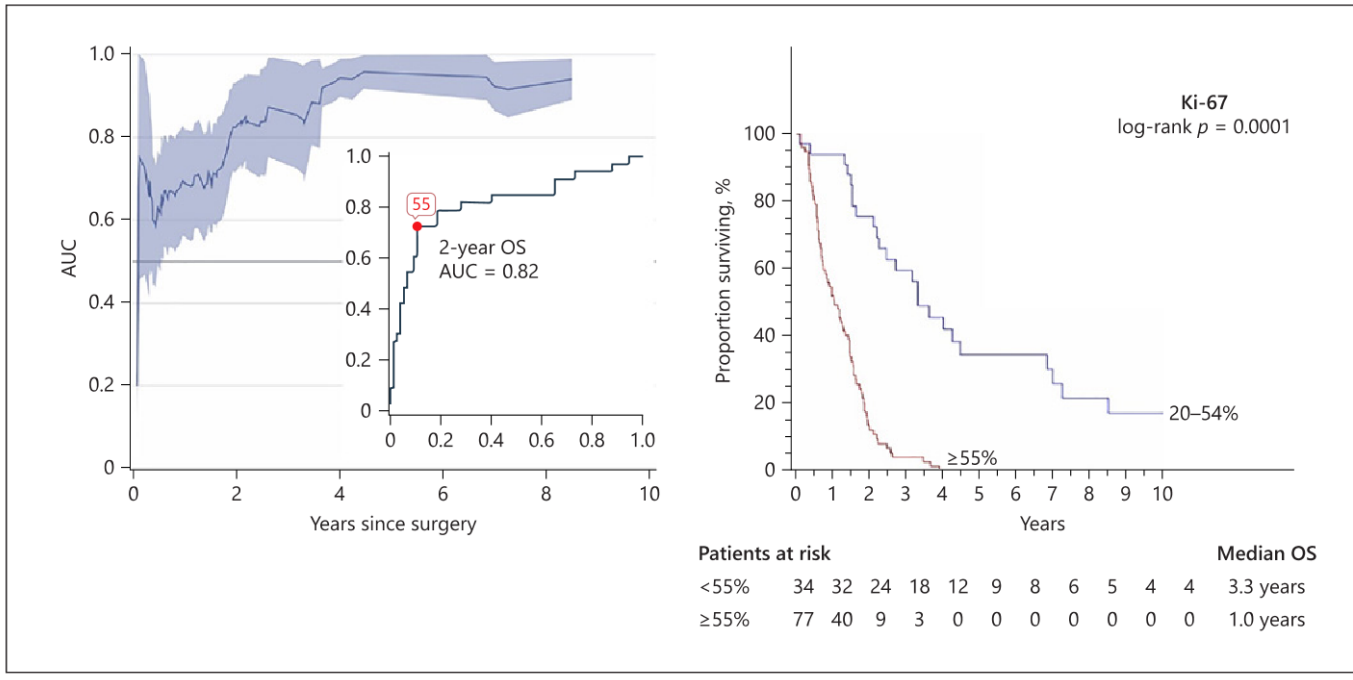


Fig. 2. Time-dependent area under the curve (AUC). Receiving operating characteristic curve for the prediction of 2-year mortality and overall survival (OS) of patients with large cell neuroendocrine carcinomas according to the Ki-67 index. The red dot indicates the optimal cutoff with the maximum Youden index value.

Morphology of Co-LCNECs. With the help of IHC for napsin A and p40 and histochemistry for Alcian blue, Co-LCNECs were further distinguished into 27 (24.3%) Co-LCNECs-ADC, 8 (7.2%) Co-LCNECs-SqCC, and 6 (5.4%) LCNECs showing only immunohistochemical napsin A positivity (LCNEC napsin A+) but no evidence of a distinct conventional ADC component [11] (Table 1; Fig. 1).

Proliferation Assessment

The median MC, evaluated in the LCNEC areas, was 34 mitotic figures per 2 mm^2, with a range from 13 to 78 mitotic figures per 2 mm^2, while the median Ki-67 value was 66%, with a range from 20 to 95% (Table 1). In all 41 Co-LCNECs, Ki-67 hot spots were found in the LCNEC component. No significant relationship was found between MC and Ki-67 (online suppl. Fig. 2). Using ROC analysis, we identified 55% as the best cutoff value for Ki-67 to predict patient survival (Fig. 2). The application of this cutoff allowed subdividing all LCNECs into two subgroups: 34 (30.6%) LCNECs-A (Ki-67 <55%) and 77 (69.3%) LCNECs-B (Ki-67 ≥55%). Likewise, 19 (46.3%) of the Co-LCNECs were reclassified as Co-LCNEC-A (Ki-67 <55%) and 22 (53.7%) as Co-LCNEC-B (Ki-67 ≥55%) (Table 1; online suppl. Fig. 3; online suppl. Tables 2, 3).

IHC Investigation

The distribution of IHC markers is reported in online supplementary Tables 2 and 3. All investigated markers showed heterogeneous expression. Positive immunoreactivity for p53 and negativity for RB1, respectively, were detected in 79 (71.2%) and 43 (38.7%) of the LCNECs; in particular, in the LCNEC-A subgroup, expression of p53 and negativity for RB1 were found in 21/34 (61.8%) and in 10/34 (29.4%) of the patients, respectively, while in the LCNEC-B subgroup, there were 58/77 (75.3%) p53-positive cases and 33/77 (42.9%) RB1-negative cases (without any statistically significant differences). MDM2 was more often expressed in the LCNEC-B subgroup (p = 0.01). TTF-1, CD44, OTP, SSTR2A, SSTR5, and mASH1 expression was not significantly different between the LCNEC-A and LCNEC-B subgroups. OTP was not expressed in any case.

SSTR2A and SSTR5 were expressed in 38 (34.2%) and 47 (42.3%) of the LCNEC cases, respectively, with no significant differences between the LCNEC-A and LCNEC-B subgroups. SSTR2A was expressed in 28/70 (40%) of

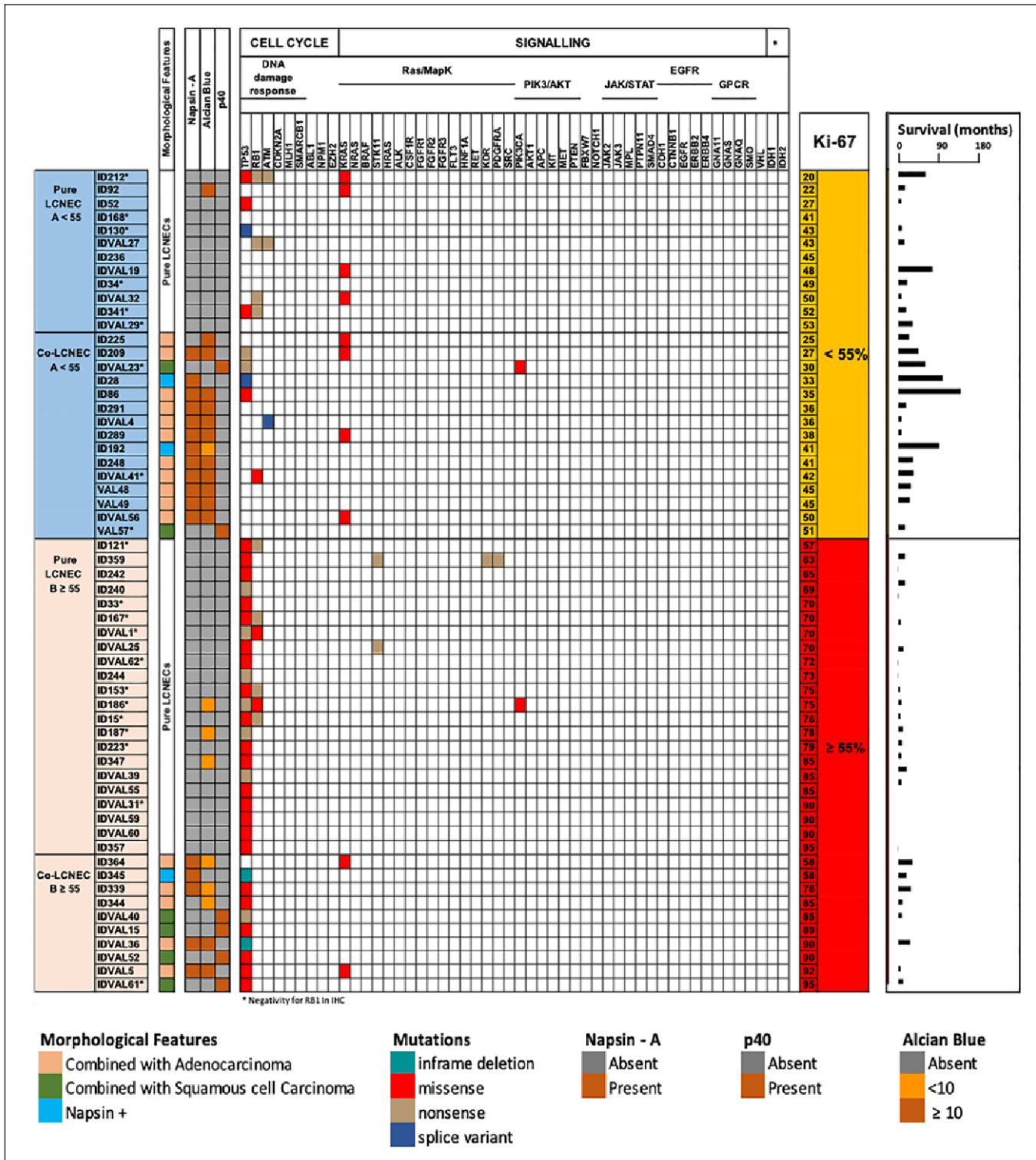
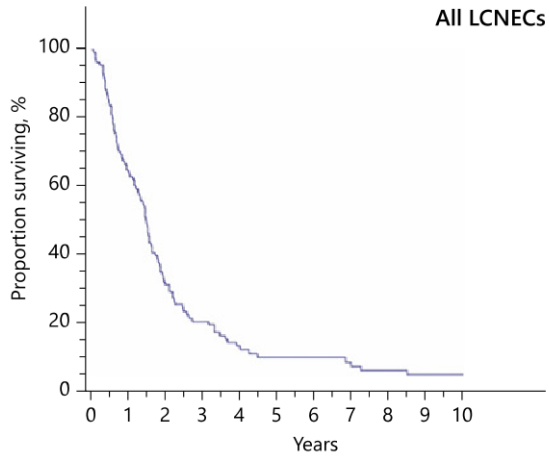
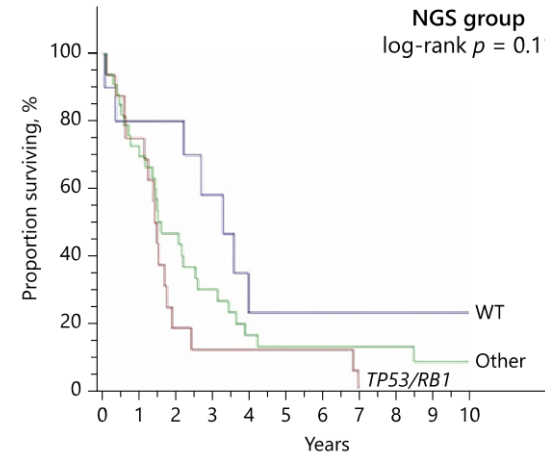


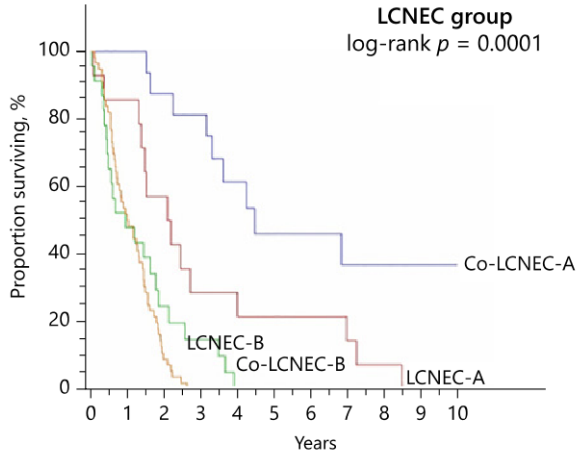
Fig. 3. Genomic alterations in large cell neuroendocrine carcinomas (LCNECs). The Ki-67 labelling index and somatic mutations are annotated for each patient grouped into pure and combined (Co-)LCNECs.



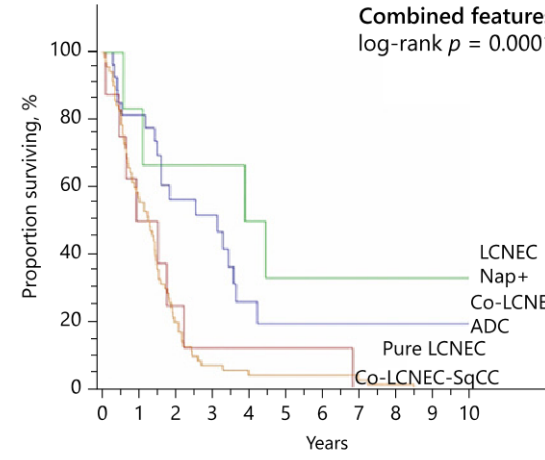
Patients at risk												Median OS
a	111	72	33	21	12	9	8	6	5	4	4	1.5 years



Patients at risk												Median OS
WT	10	8	8	5	2	1	1	1	1	1	1	3.3 years
TP53/RB1	16	12	3	2	2	2	2	–	–	–	–	1.5 years
b Other	33	24	14	9	5	4	3	3	3	2	2	1.6 years



Patients at risk												Median OS
Co-LCNEC-A	19	19	15	13	9	6	5	5	4	4	4	4.5 years
LCNEC-A	15	13	9	5	3	3	3	2	1	–	–	2.2 years
Co-LCNEC-B	22	12	4	3	–	–	–	–	–	–	–	1.1 years
c LCNEC-B	55	28	5	–	–	–	–	–	–	–	–	1.0 years



Patients at risk												Median OS
Co-LCNEC-ADC	27	22	13	11	5	3	2	2	2	2	2	3.1 years
Co-LCNEC-SqCC	8	4	2	1	1	1	1	–	–	–	–	1.2 years
LCNEC Nap+	6	5	4	4	3	2	2	2	2	2	2	4.2 years
d Pure LCNEC	70	41	14	5	3	3	3	2	1	–	–	1.3 years

	LCNEC-A napsin+	LCNEC-A napsin–	LCNEC-B napsin+	LCNEC-B napsin–
Co-LCNEC-A	16	3	–	–
LCNEC-A	–	15	–	–
Co-LCNEC-B	–	–	15	7
LCNEC-B	–	–	–	55

Fig. 4. OS in LCNECs according to selected characteristics. **a** All LCNECs. **b** NGS group. **c** LCNEC group. **d** Combined features with ADC, SqCC, or LCNEC napsin A+ (Nap+). LCNEC, large cell neuroendocrine carcinoma; OS, overall survival; ADC, adenocarcinoma; SqCC, squamous cell carcinoma; NGS, next-generation sequencing; WT, wild type.

Table 2. Univariate analysis of predictors of overall survival in patients with BP-LCNEC

Variable categories	HR (95% CI)[1]	*p* value
Age		
50–59 vs. <50 years	1.82 (0.79–4.22)	0.16
60–69 vs. <50 years	2.07 (0.96–4.43)	0.06
70+ vs. <50 years	**2.29 (1.08–4.84)**	**0.03**
Sex		
Female vs. male	0.91 (0.57–1.46)	0.70
Ki-67		
≥55 vs. <55%	**8.90 (4.31–18.4)**	**<0.0001**
Smoking		
Former vs. never-smoker	5.39 (0.53–54.4)	0.15
Current vs. never-smoker	5.80 (0.61–55.4)	0.13
Site		
Inferior vs. superior lobe	1.05 (0.54–2.06)	0.88
Hilum vs. superior lobe	1.01 (0.51–1.98)	0.98
Location		
Peripheral vs. central	0.54 (0.27–1.10)	0.09
Combined features		
Co-LCNEC-SqCC vs. Co-LCNEC-ADC	1.81 (0.67–4.93)	0.24
LCNEC napsin A+ vs. Co-LCNEC-ADC	0.59 (0.18–2.00)	0.40
Pure LCNEC vs. Co-LCNEC-ADC	**1.97 (1.05–3.71)**	**0.04**
Combined features		
Pure LCNEC vs. Co-LCNEC	**1.86 (1.10–3.14)**	**0.02**
Stage		
III/IV vs. I/II	**1.58 (1.00–2.52)**	**0.05**
Mitotic count		
>25 vs. ≤25	1.04 (0.57–1.90)	0.89
Necrosis		
Spot vs. absent	1.15 (0.56–2.38)	0.70
Map vs. absent	1.37 (0.71–2.64)	0.35
Microscopic infiltration		
Bronchus vs. air spaces	1.93 (0.94–3.95)	0.07
Pleura vs. air spaces	0.36 (0.06–2.12)	0.26
Absent vs. air spaces	**4.35 (1.77–10.7)**	**0.001**
Extra-lung vs. alveoli	0.60 (0.23–1.63)	0.32
Microscopic invasion		
Bronchus vs. no	1.84 (0.91–3.70)	0.09
Morphological pattern		
Trabecular vs. solid	1.96 (0.24–15.7)	0.53
Vascular invasion		
Present vs. absent	1.18 (0.73–1.92)	0.50
Perineural invasion		
Present vs. absent	2.31 (0.65–8.19)	0.19
Intratumoral lymphocytic infiltration		
Present vs. absent	1.43 (0.86–2.38)	0.16
Peritumoral lymphocytic infiltration		
Present vs. absent	1.03 (0.65–1.63)	0.90
Type or surgery		
Lobectomy vs. partial	1.18 (0.71–1.97)	0.53
Bilobectomy vs. partial	1.16 (0.62–2.20)	0.64
Resection margin		
R1/2 vs. R0	0.98 (0.48–2.00)	0.94
Napsin A		
Present vs. absent	**0.39 (0.21–0.70)**	**0.002**
p40		
Present vs. absent	1.99 (0.92–4.29)	0.08
TTF-1		
Present vs. absent	0.63 (0.39–1.03)	0.06
Alcian blue		
Present vs. absent	0.47 (0.21–1.07)	0.07
CD44		
Present vs. absent	1.46 (0.76–2.79)	0.25
SSTR2A		
Present vs. absent	1.03 (0.66–1.62)	0.89
SSTR5		
Present vs. absent	0.82 (0.53–1.28)	0.39
mASH1		
Present vs. absent	**0.57 (0.33–1.00)**	**0.05**
p53		
1–50% vs. absent	0.96 (0.52–1.76)	0.89
>50% vs. absent	0.69 (0.42–1.15)	0.15
p53		
Present vs. absent	0.76 (0.47–1.22)	0.25
RB1		
1–50% vs. absent	0.83 (0.43–1.60)	0.58
>50% vs. absent	0.82 (0.50–1.36)	0.45
RB1		
Present vs. absent	0.83 (0.52–1.32)	0.43
MDM2		
Present vs. absent	**0.52 (0.31–0.86)**	**0.01**
NGS		
Mutated vs. wild type	1.05 (0.33–3.33)	0.94
TP53		
Mutated vs. wild type	0.77 (0.33–1.78)	0.54
RB1		
Mutated vs. wild type	1.24 (0.58–2.69)	0.58
ATM		
Mutated vs. wild type	2.24 (0.61–8.22)	0.22
KRAS		
Mutated vs. wild type	1.20 (0.53–2.74)	0.66
STK11		
Mutated vs. wild type	1.24 (0.28–5.58)	0.78
NGS group		
Double *TP53/RB1*[2] vs. wild type	0.95 (0.27–3.30)	0.93
Other mutation vs. wild type	1.11 (0.34–3.67)	0.86

Bold type denotes significance. BP, bronchopulmonary; LCNEC, large cell neuroendocrine carcinoma; Co-LCNEC, combined large cell neuroendocrine carcinoma; SqCC, squamous cell carcinoma; ADC, adenocarcinoma; NGS, next-generation sequencing. [1] Stratified by center and adjusted for Ki-67. [2] *TP53* mutated and *RB1* mutated or immunohistochemistry for RB1 = 0.

Table 3. Multivariable analysis[1] of predictors of overall survival in patients with BP-LCNEC

Variable	Categories	Multivariable 1 HR (95% CI)	*p* value	Multivariable 2 HR (95% CI)	*p* value	Multivariable 3 HR (95% CI)	*p* value
Age (years)	Per 10-year increase	**1.33 (1.04–1.69)**	**0.02**	**1.30 (1.02–1.66)**	**0.04**	**1.27 (1.00–1.62)**	**0.05**
Sex	Female vs. male	0.63 (0.38–1.05)	0.08	0.69 (0.42–1.16)	0.16	–	
Ki-67 index	≥55 vs. <55%	**9.39 (4.33–20.4)**	**<0.0001**	**8.65 (4.00–18.7)**	**<0.0001**	**7.81 (3.71–16.5)**	**<0.0001**
Location	Central vs. peripheral	**2.52 (1.19–5.31)**	**0.02**	**2.63 (1.24–5.60)**	**0.01**	**2.48 (1.17–5.24)**	**0.02**
Combined features	Pure vs. combined	**1.73 (1.00–2.97)**	**0.049**	–	–	–	
Stage	III/IV vs. I/II	1.52 (0.91–2.53)	0.11	1.36 (0.81–2.27)	0.24	–	
Napsin A	Absent vs. present	–	–	**2.31 (1.24–4.33)**	**0.009**	**2.56 (1.41–4.67)**	**0.002**

BP, bronchopulmonary; LCNEC, large cell neuroendocrine carcinoma. [1] Stratified by center.

the pure LCNECs, 6/27 (22.2%) of the Co-LCNECs-ADC, and 1/8 (12.5%) of the Co-LCNECs-SqCC. Napsin A immunostaining was positive in 31 (27.9%) of the cases, with a higher percentage (47.1%; $n = 16$ cases) in the LCNEC-A subgroup ($p = 0.005$).

Molecular Analysis

A total of 59 (53.2%) LCNEC (27 LCNEC-A and 32 LCNEC-B) samples were analyzed by targeted NGS using the "Ampliseq cancer hotspot panel" (online suppl. Tables 2, 3; online suppl. Fig. 3).

Overall, 49 of the 59 LCNECs (83.1%) showed at least one gene mutation by NGS, mainly represented by *TP53* (64.4%), *RB1* (18.6%), and *KRAS* (18.6%) mutations, while only 10/59 (16.9%) of the cases were wild type. The cases with at least one mutation were 17/27 (63%) of the LCNECs-A and 32/32 (100%) of the LCNECs-B.

Mutations in one or more of the 50 genes examined were significantly enriched in the LCNEC-B subgroup (32/32 [100%]; $p = 0.0001$). With regard to *TP53* mutations, most occurred in the LCNEC-B subgroup (30/32 [93.8%]; $p < 0.0001$), while only 8/27 cases of LCNECs-A (26.6%) had *TP53* mutation.

RB1 mutation was observed in 5/27 (18.5%) of LCNECs-A and 6/32 (18.8%) of LCNECs-B, with no statistical difference. Mutation of *TP53* combined with *RB1* mutation or loss of expression was found in 16/59 (27.1%) of the cases, 4/27 (14.8%) belonging to subgroup A and 12/32 (37.5%) belonging to subgroup B, with a significant difference between the two groups ($p = 0.0002$).

KRAS mutation was found in 8/27 (29.6%) cases of LCNEC-A and in 3/32 (9.4%) cases of LCNEC-B, with no statistical difference. *ATM* mutations occurred in 4/27 (14.8%) of LCNECs-A and *STK11* mutations in 2/32 (6.2%) of LCNECs-B. No *JAK2* and *EGFR* mutations were found.

Molecular Features in Co-LCNECs. Mutations in one or more of the 50 genes examined were significantly enriched in the Co-LCNEC-B subgroup (10/10 [100%]) (online suppl. Table 2). The 13/25 (52%) cases with a single mutation of *TP53* were significantly ($p = 0.004$) more frequent in Co-LCNECs-B than in Co-LCNECs-A (online suppl. Table 3). Mutations of *TP53* combined with mutations or loss of expression of *RB1* were more frequently detected in pure LCNECs (87.5% of the cases) and in Co-LCNECs-SqCC (12.5%) than in Co-LCNECs-ADC and LCNECs napsin A+ (0%, respectively; online suppl. Table 3). The distribution of LCNECs-A and LCNECs-B among the different histological subtypes of cases examined by NGS is reported in Figure 3 and online supplementary Table 3.

Survival Analysis

Median OS (mOS) was 1.5 years with all LCNECs (Fig. 4a). On Kaplan-Meier analysis, patients with LCNEC-B had significantly worse OS than patients with LCNEC-A (log-rank $p = 0.0001$; Fig. 4b). This was verified both in pure LCNEC and Co-LCNEC (log-rank $p = 0.0001$; Fig. 4c). Co-LCNEC-ADC and LCNEC napsin A+ cases had longer OS than pure LCNEC and Co-LCNEC-SqCC cases (Fig. 4d). Interestingly, although not statistically significant, patients with *TP53* mutations combined with *RB1* mutation/loss of expression had a mOS of 1.5 years ($n = 16$), while those with wild-type *TP53* and *RB1* had a mOS of 3.3 years ($n = 10$; Fig. 4b).

The mOS for patients with stage I, II, III, and IV disease was 1.6, 1.4, 1.5, and 0.9 years, respectively (log-rank $p = 0.06$; online suppl. Fig. 4A). A significant difference

in OS according to stage was observed for both Co-LCNEC-A and Co-LCNEC-B, but not for pure LCNEC-A and LCNEC-B (online suppl. Fig. 4B–E).

Factors associated with poor prognosis significant on univariable analysis are reported in Table 2 and include age ≥70 years ($p = 0.03$), Ki-67 ≥55% ($p < 0.0001$), pure LCNEC histology versus Co-LCNEC histology ($p = 0.02$), stage III/IV versus I/II ($p = 0.05$), microscopic infiltration of air spaces ($p = 0.01$), absent napsin A positivity ($p = 0.002$), absent mASH1 staining ($p = 0.05$), and absent MDM2 immunostaining ($p = 0.01$). On multivariable analysis, age (per 10-year increase; HR 1.27; 95% CI: 1.00–1.62; $p = 0.05$), Ki-67 (≥55 vs. <55%; HR 7.81; 95% CI: 3.71–16.5; $p < 0.0001$), location (central vs. peripheral; HR 2.48; 95% CI: 1.17–5.24; $p = 0.02$), and expression of napsin A (absent vs. present; HR 2.56; 95% CI: 1.41–4.67; $p = 0.002$) were independent unfavorable prognostic factors (Table 3).

Discussion

Although BP-LCNECs are classified as high-grade NECs because of their morphological, phenotypic, and prognostic characteristics, similar to those of BP-SCNECs, population-based studies have demonstrated that early-stage (I/II) BP-LCNEC behaves akin to NSCLC, whereas advanced stage (IV) tumors are more similar to SCNEC [16, 34]. In early stages, surgery is recommended but does not seem to be sufficient. As for NSCLCs, platinum-based adjuvant chemotherapy may also be considered for BP-LCNECs, whilst the potential role of neoadjuvant chemotherapy is still not yet defined. For patients with advanced BP-LCNEC, the chemotherapy regimens used in BP-SCNECs, based on the combination of platinum plus etoposide, remain the gold standard, but long-term disease control is unsatisfactory [35–37]. No definite role is attributed to immunotherapy and targeted therapies for BP-LCNECs [38–40]. The poor prognosis, the lack of treatment options, and the presence of peculiar features of these neoplasms emphasize an emerging need to deepen our understanding of the biological, phenotypical, and biomolecular aspects of these tumors.

The current multicenter study includes one of the largest collections of BP-LCNECs, analyzing 111 patients with a special focus on detecting clinical, morphological, immunohistochemical, and molecular parameters of prognostic value. Of the clinicopathological parameters examined, only age, Ki-67, central location, pure versus combined histology, microscopic invasion of the bronchial wall, stage III/IV, absent napsin A immunoexpression, and absent mASH1 and MDM2 immunostaining – but not MC – were associated with shorter OS on univariate analysis, while only age, Ki-67, peripheral versus central location, pure versus combined histology, and napsin A immunostaining maintained their prognostic value on multivariable analysis.

From a clinical point of view, it is well known that elderly patients generally have a worse prognosis [34]. Moreover, the pure forms of BP-LCNEC here examined had a worse prognosis than tumors combined with NSCLC subtypes, which are generally less aggressive, a finding that has not been registered in previous studies [6, 9, 10]. Finally, the central forms are prognostically worse than the peripheral ones [41], probably due to involvement of large vessels by the disease and due to treatment choices, as central tumors are less often treated with surgery.

However, our study mostly demonstrates that the prognosis of BP-LCNEC is conditioned by Ki-67, as has been shown for LCNEC of other sites of origin. Ki-67 has proven to be significantly superior to MC, and to any other morphological (pure vs. combined histology, and invasion of air spaces or the bronchial wall), immunohistochemical (p53, RB1, napsin A, p40, TTF-1, SSTR2A, SSTR5, and CD44), or molecular (*TP53* or *RB1* or combined *TP53*/*RB1* double mutations or *TP53* mutation combined with RB1 immunonegativity, or *KRAS*, *ATM*, and *STK11* mutations) characteristic.

The 2015 WHO classification recommends Ki-67 assessment of BP-NEN as a vague complementary tool in the differential diagnosis of BP-NEN but does not use it as a prognostic grading instrument and relies very much on MC as a proliferative parameter. In our study, we found that MC is confirmed to be essential in the definition of BP-LCNEC; however, its median range resulted higher (median range 34 mitoses per 2 mm^2) than the actual suggested WHO value (>10 mitoses per 2 mm^2). Furthermore, the MC's prognostic role is statistically insignificant compared to that of Ki-67, suggesting a systematic use of Ki-67 in all BP-LCNEC cases. Moreover, no significant correlation between MC and Ki-67 was detected in our cases. Moreover, we showed that a Ki-67 threshold of 55% could be considered the main prognostic determinant in LCNEC, in agreement with previous findings reported for GEP-NECs and GEP-MANECs [26–29].

Indeed, cellular heterogeneity is a well-known phenomenon in lung cancer [42, 43], and almost 50% of pri-

mary tumors show more than one of the major histological types simultaneously [9, 44–46]. Similarly to several published studies [11, 45, 47], in our series we confirmed the diagnosis of Co-LCNEC both in combination with ADC and SqCC and described cases of napsin A+/LCNEC with no detectable histological glandular component, associated with a more favorable outcome of the patients.

SSTR2A and SSTR5 tissue distribution was also assessed, revealing a prevalence of SSTR2A in pure LCNECs, a finding that should be connected with innovative data obtained with multiple SCLC models [48, 49], and that could be reproduced also for LCNEC. Interestingly, correlations between molecular alterations, Ki-67 subclasses A and B, and pure or combined histological types in our work reveal that pure LCNECs with either co-mutation of *TP53* and *RB1*, or with mutation of *TP53* and absence of RB1 immunolabelling, demonstrate characteristics of SCLC-like LCNEC. Although several other investigations [e.g., 17] have reported similar results, our study shows that this molecular pattern is significantly enriched in cases with Ki-67 ≥55% (LCNEC-B) and associated with shorter OS; on the other hand, tumors with *KRAS* mutations are enriched in cases with Ki-67 <55% (LCNEC-A), including cases of Co-LCNEC-ADC. All these findings suggest a possible role of Ki-67 with a cutoff at 55% in predicting involvement of certain molecular alteration patterns.

In conclusion, our large series of BP-LCNECs reveals that the outcome of these neoplasms is mainly predicted by Ki-67 and that different phenotypic, molecular, and prognostic relationships exist between pure and composite LCNECs. Our results can generate some propositions. First of all, in LCNECs with Ki-67 ≥55% with advanced disease, the use of first-line platinum-based chemotherapy is mandatory, while in LCNECs with Ki-67 <55%, as previously suggested by Hijioka et al. [50] and Sorbye et al. [51], and for GEP-NECs, non-platinum-based regimens may also be considered [26]. Although few data are as yet available, given the highest number of mutations reported in NECs with Ki-67 ≥55%, BP-LCNECs with Ki-67 ≥55% could mostly benefit from immunotherapy, alone or in combination with platinum-based chemotherapy or radiotherapy [29, 52].

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Statement of Ethics

The study was performed according to the clinical standards of the 1975 and 1983 Declaration of Helsinki and was approved by the Ethics Committee of Fondazione IRCCS INT (No. INT 171/16).

Conflict of Interest Statement

The authors have no relevant conflicts of interest pertaining to this article.

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Author Contributions

M.M. and C.C.: study concept and design; G.C., G.G., and L.C.: acquisition of data; M.M., A.M., G.C., C.C., F.G., N.P., and A.B.: analysis and interpretation of data; A.M., M.M., C.C., and F.G.: drafting of the manuscript; A.M., F.G., S.P., P.B., P.S., A.P., G.C., A.D.G., S.F., K.K., G.P., L.R., E.R., L.B., A.T., M.R.B., M.S.G., R.R., A.B., and U.P.: critical revision of the manuscript for important intellectual content; P.M.: statistical analysis; M.M.: obtained funding; A.M. and G.C.: administrative, technical, or material support; M.M. and C.C.: study supervision.

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