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Lung carcinoid tumours: histology and Ki-67, the eternal rivalry

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Lung carcinoid tumours: histology and Ki-67, the eternal rivalry

WHO classification of Thoracic Tumours defines lung carcinoid tumours (LCTs) as well-differentiated neuroendocrine neoplasms (NENs) classified in low grade typical (TC) and intermediate grade atypical carcinoids (AC). Limited data exist concerning protein expression and morphologic factors able to predict

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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.

Abbreviations: AC, atypical carcinoid; Ascl1, mammalian achaete-scute homologue 1; AUC, area under the curve; CgA, chromogranin-A; CI, confidence interval; CSS, cancer-specific survival; DFS, disease-free survival; HR, hazard ratio; H&E, haematoxylin–eosin; IHC, immunohistochemical; LCTs, lung carcinoids tumours; LR−Δχ2, changes in likelihood ratio values; Lu-NET, lung neuroendocrine tumour; MC, mitotic count; NEN, neuroendocrine neoplasm; NET, neuroendocrine tumour; OS, overall survival; OTP, orthopedia homeobox protein; P adj, adjust P-values; ROC, receiver operating characteristic; Syn, synaptophysin; SSTR-2A, somatostatin receptor 2A; STAS, spread through air spaces; TC, typical carcinoid; TTF-1, thyroid transcription factor 1; WHO, world health organization.

disease aggressiveness. Though Ki-67 has proved to be a powerful diagnostic and prognostic factor for Gastro-entero-pancreatic NENs, its role in lung NENs is still debated. A retrospective series of 370 LCT from two oncology centers was centrally reviewed. Morphology and immunohistochemical markers (Ki-67, TTF-1, CD44, OTP, SSTR-2A, Ascl1, and p53) were studied and correlated with Overall Survival (OS), Cancer-specific survival (CSS) and Disease-free survival (DFS). Carcinoid histology was confirmed in 355 patients: 297 (83.7%) TC and 58 (16.3%) AC. Ki-67 at 3% was the best value in predicting DFS. Ki-67 ≥ 3% tumours were significantly associated with AC histology, stage III-IV, smoking, vascular invasion, tumour spread through air spaces OTP negativity, and TTF-1, Ascl1 and p53 positivity. After adjustment for center and period of diagnosis, both Ki-67 (≥3 versus <3) and histology (AC versus TC) alone significantly added prognostic information to OS and CSS multivariable model with age, stage and OTP; addition of both variables did not provide further prognostic information. Conversely, an improved significance of the DFS prediction model at multivariate analysis was seen by adding Ki-67 (≥3 versus <3, *P* adj = 0.01) to TC and AC histological distinction, age, lymph node involvement, residual tumour and OTP. Ki-67 ≥ 3% plays a potentially pivotal role in LCT prognosis, irrespective of histological grade.

Keywords: lung carcinoid tumours, neuroendocrine neoplasms, Ki-67 index, OTP, immunohistochemistry

Introduction

Lung neuroendocrine tumours (Lu-NETs) comprise low- and intermediate- grade well-differentiated carcinoids, distinguished as typical (TC) and atypical carcinoids (AC) according to mitotic count (MC) and necrosis.[1] In detail: TCs show <2 mitoses per 2 mm^2 and absence of necrosis, while ACs show 2–10 mitoses and/or punctate foci of necrosis.[2]

TCs and ACs represent rare entities accounting for approximately 1%–2% of all lung tumours with a TC to AC ratio of 8–10:1.[3, 4] The precise histologic distinction between TC and AC represents a crucial clinical prognostic predictor, in fact, 5-year survival rate for TC is 82%–100% while it is 50%–68% for AC.[1]

Although a few diagnostic and prognostic markers, such as Orthopedia Homeobox (OTP) and the cell surface receptor CD44,[5, 6] have emerged and correlated with patients' prognosis and survival, the role of protein expression and morphologic factors able to predict disease aggressiveness and progression still remain largely unknown. Even today, carcinoid diagnosis relies solely on morphologic parameters such as MC and/or necrosis, while Ki-67 assessment is recommended, but not mandatory. Diversely, in Gastro-entero-pancreatic neuroendocrine neoplasms (GEP-NENs), Ki-67 represents a standard marker, strongly correlated with patients' prognosis.[7] Regarding to lung carcinoids tumours (LCTs), increasing evidence has however highlighted the fundamental role of Ki-67 evaluation as a prognostic factor, providing new insights to the current World Health Organization (WHO) classification.[8]

The present study aims to evaluate the role of Ki-67 proliferation index and to examine its correlation with disease evolution and recently proposed immunohistochemical markers, including OTP, CD44, TTF-1, Ascl1, p53, and SSTR-2A on 355 cases of LCTs (297 TCs and 58 ACs).

Materials and Methods

STUDY DESIGN AND CASE SELECTION

The surgical pathology and clinical databases of two Italian oncology centers (Fondazione IRCCS Istituto Nazionale dei Tumori – INT, Milan and ASST Spedali Civili di Brescia – Brescia), between 1988 to 2018, were retrospectively searched for one of the following histologic diagnoses: "typical lung carcinoid", "atypical lung carcinoid", "lung carcinoid tumor", "peripheral carcinoid", and "bronchial carcinoid". Exclusion criteria were: (1) cases which had not undergone surgical resection with curative intent; (2) cases with poorly differentiated neuroendocrine components; (3) cases for which only bioptic samples were available; (4) cases in which the primary was of dubious lung origin (eg. lung metastases from other sites). A total of 370 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 tumour morphology were centrally and blindly reviewed by expert pathologists in Lu-NET prior to inclusion in the study (M.M. and

C.C). Carcinoid identification and morphologic characterization were based on parallel investigation of at least three consecutive sections from representative FFPE blocks, stained with haematoxylin–eosin (H&E), and for Synaptophysin (Syn) and Chromogranin A (ChgA). A total of 355 cases met all the carcinoid morphologic criteria of the current WHO Classification of Thoracic Tumours (WHO-TT 2021) and were included in the study.[1] The cases excluded from the study were: 5 tumorlets, 2 adenocarcinomas, 4 large cell neuroendocrine carcinomas, 1 combined large cell neuroendocrine carcinoma with adenocarcinoma, 2 large cell carcinomas and 1 NE-cell hyperplasia.

HISTOLOGIC ANALYSIS AND IMMUNOHISTOCHEMISTRY

Morphologic analysis considered: (a) well differentiated neuroendocrine morphology; (b) architectural pattern of the tumour registered as: (1) trabecular/nesting/organoid and (2) insular/solid; (c) MC recorded per 2 mm^2 and evaluated in the areas of highest mitotic activity in which the entire microscopic field consisted of tumour cells according the guidelines WHO-TT 2021; (d) presence/absence of necrosis; (e) pathologic tumour staging according to the Union for International Cancer Control/American Joint Committee on Cancer (UICC/AJCC) 8th edition; (f) vascular invasion (evaluated on H&E- and/or CD31-stained sections); (g) perineural invasion; (h) intra and/or peritumoral lymphocyte infiltrate; (i) microscopic invasion of bronchial wall or pleura, (j) tumour spread through air spaces (STAS).

The immunohistochemical (IHC) study included: Synaptophysin and Chromogranin-A in order to confirm the diagnosis of lung NEN; Ki-67 labeling index calculation, using the MIB antibody as a percentage of positive cells in 500–2.000 tumour cells counted in areas of strongest nuclear labeling ("hot spots") as indicated in the WHO 2019 Digestive System Tumours; thyroid transcription factor 1 (TTF-1), CD44, orthopedia homeobox protein (OTP), somatostatin receptor 2A (SSTR-2A), mammalian achaetescute homologue 1 (Ascl1), and p53 using the antibodies listed in Table S1.

To minimize assessment variability, with the exception of p53 and SSTR-2A, all markers were considered positive regardless of the number of positive cells. p53 were evaluated using 4 levels: Absent (no expression), weak heterogeneous (scatterd and weak staining in 1%–20% of tumour cells), heterogeneous (variable expression in 21%–60% of tumour cells) and overexpressed (strong p53 staining in more than 60% of tumour cells). Immunoreactivity and scores for SSTR-2A were evaluated using a two-tiered system as suggested by Volante *et al.* negative for scores of 0 and 1 and positive for 2 and 3 positivity.[9] For OTP, TTF-1 and Ascl1 only nuclear staining was considered, while for CD44 only membranous cytoplasmic staining was registered.

STATISTICAL ANALYSIS

Data were analysed by descriptive statistics. Associations between demographic characteristics, clinicopathological features and Ki-67 groups (≥3% versus <3%), were assessed using the Fisher exact test for categorical variables and the nonparametric Wilcoxon test for continuous variables.

Ki-67 cut-off values that best identify subjects with early relapse (within 4 years from surgery) were evaluated with receiver operating characteristic (ROC) curve analysis. The area under the curve (AUC) was calculated to determine the diagnostic value of the test. The optimal cut-off value for Ki-67 was determined using the Youden index, which maximizes sensitivity and specificity. OS and CSS were assessed from the date of diagnosis to date of death for any cause or tumour-associated death, respectively. DFS was assessed from the date of diagnosis to the date of first relapse, tumour-associated death or last follow-up, whichever occurred first. Deaths unrelated to cancer were censored in the CSS or DFS survival analysis. CSS and DFS curves were drawn using the Kaplan–Meier method. The log-rank test was used to assess the survival difference between patient groups. Pearson's correlation coefficients was used to correlate Ki-67 proliferative index with duration of block storage. Univariable and multivariable Cox proportional regression models were used to assess the association between clinico-pathologic characteristics and OS, DFS and CSS. Variables that had a statistically significant ($P < 0.1$) association with the outcomes in univariate were added to a Cox proportional regression analysis. Manual backward elimination was used to determine the best combination of predictors prioritizing the clinically relevant variables. Hazard ratio (HR) are presented with respective 95% confidence interval (CI). For multivariable analyses, each variable was added separately to a baseline model to determine the prognostic information added by inclusion of the variable of interest. Changes in likelihood ratio values (LR$-\Delta\chi 2$) were used to measure and compare the relative amount of information of one model compared to the other. Data analysis was performed using the R environment for statistical

computing and graphics (R Foundation, Vienna, Austria - Version 4.0.3). All tests were two-sided and *P*-values <0.05 were considered statistically significant.

Results

CLINICOPATHOLOGIC FEATURES AND TREATMENT

The flowchart and the main clinicopathological features of the 355 carcinoid patients included in the study are summarized in Figure 1 and Table S2, respectively. Overall, pathologic review identified 297 (83.7%) TCs and 58 (16.3%) ACs. The whole cohort comprised more females than males (62.3% versus 37.7%) with a median age of 60 years. The series included 264 (74.4%) stage I, 48 (13.5%) stage II, 33 stage III (9.3%) and 10 (2.8%) stage IV tumours. The most advanced surgicaly-resected tumours (stage III-IV) were ACs while TCs had the highest number of stage I cases. Former and current smokers had mostly AC (35.1% and 36.8%, respectively). All patients underwent surgical resection with curative intent, including 105 (29.6%) segmentectomies or wedge resections, 213 (60.0%) lobectomies and 37 (10.4%) bilobectomies or pneumonectomies. Data on treatment (pre- and/or postoperative) were available for 217 (61.1%) patients: 14 (6.5%) received somatostatin analogues, 5 (2.3%) chemotherapy, 2 (0.9%) radiotherapy, 3 (1.4%) combined chemo-radiotherapy and 193 (88.9%) did not receive any treatment at all.

PROLIFERATION ASSESSMENT AND MORPHOLOGIC FEATURES

Ki-67 labelling index was evaluated for 317 (89.3%) patients with a median of 1.1% and range from 0% to 26%. ACs showed statistically significant higher Ki-67 values compared to TC ($P < 0.0001$). Three cases showed a Ki-67 index >20% and were considered as highly proliferative carcinoids/grade 3 NETs. Using ROC curve analysis, we identified 3% as the best cut-off value for Ki-67 to predict disease free survival (AUC = 0.74) and time dependent AUC curve demonstrated that this cut-off was reliable throughout the duration of follow-up (Figure 2). Application of this cut-off divided the entire cohort into two groups: 260 (82.0%) with low Ki-67 (<3%) and 57 (18.0%) with high Ki-67 (≥3%) (Table 1). Tumours with high Ki-67 were associated with AC histology (n = 46, 80.7%, $P < 0.0001$), stage III-IV (n = 15, 26.3%, $P = 0.004$), former and current smoking status (n = 17, 32.7% and n = 22, 42.3%, $P = 0.001$), presence of necrosis (n = 14, 24.6%, $P < 0.0001$), vascular invasion (n = 20, 37.0%, $P = 0.01$), peritumoral lymphocyte infiltrate (n = 17, 32.7%, $P = 0.02$), presence of STAS (n = 20, 39.2%,

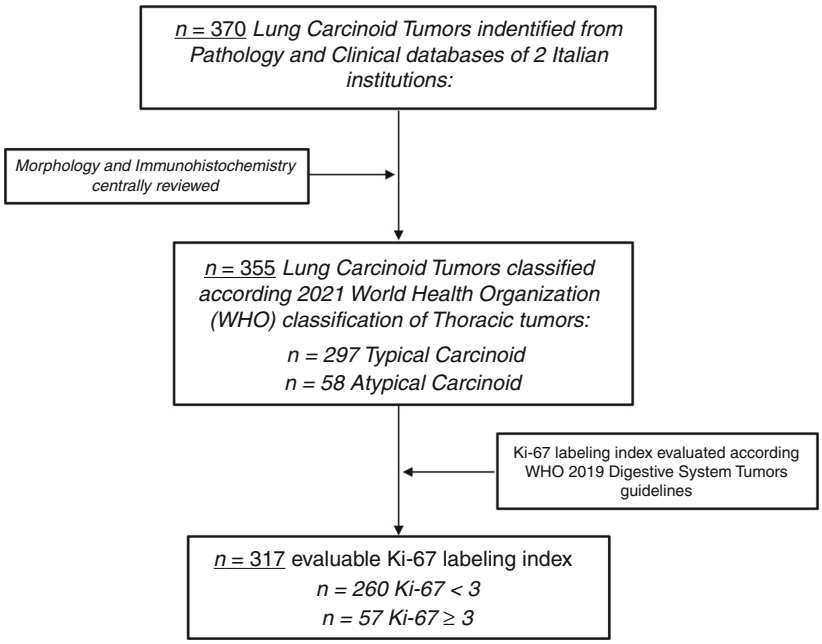

Figure 1. Workflow of the study.

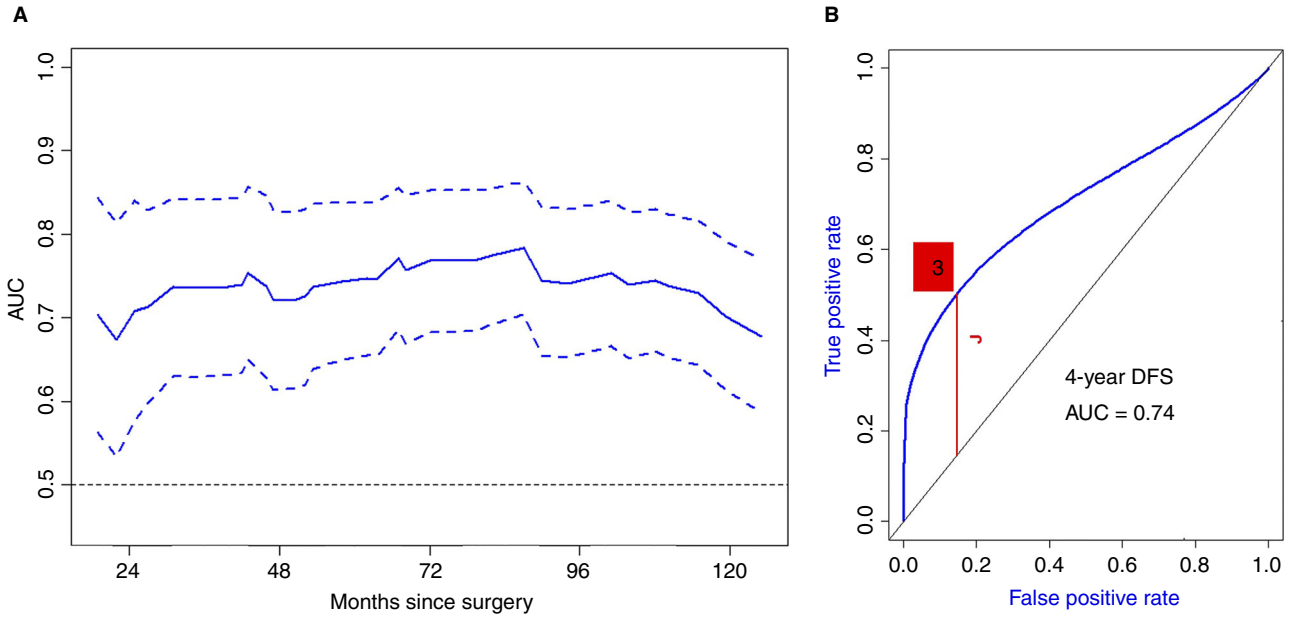

Figure 2. **A**. Time-dependent area under the curve for DFS; **B**. ROC curve for the prediction of 4-year DFS of patients with LCTs, according to Ki-67 index. The red square indicates the optimal cutoff with the maximum Youden index value. Abbreviations: DFS, disease-free survival; ROC, receiver operating characteristic; LCTs, lung carcinoids tumours.

$P < 0.0001$) and solid morphologic pattern ($n = 39$, 70.9%, $P < 0.0001$).

KI-67 LABELLING INDEX EVALUATED OVER THE TIME

Ki-67 labelling staining amounts are known to decrease over time, appearing less in older tissue blocks.[10] Indeed, in Figure S1 we reported that there was a significant mild negative correlation between Ki-67 labelling index and duration of block storage (Pearson's correlation coefficient = −0.18, $P = 0.0012$). Therefore we adjusted all the univariable and multivariable analysis for period of diagnosis, categorized in decades, in order to avoid time-dependent effects that can impact survival.

IMMUNOHISTOCHEMICAL MARKERS

The distribution of all investigated IHC markers were reported in Table 2 and Table S3. Nuclear OTP expression was more often present in the low Ki-67 group ($n = 177$, 78.7% $P < 0.0001$). Positive immunoreactivities for Ascl1 and TTF-1 were detected in 91 (32.2%) and 49 (17.1%) of all carcinoids, respectively; specifically, in the low Ki-67 group, expression of Ascl1 and TTF-1 were found in 61 (26.8%) and in 30 (13.0%) of the patients, respectively, while in the high Ki-67 group, there were 30 (54.5%) Ascl1 positive cases and 19 (34.5%) TTF-1 positive cases with statistically significant differences between groups. Expression of p53 was observed mainly in high Ki-67 group ($n = 6$, 11.1% weak heterogenus and $n = 1$ 1.9% overexpressed, $P < 0.0001$). Finally, SSTR-2A and CD44 expression were not significantly different in the two subgroups.

OVERALL, CANCER-SPECIFIC AND DISEASE-FREE SURVIVAL

Overall, 58 patients (16.3%) showed tumour-associated deaths out of 78 (22.0%) total deaths.

Survival analysis showed that patients with high Ki-67 and those with AC had significantly worse OS and CSS than patients with low Ki-67 and TC, respectively ($P < 0.0001$, Figure 3A, B, Table 3). Contrarily, patients with stage I-II and OTP expression, had a significantly better OS and CSS than patients with stage III-IV disease and absence of OTP, respectively ($P < 0.0001$; Table 3 and Figure 3C). Univariate analysis showed that 10-year age increase ($P < 0.0001$), high Ki-67 ($P < 0.0001$), positive STAS ($P = 0.05$), presence of TTF-1 ($P = 0.03$) and Ascl1 ($P = 0.01$) and absence of CD44 ($P = 0.007$) were associated with poor CSS. Interestingly, patients with SSTR-2A positivity (intensity 2–3) showed improved survival compared to patients with low or absent expression ($P = 0.008$, Table 3 and Figure 3D).

Table 1. Characteristics of patients with LCTs according to the Ki-67 cut-off 3%

	All patients[#]	Ki-67 < 3%	Ki-67 ≥ 3%	*P*-value*
Total	317 (100)	260 (100)	57 (100)	
Gender				
Female	199 (62.8)	170 (65.4)	29 (50.9)	
Male	118 (37.2)	90 (34.6)	28 (49.1)	0.05
Age				
<50 years	86 (27.1)	72 (27.7)	14 (24.6)	
50–59 years	66 (20.8)	58 (22.3)	8 (14.0)	
60–69 years	103 (32.5)	76 (29.2)	27 (47.4)	
70+ years	62 (19.6)	54 (20.8)	8 (14.0)	0.07
Histology				
Typical	259 (81.7)	248 (95.4)	11 (19.3)	
Atypical	58 (18.3)	12 (4.6)	46 (80.7)	**<0.0001**
Stage				
I	233 (73.5)	200 (76.9)	33 (57.9)	
II	44 (13.9)	35 (13.5)	9 (15.8)	
III	30 (9.5)	20 (7.7)	10 (17.5)	
IV	10 (3.1)	5 (1.9)	5 (8.8)	**0.004**
Smoke				
Never smoker	97 (45.8)	84 (52.5)	13 (25.0)	
Former smoker	56 (26.4)	39 (24.4)	17 (32.7)	
Current smoker	59 (27.8)	37 (23.1)	22 (42.3)	**0.001**
Mitoses				
Median [range]	1 [0–10]	0 [0–4]	3 [1–10]	**<0.0001**
Necrosis				
Absent	302 (95.3)	259 (99.6)	43 (75.4)	
Spot	7 (2.2)	1 (0.4)	6 (10.5)	
Extensive	8 (2.5)	0 (0.0)	8 (14.1)	**<0.0001**
Location				
Central	124 (62.3)	89 (60.5)	35 (67.3)	
Peripheral	75 (37.7)	58 (39.5)	17 (32.7)	0.4
Vascular Invasion				
Absent	225 (77.3)	191 (80.6)	34 (63.0)	
Present	66 (22.7)	46 (19.4)	20 (37.0)	**0.01**

Table 1. (*Continued*)

	All patients[#]	Ki-67 < 3%	Ki-67 ≥ 3%	*P*-value*
Perineural Invasion				
Absent	266 (91.4)	218 (92.0)	48 (88.9)	
Present	25 (8.6)	19 (8.0)	6 (11.1)	0.4
Intratumoral lymphocyte infiltrate				
Absent	242 (81.5)	199 (81.9)	43 (79.6)	
Present	55 (18.5)	44 (18.1)	11 (20.4)	0.7
Peritumoral lymphocyte infiltrate				
Absent	239 (79.4)	204 (81.9)	35 (67.3)	
Present	62 (20.6)	45 (18.1)	17 (32.7)	**0.02**
Microscopic invasion				
Absent	84 (28.9)	81 (33.8)	3 (5.9)	
Positive STAS	50 (17.2)	30 (12.5)	20 (39.2)	
Bronchus	133 (45.7)	110 (45.8)	23 (45.1)	
Extra-lung	24 (8.2)	19 (7.9)	5 (9.8)	**<0.0001**
Rindi Grade				
Grade 1	243 (78.6)	222 (87.1)	21 (38.9)	
Grade 2–3	66 (21.4)	33 (12.9)	33 (61.1)	**<0.0001**
Morphological pattern				
Insular/solid	121 (38.7)	82 (31.8)	39 (70.9)	
Trabecular/nested/organoid	184 (58.8)	171 (66.3)	13 (23.6)	
Other	8 (2.5)	5 (1.9)	3 (5.5)	**<0.0001**
Residual tumour				
R0	237 (90.5)	195 (92.4)	42 (82.4)	
R1–R2	25 (9.5)	16 (7.6)	9 (17.6)	**0.04**
Surgery				
Lobectomy	192 (60.6)	161 (61.9)	31 (54.4)	
Bilobectomy/pneumonectomy	35 (11.0)	24 (9.2)	11 (19.3)	
Partial resection	90 (28.4)	75 (28.9)	15 (26.3)	0.1
Tumour associated deaths				
No	262 (82.7)	225 (86.5)	37 (64.9)	
Yes	55 (17.4)	35 (13.5)	20 (35.1)	**0.0003**

Statistically significant *P*-value are reported in bold. LCTs, Lung carcinoids tumours; STAS, Spread trough air spaces.
[#]Patients where Ki-67 was evaluable.
**P*-value based on the Fisher's exact for categorical variables and the Wilcoxon test for continuous variables.

Table 2. Association between selected tumour biomarkers and Ki-67 cut-off 3% in patients with LCTs

	All patients[#]	Ki-67 < 3%	Ki-67 ≥ 3%	*P*-value*
Ttf1				
Absent	237 (82.9)	201 (87.0)	36 (65.5)	
Present	49 (17.1)	30 (13.0)	19 (34.5)	**0.0005**
Sstr2				
Absent	110 (38.6)	85 (37.0)	25 (45.5)	
Present	175 (61.4)	145 (63.0)	30 (54.5)	0.3
Otp				
Absent	76 (27.1)	48 (21.3)	28 (50.9)	
Present	204 (72.9)	177 (78.7)	27 (49.1)	**<0.0001**
Cd44				
Absent	120 (42.6)	93 (41.0)	27 (49.1)	
Present	162 (57.4)	134 (59.0)	28 (50.9)	0.3
Ascl1				
Absent	192 (67.8)	167 (73.2)	25 (45.5)	
Present	91 (32.2)	61 (26.8)	30 (54.5)	**0.0002**
P53				
Absent	278 (97.2)	231 (99.6)	47 (87.0)	
Weak heterogeneous	7 (2.4)	1 (0.4)	6 (11.1)	
Heterogeneous	0 (0.0)	0 (0.0)	0 (0.0)	
Overexpressed	1 (0.4)	0 (0.0)	1 (1.9)	**<0.0001**

Statistically significant *P*-value are reported in bold. Ascl1, mammalian achaete-scute homologue 1; LCTs, lung carcinoids tumours; OTP, orthopedia homeobox protein; SSTR-2A, somatostatin receptor 2A; TTF-1, thyroid transcription factor 1.
[#]Patients where Ki-67 was evaluable.
**P*-value based on the Fisher's exact Test for categorical variables.

In the entire cohort, 79 (22.3%) patients experienced a tumour-associated event. Kaplan–Meier analysis shows that patients with high Ki-67 and those with AC morphology had significantly worse DFS than patients with low Ki-67 and TC morphology, respectively (log-rank $P < 0.0001$; Figure 4A, B). In addition, patients without lymph node involvement had a significantly better DFS than patients with metastatic lymph nodes ($P < 0.0001$; Figure 4C). Furthermore, patients with OTP positive tumours had superior DFS than those without OTP expression ($P < 0.0001$; Figure 4D). At univariate analysis (Table 3), significant clinico-pathologic predictors of poorer DFS among the whole cohort were: 10-year age increase ($P < 0.0001$), pT ($P < 0.0001$), advanced tumour stage ($P < 0.0001$), positive STAS ($P = 0.008$), solid architectural pattern ($P = 0.01$) and residual tumour ($P = 0.01$). Positive immunoreactivities for TTF-1 ($P = 0.03$), Ascl1 ($P = 0.03$) and p53 ($P = 0.005$) were also correlated with worst prognosis while positivity for CD44 ($P = 0.02$) was correlated with better prognosis.

MULTIVARIABLE ANALYSIS AND PROGNOSTIC INFORMATION AMONG MODELS

Multivariable cox proportional regression analysis is reported in Table 4. After adjustment for center and period of diagnosis, AC histology (HR 3.68, 95% CI, 1.91–7.08, $P < 0.0001$) and high Ki-67 (HR 3.35, 95% CI, 1.72–6.53, $P = 0.0004$) were the strongest predictors of CSS together with age (10-year

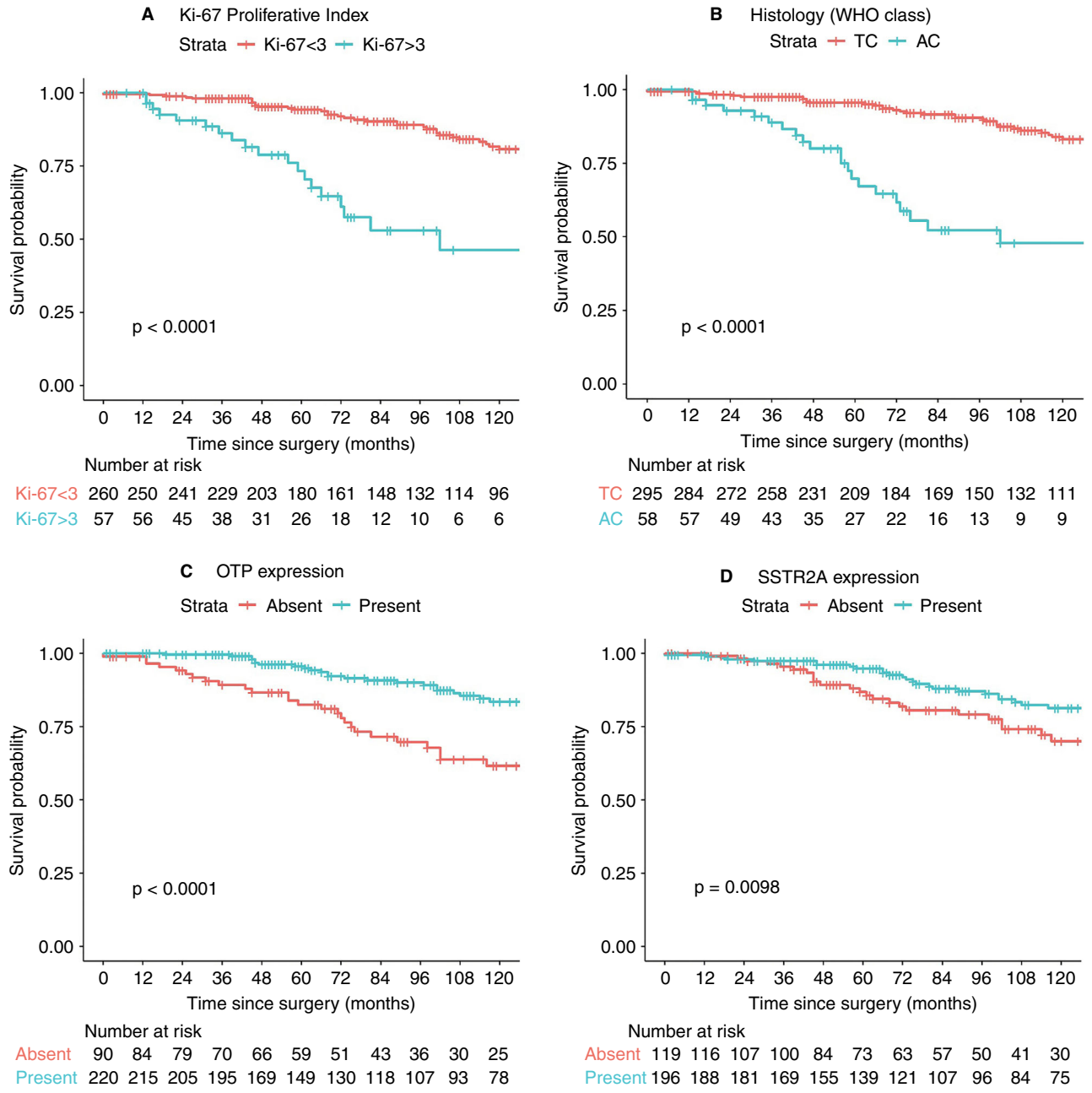

Figure 3. CSS in LCTs according to selected characteristics. **A**. Ki-67 cut-off 3%; **B**. WHO class; **C**. OTP expression; **D**. SSTR-2A expression. Abbreviations: CSS, cancer-specific survival; LCTs, lung carcinoids tumours; WHO, World Health Organization; OTP, orthopedia homeobox protein; SSTR-2A, somatostatin receptor 2A.

increase), tumour stage (III-IV versus I-II) and OTP (present versus absent). Similar results were reported for OS. In terms of DFS, AC histology (HR 4.15, 95% CI, 2.19–7.88, $P < 0.0001$) and high Ki-67 (HR 5.23, 95% CI, 2.73–10.02, $P < 0.0001$) were again the strongest prognostic factors, together with age (10-year increase), lymph node involvement (N1/2/3 versus 0), residual tumour (R1/2 versus R0) and OTP (present versus absent).

Due to the strong correlation of Ki-67 based subgroups with histologic class, we evaluated the prognostic information obtained by the addition of Ki-67, histology or both to multivariable models, in terms of OS, CSS and DFS. Specifically for CSS, either Ki-67

Table 3. Univariate* analysis of overall survival, cancer-specific survival and disease-free survival of patients with LCTs

Variable	Overall survival HR (95% CI)	*P*-value	Cancer specific survival HR (95% CI)	*P*-value	Disease free survival# HR (95% CI)	*P*-value
Sex (Male versus Female)	0.86 (0.54–1.37)	0.53	0.81 (0.47–1.39)	0.44	0.97 (0.59–1.60)	0.92
Age (10-years increase)	2.13 (1.71–2.65)	**<0.0001**	1.89 (1.48–2.40)	**<0.0001**	1.52 (1.23–1.86)	**<0.0001**
Smoke						
Never smoker	1.00		1.00		1.00	
Former smoker	1.29 (0.63–2.63)	0.48	1.50 (0.67–3.35)	0.32	0.90 (0.42–1.89)	0.77
Current smoker	0.77 (0.38–1.56)	0.46	1.02 (0.45–2.29)	0.96	1.24 (0.64–2.40)	0.53
Histotype (AC versus TC)	4.46 (2.68–7.43)	**<0.0001**	5.20 (3.01–8.99)	**<0.0001**	5.16 (3.14–8.49)	**<0.0001**
T (2–3-4 versus 1)	1.19 (0.74–1.93)	0.47	1.90 (1.12–3.22)	**0.02**	2.72 (1.68–4.41)	**<0.0001**
N (1/2/3 versus 0)	2.75 (1.63–4.64)	**0.0001**	3.32 (1.88–5.87)	**<0.0001**	3.44 (2.05–5.77)	**<0.0001**
Stage (III-IV versus I-II)	4.68 (2.67–8.18)	**<0.0001**	4.23 (2.34–7.64)	**<0.0001**	3.68 (2.06–6.59)	**<0.0001**
Mitoses (1 point increase)	1.46 (1.30–1.63)	**<0.0001**	1.45 (1.29–1.63)	**<0.0001**	1.38 (1.25–1.53)	**<0.0001**
Necrosis						
Absent	1.00		1.00		1.00	
Spot	5.02 (1.85–13.59)	**0.001**	5.04 (1.84–13.87)	**0.002**	5.26 (1.93–14.32)	**0.002**
Extensive	4.81 (1.66–13.96)	**0.004**	5.44 (1.84–16.10)	**0.002**	12.46 (4.77–32.54)	**<0.0001**
Ki-67 (≥3 versus <3)	4.96 (2.82–8.72)	**<0.0001**	5.09 (2.84–9.13)	**<0.0001**	5.95 (3.57–9.94)	**<0.0001**
Vascular Invasion (Present versus Absent)	0.86 (0.48–1.54)	0.62	0.97 (0.50–1.87)	0.92	1.26 (0.70–2.28)	0.43
Perineural Invasion (Present versus Absent)	0.59 (0.22–1.64)	0.31	0.61 (0.19–1.97)	0.41	0.68 (0.25–1.88)	0.46
Intratumoral lymphocyte infiltrate (Present versus Absent)	0.91 (0.48–1.70)	0.76	0.88 (0.42–1.81)	0.72	0.97 (0.51–1.82)	0.91
Peritumoral lymphocyte infiltrate (Present versus Absent)	1.74 (1.04–2.95)	**0.04**	1.54 (0.83–2.85)	0.17	1.12 (0.61–2.05)	0.71
Location (Peripheral versus Central)	1.20 (0.65–2.22)	0.56	0.85 (0.41–1.76)	0.66	0.69 (0.37–1.31)	0.26
Microscopic infiltration						
Absent	1.00		1.00		1.00	
Positive STAS	2.21 (1.10–4.45)	**0.03**	2.24 (1.02–4.91)	**0.05**	2.76 (1.31–5.85)	**0.008**
Bronchus	0.70 (0.37–1.34)	0.28	0.71 (0.34–1.48)	0.36	0.97 (0.49–1.92)	0.93
Extra-lung	1.01 (0.37–2.81)	0.98	0.64 (0.17–2.44)	0.52	1.17 (0.35–3.87)	0.80
Rindi Grade (Grade 2–3 versus 1)	1.77 (0.97–3.22)	0.06	2.21 (1.14–4.31)	**0.02**	1.87 (1.01–3.47)	**0.05**
Morphological pattern (Trabecular/nested/ organoid versus Insular/solid)	0.63 (0.38–1.04)	0.07	0.64 (0.36–1.12)	0.12	0.51 (0.30–0.86)	**0.01**
Residual Tumour (R1/2 versus R0)	1.83 (0.76–4.38)	0.18	1.68 (0.65–4.35)	0.29	2.55 (1.23–5.29)	**0.01**

Table 3. (*Continued*)

Variable	Overall survival HR (95% CI)	*P*-value	Cancer specific survival HR (95% CI)	*P*-value	Disease free survival[#] HR (95% CI)	*P*-value
TTF1 (Present versus Absent)	2.18 (1.14–4.17)	**0.02**	2.15 (1.09–4.27)	**0.03**	1.92 (1.07–3.47)	**0.03**
CD44 (Present versus Absent)	0.41 (0.24–0.68)	**0.0007**	0.46 (0.26–0.81)	**0.007**	0.54 (0.33–0.89)	**0.02**
OTP (Present versus Absent)	0.31 (0.19–0.52)	**<0.0001**	0.28 (0.16–0.49)	**<0.0001**	0.28 (0.17–0.48)	**<0.0001**
SSTR2 (Present versus Absent)	0.52 (0.32–0.86)	**0.01**	0.47 (0.27–0.82)	**0.008**	0.68 (0.41–1.12)	0.13
Ascl1 (Present versus Absent)	2.44 (1.48–4.03)	**0.001**	2.10 (1.20–3.69)	**0.01**	1.73 (1.04–2.87)	**0.03**
P53 (Present versus Absent)	2.29 (0.71–7.37)	0.16	1.91 (0.46–7.87)	0.37	4.45 (1.58–12.54)	**0.005**

Statistically significant *P*-value are reported in bold. Ascl1, mammalian achaete-scute homologue 1; CCS, cancer-specific survival; DFS, disease-free survival; LCTs, lung carcinoids tumours; OS, overall survival; OTP, orthopedia homeobox protein; SSTR-2A, somatostatin receptor 2A; STAS, spread trough air spaces; TTF-1, thyroid transcription factor 1.
*Adjusted for center and period of diagnosis categorized in decades (<1998, 1998–2007, 2008–2018).
[#]Evaluated on Stage I-II-III patients only.

alone (≥3 versus <3, P adj = 0.0008) or histology alone (AC versus TC, P adj = 0.0002) significantly added prognostic information to a multivariable model including center, period of diagnosis, age, stage and OTP (P adj < 0.0001). Similar results were reported for OS. In these two models, the addition of both variables did not provide further prognostic information.

For DFS, again, either Ki-67 alone (≥3 versus <3, P adj < 0.0001) or histology alone (AC versus TC, P adj = 0.0003) significantly added prognostic information to a multivariable model. Interestingly, however, multivariable analysis showed an improved significance of the prediction model by adding Ki-67 (LR−$\Delta\chi 2$ = 6.3, P adj = 0.01) to center, period of diagnosis, age, lymph involvement, residual tumour, OTP and histology, while addition of histology (LR−$\Delta\chi 2$ = 0.7, P adj = 0.40) to a model containing Ki-67 did not improve significantly prognostic information (Table 5).

Discussion

The recent WHO-2021 criteria and terminology of Lu-NETs has remained largely unchanged from the 2015 prior edition, meaning that diagnostic and prognostic challenges, debated in recent years, remain unresolved.[11] The classification of well-differentiated carcinoid tumours is still based on mitotic cut-off of 2 per 2 mm^2 and/or presence of necrosis, however no significant improvements in predicting clinical outcome have become available yet. In addition, the prognostic and diagnostic role of Ki-67 index is still much debated, although it is currently a standard marker for grading of digestive tract NENs.

In this study we characterized the morphologic, proliferative and immunophenotypic aspects of a large series of LCTs with the purpose of evaluating the morphologic factors, protein expression and role of Ki-67 index with the aim of understanding and providing new insights into the biology and aggressiveness of these rare tumours. Our study demonstrates that Ki-67 index, specifically with a 3% cut-off, is a strong prognostic marker for LCTs, strongly associated with post-operative recurrence, and therefore it should be implemented in diagnostic routine workflow.

Several studies have demonstrated the diagnostic role and predictive value of Ki-67 index in LCTs.[12–15] In particular, Clay *et al.* showed 3.5% as the best cut-off value of Ki-67 to distinguish AC from TC with excellent diagnostic performance at ROC analysis.[13] Moreover, a recent paper by Dermawan *et al.* showed that Ki-67 index was the only significant predictor of tumour recurrence on multivariate analysis among all LCTs: these authors reported a cut-off of 5% by ROC, probably due to a clearly enriched cohort of TCs, with only 11 AC samples.[15] Our results support the key role of 3% cut-off for Ki-67 as a prognostic factor in all LCTs. Interestingly, the 3% cut-off is currently a standard key-point for grading and distinguishing low grade NET G1 from intermediate grade NET G2 in GEP-NENs.[7] Similarly, this cut-off has been suggested for classifying lung well differentiated NETs in grades 1, 2 and 3 according to the unifying nomenclature proposed by the International Agency for

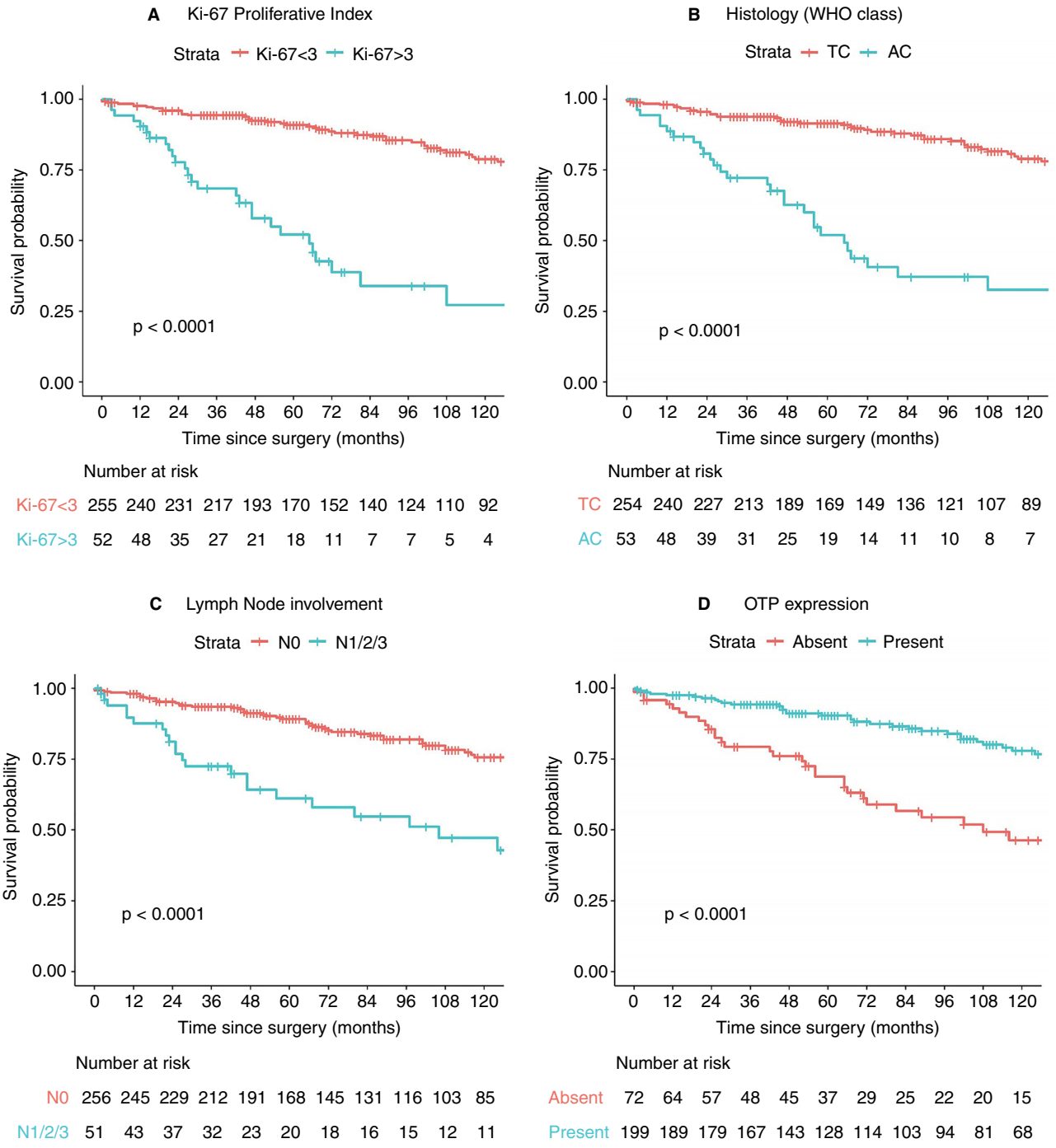

Figure 4. DFS in LCTs according to selected characteristics. **A**. Ki-67 cut-off 3%; **B**. WHO class; **C**. lymph node involvement; **D**. OTP expression. Abbreviations: DFS, disease-free survival; LCTs, lung carcinoids tumours; WHO, World Health Organization; OTP, orthopedia homeobox protein. [Color figure can be viewed at wileyonlinelibrary.com]

Research on Cancer (IARC) and the 2019 WHO Classification of Digestive System NEN (DiS NEN WHO 2019).[7,16] Moreover, a recent study demonstrated that stratifying bronco-pulmonary NENs according to DiS NEN WHO 2019 criteria results in three prognostically well-defined NET groups when grading is solely based on Ki-67 index.[17] In this setting we reported three (0.8%) highly proliferative carcinoids, specifically with Ki-67 > 20%: these cases are uncommon in the lung and mostly correspond to those classified as NET G3 in

Table 4. Multivariable* models for overall survival, cancer-specific survival and disease-free survival of patients with LCTs

Variable	OS multivariable model I HR (95% CI)	*P*-value	OS multivariable model II HR (95% CI)	*P*-value
Age (10-years increase)	2.15 (1.66–2.79)	**<0.0001**	2.20 (1.69–2.86)	**<0.0001**
Histotype (AC versus TC)	-		3.38 (1.83–6.25)	**<0.0001**
Stage (III-IV versus I-II)	2.94 (1.60–5.40)	**0.0005**	2.82 (1.54–5.19)	**0.0008**
Ki-67 (≥3 versus <3)	3.41 (1.78–6.54)	**0.0002**	-	
OTP (Present versus Absent)	0.47 (0.26–0.85)	**0.01**	0.49 (0.27–0.88)	**0.02**
Variable	CSS Multivariable Model I HR (95% CI)	*P*-value	CSS Multivariable Model II HR (95% CI)	*P*-value
Age (10-years increase)	1.92 (1.45–2.55)	**<0.0001**	1.97 (1.49–2.62)	**<0.0001**
Histotype (AC versus TC)	-		3.68 (1.91–7.08)	**<0.0001**
Stage (III-IV versus I-II)	2.71 (1.44–5.10)	**0.002**	2.58 (1.37–4.86)	**0.003**
Ki-67 (≥3 versus <3)	3.35 (1.72–6.53)	**0.0004**	-	
OTP (Present versus Absent)	0.42 (0.23–0.79)	**0.007**	0.46 (0.24–0.86)	**0.02**
Variable	DFS# Multivariable Model I HR (95% CI)	*P*-value	DFS# Multivariable Model II HR (95% CI)	*P*-value
Age (10-years increase)	1.52 (1.20–1.93)	**0.0006**	1.61 (1.26–2.05)	**0.0001**
Histotype (AC versus TC)	-		4.15 (2.19–7.88)	**<0.0001**
N (1/2/3 versus 0)	3.61 (1.94–6.72)	**<0.0001**	2.87 (1.54–5.35)	**0.0009**
Ki-67 (≥3 versus <3)	5.23 (2.73–10.02)	**<0.0001**	-	
OTP (Present versus Absent)	0.47 (0.26–0.85)	**0.01**	0.50 (0.27–0.93)	**0.03**
Residual Tumour (R1/2 versus R0)	1.93 (0.84–4.43)	0.12	2.58 (1.13–5.88)	**0.02**

Statistically significant *P*-value are reported in bold. CCS, cancer-specific survival; DFS, disease-free survival; LCTs, lung carcinoids tumours; OS, overall survival; OTP, orthopedia homeobox protein.
*Adjusted for center and period of diagnosis categorized in decades (<1998, 1998–2007, 2008–2018).
#Evaluated on Stage I-II-III patients only.

the digestive tract.[1] Studies focused on this rare entity are still scant. In particular an interesting recent study carried out by Rubino *et al.* showed that highly proliferative LCTs had a higher recurrence rate and a lower median OS than conventional lung carcinoids.[18] In our study the recurrence rate of three highly proliferative carcinoids was 67% (2/3); for the third case, data of recurrence were not available, and the CSS was 22 months.

As MC is likely proportional to Ki-67, tumours with Ki-67 ≥ 3% are strongly associated with AC histology. Indeed, both WHO tumour histology (AC versus TC) and Ki-67 (≥3 versus <3), taken individually, represent the prognostically strongest factors in terms of OS, DFS and CSS in multivariate models. Interestingly, adding both variables did not provide further significant prognostic information in CSS and OS models. These results could highlight a substantial overlap of prognostic groups which could fit in with a recent report on the association of mitotic rate and Ki-67 at gene and pathway level based on transcriptomic data.[19] In this study, the authors suggest that the integration of mitotic index and Ki-67 markers into the diagnostic framework could potentially be redundant, since both these markers govern a similar set of biological mechanisms.[19] Contrarily, we

Table 5. Prognostic information among models used in terms of overall survival, cancer-specific survival and disease-free survival

Overall survival	2 log likelihood	LR−Δχ2	DF	P-value	Adjusted *P*-value†
Without covariates	700.6	-			
Center + Period of diagnosis + Age + Stage + OTP	622.1	78.5	6	**<0.00001**	**< 0.00001**
Center + Period of diagnosis + Age + Stage + OTP + Ki-67	609.2	12.9	1	**0.0003**	**0.0005**
Center + Period of diagnosis + Age + Stage + OTP + Ki-67 + Histotype	607.3	1.9	1	0.17	0.17
Without covariates	700.6	-			
Center + Period of diagnosis + Age + Stage + OTP	622.1	78.5	6	**<0.00001**	**< 0.00001**
Center + Period of diagnosis + Age + Stage + OTP + Histotype	608.0	14.1	1	**0.0002**	**0.0003**
Center + Period of diagnosis + Age + Stage + OTP + Histotype + Ki67	607.3	0.7	1	0.40	0.40
Cancer specific survival					
Without covariates	573.4	-			
Center + Period of diagnosis + Age + Stage + OTP	516.1	57.3	6	**<0.00001**	**< 0.00001**
Center + Period of diagnosis + Age + Stage + OTP + Ki-67	504.1	12.0	1	**0.0005**	**0.0008**
Center + Period of diagnosis + Age + Stage + OTP + Ki-67 + Histotype	501.4	2.7	1	0.10	0.10
Without covariates	573.4	-			
Center + Period of diagnosis +Age + Stage + OTP	516.1	57.3	6	**<0.00001**	**< 0.00001**
Center + Period of diagnosis + Age + Stage + OTP + Histotype	501.6	14.5	1	**0.0001**	**0.0002**
Center + Period of diagnosis + Age + Stage + OTP + Histotype + Ki67	501.4	0.3	1	0.58	0.58
Disease free survival					
Without covariates	720.2	-			
Center + Period of diagnosis + Age + N + Residual Tumour + OTP	669.4	50.8	7	**<0.00001**	**< 0.00001**
Center + Period of diagnosis + Age + N + Residual Tumour + OTP + Ki67	645.7	23.7	1	**<0.00001**	**< 0.00001**
Center + Period of diagnosis + Age + N + Residual Tumour + OTP + Ki67 + Histotype	645.0	0.7	1	0.40	0.40
Without covariates	720.2	-			
Center + Period of diagnosis + Age + N + Residual Tumour + OTP	669.4	50.8	7	**<0.00001**	**< 0.00001**
Center + Period of diagnosis + Age + N + Residual Tumour + OTP + Histotype	651.3	18.2	1	**0.0002**	**0.0003**
Center + Period of diagnosis + Age + N + Residual Tumour + OTP + Histotype + Ki67	645.0	6.3	1	**0.01**	**0.01**

Statistically significant *P*-value are reported in bold. CCS, cancer-specific survival; DF, degrees of freedom; DFS, disease-free survival; LR−Δχ2, changes in likelihood ratio values; OS, overall survival; OTP, orthopedia homeobox protein.
†Correction for multiple comparisons according to Benjamini–Hochberg.

observed improved statistical significance by adding Ki-67 (≥3 versus <3) to DFS in the multivariate prediction model. This result may be related to the intrinsic nature of the Ki-67 scoring: Ki-67 proliferation evaluation is more reproducible, clear and less time-consuming compared to the scoring of mitoses,[20,21] therefore it is likely to allow a more accurate assessment of cell proliferation related to the clinical outcome, as demonstrated also by the study of Oka *et al.*[17]

OTP represents an independent prognostic marker for LCTs and this has been described before. Swarts *et al.* have previously observed that loss of expression of OTP is independently associated with shorter survival and increased risk of metastases.[6] The prognostic value of OTP has been demonstrated by Papaxoinis *et al.*, proving that loss of expression is associated with unfavourable prognosis.[22] Our study confirms this observation. Therefore, due its prognostic role as well as high sensitivity and specificity for pulmonary carcinoid tumours,[23] OTP IHC marker should be included in the diagnostic workup.

Conclusion

In conclusion, although Ki-67 index is not considered in the 2021 WHO Classification of Thoracic Tumours as an indispensable criterion for the diagnosis and prognostic evaluation of LCTs, our study proves its precise prognostic role, demonstrating that 3% cut-off is a strong predictive marker, significantly associated with post-operative recurrence. The use of groups based on the Ki-67 cut-off of 3% allows better prognostic post-surgical stratification compared to histology mainly based on MC and necrosis as showed by improved significance of the DFS prediction model. Ki-67 seems to improve the existing diagnostic histologic criteria and should be implemented in diagnostic routine workup for the evaluation of LCTs identifying patients with potential higher risk of relapse.

Authors contribution

Study concept and design – Giovanni Centonze, Patrick Maisonneuve, Carlo Capella, Massimo Milione; Methodology – Vincenzo Lagano, Giovanna Garzone, Martina Filugelli, Carlotta Pardo, Alessia Mietta; Analysis and interpretation of data – Giovanni Centonze, Patrick Maisonneuve, Carlo Capella, Massimo Milione; Drafting of manuscript – Giovanni Centonze; Critical revision of the manuscript for important intellectual content – Giovanni Centonze, Patrick Maisonneuve, Natalie Prinzi, Sara Pusceddu, Alessandro Mangogna, Alessandra Fabbri, Federica Grillo, Michele Simbolo, Aldo Scarpa, Luca Roz, Luisa Bercich, Salvatore Grisanti, Mauro Roberto Benvenuti, Alfredo Berruti, Luigi Rolli, Ugo Pastorino, Carlo Capella, Massimo Milione; Statistical analysis – Giovanni Centonze, Patrick Maisonneuve; Study supervision – Patrick Maisonneuve, Carlo Capella, Massimo Milione.

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Conflicts of interest

The authors have disclosed that they have no significant relationships with, or financial interest in, any commercial companies pertaining to this article.

Data availability statement

The data that support the findings of this study are available on request from the corresponding author.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Antibody sources and dilutions.

Table S2. Characteristics of patients with LCTs according WHO classification.

Table S3. Association between selected tumour biomarkers and WHO classification in patients with LCTs.

Figure S1. Ki-67 labeling index of the LCTs and duration of block storage.

